Overproduction of fungal endo-β-1,4-glucanase leads to characteristic lignocellulose modification for considerably enhanced biomass enzymatic saccharification and bioethanol production in transgenic rice straw

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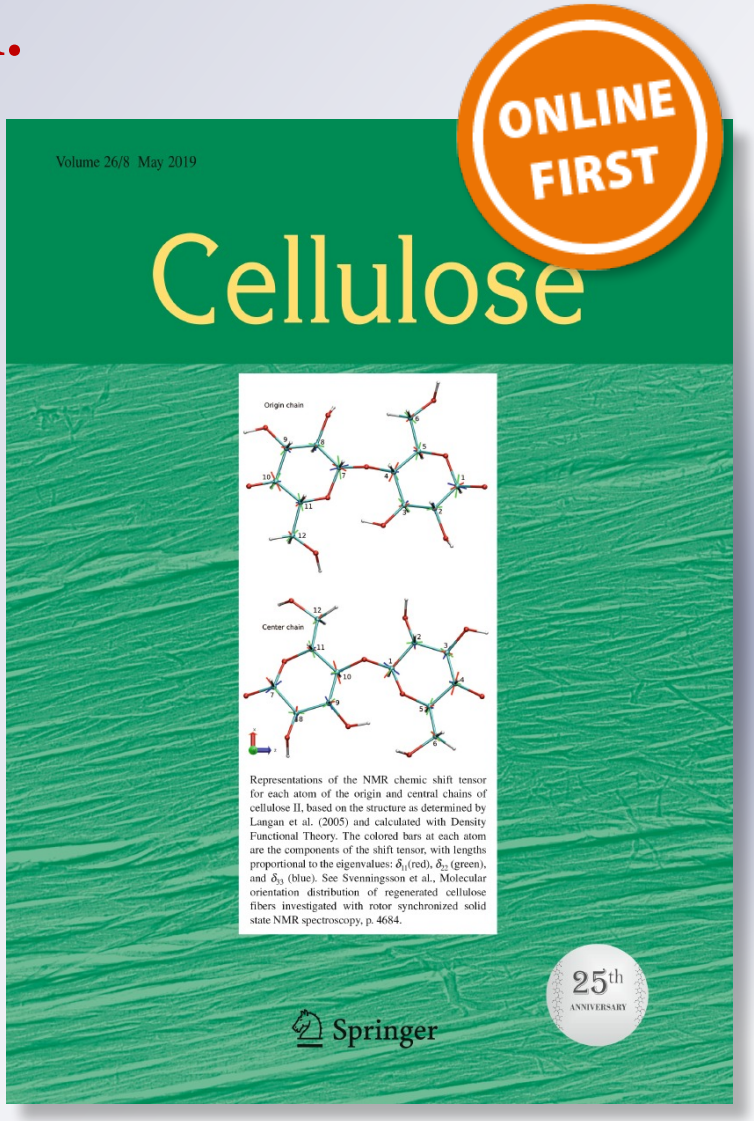

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ORIGINAL RESEARCH

Overproduction of fungal endo-β-1,4-glucanase leads to characteristic lignocellulose modification for considerably enhanced biomass enzymatic saccharification and bioethanol production in transgenic rice straw

Ying Li · Haiyan Sun · Chunfen Fan · Huizhen Hu · Leiming Wu · Xiaohuan Jin · Zhengyi Lv · Yanting Wang · Shengqiu Feng · Peng Chen · Liangcai Peng

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Abstract Genetic modification of plant cell walls has been considered to reduce lignocellulose recalcitrance for enhanced biomass enzymatic saccharification and biofuel production in bioenergy crops. Although endo-β-1,4-glucanase (EG II) secreted by fungi has been broadly applied for enzymatic hydrolysis of cellulose, it remains to explore its role in cellulose modification when the *EG II* gene is overexpressed in plant. In this study, we selected transgenic rice plants that overproduced *Trichoderma reesei* EG II enzyme specifically deposited into plant cell walls, and then examined much higher enzymatic activities by fourfold to fivefold in transgenic young seedlings than those of wild type in vitro. Notably, despite slightly altered cell wall compositions and polymer interlinkages relative to the wild type, the transgenic mature rice straw exhibited significantly reduced cellulose DP and CrI values and hemicellulosic Xyl/Ara ratio, leading to much increased biomass porosity. These should play integrated impact for enhanced biomass enzymatic saccharification and bioethanol production even under mild alkali pretreatment. Therefore, the results suggested that the EG II deposition should have enzymatic activity specific for

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Y. Li · H. Sun · C. Fan · H. Hu · L. Wu · X. Jin · Z. Lv · Y. Wang · S. Feng · P. Chen · L. Peng (✉)
Biomass and Bioenergy Research Centre, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China
e-mail: lpeng@mail.hzau.edu.cn
URL: http://bbrc.hzau.edu.cn

H. Sun
School of Biology and Food Engineering, Changshu Institute of Technology, Changshu 215500, China

C. Fan
Key Laboratory of Eco-environments of Three Gorges Reservoir Region, Ministry of Education, Institute of Resources Botany, School of Life Sciences, Southwest University, Chongqing 400715, China

H. Hu
State Key Laboratory of Biocatalysis and Enzyme Engineering, College of Life Science, Hubei University, Wuhan 430062, China

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minor-modification of cellulose microfibrils in transgenic rice plants. It has also provided a potential strategy for mild cell wall modification and optimal biomass process in rice and other bioenergy crops.

