Received: 8 June 2020 | Revised: 3 September 2020 | Accepted: 9 September 2020

DOI: 10.1111/gcbb.12764

GCB-BIOENERGY BIOPRODUCTS FOR A SUSTAINABLE BIOECONOMY Open Access WILEY

ORIGINAL RESEARCH

Ecologically adaptable *Populus simonii* is specific for recalcitrance-reduced lignocellulose and largely enhanced enzymatic saccharification among woody plants

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Funding information
Higher Education Discipline Innovation Project, Grant/Award Number: BP0820035; Fundamental Research Funds for the Central Universities, Grant/Award Number: 2662015PY168; National Key R&D Program of China, Grant/Award Number: 2662019PY054

Abstract

Woody plants provide enormous biomass resource convertible for biofuels and bioproducts, but they are of typical lignified secondary cell walls with strong recalcitrance against biomass degradation. It thus becomes critical to find out the desirable woody plant enabled for efficient biomass enzymatic saccharification. In this study, we collected totally seven biomass samples from three major hardwood species showing worldwide geographic distributions and diverse cell wall compositions. Under acid (H_2SO_4) and alkali (NaOH, CaO) pretreatments, all biomass samples showed remarkably enhanced enzymatic saccharification, but the *Populus simonii* species had the highest hexoses yields from all pretreatments performed. In particular, the mild and green-like pretreatment (10% CaO, 50°C) could lead to more than 70% cellulose degradation into fermentable hexoses in the *P. simonii* species, but only 24%–38% cellulose digestions were examined in the other six samples. Importantly, the *P. simonii* species is of the lowest ratios of lignin S/G and hemicellulose Xyl/Ara among seven samples, being two major factors accountable for much improved lignocellulose recalcitrance. These consequently caused the most reduced cellulose DP in the pretreated *P. simonii* residues for enhanced biomass saccharification. Furthermore, this study performed a genome-wide profiling of gene expression to confirm distinct wall polymer biosynthesis and biomass metabolism in the *P. simonii* species, consistent with its significantly improved lignocellulose recalcitrance. Therefore, this study has found out the desirable model of woody plants for efficient biomass enzymatic saccharification under cost-effective and green-like pretreatments, providing a powerful strategy for genetic lignocellulose modification in woody plants and beyond.

KEYWORDS

biomass saccharification, chemical pretreatment, genome-wide expression, lignocellulose recalcitrance, *Populus simonii*, woody plant

Liangcai Peng and Peng Chen should be considered joint senior author.

1 | INTRODUCTION

Woody plants provide enormous lignocellulose resource for biofuels and bioproducts. By the year 2030, the contribution of woody bioenergy plants is estimated up to 300 million tons of dry biomass (Perlack & Stokes, 2011). In general, both hardwood and softwood plants are comprised of thousands of different species across the world, accounting for 30% of total biomass resources (Perlack et al., 2005). The diverse woody species have been cultivated to adapt different geographic regions such as populus, salix, eucalyptus, alnus, loblolly, lodgepole and ponderosa, which produce distinct lignocellulose resources (Álvarez et al., 2016; Johnson et al., 2007).

Woody biomass and agricultural crop straws are considered to convert for partial replacement of starch-derived biofuels (Johnson et al., 2007). Particularly, cellulosic ethanol is evaluated as a perfect additive to petrol fuel, and its biochemical conversion involves in three major steps: initial biomass pretreatment to disrupt wall polymers, sequential enzymatic hydrolysis to release soluble sugars and final yeast fermentation to produce bioethanol (Huang et al., 2019; Li et al., 2018). However, due to its recalcitrant property, lignocellulose process basically requires a strongly chemical pretreatment and a costly enzymatic saccharification, which is unacceptable for large-scale bioethanol production with the potential secondary wastes release into the environment (Chen et al., 2016; DeMartini et al., 2013; Xie & Peng, 2011). Thus, selection of the desirable woody plants becomes a promising solution for mild and green-like biomass pretreatment and efficient enzymatic saccharification toward cost-effective bioethanol production. Furthermore, genetic modification of lignocellulose has been implemented to originally reduce recalcitrance in bioenergy woody plants (Fan et al., 2017, 2020; Huang et al., 2019; Li et al., 2018; Straub et al., 2019; Wang et al., 2016; Wu et al., 2019).

Lignocellulose recalcitrance is fundamentally determined by plant cell wall compositions, wall polymer features and polymer interaction styles (DeMartini et al., 2015). As a major compound of plant cell walls, cellulose DP (degree of polymerization) has been recently characterized as a key negative factor accounting for biomass enzymatic saccharification under various physical and chemical pretreatments (Pihlajaniemi et al., 2016; Yao et al., 2018). Meanwhile, lignin is the second largest wall polymer of plant cell walls particularly in the woody plants, and its deposition has been defined as a major contribution to lignocellulose recalcitrance (Gaur et al., 2015; Li et al., 2016). For instance, Serapiglia et al have investigated different genotypes of shrub willows (*Salix* spp.) and found that lignin contents are negatively correlated with total sugar release and ethanol yield (Serapiglia et al., 2013). Recent studies have indicated that the ratios of three lignin monomers may play dual roles in biomass enzymatic hydrolysis, mainly due to the distinct bioenergy crops examined (DeMartini et al., 2015; Herbaut et al., 2018; Li et al., 2013; Normark et al., 2014). Xylan is a major component of hemicellulose in the secondary cell walls, and its substitution degrees by arabinose or glucuronic side chain have been reported to positively affect biomass enzymatic saccharification after chemical pretreatments performed in grassy plants (Li et al., 2013, 2015; Loqué et al., 2015; Wang et al., 2015). In addition, despite pectin is a minor wall polymer, it has been also examined to significantly affect biomass enzymatic saccharification (Cheng et al., 2018; Wang et al., 2016).

