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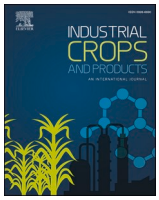

Integrated genetic and chemical modification with rice straw for maximum bioethanol production

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ABSTRACT

Crop straw provides huge lignocellulose residues that are transformable for bioethanol production and biochemicals. However, lignocellulose recalcitrance fundamentally causes a costly biomass process that is unapplicable for bioethanol conversion at industrial level with potential waste release. Here, this study selected the transgenic rice (*Oryza sativa L.*) lines that overexpressed *AtCesA6*, a typical gene involved in cellulose biosynthesis of primary cell walls in *Arabidopsis* (*Arabidopsis thaliana L Heynh.*). This work then examined significantly improved lignocellulose substrates along with much soluble sugars deposition in the transgenic rice straws. By performing green-style pretreatments with mature rice straws using two recyclable and relatively low-cost alkali chemicals ($NH_3{\cdot}H_2O$, CaO) and liquid hot water, this work determined almost complete enzymatic saccharification in the transgenic rice lines. Notably, under two optimal alkali pretreatments, the transgenic rice samples could achieve either bioethanol yields of more than 20 % (% dry matter) or bioethanol concentrations at 18.3 g/L and 19.1 g/L from one-pot relatively high solid loading saccharification, being much higher than those of wild type (*Nipponbare*). Furthermore, this study examined how the lignocellulose recalcitrance was significantly reduced for remarkably raised enzymatic saccharification in the transgenic rice straws. It also explicated that the maximum bioethanol yield obtained in the transgenic straws should mainly be subjective to near-complete enzymatic saccharification and much directly-fermentable soluble sugars accumulation. Therefore, this study has provided a novel strategy for high bioethanol production by integrating genetically-improved lignocellulose substrates with optimal one-pot-process technology in bioenergy crops.

1. Introduction

Cellulose is the most plentiful substance on the earth, and cellulosic ethanol has been considered as a favorable secondary generation of biofuel for partial alternative of petrol oils (Wang et al., 2021). However, the natural recalcitrance of lignocellulose could not avoid a high-cost process impending unexpected waste deliverance into the environment (Li et al., 2021; Yang et al., 2020). Hence, it is important to find out an integrated strategy for reduced lignocellulose recalcitrance and increased bioethanol production.

Biochemical conversion of lignocellulose for bioethanol production principally requires three main processes: physical and chemical pretreatment for effectively reduced lignocellulose recalcitrance, followed with enzymatic hydrolysis for high concentration of fermentable sugars and yeast fermentation towards high yield of bioethanol (Xu et al., 2021, 2020; Li et al., 2018). Over the past, several chemical pretreatments have been employed to overcome the biomass recalcitrance (Yao et al., 2015; Karimi and Taherzadeh, 2016). In particular, CaO as a recyclable

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alkali chemical, has been applied for a relatively low-cost and green-style pretreatment (Lv et al., 2020; Xu et al., 2010). Furthermore, ammonia is a volatile alkali liquid with advantage of chemical recovery (Ai et al., 2019; Zhao et al., 2020). In addition, the ammonia pretreatment at room temperature can lead to 73.5 % lignin removal and almost 97 % glucan conversion into fermentable sugars (Kim and Lee, 2005). In comparison, liquid hot water (LHW) is another green-style pretreatment applied in several biomass residues, due to non-chemical application (Hu et al., 2018d). Therefore, mild and green-style pretreatments are increasingly considered for the desirable lignocellulose substrates that are of lessened recalcitrance.

High solids loading saccharification is a relatively low cost of biomass processing, due to relatively less energy and water consumption and reduced disposal cost (Koppram et al., 2014). As alkaline pretreatments can selectively extract lignin and retain hexose and pentose, genetically-engineered yeast strains are attempted for co-fermentation of both sugars to maximize ethanol production (Prabakar et al., 2018a; Rawat et al., 2013; Prabakar et al., 2018b). However, because yeast strain is of the disadvantage for tolerance to high concentrations of sugars, salt, and ethanol (Cui et al., 2014), it remains to explore the optimal fermentation condition to raise bioethanol production by high solids loading along with engineered yeast strain (Raj and Krishnan, 2019).

It has been characterized that deposition and interlinkage of three major wall polymers (cellulose, hemicellulose, lignin) contribute to biomass recalcitrance (Wu et al., 2020; Li et al., 2019). To improve the recalcitrance, genetic engineering of lignocellulose substrate has been achieved in bioenergy crops (Fan et al., 2018; Li et al., 2018). As cellulose is the major polymer of plant cell wall, genetic engineering of cellulose biosynthesis has been attempted by altering cellulose crystallinity and polymerization (Li et al., 2015; Wu et al., 2019). More importantly, because the CESA6-like proteins are involved in the primary wall cellulose synthesis in *Arabidopsis*, overexpression of any of the three *AtCesA6*-like genes (*AtCesA2*, *AtCesA5,* or *AtCesA6*) can increase cellulose production in *Arabidopsis* by enhancing cell growth and secondary cell wall deposition (Hu et al., 2018b). However, it remains to test if overexpression of *AtCesA6*-like genes could significantly improve lignocellulose recalcitrance without any penalty of plant growth and biomass yield in other bioenergy crops.

