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REVIEW

Bioethanol from maize cell walls: genes, molecular tools, and breeding prospects

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Abstract

In the last decade, cellulosic ethanol has caught the growing interest of governments and private investors worldwide as it brings the promise of responsible renewable-energy and an opportunity to depart from an oil-reliant economy. Alongside advances in bioprocessing technologies, the development of specialized bioenergy crops is seen as a pressing industrial necessity, and while C4 perennials (e.g., Miscanthus, switchgrass, and sugarcane) have been coined the most promising candidates for the production of lignocellulosic biomass, maize should not be overlooked. In this review, we have addressed the benefits of advancing maize as a second-generation bioenergy feedstock. We have also analyzed current knowledge on the maize cell wall and promising genetic strategies for its modification, given that lignocellulose recalcitrance represents the most crucial breeding target in bioenergy crop research programs. In addition to lignin, a focus on the underlying genetic basis of cellulose, hemicellulose, and ferulate cross-linking patterns, as well as their regulation, has been warranted. A comprehensive overview of the state-of-art of genomic and phenotyping strategies available for bioenergy crop research is also provided. Overall, maize represents an outstanding model organism for understanding complex cell wall characteristics and defining the path for breeders looking to improve this and other promising bioenergy grasses. With an extensive array of dedicated agronomic and genomic resources at hand, we believe that breeding maize with improved processing amenability is a likely prospect but would like to remind readers that advances in high-biomass yielding properties, improved agronomic hardiness, and enhanced processing efficiency will also be necessary.

Keywords: bioethanol, breeding, cell wall, genes, lignocellulose, maize, molecular tools

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Introduction

As we enter the third millennium, it seems difficult to ignore the societal and environmental consequences of our incommensurate reliance on finite fossil fuels. Alongside the guarantee for energetic security, climate change and its detrimental effects on the environment and agriculture have instigated a global pursuit for sustainable energy alternatives (Vermerris *et al.*, 2007; Wyman, 2007; Huber & Dale, 2009). In particular, substitutes for fossil-based transportation fuels have become a pressing necessity, as our mobility sector currently accounts for over one-third of global greenhouse gas (GHG) emissions (Wyman, 2007). And so as society departs from its oil-reliant economy, researchers, governments, and private investors worldwide have grown increasingly resolute on 'cellulosic ethanol' – a viable near-term alternative to petrol (Schubert, 2006; Waltz, 2008).

Cellulosic ethanol essentially derives from lignocellulose, arguably the most abundant renewable carbon substrate on earth (Schubert, 2006; Dale, 2007; Vermerris *et al.*, 2007). As lignocellulose production requires less agricultural and energetic inputs, cellulosic ethanol could outperform gasoline- and starch-based ethanol as the transportation fuel with lowest GHG emissions and greatest net-energetic outputs (Farrell *et al.*, 2006; Dale, 2007; Wang *et al.*, 2011, 2012). This condition can only be met, however, when processing technologies have matured, as these are currently too energy intensive. Despite extensive revamps in funding and unrelenting governmental support, cellulosic ethanol is yet to transcend the demonstration plant and achieve widescale commercialization (Bacovsky, 2010; Larsen *et al.*, 2012; Saddler

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et al., 2012). With the first commercial endeavors underway (Bacovsky, 2010; Larsen *et al.*, 2012; Brown & Brown, 2013), progress in the commercialization of cellulosic ethanol could be conditioned by the instability of oil prices, market incentives, and governmental policies (Sorda *et al.*, 2010; Brown & Brown, 2013). To survive this uncertain scenario, cellulosic ethanol will need to overcome a series of technical and economic hurdles to compete neck-to-neck with fossil-based transportation fuels.

The feedstock problem

The conversion of biomass into transportation fuels can be effectively achieved through a variety of technological routes, including advanced thermochemical technologies (e.g., Fischer–Tropsch synthesis, gasification or catalytic pyrolysis; Vermerris *et al.*, 2007; Huber & Dale, 2009; Brown & Brown, 2013). Nevertheless, cellulosic ethanol production via biochemical pathways is currently the most commercially represented technology in the sector (Wyman, 2007; Brown & Brown, 2013) and therefore constitutes the referential focus of this review. By 2014, five commercial-scale cellulosic ethanol projects are expected to start operations, and their performance will crucially influence the future of cellulosic fuel policy and economic incentives (Brown & Brown, 2013).

