Green Chemistry

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c8gc00694f

Mild chemical pretreatments are sufficient for bioethanol production in transgenic rice straws overproducing glucosidase†

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Rice is a major food crop containing large amounts of lignocellulose residues usable for biofuels. In this study, we collected transgenic rice plants that over-produced *Trichoderma reesei* β-1,4-D-glucosidase (BGL I) into the cell walls in the mature straws. Without any pretreatment, the transgenic rice straws showed a consistently higher biomass enzymatic saccharification than the wild-type (WT) cultivar, in particular when 1% Tween-80 or 0.5% PEG-4000 was co-supplied into the enzymatic hydrolysis. Notably, under mild alkali pretreatment (1% NaOH at 50 °C for 2 h), the desirable transgenic line exhibited complete biomass enzymatic hydrolysis, resulting in the highest bioethanol yield of 21% (% dry matter) when compared with the rice and other bioenergy crops subjected to stronger pretreatment conditions reported in previous studies. Meanwhile, despite relatively low hexose yields obtained under 1% H_2SO_4 pretreatment, the transgenic rice straw also showed high bioethanol production at 18% due to an almost complete sugar–ethanol conversion rate. Chemical analyses indicated that the transgenic rice straw had significantly increased biomass porosity and reduced cellulose features (CrI, DP), which contributed to the largely enhanced biomass enzymatic hydrolysis. In addition, the raised arabinose level in hemicellulose and the lignin H-monomer proportion may also positively affect the biomass enzymatic saccharification in the transgenic rice straw. Hence, this study demonstrated a cost-effective and green lignocellulose conversion technology for high bioethanol production in the transgenic rice straw. It also provided a strategy for the potential genetic modification of plant cell walls in bioenergy crops.

Received 1st March 2018,
Accepted 19th March 2018

DOI: 10.1039/c8gc00694f

rsc.li/greenchem

1. Introduction

Lignocellulose represents an enormous biomass resource for biofuels and chemical production. For second generation biofuels, lignocellulose conversion requires three major steps: initial physical and chemical pretreatments for plant cell wall de-polymerization and destruction; sequential cellulase enzymatic hydrolysis of wall polymers for fermentable sugar release; and final yeast fermentation for bioethanol production.[1–4] However, because biomass recalcitrance fundamentally determines a costly lignocellulose conversion, it becomes essential to discover cost-effective biomass processing technologies applicable for biofuel industrialization.[5–7]

As an initial step, physical and chemical pretreatments have been applied to enhance sequential biomass enzymatic hydrolysis in different plant species. Although acids and alkalis are the classic agents used in chemical pretreatments, their performance normally requires extreme conditions, such as high concentration under high temperature and pressure, which is not only expensive, but may also cause secondary environmental pollution. Furthermore, the acid and alkali pretreatments play distinct roles in enhancing biomass saccharification. The alkali pretreatment can largely extract entire wall polymers by disassociating hydrogen and other bonds with

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c8gc00694f

cellulose microfibrils and lignin at high concentration, whereas the acid pretreatment can break down the major wall polymers by cleaving the chemical bonds between monosaccharides at high temperature.[5,8,9] In addition, surfactants (Tween, PEG) have been found to effectively enhance enzymatic hydrolysis of pretreated biomass residues by lessening cellulase absorption with lignin.[10–15] Hence, it is important to discover mild chemical pretreatments and efficient biomass enzymatic hydrolysis processes with less secondary pollution release.

Biomass recalcitrance is decided by plant cell wall compositions, wall polymer features and wall network styles. As the major components of biomass, plant cell walls are composed of three major polymers (cellulose, hemicelluloses, lignin). Cellulose consists of β-1,4-glucan chains that form microfibrils *via* hydrogen bonds with crystalline and amorphous regions, and the cellulose crystallinity is well demonstrated to be the key feature negatively affecting biomass enzymatic saccharification in various plant species.[14,16–19] More recently, the degree of polymerization (DP) of β-1,4-glucans has been determined to be another major cellulose feature for lignocellulose enzymatic hydrolysis.[20] In comparison, xylans are the major hemicelluloses present in grasses, and their arabinose (Ara) substitution degree has been characterized as a positive factor on biomass enzymatic saccharification by reducing the cellulose crystallinity.[19] Lignin is an amorphous wall polymer consisting of three monomers: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S);[21] recent reports suggest that lignin may play dual roles in lignocellulose digestion, depending on distinct monomer proportions in different biomass residues.[22–29] Importantly, biomass porosity has been characterized as the general parameter accounting for wall polymer features and wall network styles.[30–33]

In plants, β-glucosidase has been understood to play important roles in plant growth, stress response and bio-hydrolysis.[34,35] Recent reports have indicated that the overexpression of the β-glucosidase gene can lead to largely enhanced biomass yield and enzymatic saccharification in transgenic tobacco plants.[36–38] However, much remains unknown about the optimal biomass pretreatment for high bioethanol production in transgenic bioenergy crops exhibiting overproduction of glucosidase. Rice is a major food crop around the world and provides enormous biomass residues for biofuel production. As the genetic modification of plant cell walls can largely reduce biomass recalcitrance, it has led to much improved biomass saccharification under cost-effective pretreatments in various transgenic crops.[39–42] In this study, we collect the transgenic rice plants that over-express β-1,4-D-glucosidase (BGL I) gene of *Trichoderma reesei* and perform mild chemical pretreatments using alkali (NaOH) and acid (H_2SO_4) on the transgenic rice straws. We then measure complete biomass enzymatic saccharification and highest bioethanol production in the desirable transgenic rice line and finally, we determine major wall polymer features and biomass porosity, leading to an understanding of the high bioethanol yields achieved in this study.