As a major difference from grassy plants, the woody plants are rich at cellulose and lignin with largely varied xylan contents among the different species, leading to relatively stronger recalcitrance against biomass enzymatic hydrolysis (Álvarez et al., 2016; DeMartini et al., 2015; DeMartini & Wyman, 2011; Hou et al., 2019). On genome level, the woody plants show much more genes involved in wall polymer biosynthesis and carbohydrate metabolism for biomass production (Guerra et al., 2019; Kumar et al., 2019; Wegrzyn et al., 2010). Although genetic engineering of plant cell walls has been demonstrated to effectively reduce lignocellulose recalcitrance, it becomes crucial to select the target genes for genetic manipulation, in particular on the woody plants (Straub et al., 2019; Wang et al., 2016; Xiao et al., 2018).

Chemical pretreatments have been widely implemented as the initial steps to reduce lignocellulose recalcitrance using classic acid (H_2SO_4) and alkali (NaOH) agents. By comparison, the acid pretreatment at high temperature could partially digest hemicellulose and pectin to release soluble sugars, whereas the alkali pretreatment at high concentration leads to co-extraction of lignin and hemicellulose (Luo & Xu, 2020; van der Pol et al., 2014). However, both pretreatments under strong conditions not only produce toxic compounds that inhibit yeast fermentation but also release the secondary wastes into the environment (Franden et al., 2013; Li, Si, et al., 2014; Si et al., 2015). Due to its recycling and adsorption with toxic compounds, CaO chemical has been applied for a green-like pretreatment performed under mild condition, but the CaO pretreatment is only effective for the desirable bioenergy plants that are of relatively weak lignocellulose recalcitrance (Deng et al., 2020; van der Pol et al., 2014). In this study, we collected totally seven biomass samples from three major hardwood species (poplar, salix and eucalyptus) that are widely distributed over the world, and compared their distinct biomass enzymatic saccharification under acid (H_2SO_4) and alkali (NaOH, CaO) pretreatments at a series of concentrations. This study then identified that the *Populus simonii* is of specifically high biomass enzymatic saccharification among all woody species examined, and meanwhile sorted out how the biomass saccharification was more enhanced in this woody species by measuring major wall polymers features upon various chemical pretreatments. Using public bioinformatic data, this study finally presented genome-wide gene

expression profiling in the *Populus* species and attempted to find out the potential target genes for genetic modification of lignocellulose in woody plants.

2 | MATERIALS AND METHODS

2.1 | Woody plants geographic distribution data analysis

The geographic distribution data of the three types of woody plants (poplar, salix and eucalyptus) and *P. simonii* were collected from Global Biodiversity Information Facility (GBIF) database (https://www.gbif.org/). The dataset of the three types of woody plants including the recent 20 years of geographic distribution information (2001–2020; GBIF.org, 2020b, 2020c, 2020d), and the *P. simonii* dataset including the recent 100 years of geographic distribution information (1921–2020; GBIF.org, 2020a). All of the coordinate data were used to generate the distribution map of these woody plants types by Origin 2018 software.

2.2 | Woody biomass sample collection

Total seven hardwood biomass samples were collected including three *Populus* samples (*P. alba*, *P. deltoides* and *P. simonii*) from the experimental field of Huazhong Agricultural University; two *Salix* samples (*Salix* spp. *"zhuliu"* and *Salix* spp. *"nanhu"*) from the Shi-shou Salix germplasm field of Hubei Province; two *Eucalyptus* samples (*Eucalyptus 3229* and *Eucalyptus U6*) from the Guangdong Eucalyptus germplasm fields, Zhanjiang, Guangdong Province, P. R. China. The major branches of woody plants were chopped into 5 cm fragments, ground into powders with a high-speed plant sample homogenizer, passed through a 40-mesh screen and dried under 50°C until a constant weight. The dried biomass samples were stored in a sealed container until in use.

2.3 | Wall polymer extraction and determination

The wall polymer extraction procedure was applied as previously described (Peng et al., 2000; Wu et al., 2013). The soluble sugars, lipid, starch and pectin of biomass samples were successively extracted by means of potassium phosphate buffer (pH 7.0), chloroform–methanol (1:1, v/v), DMSO–water (9:1, v/v) and 0.5% (w/v) ammonium oxalate. The remaining solid residues were extracted with 4 M KOH containing 1.0 mg/ml sodium borohydride for 1 hr at 25°C and the supernatants were combined as the alkali-extractable hemicellulose fraction. After the following H_2SO_4 (67%, v/v) extraction, hexose of the remaining non-alkali-extractable residues was detected as cellulose level by the anthrone/H_2SO_4 method (Fry, 1988). Total hexoses and pentoses of the alkali-extractable hemicellulose fraction and pentoses of the non-alkali-extractable fraction were summed for hemicellulose content using the orcinol/HCl method (Dische, 1962) for pentose assay. Total pectin was measured as previously described (Wang et al., 2015). UV–VIS spectro-photometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd.) was used for absorbance reading. D-glucose, D-xylose and D-galacturonic acid (Sinopharm Chemical Reagent Co., Ltd.) were, respectively, applied for calibration curves of hexose, pentose and uronic acids. All experiments were conducted in independent triplicate.

2.4 | Hemicellulose monosaccharide determination by GC-MS

Monosaccharide composition of hemicellulose was determined by GC-MS as previously described (Pei et al., 2016). Restek Rxi-5ms fused silica capillary column (30 m × 0.25 mm, *df* 0.25 μm, Restek Corporation) was coupled with SHIMADZU GCMS-QP2010 Plus for monosaccharide analysis. The mass spectrometer was operated in electrospray ionization (EI) mode, and mass spectra were acquired with full scans.

2.5 | Lignin content and three monolignols determination

Total lignin level of acid insoluble lignin (AIL) and acid soluble lignin (ASL) was measured according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory as previously described by Sluiter et al. (2008). The AIL was calculated gravimetrically after correction for ash, and the ASL was measured using UV spectroscopy. Three monomers of lignin (H-, G- and S-) were determined as previously described by Li, Si, 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. All experiments were carried out in independent triplicate.

2.6 | Detection of cellulose DP

Cellulose DP was detected by the viscosity method using crude cellulose samples as previously described (Li et al., 2018). About 0.2 g dry biomass was first extracted with 4 M KOH (containing 1.0 mg/ml sodium borohydride). After centrifugation at 3,000 *g*, the pellet was re-extracted with 4 M KOH, and further extracted with 8% (w/v) $NaClO_2$ (with

1.5% acetic acid) at 25°C for 72 hr ($NaClO_2$ was changed every 12 hr). The remaining residue was washed to neutral pH with distilled water and dried at room temperature as crude cellulose sample in use. All experiments were performed in independent triplicate.

