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Review Article

Genetic engineering of grass cell wall polysaccharides for biorefining

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Summary

Grasses represent an abundant and widespread source of lignocellulosic biomass, which has yet to fulfil its potential as a feedstock for biorefining into renewable and sustainable biofuels and commodity chemicals. The inherent recalcitrance of lignocellulosic materials to deconstruction is the most crucial limitation for the commercial viability and economic feasibility of biomass biorefining. Over the last decade, the targeted genetic engineering of grasses has become more proficient, enabling rational approaches to modify lignocellulose with the aim of making it more amenable to bioconversion. In this review, we provide an overview of transgenic strategies and targets to tailor grass cell wall polysaccharides for biorefining applications. The bioengineering efforts and opportunities summarized here rely primarily on (A) reprogramming gene regulatory networks responsible for the biosynthesis of lignocellulose, (B) remodelling the chemical structure and substitution patterns of cell wall polysaccharides and (C) expressing lignocellulose degrading and/or modifying enzymes *in planta*. It is anticipated that outputs from the rational engineering of grass cell wall polysaccharides by such strategies could help in realizing an economically sustainable, grass-derived lignocellulose processing industry.

Introduction

Maize (*Zea mays*) and sugarcane (*Saccharum officinarum*) remain the world's largest biofuel-producing feedstocks (Chum *et al.*, 2014). These economic important grasses are currently utilized for respective starch and sucrose-based bioethanol production via fermentation, and accounted for ~85 billion litres of bioethanol and ~85% of global bioethanol output in 2016 (Renewable Fuels Association, 2017). These 'first-generation' biofuels offer in most cases an advantage in terms of carbon footprint compared to fossil fuels. However, with the increasing demand for agricultural land to satisfy the needs of a rapidly growing human population, alternative feedstocks for bioenergy and biorefining are required.

The utilization of abundant, diverse, carbon-neutral, and non-edible agricultural residues of grasses (*Poaceae*) including maize stover, sugarcane bagasse, rice and wheat straw, as well as the harvestable biomass of dedicated bioenergy crops including Miscanthus and switchgrass, represent crucial resources to realize the vision of a low-carbon bioeconomy with biorefining into biofuels, platform chemicals, and value-added bio-based products at its core. The opening of several lignocellulosic-based commercial-scale biofuel plants ('Beta Renewables', ~50 million Litres of bioethanol per year (L/yr); 'Project LIBERTY', ~75 million L/yr; 'DuPont', ~110 million L/yr; 'GranBio', 82 million L/yr; 'Raizen/Iogen', 40 million L/yr) has been a landmark towards the establishment of commercially viable processes for 'second-generation' biofuels. These new technology demonstrations will drive the demand for feedstocks that can fit the quality, as well as the scale required for these initiatives.

A number of crops have been explored as possible feedstock for biorefining, taking into account the carbon balance of using agricultural waste or selecting low-input/high biomass yield species. Table 1 shows the agronomical and genetic features of the main grass lignocellulosic feedstocks explored to date. Corn stover, rice and wheat straw represent the most favourable agricultural wastes available as biomass resources (Table 1). Yet focus has generally been on the effective utilization of corn stover and wheat straw, with less consideration given to rice straw which is more abundant compared to the other major agricultural wastes (Table 1) (Binod *et al.*, 2010; Sarkar *et al.*, 2012). Until recently, rice straw was considered a waste stream of rice production with little or no value and farmers often burning it in the fields, causing health and environmental problems (Oanh *et al.*, 2011). However, the potential of utilizing rice as a biorefining feedstock is increasingly being recognized (Abraham *et al.*, 2016; Liu *et al.*, 2016; Nguyen *et al.*, 2016). Amongst the dedicated biomass crops with the highest potential for biorefining are the fast-growing grasses, in particular, Miscanthus hybrids such as *Miscanthus* × *giganteus*, switchgrass (*Panicum virgatum*), and energy cane (a complex sugarcane hybrid with high lignocellulose yield) (Table 1). These C_4 photosynthesizing grasses are principally coveted for their perenniality and high field productivity across temperature and drought environments, suitability for growth on marginal and erosive land, biodiversity promoting benefits, high water use efficiency and nutrient sequestering ability (Byrt *et al.*, 2011; Carroll and Somerville, 2009; Clifton-Brown *et al.*, 2017; Feltus and Vandenbrink, 2012; Van der Weijde *et al.*, 2013).

Lignocellulosic biomass accounts for ~60%–80% of dry matter yields in grasses and is primarily composed of secondary cell walls comprised mainly of cellulose (~25%–55%), hemicellulose (~20%–50%), and lignin (~10%–35%) (Marriott *et al.*, 2015; Vogel, 2008). Secondary cell walls provide structural support,

Table 1 Grass crops with high potential for genetic engineering and biorefining activities

Species	Mechanism of photosynthesis (carbon fixation)	Type	Average yield potential (dry tonne biomass/ha/yr)*	Genome sequencing status	Genome size (Mbp)	Genetic transformation system	References
Miscanthus (*Miscanthus* × *giganteus*)	C_4	Crop	~22[†]	In progress	~7500	Not well established**	Swaminathan *et al.* (2010); Nordberg *et al.* (2014); Falter *et al.* (2015)
Sugarcane (*Saccharum officinarum*)	C_4	Bagasse and field residue	~17[‡]	In progress	~10 000	Established	Souza *et al.* (2011); De Setta *et al.* (2014); Dong *et al.* (2014); Mayavan *et al.* (2015); Wu and Altpeter (2015)
Energy cane (Saccharum complex hybrids)	C_4	Bagasse and field residue	~50[§]	In progress (see sugarcane)	>10 000	Established[††]	Bischoff *et al.* (2008); Fouad *et al.* (2015); Leon *et al.* (2015); Anderson *et al.* (2016)
Sweet sorghum (*Sorghum bicolor*)	C_4	Bagasse and field residue	~10[¶]	Complete	~730	Established	Paterson *et al.* (2009); Raghuwanshi and Birch (2010); Liu and Godwin (2012)
Switchgrass (*Panicum virgatum*)	C_4	Crop	~10[†]	In progress	~5600	Established	Xi *et al.* (2009); Ramamoorthy and Kumar (2012); Merrick and Fei (2015)
Rice (*Oryza sativa*)	C_3	Straw	~6[‖]	Complete	~390	Established	Sah *et al.* (2014)
Maize (*Zea mays*)	C_4	Stover	~2[‖]	Complete	~2400	Established	Klein *et al.* (1989); Huang and Wei (2005); Ishida *et al.* (2007); Frame *et al.* (2011); Que *et al.* (2014)
Wheat (*Triticum aestivum*)	C_3	Straw	~2[‖]	Complete	~16 500	Established	Li *et al.* (2012); Sparks *et al.* (2014)

Mbp, mega base pair.

*Yields are generally based on lignocellulosic biomass that can be harvested from fields without impacting soil fertility.

[†]Data was taken from Heaton *et al.* (2004).

[‡]The global average dry bagasse yield was calculated as described by Van der Weijde *et al.* (2013), using the global average fresh sugarcane yield for 2014 ('FAOSTAT', 2016).

[§]Average dry yield based on total aboveground portion of the energy cane plant (stalks, tops, and leaves) taken from Anderson *et al.* (2016).

[¶]Average dry sorghum bagasse and field residue yield was taken from Blümmel *et al.* (2009) and Van der Weijde *et al.* (2013).

[‖]The global average rice, maize and wheat lignocellulosic yield was calculated using residue/crop ratios according to Kim and Dale (2004) and their respective average grain yields from 2014 ('FAOSTAT', 2016).

**Transformation not well established in *Miscanthus* × *giganteus* except for a description in Falter *et al.* (2015) but established in *Miscanthus Sinensis* (Hwang *et al.*, 2014; Wang *et al.*, 2011).

[††]Transformation system established in Energy cane but with minimal transgene expression cassette (Fouad *et al.*, 2015).

resist water loss, and protect against mechanical stress and breakdown by microbes. The complexity of the major structural and chemical components of secondary cell walls, which features a variety of chemical linkages within and between the main polymers, is the basis of lignocellulosic biomass recalcitrance and plays a key role in impeding the effective utilization of lignocellulose for bioconversion into fermentable sugars and value-added products on an industrial scale. Efforts to make the deconstruction of lignocellulosic biomass economically viable and environmentally friendly have concentrated in three main areas: (i) improved pre-processing (e.g. mechanical, thermochemical); (ii) improved processing through more efficient enzymes and microbes capable of tolerating toxic inhibitors, withstanding high product and by-product concentrations during biomass digestion and the subsequent fermentation process, and (iii) developing less recalcitrant feedstocks (Agbor *et al.*, 2011; Alvira *et al.*, 2010; Balat, 2011; Klein-Marcuschamer *et al.*, 2012; Sarkar *et al.*, 2012; Sims *et al.*, 2010).

The key lignocellulose processing step in terms of energy and chemical demand is pretreatment, opening up the structure of the cell wall matrix, facilitating enzymes to access their substrates and improving hydrolysis of biomass polysaccharides (Galbe and Zacchi, 2012). Pretreatments modify the composition and architecture of the cell wall and can result in the production of fermentation inhibitors such as formic acid, acetic acid, or furfural, which often require removal prior to fermentation (Jönsson *et al.*, 2013; Phitsuwan *et al.*, 2013). While a wide range of pretreatments have been assessed, few have been implemented in commercial operations. These include the advanced steam explosion pretreatment technology by ANDRITZ Inc. and Proesa® for Project LIBERTY and GranBio or Beta Renewables, respectively, the dilute acid pretreatment technology by Iogen for the Raizen project, and the more exploratory ones such as ionic liquids or the mild alkali pretreatment technology developed by the National Renewable Energy Laboratory for DuPont.