2. Materials and methods

2.1. Plant samples

The homozygous transgenic rice plants and wild-type rice plants (Zhonghua 11, a japonica rice cultivar) were grown in the experimental field of Huazhong Agricultural University, Wuhan, China. The mature straws were dried, mechanically crushed using a knife-mill and stored in a dry container until further use.

2.2. Collection of transgenic rice plants

The *BGL I* (U09580) gene was amplified by PCR using cDNA of *Trichoderma reesei* and then inserted in the vector containing the rice rbcS promoter, tobacco mosaic virus translational enhancer (Ω) and tobacco pathogenesis-related protein signal peptide (prla) encoding sequence for apoplast targeting. The construct carrying the *Trichoderma reesei BGL I* gene was transformed into the *Agrobacterium* strain (EHA105) with minor modifications,[43,44] and the *Agrobacterium* strain was cultured for 24 h in an LB medium for transformation with rice WT. The transformation and regeneration of transgenic plants were performed as previously described by Fan *et al.*[41] The homozygous transgenic lines were selected based on the hygromycin selection and PCR analysis and were confirmed by the Western blot analysis (Fig. S1†). Total RNA was extracted from leaf tissues at the seedling stage using the TRIzol method according to the instructions of the manufacturer (Invitrogen). cDNA synthesis and semi-quantitative RT-PCR were performed as described by Crépineau *et al.*[45] RT-PCR primers specific to each rice gene are listed in Fig. S1.†

2.3. Cellulase activity assay *in vitro* and Western blot analysis

The transgenic rice plants were grown for 4 weeks in the field, and their leaf tissues were collected for total enzyme extraction. The fresh leaf tissues were ground into powders with liquid nitrogen and extracted with 100 mM sodium acetate trihydrate buffer (pH 5.5) for BGL I activity assay. After centrifugation at 12 000*g* for 10 min at 4 °C, the entire supernatant was used for the cellulase activity assay, and the assay was conducted as described by Jin *et al.*[36] and by using Western blot analyses. The soluble protein was determined by the Bradford method[46] with BSA as the standard. All cellulase activity assays were conducted with biological triplicates.

Total proteins were obtained from supernatants extracted with fresh 2nd internode tissues at the rice heading stage. The protein samples were loaded into 12% SDS-PAGE gel, and Western blot analysis was performed as previously described by Li *et al.*[42] For primary antibody detection, an antibody of BGL I was used at 1 : 1000 dilution. All experiments used anti-mouse secondary antibodies (IgG) at 1 : 5000 concentration. The reactions were measured by the ECL Plus Western Blotting Detection. Protein immunoblot bands were scanned with a GeneGnome XRQ instrument (Syngene Inc., Maryland, US).

2.4. Direct biomass enzymatic hydrolysis co-supplied with Tween-80 and PEG-4000

Biomass powders were incubated with 0.16% (w/v) mixed cellulases (Imperial Jade Biotechnology Co., Ltd Ningxia 750002, China) with the final concentrations of cellulases at 10.60 FPU g^{-1} biomass and xylanase at 6.72 U g^{-1} biomass and co-supplied with 1% Tween-80 (v/v) or 0.5% PEG-4000 (w/v). The measurement of the mixed-cellulase activity was based on the filter paper assay following the International Union of Pure and Applied Chemistry (IUPAC) guidelines; 1FPU = 1 μmol min^{-1} of "glucose" (reducing sugars as glucose) formed during the hydrolysis reaction. In the measurement of the xylanase activity, we used 1% (w/v) xylan (Sigma-Aldrich Co. LLC, California, USA) as the substrate; 1 U = 1 μmol min^{-1} of "xylose" (reducing sugars as xylose) formed during the hydrolysis reaction. The sealed samples were shaken at 150 rpm for 48 h at 50 °C. After centrifugation at 3000*g* for 5 min, the supernatants were collected for the pentose and hexose assays. Control samples were incubated with reaction buffer only (0.2 M acetic acid-sodium acetate, pH 4.8) under shaking for 48 h at 50 °C, and the released soluble sugars were determined in the samples by cellulase enzymatic hydrolysis. All experiments were performed with biological triplicates.

2.5. Acid and alkali pretreatment and biomass enzymatic hydrolysis

Acid (H_2SO_4) or alkali (NaOH) pretreatment and sequential enzymatic hydrolysis were performed as previously described by Fan *et al.*[41] For 1% NaOH pretreatment, biomass powders were incubated with 6 mL 1% NaOH (w/v) under shaking (150 rpm) at 50 °C for 2 h. For 1% H_2SO_4 pretreatment, the biomass powder was added to 6 mL 1% H_2SO_4 (v/v) and heated at 121 °C for 20 min in an autoclave. After centrifugation at 3000*g* for 5 min, the pretreated biomass residues were incubated with the mixed-cellulase enzymes as described above. All experiments were performed in biological triplicates.

2.6. Yeast fermentation and ethanol measurement

Yeast fermentation was performed as previously described by Jin *et al.*[13] and Fan *et al.*[41] with minor modifications. The yeast strain *Saccharomyces cerevisiae* (Angel Yeast Co., Ltd, Yichang, China) was used in all fermentation reactions, and the yeast powder was dissolved in 0.2 M phosphate buffer (pH 4.8) for 30 min for activation prior to use. The yeast powder was suspended in pH 4.8 phosphate buffer to achieve a final concentration of 0.5 g L^{-1} in all fermentation tubes, and the fermentation was performed at 37 °C for 48 h. Ethanol was measured using the $K_2Cr_2O_7$ method as described by Li *et al.*[42] and Zahoor *et al.*[47] The fermentation liquid was distilled at 100 °C for 10 min, and an appropriate amount of ethanol sample was heated in 2 mL 5% $K_2Cr_2O_7$ for 10 min in a boiling water bath. The samples were cooled, and distilled water was added up to 10 mL volume. All experiments were conducted in biological triplicates.