Rice is a major agricultural crop worldwide and provides substantial biomass residues (Li et al., 2018; Huang et al., 2019). This study generated the transgenic rice lines that overexpressed *AtCesA6,* and determined much changed cell wall composition and cellulose features in the transgenic rice straws. This study then performed three green-style pretreatments with rice straws for near-complete lignocellulose enzymatic saccharification by using $NH_3{\cdot}H_2O$, CaO and LHW under various conditions. Finally, this study combined soluble sugars and lignocellulose hydrates with engineered yeast strain to achieve maximum bioethanol yield in the transgenic rice straws, providing an applicable strategy for bioethanol production in rice and beyond.

2. Material and methods

2.1. Selection of transgenic rice straw samples

Full *AtCesA6* coding cDNAs were constructed with the binary vector pD1301s plasmid as described (Hu et al., 2018b). *Agrobacterium tumefaciens* strain (EHA105) was applied for the *AtCesA6* gene transformation into rice cultivar (NPB; Lin and Zhang, 2005). Selection of transgenic rice lines were completed as previously described (Fan et al., 2017). T1 transgenic rice lines were initially identified by growing seedlings with 1/2 MS medium added with 50 mg/L hygromycin, and confirmed by RT-PCR and qRT-PCR analyses. More than two independent hygromycin-resistant lines were propagated to homozygous generations. All rice samples were collected in HZAU experimental station from 2016 to 2020.The dried and ground powders of the mature rice straws (without leaves) were passed by a 40-mesh screen and stored in a dry container until in use.

2.2. RNA extraction and qRT-PCR measurement

Trizol reagent (Invitrogen, Carlsbad, CA) was used for total RNA extraction and the qRT-PCR was run at independent triplicates as described (Huang et al., 2019). The gene expression levels were normalized relative to the UBQ expression level. The primers applied in this study were described in Table S1.

2.3. Wall polymer extraction and assay

The wall polymer extraction was completed as previously described (Peng et al., 2000; Gao et al., 2021; Madadi et al., 2021a, b). The anthrone /H_2SO_4 method (Lv et al., 2020; Zhang et al., 2020) and orcinol/HCl method were respectively applied to measure hexoses and pentose of the hemicellulose fraction or hexose of cellulose fraction. Crystalline cellulose sample was obtained by extracting (0.1000 g) biomass sample with 5 mL acetic acid–nitric acid–water (8: 1: 2, v/v/v) in a boiling water bath for 1 h. The experimental analyses were completed from independent triplicate.

2.4. Soluble sugars analysis

The biomass sample (0.3000 g) was incubated with 8 mL distilled water and shaken at 150 rpm for 24 h at 50 °C. After centrifugation at 3000 *g* for 5 min, the supernatant was collected to detect the pentoses and hexoses of soluble sugars, respectively (Wu et al., 2019).

2.5. Biomass pretreatment and enzymatic hydrolysis

Biomass pretreatments and enzymatic hydrolysis were generally completed as previously described (Wu et al., 2019). In terms of liquid hot water (LHW) pretreatment, the well-mixed biomass powder (0.3000 g) was loaded with steel capsule under 12.5 % solid loading, and treated at 200 °C under 15 rpm shaking for a time course. For CaO pretreatment (Lv et al., 2020), various concentrations of CaO (0%, 5%, 10 %, 15 % w/w) were added into biomass samples and treated at 50 °C for 48 h, with the solid/liquid ratio at 5%. For aqueous ammonia pretreatment (Zhao et al., 2020; Wu et al., 2019; Kim et al., 2008), the aqueous ammonia chemicals at different concentrations (0.0 %,10.0 %, 12.5 %, 15.0 % v/v) were supplemented with biomass samples under shaken at 50 °C for 48 h.

After washing with the reaction buffer (0.2 M acetic acid–sodium acetate, pH 4.8), the pretreated biomass sample was supplemented with 0.012 g mixed-cellulases (containing cellulases at 10.60 FPU g^{-1} biomass and xylanase at 6.72 U g^{-1} biomass from Imperial Jade Biotechnology Co., Ltd) with 1% Tween-80 at 5% solid loading (Huang et al., 2019). The enzymatic hydrolysis reaction (6 ml) was completed under 150 rpm shaking for 48 h at 50 °C, and the supernatant was harvested to determine pentoses and hexoses at independent triplicate.

2.6. Yeast fermentation and ethanol measurement

Engineered *Saccharomyces cerevisiae* strain (CBC1, provided by Zhu et al., 2020) was applied for yeast fermentation, and ethanol content was measured by dichromate oxidation method at independent triplicate as described (Li et al., 2018). The yeast fermentation was conducted under 30 °C at pH 4.8 for 48 h, and the fermentation liquid was then distilled at 90 °C for 10 min. After cooling at room temperature, the absorbance was measured at 600 nm, and the initial OD_{600} for the fermentation was 1.0. Meanwhile, absolute ethanol (99.9 %) was used as the standard.

2.7. Characterization of cellulose features

The crystalline index (CrI) detection of biomass sample was completed by X-ray diffraction (XRD) method using Rigaku-D/MAX instrument (Ultima III, Japan) as described (Fan et al., 2017). The degree of polymerization (DP) of crude cellulose sample was detected as described (Wang et al., 2016). Congo red (CR) staining was performed to estimate cellulose accessibility at independent triplicate as described (Wiman et al., 2012).

2.8. Fourier transform infrared spectroscopy scanning

FTIR spectroscopy was applied for observation of chemical linkages in biomass samples by using PerkinElmer spectrophotometer (NEXUS 470, Thermo Fisher Scientific, Waltham, MA, USA) as described (Cheng et al., 2019).

2.9. Microscopic observation

Scanning electron microscope (SEM JSM-5610/LV, Hitachi, Tokyo, Japan) was applied to observe biomass surfaces and representative samples were imaged as described (Lv et al., 2020). Transmission electron microscopy was applied for views of cell wall structures as described (Hu et al., 2018b; Zhang et al., 2020).