2.7 | Scanning electron microscopy

Scanning electron microscopy was performed according to Li et al. (2013). Residues after pretreatments or enzymatic hydrolysis were rinsed with distilled water until neutral pH, dried and sputter-coated with gold in a JFC-1600 ion sputter (Mito City, Japan). Sample surface morphology was recorded by scanning electron microscopy model MERLIN Compact (Zeiss) at different resolutions, five to eight images were taken for each condition to acquire representative images.

2.8 | Biomass pretreatment and enzymatic hydrolysis

Chemical pretreatment and sequential enzymatic hydrolysis were performed as previously described (Deng et al., 2020; Huang et al., 2012). Biomass samples (0.3 g) were, respectively, incubated with H_2SO_4 or NaOH at five concentrations (0.5%, 1%, 2%, 4%, 8%, v/v for H_2SO_4, w/v for NaOH, respectively), or with CaO at five concentrations (1%, 2.5%, 5%, 10%, 20%, w/w). After pretreatments, pentoses and hexoses of the supernatants were, respectively, measured and the remaining residues were used for enzymatic hydrolysis. The remaining residues were washed at least five times with distilled water until neutral pH, once with enzymatic hydrolysis buffer (0.2 M acetic acid-sodium acetate, pH 4.8), and then incubated with mixed-cellulases (Imperial Jade Biotechnology Co., Ltd, containing β-glucanase $\geq 2.98 \times 10^4$ U, cellulase $\geq$ 298 U and xylanase $\geq 4.8 \times 10^4$ U) at a final concentration of 3.2 g/L for enzymatic hydrolysis at 50°C, 150 rpm for 48 hr, while co-supplied with 1% (v/v) Tween-80. Biomass saccharification was calculated by measuring hexoses released from the enzymatic hydrolysis on the basis of cellulose content derived from the same biomass sample. All experiments were conducted in independent triplicate.

2.9 | GEO data collection and gene expression profiling

GEO data of cell-wall-related genes in three *Populus* species (*P. deltoides*, *P. alba* and *P. simonii*) were downloaded from Gene Expression Omnibus (GEO) portal (http://www.ncbi.nlm.nih.gov/gds/). The dataset includes GSM2561884 and GSM2561885 from GSE97335 series for *P. alba*, GSM600307, GSM600308 and GSM600309 from GSE24349 series for *P. deltoides*, and GSM1072613 and GSM1072614 from GSE43872 series for *P. simonii*. All datasets were recorded using GPL4359 platform, which contain 61,413 probe sets representing over 56,000 transcripts and gene predictions. RMA normalization was performed using the RMAExpressGUI (v1.20.0-alpha-4) to remove local biases among samples (Bolstad et al., 2003; Irizarry, Bolstad, et al., 2003; Irizarry, Hobbs, et al., 2003). Probe IDs were converted to official gene symbols with Affymetrix (http://www.affymetrix.com/site/mainPage.affx), and an independent probe filtering was performed to remove the unmatched probes and the multi-matched probes which matched to more than one gene symbol by checking the GPL4359 platform annotation file. For the genes that have more than one matched probes, the Collapse Dataset tool of GSEA 4.0.3 software (https://www.gsea-msigdb.org/gsea/downloads.jsp) was used to select one probe set per gene, and median was selected as the Collapsing mode (Mootha et al., 2003; Subramanian et al., 2005). After these filtering processes, about 27,507 gene symbols were obtained with the median expression values. BART (Bioinformatics Array Research Tool, http://igc1.salk.edu:3838/bart/) was used to acquire differential expression data among the three *Populus* species (Amaral et al., 2018). The following screening criteria was deemed as differentially expression: $\log_2$Fold-Change > 2 or <-2 (upregulation or downregulation) and the corrected p value, p.adjust $< .01$ were defined as the significant. We obtained the co-upregulated and co-downregulated differentially expressed genes (DEGs) lists between *P. alba* versus *P. simonii* and *P. deltoids* versus *P. simonii* from the upset plot. These DEGs were annotated according to Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and UniProt (https://www.uniprot.org) database. The Gene Ontology (GO) enrichment analysis was then conducted on PANTHER (http://www.pantherdb.org/) with the latest version 15.0. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was processed on STRING (https://string-db.org/cgi/input.pl). FDR < 0.05 were considered as the GO and KEGG pathway analysis significant cut-offs. Heat map was generated on these DEGs with hierarchical clustering analysis. The Volcano plot, Bubble Chart and Histogram were generated by R x64 3.5.1 software. The Upset plot and Heat map were generated by TBtools software (Chen et al., 2020).

2.10 | Correlation analysis between wall polymer features and biomass saccharification

Correlation coefficients between wall polymer features and hexoses yields (biomass saccharification) were calculated by SPSS software (SPSS 17.0). The correlation graphs were generated by GraphPad software (Prism 7.0.4). The box plots were generated using Origin 2018 software.

3 | RESULTS AND DISCUSSION

3.1 | Wide geographic distribution and diverse cell wall composition of seven woody plants

In this study, we collected totally seven biomass samples from three major woody species such as *Populus*, *Salix* and *Eucalyptus*, which are widely distributed around the world (Figure 1). As one of the distinctive plant species in Australia, *Eucalyptus* is also found in South Africa, South America, Europe and Southern coast of China. By comparison, *Salix* species occupies most of the north temperate zone, including North America, Europe and many Asia countries. Some *Salix* species are also found in New Zealand and Southeast Australia. *Populus* species almost overlaps the salix distribution, but it exhibits less distribution than salix in North America. Notably, a *Populus* species, *P. simonii*, is mainly growing in China and European countries (Figure 1).

