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Genetic modification of plant cell walls to enhance biomass yield and biofuel production in bioenergy crops

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ABSTRACT

Plant cell walls represent an enormous biomass resource for the generation of biofuels and chemicals. As ligno-cellulose property principally determines biomass recalcitrance, the genetic modification of plant cell walls has been posed as a powerful solution. Here, we review recent progress in understanding the effects of distinct cell wall polymers (cellulose, hemicelluloses, lignin, pectin, wall proteins) on the enzymatic digestibility of biomass under various physical and chemical pretreatments in herbaceous grasses, major agronomic crops and fast-growing trees. We also compare the main factors of wall polymer features, including cellulose crystallinity (CrI), hemicellulosic Xyl/Ara ratio, monolignol proportion and uronic acid level. Furthermore, the review presents the main gene candidates, such as *CesA*, *GH9*, *GH10*, *GT61*, *GT43* etc., for potential genetic cell wall modification towards enhancing both biomass yield and enzymatic saccharification in genetic mutants and transgenic plants. Regarding cell wall modification, it proposes a novel groove-like cell wall model that highlights to increase amorphous regions (density and depth) of the native cellulose microfibrils, providing a general strategy for bioenergy crop breeding and biofuel processing technology.

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1. Introduction

Bioenergy is a renewable form of energy derived from biological sources and can be used to generate heat, electricity and fuels (Himmel and Bayer, 2009; Yuan et al., 2008). By 2050, it is estimated that the use of bioenergy must increase at least four folds to reduce worldwide CO_2 emissions by 50%. While the use of first-generation biofuels, such as starch- and sugar-derived ethanol and plant oil-derived biodiesel, has already made small but significant contributions to the global energy supply, the increase in bioenergy will most likely stem from the use of lignocellulose, which produces 1.5×10^{10} tons/year of biomass, with a 60% conversion efficiency (Chen and Peng, 2013; Pauly and Keegstra, 2008; Ragauskas et al., 2006; Rubin, 2008). Lignocellulosic ethanol, a second-generation biofuel, has the potential to fill most global transportation fuel needs and does not present a conflict between energy demand and food supply (Peng, 2012; Sims et al., 2010).

Plant cell wall represents the most abundant renewable biomass resource for biofuels on the earth. The conversion of lignocellulose to ethanol involves three major steps: physical and chemical pretreatments to enhance cell wall destruction, enzymatic digestion to release soluble sugars, and yeast fermentation to produce ethanol (Carroll and Somerville, 2009; Xu et al., 2012). Because plant cell walls have evolved a complex structure and the mechanical strength to resist physical and biochemical digestion in nature, lignocellulose recalcitrance greatly leads to an unacceptably costly biomass process (Himmel et al., 2007; Lynd et al., 2008). In principal, biomass recalcitrance is determined by cell wall composition, wall polymer characteristics, and wall polymer network structures. Hence, genetic modification of plant cell walls represents a powerful solution to biomass recalcitrance (Chen and Dixon, 2007; Demura and Ye, 2010; Torney et al., 2007; Vega-Sánchez and Ronald, 2010; Wang et al., 2014b; Xie and Peng, 2011).

The cell wall is a layer of structural material that surrounds the plant cell and extends to the protoplast (Fig. 1A, B). The plant cell wall can be divided into primary and secondary cell walls (Fig. 1C, D), which are systematically formed during plant cell growth and development (Cosgrove, 2005; Dumville and Fry, 2000; Hall and Cannon, 2002; Motose et al., 2004; Wang et al., 2014b). Plant cell walls are mainly composed of cellulose, hemicelluloses and lignin, as well as minor pectic polysaccharides and wall proteins (Fig. 1E–H), and they have distinct compositions in different plant species (Table 1). As plants comprise of numerous cell types with extremely complicated cell wall structures and diverse biological functions, the genetic modification of plant cell walls is often associated with defects in plant growth and development, which could affect important agronomic traits in crops such as plant lodging resistance, biomass yield and environmental stress tolerance (Casler et al., 2002; Li et al., 2015b; Pedersen et al., 2005; Vega-Sánchez and Ronald, 2010). It thus becomes critical to find out an optimal genetic engineering approach that not only maintains normal plant growth but also enhances biomass yield and lignocellulose enzymatic digestibility (Wang et al., 2014b; Xie and Peng, 2011). In the present review, we describe recent research progress about cell wall polymer distinct effects on biomass enzymatic saccharification under different physical and chemical pretreatments in various plant species, including herbaceous grasses of high biomass yield (*Miscanthus*, switchgrass, and reed), major food crops (rice, wheat, maize, sweet sorghum, and cotton) with large biomass residues, and fast-growing woody trees rich in cellulose. We then discuss the key genes involved in cell wall modifications that might enhance both biomass yield and biofuel production. Finally, we present a hypothetical model for bridging plant cell wall engineering and biomass processing technology.

2. Effects of wall polymer features on biomass saccharification

Plant cell wall composition is highly variable among different plant cell types and tissues. It therefore remains technically challenging to identify individual wall polymers that impact the enzymatic digestibility of biomass. For example, selection of one genetic mutant or a one-gene transgenic plant could result in multiple wall polymer alterations. However, based on systems biology analyses of large populations of biomass samples, three major wall polymers (cellulose, hemicelluloses, and lignin) have been characterized in several plants used primarily for biofuel production, as shown in Table 2 and Fig. 2. In addition, we discuss recent studies of pectin and wall proteins in this section.

2.1. Cellulose

Cellulose is a major component of plant cell walls that accounts for approximately 2–4% of cereal endosperm walls to 95% of the secondary cell walls of cotton fibers (Delmer, 1999; Huang et al., 2015b; Li et al., 2013a; Ragauskas et al., 2006). Among herbaceous grasses, *Miscanthus* accessions exhibit a large variation in cellulose levels, from 20% to 46% (Huang et al., 2012; Magid et al., 2004; Meineke et al., 2014), whereas switchgrass and reed have cellulose levels of approximately 32–39% (David and Ragauskas, 2010; Lindsey et al., 2013). In agronomic crops, cellulose variation has been observed among rice genetic mutants (Li et al., 2015b), sweet sorghum germplasms (Li et al., 2014a; Wu et al., 2015), maize and wheat cultivars (Chundawat et al., 2007; Jia et al., 2014; Wu et al., 2014b), and cotton and rapeseeds varieties (Huang et al., 2015b). By comparison, economical crops (e.g., ramie, jute, kenaf, and agave) and woody trees (e.g., poplar and pinus) are extremely rich in cellulose, with levels ranging from 42% to 80% (Escamilla-Treviño, 2012; Leple et al., 2007; Vieira et al., 2002).

Cellulose is a high molecular weight polymer found in plant cell walls that is composed of β-1,4-glucan chains. Intermolecular hydrogen bonding between these parallel chains leads to a compact cellulose structure that is crystalline, fibrous, and mechanically strong (Brown and Emsley, 2004). Cellulose crystallinity is thus a key feature of cell walls that not only accounts for the amorphous and crystalline regions of native cellulose but also reflects cellulose interactions with other wall polymers (Kaida et al., 2009). Using X-ray diffraction, the crystalline index (CrI) has been applied to detect cellulose crystallinity in various biomass materials (Bansal et al., 2010; Park et al., 2010). Cellulose CrI has thereby been determined to be a primary negative factor on biomass enzymatic digestibility under various physical and chemical pretreatments in almost all plant species examined (Zhang et al., 2013). Recently, Li et al. (2015b) have reported that cellulose CrI is the major factor negatively affecting rice lodging resistance, a major agronomic trait significantly associated with grain yield and biomass production in crops. In addition, the cellulose level is known to have a negative impact on biomass enzymatic saccharification in most plant species examined, likely due to its positive correlation with cellulose CrI (Table 2).

The degree of polymerization (DP) is another important feature of cellulose that is highly variable among different plant species (Hallac
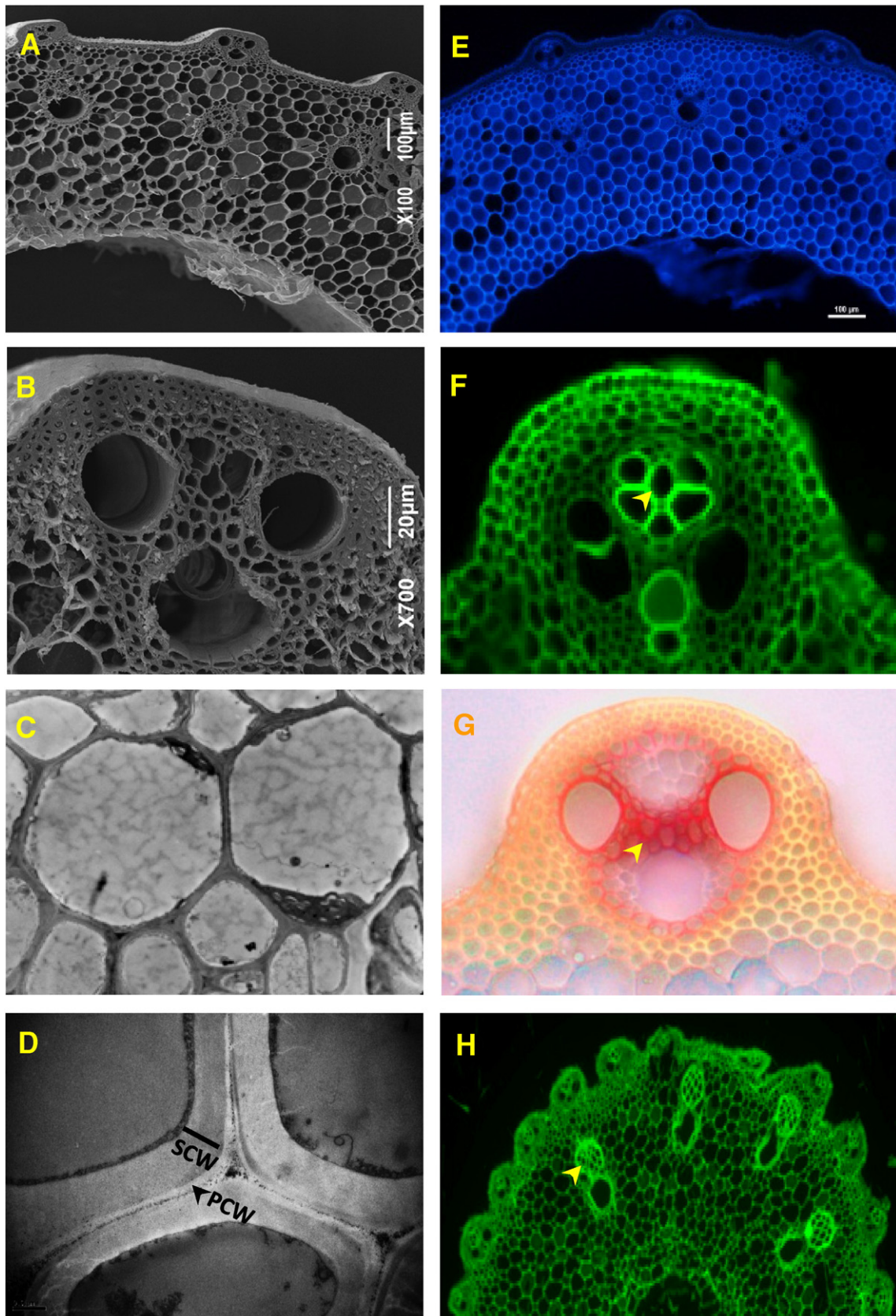
Fig. 1. Plant cell wall structures in rice. (A, B) Scanning electron microscopic images of stem tissues at heading stage; (C, D) transmission electron microscopy observations of cell walls in leaf veins (PCW: primary cell wall, SCW: secondary cell wall); (E) calcofluor staining with stem tissues on cellulose; (G) Wiesner's staining on lignin (arrow highlights lignin); (F, H) staining with anti-xylogulucan (F, CCRC-M88) and anti-rhamnogalacturonan (H, CCRC-M35,); Arrows highlight high level wall polymers as indicated, and images are provided by Li FC and Zhuo JD.

Table 1
Three major wall polymer composition in bioenergy plants[*].