2.10. Monosaccharide composition analysis

Monosaccharide detection of biomass sample was completed by GC/MS running (SHIMADZU GCMS-QP2010 Plus) as described (Fan et al., 2018). The analytical conditions: Restek Rxi-5 ms, 30 m × 0.25 mm ID × 0.25 um df column; carrier gas: He; injection port: 250 °C; interface: 250 °C; Injection volume: 1.0 μL. The temperature program: from 170 °C (held for 12 min) to 220 °C (held for 8 min) at 3 °C/min. Ion source temperature: 200 °C, ACQ Mode: SIM. The mass spectrometer was handled in the EI mode with ionization energy of 70 eV. Mass spectra were acquired with full scans based on the temperature program from 50 to 500 *m/z* in 0.45 s.

2.11. Lignin and three monolignols assay

Total lignin content was evaluated by two-step acid hydrolysis method as described (Alam et al., 2019; Deng et al., 2020). The acid-soluble lignin was solubilized during the hydrolysis process, and measured by UV spectroscopy at 205 nm. The remaining residues were loaded in a muffle furnace at 575 ± 25 °C for 4 h for the acid-insoluble lignin assay. Three monomers (H, G, S) of lignin were determined as previously described (Li et al., 2014). A Kromat Universil C18 column (4.6 mm × 250 mm, 5 μm) was used for HPLC analysis on LC-20A (SHIMADZU) HPLC with a UV-detector at 280 nm. Lignin level was determined in independent triplicate.

2.12. Statistical analysis

The SPSS statistical software was applied in terms of data analysis. Statistical analysis was implemented by Student's t tests as $*P < 0.05$ and $**P < 0.01$.

3. Results and discussion

3.1. Altered lignocellulose composition and raised soluble sugars accumulation in transgenic rice straw

In previous studies, it has been characterized that overexpression of *AtCesA6,* a typical cellulose synthesis gene of primary cell wall in *Arabidopsis*, can specifically enhance cellulose biosynthesis and biomass production in transgenic *Arabidopsis* (Hu et al., 2018a, b, c). Because the phylogenetic analysis showed a high similarity between the AtCESA6 and the OsCESA3, A5, A6 among rice OsCESAs members (Fig. S1A), this study generated the transgenic rice lines that overexpressed *AtCesA6* driven by 35S promoter in wild type (WT, Nipponbare/NPB) background (Fig. S1B; Table S1), and selected their homozygous transgenic progeny, which were verified by genetic analyses (Fig. S1C–F).

In two-years field experiments, this study detected slightly reduced plant heights in two independent transgenic lines (*AtA6-1, -2*) relative to the WT(NPB), but they had similar biomass and grain yields (Figs. 1A; S2). Notably, both transgenic lines were of reduced lodging indexes accountable for significantly raised lodging resistance (Fig. S2). Accordingly, this study observed a slightly reduced plant cell wall thickness in the transgenic lines (Fig. 1B–D), being different from previous report about increased cell wall widths and biomass yields in the transgenic *Arabidopsis* plants of overexpressing *AtCesA6* (Hu et al., 2018b). However, this finding was consistent with observation of the transgenic Aspen (*Populus alba L.*) that overexpressed *PtdCesA8* (Joshi et al., 2011), suggesting that micro-RNA silence may occur in the transgenic rice lines.

Furthermore, this study determined significantly reduced cellulose and hemicellulose levels by 29 % and 28 % with increased pectin content up to four folds in two transgenic lines relative to the WT, but both transgenic and WT samples had a similar lignin content (Fig. 1E). Accordingly, this study examined remarkably raised soluble sugars (pentoses, hexoses) accumulation in the mature straws of two transgenic rice straws (Fig. 1F), which was assumed from the extra monosaccharides that are not assimilated for wall polysaccharides (cellulose, hemicellulose) syntheses in the transgenic rice lines. Therefore, the results indicated that transgenic rice straws should be of significantly improved carbon assimilation (Fan et al., 2020) for much soluble sugars accumulation (Wu et al., 2019; Xu et al., 2013), which could be directly fermentable for bioethanol production. In addition, the cellulose defective phenotype was probably induced by co-suppression of the transgene (*AtCesA6*) and native genes (*OsCesA6*-like genes), resulting in a post-transcriptional gene silencing event that may occur in the transgenic rice lines (Tan et al., 2015; Joshi et al., 2011).

3.2. Remarkably raised biomass saccharification under optimal green-style pretreatments

As liquid and solid alkali chemicals ($NH_3{\cdot}H_2O$, CaO) are recyclable and relatively low-priced, they have been applied as green-style pretreatments (Ai et al., 2019; Chandel et al., 2013; Zhao et al., 2020; Wu et al., 2019; Zahoor et al., 2017). This study performed both alkali pretreatments with rice straws at 50 °C for 24 h under different chemical concentrations, and determined enzymatic saccharification of pretreated biomass residues using previously-established approach (Fig. 2). As a comparison with the WT, two transgenic samples exhibited significantly higher hexoses yields (% cellulose) obtained from enzymatic hydrolysis after pretreatments were conducted with $NH_3{\cdot}H_2O$ and CaO at series of concentrations (Fig. 2A, B). Notably, the transgenic rice straws were of near-complete enzymatic saccharification with hexoses yields of 100 % under 10 % $NH_3{\cdot}H_2O$ and 10 % CaO pretreatments, whereas the WT had the hexoses yields of less than 85 % (% cellulose) even though under higher alkali concentrations (15 % $NH_3{\cdot}H_2O$, 15 % CaO). In terms of the raw straw materials that were not applied for any pretreatment, the transgenic rice lines even remained significantly higher enzymatic saccharification than that of the WT with the increased rates of hexoses yields by 60 %.