Keywords Cellulose features · Biomass porosity · Endo-β-1,4-glucanase · Biomass saccharification · Bioethanol

Abbreviations

EG II	Endo-β-1,4-glucanase
CrI	Cellulose crystallinity index
DP	Degree of polymerization
Ara	Arabinose
Xyl	Xylose
SEM	Scanning electron microscopy
FTIR	Fourier transforms infrared

Introduction

Lignocellulose is increasingly considered for the development of secondary bioenergy generation (Kumar et al. 2008; Ge et al. 2011; Xie and Peng 2011; Lee and Kuan 2015). In particular, food crops could provide significant amounts of lignocellulosic straw for biofuels without creating a conflict between energy demand and food security (Lynd et al. 2008; Arai-Sanoh et al. 2011). Lignocellulose ethanol conversion involves in three major steps: physical and chemical pretreatment for lignocellulose destruction, sequential enzymatic hydrolysis for soluble sugars release, and final yeast fermentation for bioethanol production (Ciolacu et al. 2011; Wu et al. 2013). However, biomass processing is largely hindered by the lignocellulose recalcitrance, due to the high heterogeneity of lignocellulose and the intricate linkages within and between wall polymers (Himmel et al. 2007; Pauly and Keegstra 2010; Chundawat et al. 2011). To reduce recalcitrance, genetic modification of plant cell walls has been considered vital for enhancing biomass enzymatic saccharification in transgenic plants (Taylor et al. 2008).

Plant cell walls are mainly composed of cellulose, hemicelluloses and lignin. Cellulose is a linear β-1,4-glucan that forms microfibrils by hydrogen bonds and van der Waals forces. These microfibrils are thought to have both crystalline and amorphous zones, and the crystalline regions are characterized as a negative factor on biomass saccharification. However, the degree of polymerization (DP) of β-1,4-glucans has recently been found as a feature that directly affects cellulose enzymatic hydrolysis (Li et al. 2015; Pei et al. 2016; Zahoor et al. 2017). Hemicelluloses are a class of heterogeneous polysaccharides containing diverse monosaccharides, and xylans are the major hemicelluloses in grasses. It has been recently reported that the xylose/arabinose ratio (Xyl/Ara) of xylan is a negative factor on biomass enzymatic digestibility by reducing cellulose crystallinity (Li et al. 2013a), probably due to Ara interaction with the β-1,4-glucan chains in the amorphous regions of cellulose microfibrils (Wang et al. 2016). More importantly, lignocellulose is featured with a porous medium and its porosity is characterized as a general parameter accounting for lignocellulose enzymatic saccharification (Tanaka et al. 1988; Divne et al. 1994; Sun et al. 2017; Kim and Lee 2019).

For cellulose hydrolysis into fermentable glucose, at least three types of hydrolytic enzymes are required including endo-β-1,4-glucanase (EG; E.C. 3.2.1.4), β-1,4-exoglucanase (cellobiohydrolase/CBH; E.C. 3.2.1.91) and β-1,4-D-glucosidase (BGL; E.C. 3.2.1.21) (Henrissat 1994; Tomme et al. 1995; Barnard et al. 2010). In comparison, EGs mainly digest β-glycosidic bonds within sugar chains with high activity on amorphous rather than crystalline cellulose, whereas CBHs specifically act on the ends of the cellulose chains to cleave off cellobiose sugars. *Trichoderma reesei* is one of the fungi best known for producing cellulolytic enzymes, including a set of five endoglucanases and two exoglucanases (Rosgaard et al. 2007; Peterson and Nevalainen 2012). Although transgenic plants can produce endo-β-1,4-glucanase enzymes, little is yet reported about its potential modification on plant cell wall composition and carbohydrate polymer feature in transgenic plant (Dai et al. 2000, 2005; Hood et al. 2007; Klose et al. 2013, 2015).

Rice is a major food crop around the world with enormous biomass residues for biofuel production. In this study, we selected transgenic rice plants that overexpressed endo-β-1,4-glucanase genes (*EG II*) derived from *Trichoderma reesei*, and detected cell wall compositions, wall polymer feature and biomass porosity in transgenic rice plants. Finally, we examined biomass enzymatic saccharification under mild

alkali pretreatment, and measured bioethanol production in the transgenic rice plants. Hence, this study demonstrated how endo-β-1,4-glucanase could specifically modify lignocellulose for enhanced biomass saccharification in the transgenic rice straw.

Materials and methods

Transformation vector construction

The coding sequence of EG II (M19373) was amplified by PCR from a cDNA of *Trichoderma reesei*. For the generation of EG II fusion constructs, the rice rbcS promoter, the tobacco mosaic virus translational enhancer (Ω) and the tobacco pathogenesis-related protein signal peptide (prla) encoding sequence for apoplast targeting as shown in Fig. S1. The vector also contains the bar gene encoding hygromycin B phosphotransferase (*HPT*) as a selectable marker. The subcellular localization of EG II fluorescence was observed by fusing EG II with C-terminus of EGFP protein.

Selection of plant transgenic lines

Construct carrying *Trichoderma reesei EG II* gene was transformed into *Agrobacterium* strain (EHA105) and selected on LB medium plates supplemented with kanamycin (50 mg/mL). The transgenic rice plants were selected as previously described by Li et al. (2018a). The homozygous transgenic lines were detected based on the PCR analysis as shown in Table S1, and then confirmed by Western analysis (Fig. 1).

Gene expression analysis

The total RNA of leaf tissues was extracted using the TRIzol method based on the instructions of the manufacturer (Invitrogen). cDNA synthesis and semi-quantitative RT-PCR were performed use the PCR primers showed in Table S1.