2.7. Total protein extraction of crude cell walls

Total crude cell walls were obtained by extracting soluble sugar, lipids and starch from biomass powders as described by Peng *et al.*[48] and Li *et al.*[42] The crude cell wall materials were washed with 1% Tween-80 at room temperature for 48 h, centrifuged at 3000*g* for 5 min, and the supernatants were collected for the total protein assay. To reduce Tween-80 interference, the protein samples were washed with 80% ethanol, and the precipitated proteins were determined using the Bradford method.

2.8. Analysis of average diameter of pores in crude cell walls

The crude cell walls were suspended in 5.0 mL 0.2 M acetic acid-sodium acetate (pH 4.8) with 1% Tween-80 (v/v). The sealed samples were shaken under 150 rpm for 48 h at 50 °C. After centrifugation, the pellet samples were washed with distilled water six times until the pH of the samples was 7.0 and then, the samples were dried at 50 °C for 24 h. Measurements of specific surface area, accumulative volume and mean pore radius were conducted using the multipurpose apparatus Micrometrics ASAP 2460 (USA) as described by Brunauer *et al.*[49] and Liu *et al.*[50] The nitrogen adsorption/desorption isotherms were measured at 77.35 K on a Micromeritics ASAP 2460 analyzer. The samples were degassed in vacuum at 70 °C before measurements. The specific surface area was calculated by the Braunauer–Emmett–Teller (BET) method with the adsorption data at the relative pressure (P/P_0) range of 0.05–0.3. The total pore volumes were measured at $P/P_0 = 0.95$. The average pore diameter was obtained using the iterative method of Barrett–Joyner–Halenda (BJH) and BET.

2.9. Electron microscopic observation

The biomass residues were directly incubated with the mixed-cellulases co-supplied with 1% Tween-80 as described above. After enzymatic hydrolysis, the samples were dried at 50 °C to constant weight, and the surfaces of biomass samples were observed using a scanning electron microscope (SEM JSM-5610/LV, Hitachi, Tokyo, Japan). Each sample was observed 8–10 times, and a representative image was used in this study.

2.10. Detection of cellulose features (CrI, DP)

For cellulose CrI and DP measurements, the dry biomass powders (0.2 g) were extracted with 4 M KOH (containing sodium borohydride at 1.0 mg mL^{-1}) at 25 °C for 1 h. After centrifugation at 4000*g* for 5 min, the pellet was re-extracted with 4 M KOH and washed six times with distilled water until pH 7.0. The pellet was further extracted with 10 mL 8% (w/v) $NaClO_2$ (containing 1.5% acetic acid) at 25 °C for 72 h ($NaClO_2$ was changed every 12 h). After centrifugation, the residues were washed seven times with distilled water until their pH was 7.0 and dried with vacuum suction filtration. The crystalline index (CrI) of cellulose was detected using the X-ray diffraction (XRD) method (Rigaku-D/MAX instrument, Uitima III, Japan) as previously described by Li *et al.*[42] DP of the crude

cellulose sample was measured using the viscosity method[51] with minor modifications.[42] In detail, DP of the crude cellulose samples was measured according to the equation $DP^{0.905} = 0.75\ [\eta]$, where $[\eta]$ is the intrinsic viscosity of the solution. All experiments were carried out at 25 ± 0.5 °C using an Ubbelohde viscosity meter and bis(ethylenediamine) copper(II) hydroxide solution (Sigma-Aldrich Co. LLC, California, USA) as the solvent. The intrinsic viscosity was calculated by interpolation using the USP table (USP, 2002) that illustrated predetermined values of the product of intrinsic viscosity and concentration. Intrinsic viscosity, $[\eta]C$, at different values of relative viscosity for the crude cellulose samples exhibiting relative viscocity (η_{rel}) values between 1.1 and 9.9. η_{rel} was calculated using the equation $\eta_{rel} = T/T_0$, where T and T_0 are the efflux times for the cellulose solution and Cuen (blank) solvent, respectively. All experiments were performed in biological triplicates.

2.11. Hemicellulose monosaccharide determination

Polysaccharides (cellulose and hemicellulose) of biomass samples were extracted as described by Peng *et al.*[48] and Li *et al.*[42] After removal of soluble sugar, lipids, starch and pectin, the remaining pellet was hydrolyzed by 2 M TFA at 120 °C for 1 h for determining the monosaccharide composition of hemicellulose using GC-MS.

2.12. Lignin and three monomer assays

The total lignin content was measured by the two-step acid hydrolysis method according to Laboratory Analytical Procedure of the National Renewable Energy Laboratory with minor modifications as described by Sun *et al.*[33] The three monomers of lignin were determined by HPLC as described by Jin *et al.*[13] and Sun *et al.*[33]

3. Results and discussion

3.1. Collection of transgenic rice plants overexpressing fungal *BGL I* gene

This study collected three independent homozygous transgenic rice lines (B1, B2, B3) that over-expressed *Trichoderma reesei BGL I* gene, and RT-PCR studies and Western blot analyses were then conducted (Fig. S1†). Because BGL I influences cellobiose or glucoside digestion into glucose, this study performed an *in vitro* assay of enzymatic activity by incubating standard substrate with total protein extracts obtained from young seedlings of transgenic rice samples (Fig. 1A). Both wild-type (WT) rice and the empty vector (EV) transgenic line showed very low enzymatic activities (Fig. 1B), suggesting that the activity could be derived from endogenous plant enzymes. The enzymatic activities of the three transgenic rice lines (B1–B3) significantly increased by 135%–571% when compared with those of the wild-type and EV controls, indicating that the raised enzyme activity may be due to the exogenous *T. reesei BGL I* gene expression in the transgenic rice plants. In addition, as the heterologous BGL I protein was mainly deposited into plant cell walls (Fig. 6B), the results also suggested that the active BGL I enzyme may play a modification role in the cellulose microfibril formation and cell wall remodeling in the transgenic rice plants.