During the production of cellulosic ethanol, the polysaccharide fraction (cellulose and hemicellulose) of plant lignocellulose is enzymatically depolymerized and much like in starch-based ethanol platforms, the resulting sugars are fermented into hydrous ethanol. Lignocellulose, however, has evolved to resist enzymatic degradation, and its efficient depolymerization into fermentable sugars is the predominant technical bottleneck in the system (Wyman, 2007). To circumvent this problem, thermochemical pretreatments are typically employed to increase the accessibility of biomass polysaccharides to hydrolytic enzymes (Mosier *et al.*, 2005). This accessory procedure greatly improves fermentable sugar yields, but it also significantly increases production costs and reduces the energetic and environmental performance of the conversion system (Mosier *et al.*, 2005; Wyman, 2007).

Experts ultimately coincide that the commercial future of cellulosic ethanol is pending on innovations that can reduce the use of costly pretreatments, while simultaneously improving fermentable sugar yields (Wyman, 2007; Huber & Dale, 2009). At its core, research in the field has prioritized advances in the techno-economic efficiency of thermochemical pretreatments, as well as biotechnological endeavors aimed at increasing the yields of enzymatic hydrolysis and fermentation processes. Alongside these advances, however, the choice of feedstock used in the industry will also play a determinant role in the efficiency and profitability of the industry (Vermerris *et al.*, 2007; Wyman, 2007; Carroll & Somerville, 2009; Torres *et al.*, 2013).

Based on the constraints faced by current conversion technologies (including thermochemical routes), cellulosic ethanol will need to be produced from an inexpensive, readily abundant and sustainable substrate (Farrell *et al.*, 2006; Vermerris *et al.*, 2007; Huber & Dale, 2009). In addition, because lignocellulose recalcitrance is a critical barrier to the efficient production of cellulosic fuels, improving the ease with which lignocellulosic materials are consumed in processing facilities would lead to higher energetic yields and greater economic gains. Crops that entirely meet this criterion are not yet available, but genetic improvement programs are underway and optimistic prospects exist for the creation of lignocellulosic feedstocks that can effectively accommodate the needs of the fast-growing cellulosic ethanol industry.

Maize makes sense

Fast-growing C4 perennials, like Miscanthus, switchgrass, and sugarcane, have been coined the most promising candidates for the industrial production of lignocellulosic biomass. These species are principally coveted for their high biomass yields (Fig. 1), broad geographic adaptation, superior carbon sequestration, and efficient nutrient utilization (Carroll & Somerville, 2009; Weijde *et al.*, 2013). Additionally, when used for the production of bio-based fuels, C4 perennials will expectedly offer the greatest net-energetic outputs in relation to other bioenergy feedstocks (Farrell *et al.*, 2006; Von Blottnitz & Curran, 2007; Yuan *et al.*, 2008). The commercial success of upcoming perennials, however, will rely on the availability of superior cultivars that increase the competitiveness of the industry, while sustainably meeting projected market volumes (Weijde *et al.*, 2013). Breeding objectives include increasing biomass yields and yield stability under low-input agricultural systems, enhancing pest and disease resistance, and modifying biomass composition for improved industrial processing (Carroll & Somerville, 2009; Weijde *et al.*, 2013).

With the first cellulosic ethanol commercial plants on the way (Brown & Brown, 2013), a reliable and abundant feedstock is a pressing necessity (Zegada-Lizarazu *et al.*, 2013). Because C4 perennials cannot be readily implemented on a wide-commercial scale, maize will prove instrumental to the development and commercial success of the cellulosic ethanol industry (Schubert, 2006; Vermerris *et al.*, 2007; Carpita & Mccann, 2008;