Cellulase activity assay in vitro

The fresh leaf tissues of rice plants grown for 4 weeks were ground into the powders by liquid nitrogen and extracted using 100 mM sodium acetate trihydrate buffer (pH 5.5) for EG II activity assay. The total supernatants were applied for cellulase activity assay as described by Dai et al. (2000) and Western blot analyses after centrifugation twice at 12,000×*g* for 15 min at 4 °C. The activity of cellulases against 4-methylumbelliferyl-β-D-cellobioside (4-MUC) was carried out as previously described by Klose et al. (2013). The fluorescence was determined at 465 nm using an excitation wavelength of 360 nm. All cellulases activity assays were conducted with biological triplicates.

GFP fluorescence observation

The 60-μm agarose embedded sections were prepared from leaf vein materials using a Leica VT1000S (Leica Microsystems, Wetzlar, Germany). To visualize the EG II-EGFP distribution within the leaf vein, CLSM imaging was obtained using a Leica TCS SP8 confocal laser scanning system (Leica Microsystems, Wetzlar, Germany). Fluorescence was examined using the following setting: GFP signal (488-nm excitation and 498- to 540-nm emission). Chloroplast autofluorescence was excited at 633 nm by a helium–neon laser.

SDS-PAGE and Western blot analysis

Total proteins were collected from supernatants extracted with fresh 2nd internode tissues at heading stage of rice plants. The SDS-PAGE and Western blot analysis were performed as described by Li et al. (2017) and Fan et al. (2017). The primary antibody of EG II and anti-mouse antibody (IgG) were diluted 1:1000 and 1:5000 respectively. The reactions were measured by the ECL Plus Western Blotting Detection. The signal was visualized with a GeneGnome XRQ (Syngene Inc., Maryland, USA).

Biomass sample collection

The transgenic rice plants and wild type (Zhonghua 11, a japonica rice cultivar) were grown in the experimental field of Huazhong Agricultural University, Wuhan, China. The mature stem tissues of rice plants were collected, dried and ground into powders through 40 mesh (0.425 mm × 0.425 mm).

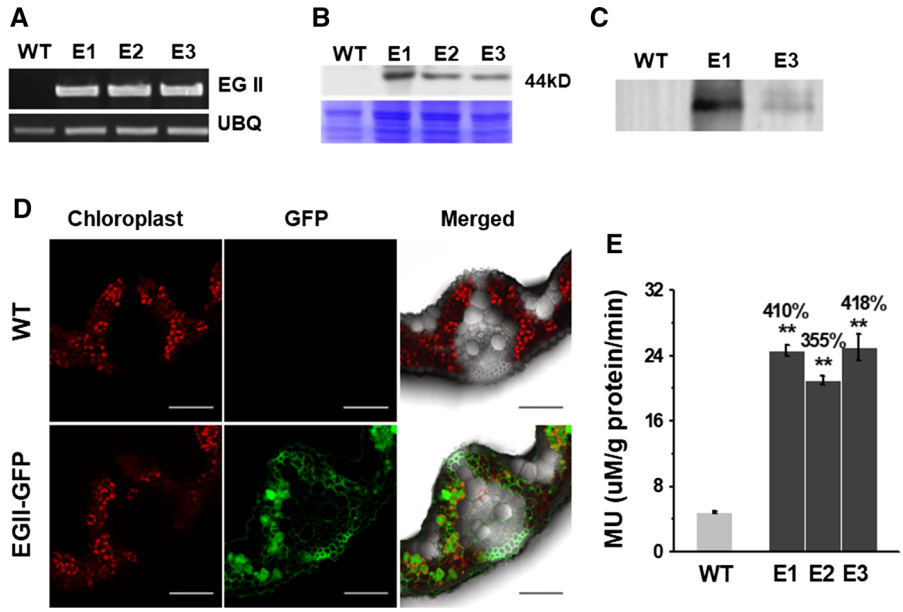

Fig. 1 Selection of transgenic rice plants: **a** RT-PCR analysis of *Trichoderma reesei EG II* expressions in leaf tissues of three independent homozygous transgenic rice lines (E1–E3) and wild type (WT, Zhonghua 11) at seeding stages using primers shown in Fig. S1 and Table. S1; **b** western blot analysis of EG II proteins extracted from the 2nd internodes of stem tissues at rice heading stages; **c** western blot analysis of EG II proteins obtained from 1% Tween-80 extraction with total crude cell walls of the stem tissues at mature stages; **d** GFP fluorescence observation in situ in the transgenic rice plant that expressed *EG II::GFP* fusing gene and WT; Scale bar as 50 μm; **e** cellulases enzymatic activity assay in vitro in young seedlings of transgenic rice lines and wild type (WT) using 4-methylumbelliferyl-β-1,4-D-cellobioside (4-MUC) as substrate; Student's *t*-test between WT and transgenic lines as $^{**}p < 0.01$; increased percentage obtained by subtraction between transgenic line and WT divided by WT, with detailed data in Table S2

Cell wall fractionation and monosaccharide determination

Polysaccharides (cellulose and hemicellulose) of biomass samples were extracted as described by Li et al. (2017) and Peng et al. (2000). After removals of soluble sugar, lipids, starch and pectin, the remaining pellet was hydrolyzed by 2 M TFA at 120 °C for 1 h for determining monosaccharide composition of hemicelluloses using GC–MS. In addition, total lignin content was measured by two-step acid hydrolysis method according to Laboratory Analytical Procedure of the National Renewable Energy Laboratory. Acid-insoluble lignin was measured gravimetrically after correction for ash, and then acid-soluble lignin was detected using UV spectroscopy at 205 nm.

Detection of cellulose CrI and DP

Crystallinity index (CrI) of crystalline cellulose was detected using X-ray diffraction (XRD) method (Rigaku-D/MAX instrument, Uitima III, Japan) as previously described by Li et al. (2017). The DP of crude cellulose sample was measured using the viscosity method as described by Li et al. (2018a).