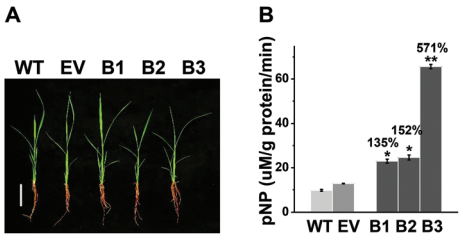

Fig. 1 Glucosidase enzymatic activity assay *in vitro* in transgenic rice plants. (A) Images of two-week-old transgenic rice plants and controls (WT and EV as empty vectors), scale bar is 5 cm; (B) enzymatic activities of transgenic rice lines (B1–B3) and controls using *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) as substrate. Student's *t*-test between WT and transgenic plants as $^{**}P < 0.01$ and $^{*}P < 0.05$; increased percentage obtained by subtraction between the results of transgenic line and WT divided by the result of WT.

3.2. Distinct enhanced biomass enzymatic saccharification under various chemical pretreatments

Although the transgenic young seedlings exhibited BGL I enzymatic activity *in vitro* as described above, we did not detect much increased enzymatic activity in the mature straws of transgenic rice plants when compared with the WT and EV control (data not shown); this was probably due to a tight association between over-produced *T. reesei* BGL I protein and wall polymers or due to ultrastructural alteration of BGL I within plant cell walls. Moreover, this study detected significantly increased biomass enzymatic saccharification (digestibility) in the transgenic rice by calculating hexose yields (% cellulose or % dry matter) obtained from the commercial mixed-cellulase hydrolysis reactions of mature rice straws under chemical pretreatments (Fig. 2; Fig. S2†). Without any pretreatment, the three transgenic lines exhibited hexose yields from 31% to 39% (% cellulose) obtained from the enzymatic hydrolysis of crude straw biomass, and these yields were higher than those of WT by 30%–65% at $P < 0.01$ levels (Fig. 2A). When 1% Tween-80 was co-supplied into the enzymatic hydrolysis, the transgenic lines showed significantly enhanced hexose yields by 38%–103% when compared with the WT samples (Fig. 2B). Furthermore, under 1% H_2SO_4 pretreatment, the three transgenic lines had hexose yields at more than 60% (% cellulose), which were higher than those of the WT samples by 42%–52% (Fig. 2C). Notably, under 1% NaOH pretreatment at 50 °C for 2 h, one transgenic line (B3) exhibited a complete biomass saccharification with hexose yields at 100% (% cellulose) and 36% (% dry matter), whereas the other two transgenic lines exhibited hexose yields at 80%–95% (% cellulose). By comparison, the WT samples only produced hexose yields at 67% (% cellulose) and 25% (% dry matter), which were significantly lower than those of the three transgenic lines by 15%–47% (Fig. 2D; Fig. S2D†). To re-confirm the

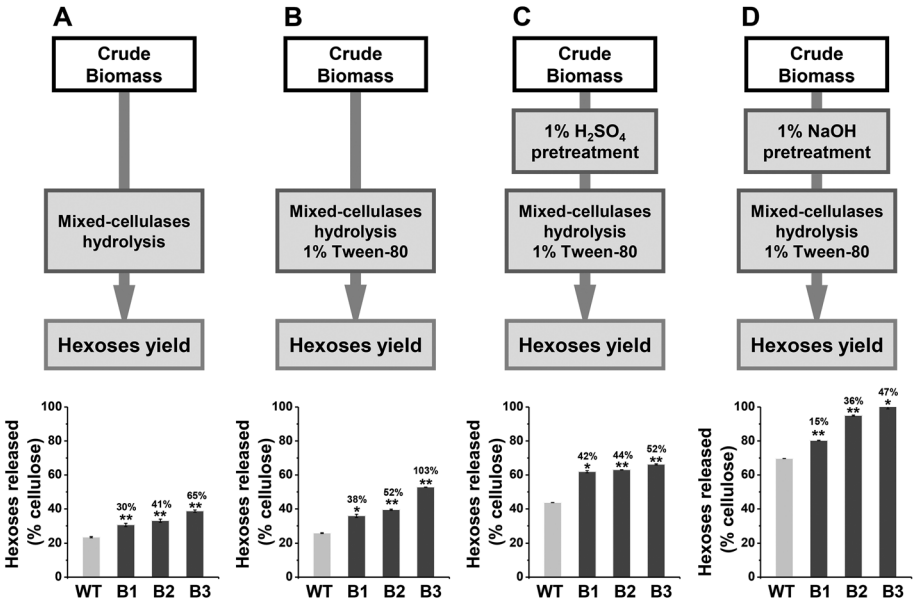

Fig. 2 Biomass enzymatic saccharification in the mature straws of transgenic rice lines and WT. (A) Hexose yields released from direct biomass enzymatic hydrolysis using commercial mixed-cellulases. (B) Hexose yields released from direct biomass enzymatic hydrolysis using commercial mixed-cellulases co-supplied with 1% Tween-80. (C) Hexose yields released from enzymatic hydrolysis co-supplied with 1% Tween-80 after 1% H_2SO_4 pretreatment. (D) Hexose yields released from enzymatic hydrolysis co-supplied with 1% Tween-80 after 1% sodium hydroxide pretreatment. Student's *t*-test between WT and transgenic plants as $^{**}P < 0.01$ and $^{*}P < 0.05$; increased percentage obtained by subtraction between the results of transgenic line and WT divided by the result of WT.

surfactant role in the biomass enzymatic hydrolysis, we also considered PEG-4000 as a replacement for Tween-80, and all three transgenic lines exhibited significantly enhanced hexose yields under H_2SO_4 and NaOH pretreatments when compared with the WT samples (Fig. 3). Similarly, the 1% NaOH pretreatment led to much higher hexose yields (% dry matter or % cellulose) than the 1% H_2SO_4 pretreatment when 0.5% PEG-4000 was co-supplied into the enzymatic hydrolysis. However, the PEG-4000 co-supply caused relatively less hexose yields than the co-supply of Tween-80. Hence, the three transgenic rice lines showed consistently enhanced biomass enzymatic saccharification.