Plant species	Cellulose	Hemicelluloses	Lignin	Reference
I. Herbaceous grasses				
Miscanthus				
(*M. sinensis*)	24–40	24–38	22–29	Huang et al. (2012),
(*M. floridulus*)	22–38	24–34	19–26	Magid et al. (2004),
(*M. sacchariflorus*)	20–44	23–35	24–30	Meineke et al. (2014)
(*M. lutarioriparius*)	28–46	27–35	25–31	
(*M. x giganteus*)	40	34	26	
Switchgrass				David and Ragauskas (2010),
(*P. virgatum*)	32–39	25–33	17–22	Lindsey et al. (2013)
Reed (*P. australis*)	34–36	26–27	21	Jin et al. (2016)
II. Agronomic crops				
Rice (*O. sativa*)	14–30	8–18	11–19	Li et al. (2015b),
				Zhang et al. (2015)
Wheat (*T. aestivum*)	34–38	20–35	22–24	Garcia-Cubero et al. (2009),
				Wu et al. (2014b)
Maize (*Z. may*)	20–38	21–32	13–21	Chundawat et al. (2007),
				Jia et al. (2014)
Sugarcane (*S. officinarum*)	42–46	25–27	20–21	Kim and Day (2011),
				Rocha et al. (2011)
Sweet sorghum (*S. bicolor*)	26–38	25–38	14–24	Li et al. (2014a)
Cotton				
(*G. hirsutum*)	32	14	25	Huang et al. (2015)
(*G. barbadense*)	40	17	26	
Rye (*L. perenne*)	31	22	25	Garcia-Cubero et al. (2009)
Rapeseed				
(*B. napus*)	28–32	16–20	15–20	
(*B. rapa*)	20–35	15–22	16–20	Pei et al. (2016)
(*B.carinata*)	27–32	19–20	21–23	
(*B. junjea*)	24–35	20	22–24	
III. Fiber plants				
Ramie (*B. nivea*)	48	26	26	
Jute (*C. capsularis*)	44	28	28	Wei et al. (2016)
Kenaf (*H. cannabinus*)	42	29	29	
Agave				
(*A. lechuguilla*)	80	3	15	Escamilla-Treviño (2012), Vieira et al. (2002)
(*A. fourcroydes*)	78	5	13	
Poplar (*P. trichocarpa*)	48	31	21	Leple et al. (2007)

[*]: indicates wall polymer level (% of dry weight, w/w).

and Ragauskas, 2011; Huang et al., 2015b). In the three plant species examined (*Miscanthus*, sweet sorghum, and cotton), cellulose DP is the factor that significantly negatively affects biomass digestibility (Table 2). Recently, Zhang et al. (2013) have shown that cellulose DP is positively correlated with cellulose CrI in 80 distinct *Miscanthus* germplasm accessions, indicating that cellulose CrI and DP are both important features of cellulose that impact biomass saccharification.

2.2. Hemicelluloses

Hemicelluloses are a class of heterogeneous polysaccharides with diverse monosaccharide subunits with distinct compositions in different plant species (Scheller and Ulvskov, 2010). For example, xylans are major hemicelluloses found in the mature tissues of grasses; arabinogalactan and glucuronoxylan mainly occur in woody plants;

Table 2
Major wall polymer impacts on biomass enzymatic digestibility under pretreatment.

Wall polymer feature	Plant species							
	Rice[a]	Wheat[b]	Maize[c]	*Miscanthus*[d]	Sweet sorghum[e]	Switchgrass[f]	Cotton[g]	Poplar[h]
Cellulose	−	−	−	−	−		−	/
CrI	−	−					−	−
DP				−	−		−	/
Hemicelluloses	+	−	−	+	/	+	−	+
Xyl	+		+	+	/		/	/
Ara	+		+	+	/		/	/
Xyl/Ara	−	−	−	−	−		−	/
Lignin	+	−	−	−	−	−	−	−
G	+	/	−	−	−	−	/	+
S	+	−	−	−	−	−	/	/
H	+	/	−	−			/	/
S/G	/	/	+	−	/	−	/	+
H/G	+	+	/	−	/	+	/	/
S/H	/	/	/	−	/		/	/

+ and −: respectively indicated significantly positive and negative effects on biomass enzymatic digestibility; / indicated no significant effect; Blank: not determined; Data modified from [a]Li et al. (2015b) and Wu et al. (2013); [b]Wu et al. (2013) and Wu et al. (2014b); [c]Guillaumie et al. (2008), Jia et al. (2014), and Kim et al. (2003); [d]Li et al. (2013b, 2014c), and Zhang et al. (2013); [e]Li et al. (2014a); [f]Baxter et al. (2014), Fu et al. (2011), Shen et al. (2012b), and Xu et al. (2011); [g]Huang et al. (2015b); [h]Studer et al. (2011); Van Acker et al. (2014), and Zhu et al. (2008).
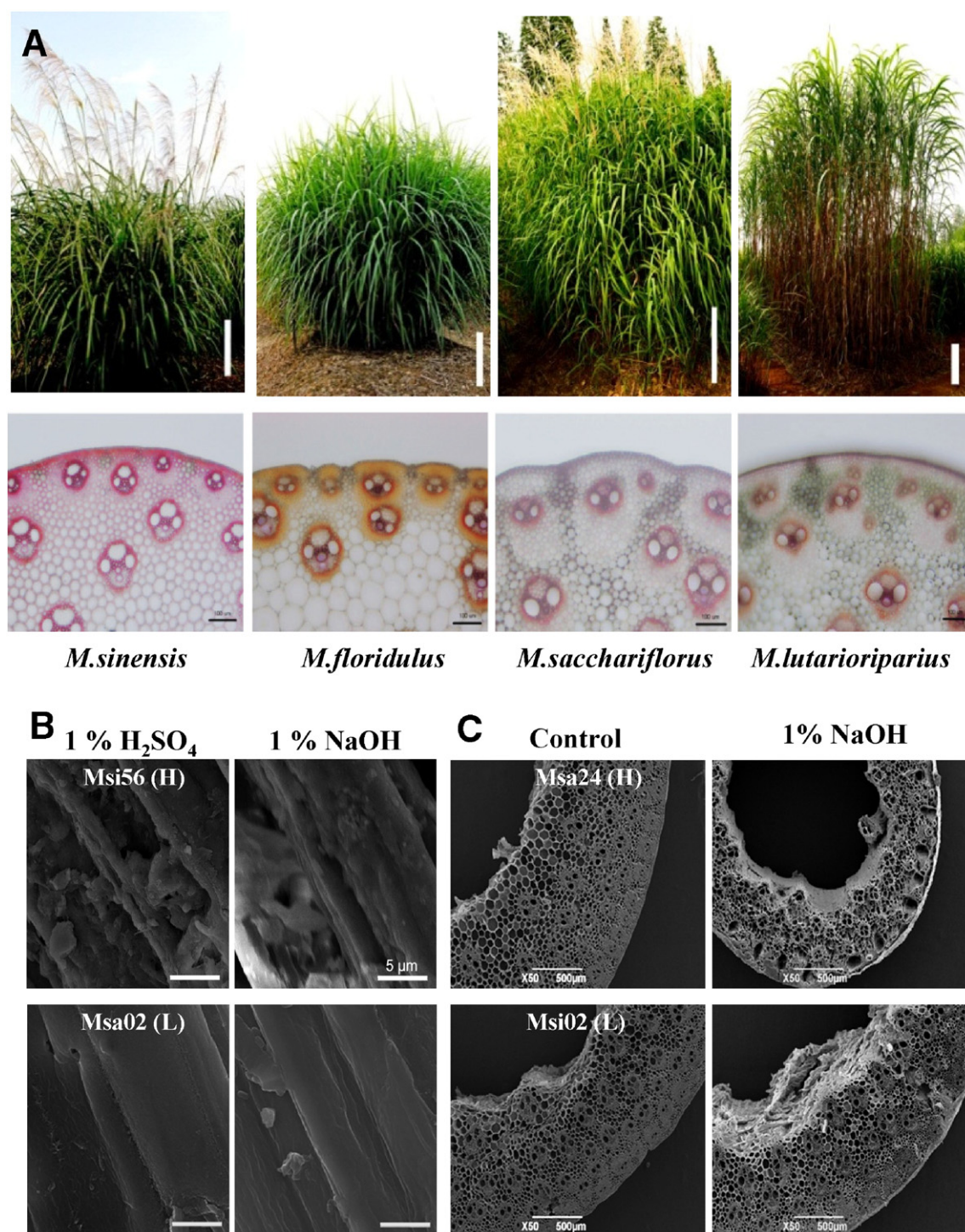
Fig. 2. Wall polymer feature impacts on biomass enzymatic saccharification in *Miscanthus*. (A) Four major *Miscanthus* species have distinct biomass yields (upper lane) and cell wall features (below lane: Wiesner's staining on lignin); (B) Ara-rich (relatively lower Xyl/Ara) *Miscanthus* accession (Msi56) shows much rougher face in vitro than that of its paired sample (Msi02) after pretreatment and enzymatic hydrolysis (Li et al., 2013b); (C) uronic acids-rich *Miscanthus* accession (Msa24) exhibits much more destructed and lost parenchyma cells in situ after 1% NaOH pretreatment and sequential enzymatic hydrolysis (Wang et al., 2015a); (H) and (L) indicate the samples with relatively high and low biomass saccharification, respectively.

and xyloglucan is found in both grasses and woody plants (Girio et al., 2010). Xylans are most commonly substituted by α-L-arabinofuranosyl units in arabinoxylan, while they are substituted by α-D-glucopyranosyl uronic units or 4-O-methyl derivative side chains in glucuronoarabinoxylan. Furthermore, hemicellulose content varies greatly among different plant species. For example, different high-level hemicelluloses are detected in particular samples of *Miscanthus* accessions, sweet sorghum germplasm, and wheat cultivars

at approximately 35%–38% (Huang et al., 2012; Li et al., 2014a; Meineke et al., 2014; Wu et al., 2014b), whereas agave species and typical rice mutants have extremely low hemicellulose contents at approximately 3%–8% (Li et al., 2015b; Escamilla-Treviño, 2012; Vieira et al., 2002).

The major biological role of hemicelluloses is its cross-linkage with cellulose and lignin. These interactions embed crystalline cellulose elementary fibrils to strengthen the cell wall and form a wall barrier against enzymes accessible to the cellulose surface, a major cause of

Table 3

Main gene candidates and appropriate genetic approaches applicable for plant cell wall modification.