Furthermore, this study determined cell wall compositions of seven biomass samples including three *Populus* species (*P. alba*, *P. deltoides* and *P. simonii*), two *Eucalyptus* cultivars (*Eucalyptus urophylla* × *grandis*) and two *Salix* cultivars (*Salix* spp. *"zhuliu"* and *Salix* spp. *"nanhu"*). In general, seven biomass samples showed diverse cell wall compositions including cellulose, hemicellulose, lignin and pectin, and three biomass samples of the *Populus* species remained largely varied cellulose (33%–42%, w/w of total dry matter) and hemicellulose (19%–26%) contents (Table 1). In particular, all biomass samples contained high cellulose contents over 30% and the lignin levels of 27% on average, which were much higher than those of grassy plants (DeMartini et al., 2015; DeMartini & Wyman, 2011; Wang et al., 2016). In addition, the average hemicellulose level remained 23%, whereas the average pectin content was less than 1%, suggesting typical lignified secondary cell walls of seven woody plants examined in this study. Hence, the selected seven samples are of representative woody biomass materials for the following lignocellulose saccharification experiments, probably due to their wide geographic distributions and diverse genetic germplasms.

3.2 | Remarkably enhanced enzymatic saccharification under chemical pretreatments in *P. simonii*

Biomass enzymatic saccharification refers to hexoses yield against cellulose (% cellulose) released from enzymatic hydrolysis of the pretreated biomass residues (Li et al., 2013; Wu et al., 2013; Xu et al., 2012). Using our previously established approaches (Deng et al., 2020; Huang et al., 2012), this study performed acid (H_2SO_4) and alkali (NaOH, CaO) pretreatments at series of concentrations and determined the

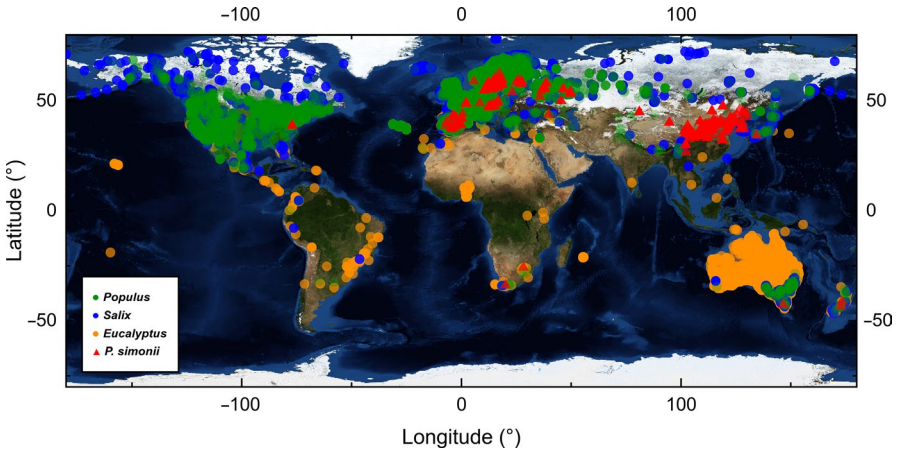

FIGURE 1 Geographic distribution of three types of woody plants used in this study. The yellow, blue and green dots represent *Eucalyptus* spp., *Salix* spp. and *Populus* spp. respectively. The *Populus simonii* locations are showed by red triangles. Data collected from the Global Biodiversity Information Facility database (http://www.gbif.org/)

TABLE 1 Cell wall composition of seven woody biomass samples (% of total dry matter)

	Pectin	Cellulose	Hemicellulose	Lignin
Populus alba	1.40 ± 0.64	41.65 ± 0.53	26.05 ± 1.01	24.71 ± 1.35
Populus deltoides	0.49 ± 0.09	36.12 ± 0.85	19.08 ± 0.24	26.25 ± 0.44
Populus simonii	1.34 ± 0.16	32.74 ± 0.81	21.33 ± 0.83	27.99 ± 0.59
Eucalyptus 3229	0.46 ± 0.07	32.92 ± 2.16	20.74 ± 0.68	29.14 ± 0.28
Eucalyptus U6	0.53 ± 0.15	30.61 ± 3.03	20.20 ± 1.37	31.03 ± 0.69
Salix "zhuliu"	0.88 ± 0.11	38.59 ± 2.76	20.06 ± 0.26	23.34 ± 0.40
Salix "nanhu"	0.40 ± 0.02	36.01 ± 0.75	23.81 ± 0.10	28.74 ± 0.51

Note: ±Standard deviation of three technical replicates.

hexoses yields released from enzymatic hydrolysis while co-supplied with 1% Tween-80 (Figure 2). As a result, the *P. simonii* sample showed consistently higher hexoses yields than those of other six biomass samples under all chemical pretreatments performed in this study. Notably, under the optimal CaO (10% at 50°C) pretreatment, the *P. simonii* sample showed much more enhanced hexoses yield up to twofold than those of four biomass samples even though under 20% CaO pretreatments. Meanwhile, under the optimal NaOH (4% at 50°C) pretreatment, the *P. simonii* sample showed a hexoses yield of more than 80%, but the other six biomass samples less than 60%. Furthermore, among the other six biomass samples, the hexoses yields remained slightly varied under their optimal acid and alkali pretreatments, indicating that the *P. simonii* species is of specifically high biomass enzymatic saccharification under mild and green-like pretreatments, probably due to its distinct cell wall composition and wall polymer feature as described below. In addition, this study examined that all seven biomass samples had significantly higher hexoses yields under two alkali pretreatments than those of the acid pretreatments, consistent with the previous findings that the alkali pretreatments could effectively co-extract lignin and hemicelluloses for much more increased biomass porosity and cellulose accessibility, whereas the acid pretreatments only specifically digest partial hemicelluloses in grassy plants (Alam et al., 2019; Li, Feng, et al., 2014; Li et al., 2018; Pei et al., 2016; Wu et al., 2013; Xu et al., 2012). Further analyses confirmed distinct biomass balance and pentoses release between the acid and alkali pretreatments (Tables S1 and S2). Hence, the mild alkali pretreatments remained more effective for enhancing biomass enzymatic saccharification in the woody plants, mainly due to an efficient co-extraction of lignin and hemicellulose in the lignin-rich woody plants.