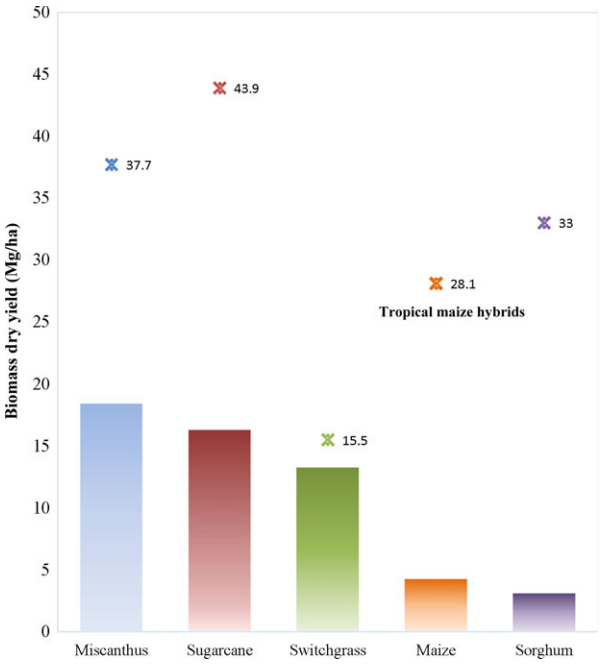

Fig. 1 Mean and potential annual dry biomass yields (Mg ha^{-1}) for relevant C4 energy grasses. For Miscanthus and switchgrass, delayed harvest yields (after winter) are reported. Colored asterisks correspond to highest reported yields in literature, except for sugarcane, for which highest reported yields were extracted from Faostat (2011). Mean and maximum yield values were calculated or extracted from (Clifton-Brown *et al.*, 2004; Heaton *et al.*, 2008) for Miscanthus (Faostat, 2011) for Sugarcane (Heaton *et al.*, 2008; Dweikat *et al.*, 2012) for switchgrass (Faostat, 2011; White *et al.*, 2012) for maize and (Faostat, 2011; Dweikat *et al.*, 2012) for Sorghum. Reported yields do not correspond to comparative trials using standardized conditions (e.g., soil, temperature, season, etc.) and should be regarded as potentiality indicators.

Dweikat *et al.*, 2012; Torres *et al.*, 2013; Weijde *et al.*, 2013). Currently, around 1300 million tons of dry maize stover are produced worldwide; and after factoring the effects of stover removal on soil erosion and nutrient depletion, experts believe that between 40 and 65% of all maize agricultural residues can be sustainably harvested for advanced fuel production (Kim & Dale, 2004; Graham *et al.*, 2007; Youngs & Somerville, 2012). Combined with agricultural crop residues from Sorghum (another promising annual bioenergy grass), this much biomass can contribute significantly to the industry's present and future feedstock needs (Vermerris *et al.*, 2007; Weijde *et al.*, 2013). Furthermore, implementing the technology required for cellulosic fuel production entails significant capital investments and financial risks (Schubert, 2006; Waltz, 2008; Huber & Dale, 2009). Experts have also envisioned that the first commercial cellulosic fuel plants should operate in the vicinity of starch-based ethanol facilities and use maize stover as their lignocellulosic substrate (Schubert, 2006; Vermerris *et al.*, 2007; Carpita & Mccann, 2008). By doing so, nascent enterprises will reduce financial burdens by benefitting from the commercially effective maize-farming, processing and transportation infrastructure (Carpita & Mccann, 2008; Dweikat *et al.*, 2012).

In the future, grower's acceptance of bioenergy perennials will also impact the prevalence of maize as a lignocellulosic feedstock (Carpita & Mccann, 2008). This perspective takes into consideration the high costs and financial risks associated with the setup of new plantations, the amount of years needed before these reach maximum productivity, the loss of growing flexibility that only comes with the choice of annual bioenergy crops like maize and sorghum and the subjective preferences/prejudices of farmers (Carpita & Mccann, 2008; Dweikat *et al.*, 2012; Zegada-Lizarazu *et al.*, 2013). Ultimately, this factor can open unexplored avenues for the production of 'energy-dedicated' maize varieties that could potentially compete with other promising C4 species. With a wealth of agronomic and genomic resources, advancing maize with high-biomass yielding properties and improved nutrient use efficiency is a likely prospect (Carpita & Mccann, 2008; White, 2010; Dweikat *et al.*, 2012; White *et al.*, 2012). Photoperiod-sensitive hybrids derived from crosses between temperate and tropical varieties, for instance, are a proof-of-concept example for the derivation of maize into an energy-dedicated species (Fig. 1). These temperate × tropical maize (TTM) hybrids typically allocate the majority of their biomass into the stover and can yield up to 28.1 Mg ha^{-1} annual dry biomass in cropping systems supplemented with nitrogen (N) fertilizer (White *et al.*, 2012), and up to 21.3 Mg ha^{-1} annual dry biomass without supplemental N fertilization (White, 2010). Because TTM hybrids can also accumulate high amounts of soluble sugars in their stems (~50% more when compared to commercial hybrids), these can expectedly yield comparable amounts of ethanol (~8000 L ha^{-1}) per hectare under no supplemental N fertilization as commercial grain hybrids supplemented with N (~10 500 L ha^{-1}; White *et al.*, 2012). Although preliminary in nature, these results demonstrate the potential behind breeding endeavors looking to advance maize outside its classical framework. Understandably, before TTM hybrids can be considered for dedicated lignocellulose production, major advances in nutrient use efficiency, climatic hardiness, biotic resistance, and seed productivity will need to be achieved through genetic improvement and crop management (Dweikat *et al.*, 2012; White *et al.*, 2012). In particular, claims regarding enhanced biomass productivity in the absence of fertilization should be evaluated carefully,