Fourier transform infrared (FTIR) spectra analysis

FTIR spectroscopy was performed to observe the structural constituents and chemical linkages in the representative raw rice stem samples. A Fourier transform infrared spectroscopy (FTIR, Nicolet iS50, Thermo Fisher, USA) was used to qualitatively monitor the samples at room temperature. The well-dried powders were positioned in the path of IR light and the spectra were recorded in absorption mode over 32 scans within a range of 4000–400 cm^{-1}.

Total protein extraction of crude cell walls

After 1% Tween-80 washing at room temperature for 48 h, total crude cell walls were centrifuged at

3000×*g* for 5 min. After centrifugation, the supernatants were collected for total protein assay. To reduce the interference of Tween-80, the collected protein samples were washed with fourfold volume ethanol and precipitated at room temperature. The dried protein samples were redissolved with distilled water and detected using Bradford method.

Analysis of adsorption average diameter of pores in crude cell walls

The remaining crude cell walls were suspended in 5.0 mL 0.2 M acetic acid-sodium acetate (pH 4.8) with and without 1% Tween-80 (v/v), respectively. Then the samples were shaken under 150 rpm at 50 °C for 48 h. The pellet samples were washed with distilled water until pH 7.0, dried at 50 °C until constant weight and then through 40 mesh. The specific surface area, accumulative volume and mean pore radius were measured using multipurpose apparatus Micrometrics ASAP 2460 (USA) (Brunauer et al. 1938; Liu et al. 2011; Li et al. 2018a).

Measurement of biomass enzymatic hydrolysis with 1% Tween-80 supply

Biomass powder samples were incubated with 6 mL (1.6 g/L) of a mixed-cellulases (purchased from Imperial Jade Bio-technology Co., Ltd.) dissolved in reaction buffer (0.2 M acetic acid-sodium acetate, pH 4.8) containing β-glucanase ($\geq 6 \times 10^4$ U), cellulase ($\geq$ 373 U) and xylanase ($\geq 6 \times 10^4$ U) supplied with or without 1% Tween-80. The sealed samples were shaken under 150 rpm for 48 h at 50 °C. Control samples containing 6 mL of reaction buffer were shaken for 48 h at 50 °C and were used for calculation of soluble sugars. The soluble sugars would be deducted from the total sugars after enzymatic hydrolysis. After centrifugation at 3000 *g* for 5 min, the supernatants were collected for pentose and hexose assay. All experimental analyses were performed with biological triplicate.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM JSM-5610/LV, Hitachi, Tokyo, Japan) was used for observations of the biomass residues. The biomass residues were collected after enzymatic hydrolysis with mixed-cellulases with 1% Tween-80 as described above, and the remaining residues were washed with distilled water until pH 7.0 as described by Li et al. (2018a).

Biomass pretreatment and bioethanol fermentation

The NaOH pretreatment and sequential enzymatic hydrolysis were performed as described previously by Fan et al. (2017). The ground biomass powder was incubated with 6 mL 1% NaOH (w/v), shaken at 150 r/min for 2 h at 50 °C, and centrifuged at 3000×*g* for 5 min. After pretreatments, the biomass residues and supernatants were neutralized to pH 4.8 using appropriate 60 μL of 50% H_2SO_4. The remaining residue was collected for enzymatic hydrolysis supplied with 1% Tween-80 as described above. After enzymatic hydrolysis for 48 h, the supernatants were collected for pentose and hexose assay. Samples with 6 mL distilled water only were shaken for 2 h at 50 °C as control. All experiments were performed in the biological triplicates.

All the supernatants after pretreatments and enzymatic hydrolysis were collected for the yeast fermentation and ethanol measured (Jin et al. 2016; Fan et al. 2017). *Saccharomyces cerevisiae* (Angel yeast Co., Ltd., Yichang, China) was used in all fermentation reactions. The yeast powder was suspended in 0.2 M phosphate buffer (pH 4.8) to achieve a final concentration of 0.5 g/L 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 following: 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. After cooling, distilled water was added up to 10 mL volume, and the absorbance was measured at 600 nm. Absolute ethanol was used as the standard. All experiments were performed in the biological triplicates.

Correlation coefficient calculation

Correlation coefficients were performed using spearman rank correlation analysis, and Student's *t*-test was conducted as described by Fan et al. (2017). The analysis used average values calculated from all original determination values.

Results

Identification of transgenic rice plants overexpressing *EG II* gene

In this study, the typical endo-β-1,4-glucanase (EG II) gene isolated from *Trichoderma reesei* was inserted into the transformation vectors constructed with the rice constitutive rbcS promoter, tobacco mosaic virus translational enhancer (Ω) and the tobacco pathogenesis-related protein signal (prla) specific for apoplast/-cell wall targeting (Fig. S1). Following *Agrobacterium*-mediated transformations into a japonica rice cultivar (Zhonghua 11, wild type-WT), we selected at least three independent homozygous transgenic rice lines (E1–E3), based on RT-PCR and Western blotting analyses (Fig. 1a, b). To examine the EG II localization, we extracted the proteins from total crude cell wall pellets of mature stem tissues after Tween-80 washing, and then found that transgenic lines showed detectable band that corresponded to EG II proteins via Western analysis (Fig. 1c). Furthermore, we selected transgenic rice plants that expressed *EG II::GFP* fusing gene, and observed strong GFP fluorescence in situ in plant cell walls (Fig. 1d). Hence, the data suggest that the endo-β-1,4-glucanase protein of *T. reesei* was mainly localized in plant cell walls of transgenic rice.