Wall polymer	Species	Gene (source)	Approach	Phenotype	Yield	Digestibility	Reference
					Biomass		
Cellulose synthesis	*Arabidopsis*	*AtCesA1, 3*	*Aegeus*(A903V), *ixr1-2* (T942I)	Reduce cellulose, CrI	/	↑	Harris et al. (2012)
		AtCesA6	*prc1-1* (Q720stop), *prc1-9* (K720stop), *prc1-4* (W777stop), *prc1-19* (Y275stop)	Reduce cellulose, CrI; affect growth	↓		Fagard et al. (2000)
		AtCesA4	*irx5-1, irx5-2* (W995stop), *irx5-3* (Q263stop)	Reduce cellulose; affect growth	↓		Taylor et al. (2003)
		AtCesA7	*p35S::CesA7fra5* (OE)	Reduce cellulose; affect growth	↓		Zhong et al. (2003)
		AtCesA8	*irx1-1* (D683N), *irx1-2* (S679L)	Reduce cellulose; affect growth	↓		Taylor et al. (2000)
		AtCOBL2	*cobl2-1, cobl2-2* (T-DNA)	Reduce crystalline cellulose in seed mucilage	/		Ben-Tov et al. (2015)
		AtCesA2, 5	*pAtCesA6::CesA2, 5 /prc1-1* (OE)	Increase cellulose	↑		Desprez et al. (2007), Persson et al. (2007b)
	Rice	*OsCesA4*	*bc11* (G858R), *NE1031, ND5658* (Tos17)	Reduce cellulose; affect growth	↓		Zhang et al. (2009), Tanaka et al. (2003)
		OsCesA7	*NC0259, ND8759* (Tos17)	Reduce cellulose; affect growth	↓		Tanaka et al. (2003)
	B. distachyon	*BdCesA4, 7*	*pUBI::CESA4, 7* (amiR)	Reduce cellulose, CrI; affect growth	↓		Handakumbura et al. (2013)
		HvCesA4	*fs2* (Copia-like retroelement)	Reduce cellulose; affect growth	/		Burton et al. (2010)
	Barley	*HvCesA4, 8*	*p35S::CesA4, 8* (OE)	Reduce cellulose; affect growth	↓		Tan et al. (2015)
	Tobacco	*AtCESA3*	*pM24-GFP::CESA3ixr1-2* (OE)	Affect growth	↓	↑	Sahoo et al. (2013)
	Poplar	*PtdCesA8*	*p35S::CesA8* (OE)	Reduce cellulose; affect growth	↓		Joshi et al. (2011)
Cellulose degradation	*Arabidopsis*	*PttCel9A1*	*p35S::Cel9A1 / kor1-1* (OE)	Reduce CrI	↑		Takahashi et al. (2009)
		AtKORRIGAN1	*irx2-2*(P553L), *kor1-1* (T-DNA)	Reduce primary cellulose; increase CrI; affect growth	↓		Szyjanowicz et al. (2004), Takahashi et al. (2009)
		AtCel1	*p35S::Cel1* (antisense)	Reduce cellulose; affect growth	↓		Tsabary et al. (2003)
		PtGH9B5, C2	*pAtCesA8::GH9B5, C2* (OE)	Increase CrI; affect growth	↓		Glass et al. (2015)
		AtGH9C2	*p35S::GH9C2* (RNAi)	Reduce CrI	↑		Glass et al. (2015)
Cellulose regulation	*Arabidopsis*	*AtVND6, 7*	*p35S::VND6, 7* (OE)	Reduce secondary wall thickness; affect growth	↓		Kubo et al. (2005)
		AtMYB46, 83	*p35S::VMYB83* (OE)	Increase cellulose; affect growth	↓		McCarthy et al. (2009)
		AtMYB46, 83	*myb46-1, b83* (T-DNA)	Reduce cellulose; affect growth	↓		McCarthy et al. (2009)
	Poplar, *Arabidopsis*	*PdC3H17,18 AtC3H14,15; PdMYB10, 28*	*p35S::C3H17, 18; p35S::C3H14, 15; p35S::C3H10, 28* (OE)	Increase secondary wall thickness; affect growth	↓		Chai et al. (2014a, 2014b, 2015),
	Rice	*OsMYB103L*	*pUbi::MYB103L* (OE)	Increase secondary wall thickness	/		Yang et al. (2014)
		OsMYB61; NAC29, 31	*pUbi::MYB61; NAC29, 31* (OE)	Increase secondary wall thickness	/		Huang et al. (2015a)
Hemicelluloses synthesis	*Arabidopsis*	*AtIRX9, 9L, 14,4L*(GT43)	*irx9, 9l, 14, 14l* (T-DNA)	Reduce Xyl, GX; affect growth	↓		Keppler and Showalter (2010), Wu et al. (2010), Lee et al. (2010)
		AtESK1(DUF231)	*esk1* (T-DNA)	Reduce xylan accetylation; affect growth	↓	↑	Yuan et al. (2013)
		AtGXMT(DUF579)	*gxmt1-1, 1-2* (T-DNA)	Reduce 4-O-methyl-GlcA substituents		↑	Urbanowicza et al. (2012)
		AtTBL129	*tbl29-1, l29-2* (T-DNA)	Reduce acetate content; affect growth		↓	Xiong et al. (2013)
		AtIRX10, 10L(GT47)	*irx10/irx10l* (T-DNA)	Reduce GX; affect growth	↓		Wu et al. (2009), Brown et al. (2009)
		AtGUX1/PGSIP1, AtGUX2/PGSIP3(GT8)	*gux1-1, 1-2, 2-1, 2-2* (T-DNA)	Reduce in [Me]GX		↑	Mortimer et al. (2010), Bromley et al. (2013)
		AtIRX15, 15L(DUF579)	*irx15/15l* (T-DNA)	Reduce xylan, Xyl; mild collapsed xylem		↑	Jensen et al. (2011), Brown et al. (2011)
		AtFRA8, AtF8H(GT47)	*fra8* (C1296T), *f8h* (T-DNA); *p35S::F8H/fra8*	Reduce GX; affect growth	↓		Zhong et al. (2005), Lee et al. (2009)
		AnAXE1	*p35S::AXE1-eGFP, pGT43B::AXE1*	Reduce xylan acetyl; increase ethanol yields	/	↑	Pawar et al. (2015)
		AtIRX8(GT8), *9*(GT43)	*irx8, 9* (T-DNA)	Reduce GX; affect growth	↓		Peña et al. (2007), Lee et al. (2007), Persson et al. (2007a)
		AtXXT1, 2	*xxt1, 2* (T-DNA)	Reduce xyloglucan; affect growth	↓		Cavalier et al. (2008)
		AtMUR3/AtMURUS3 (GT47)	*mur3* (T-DNA)	Reduce xyloglucan; affect growth	↓		Kong et al. (2015)
		AtAXY3	*axy3.1* (E630L), *3.2, 3.3* (T-DNA)	Reduce xyloglucan, arabinose; affect growth	↓		Günl and Pauly (2011)
		AtAXY9(TBL)	*axy9.1* (stop codon at 276th), *9.2*(T-DNA)	Reduce xyloglucan acetylation; affect growth	↓		Schultink et al. (2015)
	Rice	*OsXAX1*(GT61)	*axa1* (T-DNA)	Reduce Xyl, ferulic, coumaric acid	↓	↑	Chiniquy et al. (2012)
		OsIRX10(GT47)	*Osirx10* (RGT6229D)	Reduce X/A; affect growth	↓	↑	Chen et al. (2013)

Process	Species	Gene	Construct	Phenotype			Reference
	Wheat	*TaXAT1, 2(GT61)*	*p35S::XAT1,2* (RNAi)	Increase X/A	/		Anders et al. (2011)
	Poplar	*PtGAUT12.1,12.2(GT8)*	*pUBi3:: GAUT12.1, 12.2* (RNAi)	Reduce xylan content	↑	↑	Biswal et al. (2015)
Hemicelluloses degradation	Maize	*XynB, ThiXynB(GH11)*	*pUbi3::XynB, iXynB* (OE)	Affect growth	/		Shen et al. (2012a)
	Poplar	*PtxtXyn10A(GH10)*	*p35S::xtXyn10A* (RNAi)			↑	Derba-Maceluch et al. (2014)
		AtKNAT7	*knat7-1* (T-DNA)	Increase IRX8, IRX9, FRA8; affect growth	↓		Li et al. (2012)
Hemicelluloses regulation	*Arabidopsis*	*VND7*	*pVND7::IRX9* (IRX7, IRX8)/*irx9* (*irx7, irx8*)	Partially complement the irx phenotype	↑	↑	Petersen et al. (2012)
Lignin synthesis	Alfalfa	*MsHCT*	*pPvPAL2::HCT* (RNAi)	Increase H, reduce G, S		↑	Pu et al. (2009)
		MsHCT	*pPvPAL2::HCT* (RNAi)	Reduce lignin; increase H; affect growth	↓		Gallego-Giraldo et al. (2014)
		MsC3H	*pPvPAL2::C3H* (RNAi)	Reduce lignin, G, increase H; affect growth		↑	Reddy et al. (2005)
		MsC4H	*pPvPAL2::C4H* (RNAi)	Reduce lignin, S, increase G		↑	Reddy et al. (2005)
		MsF5H	*pPvPAL2::F5H* (RNAi)	Increase G, reduce S		↑	Reddy et al. (2005)
		MsCCR1	*p35S::CCR1* (RNAi)	Reduce lignin, S, increase S/G	↓		Jackson et al. (2008)
		MsCAD1	*p35S::CAD1* (RNAi)	Reduce lignin, S, S/G	↓		Jackson et al. (2008)
		MsC4H	*pPvPAL2::C4H* (RNAi)	Reduce lignin, H, G		↑	Chen and Dixon (2007)
		MsHCT	*pPvPAL2::HCT* (RNAi)	Reduce lignin, G , S, increase hemicellulose	↓	↑	Chen and Dixon (2007)
		MsC3H	*pPvPAL2::C3H* (RNAi)	Reduce lignin, G, S, increase hemicellulose	↓	↑	Chen and Dixon (2007)
		MsCCoAOMT	*pPvPAL2::CCoAOMT* (RNAi)	Reduce lignin, G, S	/	↑	Chen and Dixon (2007)
		MsF5H	*pPvPAL2::F5H* (RNAi)	Reduce lignin, H, S, S/G, increase G		/	Chen and Dixon (2007)
		MsCOMT	*pPvPAL2::COMT* (RNAi)	Reduce lignin, H, G, increase hemicellulose	/	↑	Chen and Dixon (2007)
	Arabidopsis	*AtCAD*	*Atcad-c/cad-d* (T-DNA)	Reduce lignin, S, β-O-4 linkage, increase H, G; affect growth	↓	↑	Sibout et al. (2005)
		AtLAC4-1	*lac4-1* (T-DNA)	Reduce lignin	↓	↑	Berthet et al. (2011)
		AtlLAC4-2/17	*lac4-2/17* (T-DNA)	Reduce lignin, increase S/G; affect growth	↓	↑	Berthet et al. (2011)
		AtCSE-1	*cse-1* (T-DNA)	Reduce lignin, G, increase H	↓	↑	Vanholme et al. (2013c)
		AtMOMT	*momt3* (T133L-E165I-F175I)	Reduce lignin, G, S	/	↑	Zhang et al. (2012)
		AtCCR1	*pAtCESA7::CCR1* (miRNAi)	Reduce lignin, G, S/G, increase S; affect growth	↓		Smith et al. (2013)
	B. distachyon	*BdCAD1*	*cad1* (G192D)	Reduce lignin, S, increase H, G	/	↑	Bouvier d'Yvoire et al. (2013)
	L. leucocephala	*LlOMT*	*p35S::LlOMT* (RNAi)	Reduce lignin, S, increase cellulose		↑	Rastogi and Dwivedi (2006)
	Maize	*ZmCOMT*	*pZmAdh1::COMT* (RNAi)	Reduce lignin, S		↑	Guillaumie et al. (2008)
	Poplar	*PtCCR*	*p35S::CCR* (RNAi)	Reduce lignin, increase G, hemicellulose	↓	↑	Van Acker et al. (2014)
		AtF5H	*pAtC4H::AtF5H* (OE)	Reduce lignin and G, increase H, S, S/G, resinol (β- β) and spirodienone (β-1)	/		Stewart et al. (2009)
	Rice	*Os4CL3*	*p35S::4CL3* (RNAi)	Reduce lignin; affect growth	↓		Gui et al. (2011)
	S. miltiorrhiza	*SmPAL1*	*p35S::PAL1* (RNAi)	Reduce lignin	↓		Song and Wang (2011)
	Sorghum	*SbCOMT*	*comt* (A71V, P150L, G225D, G325S)	Reduce lignin		↑	Sattler et al. (2012)
	Sugarcane	*SbCOMT*	*pOsC4H::COMT* (RNAi)	Reduce lignin	↓	↑	Jung et al. (2012)
	Switchgrass	*PvCOMT*	*pUbi::COMT* (RNAi)	Reduce lignin		↑	Fu et al. (2011)
		Pv4CL1	*pUbi::4CL1* (RNAi)	Reduce lignin, G, increase H, S/G	/	↑	Xu et al. (2011)
		SbCOMT	*pUbi::COMT* (RNAi)	Reduce lignin, S/G, increase hemicellulose	/	↑	Baxter et al. (2014)
Lignin interlinking	Poplar	*AsFMT*	*pPtCesA8/35S::FMT* (OE)	Increase ferulate conjugaes, ester bonds, reduce β-ether bonds	/	↑	Wilkerson et al. (2014)
	Alfalfa	*MsC3H*	*c3h* (G444D)	Increase β-β, aryglycerol endgroup; affect growth	↓		Ralph et al. (2006)
Lignin regulation	*Arabidopsis*	*AtREF4/AtRFR1*	*med5a/5b* (G383S, T-DNA)	Increase G, reduce S, S/G		↓	Bonawitz et al. (2014)
		AtC3′H	*ref8-1* (EMS missense mutant)	Reduce lignin; affect growth	↓		Bonawitz et al. (2014)
			med5a/5b ref8-1 (triple mutant)	Increase β-β, β-5; reduce β-O-4; affect growth	↓		Bonawitz et al. (2014)
	Rice	*OsSWN2S*	*pOsSWN1::OsSWN2S-SRDX*	Reduce lignin, Xyl; affect plant growth		↑	Yoshida et al. (2013)
	Switchgrass	*PvMYB4*	*p35S::MYB4* (OE)	Reduce lignin, G, S		↑	Shen et al. (2012b)
Pectin synthesis	*Arabidopsis*	*AtQUA2-1*	*qua2-1* (R2389stop)	Reduce GalA and de-methyl-esterified HG, increase Gal and Xyl	↓	↑	Francocci et al. (2013)
		AtPME3	*pme3* (T-DNA)	Reduce de-methyl-esterified HG, increase DM; increase resistant to pathogens	/	↑	Francocci et al. (2013), Raiola et al. (2011)
		AtPMEI	*p35S::PMEI* (OE)	Reduce de-methyl-esterified HG; increase resistant to pathogens	↑	↑	Lionetti et al. (2010), Lionetti et al. (2007)
	Wheat	*AcPMEI*	*p35S::PMEI* (OE)	Reduce de-methyl-esterified HG	↓	↑	
	Poplar	*PtGAUT12*	*pUBi3::GAUT12* (RNAi)	Reduce Xyl, GalA, HG, RG; increase Man and Gal	↑	↑	Biswal et al. (2015)
Pectin degradation	*Arabidopsis*	*PcPL1*	*pMDC7_SpPcPL1-HA* (OE)	Increase Glc; affect growth	↓	↑	Tomassetti et al. (2015)