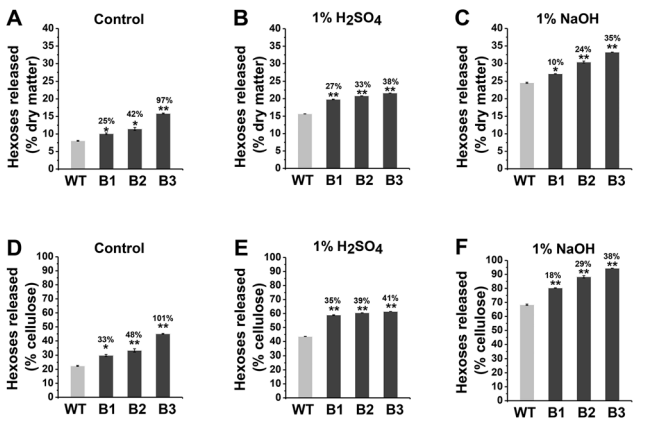

Fig. 3 Biomass enzymatic saccharification co-supplied with 0.5% PEG-4000 in the mature straws of transgenic rice lines. (A) Hexose yields (% dry matter) released from direct biomass enzymatic hydrolysis in transgenic rice lines and WT using commercial mixed-cellulases. (B) and (C) Hexose yields (% dry matter) released from enzymatic hydrolysis after 1% sulfuric acid pretreatment or 1% sodium hydroxide pretreatment in transgenic rice lines and WT. (D–F) Hexose yields (% cellulose) released from enzymatic hydrolysis after different pretreatments as shown in (A–C). Student's *t*-test between WT and transgenic plants as $^{**}P < 0.01$ and $^{*}P < 0.05$; increased percentage obtained by subtraction between the results of transgenic line and WT divided by the result of WT.

3.3. High bioethanol yields and sugar–ethanol conversion rates under acid and alkali pretreatments

As the transgenic rice straws showed much enhanced biomass enzymatic saccharification, this study sequentially detected bioethanol production generated by the yeast fermentation of soluble hexoses obtained from the enzymatic hydrolysis of pretreated biomass residues when 1% Tween-80 (Fig. 4) or 0.5% PEG-4000 was co-supplied (Fig. 5). Under 1% H_2SO_4 pretreatment, the three transgenic rice lines showed higher bioethanol yields (% dry matter) by 11%–29% than the WT samples (Fig. 4A); also, they exhibited even more increased bioethanol yields by 17%–45% under the 1% NaOH pretreatment when 1% Tween-80 was co-supplied into the enzymatic hydrolysis (Fig. 4B). Further calculations indicated that the transgenic lines exhibited much higher sugar–ethanol conversion rates under both acid and alkali pretreatments when compared with the WT samples (Fig. 4C and D). Notably, due to complete biomass saccharification under 1% NaOH pretreatment, the highest bioethanol yield at 21% (% dry matter) was produced by the desirable transgenic rice (B3) with the sugar–ethanol

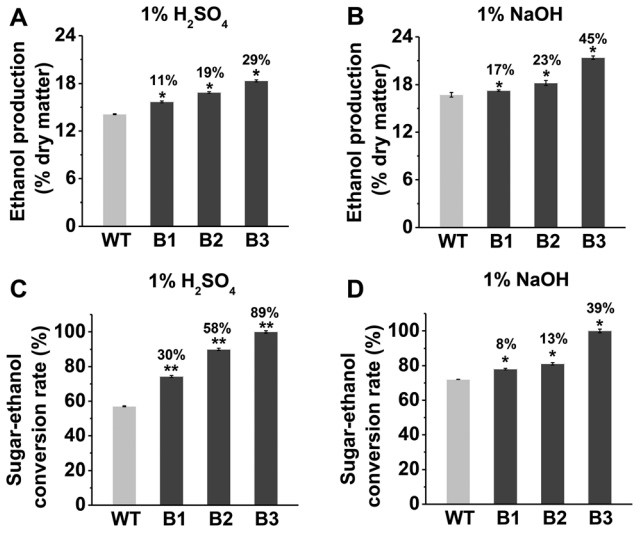

Fig. 4 Bioethanol yield and sugar–ethanol conversion rate under mild acid and alkali pretreatment co-supplied with 1% Tween-80 in the mature straws of transgenic rice lines and WT. (A) and (B), Bioethanol yields obtained from yeast fermentation using total hexose contents released from enzymatic hydrolysis after acid pretreatment and alkali pretreatment. (C) and (D), Sugar–ethanol conversion rates under mild acid and alkali pretreatment. Student's *t*-test between WT and transgenic plants as $^{**}P < 0.01$ and $^{*}P < 0.05$; increased percentage obtained by subtraction between the results of transgenic line and WT divided by the result of WT.