According to previously-established conditions (Wu et al., 2019), this work conducted liquid hot water (LHW) pretreatments with rice straws. Similarly, two transgenic rice samples showed consistently increased hexoses yields during a time-course incubation (Fig. 2C). Particularly treated with 10 min LHW, two transgenic rice samples respectively achieved the hexoses yields of 95 % and 97 % (% cellulose), while the WT had the hexoses yield of less than 80 % even though under

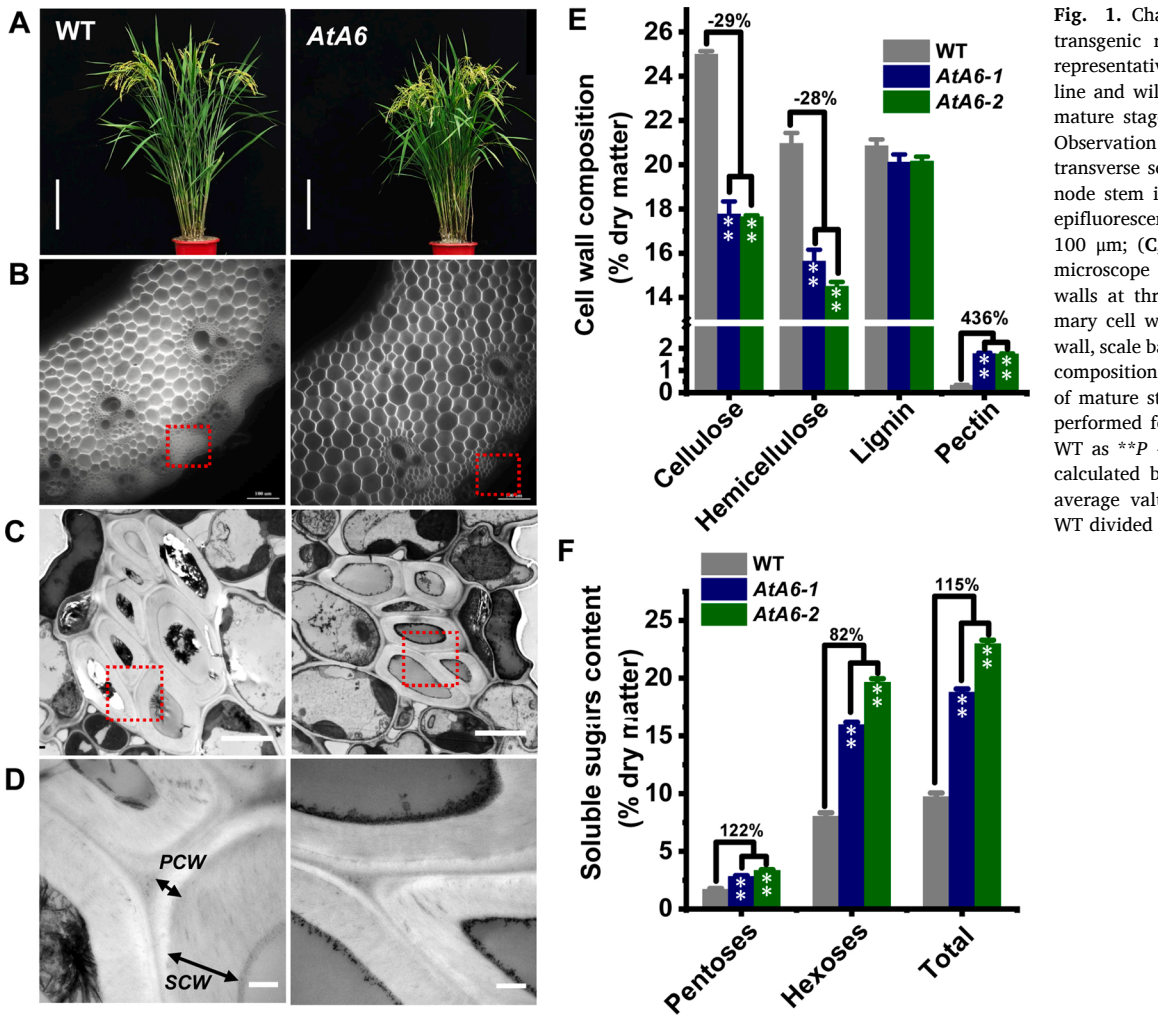

Fig. 1. Characterization of *AtCesA6* transgenic rice lines. (**A**) Pictures of representative *AtCesA6* transgenic rice line and wild type (WT/*Nipponbare*) at mature stage, scale bar as 25 cm; (**B**) Observation of calcofluor staining at transverse sections of the second internode stem in the heading stage under epifluorescence microscopy, scale bar as 100 μm; (**C**, **D**) Transmission electron microscope observation of plant cell walls at three-leaf stage, PCW as primary cell wall, SCW as secondary cell wall, scale bar as 0.5 um; (**E**, **F**) Cell wall composition and soluble sugars content of mature stem tissues. Student's *t*-test performed for the transgenic lines and WT as $^{**}P < 0.01$ and the percentage calculated by subtraction between the average value of transgenic lines and WT divided by WT.