Table 3 (*continued*)

Wall polymer	Species	Gene (source)	Approach	Phenotype	Biomass		Reference
					Yield	Digestibility	
		AnPG	pSAG12:: PG (OE)	Reduce GalA and de-methyl-esterified HG	/	↑	
		AnPGII	p35S::PGII (OE)	Reduce de-methyl-esterified HG; affect growth	↓	↑	Capodicasa et al. (2004), Lionetti et al. (2010)
	Tobacco	AnPGII	p35S::PGII (OE)	Reduce GalA and de-methyl-esterified HG; affect growth	/	↑	Capodicasa et al. (2004), Lionetti et al. (2010)
	Apple	MdPG	p35S:: PGS-2, PGS-3, PGS-4 (OE)	Reduced Gal, Ara, DM, and length of pectin molecular; change cell adhesion	/		Atkinson et al. (2002)
	Poplar	PtxtPL1-27	p35S:: PL1-27 (OE)	Increase solubility of pectin, xylans		↑	Biswal et al. (2015)
Wall protein	Arabidopsis	FaEXP2	p35S::CBMEXP2 (OE)	Reduce cellulose, hemicellulose; increase pectin, stress tolerance	↑		Nardi et al. (2015)
		AtEXPA1,3, 5,10	pOpON::EXPA1,3,5,10 (amiRNA)	Affect growth	↓		Goh et al. (2012)
		AtEXPA7	pEXPA7::EXPA7 (RNAi)	Affect growth	↓		Lin et al. (2011)
		AtEXT3	EXT3 (Enhancer-trap insertion)	Affect growth	↓		Hall and Cannon (2002), Cannon et al. (2008), Saha et al. (2013)
		AtEXT18	EXT18 (Gene-trap insertion)	Affect growth	↓		Choudhary et al. (2015)
		AtEXT1	p35S::EXT1 (OE)		↑		Roberts and Shirsat (2006)
	Rice	OsEXPA8	p35S::EXPA8 (RNAi)	Affect growth	↓		Wang et al. (2014a)
		OsEXPA8	p35S::EXPA8 (OE)		↑		Ma et al. (2013)
		OsEXPA3	p35S::EXPA3 (RNAi)	Affect growth	↑		Qiu et al. (2014)
	Tobacco	TaEXPB23	p35S::EXPB23, pPYK10::EXPB23 (OE)	Increase pectin; increase stress tolerance	↑		Li et al. (2015a)
		TaEXPB23	p35S::EXPB23 (OE)	Increase stress tolerance	↑		Han et al. (2015)
		PpEXP1	p35S::EXP1 (OE)	Increase stress tolerance	↑		Xu et al. (2014)
	Cotton	GhEXPA1	p35S::EXPA1 (OE)		↑		Xu et al. (2013)
		GbEXPATR	p35S::EXPATR (OE)	Increase noncellulosic polysaccharides	↑		Li et al. (2015c)
	Soybean	GmEXPB2	p35S::EXPB2 (OE)		↑		Zhou et al. (2014)

↑ and ↓: respectively indicated significantly positive and negative impacts on biomass enzymatic digestibility under pretreatment; ∕: as no significant effect; blank: as not determined.

lignocellulose recalcitrance (Himmel et al., 2007; Scheller and Ulvskov, 2010). Recent findings indicate that hemicelluloses positively affect biomass enzymatic digestibility under various physical and chemical pretreatments by reducing cellulose crystallinity in *Miscanthus* and rice (Table 2). Notably, the Ara substitution degree of xylan (i.e., the reverse Xyl/Ara ratio) is a mainly positive factor on biomass saccharification in all grass species examined (Fig. 2B), in particular on the non-KOH-extractable residues that comprise 10%–30% of total hemicelluloses. Analysis of the digestion of individual cellulases further indicates that the substituted Ara may interact with the β-1,4-glucan chains in amorphous regions of cellulose microfibrils via hydrogen bonding, thereby significantly reducing cellulose crystallinity (Li et al., 2013b). This finding also suggests that the remaining Ara in non-KOH-extractable residues could aid in maintaining cellulose microfibrils in the native state accessible to amorphous regions for efficient biomass enzymatic hydrolysis, because classic physical and chemical pretreatments performed could only remove partial hemicelluloses and lignin by 10%–60% (Li et al., 2013b; Wang et al., 2014b). In contrast, the removal of nearly all hemicelluloses leads to the reassembly of highly crystalline cellulose macrofibrils that are not digestible by enzymes. In addition, Li et al. (2015b) have reported that the Ara level in non-KOH-extractable residues positively affects plant lodging resistance by reducing the crystallinity of cellulose in rice mutants.

2.3. Lignin

Lignin is an amorphous polymer containing phenylpropane units primarily consisting of three monomers: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Grabber et al., 2004; Sun et al., 2013). It is mainly deposited in secondary cell walls in amounts that vary largely between different plant species. In general, grass and woody plants have lignin contents by weight ranging from 15% to 30%, accounting for 30%–40% of the energy content of the biomass (Boerjan et al., 2003; Table 1). In particular, several rice mutants show low lignin levels at 11%, whereas special germplasm accessions of *Miscanthus* species have high lignin contents up to 28%–31% (Huang et al., 2012; Magid et al., 2004; Meineke et al., 2014)

Because lignin tightly associates with hemicelluloses to maintain plant mechanical strength and biomass recalcitrance (Li et al., 2015b), it is thought to play a vital role in the adaptation of plants to terrestrial environments (Abramson et al., 2010). Lignin is thus considered to exert two primary effects on biomass enzymatic hydrolysis: preventing cellulose microfibril swelling to reduce surface area access for cellulase enzymes, and preventing cellulase action on the cellulose surface (Keating et al., 2006). For instance, lignin typically has a negative impact on biomass enzymatic digestibility under various chemical pretreatments through the indirect increase of cellulose crystallinity in *Miscanthus* accessions (Fig. 2A; Table 2). However, recent findings have suggested that lignin could enhance biomass yield and lignocellulose enzymatic digestion in rice mutants (Wu et al., 2013; Li et al., 2015b). In addition, dual effects of lignin were also observed in other species and transgenic plants (Table 2), and the positive impacts of lignin could be interpreted in three ways: (1) After lignin is extracted via physical and chemical pretreatments, the remaining non-KOH-extractable lignin-hemicellulose complexes could maintain cellulose microfibrils in the native state that are accessible by enzymes in amorphous regions (Li et al., 2014c; Wu et al., 2013); (2) Lignin composed of relatively high proportions of G and H monomers is more extractable after alkaline pretreatment (Li et al., 2014c); (3) The increased monolignol levels in genetic mutants and transgenic plants may not be interlinked to form a complete lignin-carbohydrate complex, leading to more cell wall pores for cellulase enzyme loading and accession (Chandra et al., 2008; Li et al., 2014c; Wang et al., 2014b).

2.4. Pectin

Pectin is the most structurally complex polysaccharide that comprises a major component of primary cell walls and is found in smaller amounts in the secondary walls of both monocots and dicots (Mohnen, 2008) (Fig. 1H). In principle, pectic polysaccharides have been classified into so-called "domains" that include homogalacturonan, xylogalacturonan, apiogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan- II (Atmodjo et al., 2013; Mohnen, 2008). Uronic acids, which are typical components of pectin, are primarily found in glucuronoarabinoxylans and galactosyluronic acid-rich pectin (Kim and Carpita, 1992), and they can be extracted with ammonium oxalate (AO) and other chelating agents. Pectin can form a macromolecular wall-network through molecular substitution with side chains associated with cellulose or hemicelluloses (Marcus et al., 2008; Popper and Fry, 2008; Thompson and Fry, 2000; Zykwinska et al., 2005; Wang et al., 2015b), suggesting that it plays a role in maintaining cell wall structure and biomass recalcitrance. Transgenic plants expressing genes involved in pectin synthesis, modification and degradation exhibit improved biomass digestibility (Biswal et al., 2014, 2015; Francocci et al., 2013; Lionetti et al., 2010; Tomassetti et al., 2015; Xiao and Anderson, 2013), but it is difficult to determine whether this is due to a change in pectin alone rather than in other associated wall polymers. Because of the extremely low level and complicated structure of pectin in mature tissues, it is technically difficult to identify the specific effects of pectin on lignocellulosic enzymatic digestibility after physical or chemical pretreatment. However, based on systems biological analyses of a large population of *Miscanthus* accessions, Wang et al. (2015b) have recently shown that AO-extractable uronic acids are predominately accounting for the positive impact of pectin on biomass enzymatic digestibility by significantly reducing cellulose crystallinity (Fig. 2C). These reports also suggest that uronic acid-rich pectin may interact with the β-1,4-glucan chains that reduce cellulose CrI for high biomass saccharification in *Miscanthus* and beyond.

2.5. Wall proteins

Wall proteins are minor and essential components of plant cell walls (Cassab, 1998; Roberts, 1989, 1990; Showalter, 1993). Despite of wall proteins role in maintaining cell wall strength and flexibility (Choi et al., 2006; Cosgrove, 2000; Jamet et al., 2006; Sampedro and Cosgrove, 2005), little is known about their effects on lignocellulose recalcitrance and biomass enzymatic digestion. Of the numerous cell wall proteins, expansins are responsible for loosening plant cell walls by targeting wall polymers and reducing hydrogen bonding (Cosgrove, 2000; Li et al., 2015a, 2015b; Nardi et al., 2015), suggesting that they should affect cell wall destruction during biomass pretreatment. In addition, extensins are important hydroxyproline-rich wall proteins (Cassab, 1998; Showalter, 1993) and likely form a cross-linked wall network (Cannon et al., 2008; Fry, 1986; Lamport et al., 2011; Velasquez et al., 2011). The mutation of extensins genes leads to reduced biomass yields, whereas overproduction of extension could enhance biomass production in *Arabidopsis* (Cannon et al., 2008; Choudhary et al., 2015; Roberts and Shirsat, 2006; Saha et al., 2013). Furthermore, the overexpression of extensins genes in transgenic tobacco cells can either decrease cellulose crystallinity and increase xyloglucan or raise hydration capacity and wall biomass (Tan et al., 2014). Hence, it remains to examine whether expansins and extensins have significant effects on biomass enzymatic digestibility in bioenergy crops.

3. Genetic modification of plant cell walls

Selection of genetic mutants and transgenic plants that targets cell wall modification has been considered to enhance biomass enzymatic digestibility. However, as plants have to generate a complex wall-network enabling to complete life cycles rather than to meet biofuel purpose, it remains a challenge to maintain plant normal growth and mechanical strength with high biomass yields (Wang et al., 2014b; Xie and Peng, 2011). To address this challenge, the identification of appropriate genes is a crucial initial step, and the genetic engineering

approach used must include appropriate promoters and effective systems for gene transformation and genetic mutagenesis. To date, more than one thousand genes are thought to involve in cell wall biosynthesis, degradation and modification (Guo et al., 2014), but only a small number of these genes have been characterized, as shown in Table 3. Based on the functions of these genes, potential genetic approaches are described in this section.