Lignocellulose depolymerisation enzyme discovery and improvement programmes have resulted in new generations of commercial enzyme cocktails that have improved the price competitiveness of cellulosic ethanol (Chandel *et al.*, 2012). These programmes include: surveying enzymes produced by microbes isolated from a diverse range of environments including the rumen, compost heaps, hot springs and tropical forests as well as from 'omic' databases; modification of enzymes through computational biology and forced evolution; and genetic, metabolic and protein engineering techniques aimed at designing industrial microbial strains with proficient cellulolytic and hemicellulolytic activities (Banerjee, 2010).

Another option to increase the efficiency of lignocellulosic deconstruction and processing is the development of biomass tailored for these applications. Choices of feedstock species and breeding for less recalcitrant biomass while maintaining field performance including grain yield in dual-purpose crops represent attractive approaches to improve process techno-economics. Although breeding programmes on C_4 grasses have been a time-consuming and immensely complicated task due to screening of thousands of variants, chromosomal architecture, or multiplicity of alleles, the availability of modern genomic tools to deal with these complications opens the possibility of accurate mapping of genes and/or traits of interest that can be introduced in breeding strategies (Feltus and Vandenbrink, 2012; Slavov *et al.*, 2013, 2014).

Alongside the progress in bioprocessing technologies, enzyme efficiencies, improved microbial strains, and feedstock choices, a complementary prospect to expedite biorefining of grass polysaccharides is via genetic engineering, which is the focus of this review. Although decoding the genetic and structural features that underpin cell wall recalcitrance remains complex, there has been a great deal of interest and progress in this area over the last 10 years. Here, we provide a brief overview of gene targets for genetic engineering of grass polysaccharides and highlight outcomes and perspectives of three different engineering strategies (A) reprogramming gene regulatory networks responsible for the biosynthesis of lignocellulose, (B) remodelling the chemical structure and substitution patterns of cell wall polysaccharides, and (C) expressing microbial lignocellulose degrading and/or modifying enzymes *in planta*. This review does not encompass all engineering efforts to date and does not focus directly on lignin modification or metabolism (covered elsewhere, (Furtado *et al.*, 2014; Poovaiah *et al.*, 2014; Cesarino *et al.*, 2016)) due to the expanse of information on lignin biosynthesis genes and the effects of their manipulation on cell wall properties and digestibility (Eudes *et al.*, 2014; Mottiar *et al.*, 2016).

The distinct features of grass cell walls

The cell walls of grasses consist of a complex composite framework composed mainly of polyphenol lignin (~10%–30%), cellulose (~35%–45%), and hemicellulose (~40%–50%) (for a review on lignocellulosic cell walls, their constituents and synthesis, see Marriott *et al.* (2015)). During the cell cycle in plants, dividing, expanding, or elongating cells have a distinctive primary cell wall. In the *Poaceae* family, the primary wall is thin, aqueous (~60%–70% water), and flexible, and is composed of ~1%–5% hydroxycinnamic acids (HCAs) such as ferulic acids (FA) and *p*-coumaric acids (*p*-CA), pectins (5%), and a few layers of cellulosic microfibrils (~20%–30%) embedded in a matrix of hemicelluloses such as mixed-linkage glucans (MLGs) (~10%–30%) and highly substituted glucuronoarabinoxylans (GAXs) (~20%–40%) (O'Neill and York, 2003; Vogel, 2008). Upon cessation of cell enlargement, an additional and rigid secondary wall is deposited inside of the primary wall. This secondary cell wall, while containing negligible amounts of pectin (~0.1%), minor structural proteins and MLGs, HCAs (~0.5%–1.5%) and a small proportion of water (~5%), is primarily made up of hundreds of layers of cellulose microfibrils (~35%–45%) embedded in GAXs (~40%–50%) which in turn are covalently cross-linked with hydrophobic polyphenol lignin (~20%) (Albersheim *et al.*, 2011; Ebringerová *et al.*, 2005; Vogel, 2008).

Depending on the tissue, cell type, cell wall layer, developmental stage, and plant taxa, the overall amount, architecture, and chemical composition of cell walls can vary significantly (Pauly and Keegstra, 2010). A characteristic feature of grass walls is the presence of particular polysaccharides such as GAX and MLG not found in the cell walls of woody biomass. Up to 40%–80% of the xylose residues of the xylan backbone can be substituted with *O*-acetyl groups (Pauly *et al.*, 2013). Another characteristic feature is the high amount of total FA (~4%) and *p*-CA (~3%) as unbound acids or esterified to GAXs and ester- and ether-linked to lignin in the primary and secondary walls of grasses, thereby cross-linking these components (De Oliveira *et al.*, 2015; Ishii, 1997; Lam *et al.*, 2001; Ralph *et al.*, 2004; Saulnier *et al.*, 1999). Lignin is one of the main carbon components (~20%) of grass secondary walls and typically polymerized from three

different 4-hydroxyphenylpropanoids known as monolignols: *p*-hydroxyphenyls (H) (~4%–15%), guaiacyl (G) (~35%–49%), and syringyl (S) (~40%–61%) (Boerjan *et al.*, 2003). Such monolignols form diverse chemical bonds with each other at multiple positions (Boerjan *et al.*, 2003), thereby crafting lignin as a heterogeneous aromatic and hydrophobic polymer that may lack a repeat structure. Hence, lignin tends to play a critical role in conferring cell wall rigidity and compactness by filling the voids between and around the cellulose and hemicellulose complexion, as well as fortifying the plant cell wall against biotic and abiotic responses. Collective evidence suggests that lignocellulosic biomass recalcitrance is dictated by several of the described cell wall components, their relative abundances, and interactions within the cell wall matrix.

Efforts over the past decade have shown that engineering of grass cell walls using transgenic approaches can help overcome traits associated with cell wall recalcitrance. Researchers identified the need to select gene targets based on the different cell wall polymer targets they act upon, or different functionalities during cell wall construction or deconstruction, as categorized in Figure 1. These targets have driven most efforts to alter grass cell wall characteristics for effective downstream bioconversion, as reflected in the number of publications on this subject over the last decade (Tables 2, 3 and 4). We discuss the progress and perspectives of three different engineering strategies aimed at tailoring grass cell wall polysaccharides for biorefining applications.

A. Reprogramming grass cell wall gene regulatory networks

There are several major plant transcription factor (TF) families, including basic Helix-Loop-Helix (bHLH), Homeobox (HB), basic-region leucine zipper (bZIP), Auxin/indole-3-acetic acid (AUX/IAA) and APETALA2/Ethylene Responsive Factor (AP2/ERF), potentially implicated in regulating secondary cell wall biosynthesis (Cassan-Wang *et al.*, 2013; Hirano *et al.*, 2013). Within the secondary cell wall TF network, two favourable targets for grass cell wall engineering have been the R2R3-MYB (MYELOBLASTOSIS) and NAC (NAM, ATAF, CUC) TF family members (Table 2). These proteins form one of the largest plant-specific TF families and play a key role in regulating cell wall formation (Dubos *et al.*, 2010; Olsen *et al.*, 2005). Hence, modified expression of *MYB* and *NAC* TF genes are expected to reprogram cell wall biosynthesis, providing a route towards improving relevant grass cell wall traits (Bhatia and Bosch, 2014). TFs are sequence-specific DNA binding proteins that *trans*-modify the transcription of target genes quantitatively, temporally (developmental stage-specific), spatially (tissue-specific) or in a stimulus-dependent manner. Thus, understanding the biological role of TFs is important to fully harness their potential as a genetic tool for the improvement of grass wall characteristics. Research efforts have revealed an extensive, complex, hierarchical, and multilevel regulatory network of *MYB* and *NAC* TF genes in the dicot model species *Arabidopsis* (Hussey *et al.*, 2013; Taylor-Teeples *et al.*, 2015). Although some grass MYB and NAC TFs have been shown to regulate secondary cell wall biosynthesis (Fornalé *et al.*, 2010; Sonbol *et al.*, 2009; Valdivia *et al.*, 2013; Zhong *et al.*, 2011), the model of the grass cell wall transcriptional regulatory network is still not as well defined (Handakumbura and Hazen, 2012).

There have been relatively few but valuable attempts in the reprogramming of grass cell wall gene regulatory networks (GRNs) by transgenic approaches (Table 2). For instance, overexpression (OX) of *PvMYB4* in switchgrass not only reduced lignin content and ester-linked *p*-CA:FA ratio by ~50%, but also improved cellulosic ethanol yield by ~2.5-fold (Shen *et al.*, 2012a, 2013). Conversely, overexpression of *SbMYB60* in sorghum was associated with increased lignin biosynthesis, resulting in a higher energy content of the biomass (Scully *et al.*, 2016). However, both overexpression of *PvMYB4* and *SbMYB60* altered several plant growth characteristics, including a significant reduction in plant height (~40% and ~30% respectively). These findings suggest that there is a limit in the plasticity of grasses to tolerate TF-based manipulations in biomass composition without significant impediments in cell wall expansion during plant growth and development. Overexpression of *PvKN1* (Knotted1-like) and *PvERF001* (AP2/ERF) TF genes in switchgrass enhanced saccharification (Wuddineh *et al.*, 2015, 2016), with the former altering the expression of lignin, cellulose and hemicellulose biosynthetic genes, as well as the gibberellin biosynthesis pathway (Wuddineh *et al.*, 2016), while no significant changes in lignin content and composition were detected for the latter (Wuddineh *et al.*, 2015). However, as before, transgenic plants exhibited altered growth phenotypes, with *PvKN1*-OX lines often showing inhibited shoot and root elongation while *PvERF001*-OX lines showed a ~20%–100% increase in dry biomass yield. Despite the apparent absence of a direct association with cell wall regulatory and biosynthetic pathways, the *PvERF001*-OX study shows that TFs can simultaneously improve enzymatic saccharification and biomass yield. Interestingly, transgenic sugarcane overexpressing the maize transcription factor *ZmMYB42* showed a significant reduction in lignin content (8%–21%) and released ~30% more glucose with minimal phenotypic effects (Poovaiah *et al.*, 2016). Besides highlighting the potential of using TFs to increase sugar release by a modest reduction in lignin content, this study also emphasized the difficulties in predicting outcomes of modifying gene expression levels, particularly in grasses with large complex polyploid genomes, and the need to better understand metabolic fluxes through the cell wall biosynthesis pathways.