Furthermore, to test *T. reesei* cellulase activity of the selected transgenic rice lines, we performed in vitro assays by incubating standard substrate with total protein extracts obtained from transgenic young seedlings of rice (Fig. 1e). As a result, WT showed low cellulase activities (Fig. 1e; Table S2), which should be derived from plant endogenous cellulase enzymes. In comparison, three transgenic rice plants exhibited much higher cellulase activities by four to fivefold than those of WT, indicating that the overproduced exogenous EG II should have high enzymatic activity in transgenic rice plants. Since the *T. reesei* EG II enzyme was mainly deposited into plant cell wall with high enzymatic activity, it suggested that the EG II deposition may affect plant cell walls in the transgenic rice plants.

Distinct alteration of lignocellulose features in transgenic rice straw

Despite of detected enzymatic activity of *T. reesei* EG II in young rice leaves as described above, we observed that the homozygous transgenic rice plants exhibited a normal growth with mature biomass yields similar to the WT (data not shown). To confirm this finding, we examined three major wall polymer levels in mature straw tissues of two representative transgenic lines (E1, E3). Compared to the WT, both transgenic lines only showed slightly increased hemicelluloses levels, but remained similar cellulose and lignin contents (Table S3), consistent with their similar biomass yields. However, because the overproduced EG II had enzymatic activity for β-1,4-glucan hydrolysis as described above, this study further examined cellulose DP in the mature transgenic rice plants. As a result, two representative transgenic lines had significantly lower DP values than those of the WT by 5–10% at $p < 0.01$ levels (Fig. 2a). As cellulose DP and hemicelluloses level could affect cellulose crystallinity (Zhang et al. 2013;

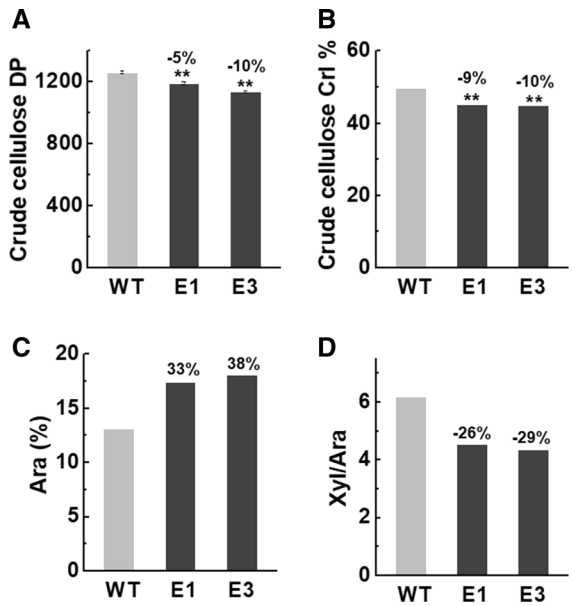

Fig. 2 Major wall polymer features alteration in transgenic rice plants: **a** degree polymerization (DP) of crude cellulose; **b** cellulose crystalline index (CrI); **c** arabinose (Ara) proportion of hemicelluloses; **d** ratio (Xyl/Ara) between xylose (Xyl) and arabinose of hemicellulose. Student's *t*-test between WT and transgenic lines as $^{**}p < 0.01$; increased percentage obtained by subtraction between transgenic line and WT divided by WT

Huang et al. 2015; Zahoor et al. 2017), this study used X-ray diffraction method (French 2014) to detect cellulose crystalline index (CrI) in the transgenic rice plants (Fig. S2). Compared to the WT, two transgenic lines had significantly reduced CrI values by 9–10% at $p < 0.01$ levels (Fig. 2b). Hence, the data suggested that the overproduced EG II should have enzymatic activity for β-1,4-glucan digestion, leading to significantly reduced cellulose DP and CrI in the transgenic rice plants.

With respect to slightly increased hemicelluloses levels in the transgenic plants, this study also determined monosaccharide composition of hemicelluloses. Despite the two transgenic lines did not show much altered xylose (Xyl) proportions, they had increased arabinose (Ara) contents, leading to the Xyl/Ara ratios reduced by 26% and 29%, compared to the WT (Fig. 2c, d; Table S4). In addition, although the transgenic rice plants exhibited cell wall compositions and total dry biomass yields similar to the WT, this study applied Fourier transform infrared (FTIR) spectroscopy to detect potential alterations of wall polymer interlinkages in the representative transgenic rice line. Consistently, both transgenic line (E3) and WT presented a similarity of all peaks for characteristic linkages among three major wall polymers (Fig. S3; Table S5), suggesting that the EG II deposition should not much alter wall network styles in the transgenic rice plants.

Raised biomass porosity under Tween-80 washing

As the overproduced EG II protein was mainly deposited into plant cell walls as described above, this study detected total protein levels extracted with 1% Tween-80 in biomass samples, and the representative transgenic line (E3) had much more extracted proteins than those of the WT by 126% at $p < 0.05$ level (Fig. 3a). Meanwhile, based on Western-blot analysis, this study detected the protein band corresponding to the *T. reesei* EG II enzyme only in the transgenic line (E3) rather than in the WT (Fig. 3b), indicating that the increased protein should be mainly derived from the *T. reesei* EG II enzyme deposited into the cell walls. To confirm this, we measured biomass porosity alteration from Tween-80 washing using nitrogen porosimetry method. Compared to the WT, the transgenic line showed much increased porosity including average diameter of pore and adsorption