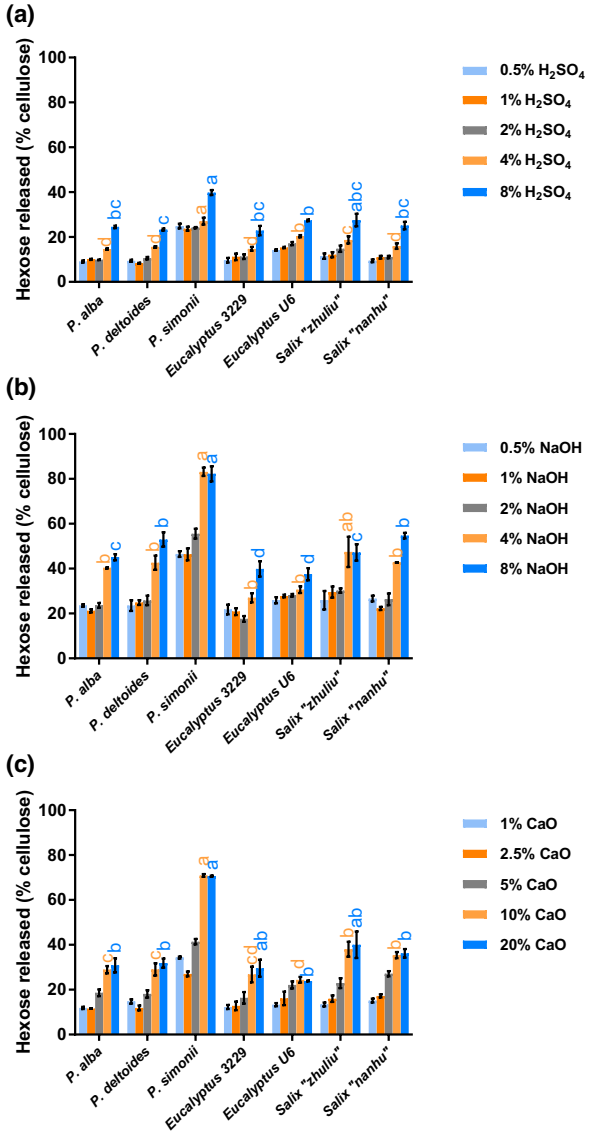

FIGURE 2 Hexose yields released from enzymatic hydrolysis after acid and alkali pretreatments in seven woody biomass samples. (a) Acid (H_2SO_4) pretreatments at various concentrations. (b, c) alkali (NaOH, CaO) pretreatments at series of concentrations, respectively. Letters above the bars (a, b, c and d) represent the results of significant tests conducted with ANOVA and multiple comparing at $p < .05$ ($n = 3$). The colors of the letters were consistent with the corresponding bars

3.3 | Lignin G- and S-monomers distinctively affect biomass enzymatic saccharification

It has been recently characterized that lignin monomers play dual roles in biomass enzymatic hydrolysis (Hu et al., 2018; Li, Si, et al., 2014; Pei et al., 2016; Studer et al., 2011; Yoo et al., 2017). To understand why the *P. simonii* sample is of the higher biomass enzymatic saccharification, this study examined three lignin monomer proportions and found that G- and S-lignin together covered almost 98% of total in seven biomass samples (Figure 3a). Notably, the *P. simonii* sample showed relatively higher G-monomer (41%, w/w%) and lower S-monomer (59%) proportions, leading to the lowest S/G ratio at 1.19, compared to the other six samples with the S/G values ranged from 1.38 to 1.84. As correlation analysis has been well applied to account for major wall polymer impacts on biomass enzymatic saccharification (Li, Feng, et al., 2014; Pei et al., 2016; Wu et al., 2013; Xu et al., 2012), this study further performed the correlation analysis between three major monomer proportions and hexoses yields released from enzymatic hydrolysis after two alkali (NaOH, CaO) chemical pretreatments in seven biomass samples (Figure 3b; Figure S1). By comparison, the G-monomers showed a positive correlation with the hexoses yields, whereas the S-monomers were negatively correlated with the hexoses yields at $p < .01$ levels, leading to the S/G ratio as the significantly negative factor accounting for biomass enzymatic saccharification in seven biomass samples

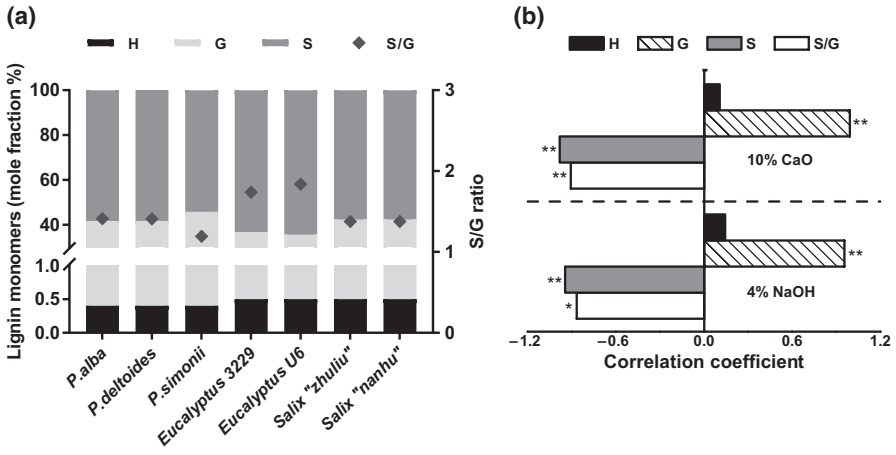

FIGURE 3 Lignin monomers impacts on biomass enzymatic saccharification in seven biomass samples. (a) Mole fraction (% of total) of three lignin monomers (S-, G-, H-) and S/G ratios. (b) Correlation analyses between three monomers or S/G ratios and hexoses yields released from enzymatic hydrolysis after alkali pretreatments. * and ** indicate the significant correlation coefficient values at $p < .05$ and 0.01, respectively ($n = 7$)