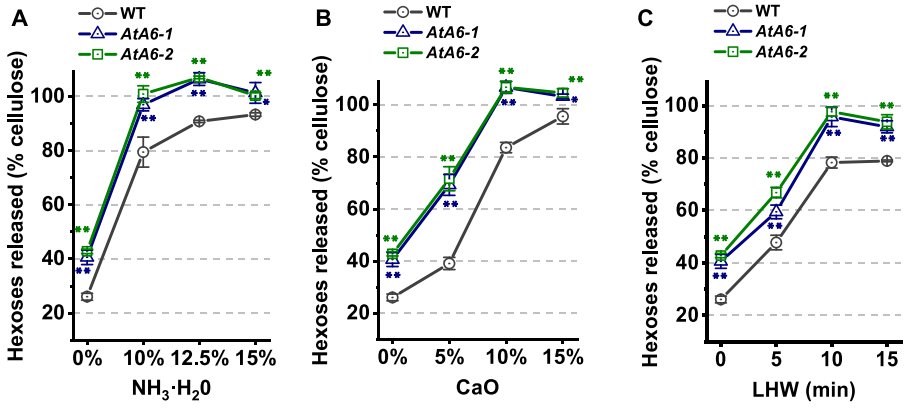

Fig. 2. Measurement of hexose yields (% cellulose) obtained from enzymatic hydrolyses after three pretreatments were completed with mature rice straws in *AtCesA6* transgenic rice lines and WT. (**A**, **B**) $NH_3{\cdot}H_2O$ and CaO pretreatments at different concentrations; (**C**) LHW pretreatment under a time course. Data as mean ± SD ($n \geq 3$). * and ** indicated significant difference between transgenic lines and WT by *t*-test as $P < 0.05$ and 0.01, respectively.

a longer incubation. Therefore, this study demonstrated that the transgenic rice straws are of remarkably enhanced biomass saccharification under mild pretreatments performed at relatively low temperature and short incubation time, which should be mainly subjective to much improved lignocellulose recalcitrance as described below.

3.3. Integrated enhancement of bioethanol production by engineered yeast strain

As the transgenic rice straws accumulated much soluble sugars and had high lignocellulose enzymatic saccharification under three green-style pretreatments, this study performed an integrated ethanol fermentation by using engineered yeast strain enabled to consume all

xylose and hexoses obtained from soluble sugars, alkali pretreatments and enzymatic hydrolyses (Fig. 3). Under three optimal green-style pretreatments, two transgenic rice straw samples showed consistently higher ethanol yields (% dry matter) than those of the WT, with increased rates of bioethanol yields by 21 %, 20 %, and 55 %, respectively. Despite the optimal LHW pretreatment led to much higher increased rates (55 %), it could achieve the bioethanol yields at less than 13 % (% dry matter), being much lower than those of two optimal alkali pretreatments with the bioethanol yields at more than 20 % in the transgenic rice samples. This may be mainly due to the LHW pretreatment that releases much more toxic compounds enabled to inhibit yeast fermentation (Alam et al., 2019; Yu et al., 2010).

Further statistical analysis indicated that the transgenic rice samples had significantly higher ethanol yields than those of the WT at $P < 0.01$ level only from yeast fermentation with soluble sugars, rather than with the ones from pretreatments and enzymatic hydrolyses. Although the transgenic rice straws had higher hexoses yields (% cellulose) than those of the WT under three optimal pretreatments as described above, they contained relatively lower cellulose levels on the basis of dry matter, which should cause a similar bioethanol yield (% dry matter) between the transgenic rice samples and WT. Hence, the transgenic rice straw samples enabled to produce higher bioethanol yields under optimal alkali pretreatments, mainly due to much soluble sugars accumulation and efficient co-fermentation with xylose and hexoses by engineered yeast strain.

3.4. One-pot high solids loading saccharification for raised bioethanol productivity

Since biomass enzymatic saccharification and ethanol production were conducted under low dosage of rice straw samples (5% solid-liquid ratio) as described above, this study further used high dosage of rice straws to conduct one-pot biomass process under two optimal alkali pretreatments (Fig. 4). Upon 12.5 % $NH_3{\cdot}H_2O$ pretreatments with high dosages of rice straws at 12 % solid-liquid ratio, two transgenic rice samples respectively produced bioethanol products at 18.3 g/L and 19.1 g/L, whereas the WT only reached to 10.8 g/L (Fig. 4A, B). Furthermore, pretreated with 10 % CaO at 20 % solid-liquid ratio, two transgenic rice samples could even achieve higher concentrations of bioethanol at 28.0 g/L and 29.4 g/L, but the WT remained low at 20 g/L (Fig. 4C, D). Therefore, loading of high dosages of biomass residues has demonstrated consistently higher bioethanol productivity in the transgenic rice straws. In addition, the one-pot biomass process technology has also provided an applicable approach for cellulosic ethanol productivity in bioenergy crops.

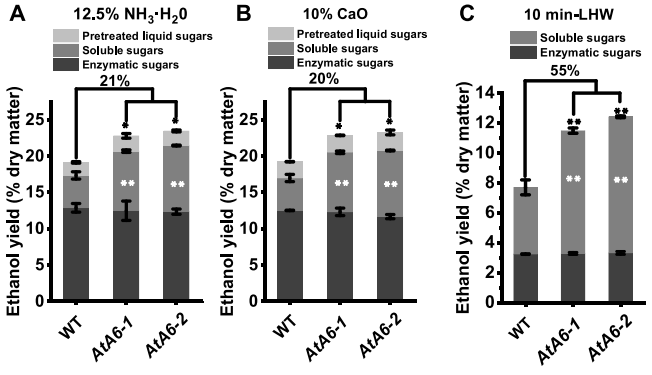

Fig. 3. Detection of bioethanol yields (% dry matter) achieved in transgenic rice lines and WT by engineered yeast strain co-fermentation with xylose and hexoses from soluble sugars, pretreated supernatants and enzymatic hydrolyses of pretreated residues. (**A**, **B**) $NH_3{\cdot}H_2O$ and CaO pretreatments under different concentrations; (**C**) LHW pretreatment under a time course. Data as mean ± SD ($n \geq 3$). ** as significant difference between transgenic lines and WT by *t*-test as $P < 0.01$.