given that such cropping systems would rapidly deplete nutrient soil reserves when the crop fails to return nutrients back to the soil. As of today, however, the extensive genetic diversity of maize remains largely unexploited (Box 1) and opportunities exist for the introgression of useful exotic traits that can expedite the advance of dual-purpose and energy-dedicated maize cultivars for the cellulosic ethanol industry (Lewis *et al.*, 2010; Lorenz *et al.*, 2010; Cairns *et al.*, 2012; Muttoni *et al.*, 2012).

Box 1.
The unexploited diversity of maize

Progress in the development of maize for cellulosic fuel production should not be confined to the exclusive utilization of commercially available germplasm. Breeding endeavors in maize have predominantly focused on advancing grain yield and yield stability, and only a minority have specialized on exploiting useful biomass characteristics (Lauer *et al.*, 2001). A natural outcrosser, maize is remarkably diverse, with most of its desirable traits yet to be utilized (Yan *et al.*, 2009; Hallauer *et al.*, 2010; Muttoni *et al.*, 2012). This unexploited diversity has been preserved in gene bank collections at numerous international research centers and is publically available upon request. In addition, public and private efforts like the Latin American Maize Project (Salhuana *et al.*, 1991), the Germplasm Enhancement of Maize project (Pollak, 2003), and on-going work at the International Maize and What Improvement Centre (CIMMYT) are making immense contributions towards the evaluation and classification of exotic germplasm, as well as its adaptation into elite material. The success of these and similar projects will prove indispensable to the incorporation of novel characteristics; all of which offer possibilities to improve the biomass potential and agronomic sustainability of this crop species.

Building upon the maize cell wall: from gene to phenotype

A comprehensive recount of the state-of-art of maize breeding for the cellulosic ethanol industry would encompass a broad range of subjects spanning over the allotted length of this article. We have focused on the maize cell wall, nonetheless, as we firmly believe that advancing biomass feedstocks that best match the processing conditions used in the industry can improve the commercial and environmental performance of cellulosic ethanol production (Torres *et al.*, 2013). This section describes current knowledge on the genes involved in the synthesis of the main components of maize cell walls (cellulose, hemicellulose and lignin), their regulation and strategies to optimize biomass composition for bioethanol production.

Cellulose

Improving the relative content and industrial quality of cellulose is a pivotal strategy toward the development of advanced lignocellulosic feedstocks. On the one hand, a higher abundance of cell wall polysaccharides per unit of biomass will conceivably increase the amount of harvestable energy per unit of land. Alterations in cellulose ultrastructure, which simplify its enzymatic depolymerization, on the other hand, are expected to improve the processing efficiency and economics of biomass-to-ethanol conversion technologies. Cellulose is a highly recalcitrant substrate and properties presumed to limit its enzymatic degradability include its high degree of polymerization and high crystallinity index (Mansfield *et al.*, 1999; Park *et al.*, 2010).