Some of our knowledge of grass-specific secondary cell wall-related TFs comes from the study of the rice TFs, *OsMYB103L* and *OsSWN1*, which were characterized by overexpression and RNA interference (RNAi) techniques (Chai *et al.*, 2015; Yang *et al.*, 2014) (Table 2). The expression levels of several cellulose synthases (*CesAs*) in *OsMYB103L*-OX lines were significantly increased along with cellulose content (~13%). Concordantly, RNAi of *OsMYB103L* led to a reduction in cellulose content (~15%–30%) and expression levels of *CESA* genes as well as impaired mechanical strength in leaves (Yang *et al.*, 2014),

Figure 1 Cell wall polymer and associated gene targets for genetic engineering of grass biomass. Cell wall polymer targets were adapted from Rubin (2008), Harholt *et al.* (2010a), Scheller and Ulvskov (2010) and Marriott *et al.* (2015) and used with permission. ABC, ATP-binding cassette transporters; AX, arabinoxylan; CESAs, cellulose synthase genes; CSL, cellulose synthase-like gene; CWDs, cell wall degrading enzymes; FA, ferulic acid; G, guaiacyl units; GAUT, α-(1,4) galacturonosyltransferase; GH, glycosylhydrolase; GT, glycosyltransferase; H, *p*-hydroxyphenyl units; HCAs, hydroxycinnamic acids; MLG, mixed-linkage glucan; MYB, Myeloblastosis; NAC, NAM,ATAF1,2 and CUC2; *p*-CA, *p*-coumaric acid; PME, pectin methylesterase; PMEI, pectin methylesterase inhibitor; RLK, receptor-like kinase; S, syringyl units; TFs, transcription factors.

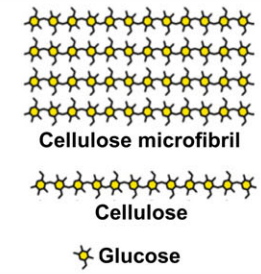
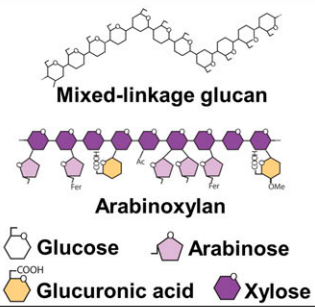
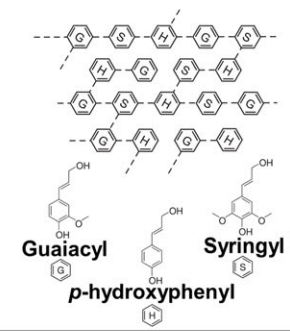
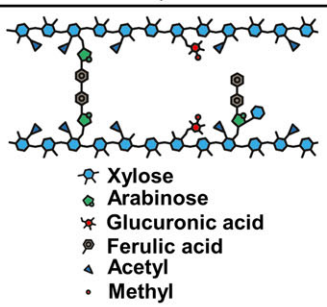
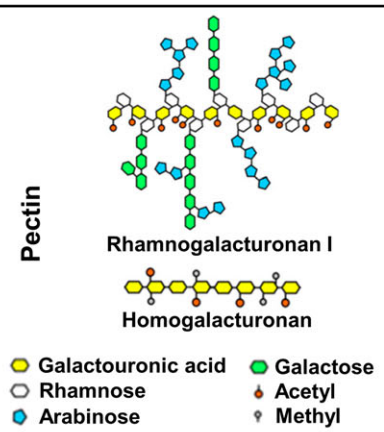
Cell wall polymer targets	Gene category	Gene targets	Modifications
Cellulose 	Biosynthesis; assembly; deposition; regulation; deconstruction	TFs (e.g. NAC, MYB); CESA; CWDs	More cellulose; less crystalline cellulose; polymerization
Hemicellulose 	Biosynthesis; secretion; cross-linking; regulation; deconstruction	TFs (e.g. NAC, MYB); GTs (e.g. GT43, GT47, GT61); CSL (e.g. CSLF6, CSLH1); CWDs	More MLG; less xylan; AX backbone with fewer decorations; reduced acetylation
Lignin 	Biosynthesis; secretion; polymerization; regulation; deconstruction	TFs (e.g. NAC, MYB); monolignol genes; ABC transporters; laccases; peroxidases; CWDs	Less lignin; quality; altered S:G:H monomer ratios; polymerization; secretion
HCA 	Biosynthesis; cross-linking; deposition; regulation; deconstruction	TFs (e.g. MYB); GTs (e.g. GT61); lignin biosynthesis genes; CWDs	Quantity; altered *p*-CA:FA ratio
Pectin 	Biosynthesis; remodelling; deconstruction	GTs (e.g. GAUT); methyl- and acetyltransferases; GHs, RLKs, PMEs, PMEIs; CWDs	Quantity; modified pectin

Table 2 Literature related to transcriptional regulation of the cell wall by transcription factors

Transformed Gene	TF	ID	Source of transgene	Species	Transgenic approach	Promoter	Function/Results*	Plant phenotype*	References
OsMYB46 *ZmMYB46*	MYB	Os12g0515300/Os12g33070 JN634085	*Oryza sativa* *Zea mays*	*Arabidopsis thaliana*	Heterologous expression	35S	Activates cellulose, lignin, and xylan biosynthesis; induces ectopic deposition of lignin and xylan; increases cellulose accumulation	Strong curly leaves	Zhong *et al.* (2011)
ZmMYB31	MYB	GRMZM2G050305	*Zea mays*	*Arabidopsis thaliana*	Heterologous expression	35S	Directly represses lignin biosynthesis; decreases lignin content by 70%; 4-fold increase in H monomer	Dwarfed with smaller leaf, stalk and flower size and delayed flowering	Fornalé *et al.* (2006, 2010)
ZmMYB42	MYB	GRMZM2G419239	*Zea mays*	*Arabidopsis thaliana*	Heterologous expression	35S	Represses lignin biosynthesis; decreases lignin content by 60%; 4-fold increase in H monomer	Dwarfed with smaller leaves	Fornalé *et al.* (2006); Sonbol *et al.* (2009)
ZmMYB31 *ZmMYB42*	MYB	GRMZM2G050305 GRMZM2G419239	*Zea mays*	*Saccharum spp. hybrids*	Overexpression	ZmUbi1	Represses lignin biosynthesis; decreases lignin content by ~13% in some lines; improves glucose release by ~30% in all *ZmMYB46* plants and by ~25% in two *ZmMYB31* plants	Little difference in plant height and number of internodes	Poovaiah *et al.* (2016)
PvMYB4	MYB	JF299185	*Panicum virgatum*	*Panicum virgatum*	Overexpression	ZmUbi1	Represses lignin biosynthesis; decreases lignin content by ~40%–50%; reduces *p*-CA: FA ratio by ~50%; improves sugar release by ~3-fold and ethanol yield by ~2.5-fold	Reduced plant stature (~40%); increased tillering (~2.5-fold)	Shen *et al.* (2012a, 2013)
PvMYB46A	MYB	AP13ISTG55477	*Panicum virgatum*	*Arabidopsis thaliana*	Heterologous expression	35S	Induces ectopic deposition of cellulose, lignin and xylan	Smaller rosette size, curly leaves	Zhong *et al.* (2015)
SbMYB60	MYB	Sb004G273800	*Sorghum bicolor*	*Sorghum bicolor*	Overexpression	35S	Increases lignin biosynthesis; 1.25–2.5-fold increase in S monomer; ~2%–4% increase in energy content	Reduced plant height (~30%); delayed flowering	Scully *et al.* (2016)
OsMYB103L	MYB	Os08g05520	*Oryza sativa*	*Oryza sativa*	Overexpression RNA interference	ZmUbi1	*OsMYB103L* overexpression increases cellulose content by~13%; *OsMYB103L* RNAi decreases cellulose content by ~15%–30%	*OsMYB103L* overexpression causes inward rolled leaf; *OsMYB103L* RNAi reduces mechanical strength in leaves	Yang *et al.* (2014)
TaMYB4	MYB	JF746995	*Triticum aestivum*	*Nicotiana tabacum*	Heterologous expression	35S	Represses lignin biosynthesis; decreases lignin content by ~16%–23%; increases S/G ratio by 36%–66% and leaf flavonoid content by 22% –29%	No morphological alterations except for dark green patches in leaves	Ma *et al.* (2011)

Table 2 Continued

Transformed Gene	TF	ID	Source of transgene	Species	Transgenic approach	Promoter	Function/Results*	Plant phenotype*	References
OsSWN1 *OsSWN3* *OsSWN7* *ZmSWN1* *ZmSWN3* *ZmSWN7*	NAC	Os06g04090/Os06g0131700 Os08g01330/Os08g0103900 Os06g01480/Os06g0104200 JN634077 JN634079 JN634083	*Oryza sativa* *Zea mays*	*Arabidopsis thaliana*	Heterologous expression	35S	Activates cellulose, lignin, and xylan biosynthesis; induces ectopic deposition of cellulose, xylan and lignin	Strong curly leaves	(Zhong *et al.*, 2011)
OsSWN1 *OsSWN2S*	NAC	Os06g0131700 Os08g0115800	*Oryza sativa*	*Arabidopsis thaliana* *Oryza sativa*	Heterologous expression Chimeric repression	35S SRDX	Only *OsSWN1* heterologous expression induces secondary wall formation; *OsSWN2S* chimeric repression reduces wall thickening, lignin and xylose contents and increases digestibility by ~3%–4%	*OsSWN2S* chimeric repression results in drooping leaf phenotype	Yoshida *et al.* (2013)
OsSWN1	NAC	Os06g04090	*Oryza sativa*	*Oryza sativa*	Overexpression RNA interference	ZmUbi1	*OsSWN1* overexpression enhances lignin content by ~2-6% and reduces saccharification yields by ~30%; *OsSWN1* silencing reduces lignin content by ~7%–20% and enhances saccharification yields by ~14%–43%	Most *OsSWN1* overexpression lines are semi-dwarfed, sterile and have erect leaves; *OsSWN1* RNAi lines are normal but sterile	Chai *et al.* (2015)
PvSWN1-8	NAC	KT075080-93	*Panicum virgatum*	*Arabidopsis thaliana*	Heterologous expression	35S	Activates cellulose, lignin and xylan biosynthesis; induces ectopic deposition of cellulose, lignin, and xylan	Smaller rosette size; curly leaves	Zhong *et al.* (2015)
BdSWN5	NAC	JQ693422–JQ693429	*Brachypodium distachyon*	*Brachypodium distachyon*	Overexpression	Oestradiol-inducible	Activates secondary wall gene synthesis and cell death	Normal	Valdivia *et al.* (2013)
AtSHN2	SHN	At5g11190	*Arabidopsis thaliana*	*Oryza sativa*	Heterologous expression	35S	34% increase in cellulose; 45% reduction in lignin	Normal	Ambavaram *et al.* (2011)
PvERF001	AP2/ERF	NR	*Panicum virgatum*	*Panicum virgatum*	Overexpression	ZmUbi1	Increases glucose release by ~10%–16%	~20%–100% increase in dry biomass yield	Wuddineh *et al.* (2015)

*May not encompass complete research findings.