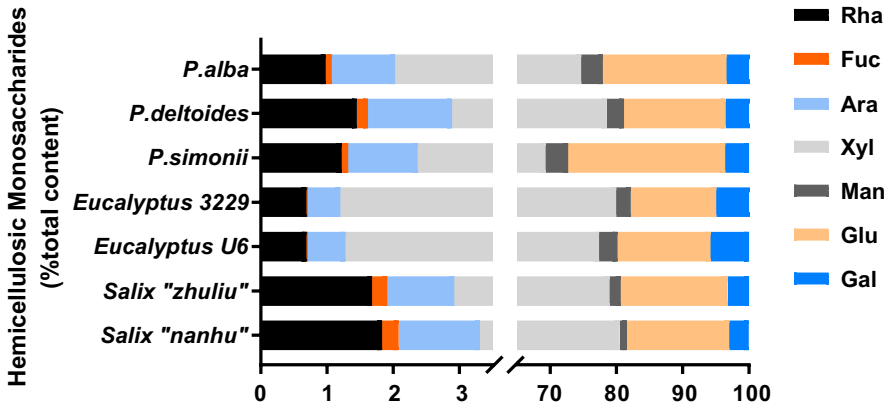

FIGURE 4 Hemicellulose monosaccharide composition (% of total) in seven biomass samples. Rhamnose/Rha; Fucose/Fuc; Arabinose/Ara; Xylose/Xyl; Mannose/Man; Glucose/Glu; Galactose/Gal

examined. In addition, the H-monomers did not show any significant correlation, probably due to its minor proportions in woody biomass samples (Figure 3b; Figure S1). Therefore, the finding about the lowest S/G ratio of *P. simonii* sample should be one of the major factors about why it was of the highest biomass enzymatic saccharification among all seven biomass samples examined. On the other hand, although the S/G ratios could play dual roles in biomass enzymatic saccharification mainly due to distinct lignocellulose residues examined among different types of bioenergy plants and diverse physical and chemical pretreatments performed in biomass processing (Li, Si, et al., 2014; Pei et al., 2016; Studer et al., 2011; Wang et al., 2016; Yoo et al., 2017), this study has well demonstrated that the S/G should be a major negative factor accounting for biomass enzymatic saccharification under mild chemical pretreatments in seven woody plants.

3.4 | Hemicellulosic monosaccharides may play an enhancement role for biomass enzymatic saccharification

Because hemicellulose is also a major compound of plant cell walls in woody plants, this study detected hemicellulose monosaccharide composition, and found largely varied fucose, rhamnose and arabinose proportions among seven biomass samples (Figure 4; Table S3). In particular, the *P. simonii* sample had relatively low xylose/arabinose (Xyl/Ara) ratio, suggesting that it should be of relatively high Ara substitution degree. Importantly, the *P. simonii* sample contained the highest proportion of glucose (24% of total), whereas the other six biomass samples had the glucose proportions ranged from 13% to 19%. It has been recently proposed that the glucose of hemicellulose fraction may derive from the non-crystalline cellulose co-extracted with hemicellulose, the Ara substitution on xylan side chain could enhance biomass enzymatic saccharification by interacting with non-crystalline cellulose (Li et al., 2013, 2015; Wang et al., 2016; Xu et al., 2012). Therefore, the data obtained in this study suggest that the reduced Xyl/Ara ratio and increased glucose proportion should be another major cause for the higher biomass enzymatic saccharification rates observed in the *P. simonii* sample.

3.5 | Mechanism of enhanced biomass enzymatic saccharification in *P. simonii*

To understand the mechanism of much enhanced biomass enzymatic saccharification under mild alkali pretreatment in the *P. simonii* sample, this study measured cellulose DP of raw materials and 10% Cao-pretreated biomass residues among

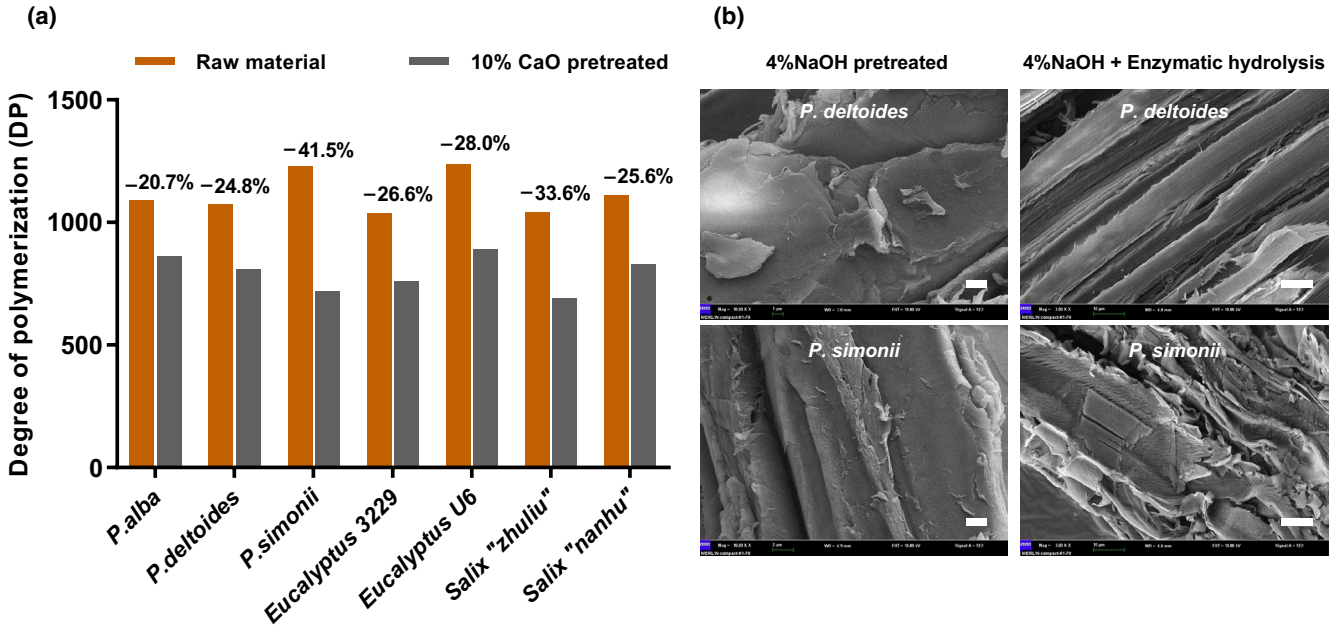

FIGURE 5 Characterization of lignocellulose features under alkali pretreatments. (a) Detection of cellulose DP in seven biomass samples. Percentages indicate the reduced cellulose DP from the alkali pretreatment. (b) Observation of the alkali-pretreated lignocellulose surfaces of two *Populus* biomass samples under scan electronic microscopy. Scale bars, 2 μm for 4% NaOH pretreatment, 10 μm for 4% NaOH + Enzymatic hydrolysis, respectively