Modifying cellulose assembly and deposition patterns in maize, however, is a challenging undertaking that will require a thorough understanding of its complex biosynthetic machinery. At present, 12 members of the maize cellulose synthase (*CesA*) gene family have been annotated and characterized (Holland *et al.*, 2000; Appenzeller *et al.*, 2004). Based on sequence orthology, these genes presumably encode the catalytic subunits of the maize cellulose synthase complex (CSC; Holland *et al.*, 2000; Appenzeller *et al.*, 2004; Penning *et al.*, 2009). In accordance with the functional specialization of *CesA* isoforms in Arabidopsis (Taylor *et al.*, 2003; Desprez *et al.*, 2007; Persson *et al.*, 2007), rice (Tanaka *et al.*, 2003) and barley(Burton *et al.*, 2004), expression studies reveal that at least three specific maize *CesA*s (namely *ZmCesA*10, 11 and 12) are required during secondary cell wall formation, while the rest are assumed to be involved for primary cell wall deposition (Appenzeller *et al.*, 2004; Penning *et al.*, 2009).

The CSC also appears to interact with a wide array of plasma membrane-associated proteins; most suspected necessary for normal cellulose microfibril assembly, crystallization, orientation, and patterning (Somerville, 2006). In maize, a gene orthologous to the Arabidopsis *Cobra-Like4* isoform was cloned from the *brittle stalk-2* (*bk-2*) mutant (Ching *et al.*, 2006; Sindhu *et al.*, 2007); a naturally occurring phenotype characterized by stalks which break easily under mechanical pressure. This finding and other recent breakthroughs in fundamental cell wall research would suggest that cellulose content and ultrastructure are targets of multiple regulatory mechanisms extending further than the CSC and its associated pro-

teins. Targeted alterations in cellulose content or molecular quality are yet to be reported for maize. Genetic engineering approaches will need to be carefully evaluated, however, as perturbations to the cellulose synthesis machinery could lead to phenotypes with decreased vigor or other undesirable biomass characteristics. Alternatively, allelic variants of crucial cellulose biosynthesis genes could be characterized and used directly in classical breeding schemes. Harris *et al.* (2009) uncovered an Arabidopsis mutant (*irx 1-2*) exhibiting a point-mutation at the C-terminal transmembrane region of the *CesA*3. The resulting phenotype displayed lower cellulose crystallinity (<~30%) and improved cell wall digestibility relative to wild type, but no profound perturbation on growth and fitness (Harris *et al*., 2009, 2012).

Hemicellulose

Research efforts looking to improve the yields and industrial quality of lignocellulosic crops have paid less recognition to the benefits that could arise from modifying the hemicellulosic fraction of plant cell walls. Current advances in the development of novel xylanases and C5-fermenting microorganisms, however, have opened the possibility to exploit this polysaccharide for the production of cellulosic ethanol and other side-stream bio-commodities (Becker & Boles, 2003; Bera *et al*., 2011; Zhang *et al*., 2011). In addition, because hemicellulose binds to cellulose microfibrils and threads them via cross-links with lignin (Carpita, 1996; Grabber *et al*., 2000), hemicellulose plays a crucial role in the structural integrity and recalcitrant nature of the cell wall. By elucidating the genetic mechanisms controlling hemicellulose biosynthesis, it should be possible to identify genetic variants that improve cell wall digestibility.

Although it is well recognized that plant hemicelluloses are synthesized in the ER/Golgi and mobilized to the growing cell wall via secreted vesicles (Carpita, 1996; Carpita & Mccann, 2010), limited information exists with respect to the enzymatic complexes directing their biosynthesis. Hemicellulosic cell wall polysaccharides appear to be synthesized by members of the *Cellulose Synthase Like* (*Csl*) gene family; a multigene complex highly homologous to the *CesA* family. Richmond and Somerville (2000, 2001) originally ascribed *Csl* gene products a processive glycosyltransferase (GT) function after observing that all *Csl* proteins possess a conserved domain defining their ability to catalyze the characteristic β-linkage common to cell wall polysaccharides. Thus far, expression studies suggest that primary wall xyloglucans (Cocuron *et al*., 2007), (gluco)mannans (Dhugga *et al*., 2004; Liepman *et al*., 2005) and grass-specific mixed linkage glucans (Doblin *et al*., 2009) are all synthesized by *Csl* encoded enzymes. By contrast, extensive evidence indicates that the xylan backbones of secondary wall glucuronoxylan (GX) in dicots and (glucurono) arabinoxylan (GAX) in grasses are synthesized by non-processive GTs (Table 1; Aspeborg *et al*., 2005; Brown *et al*., 2005; Persson *et al*., 2005; Mitchell *et al*., 2007; Bosch *et al*., 2011). Advances in our understanding of the synthesis of GX in model dicots (e.g., Arabidopsis) will prove fundamental to the development of bioenergy grasses with tailored hemicellulose composition, as GAX represents the major noncellulosic polysaccharide in monocots. In maize, Bosch *et al.* (2011) have identified two GT47 sequences (*GRMZM2G100143* and *GRMZM2G059825*) displaying preferential expression in internodes undergoing secondary cell wall deposition. Both genes are homologous to the reduced-xylan deposition *IRX10* and *IRX10L* mutants of Arabidopsis and are likely candidates for the biosynthesis of GAX.