ID, identifier; NR, not reported; RNAi, RNA interference; SRDX, EAR-repression domain; ZmUbi1, maize ubiquitin 1 promoter; 35S, cauliflower mosaic virus promoter.

Table 3 Literature related to remodelling of grass cell wall polysaccharides

Transformed Gene	Annotation	ID	Source of transgene	Species	Transgenic approach	Promoter	Function/Results*	Plant phenotype*	References
OsIRX9 *OsIRX9L* *OsIRX14*	GT43	Os07g49370 Os01g48440 Os06g47340	*Oryza sativa*	*Arabidopsis thaliana*	Heterologous expression	35S	Increases xylan synthase activity	Restores *irx14* and *irx9* mutants	Chiniquy *et al.* (2013)
OsGT43	GT43	Os05g03174 Os05g48600 Os04g01280 Os06g47340	*Oryza sativa*	*Arabidopsis thaliana*	Heterologous expression	35S	Xylan backbone synthesis	Restores *irx14* and *irx9* mutants	Lee *et al.* (2014)
MIGT43A-G	GT43	KX082754-KX082760	*Miscanthus lutarioriparius*	*Arabidopsis thaliana*	Heterologous expression	35S	Xylan biosynthesis	*MIGT43A-E* restores *irx9* mutant; *MIGT 43F-G* restores *irx14* mutant	Wang *et al.* (2016)
TaGT43 *TaGT47*	GT43 GT47	HF913567-9 HF913570-2	*Triticum aestivum*	*Triticum aestivum*	RNA interference	HMW1Dx5	Decreases AX content by 40%–50%; increases degree of arabinosylation by 25%–30%; 50% decrease in cell wall thickness	Normal	Lovegrove *et al.* (2013)
OsGT47A	GT47	Os01g0926600/Os01g70190	*Oryza sativa*	*Arabidopsis thaliana*	Heterologous expression	35S	Restores secondary wall thickness and monosaccharide content	Restores plant growth in *irx10 irx10L* double mutant	Zhang *et al.* (2014)
TaXAT1 *TaXAT2* *OsXAT3*	GT61	FR873610.1 FR846232.1	*Triticum aestivum* *Oryza sativa*	*Arabidopsis thaliana*	RNA interference	HMW1Dx5	Decreases Ara*f* substitution of xylan	Normal	Anders *et al.* (2012)
OsUAM1	UDP-arabinopyranose mutase	Os03g0599800	*Oryza sativa*	*Oryza sativa*	RNA interference	ZmUbi1	Reduces arabinose by up to 44% and extent of xylan substitution; reduces FA and *p*-CA contents by 25%–80%	Plants with >25% reduction in arabinose were dwarfed and infertile	Konishi *et al.* (2011)
PvUAM1	UDP-arabinopyranose mutase	Pavirv000Ib03909	*Panicum virgatum*	*Panicum virgatum*	RNA interference	ZmUbi1	Reduces stem arabinose by up to 39%; increases level of stem cellulose by up to 38% and lignin by up to 13%; unchanged saccharification efficiency	Phenotypic differences between RNAi lines	Willis *et al.* (2016b)
OsARAF1 *OsARAF3*	Arabinofuranosidase	Os07g0686900 Os11g0131900	*Oryza sativa*	*Oryza sativa*	Overexpression	ZmUbi1	~20%–25% decrease in arabinose content; ~28%–34% increase in glucose; ~46%–70% increase in saccharification	Normal	Sumiyoshi *et al.* (2013)
OsCslF2 *OsCslF4* *OsCslF9*	Cellulose synthase	Os07g0552800 Os07g0553300 Os07g0551500	*Oryza sativa*	*Arabidopsis thaliana*	Heterologous expression	35S	Accumulation of MLG <0.1% of total wall	Normal	Burton *et al.* (2006)
HvCesA4 *HvCesA8*	Cellulose synthase	HM222644 KM45970	*Hordeum vulgare*	*Hordeum vulgare*	Overexpression	35S	Reductions in cellulose content by 40%; decrease in cell wall thickness	Dwarfism; early-stage leaf necrosis; stunted; brittle nodes	Tan *et al.* (2015)

Table 3 Continued

Transformed Gene	Annotation	ID	Source of transgene	Species	Transgenic approach	Promoter	Function/Results*	Plant phenotype*	References
HvCSLH1	Cellulose synthase	FJ459581	*Hordeum vulgare*	*Arabidopsis thaliana*	Heterologous expression	35S	Accumulation of MLG between 0.00015% and 0.016% of total wall	Normal	Doblin *et al.* (2009)
HvCslF6	Cellulose synthase	AB621333.1	*Hordeum vulgare*	*Hordeum vulgare*	Overexpression	35S	Up to 6-fold higher MLG content in leaves	Often lethal; surviving plants have necrotic leaf tips	Burton *et al.* (2011)
OsCslF6	Cellulose synthase	Os08g0160500	*Oryza sativa*	*Arabidopsis thaliana*	Heterologous expression	SAG12	4-times more glucose in the cell wall and ~42% increase in saccharification	Normal	Vega-Sánchez *et al.* (2015)

*May not encompass complete research findings.
HMW1Dx5, starchy endosperm-specific promoter; ID, identifier; SAG12, senescence-associated gene 12; UDP, Uridine diphosphate; ZmUbi1, maize ubiquitin 1 promoter; 35S, cauliflower mosaic virus promoter.

common phenotypes associated with *CESA* mutants such as *brittle culm13* (*bc13*) in rice and *irregular xylem* (*irx1* to *irx3*) in *Arabidopsis* (Song *et al.*, 2013; Tanaka *et al.*, 2003; Turner and Somerville, 1997). Overexpression of the NAC TF *OsSWN1* increased lignin content by ~2%–6% and decreased the glucose yield by ~30%, while RNAi lines showed a concomitant decrease in lignin content by ~7%–20% and increase in glucose yield by ~14%–43% (Chai *et al.*, 2015). Both OX and RNAi lines showed abnormal developmental phenotypes with most *OsSWN1*-OX lines displaying a semi-dwarfed and nearly sterile phenotype, while RNAi lines had a relative normal growth phenotype but were sterile.

It is evident that manipulation of cell wall composition and sugar release by altering the expression of certain TFs is often accompanied by aberrant plant growth and fitness penalties (Table 2). Such phenotypic effects can either be a direct result of TF-induced changes in cell wall composition or due to pleiotropic effects as a cell wall-associated TF may also be involved in the regulation of developmental processes or in the response to biotic and abiotic stresses (Fornalé *et al.*, 2010; Zhong *et al.*, 2010). Overexpression studies can also lead to metabolic spillover into related pathways, and TFs may lose some target specificity when expressed at high levels (Martin *et al.*, 2012). Such off-target effects may make TFs perhaps less tractable and more challenging as tools for grass cell wall engineering. In this context, TF-based genetic engineering studies require additional supporting data for interpretations. Only a limited number of studies have deepened into the evidence behind gene targets and protein–protein interactions of grass-specific TFs involved in secondary cell wall transcriptional regulation. Shen *et al.* (2012a) for instance, identified *cis*-regulatory elements (i.e. TF-binding motifs) such as AC-rich elements of monolignol pathway genes recognized by *PvMYB4*. Chromatin immunoprecipitation (ChIP) followed by microarray (ChIP-chip) or sequencing (ChIP-seq) could be key techniques to uncover direct or indirect target genes and binding sites of TFs (Agarwal *et al.*, 2016; Zhu *et al.*, 2012) to increase our understanding of the network dynamics and functionality for secondary wall formation. Additionally, yeast one-hybrid (Y1H) assays represent powerful complements to ChIP for identifying and constructing transcriptional GRNs (Kim *et al.*, 2013; Zhang *et al.*, 2016), though Y1H assays have their own set of limitations (Reece-Hoyes and Walhout, 2012). For a summary of the pros and cons of TF-based genetic engineering and advantages and challenges of the methodologies used to infer transcriptional regulatory networks, see Zhang, 2003; Broun, 2004; Grotewold, 2008 and Hussey *et al.*, 2013.

Much of the initial work on the transcriptional regulation of secondary wall biosynthesis has been based on *Arabidopsis*, with ~45% of the systematic analysis of grass TFs conducted using heterologous studies in transgenic *Arabidopsis* (Table 2). Given the relatively large genome size and TFs family divergence in grass species (Du *et al.*, 2012; Pereira-Santana *et al.*, 2015), it remains questionable whether cell wall biosynthesis GRNs are equally conserved and wholly generalizable amongst dicot and monocot plant species. For example, while MYB58 and MYB63 act as lignin-specific transcriptional activators in *Arabidopsis* (Zhou *et al.*, 2009), the putative rice *(Oryza sativa)* orthologue OsMYB58/63 also regulates cellulose biosynthesis (Noda *et al.*, 2015). Promoter analysis suggested that differences and similarities in the transcriptional regulation of lignocellulose biosynthesis genes between rice and *Arabidopsis* may be due to the distinct *cis*-element composition of their promoters (Noda *et al.*, 2015).