seven biomass samples. By comparison, the *P. simonii* and *Eucalyptus U6* samples had the highest cellulose DP values of raw materials among seven biomass samples examined (Figure 5a). However, after 10% CaO pretreatment, the *P. simonii* sample showed the most reduction by 42%, resulting in the lowest cellulose DP value measured in the CaO pretreatment residue from *P. simonii* sample. Recent studies have indicated that reduced cellulose DP could provide more reducing ends of β-1,4-glucans for efficient enzymatic hydrolysis of lignocellulose in both grassy plants and woody plants (Alam et al., 2019; Cheng et al., 2018; Fan et al., 2020; Huang et al., 2015; Li et al., 2018), the most reduction on cellulose DP is consistent with the highest hexoses yield achieved in the *P. simonii* sample. To confirm this finding, we observed lignocellulose morphogenesis of two *Populus* species (*P. simonii* and *P. deltoides*) using scanning electronic microscopy (Figure 5b). Under the alkali (NaOH) pretreatment, the *P. simonii* sample exhibited much rougher surfaces than those of the *P. deltoides* sample, consistent with their distinct cellulose DP values. After sequential enzymatic hydrolysis, the *P. simonii* sample showed the most destructed lignocellulose macrofibrils accounting for its cellulose hydrolysis rate by 80%, whereas the *P. deltoides* samples remained mostly undigested, consistent with its cellulose hydrolysis rate by 42% (Figure 2). Accordingly, this cellulose surface observation should also explain why the alkali pretreatments performed in this study could lead to the most enhancement on biomass enzymatic saccharification in the *P. simonii* sample as described above.

Furthermore, based on the data obtained in this study and the related advance recently achieved, we proposed our hypothesis to interpret why the *P. simonii* sample could achieve the highest biomass enzymatic saccharification among seven biomass samples examined. As it has been reported that the G-rich and S-less lignin could be effectively co-extracted with hemicellulose under alkali pretreatment for enhanced biomass enzymatic hydrolysis (Li, Si, et al., 2014), the lowest S/G ratio of the *P. simonii* sample should be one major cause for its highest biomass enzymatic saccharification. Because the branched Ara of xylan in the non-alkali-extractable hemicellulose may interact with the non-crystalline cellulose (Li et al., 2013, 2015; Wang et al., 2016; Xu et al., 2012), the low Xyl/Ara ratio is consistent with its most reduced cellulose DP after alkali pretreatments. Meanwhile, as the dominated Ara of xylan in the alkali-extractable hemicellulose has been considered to interlink with lignin and pectin to form dynamic wall-networks (Wang et al., 2016), the low Xyl/Ara ratio may also result in an efficient co-extraction of hemicellulose and lignin/pectin complexes. Hence, relatively higher Ara level or lower Xyl/Ara ratio in the *P. simonii* sample should be accountable for its increased non-crystalline cellulose level and co-extraction of wall polymers, leading to an integrated enhancement on biomass enzymatic hydrolysis. Taken together, we conclude that the *P. simonii* sample is of distinctively altered lignin and hemicellulose features, leading to significantly reduced cellulose DP for largely enhanced biomass enzymatic saccharification under mild and green-like alkali pretreatments.

3.6 | Genome-wide gene expression profiling for potential genetic modification of lignocellulose in woody plants

As the *P. simonii* species is of distinctive features for significantly improved saccharification rates, we attempted to find out the endogenous causes behind these unique features as guide for potential genetic modification of lignocellulose recalcitrance in woody plants. Using the public data, we thus performed genome-wide gene expression analyses associated with wall polymer production in *P. simonii* and the other two *Populus* species (*P. deltoides*, *P. alba*). Based on the GEO database, we compared DEGs among three *Populus* species (Figure 6). We obtained 2,153 DEGs (551 upregulate and 1,602 downregulate) from the *P. alba* versus *P. simonii*, and 1,923 DEGs (750 upregulate and 1,173 downregulate) from the *P. deltoids* versus *P. simonii* ($|\log_2 FC| > 2$, $p.adjust < .01$; Figure 6a), respectively. The overlap between these two comparisons was illustrated in an Upset plot (Figure 6b). A total number of 912 overlapping genes were found in the common region, containing 200 co-upregulated and 667 co-downregulated DEGs.

Since both *P. alba* and *P. deltoides* samples showed less hexoses yields than those of the *P. simonii*, this study focused to analyze those 867 (200 and 667 as above) co-regulated DEGs and generated their expression heat map (Figure 6c).