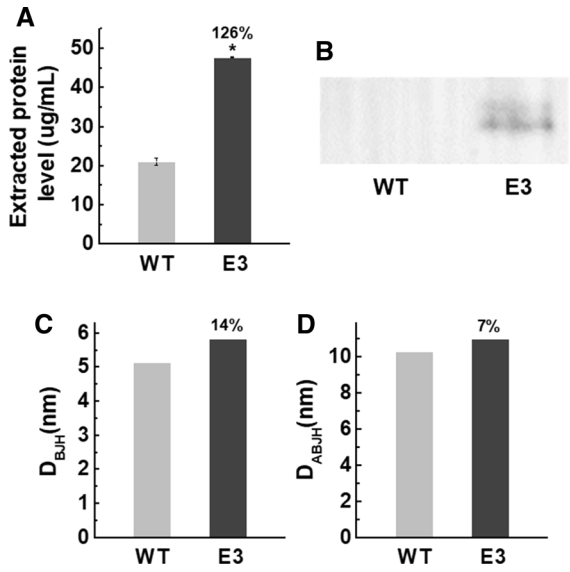

Fig. 3 Biomass porosity increased from Tween-80 washing in transgenic rice plant: **a** total protein extracted from 1% Tween-80 washing, data as mean ± SD (n = 3), Student's *t*-test between WT and transgenic lines as $*p < 0.05$, increased percentage obtained by subtraction between transgenic line (E3) and WT divided by WT; **b** western blot analysis of EG II proteins extracted from 1% Tween-80 washing as described in (**a**); **c**, **d** average diameter of pore and adsorption average diameter of pore after 1% Tween-80 washing, increased percentage obtained by subtraction between transgenic E3 and WT divided by WT. Student's *t*-test between WT and E3 as $*p < 0.05$

average diameter of pore (Fig. 3c, d). Therefore, despite the overproduced EG II enzyme was deposited into the cell walls, it was extractable for increased biomass porosity in the transgenic rice plants. On the other hand, it also indicated that the EG II enzyme was not tightly associated with wall polymers, consistent with the findings of unaltered polymer interlinkages in the transgenic rice plants.

Enhanced biomass enzymatic saccharification by Tween-80 supply

With respects to the biomass porosity increased from Tween-80 washing with the cell wall residues, this study detected biomass enzymatic saccharification (digestibility) by calculating hexose yields (% cellulose) released from a direct commercial mixed-cellulases hydrolysis of mature rice straw without any pretreatment (Table 1). Compared to the WT, two transgenic lines (E1, E3) exhibited significantly

Table 1 Direct biomass enzymatic saccharification in mature straw of transgenic rice plants

	Hexose yield (% cellulose)				Total sugars yield (% dry weight)			
	− Tween		+ Tween		− Tween		+ Tween	
WT	22.44 ± 0.48		23.54 ± 0.38		10.04 ± 0.17		10.24 ± 0.14	
E1	25.14 ± 0.68**	12%[a]	29.66 ± 1.15**	26%	11.14 ± 0.24**	11%	12.77 ± 0.26**	24%
E3	26.37 ± 0.22**	17%	30.6 ± 0.73**	30%	13.21 ± 0.09**	31%	14.33 ± 0.15**	39%

Hexose yields released from enzymatic hydrolysis of biomass residues after washing without and with 1% Tween-80; Data as mean ± SD (n = 3); Student's *t*-test between WT and transgenic lines as $^{**}p < 0.01$

[a]Percentage of the increased level between the mutants and wild type

enhanced hexose yields by 12% and 17% from enzymatic hydrolysis of crude straw biomass at $p < 0.01$ levels. Notably, while 1% Tween-80 was co-supplied into enzymatic hydrolysis, the transgenic lines had much higher hexose yields than those of the WT by 26% and 30%, respectively (Table 1). Furthermore, we observed that the representative transgenic line (E3) exhibited much rougher surfaces of the undigested lignocellulose residue than those of the WT under scanning electron microscopy (Fig. S4), consistent with previous observations in different biomass samples (Helle et al. 1993; Xu et al. 2012; Cao and Aita 2013; Li et al. 2017). Hence, despite Tween-80 is effective for enhancing biomass enzymatic saccharification by lessening cellulase enzyme adsorption with lignin (Jin et al. 2016), the data obtained in this study suggested that much higher hexose yields in the transgenic plants should be mainly due to their increased biomass porosity from Tween-80 washing, compared with the WT.

High bioethanol production under mild alkali pretreatment

Biomass saccharification is not only enhanced by biomass porosity, but also largely affected by wall polymer features under various chemical pretreatments (Xu et al. 2012; Huang et al. 2015; Jin et al. 2016), this study performed alkali pretreatment to enhance sequential biomass enzymatic saccharification for final bioethanol production from yeast fermentation in the transgenic rice plants (Table 2). Under mild alkali pretreatment (1% NaOH at 50 °C), the representative transgenic rice line (E3) showed significantly higher hexose yields by 19% than that of the WT released from enzymatic hydrolysis co-supplied with 1% Tween-80. As a consequence, the transgenic line exhibited much increased bioethanol yield by 38% obtained from yeast fermentation, compared with the WT (Table 2). Hence, even though under mild alkali pretreatment, the bioethanol yield reached to 20% (% dry matter) in the transgenic rice line, which should be much high bioethanol production compared with the previously reported ones in other grass species subjected to extreme physical and chemical pretreatments (Zhong et al. 2009; Li et al. 2013b; Jin et al. 2016). On the other hand, without any alkali pretreatment as control, the transgenic rice line showed significantly higher hexose and bioethanol yields than those of the WT, but they both remained low compared with the alkali pretreatment.