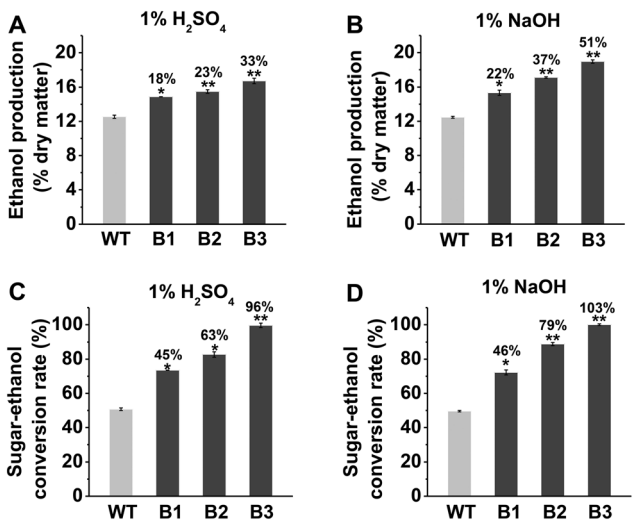

Fig. 5 Bioethanol yield and sugar–ethanol conversion rate under mild acid and alkali pretreatment co-supplied with 0.5% PEG-4000 in the mature straws of transgenic rice lines and WT. (A) and (B), Bioethanol yields obtained from yeast fermentation using total hexose contents released from enzymatic hydrolysis after acid pretreatment and alkali pretreatment. (C) and (D), Sugar–ethanol conversion rates under mild acid and alkali pretreatment. Student's *t*-test between WT and transgenic plants as $^{**}P < 0.01$ and $^{*}P < 0.05$; increased percentage obtained by subtraction between the results of transgenic line and WT divided by the result of WT.

conversion rate close to the theoretical value (100%) when compared with either the bioethanol yield of the WT samples at 14% or with the previously reported bioethanol yields at 17%–20% in rice and other grass species subjected to stronger physical and chemical pretreatments (Table 1).[13,47,52–56] Meanwhile, despite relatively low hexose yield from 1% H_2SO_4 pretreatment (Fig. 2C), the desirable transgenic rice (B3) also showed high bioethanol production at 18% (% dry matter), which was probably due to fewer inhibitory compounds released from the acid pretreatment (Table 1). Similarly, when 0.5% PEG-4000 was co-supplied into the enzymatic hydrolysis after 1% H_2SO_4 or 1% NaOH pretreatment, the three transgenic lines also exhibited much higher bioethanol yields and sugar–ethanol conversion rates than the WT samples (Fig. 5). Furthermore, due to the relatively higher hexose yields obtained from the enzymatic hydrolysis as described above, the 0.5% PEG-4000 co-supplement caused relatively lower bioethanol yields than 1% Tween-80 (Fig. 4). However, among the three transgenic lines examined, the desirable transgenic line (B3) exhibited the highest bioethanol yield and conversion rate for both Tween-80 and PEG-4000 co-supplements.

Hence, the desirable transgenic rice line B3 could exhibit complete biomass enzymatic saccharification and highest bioethanol productivity even though only mild pretreatment (1% NaOH at 50 °C for 2 h) was performed, which was probably due to much improved wall polymer features and biomass porosity in the transgenic rice straws.

3.4. Increased biomass porosity in transgenic rice straws

To understand the considerably enhanced biomass saccharification and bioethanol production in the mature transgenic rice straws, we first detected total protein levels extracted with 1% Tween-80 from crude cell wall materials, and the two representative transgenic lines (B2, B3) showed an increase in the extracted proteins than those of WT by 66% and 79% at $P < 0.05$ levels (Fig. 6A). Based on the Western blot analysis, we then detected the protein bands corresponding to the *T. reesei* BGL I protein in transgenic lines only rather than in the WT samples (Fig. 6B), and the results indicated that the *T. reesei* BGL I protein could deposit into plant cell walls and the increased protein from Tween-80 extracts was mainly derived from the *T. reesei BGL I* gene in the transgenic rice lines. Then, we measured the porosity of the biomass residues obtained from 1% Tween-80 washing with total crude cell wall materials (Fig. 6C). When compared with the WT samples, the two transgenic lines exhibited much higher biomass porosity including higher pore diameter (Fig. 6C), specific surface area, cumulative volume of pores and average pore diameter (Table 2). Because biomass porosity has been defined to provide space and position for cellulase enzyme loading and access to the cellulose microfibril surface,[57–60] the increased biomass porosity could be a major cause for the largely enhanced biomass enzymatic hydrolysis in the transgenic rice plants. To further confirm this, we observed that the desirable transgenic line (B3) exhibited a much

Table 1 Bioethanol production in rice and other crops

Plant species	Pretreatment	Ethanol production (% dry matter)	Ref.
Rice			
WT	1% Sulfuric acid + 1% Tween-80	14%	This study
	1% Sodium hydroxide + 1% Tween-80	15%	
B3	1% Sulfuric acid + 1% Tween-80	18%	
	1% Sodium hydroxide + 1% Tween-80	21%	
Rice	Ammonia fiber expansion	17%	52
Wheat	Steam explosion + 0.5% sulfuric acid + 1% Tween-80	19%	47
Cotton	Liquid hot water pretreatment (LHWP)	18%	53
Rapeseed	Alkaline peroxide and stream pretreatment	15%	54
Maize	Ammonia fiber expansion	19%	55
Miscanthus	Liquid hot water	15%	56
Reed	Steam-explosion + 5% CaO + 1% Tween-80	19%	13