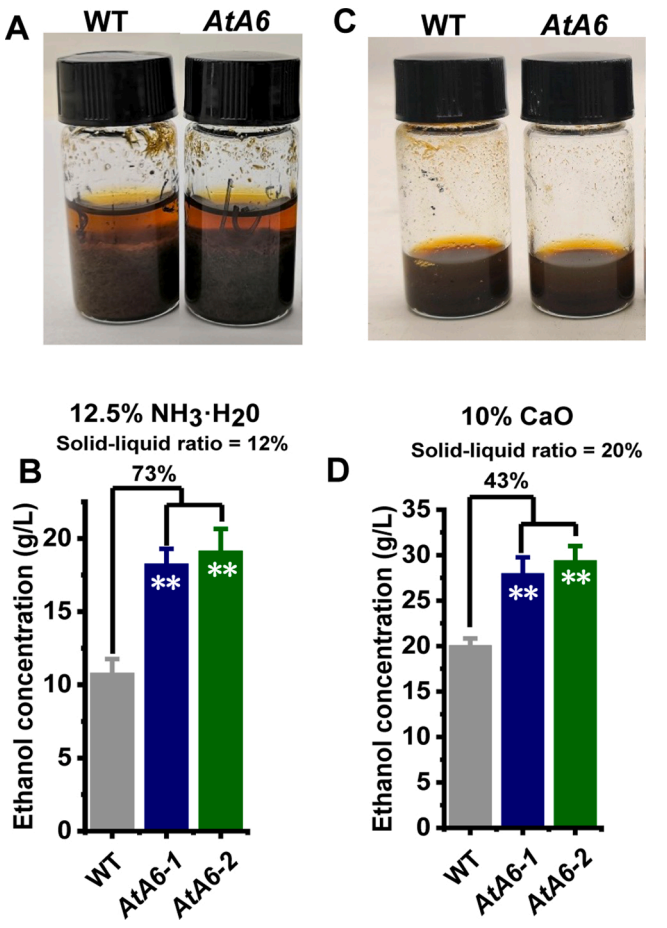

Fig. 4. Detection of bioethanol products (g/L) achieved in *AtCesA6* transgenic rice lines and WT by engineered yeast strain co-fermentation with xylose and hexoses from one-pot high solids loading saccharification under two optimal alkali pretreatments. (**A**, **B**) $NH_3{\cdot}H_2O$ pretreatment; (**C**, **D**) CaO pretreatment. Data as mean ± SD ($n \geq 3$). ** indicated significant difference between transgenic lines and WT by *t*-test as $P < 0.01$, and the percentage calculated by subtraction between the average value of transgenic lines and WT divided by WT.

3.5. Distinctively raised cellulose accessibility by two optimal alkali pretreatments

To understand how two optimal alkali pretreatments could largely enhance biomass enzymatic saccharification, this study initially observed surfaces of biomass residues in the transgenic rice straws (Fig. 5A). In general, two alkali pretreatments could obviously destruct lignocellulose residues in both transgenic lines and WT, as a comparison with the raw materials (control/without pretreatment). In particular, compared with the WT, the transgenic rice samples were of much rougher surfaces after two optimal pretreatments were conducted, which should more increase cellulose accessibility for relatively higher biomass saccharification examined in the transgenic lines. To confirm this finding, this study detected cellulose accessibility using previously-established Congo Red staining method (Alam et al., 2019; Deng et al., 2020). Compared with the WT, the transgenic rice samples were of significantly raised cellulose accessibility by 64 % and 80 % at $P < 0.01$ levels (Fig. 5B). Even though with non-pretreatment, the transgenic rice line had relatively higher cellulose accessibility than that of the WT by 26 %, which confirmed an improved lignocellulose recalcitrance in the transgenic sample.