Lignocellulose feature impacts on biomass enzymatic saccharification

Correlation analysis has been well applied to explore lignocellulose feature impacts on biomass enzymatic saccharification in different biomass residues (Sheikh et al. 2013; Kim et al. 2014; Huang et al. 2015; Thomsen et al. 2015; Zahoor et al. 2017). Because lignocellulose features were largely altered in the transgenic rice plants, this study analyzed the correlation between major wall polymer features and hexose yields released from enzymatic hydrolysis (Fig. 4). As a result, cellulose DP and CrI values exhibited a significant negative correlation with hexose yields, with high R^2 values at 0.74 and 0.96 (Fig. 4a, b), consistent with previous findings of two cellulose features that negatively affect biomass enzymatic saccharification in different plant species

Table 2 Hexose and bioethanol yields in transgenic lines after 1% NaOH pretreatment

	Hexose yield (% cellulose)		Ethanol yield (% dry matter)		Sugar-ethanol conversion rate (%)	
WT	57.7 ± 0.42		14.87 ± 0.18		97.8 ± 4.12	
E1	64.11 ± 0.03*	11%[a]	17.93 ± 0.13**	21%	98.08 ± 1.57**	23%
E3	69.07 ± 0.93*	19%	20.46 ± 0.22**	38%	100 ± 2.95*	25%

[a]Percentage of the increased level between the mutants and wild type

Data as mean ± SD (n = 3); Student's t-test between WT and transgenic lines as *$p < 0.05$ or **$p < 0.01$

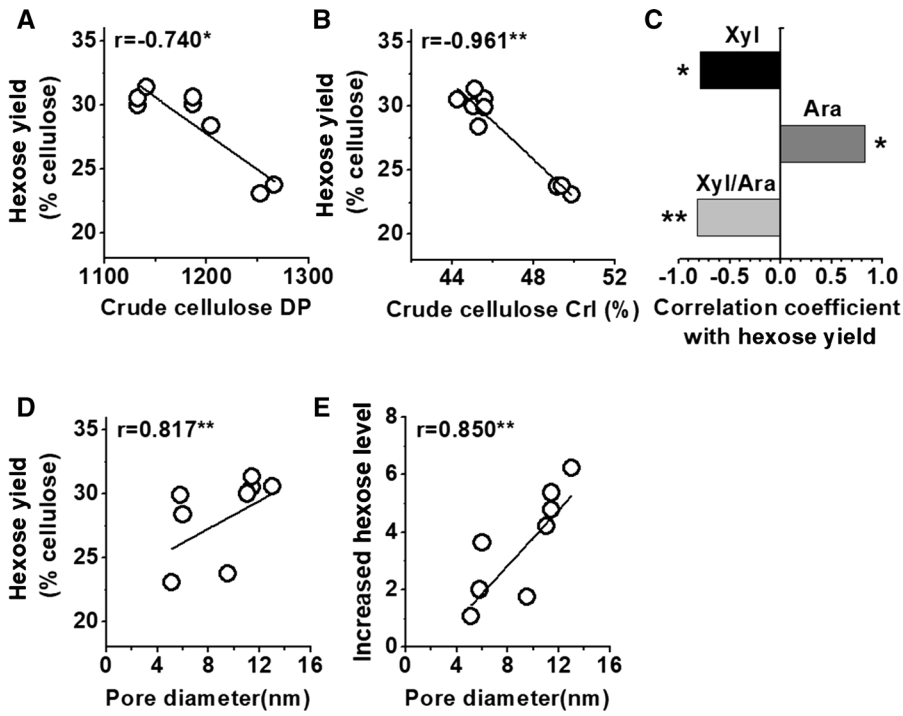

Fig. 4 Correlation analysis among major wall polymer features and hexose yields in all transgenic rice lines and WT: **a** correlations between cellulose DP values and hexose yields; **b** correlations between cellulose CrI values and hexose yields; **c** correlation coefficient between hemicellulose features and hexose yields; **d** correlation between average diameter of pore and hexose yields. **e** Correlation between average diameter of pore and increased hexose level by subtraction transgenic lines with WT; **d**, **e** **As significant positive correlation at $p < 0.01$ (n = 8). * and **As significant correlations at $p < 0.05$ and 0.01, respectively

(Baba et al. 2001; Wu et al. 2013; Zhang et al. 2013; Li et al. 2014a, b, 2015; Pei et al. 2016). Hence, the reduced cellulose DP and CrI should be the major factors for largely enhanced biomass saccharification in the transgenic rice plants. Meanwhile, the hemicellulose Ara level was positively correlated with hexose yields, whereas the Xyl level and Xyl/Ara ratios showed a negative correlation at $p < 0.05$ and 0.01 levels (Fig. 4c), in supporting of the previous findings that the Ara substitution degree of xylans (reverse Xyl/Ara ratio) could negatively affect biomass enzymatic saccharification in grass plants (Wu et al. 2013; Li et al. 2015). Expectedly, two parameters of biomass porosity were highly correlated with hexose yields at $p < 0.01$ levels, with the R^2 values at 0.82 and 0.85 (Fig. 4d, e), consistent with the previous findings that the biomass porosity is the general positive factor accounting for biomass enzymatic saccharification in the pretreated biomass residues (Sun et al. 2017; Li et al. 2018a). Taken all together, it has demonstrated

that the EG II deposition should lead to an integrated impact on biomass enzymatic saccharification in the transgenic rice plants including a positive factor of biomass porosity and two negative factors of cellulose features (DP, CrI) and hemicellulosic Xyl/Ara ratio.

Discussion

Cellulases production has a wide range of applications in many industries such as forest, food, textile, cloth, fuels and other chemicals. Selection of transgenic plants that overproduced cellulases enzymes has been attempted as a large-scale bioreactor with cost-effective and environment-benign advantages (Dai et al. 2000, 2005; Baba et al. 2001; Oraby et al. 2007; Mei et al. 2009; Fan and Yuan 2010; Jung et al. 2010; Chou et al. 2011). In this study, the endo-β-1,4-glucanase (EG II) of *T. reesei*, one of the major cellulases, could be overproduced in cell walls of transgenic rice plants, leading to increased enzymatic activities by fourfold to fivefold in vitro in young seedlings (Fig. 1e), but the mature transgenic rice plants show much lower cellulase activity (data not shown), probably due to ultrastructural alteration of cellulases within plant cell walls, suggesting that plant cell walls may not be the optimal compartment for overproducing heterologous EG II enzyme in transgenic crops.