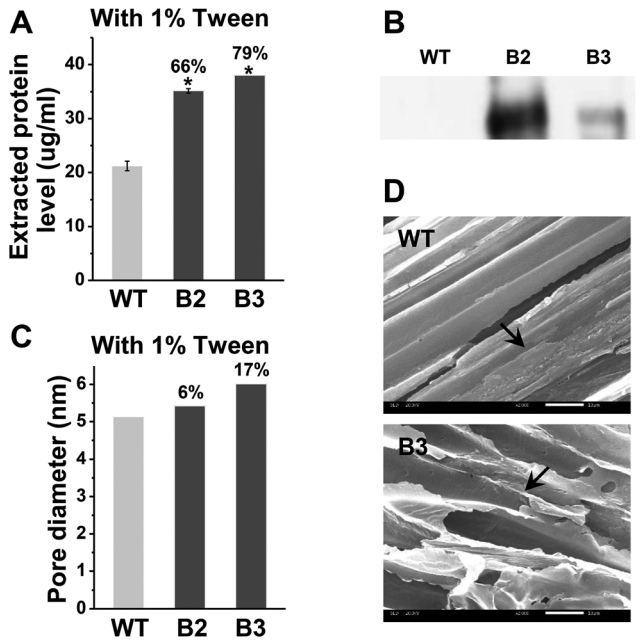

Fig. 6 Wall protein and biomass porosity obtained from extraction with 1% Tween-80. (A) Protein levels extracted from 1% Tween-80 washing; data as mean ± SD (n = 3); Student's t-test between WT and transgenic lines as *P < 0.05; increased percentage obtained by subtraction between transgenic line and WT results divided by the result of WT. (B) Western blot analysis of BGL I proteins extracted from 1% Tween-80 washing as described in (A). (C) Average diameter of pore after 1% Tween-80 washing; increased percentage obtained by subtraction between the results of transgenic line and WT divided by the result of WT. (D) SEM images of biomass residues obtained from enzymatic hydrolysis co-supplied with 1% Tween-80. Scale bar is 5 μm; allow as rough point.

Table 2 Pore characteristic of the crude biomass after 1% Tween-80 washing with the mature straws of transgenic rice lines

	S_{BET} (m^2 g^{-1})	V_{BJH} (cm^3 mg^{-1})	D_{BJH} (nm)
WT	1.1827	1.5	5.1316
B2	2.1447	2.9	5.4191
B3	1.7339	2.6	6.0108

S_{BET}, specific surface area; V_{BJH}, cumulative volume of pores; D_{BJH}, average diameter of pore.

rougher surface than the WT samples in the biomass residue obtained from direct cellulase enzymatic hydrolysis co-supplied with 1% Tween-80 (Fig. 6D). As the rough lignocellulose surfaces can regulate high biomass enzymatic saccharification in various biomass samples examined,[61,62] this phenomenon can also be applied for the transgenic rice lines used in this study.

3.5. Distinctively altered wall polymer features in transgenic rice straws

It has been found that wall polymer features distinctively affect biomass enzymatic saccharification and sequential bioethanol productivity in different plant species.[47,63–66] Due to the greatly enhanced biomass enzymatic saccharification in the transgenic rice plants, this study detected their major wall polymer features (Fig. 7). First, we examined significantly reduced cellulose DP and CrI values in the three transgenic lines, and we compared these values with the results of the WT samples (Fig. 7A and B). Because cellulose DP and CrI have been well characterized to negatively affect the biomass enzymatic saccharification under various physical and chemical pretreatments in different biomass residues,[2,41,42] the reduced cellulose features mainly contribute to the greater biomass enzymatic saccharification in the transgenic rice plants.

Furthermore, this study focused on the cell wall composition and other wall polymer features in the desirable transgenic line (B3). In general, the desirable transgenic B3 line showed a slightly altered cell wall composition, when compared with the WT samples (Table S1†), which was consistent with our observations of the transgenic rice plants showing normal growth and development (data not shown). In detail, in the B3 transgenic line, the cellulose and lignin levels slightly reduced by 6% and 4%, respectively, and the hemicellulose content relatively increased by 14%, supporting the assumption that *T. reesei* BGL I may have enzymatic activity for the post-modification of cellulose microfibrils, in particular for reduced cellulose DP in the transgenic rice plant. On the other hand, based on previous reports,[19,42] the reduced cellulose CrI may be mainly due to the decreased cellulose DP and the increased hemicellulose level in the transgenic rice plants.

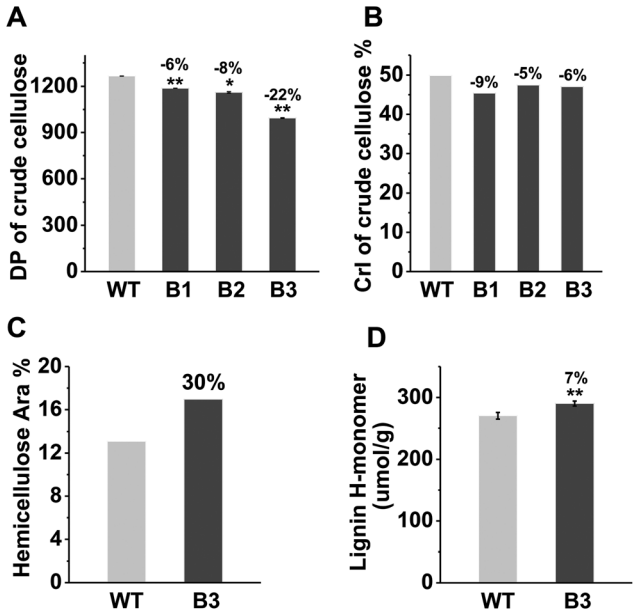

Fig. 7 Detection of wall polymer features in the mature straws of transgenic rice lines and WT. (A) Degree of polymerization (DP) of crude cellulose. (B) Crystalline index (CrI) of crude cellulose. (C) Arabinose (Ara) proportion of hemicellulose. (D) H-monomer proportion of lignin; * or ** as significant difference between WT and transgenic plants by *t*-test at $P < 0.05$ or $P < 0.01$ ($n = 3$); increased percentage obtained by subtraction between the results of transgenic line and WT divided by the result of WT.