3.1. Wall polymer synthesis

Increasing the biomass yield of bioenergy crops by enhancing wall polymer synthesis, in particular on cellulose, is a major goal of bioenergy engineering. In higher plants, cellulose is synthesized by cellulose synthase (CESA) complexes and deposited into the cell wall in a directional manner (Brabham and DeBolt, 2013; Mueller et al., 1976; Somerville, 2006). These CESA complexes are assembled in the Golgi apparatus and then exported to the plasma membrane for cellulose biosynthesis in three major steps: β-1,4-glucan chain initiation, elongation, and termination (Peng et al., 2002). Since the cellulose synthase (CesA) gene was first identified (Arioli et al., 1998), the CesA superfamily have been characterized in many plant species, including Arabidopsis (Taylor et al., 2003; Persson et al., 2007b; Desprez et al., 2007), rice (Tanaka et al., 2003; Wang et al., 2010), maize (Appenzeller et al., 2004), cotton (Li et al., 2013a), barley (Burton et al., 2004) and poplar (Joshi et al., 2004; Djerbi et al., 2005). For example, OsCesA1, OsCesA3, and OsCesA8 form the CESA complex typical for primary cell wall biosynthesis, whereas OsCesA4, OsCesA7, and OsCesA9 are involved in secondary cell wall formation in rice (Wang et al., 2010). To dissect the biological functions of CesAs, dozens of distinct CesA mutants have been identified through multiple genetic approaches, but most mutants exhibit reduced cellulose levels and defective plant growth (Table 3). Due to this reduction in cellulose, several mutants have exhibited low cellulose crystallinity and high biomass

enzymatic digestibility, but one Osbc13 rice mutant with an amino acid substitution in OsCesA9 exhibits normal plant growth and cadmium tolerance (Song et al., 2013). Furthermore, our recent data indicate that conserved-site mutation of OsCesA9 could not only increase plant lodging resistance and biomass yield but also enhance biomass enzymatic digestibility in rice by reducing cellulose CrI and DP (unpublished), thereby providing a potential genetic engineering approach for cellulose modification. Surprisingly, the overexpression of CesA genes results in defective plant growth and reduced biomass yield in transgenic poplar and barley (Joshi et al., 2011; Tan et al., 2015), suggesting that an inducible promoter or a particular CesA gene may be required for transgenic plant selection.

Hemicellulose, another group of abundant cell wall polysaccharides, is synthesized in the Golgi apparatus by a plethora of glycosyltransferases (GTs). There are seven GT gene families associated with hemicelluloses biosynthesis (Guo et al., 2014; Pauly et al., 2013; Scheller and Ulvskov, 2010) and cellulose synthase-like (Csl) genes encoded by Golgi-localized glycan synthases are also involved in hemicellulose synthesis. Hemicellulose mainly consists of C5 sugars such as Xyl and Ara, which cannot be fermented into bioethanol by classic yeast strains. However, as xylan has a positive effect on biomass enzymatic digestibility by reducing cellulose CrI, particularly on Ara substitution degree in non-KOH-extractable hemicelluloses (Li et al., 2013b, 2015b), increasing hemicellulose represents another promising strategy for enhancing both biomass yield and lignocellulose enzymatic saccharification in bioenergy crops. Recently, several genetic mutants and transgenic plants associated with hemicellulose biosynthesis have exhibited a reduced biomass yield and an increased biomass digestibility in rice, wheat, maize and poplar (Biswal et al., 2015; Chiniquy et al., 2012; Chen et al. and Peng, 2013; Derba-Maceluch et al., 2014; Shen et al., 2012a; Yoshida et al., 2013). Among the identified mutants, the GT43 and GT47 enzymes catalyze xylan backbone elongation (Brown et al., 2007, 2009; Chiniquy et al., 2013; Chen et al., 2013; Keppler and
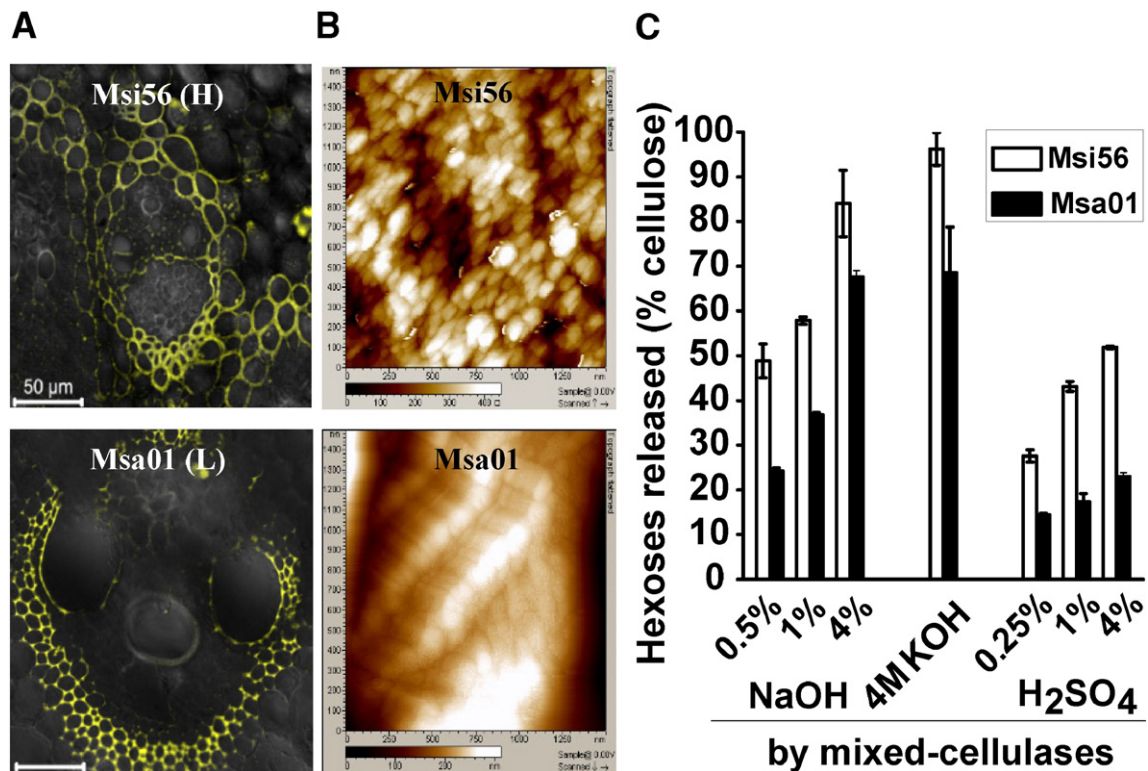

Fig. 3. Potential cellulase (GH9) gene engineering on lignocellulose modification towards enhanced biomass digestibility. (A) Observation of cellulase activities in situ of two Miscanthus accessions; (H) and (L) indicate relatively high and low cellulase activities (provided by Yang B); (B) images of macrofibrils faces in two accessions under atomic force microscope (AFM) after hemicellulose and lignin of biomass are almost extracted; Msi56 shows relatively short macrofibrils, corresponding to its low cellulose CrI and DP, compared with Msa01 (provided by Xu ZD); (C) Msi56 and Msa01 exhibit distinct hexoses yields after chemical (NaOH, H₂SO₄) pretreatments and sequential enzymatic hydrolysis of biomass (Li et al., 2013b).
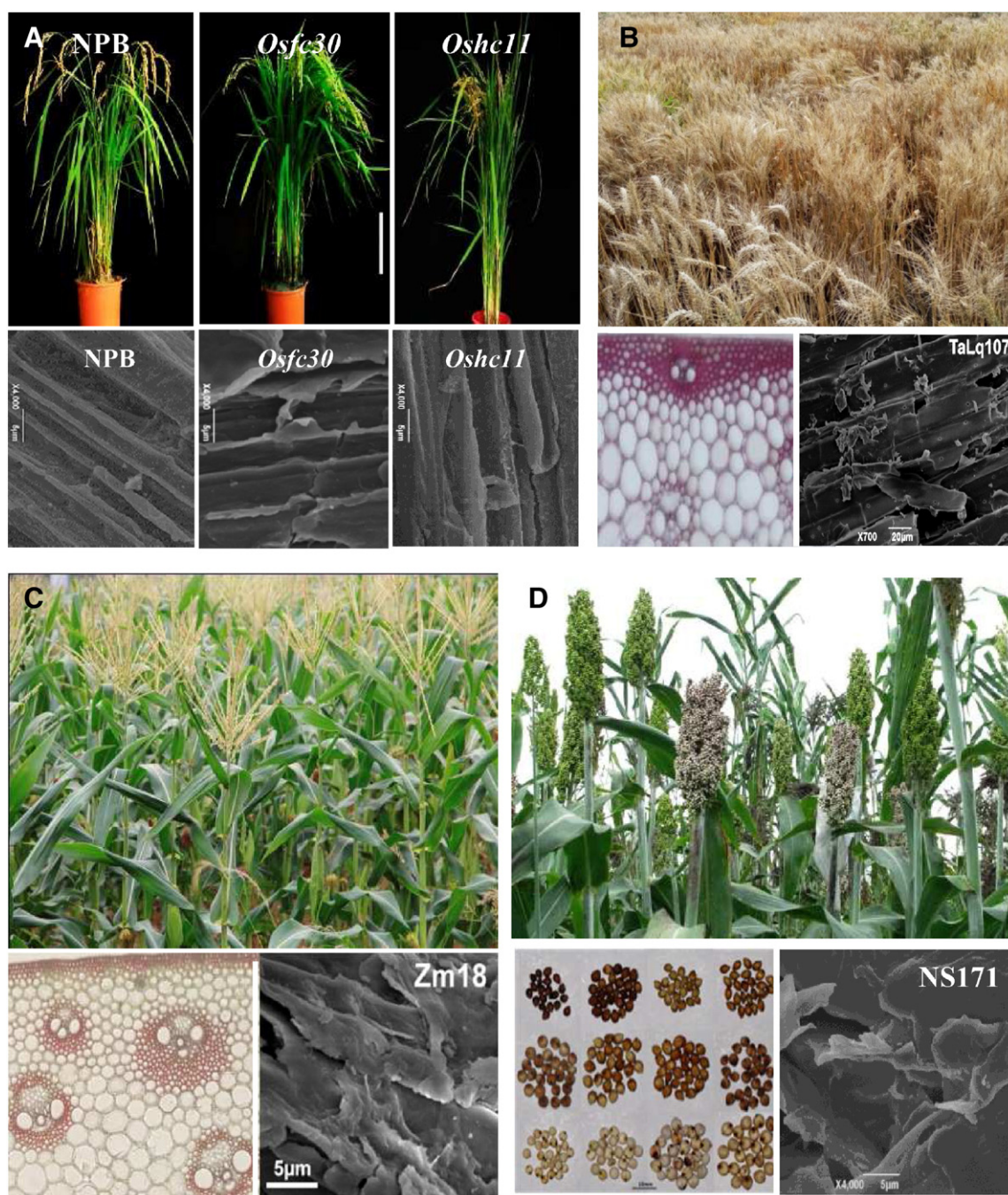
Fig. 4. Dedicated bioenergy crops. (A) Two distinct rice mutants (*Osfc30*, *Oshc11*) show enhanced lodging resistance, high biomass yields and effective enzymatic saccharification (down rights: rougher biomass residue faces) by either enhancing hemicellulosic Ara (*Osfc30*; Li et al., 2015b) or adding silicon into plant growth culture and land field (*Oshc11*; Zhang et al., 2015), compared with wild type (NPB); (B) wheat variety (TaLq107) has relatively low lignin level (down left: Wiesner's staining) and high biomass enzymatic digestibility (down right: rougher residue face) under alkali pretreatment compared with other cultivars (Wu et al., 2013, 2014b); (C) selected maize variety (Zm18) shows high seed yield and biomass enzymatic saccharification upon 4% NaOH pretreatment (Jia et al., 2014); (D) selected sweet sorghum variety (NS171) from large population of cultivars (down left: seeds of different accessions) exhibits much high hexoses yield under 1% NaOH pretreatment and enzymatic hydrolysis, coupled with high soluble sugars in stem and high seed yield (Li et al., 2014a).