Regardless of fungi EG II deposition into cell walls, little has been reported about EG II modification roles in plant cell wall formation and wall network construction. Endo-β-1,4-glucanases are found in both prokaryotic and eukaryotic organisms. In plants, the native endo-β-1,4-glucanases are thought to play an important role in cell wall modification and remodeling, in particular for lignocellulose crystallinity reduction (Kim et al. 1982). Nevertheless, the plant *KORRIGAN* gene, encoding an endo-β-1,4-glucanase, has been characterized to associate with cellulose synthase complexes for cellulose production, and commercial fungal endo-cellulase could enhance cellulose biosynthesis in vitro, probably by its cleavage of a primer for promoting β-1,4-glucan elongation (Peng et al. 2002). Very surprisingly, this study has detected that the overproduced EG II could significantly reduce cellulose DP in the transgenic rice plants (Fig. 2), probably due to the EG II having enzymatic activity for partial β-1,4-glucan digestion in the amorphous regions of cellulose microfibrils (Lynd et al. 2002; Zhang and Lynd 2004; Klose et al. 2015; Li et al. 2018b). However, it remains to determine if the EG II also involves in cellulose biosynthesis in the future studies. Meanwhile, this study has determined significantly reduced cellulose CrI values in the transgenic rice plants, which may be due to three major causes: (1) The reduced cellulose DP positively affects cellulose CrI (Zhang et al. 2013; Huang et al. 2015; Zahoor et al. 2017); (2) The EG II enzyme may interact with cellulose microfibers via hydrogen bonds for reduced cellulose crystallinity; (3) Relatively increased hemicellulose and Ara may associate with cellulose microfibers via hydrogen bonds as previously reported in grass plants (Wu et al. 2013; Zhang et al. 2013; Li et al. 2015, 2018b; Sun et al. 2017).

In our recent study, the fungi β-glucosidase deposition into plant cell wall could not only largely reduce cellulose features (DP, CrI), but also significantly alter cell wall composition and non-cellulosic polymers features including reduced cellulose and lignin by 4% and 6%, much increased hemicelluloses by 14% at $p < 0.05$ and 0.01 levels, and increased two monomers proportions of lignin (Li et al. 2018a), which should be subjective to the β-glucosidase having enzymatic activity for multiple and non-specific substrates of plant cell walls. By comparison, this study only examined slightly increased hemicellulose level by 3–4% in the transgenic rice plants, but their cellulose and lignin contents were not significantly altered (Table S3), indicating that the EG II should have the enzymatic activity specific for the substrate of β-1,4-glucans in the amorphous regions of cellulose microfibrils. Furthermore, based on our previous findings (Xu et al. 2012; Wu et al. 2013; Li et al. 2015; Sun et al. 2017; Zahoor et al. 2017), the EG II deposition may consequently regulate hemicellulose and Ara levels to associate with the amorphous regions of cellulose microfibrils for maintaining plant cell wall integrity in the transgenic rice plants. Taken together, it suggests that the EG II deposition should lead to a slight post-modification specific for the amorphous regions of cellulose microfibrils, which may also interpret why the transgenic rice plants showed a normal plant growth, similar biomass yield and unaltered wall polymer interlinkage compared to the WT.

As plant cell walls fundamentally determine biomass recalcitrance, genetic modification of plant cell walls has been considered as a powerful solution

for enhancing biomass enzymatic saccharification and bioethanol productivity in transgenic crops (Li et al. 2015, 2017; Fan et al. 2017). Regardless of slight lignocellulose modification in the EG II-overproduced transgenic rice plants, this study has examined that the reduced cellulose features (DP, CrI) and hemicellulose Xyl/Ara could positively affect biomass enzymatic saccharification under mild alkali pretreatment in the transgenic rice plants (Fig. 4). Furthermore, it has been well characterized that biomass porosity could positively affect biomass enzymatic digestibility by providing space for cellulases loading and access to cellulose microfibril surface (Kim et al. 1982; Sun et al. 2017). Although Tween-80 could effectively extract *T. reesei* cellulases proteins (Fig. 3), the 1% NaOH pretreatment should be also able to remove partial cellulases for increased biomass porosity in the transgenic rice plants. More importantly, our recent report has indicated that the biomass porosity is distinctively affected by major wall polymer features (Sun et al. 2017; Li et al. 2018a). Hence, both significantly increased biomass porosity and distinctively altered cellulose and hemicellulosic features should play accumulatively enhancement roles in biomass enzymatic saccharification, which may somehow interpret why the highest bioethanol yield was obtained in the transgenic rice plants even though mild pretreatment was performed in this work. Hence, this study demonstrates that overproduction of *T. reesei* EG II into plant cell walls should be a powerful approach for cost-effective bioethanol production in transgenic crops.

Conclusion

Using transgenic rice plants that overproduced fungi EG II enzyme into plant cell walls, this study examined that the EG II had enzymatic activity specific for minor modification of cellulose microfibrils, leading to either significantly reduced cellulose DP and CrI values and hemiellulosic Xyl/Ara ratio or much increased biomass porosity in the mature transgenic rice straw. Due to the lignocellulose modification, the transgenic rice plants exhibited largely enhanced hexose yields released from enzymatic hydrolysis, leading to high bioethanol yield under mild alkali pretreatment. Hence, this study has demonstrated a slight cellulose modification for high biomass saccharification and bioethanol production in bioenergy crops.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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