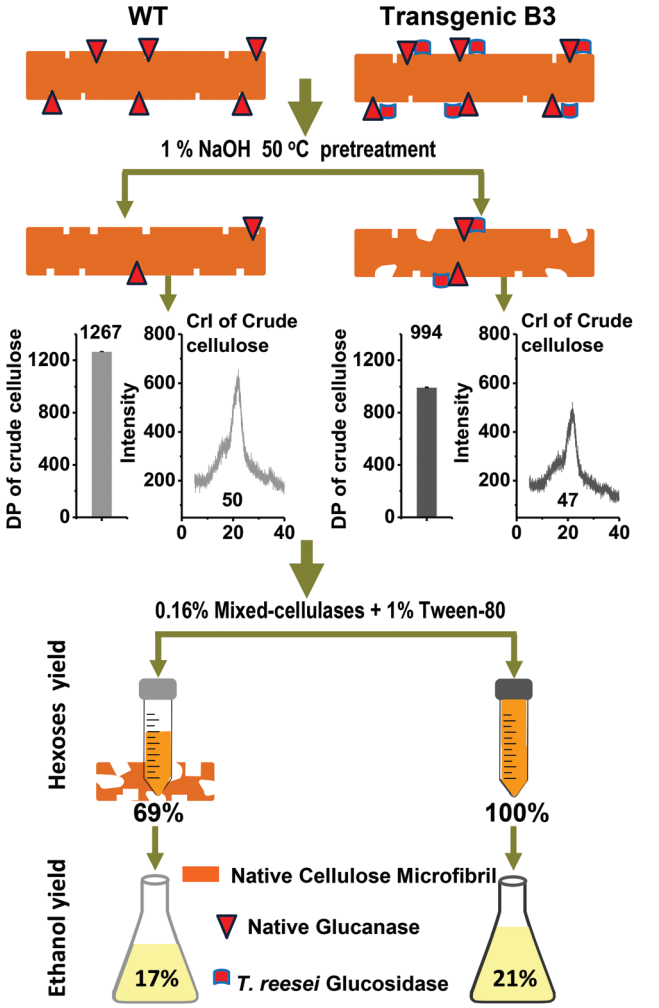

Fig. 8 A hypothetical model outlining that the over-produced *T. reesei* β-glucosidase may be involved in the post-modification of cellulose microfibrils for specifically reducing cellulose CrI and DP and significantly increasing biomass porosity, which leads to largely enhanced biomass enzymatic saccharification and bioethanol production in the desirable transgenic rice B3 line.

In addition, this study detected the monosaccharide compositions of hemicellulose and monomer constitution of lignin, and the desirable transgenic rice line (B3) exhibited increased Ara proportion by 30% and H-monomer level by 7% when compared with the WT samples (Fig. 7C and D; Tables S2 and S3†). As Ara present in hemicellulose and H-monomer have been reported as positive factors for biomass enzymatic hydrolysis,[19,67–69] the results suggest that the increased Ara and H levels may also be minor factors contributing to the enhanced biomass enzymatic saccharification in the transgenic rice plants.

3.6. Mechanism of the over-produced β-glucosidase enhancement on lignocellulose saccharification and bioethanol production

To elucidate how the over-production of *T. reesei* β-glucosidase enzyme could lead to largely enhanced biomass enzymatic saccharification and bioethanol yield under mild alkali pretreatment in the desirable transgenic line, we proposed a hypothetical model based on the novel findings of this study and information obtained from previously published articles (Fig. 8). (1) As the *T. reesei* β-glucosidase (BGL) enzyme has been found to deposit into plant cell walls (Fig. 6), the BGL enzyme may join the native glucanases to attack the amorphous/non-crystalline regions of cellulose microfibrils in the desirable transgenic B3 line.[2,42] (2) Because the over-produced β-glucosidase has enzymatic activity *in vitro* (Fig. 1), it can join the native glucanases in post-modification of the cellulose microfibrils to increase the surface and depth of the amorphous regions;[70] this leads to specifically reduced cellulose DP and CrI along with relatively increased porosity in the transgenic line when compared with that in the WT samples (Fig. 7). (3) Based on the property of protein, the over-produced β-glucosidase can simply disassociate under mild alkali pretreatment (1% NaOH at 50 °C for 2 h), resulting in higher biomass porosity in the transgenic line (Fig. 6). (4) The co-supplement with 1% Tween-80 can either extract the remaining β-glucosidase on the microfibrils or specifically lessen the commercial mixed-cellulase absorption with both lignin and the disassociated glucodsidase,[13,33] leading to much higher hexose and bioethanol yields in the transgenic B3 line than those of the WT samples. Therefore, this model highlighted that the over-production of *T. reesei* BGL could mainly improve cellulose features (DP, CrI) and biomass porosity for accumulative enhancement of biomass

enzymatic saccharification and sequential bioethanol production in the transgenic rice plants.

4. Conclusions

Using transgenic rice plants that over-produced *T. reesei* β-glucosidase enzyme into cell walls, this study detected significantly increased biomass porosity and reduced cellulose crystallinity and DP in mature straws, leading to highly enhanced biomass enzymatic saccharification as a result of various chemical pretreatments, in particular with the co-supplement 1% Tween-80 or 0.5% PEG-4000. Notably, the desirable transgenic rice line showed complete biomass digestibility and highest bioethanol yield at 21% (% dry matter) under mild alkali pretreatment (1% NaOH at 50 °C for 2 h). Therefore, this study provides a cost-effective technology for biomass process and bioethanol production in the transgenic rice plants and other crops.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

This study was in part supported by grants from the National Key Research and Development Program (2016YFD0800804), the National 111 Project (B08032), the National Science Foundation of China (31670296; 31571721) and the National Transgenic Project (2009ZX08009-119B).

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