Showalter, 2010; Lee et al., 2007, 2009, 2010; Persson et al., 2007a; Wu et al., 2009, 2010; Zhong et al., 2005), whereas GT8 and GT61 are involved in side-chain substitution (Anders et al., 2011; Bromley et al., 2013; Chiniquy et al., 2012; Lee et al., 2012; Mortimer et al., 2010). The expression of these four genes under appropriate promoters, in particular on the *GT8* and *GT61* genes, should enhance both biomass yield and lignocellulose enzymatic digestibility in transgenic plants (Bromley et al., 2013; Biswal et al., 2015; Chiniquy et al., 2012; Mortimer et al., 2010; Petersen et al., 2012).

Lignin biosynthesis involves in two major processes with various types of enzymes that enable the catalysis of the synthesis of three monomers (Boerjan et al., 2003). In the first process, more than ten enzymes are involved in monolignol biosynthesis: phenylalanine ammonia lyase (PAL) (Sewalt et al., 1997; Song and Wang, 2011), cinnamate 4-hydroxylase (C4H) (Blee et al., 2001; Reddy et al., 2005; Sewalt et al., 1997), 4-coumarate-CoA ligase (4CL) (Gui et al., 2011; Sun et al., 2013; Xu et al., 2011), shikimate hydroxycinnamoyl transferase (HCT) (Gallego-Giraldo et al., 2014; Vanholme et al., 2013a), coumarate 3-hydroxylase (C3H) (Kim et al., 2014a; Pu et al., 2009; Reddy et al., 2005), caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) (Li et al., 2013c; Pang et al., 2014), cinnamoyl-CoA reductase (CCR) (Van Acker et al., 2014; Chabannes et al., 2001; Jackson et al., 2008; Smith et al.,

Table 4
Physical and chemical pretreatment approach for altering wall polymer feature and enhancing biomass enzymatic digestibility.

Wall polymer alteration	Species	Pretreatment	Biomass digestion	Reference
Cellulose level	Maize straw	NaOH	36%–98%	Jia et al. (2014)
CrI	Wheat & Rice straw	NaOH, H₂SO₄	60%–93%	Wu et al. (2013)
	Kenaf core fiber	Microwave, ionic liquid	60%–90%	Ninomiya et al. (2014)
	Rice straw	(NH₄)₂CO₃	72%	Kim et al. (2014b)
	Switchgrass stem	Ionic liquid	80%	Sathitsuksanoh et al. (2015)
	Miscanthus strem	NaOH, H₂SO₄	26%–86%	Zhang et al. (2013)
	Sugarcane bagasse	Ionic liquid	98%	Gao et al. (2013)
	Sugarcane bagasse	Dilute acid, ionic liquid	96%	Jiang et al. (2013)
	Poplar wood	Ionic liquid	97%	Wu et al. (2014a)
	Bamboo chips	Subcritical water	42%	Mohan et al. (2015)
	Bamboo chips	Steam explosion, alkaline	46%–64%	Sun et al. (2014)
	Pinus chips	Ionic liquid	90%	Trinh et al. (2015)
	Switchgrass	Ionic liquid	96%	Li et al. (2010a)
	Rice straw	Ultrasonic irradiation , ionic liquid	96%	Yang and Fang (2014)
	Mustard stalk	Steam explosion, alkaline, dilute acid	81%	Kapoor et al. (2015)
DP	Sweet sorghum bagasse	NaOH, H₂SO₄	40%–100%	Li et al. (2014a)
	Miscanthus stem	NaOH, H₂SO₄	26%–86%	Zhang et al. (2013)
	Cotton stalk	Steam explosion, NaOH	78%	Huang et al. (2015b)
Hemicellulose level	*Miscanthus* stem	NaOH	99%	Xu et al. (2012)
Xyl	Sugarcane bagasse	Liquid hot water, NaOH, HCl	72%–77%	Yu et al. (2013)
	Barley straw	Alkali, enzymatic extrusion	75%	Duque et al. (2014)
Ara	Wheat bran	Hydrothermal microwave	91%	Aguedo et al. (2015)
Xyl/Ara	Sweet sorghum bagasse	NaOH, H₂SO₄	40%–100%	Li et al. (2014a)
	Wheat bran	Hydrothermal microwave	91%	Aguedo et al. (2015)
	Miscanthus stem	NaOH, H₂SO₄	96%	Li et al. (2013b)
	Wheat & Rice straw	NaOH, H₂SO₄	60%–93%	Wu et al. (2013)
Lignin level	Maize straw	NaOH, H₂SO₄	98%	Jia et al. (2014)
	Rice straw	Microwave, alkali	70%	Ma et al. (2009)
	Rice straw	*Trichoderma viride*	56%	Ghorbani et al. (2015)
	Miscanthus stem	NaOH, H₂SO₄	100%	Si et al. (2015)
	Sweet sorghum bagasse	NaOH, H₂SO₄	40%–100%	Li et al. (2014a)
	Spruce, Birch, Sugarcane bagasse	Alkaline oxidation	80%–97%	Kallioinen et al. (2013)
	Poplar wood	White rot fungus	85%	Wang et al. (2013)
	Eucalyptus chips	Endophytic fungi	55%	Martín-Sampedro et al. (2015)
	Eucalyptus chips	Steam	47%	Martin-Sampedro et al. (2014)
G	Switchgrass stem	Ionic liquid	96%	Li et al. (2010a)
	Corncob	Hot compressed water	81%	Daorattanachai et al. (2013)
	Miscanthus stem	NaOH	93%–100%	Li et al. (2014b)
S	*Miscanthus* stem Empty palm fruit bunch, Typha straw	Organosolv,Hydrotherma, 2-naphthol	36%–45%	Timilsena et al. (2013)
H/G	Wheat & Rice straw	NaOH	60%–93%	Wu et al. (2013)
S/G	*Miscanthus* stem	NaOH, H₂SO₄	99%	Xu et al. (2012)
	Maize straw	NaOH	36%–98%	Jia et al. (2014)
	Poplar wood	Hot water	91%	Studer et al. (2011)
	Mixed hardwood chips	H₂SO₄	55%	Lim et al. (2013)
Pectic level	Oilseed rape straw	Steam explosion	86%	Wood et al. (2014)
Uronic acids	*Miscanthus* stem	NaOH, H₂SO₄	39%–63%	Wang et al. (2015b)

2013), cinnamyl alcohol dehydrogenase (CAD) (Bouvier d'Yvoire et al., 2013; Jackson et al., 2008; Sibout et al., 2005), ferulate 5-hydroxylase (F5H) (Stewart et al., 2009) and caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT) (Baxter et al., 2014; Fu et al., 2011; Guillaumie et al., 2008; Jung et al., 2012; Sattler et al., 2012). Subsequent enzymatic steps that catalyze hydroxylation and methylation reactions then produce lignin monomers (Shi et al., 2009). To date, many genetic mutants and transgenic plants involved in lignin biosynthesis have been identified in different plant species (Table 3). For example, sorghum *SbCOMT*s, which include four site mutations of *COMT*, exhibit lower levels of lignin and improved biomass digestibility (Sattler et al., 2012), whereas RNAi silencing of the *Pv4CL1* gene in transgenic switchgrass plants reduces lignin content and increases H-monomer, leading to enhanced biomass digestibility (Xu et al., 2011). Although the down-regulation of these genes in genetic mutants and transgenic plants could enhance biomass enzymatic digestibility in almost all samples examined, it also affects normal growth and stress tolerance with a significantly reduced biomass yield in these plants (Table 3). It has thus suggested that a reduction of lignin level is not an appropriate approach to cell wall modification in bioenergy crops.

Pectin biosynthesis and modification are highly associated with plant growth and stress response, due to its complicated structures in plant cell walls (Caffall and Mohnen, 2009; Ridley and O'Neill, 2001; Wolf et al., 2009). Despite that many genes are known to play a role in pectin biosynthesis, it remains to explore their effects on biomass digestibility (Xiao and Anderson, 2013). However, recent progress has indicated that expression of a fungal polygalacturonase or of a pectin methylesterase inhibitor could lead to a reduced de-methylesterified homogalacturonan and increased enzymatic saccharification in transgenic *Arabidopsis* plants (Lionetti et al., 2010). In addition, down regulation of pectin methylesterase (PME) could drastically increase biomass production and improve saccharification in transgenic *Arabidopsis* (Chen and Peng, 2013). By comparison, the down regulation of *PME3*, *QUA2* and *GAUT12* enhances biomass digestibility but also affects plant growth in *Arabidopsis* mutants and poplar transgenic plants (Biswal et al., 2014; Francocci et al., 2013). In terms of cell wall proteins, the overexpression of *EXP* and *EXT* genes could enhance stress tolerance and biomass yield in transgenic plants, whereas the down-regulation of these genes in most cases leads to defective plant growth and a reduced biomass yield in mutants (Cannon et al., 2008; Choudhary et al., 2015; Goh et al., 2012; Lin et al., 2011; Saha et al., 2013). But, it remains to test whether altering wall protein expression affects biomass digestibility in transgenic plants.
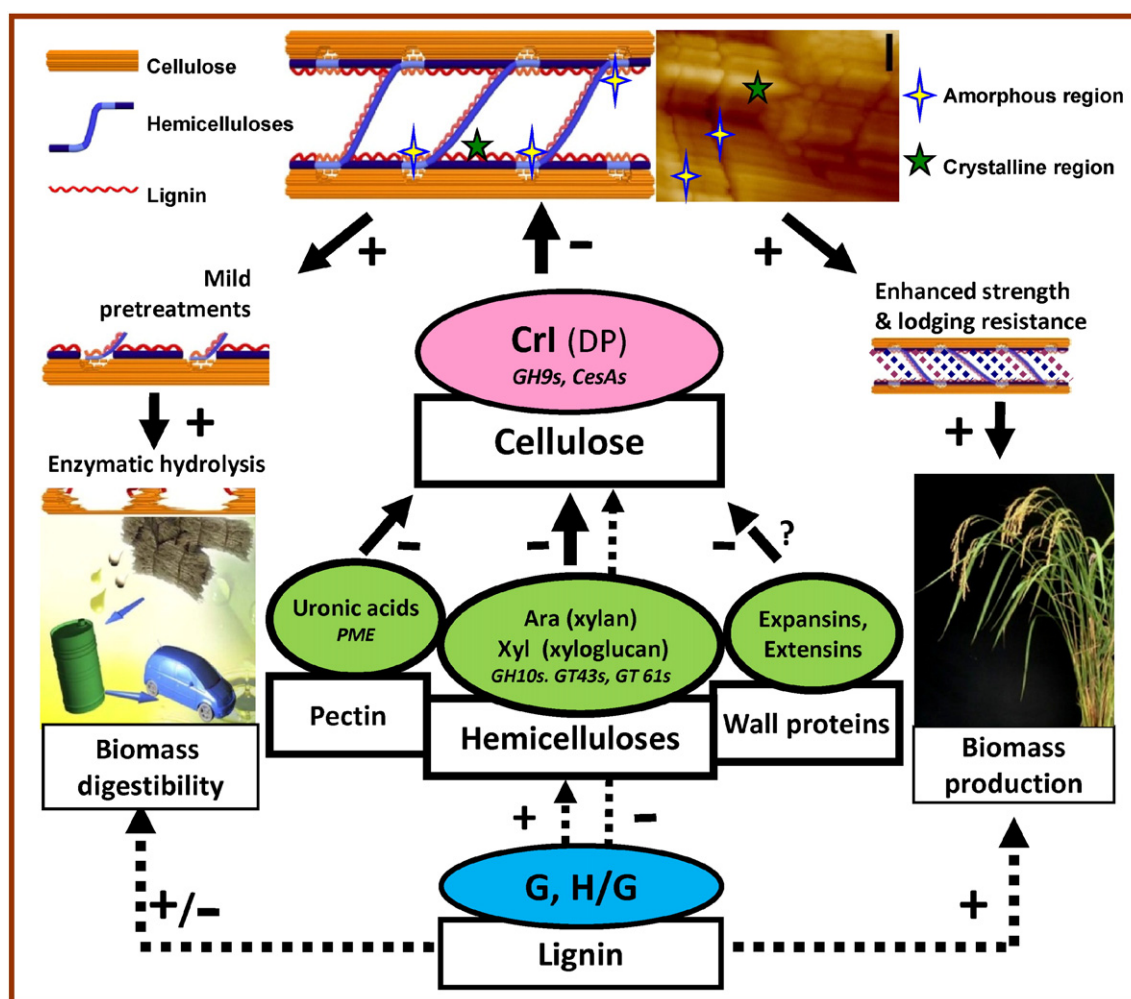
Fig. 5. A model on cell wall modification for enhancing biomass yield and biofuel production. Based on the cell wall structure (top), increasing of amorphous regions (density and depth, left on the top) of native cellulose microfibrils, presumed as AFM observed groove-like regions of the extracted cellulose macrofibrils (right on the top), could largely enhance both biomass saccharification and plant lodging resistance by reducing cellulose CrI (and DP). (1) Xylan's Ara, xyloglucan's Xyl, galacturonic acids and specific wall protein structures may negatively affect cellulose CrI probably by their direct interactions with internal molecules of amorphous regions, whereas lignin and G-monomer may either have direct effects or play an indirect role by its association with hemicelluloses (Li et al., 2013b, 2014b, 2015a, 2015b, 2015c; Wang et al., 2015b); (2) The groove-like amorphous regions are rich in branched-Ara of xylan (secondary cell walls) or branched-Xyl of xyloglucan and uronic acids of pectin (primary cell walls), while crystalline regions may be tightly interlinked with lignin and the backbone of xylan with less branched-Ara (Wang et al., 2014b); (3) According to the lignocellulose structure observation (right on the top) by AFM (bar as 200 nm) in the *Miscanthus* stem, in which hemicelluloses and lignin are almost extracted, a related groove-like cell wall structure (left on the top) is modified subjective to the main findings reported by Li et al., 2015b; Wang et al., 2014b and Wang et al., 2015a. . +/−: indicate positive/negative impacts on biomass yield and lignocellulose enzymatic digestibility, respectively.