This highlights the importance of characterizing TFs regulating secondary cell wall biosynthesis in grasses as the functionality of such TFs cannot be derived solely from functions defined by their dicotyledonous orthologs. The two genetic grass model systems *Brachypodium distachyon* and *Setaria viridis* could be alternative complementary resources to mine and validate genes and GRNs for grasses (Brutnell *et al.*, 2015). Moreover, reprogramming approaches of grass cell wall GRNs have so far mostly been crude with not much variety in the selection of promoters for TFs to modify transcription of downstream target genes temporally, spatially or in a stimulus-dependent manner (Table 2). Therefore, despite the potential of TF-based genetic engineering strategies to reprogram grass cell wall GRNs, ample work is still necessary to fully dissect the roles of grass-specific TFs in cell wall biosynthesis and to eliminate or at least mitigate against possible plant phenotype drawbacks.

B. Remodelling grass cell wall polysaccharides

Cellulose

Cellulose is the main component of plant lignocellulosic biomass and the most abundant terrestrial source of carbon. As a tightly packed microfibril of linear chains of β-(1,4)-linked glucose residues predominantly composed of crystalline domains that exhibit strong intra- and inter-molecular bonding, cellulose has remarkable structural properties with a tensile strength equivalent to that of steel (Cosgrove, 1997). The strong inter-chain hydrogen bonding network that gives cellulose its sturdy structural properties also makes it resistant to enzymatic hydrolysis, with an inverse correlation between cellulose crystallinity and the initial rate of cellulose hydrolysis (Hall *et al.*, 2010). Hence, engineering approaches rendering crystalline cellulose more amorphous are a major research focus (for a comprehensive review see: Abramson *et al.*, 2010). Initial studies, however, showed that such a target compromised other important plant agronomic traits. Harris *et al.* (2012) showed that in *Arabidopsis* two CESA mutants reduced the crystallinity of the cellulose microfibrils compared to the wild type. Lignocellulosic extracts of these mutants showed less recalcitrance in saccharification assays (49% increase in sugar release for the double mutant). However, the mutants, in particular the double mutant, exhibited dwarfed phenotypes. To this end, it seems that the targeted expression of exogenous cell wall degrading or modifying enzymes, explained in more detail in Section C, could provide a better route to alter cellulose crystallinity without compromising plant performance (Table 4).

Another biotechnological target has been to increase the amount of cellulose per unit of biomass, increasing the ratio of more easily fermented glucose monosaccharides (hexoses) compared to pentoses (mainly xylose derived from xylans). As cellulose is synthesized by hexameric rosette CESA complexes located at the plasma membrane (Carpita, 2012), increasing the amount and activity of grass-specific CESA's, such as of OsCESA4, 7 and 9 that form the CESA complex typical for secondary cell wall biosynthesis in rice (Tanaka *et al.*, 2003), appears as a logical approach. Attempts to implement such a strategy in barley (*Hordeum vulgare*) resulted in pleiotropic phenotypes and transcript silencing (Tan *et al.*, 2015). An alternative approach would be to specifically target the transcriptional regulation of secondary cell wall cellulose synthases. This could theoretically lead to variations in cellulose synthesis with consequences on the orientation/organisation of cellulose microfibrils, possibly improving biorefining capabilities. However, there are no reports on the existence of such TFs. Overall, it remains questionable if reducing cellulose crystallinity and increasing cellulose abundance in grasses by altering the expression of endogenous genes can be achieved without a significant penalty on plant growth and performance.

Xylan

The major grass hemicellulose sugar, xylan, varies in the number of substituents and side chains but is predominantly composed of a linear backbone of β-(1,4)-linked xylose residues often substituted with single residues of α-(1,2)-linked glucuronic acid (GlcA)/4-*O*-methylglucuronic acid (MeGlcA), α-(1,2)- and/or α-(1,3)-linked arabinofuranosyl (Ara*f*), as well as less frequent disaccharide side chains including α-(1,3)-linked Ara*f* substituted with α-(1,3)-linked Ara*f* or β-(1,2)-linked xylose (Ebringerová and Heinze, 2000). In addition to sugar substitutions, xylosyl residues of xylan may also be *O*-acetylated, and Ara*f* residues on the xylan backbone may be esterified with FA or *p*-CA, the former covalently cross-linking with lignin or adjacent xylan chains to strengthen secondary walls (Faik, 2010) (for a review on the detailed structure of hemicelluloses, see Scheller and Ulvskov (2010); for a xylan biosynthesis review, see Rennie and Scheller (2014)). This diverse pattern of possible xylan substitutions affects xylan conformation and solubility, and consequently grass cell wall architecture, all key determinants of saccharification yields. It also has implications regarding the need for complex enzyme mixtures to completely hydrolyse this polysaccharide to fermentable sugars.

Xylan acetylation is one of the main factors determining the insolubility and assembly of the xylans *in muro*. Deacetylation of maize stover by dilute alkaline extraction improves xylose monomer yields by ~10% upon pretreatment (Chen *et al.*, 2012). The same study also showed that deacetylation of maize stover prior to dilute acid pretreatment results in ~20% higher saccharification yield compared to the same material acid pre-treated. Studies in *Arabidopsis* likewise showed *O*-acetylation levels to affect the physicochemical properties of xylan, plant growth and the enzymatic degradation of wall polymers (Schultink *et al.*, 2015; Yuan *et al.*, 2016). The presence of acetyl groups not only appears to be an impediment to enzymatic degradation but the release of acetate, mainly derived from deacetylation of xylan and pectins, may also act as yeast fermentation and enzyme digestion inhibitors (Helle *et al.*, 2003; Pawar *et al.*, 2016; Selig *et al.*, 2009). Genes involved in xylan acetylation have not yet been characterized in grasses and understanding the mechanisms of polysaccharide *O*-acetylation or modulating acetyltransferase activities might provide routes to enhance the conversion efficiency of lignocellulosic grasses to biorefining.

Given the diverse structural features of xylan, multiple modifying enzymes such as acetyltransferases and methyltransferases along with at least five glycosyltransferase (GT) enzyme activities, namely β-(1,4) xylan synthase, α-(1,2) glucuronyltransferase (GlcAT), α-(1,2) or α-(1,3) arabinofuranose transferase (AraT) and β-(1,2) xylosyltransferase (XylT), are assumed to be involved in the xylan biosynthetic mechanism within the Golgi apparatus (Faik, 2010). Concurrently, these enzymes represent added targets and hold promise for engineering grass cell wall xylan. The importance of xylan side branches in changing the accessibility of lignocellulolytic enzymes is demonstrated by the dramatic effect of arabinofuranosidase (*OsARAF*) overexpression in rice, where the arabinose content decreased by 20%–25% while the glucose content increased by ~28%–34%, resulting in ~46%–70% improvement in saccharification efficiency with no visible phenotype (Sumiyoshi

et al., 2013). Another report explored the significance of xylan backbone substitutions in transgenic rice via RNAi to suppress uridine diphosphate (UDP)-arabinopyranose mutase (*OsUAM1*) expression, an enzyme that catalyses the formation of UDP-Ara*f* from UDP-arabinopyranose (UDP-Ara*p*) (Konishi *et al.*, *2011*). Although a reduction of 6%–44% in Ara*f* as well as 25%–80% reductions in the FA and *p*-CA contents of the cell wall was observed, those transgenic rice plants with a >25% reduction in Ara*f* content were dwarfed and infertile (Konishi *et al.*, *2011*). UAM's potential role in the recalcitrance of grass cell walls was recently investigated using RNAi to down-regulate the expression of *PvUAM1* in switchgrass (Willis *et al.*, 2016b). While there was an up to 39% decrease in cell wall-associated arabinose from stem, a concurrent increase in cellulose (up to 38%) and lignin (up to 13%) content was observed in stems of *PvUAM*-RNAi transgenic lines. This potential compensation response to maintain cell wall integrity may be the reason why enzymatic saccharification efficiency was unchanged (Willis *et al.*, 2016b). However, it must be noted that reducing the number of xylan side chains with the aim of reducing wall cross-linking and recalcitrance might also lead to structural changes and perhaps a denser cell wall matrix. Indeed, removal of arabinofuranose side chains decreased arabinoxylan (AX) solubility (Anders *et al.*, 2012), possibly induced by increased hydrogen bonding between neighbouring AX chains.

A role in xylan biosynthesis for rice and Miscanthus GTs, mainly belonging to the GT43 and GT47 families, has been confirmed by their overexpression in *Arabidopsis irx* mutants. The complementation of the mutant phenotypes verified the function of each GT (Table 3). Other candidate genes with the same function in grasses have also been identified and characterized. For example, in wheat, the *IRX9* homologue *TaGT43_2* and the *IRX10* homologue *TaGT47_2* have been implicated in the biosynthesis of AX (Lovegrove *et al.*, 2013). Additionally, two maize GT47 genes (*GRMZM2G100143* and *GRMZM2G059825*) identified via differential gene expression profiling in internodes undergoing secondary wall deposition represent likely candidates for involvement in the biosynthetic process of grass cell wall xylan (Bosch *et al.*, 2011). Although modification of cell wall xylan content, composition and assembly/cross-linking have been explored using grass-specific and Golgi-localized GT enzymes, less attention has been paid to enzymatic saccharification benefits that could arise from such transgenic modifications (Anders *et al.*, 2012; Chiniquy *et al.*, 2013; Lee *et al.*, 2014; Lovegrove *et al.*, 2013; Zhang *et al.*, 2014).