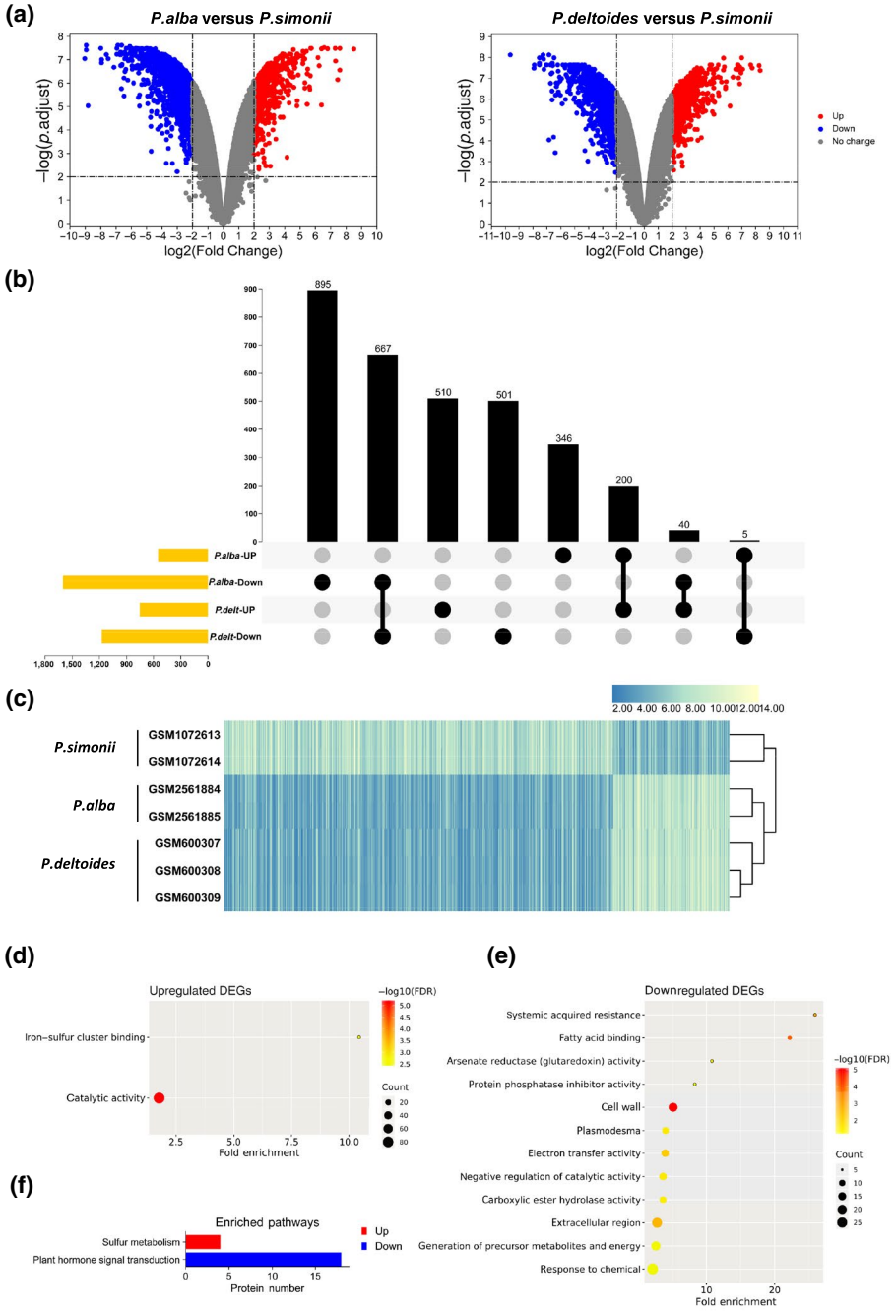

FIGURE 6 Microarray analysis of gene expression associated with cell wall biosynthesis and biomass production in three *Populus* species. (a) Volcano plots and (b) Upset plot of *Populus alba* versus *Populus simonii* and *Populus deltoids* versus *P. simonii* differentially expressed genes (DEGs). Red and blue dots represent upregulated or downregulated genes, with $|\log_2 FC| > 2$ and $p.adjust < .01$ as threshold. (c) Hierarchical clustering heat map of the co-upregulated and co-downregulated DEG expression levels, with the normalized expression values (log2 scale) from 2 to 14 (blue to primrose yellow). Gene Ontology (GO) enrichment analysis (d and e) and KEGG pathway analysis (f) considered with the significant cutoffs by FDR < 0.05

To sort out potential functions of those DEGs, we conducted the GO and KEGG pathway enrichment analyses (Figure 6d–f). Notably, only one specific molecular function (MF) term, iron–sulfur cluster binding, which was associated with iron–sulfur storage, electron transfer, substrate activation, regulation of enzyme activity and gene expression (Johnson et al., 2005), was identified from the co-upregulated DEGs (Figure 6d), but 12 specific GO terms were enriched for the co-downregulated DEGs (Figure 6e). Of all GO terms, the cell walls within cellular component term had the lowest FDR value, whereas the other biological process and MF terms were enriched for enzyme catalytic activity regulation, precursor metabolites, energy generation and dynamic responses to chemical or stress. With respect to the KEGG analysis, co-upregulated genes showed an enrichment on sulfur metabolism, while co-downregulated DEGs were enriched for plant hormone signal transduction (Figure 6f). Taken together, those GEO analyses have suggested that the distinct lignocellulose recalcitrance between *Populus* species may be resulting from characteristic gene expression profiles. Therefore, analyzing DEGs could provide candidate genes for potential genetic modification of woody plants toward reduced lignocellulose recalcitrance and improved biomass utilization.

4 | CONCLUSIONS

Using seven representative hardwood biomass samples showing distinct cell wall compositions and wide geographic distributions, this study identified that the *P. simonii* species is of specifically higher biomass enzymatic saccharification under acid and alkali pretreatments. Chemical analyses indicated that the *P. simonii* biomass sample had distinctively reduced lignocellulose recalcitrance such as the lowest ratios of both lignin S/G and hemicellulose Xyl/Ara among seven biomass samples examined, leading to the most reduced cellulose DP for remarkably enhanced biomass enzymatic hydrolysis upon green-like pretreatment. Further genome-wide profiling of gene expression confirmed a distinct wall polymer biosynthesis and biomass metabolism for improved lignocellulose recalcitrance in the *P. simonii* biomass sample. Hence, this study has sorted out the desired model of woody plant that are of low lignocellulose recalcitrance and high biomass enzymatic saccharification, providing the potential targets for genetic lignocellulose modification in woody plants.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Key R&D Program of China (2662019PY054), the National 111 Project (BP0820035) and the Fundamental Research Funds for the Central Universities, China (2662015PY168).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Lv Z, Liu F, Zhang Y, Tu Y, Chen P, Peng L. Ecologically adaptable *Populus simonii* is specific for recalcitrance-reduced lignocellulose and largely enhanced enzymatic saccharification among woody plants. *GCB Bioenergy*. 2021;13:348–360. https://doi.org/10.1111/gcbb.12764