3.2. Wall polymer degradation

There are different types of enzymes associated with wall polymer degradations that involve in cell wall remodelling and wall-network construction in plant cells. Endo-β-1,4-glucanases (EGases, EC3.2.1.4) cleave the internal β-1,4-glycosidic bonds between two glucose moieties at the center of a polysaccharide chain. Plant EGase enzymes belong to the glycoside hydrolase family 9 (GH9) that consists of three subclasses (A, B, C), each with specificity for different substrates (Urbanowicz et al., 2007; Xie et al., 2013). KORRIGAN protein, a *GH9A* family member, can play a role in cellulose biosynthesis by either cleaving a sterol-cellodextrin substrate (Peng et al., 2002) or removing glucan chains incorrectly assembled in the growing microfibrils (Lane et al., 2001). By comparison, *KORRIGAN* (*kor*) mutants exhibit reduced cellulose levels and defective plant growth, whereas overexpression of *PtCel9A1* (*GH9A*) leads to reduced cellulose CrI and increased biomass yield (Glass et al., 2015). In the *GH9B* and *GH9C* families, the enzymes have cello-oligosaccharide release and xyloglucan cleavage properties in plants. In rice mutants, the expression levels of *OsGH9B* family

genes are significantly correlated with cellulase activity and cellulose CrI, suggesting that the *GH9B* family should play a role in cellulose degradation (Fig. 3). Recent reports have indicated that the overexpression of *PtGH9B5/C2* genes leads to defective plant growth and biomass yield, but RNAi silencing of the *AtGH9C2* gene could reduce cellulose CrI and enhance biomass yield (Glass et al., 2015).

In addition to the GH9 enzymes having activities on xyloglucan digestion, GH10 and GH11 enzymes are also involved in hemicelluloses (such as xylan) degradation. Overexpression of the *xynB* (*GH11*) gene results in enhanced biomass digestibility in transgenic maize (Shen et al., 2012a), whereas RNAi silencing of the *xyn10A* (*GH10*) gene increases biomass yield in poplar (Derba-Maceluch et al., 2014). With respect to pectin degradation, overexpression of the *PL* and *PG* genes enhances biomass enzymatic digestibility but also leads to reduced biomass yield in transgenic *Arabidopsis* and poplar (Lionetti et al., 2010; Tomassetti et al., 2015). Therefore, a feasible approach is required to enhance both biomass yield and lignocellulose digestibility via the genetic manipulation of the *GH9*, *GH10*, *GH11* genes involved in cellulose and hemicelluloses modification but not in pectin degradation.

3.3. Wall network construction

Wall network construction involves the coordinated deposition and interlinking of wall polymers, which is dependent on the dynamic regulation of wall polymer synthesis and degradation. Although dozens of transcription factors (e.g., NAC, MYB, SND, VND, C3H, and SWN) have been shown to regulate wall polymer biosynthesis (Zhong and Ye, 2007), the overexpression or silencing of most genes leads to defective plant growth and reduced biomass yield in transgenic plants or genetic mutants (Chai et al., 2014a, 2014b, 2015; Kubo et al., 2005; McCarthy et al., 2009; Ralph et al., 2006; Yoshida et al., 2013). Expression of the *IRX* gene via the *AtVND7* promoter in *Atirx* mutants defective in hemicelluloses biosynthesis could enhance both biomass yield and lignocellulose enzymatic digestibility (Petersen et al., 2012). In addition, expression of *OsSWN1* via the *OsACT* promoter enhances biomass saccharification in transgenic rice (Yoshida et al., 2013). More recently, our results have suggested that site-specific mutation of MYB transcription factors could maintain normal plant growth with enhanced biomass digestibility by altering cell wall composition in rice mutants (unpublished). In conclusion, using appropriate promoter(s) or site-specific mutation should be considered in the genetic manipulation of transcription factors for cell wall modification.

Three monomers (H, G, S) are linked by ether-, ester- and C—C bonds that are irregularly repeated in lignin structure, which determines the pattern of lignin interlinkage with wall polysaccharides in cell wall network. Ralph et al. (2006) have shown that reducing the β-aryl ether level in the lignin structure affects plant growth and biomass yield in alfalfa mutants upon site-specific mutation of the *C3H* gene. However, Wilkerson et al. (2014) have reported that replacing β-ether bonds with ferulate conjugates and ester bonds could maintain normal plant growth and enhance biomass saccharification in the transgenic poplar lines by overexpressing the *AsFMT* gene (Table 3). In addition, Li et al. (2014c) have noted that the minor non-KOH-extractable lignin-polysaccharides-interlinking complex is a target for a simple genetic modification of plant cell walls.

In addition to cell wall homologous manipulation, attempts to overexpress heterologous cell wall degradation enzymes have been made with success in transgenic plants. For example, cellulases or xylanases native to micro-organisms have been thermo-stably expressed in various cellular compartments without adverse effects on plant growth, but enhance the hydrolysis activities of transgenic plants (Shen et al., 2012a). In addition, introducing soluble polysaccharides into plant cell walls could improve biomass solubility with little impact on plant growth. For instances, introducing hyaluronan synthase from chlorella viruses into tobacco has led to enhanced cellulose hydrolysis upon heat and acid pretreatment in transgenic plants (Abramson et al., 2010). More recently, Vega-Sánchez et al. (2015) have shown that introducing β-1,3-1,4-glucans into *Arabidopsis* by expressing the rice *MLG* synthase gene driven by a senescence-associated promoter could increase glucose levels up to 4-fold and biomass saccharification by 42% in transgenic plants.

4. Evaluation of bioenergy crop breeding

Lignocellulosic biomass can be collected from herbaceous grasses, food crop residues and fast-growing trees. In terms of the bioenergy crop breeding, in particular for the development of lignocellulosic biofuels, the main challenges include maximization of biomass yield; improvement of biomass quality and maintenance of sustainability while minimizing agricultural inputs; as well as prevention of competition with food production (Chen and Peng, 2013). In this section, genetic breeding approaches are considered through the selection of natural germplasm accessions, genetic mutants and transgenic plants in major bioenergy crops.

4.1. Herbaceous grasses

Among herbaceous grass plants, *Miscanthus* has been considered one of the leading lignocellulose feedstock candidates for biofuel production (Brosse et al., 2012). As a C4 perennial plant, *Miscanthus* is of high biomass yield and well adaptation to various environment conditions with minor requirements of water and fertilizers (Lewandowski et al., 2003). It also exhibits a high combustion quality due to low moisture and ash content in dry biomass matter (Hodgson et al., 2010). *Miscanthus* is mainly originated from East Asia and nearby Pacific islands with more than 17 species identified worldwide (Jakob et al., 2009). As thousands of natural germplasm accessions from four major *Miscanthus* species exhibit diverse cell wall compositions and features, as well as varied biomass enzymatic digestibility and bioethanol productivity (Huang et al., 2012; Li et al., 2013b; Zhang et al., 2013) (Fig. 2), several *Miscanthus* accessions have been selected as the desire bioenergy plants under development in large-scale breeding (Fig. 3). In addition, genetic modification of plant cell walls could lead to an enhancement on biomass yield and enzymatic saccharification in the transgenic *Miscanthus* accessions by expressing the key genes that correspond to alterations of the major wall polymer features such as lignocellulose crystallinity (CrI), cellulose DP and hemicellulosic Xyl/Ara.

Switchgrass is a widely adapted endemic species of North American native ecosystem with two ecotypes (Parrish and Fike, 2005). Despite that it has relatively less biomass yield than *Miscanthus*, switchgrass has been ranked on the top list among the 18 perennial grass species due to its considerable within-species variability for the potential increase of biomass yield through further breeding (Lewandowski et al., 2003). Recent reports indicate that four switchgrass genotypes have significant difference in lignin content, especially the ratio of three monomers and considerable amounts of *p*-coumaric acid and ferulic acid (Yan et al., 2010), suggesting the potential genetic modification of lignin structures in transgenic plants that favors the use of alkaline-based pretreatment for subsequently efficient enzymatic hydrolysis (Alizadeh et al., 2005; Galbe and Zacchi, 2007; Sun and Cheng, 2002). In addition, as the full genome sequence of switchgrass is accomplished, molecular breeding becomes available to improve biomass yield and quality.

4.2. Food crops

Rice, wheat and maize are major annual food crops that can provide approximately 75% of total agricultural lignocellulose residues over the world. Although significant amounts of crop residues need to remain in the field for soil conservation and sustainable grain production, approximately 20%–50% of total lignocellulose could be applied to biofuel production (Antizar-Ladislao and Turrion-Gomez, 2008; Liu et al., 2010). Thereby, the breeding of bioenergy food crops becomes important to enhance both biomass yield and enzymatic digestibility by selecting genetic mutants and transgenic plants (Fig. 4). For instance, among the selected dozens of rice cell wall mutants, two distinct mutants exhibit enhanced plant lodging resistance, high biomass yield and effective lignocellulose enzymatic saccharification (Fig. 4A), indicating potential genetic modification of hemicelluloses composition and structure by increasing Ara level in transgenic plants (Li et al., 2015b). It also suggests an approach for enhancing plant mechanic strength and biomass digestibility in the mutants by applying silicon fertilizer to field (Zhang et al., 2015). Furthermore, Wu et al. (2013, 2014) and Jia et al. (2014) have respectively identified several varieties with high biomass enzymatic saccharification from large populations of wheat and maize cultivars (Fig. 4B, C). Those selected varieties could be used either as genetic parents of breeding or in transgenic plant selection.

Among the annual food crops, sweet sorghum has emerged as an exceptional bioenergy crop because it contains both large amounts of fermentable soluble sugars at stalk and degradable lignocellulose at bagasse (Zegada-Lizarazu and Monti, 2012; Zegada-Lizarazu et al., 2012). It grows fast and has the high tolerance to drought, submergence and

salt stresses. In addition to its high grain yield of seeds, sweet sorghum can potentially produce 20 million tons of bioethanol per year in the alkaline soils in the north of China (Shi, 2008). Based on integrative analysis of 63 representative sweet sorghum accessions, Li et al. (2014a) find that both soluble sugar content and dry bagasse level are not significantly correlated with lignocellulose enzymatic saccharification, indicating that germplasm collection and genetic breeding should be used to screen out the desirable sweet sorghum accessions as bioenergy crop (Fig. 4D). It also suggests potential genetic approach for enhancing both soluble sugar level and lignocellulose digestibility with reduced yeast fermentation inhibitors in transgenic sweet sorghum plants.