Another defining feature of grass cell walls is the presence of FA substitution that allows cross-linking of AX chains or AXs to lignin monomers (Buanafina, 2009; Burr and Fry, 2009). Not surprisingly, an increasing volume of evidence points to the impact of FA-mediated cross-linking in saccharification yields as well as in the *in vitro* wall digestibility of grasses (Grabber *et al.*, 1998a,b; Iiyama and Lam, 2001; Jung *et al.*, 1991; Lam *et al.*, 2003). Studies have shown grass-specific GT61 family members to be involved in mediating such xylan substitutions. Mutants in these genes have little or no arabinofuranose side chains, lower feruloylation and HCAs cross-linking (Anders *et al.*, 2012; Chiniquy *et al.*, 2012), in many cases exhibiting increased saccharification, such as *xax1* mutant plants (Chiniquy *et al.*, 2012). Even if the pathway for feruloyl esterification is not fully understood, it appears to involve acyltransferases from the BAHD family (Bartley *et al.*, 2013). Overexpression of the BAHD acyltransferase *OsAt10* in rice resulted in increased *p*-CA esterification and reduced FA esterification, and a ~20%–40% increase in saccharification efficiency (Bartley *et al.*, 2013). Although the properties of xylan have been changed using transgenic approaches involving GTs (Table 3), one of the potential caveats of overexpressing GTs is that it might lead to saturation of catalytically active GTs in the Golgi apparatus, thereby possibly (i) remodelling xylan formation and/or cross-linking due to substrate competition and (ii) limiting the availability of other Golgi transmembrane proteins responsible for different xylan substitution patterns.

Despite at least a third of grass cell wall-related genes having no or few orthologs in *Arabidopsis* (Carpita and McCann, 2008), bioinformatic analysis, transcriptome profiling, and complementation studies using *irx* mutants indicate that several members of the GT43, GT47, and GT61 family have conserved functions in the xylan biosynthetic process across the dicots and monocots (Mitchell *et al.*, 2007; Pellny *et al.*, 2012). In this context, definitive and direct proof of biochemical function of putative GT43, GT47, GT61, and BAHD grass candidate gene products remain to a greater part unexplored (Table 3). The mechanisms that control the chain length and assembly of the xylan backbone into a functional cell wall are yet unidentified. Discoveries in this research area are appealing and may boost grass cell wall xylan engineering efforts for improved biorefining.

Mixed-linkage glucan

Grasses accumulate large amounts (~10%–30%) of non-branched β-(1,3;1,4)-linked glucose residues, also known as mixed-linkage glucan (MLG), in their primary cell walls (Vogel, 2008). Because of their high and transient accumulation during cell elongation in growing tissues, MLGs have primarily been associated with cell expansion (Carpita and McCann, 2010). However, a higher abundance of MLGs in mature tissues, particularly in the vasculature and sclerenchyma (Vega-Sánchez *et al.*, 2013), and a structural role for MLGs in such tissues (Vega-Sánchez *et al.*, 2012), suggests a broader role for MLG in grasses. The amorphous characteristics of MLG, entirely composed of unbranched and unsubstituted glucose residues yet relatively soluble with low recalcitrance (Burton and Fincher, 2009), make it an attractive target for cell wall engineering aimed at reducing recalcitrance by increasing the amount of easily hydrolysable glucose polymers as well as the ratio of hexose to pentose sugars.

The biosynthesis of MLG involves cellulose synthase-like proteins CSLF and CSLH (Burton *et al.*, 2006; Doblin *et al.*, 2009). Recent work has shown that the mutation of a single cellulose synthase-like gene (*CSLF6*) resulted in a severe reduction or even apparent lack of MLG in rice and barley (Taketa *et al.*, 2012; Vega-Sánchez *et al.*, 2012, 2013), demonstrating that *CSLF6* is a dominant gene for controlling the biosynthesis of MLG. Overexpression of the barley *CSLF6* gene under control of the constitutive 35S promoter resulted in a 6-fold increase of β-(1,3;1,4) glucans in leaves but also in high mortality as many transgenic barley plants did not survive the transformation process or growth in subsequent generations (Burton *et al.*, 2011). This accentuates the need of spatiotemporal regulation when targeting the biosynthesis of MLG. Indeed, heterologous expression of the rice CSLF6 MLG synthase in *Arabidopsis* using a senescence-associated promoter resulted in up to four times more glucose in the matrix cell wall fraction (without competing with cellulose accumulation) and up to 42% increase in saccharification compared to control lines (Vega-Sánchez *et al.*, 2015) without apparent defects in growth and development. This provides proof of concept that increasing the levels of MLG in grasses when using a promoter that

Table 4 Literature related to genetic engineering of grass cell walls by *in planta* expression of cell wall degrading or modifying enzymes

Transformed Gene	Annotation	ID	Source of transgene	Species	Transgenic approach	Promoter	Function/Results*	Plant phenotype*	References
EG *CBH1*	Endoglucanase Cellobiohydrolase	E.C. 3.2.1.4 E.C. 3.2.1.91	*Acidothermus cellulolyticus* *Trichoderma reesei*	*Zea mays*	Heterologous expression	35S	Enzyme accumulated up to 2.1% TSP; enzymatic activity of 0.845 nmol/μg/min in leaf	Normal	Biswas *et al.* (2006)
EG *CBH1*	Endoglucanase Cellobiohydrolase	U33212 X69976	*Acidothermus cellulolyticus* *Trichoderma koningii*	*Zea mays*	Heterologous expression	Glob-1	Enzyme accumulated >16% TSP	Normal	Hood *et al.* (2007)
EG *CBH1* *Bg1A*	Endoglucanase Cellobiohydrolase Cellobiase	E.C. 3.2.1.4 E.C. 3.2.1.91 E.C. 3.2.1.21	*Acidothermus cellulolyticus* *Trichoderma reesei* *Butyrivibrio fibrisolvens*	*Zea mays*	Heterologous expression	RbcS1 35S	Ratio of 1:4:1 (EG:CBH1:Bg1A) shows efficient conversion of pre-treated corn stover	Normal	Park *et al.* (2011)
CBH1 *CBH2* *EG*	Cellobiohydrolase Endoglucanase	FR719150 FR719151 FR719152	*Penicillium sp.* *Trichoderma sp.*	*Saccharum officinarum*	Heterologous expression	ZmPepC ZmUbi1	Endo- and Exoglucanase activity achieved in the leaves	Normal	Harrison *et al.* (2011, 2014b)
CBH1	Cellobiohydrolase	E.C. 3.2.1.91	*Penicillium sp.*	*Zea mays*	Heterologous expression	ZmPepC	Use of recombinant CBH1 enhanced performance of commercial cellulase mixture by up to 4-fold on pre-treated sugarcane bagasse	Normal	Harrison *et al.* (2014a)
Cel-Hyb1 (CelA and Cel6G)	Endoglucanase	AY206451	*Neocallimastix patriciarum* *Piromyces sp.*	*Hordeum vulgare*	Heterologous expression	GluB-1	Endoglucanase production of up to 1.5% of total grain protein remains stable post-harvest	Normal	Xue *et al.* (2003)
EG	Endoglucanase	E.C. 3.2.1.4	*Acidothermus cellulolyticus*	*Oryza sativa*	Heterologous expression	35S	Enzyme accumulated up to 4.9% TSP; ~22%–30% of the cellulose converted into glucose	Normal	Oraby *et al.* (2007)
EG	Endoglucanase	E.C. 3.2.1.4	*Acidothermus cellulolyticus*	*Zea mays*	Heterologous expression	35S	Enzyme accumulated up to 1.13% TSP; Enhanced auto-hydrolytic efficiency	Normal	Ransom *et al.* (2007)
EG	Endoglucanase	E.C. 3.2.1.4	*Acidothermus cellulolyticus*	*Zea mays*	Heterologous expression	RbcS1	Endoglucanase converts cellulose into fermentable glucose	Normal	Mei *et al.* (2009)
AcCel5A	Endoglucanase	E.C. 3.2.1.4	*Acidothermus cellulolyticus*	*Zea mays*	Heterologous expression	35S	Improves saccharification by 10%–15% after mild-pretreatment	Normal	Brunecky *et al.* (2011)
EG	Endoglucanase	E.C. 3.2.1.4	*Acidothermus cellulolyticus*	*Oryza sativa*	Heterologous expression	Mac	Enzyme accumulated up to 6.1% TSP; enhances hydrolysis of cellulose to reducing sugars by 43%	Normal; high AcE1 expression reduces plant stature and delays flowering	Chou *et al.* (2011)