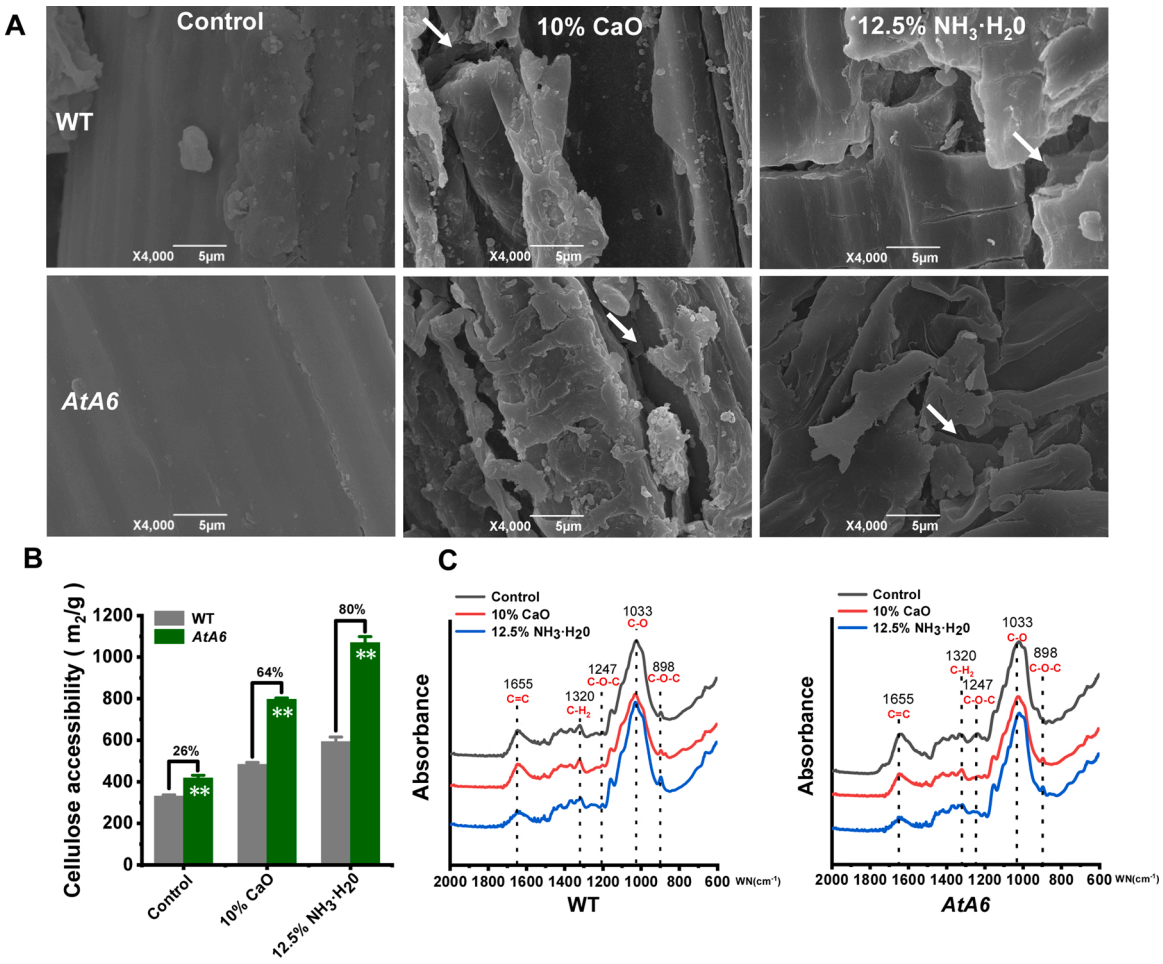

Fig. 5. Biomass characterization in *AtCesA6* transgenic rice lines and WT under two optimal alkali pretreatments (control/without pretreatment). (**A**) Observation of biomass surface under SEM, scale bars as 5 μm and allow highlighted the rough zone; (**B**) Detection of cellulose accessibility with Congo Red staining, data as mean ± SD ($n \geq 3$). ** indicated significant difference between transgenic lines and WT by *t*-test as $P < 0.01$, and the percentage calculated by subtraction between the average value of transgenic lines and WT divided by WT; (**C**) Fourier transform infrared spectroscopic profiling along with characteristic peaks corresponding for chemical bonds assigned in Table S2.

Using fourier transform infrared spectroscopy, this study further examined the alteration of wall polymer interlinkages caused by the optimal alkali pretreatments performed in this study (Fig. 5C; Table S2). As a comparison, the absorption peaks located at 1247 cm (Wu et al., 2019) showed obviously decreased intensities for the C-O-C bonds between hemicelluloses and lignin in the pretreated residues of transgenic rice samples, but change was little found in the WT. Similarly, other bonds (C=C stretching, C-H_2, C-O, C-H vibration) were more altered in the pretreated transgenic samples relative to the WT (Alam et al., 2020; Faix, 1991). In addition, the absorption peaks located at 1655 cm assigned to the C=C stretching bonds, showed much decreased intensities in the alkali-pretreated residues of both transgenic sample and WT, especially from the $NH_3{\cdot}H_2O$ pretreatment. Taken together, the optimal alkali pretreatments should effectively extract wall polymers to alter wall networks for much increased cellulose accessibility in the transgenic rice straws. The results also suggested that the optimal alkali pretreatments with recalcitrance-reduced transgenic rice straws should cause an integrating impact on biomass enzymatic saccharification examined.

3.6. Decreased cellulose crystallinity and polymerization in the transgenic rice straws

To sort out how the lignocellulose recalcitrance is lessened in the transgenic rice lines examined, this study further detected major lignocellulose recalcitrant factors. As cellulose crystallinity is an integrated parameter negatively accounting for lignocellulose recalcitrance, we first examined obviously reduced crystalline index (CrI) values in the transgenic rice straws, as a comparison with the WT (Fig. 6A). This work then determined significantly reduced degree of polymerization (DP) values by 18 % and 13 % in two types of cellulose substrates (crude and crystalline cellulose) of transgenic rice straws (Fig. 6B, C), suggesting that much more reducing ends of cellulose substrate should occur in the transgenic rice sample. Because cellobiohydrolase (CBH) enzyme is of specific activity for digestion of the reducing ends of β-1,4-glucan chains (Huang et al., 2019), this study performed CBHI hydrolysis with crude cellulose substrate *in vitro* under a time course (Fig. 6D). Significantly, the transgenic rice sample showed consistently higher glucose yields released from time course of CBHI hydrolysis at $P < 0.01$ level, compared with the WT, which was consistent with much decreased cellulose DP in the transgenic rice samples.