4.3. Woody trees

Woody plants are rich in lignocellulose for high biofuel production. In particular, short-rotation woody trees are considered as ideal energy plants due to their fast growth, low land occupation, high disease resistance and low management cost (Sims et al., 2001). Among the bioenergy woody trees, poplar is one of leading candidates for selecting plants with interesting traits via molecular breeding (Karp and Shield, 2008) subjective to its rather mature transgenic technique. In addition, willow, pine and eucalyptus are also considerable lignocellulose sources, but the appropriate genetic engineering approaches to enhance biomass enzymatic saccharification and biofuel conversion rates remain under development (Chen and Peng, 2013).

5. Biotechnology of biofuel process

Biomass processing principally involves three major steps: biomass pretreatment, enzymatic digestion and yeast fermentation (Rubin, 2008). To reduce biomass processing cost and to raise bioethanol conversion efficiency, optimal technologies comprising three steps are discussed in this section, based on their distinct actions in wall polymer extraction and wall polymer feature alteration in major bioenergy crops.

5.1. Biomass pretreatment

As an initial step in enhancing biomass saccharification, a variety of pretreatments have been applied including physical, chemical and biological approaches (Trinh et al., 2015). Different pretreatments appear to play distinct roles by selectively removing wall polymers or increasing the porosity and accessibility of biomass particles (Wu et al., 2014b). Over the past several years, pretreatments applicable for enhancing biomass enzymatic digestibility have been performed in different plant species, resulting in distinct alterations of wall polymer levels and features, as shown in Table 4. Chemicals such as acids (H_2SO_4, H_3PO_4), alkalis (NaOH, CaO, $NH_3·H_2O$) and salts or ionic liquids are often used as effective pretreatments. In principal, alkali pretreatment extracts wall polymers by disassociating hydrogen and other covalent bonds with cellulose microfibrils (Hendriks and Zeeman, 2009), whereas acid pretreatment often digests wall polymers by splitting strong chemical bonds under high temperatures (Li et al., 2010a; Xu et al., 2012; Zheng et al., 2009). In addition, strong salts or ionic liquid pretreatments mainly dissolve and separate cellulose from other wall polymers (da Costa Lopes et al., 2013). As a result, alkali pretreatment at relatively low temperatures could lead to much higher biomass enzymatic digestibility, compared to acid pretreatment performed in the biomass samples (Jia et al., 2014; Wang et al., 2015b; Wu et al., 2013; Xu et al., 2012). Furthermore, Li et al. (2014b) report that mild alkali pretreatment effectively extracts G-rich lignin for high biomass digestibility in *Miscanthus*. By comparison, ionic liquid pretreatment could enhance lignocellulose enzymatic saccharification in biomass samples with low cellulose CrI (da Costa Lopes et al., 2013; Trinh et al., 2015) and low lignin levels (Li et al., 2010a). More recently, Si et al. (2015) have shown that two-step pretreatments with 2% NaOH and 1% H_2SO_4

are optimal for enhancing biomass digestibility in hemicelluloses-rich samples via the effective co-extraction of hemicelluloses and lignin.

Steam explosion, heat water and hydrothermal microwave have been applied as physical pretreatments in different biomass materials (Aguedo et al., 2015; Kapoor et al., 2015; Yu et al., 2013). For instance, Wood et al. (2014) and Huang et al. (2015b) find that stem explosions are powerful for enhancing biomass digestibility in cotton stalks or rapeseeds residues by reducing cellulose DP and extracting wall polymers. In addition, Li et al. (2013c) report that hot water is effective for high bioethanol production in *Miscanthus* accessions rich in cellulose level, whereas Aguedo et al. (2015) find that hydrothermal microwave pretreatment could increase biomass hydrolysis by 91% in the wheat bran samples with relatively high arabinoxylans. Regardless of being time-consuming, biological pretreatment is another approach for specific wall polymer extraction (Martín-Sampedro et al., 2015; Ishola et al., 2014). For instance, white rot fungi can be used for specific lignin digestion and white termite can effectively disassemble lignocellulose structures, leading to an enhanced biomass enzymatic saccharification in woody plants (Ghorbani et al., 2015; Wang et al., 2013).

5.2. Lignocellulose enzymatic hydrolysis

Enzymatic hydrolysis is a typical biochemical conversion of lignocellulose into monomer sugars for use in subsequent yeast fermentation into bioethanol (Balat, 2011; Singh et al., 2015). The cocktail-like multiple enzymes are typically applied for the biochemical degradation of pretreated biomass residues, mainly via cellobiohydrolases-I (CBHI), endo-1,4-β-glucanases (EGII), β-glucosidases (BG) and xylanases (Li et al., 2010b; Vanholme et al., 2013b). In particular, three types of enzymes (CBHI, EGII, BG) are jointly required for cellulose degradation into glucose, and xylanases are involved in xylan digestion into pentose sugars (Kumar and Wyman, 2009; Nakamura et al., 2011). Progress has been made in improving mixed-cellulase activities for biomass enzymatic hydrolysis, including genetic engineering of microorganism strains to increase enzyme secretion and productivity, multiple biochemical approaches for efficient enzyme purification and stable storage, and supplying enzyme cofactors and diminishing inhibitors for high enzyme activity (Yang and Fang, 2014). However, the efficiency of biomass enzymatic hydrolysis requires an optimal ratio or proportion of enzymes, as well as their synergistic cooperation, which is highly dependent on the properties of biomass substrates, including the porosity and accessibility of biomass particles; the surface structure of cellulose microfibrils; and cell wall network features (Hendriks and Zeeman, 2009; Zhang et al., 2009). As previously described, appropriate pretreatments could improve biomass substrate properties, but the genetic modification of cell walls may lead to more mild and cost-effective pretreatments for high enzymatic hydrolysis (Xie and Peng, 2011). It is generally accepted that cellulase enzymes are accessible to amorphous (non-crystalline) regions of cellulose microfibrils for the initial digestion of β-1,4-glucans chains (Song et al., 2015). Thus, appropriate genetic modifications of the surface structure of cellulose microfibrils by increasing the density and depth of the amorphous region might be optimal for both biomass pretreatment and enzymatic hydrolysis in biofuel generation (Wu et al., 2014a).

5.3. Bioethanol fermentation

Yeast fermentation is the final step in bioethanol production by using soluble sugars released by the enzymatic hydrolysis of biomass. *Saccharomyces cerevisiae* is one of the most effective ethanol-producing organisms (Li et al., 2010b), but it can only use hexose as a carbon source. Over the past several years, genetic engineering of yeast strains has not only enhanced hexose fermentation capacity but also converted xylose into bioethanol by introducing pentose fermentation genes (*XYL1*, *XYL2*, and *XKS1*) into transgenic yeast strains (Gonçalves et al., 2014). However, the yeast fermentation process is strongly affected by

a wide range of inhibitory compounds that are released during various physical and chemical pretreatments (Yu et al., 2014). As yeast fermentation inhibitors contain many compounds, such as weak acids, furan derivatives and phenolics, it is technically difficult to prevent the release of inhibitors during biomass processing (Li et al., 2014b). The selection of mild pretreatments seems to be a simple and feasible approach to reducing yeast fermentation inhibitor release or to removing the compounds prior to yeast fermentation. For example, Li et al. (2014b) have shown that mild alkali pretreatment effectively extracted G-rich lignin coupled with diminishing yeast fermentation inhibitors in *Miscanthus*. Therefore, the genetic modification of plant cell walls is suitable for mild biomass pretreatment and is also beneficial for efficient bioethanol production due to the reduction of yeast fermentation inhibitors.

6. A model for cell wall modification and biofuel application

Based on the impacts of major wall polymer features on biomass enzymatic digestibility in different plant species described above, a groove-like cell wall structure model (Fig. 5) is posed to enhance both biomass yield and biofuel production in bioenergy crops through a critical cell wall modification. The groove-like structure has been observed by atomic force microscopy (AFM) on the extracted cellulose macrofibrils in vitro after hemicelluloses and lignin are most removed from physical and chemical pretreatments of biomass (Fig. 3B; Wang et al., 2014b). Therefore, the structure model could somehow reflect the density and depth of amorphous regions of native cellulose microfibrils. Since the amorphous regions of native cellulose microfibrils are regarded as the initial places for cellulase enzyme digestion (Wang et al., 2014b), this model could interpret the major wall polymer feature impacts on lignocellulose enzymatic digestibility under various physical and chemical pretreatments. (1) The density and depth of amorphous regions of native cellulose microfibrils could be negatively accounting for the cellulose crystallinity (CrI), which is the key factor that negatively affects biomass enzymatic saccharification under various physical and chemical pretreatments in almost all plant species examined (Harris et al., 2012; Xu et al., 2012; Zhang et al., 2013; Li et al., 2014a; Pei et al., 2016). (2) Hemicellulosic Ara level or the Ara substitution degree (i.e., reverse Xyl/Ara) of xylan plays a major positive impact on biomass enzymatic hydrolysis in most plant species (Li et al., 2013b; Aguedo et al., 2015) examined by negatively affecting cellulose CrI, probably through the interlinking of branched Ara with inside β-1,4-glucans of the amorphous regions (groove-like regions) of native cellulose microfibrils via hydrogen bonds and other bonds. (3) Pectin and uronic acids also positively affect biomass enzymatic hydrolysis by reducing cellulose CrI in *Miscanthus* (Wang et al., 2015b), and the branched sugars of pectin have been proposed to interlink with inside cellulose microfibrils (Wang et al., 2015a), probably with β-1,4-glucans in the amorphous regions. (4) G-rich lignin exhibit dual effects on biomass digestibility (Li et al., 2013b, 2015b), probably due to the diverse associations of G-monomer with hemicelluloses that indirectly affect Ara interaction with native amorphous regions (negative impact) or that lead to an effective extraction in vitro of lignin-hemicelluloses complex for more cellulase access and loading after physical and chemical pretreatment (positive impact). (5) Cellulose CrI is also a negative factor on biomass yield and plant lodging resistance in rice mutants (Li et al., 2015b), suggesting that the increased amorphous regions (low CrI) may lead to a tight association between cellulose microfibrils and hemicelluloses/lignin to maintain cell wall integrity and plant mechanical strength. In addition, this model refers that specific structure of wall proteins, like hemicellulose's Ara and pectin's uronic acids, may also play a positive role in biomass yield and lignocellulose enzymatic hydrolysis under physical and chemical pretreatments (Cannon et al., 2008; Li et al., 2015a; Nardi et al., 2015; Roberts and Shirsat, 2006; Saha et al., 2013).

Genetic modification of plant cell walls must meet the needs of reducing biomass recalcitrance and maintaining normal plant growth. The proposed model highlights the major genes known to enhance both biomass yield and enzymatic digestibility. With respect to the cellulose crystallinity, a key negative factor, reducing cellulose CrI (and DP) is a critical cell wall modification probably by coexpression and mutation of major genes (*GH9*, *CesA*, *GH10*, *GT43*, *GT61* and *PME*) in transgenic plants. GH9 enzymes and modified CESA enzymes should have activities on producing amorphous regions on the surface of cellulose microfibrils, whereas GH10, GT43, GT61, PME enzymes may catalyze Ara and uronic acids production to have more associations with the amorphous regions that maintain cell wall integrity and strength for normal plant growth and high biomass yield (Wang et al., 2014b; Li et al., 2015b). In addition, the enhanced G-monomer and lignin contents may aid to enhance cell wall integrity and strength in genetic mutants and transgenic plants.

As the amorphous regions of native cellulose microfibrils are accessible to initial cellulase hydrolysis of β-1,4-glucans, it should fundamentally determine biomass enzymatic saccharification. In the proposed model (Fig. 5), increase in the amorphous density and depth on the surfaces of cellulose microfibrils in transgenic plants, should lead to effective biomass pretreatment and subsequently efficient enzymatic hydrolysis due to more spaces or choices available for cellulases loading and access. In addition, the increase in amorphous regions of cellulose microfibrils may result in relatively tight associations with noncellulosic polymers in plant cell walls, thereby positively affecting plant strength, mechanical flexibility and lodging resistance in mutants and transgenic plants. It may also explain why cellulose CrI has a significant negative effect on plant lodging resistance in rice mutants.

In conclusion, cell wall modifications that increase the amorphous density and depth of cellulose microfibrils could represent a powerful solution to both biomass recalcitrance and plant strength (or lodging resistance) through the coexpression and/or silencing of different types of genes involved in forming and modifying amorphous regions in genetic mutants and transgenic plants. Meanwhile, an appropriate mild pretreatment could be applied for cost-effective biofuel production subjective to cell wall modification in amorphous regions of cellulose microfibrils in bioenergy crops.

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