Table 4 Continued

Transformed Gene	Annotation	ID	Source of transgene	Species	Transgenic approach	Promoter	Function/Results*	Plant phenotype*	References
EG	Endoglucanase	E.C. 3.2.1.4	*Acidothermus cellulolyticus*	*Oryza sativa*	Heterologous expression	Gt1	Endoglucanase activity at ~830 U/g of dried seeds	Seeds smaller; plant dwarfing and early flowering	Zhang *et al.* (2012)
Bgl7A	Endoglucanase	EC 3.2.1.73	*Bispora sp. MEY-1*	*Zea mays*	Heterologous expression	ZM-leg1A	Endoglucanase activity at ~780 U/g of dried seeds	Normal	Zhang *et al.* (2013)
EXG1 *ENG1* *BEG1*	Exoglucanase Endoglucanase Cellobiase	AK108835 AK102748 AK070962	*Oryza sativa*	*Oryza sativa*	Overexpression	ZmUbi1 Act1P	Enhances saccharification of transgenic *EXG1* rice stems by ~32%–58%; no activity detected for ENG1 and BEG1	Abnormalities in leaf and sterility; no transgenic *ENG1* plants regenerated; *BEG1* transgenic plants grow normal	Nigorikawa *et al.* (2012)
EXG1	Exoglucanase	AK108835	*Oryza sativa*	*Oryza sativa*	Overexpression	SGR	Enhances saccharification of transgenic *EXG1* rice by ~4-8%	Normal	Furukawa *et al.* (2014)
XynA	Xylanase	E. C. 3.2.1.8	*Neocallimastix patriciarum*	*Hordeum vulgare*	Heterologous expression	GluB-1 Hor2-4	Xylanase remains stable post-harvest	~90% fertile transgenic lines	Patel *et al.* (2000)
XynA1	Xylanase	E. C. 3.2.1.8	*Clostridium thermocellum*	*Oryza sativa*	Heterologous expression	35S	Xylanase activity at ~250 U/g detected in leaves and seed grains	Normal	Kimura *et al.* (2003)
XynBM	Xylanase	E.C. 3.2.1.8	*Clostridium stercorarium*	*Oryza sativa*	Heterologous expression	Act1	~80% xylanase activity maintained in leaves	Normal	Kimura *et al.* (2010)
XynB *BSX*	Xylanase	E.C. 3.2.1.8	*Clostridium stercorarium* *Bacillus sp.*	*Zea mays*	Heterologous expression	GluB-4 rubi3	Enzyme accumulated up to 0.1% TSP; BSX and XynB accumulated up to 4.0% TSP and 16.4% TSP respectively in grains	Stunted plants; sterile grains	Gray *et al.* (2011)
Xyn2	Xylanase	E.C. 3.2.1.8	*Trichoderma reesei*	*Festuca arundinacea*	Heterologous expression	Act1 LmSee1	Modifies cell wall structure and reduces sugar release by 30%	Reduced plant growth; 10%–60% reduction in biomass accumulation	Buanafina *et al.* (2012)
iXynB	Xylanase	E.C. 3.2.1.8	*Dictyoglomus thermophilum*	*Zea mays*	Heterologous expression	NR	Improves glucose and xylose release by ~20%	Normal seeds and fertility	Shen *et al.* (2012b)
ATX	Xylanase	AY949 844 E.C. 3.2.1.8	*Thermobifida fusca*	*Oryza sativa*	Heterologous expression	35S	Xylanase activity at ~3 U/g in fresh leaves	Normal	Weng *et al.* (2013)
AnAXE	Xylan acetyltransferase	AN6093.2 EC 3.1.1.72	*Aspergillus nidulans*	*Brachypodium distachyon*	Heterologous expression	ZmUbi1	Reduces cell wall acetylation by 1.3-fold	Normal	Pogorelko *et al.* (2013)
XynA *FAE*	Xylanase Ferulic acid esterase	NC000964.2 Y09330.2	*Bacillus subtilis* *Aspergillus niger*	*Triticum aestivum*	Heterologous expression	1DX5	8%–20% increase in AX content in all transformants; 10%–15% increase in arabinose to xylose ratio in *XynA* grain cell walls; 13%–34% decrease in FA content in *FAE* grain cell walls	Mostly sterile; transgenic offspring kernels are shrivelled	Harholt *et al.* (2010b)
XynA *XynB*	Xylanase Endoglucanase		*Dictyoglomus Thermophilum*	*Zea mays*	Heterologous expression	NR	Plants expressing one or two CWD enzymes show improved sugar release;	NR	Zhang *et al.* (2011)

Table 4 Continued

Transformed Gene	Annotation	ID	Source of transgene	Species	Transgenic approach	Promoter	Function/Results*	Plant phenotype*	References
EG *FAE*	Ferulic acid esterase	E.C. 3.2.1.8 E.C. 3.2.1.4 E.C. 3.1.1.73	*Thermomyces Lanuginosus* *Nasutitermes Takasagoensis* *Acidothermus Cellulolyticus* *Aspergillus niger*				*EGA* and *EGA/XynA* plants show 55% improvement in ethanol production		
FAE *Xyn2*	Ferulic acid esterase Xylanase	E.C. 3.1.1.73 E.C. 3.2.1.8	*Aspergillus niger* *Trichoderma reesei*	*Festuca arundinacea*	Heterologous expression	Act1 LmSee1	Increases lignin by 23% and saccharification by 31%	Narrow and short leaves; ~70% decrease in biomass	Buanafina *et al.* (2015)
FAE	Ferulic acid esterase	E.C. 3.1.1.73	*Aspergillus niger*	*Festuca arundinacea*	Heterologous expression	Act1 LmSee1	Ferulic acid esterase activity at ~100–400 U/g in fresh leaves; reduces cell wall ferulates by ~14%–25%; increases *in vitro* dry matter digestibility by up to 4% in *FAE* plants with lower ferulate levels	Normal	Buanafina *et al.* (2010)
FAE	Ferulic acid esterase	E.C. 3.1.1.73	*Aspergillus niger*	*Festuca arundinacea*	Heterologous expression	Act1 ZmUbi1 35S HS LmSee1	Ferulic acid esterase activity at ~400-500 U/g in fresh leaves with heat shock and senescence promoters respectively; increases *in vitro* dry matter digestibility by up to 14% in *FAE*-Act1 plants	Normal	Buanafina *et al.* (2008)
FAE	Ferulic acid esterase	E.C. 3.1.1.73	*Aspergillus niger*	*Lolium multiflorum*	Heterologous expression	Act1	Ferulic acid esterase activity at ~25–400 U/g in fresh leaves; reduces cell wall ferulates by ~50%–85%; increases *in vitro* dry matter digestibility by up to 14%	Normal	Buanafina *et al.* (2006)
AcPMEI	Pectin methylesterase	E.C. 3.1.1.11	*Actinidia chinensis*	*Triticum durum cv. Svevo*	Overexpression	ZmUbi1	~2.5-fold higher saccharification efficiency	Normal	Lionetti *et al.* (2010)
Man5A	Mannase	EC 3.2.1.78	*Bispora sp. MEY-1*	*Zea mays*	Heterologous expression	ZM-leg1A	Mannase activity at ~20–26 U/g of dried seeds	Lower plant height by ~3%	Xu *et al.* (2013)
Aga-F75	Galactosidase	EC3.2.1.22	*Gibberella sp. strain F75*	*Zea mays*	Heterologous expression	ZM-leg1A	Galactosidase activity at 10 U/g of dried seeds	Normal	Yang *et al.* (2015)
OsEXP4	Expansin	Os05g0477600	*Oryza sativa*	*Oryza sativa*	Overexpression and RNA interference	ZmUbi1	Expansin expression affects growth and development	Pleiotropic phenotypes in plant height, leaf number, flowering time, and seed set	Choi *et al.* (2003)

Table 4 Continued

Transformed Gene	Annotation	ID	Source of transgene	Species	Transgenic approach	Promoter	Function/Results*	Plant phenotype*	References
OsEXPA8	Expansin	Os01g0248900	*Oryza sativa*	*Oryza sativa*	Overexpression	35S	Enhances cell size of leaf and root vascular bundles in transgenic rice	Increased plant height (~10%), leaf size (~16%) and root length (~36%)	Ma *et al.* (2013)
OsEXPA8	Expansin	Os01g0248900	*Oryza sativa*	*Oryza sativa*	RNA interference	ZmUbi1	Reduces cell size; increases cell wall stiffness; inhibits cell growth	Reduction in plant height and growth	Wang *et al.* (2014)

*May not encompass complete research findings.

Act1, rice actin 1 promoter; Glob-1, maize embryo-preferred globulin-1 promoter; GluB-1, barley glutelin B-1 promoter; GluB-4, rice glutelin 4 promoter; Gt1, rice glutelin 1 promoter; Hor2-4, hordein gene promoter; HS, soybean heat shock promoter; ID, identifier; LmSee1, Lolium multiflorum senescence-enhanced gene promoter; Mac, a hybrid promoter of mannopine synthase promoter and cauliflower mosaic virus 35S promoter enhancer region; NR, not reported; RbcS1, rubisco small subunit promoter; rubi3, rice ubiquitin 3 promoter; SGR, stay green promoter; TSP, total soluble plant protein; ZM-leg1A, maize legumin 1A (leg1) promoter; ZmpepC, maize phosphoenolpyruvate carboxylase promoter; ZmUbi1, maize ubiquitin 1 promoter; 1DX5, endosperm-specific 1DX5 glutenin promoter; 35S, cauliflower mosaic virus promoter.

controls the timing of increases in gene expression levels (e.g. employing chemical- or temperature-inducible promoters, or a developmentally regulated promoter), should be feasible. However, as highlighted before, such interventions should be accompanied by careful evaluation of the impact of increasing MLG content on the overall crop fitness. It is also important to highlight that, based on glycome profiling data with the BG1 monoclonal antibody (Meikle *et al.*, 1994), some MLGs are firmly integrated into the cell wall matrix as they can only be released after delignification of the cell wall fraction. This has been observed for switchgrass (Shen *et al.*, 2013), sugarcane (De Souza *et al.*, 2013), maize stover (Li *et al.*, 2014), and Miscanthus (da Costa *et al.*, 2017), underlining the need to improve our knowledge of the structural associations of MLGs with other cell wall constituents (Kiemle *et al.*, 2014; Smith-Moritz *et al.*, 2015) to device engineering strategies based around MLGs.

Pectin

Pectins are complex, galacturonic acid-rich, plant cell wall polysaccharides, with homogalacturonan (HG) (~65%) as the most abundant form. For a comprehensive review on the structure and biosynthesis of pectin, we refer to Harholt *et al.* (2010a) and Mohnen (2008). Pectin polysaccharides only constitute a minor component of the cell wall biomass in grasses (~5% of growing cell walls and ~0.1% of mature cell walls (Ishii, 1997)) and have therefore received little attention as a target for optimizing lignocellulosic biomass for biorefining purposes. However, several studies involving ELISA-based glycome profiling approaches have shown that a proportion of pectin epitopes cannot be released before delignification of the cell wall fraction, including for Miscanthus and switchgrass (da Costa *et al.*, 2017; De Souza *et al.*, 2015; DeMartini *et al.*, 2013; Shen *et al.*, 2013), suggesting tight associations between pectin and lignin. It has been postulated that lignin polymerization initiates in the pectin-rich middle lamella that lies between the walls of adjacent cells and *in vitro* model studies provide evidence that pectin is important for lignin deposition in the cell wall and lignin-pectin associations can indeed occur (Achyuthan *et al.*, 2010; Lairez *et al.*, 2005; Wang *et al.*, 2013). Additional research is required to address the various hypotheses concerning the exact functional role of pectin during lignification.