Furthermore, this study measured the transcript levels of native *OsGH9* family genes encoded the enzymes for cellulose hydrolysis and modification in rice (Xie et al., 2013; Huang et al., 2019). Significantly, all four representative genes were examined at higher expression up to several folds in two transgenic rice samples, compared with the WT (Fig. 7), indicating that those enzymes may involve in cellulose modification towards much decreased cellulose DP and CrI values examined in the transgenic rice samples. It also suggested that cellulose modification should be the major cause for improved lignocellulose

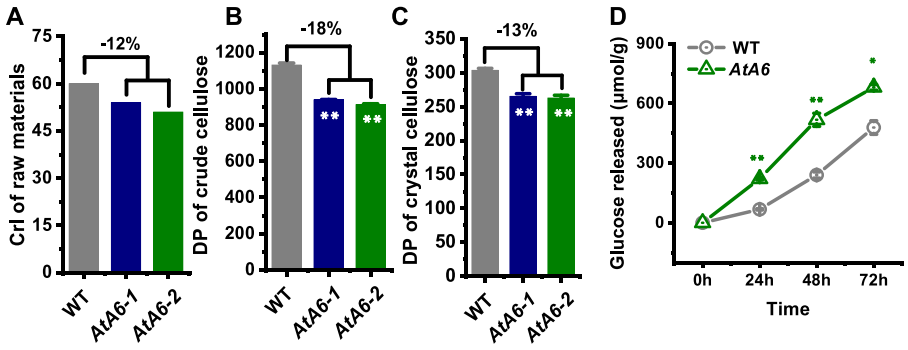

Fig. 6. Lignocellulose features detected in transgenic rice lines and WT. (**A**) Crystalline index of raw materials; (**B**, **C**) Degree of polymerization of crude cellulose and crystal cellulose, data as means ± SD ($n \geq 3$); (**D**) Glucose yield released from time-course CBHI hydrolysis with crude cellulose substrate, data as means ± SD ($n = 2$). * and ** indicated significant difference between transgenic line and WT by *t*-test as $P < 0.05$ and 0.01, respectively.

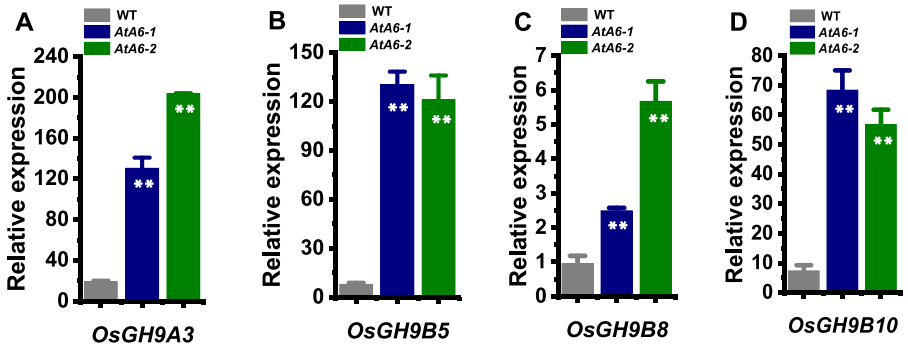

Fig. 7. Quantitative real time-PCR analyses of four *OsGH9* family genes in transgenic rice lines and WT.

recalcitrance in the transgenic rice straws. Therefore, it has demonstrated that the optimal alkali pretreatments with cellulose-modified rice straws should play a synergistic role in biomass enzymatic hydrolysis by remarkably raising cellulose accessibility. Importantly, it has not only led to complete lignocellulose enzymatic saccharification, but also caused much direct-fermentable soluble sugars accumulation, which should be combined to achieve maximum bioethanol production in *AtCesA6* transgenic rice straws.

4. Conclusion

By selecting transgenic rice lines that overexpressed *AtCesA6* gene, this study examined significantly reduced cellulose crystallinity and polymerization with improved carbon assimilation for remarkably soluble sugars accumulation in the transgenic rice straws. Using two recyclable alkali chemicals ($NH_3{\cdot}H_2O$, CaO) and liquid hot water (Wu et al., 2019; Zhao et al., 2020), this work performed three mild green-style pretreatments with mature rice straws, leading to either further reduced lignocellulose recalcitrance or much raised cellulose accessibility detected in the transgenic rice samples. Under two optimal alkali pretreatments, the transgenic rice samples achieved the bioethanol yields of more than 20 % (% dry matter) or the bioethanol concentrations at 18.3 g/L and 19.1 g/L from one-pot high solid loading saccharification, which were much higher than those of the WT. Notably, this study attempted to explain how the maximum ethanol was achieved in *AtCesA6* transgenic rice samples, mainly due to near-complete enzymatic hydrolysis and much directly-fermentable soluble sugars accumulation. Therefore, this work has provided an integrated strategy for genetic lignocellulose improvement and optimal biomass process technology in bioenergy crops.

CRediT authorship contribution statement

Yuanhang Ai: Conceptualization, Investigation, Visualization, Writing - original draft. **Shengqiu Feng:** Project administration, Conceptualization, Validation. **Youmei Wang:** Conceptualization, Methodology, Validation. **Jun Lu:** Data curation, Supervision, Funding acquisition. **Mengdan Sun:** Investigation, Resources. **Huizhen Hu:** Resources. **Zhen Hu:** Supervision, Software, Methodology. **Ran Zhang:** Conceptualization, Methodology. **Peng Liu:** Software, Resources. **Hao Peng:** Data curation. **Yanting Wang:** Data curation, Validation. **Limin Cao:** Resources. **Tao Xia:** Supervision, Project administration. **Liangcai Peng:** Conceptualization, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2021.114133.

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