One surprising finding was that increasing the ratio of methyl-esterified pectin to demethyl-esterified pectin in wheat, through the expression of a kiwifruit pectin methylesterase inhibitor (PMEI), more than doubled saccharification efficiency without adverse effects on plant growth or cell wall deposition (Lionetti *et al.*, 2010). PMEIs are inhibitors of pectin methylesterases (PMEs), enzymes that demethyl-esterify pectins *in muro*, exposing carboxyl residues which can be cross-linked by calcium (Bosch and Hepler, 2005). Hence, PMEI induced increases in saccharification efficiencies may result from a higher proportion of methyl-esterified pectins, leading to reduced cell wall cross-linking and improved accessibility of hydrolytic enzymes to their substrates. Indeed, it appears that the pattern and degree of pectin methyl-esterification are important in determining the cell wall porosity (Willats *et al.*, 2001). It is becoming clear that despite its low content in grass secondary cell walls, pectin polysaccharides can somehow contribute to the cell wall recalcitrance to hydrolysis. Genetic engineering approaches targeting changes in pectin content and/or its substitution pattern might, therefore, provide interesting routes for generating biomass more amenable to saccharification (Latarullo *et al.*, 2016). However, more studies are required to

establish how pectin modifications affect cell wall recalcitrance in grasses before such approaches can be implemented.

C. *In planta* production of cell wall degrading or modifying enzymes

The three major cost components associated with the bioconversion of lignocellulosic biomass for use by the biorefining industry are the production of microbial enzymes, feedstocks, and their biochemical processing. The *in planta* production of lignocellulolytic enzymes is a way of tackling all these three important aspects at the same time and has concentrated a lot of research effort. High-level expression of cell wall degrading (CWD) or modifying enzymes *in planta* is an attractive strategy to alter cell wall architecture, reduce exogenous enzyme production costs, and/or improve plant auto-hydrolysis for biomass saccharification (Table 4). This approach requires a careful consideration of the strategy for the expression of active enzymes such as the subcellular or tissue targeting, the number of enzymes with different functionalities expressed, and the timing of the expression or activation of the heterologous enzymes.

A range of microbial CWD enzymes including xylanases, cellobiohydrolases (*CBH*) sometimes referred to as exoglucanases (*EXG*), endoglucanases (*ENG*) and β-glucosidase have been assessed via heterologous production or overexpression in several transgenic grasses, generally yielding no observable negative phenotypic differences and several resulting in enhanced saccharification (Table 4). One iconic example led by the industrial company Agrivida was the expression of an engineered thermostable xylanase gene (*iXynB*) from *Dictyoglomus thermophilum* that remains stable in transgenic maize post-harvest and only activates upon mild thermochemical pretreatment (Shen *et al.*, 2012b). Subsequent enzymatic saccharification of the transgenic plants resulted in ~20% higher glucose and xylose release (Shen *et al.*, 2012b). This transgenic modulation demonstrates the feasibility and efficiency of expressing thermostable wall degrading enzymes *in planta* without causing premature auto-hydrolysis or limiting biomass yield via negative phenotypic impacts. Transgenic rice plants expressing a rice exoglucanase (*EXG1*) under the control of a senescence-inducible promoter also exhibited ~4%–8% higher saccharification ability of rice straw after senescence and successfully eliminated morphological abnormality or sterility (Furukawa *et al.*, 2014), which was observed when *EXG1* was constitutively overexpressed in transgenic rice plants (Nigorikawa *et al.*, 2012). In addition to the list of glycosyl hydrolases (Table 4), an *Aspergillus niger* ferulic acid esterase (FAE) has been expressed aimed at altering cell wall composition and reducing recalcitrance during saccharification. The targeted expression of this FAE to the Golgi in *Festuca arundinacea* had no other impact than reduced cell wall ferulates (~14%–25%) and an up to 4% increase in *in vitro* dry matter digestibility on the transgenic plants (Buanafina *et al.*, 2010). This effect is likely due to disruption of the ester bonds linking FA to cell wall polysaccharides. For a complete review on *in planta* expression of CWD, please see Furtado *et al.* (2014), Park *et al.* (2016), and Willis *et al.* (2016a).

Although most *in planta* CWD enzyme expression studies have assessed the effect of a single gene encoding for single enzyme activity, complete depolymerisation of lignocellulose requires a suite of CWD enzymes including cellulases, hemicellulases, pectinases, polysaccharide lyases, carbohydrate esterases, laccases, peroxidases, and lytic polysaccharide monooxygenases (LPMOs) with synergistic activities. The principle of producing a cocktail of enzymes as an auto-hydrolysis system has been applied to tobacco, with the *in planta* production of effective enzymes in the chloroplast that can be used for the generation of fermentable sugars when applied to lignocellulosic biomass (Verma *et al.*, 2010). However, there are only a few reports on gene stacking or expression of multiple enzymes aimed at *in planta* hydrolysis. Agrivida employed the co-expression of an β-(1,4) endoxylanase with either FAE or an β-(1,4) endoglucanase to significantly improve hydrolysis (glucose and xylose; and glucose, respectively) of transgenic maize plants compared to controls (Zhang *et al.*, 2011), although details about potential effects on plant growth and biomass yield were not reported. An increase in sugar release (31%) was also reported when a FAE was co-expressed with a senescence-induced β-(1,4) endoxylanase in *Festuca arundinacea* but this was accompanied by a 71% decrease in biomass (Buanafina *et al.*, 2015). Considerations around the subcellular targeting of CWD enzymes and spatial and temporal control of synthesis and/or activation, coupled with *in planta* expression of multifunctional chimeric genes provide possible routes to mitigate against plant growth issues associated with *in planta* expression of CWD enzymes.

Non-hydrolytic disruption of lignocellulose (termed amorphogenesis) also provides a viable platform to potentially interfere with cell wall polysaccharide networks and facilitate the accessibility of cellulose to hydrolytic enzymes. Several non-hydrolytic proteins such as swollenin, carbohydrate binding modules (CBM), loosenin and expansins are thought to induce amorphogenesis through swelling, breaking hydrogen bonding networks and/or pH-dependent loosening of the cellulose microfibrils or between cellulose and hemicelluloses without lysis of wall polymers (Arantes and Saddler, 2010). Some of these proteins have already been shown to act synergistically when supplemented with hydrolytic enzyme cocktails and to significantly enhance the efficiency of grass cell wall digestibility (Bunterngsook *et al.*, 2014; Kim *et al.*, 2014; Liu *et al.*, 2015). Despite the clear potential of amorphogenesis-related proteins for improving cellulose accessibility through *in planta* expression, studies, to this end, are merely confined to the expression of plant expansins. The altered expression of endogenous plant expansins *OsEXP4* and *OsEXPA8* in transgenic rice was shown to cause pleiotropic changes in plant growth and development (Choi *et al.*, 2003; Ma *et al.*, 2013; Wang *et al.*, 2014) (Table 4). The authors rationalized this to be a function of altered cell wall compositions, mechanical properties and extensibility from the wall loosening action of expansins. There have been no reports thus far concerning their effect on saccharification and fermentation yields (Table 4).

Recently discovered LPMOs, now classified as auxiliary activity (AA) enzymes in the CAZy database (Levasseur *et al.*, 2013), have emerged as key enzymes for the effective degradation of lignocellulosic biomass and have made a significant contribution to the improvement of commercial enzyme cocktails. The two best-characterized families are AA9 (formerly GH61), mostly fungal enzymes that cleave cellulose chains; and AA10 (formerly CBM33), mostly bacterial enzymes acting on chitin or cellulose. AA9 and AA10 LPMOs share similar 3D structural features and are capable of cleaving polysaccharide chains in their crystalline contexts using an oxidative mechanism that depends on the presence of divalent metal ions and an electron donor (Horn *et al.*, 2012; Vaaje-Kolstad *et al.*, 2010). The new chain-ends generated by LPMOs makes the substrates more susceptible to the activity of glycosyl hydrolases, thus speeding up enzymatic

conversion of biomass (Horn *et al.*, 2012). Plant cell walls most likely contain sufficient concentrations of electrons delivered by lignin (Dimarogona *et al.*, 2012; Westereng *et al.*, 2015) and of divalent metal ions (Krzesłowska, 2011) to allow for effective LPMOs activity. Thus, LPMOs could potentially broaden the range of cell wall degrading enzymes for *in planta* expression to facilitate the degradation of cell wall polysaccharides. The identification of new LPMO families and their polysaccharide substrates, which besides cellulose and chitin, now also includes xyloglucan, glucomannan, xylan, MLG, and starch (Hemsworth *et al.*, 2015), widens the scope for the oxidative *in planta* 'pretreatment' of plant biomass by LPMOs.

Concluding remarks

The prospect of targeted genetic engineering approaches to improve cell wall biorefining properties of grasses, without significant growth penalties seems complex and challenging. It is important that the research devoted to the biotechnological uses of grasses becomes proportional to their vital significance for the production of food, feed, and materials, as well as feedstock for biorefining. With few exceptions, to date, most genetic engineering approaches to modify cell wall polysaccharides in grasses with the aim of making its biomass more amenable to bioconversion have been fairly crude. Irrespective of the strategy (A, B or C), the development of refined mature genetic engineering approaches in grasses requires (i) a better understanding of grass secondary cell wall biosynthesis, including the roles of the individual cell wall-associated enzymes and their substrate identities, and the fine cross-links and structures of secondary cell wall components, and (ii) improved control of the spatiotemporal expression of transgenes encoding enzymes with synergistic or complemental functionalities. With this in mind, rational engineering of cell wall polysaccharides can contribute to an economically sustainable, grass-derived lignocellulose processing industry.

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