Efforts have also been devoted toward characterizing enzymes mediating GX and GAX branching reactions. Recent breakthroughs include the identification of the reduced wall acetylation (Lee *et al*., 2011) and Glucuronic Acid Substitution of Xylan (*GUX*; Mortimer *et al*., 2010) genes from Arabidopsis, as well as the Xylan Arabinosyltransferase (*XAT*) genes from rice and wheat (Anders *et al*., 2012) and the Xylosyl Arabinosyl Substitution of Xylan (*XAX*) gene from rice (Chiniquy *et al*., 2012). Exciting new evidence would also suggest that homologous sidegroup transferases differ in their enzymatic affinities and produce unique branching patterns. The functionally divergent *GUX1* and *GUX2* homologues from Arabidopsis appear to decorate distinct domains of the same xylan molecule either in evenly spaced long intervals or in tightly clustered patterns (Bromley *et al*., 2013). It is yet to be determined, however, whether GXs differ in the proportion, length, and distribution of substitution patterns (Bromley *et al*., 2013), or whether heterogeneous GXs have different affinities and functions in the plant cell wall.

Research on the genetic mechanisms controlling hemicellulosic branching is increasingly appealing for the production of advanced lignocellulosic feedstocks. Presently, a prominent view suggests that reducing the complexity of hemicelluloses would enhance their extractability and improve the overall degradability of lignocellulosic biomass (Appeldoorn *et al*., 2010; Mortimer *et al*., 2010; Van Eylen *et al*., 2011). In maize, the high substitution frequency of GAX has proven detrimental to the enzymatic conversion of cell wall polysaccharides following dilute acid pretreatment (Appeldoorn *et al*., 2010; Van Eylen *et al*., 2011). Based on the work of Van Eylen *et al.* (2011) and Appeldoorn *et al.* (2010), reductions in the frequency of acetic acid, uronic acid and arabinose side groups in GAX would concomitantly lead to a reduction in the use of costly enzymatic cocktails

Table 1 Genes involved in cell wall xylan biosynthesis

Gene	Species	GT sub-class	Presumed function	References
IRX8, *FRA8*, *FH8*, *PARVUS*	Arabidopsis	GT47 (*Fra-8*, *FH8*) GT8 (*IRX-8*, *Parvus*)	Synthesis of a unique β-d-Xyl-(1 → 3)-α-l-Rha-(1 → 2)-α-d-GalA-(1 → 4)-d-Xyl tetrasaccharide commonly found at the reducing end of glucuronoxylan (GX). This tetrasaccharide may act as a primer sequence for the initiation of short xylan chains which presumably splice together to form longer xylan polymers	(Brown *et al.*, 2007; Peña *et al.*, 2007; York & O'neill, 2008; Lee *et al.*, 2009)
IRX9, *IRX9-L*, *IRX-10*, *IRX-10L*, *IRX14*, *IRX14-L*	Arabidopsis	GT43 (*IRX9*, *IRX9-L*, *IRX14*, *IRX14-L*) GT47 (*IRX-10*, *IRX-10L*)	Elongation of GX oligomeric backbones	(Brown *et al.*, 2009; Wu *et al.*, 2009; Keppler & Showalter, 2010; Wu *et al.*, 2010)
GUX-1, *GUX-2*	Arabidopsis	GT8	Addition of glucuronic acid and 4-O-methylglucuronic acid side branches on GX backbone	(Mortimer *et al.*, 2010; Bromley *et al.*, 2013)
TaGT43-4, *TaGT47-13*, *TaGT75-4*	Wheat	GT43 (*TaGT43-4*) GT47 (*TaGT47-13*) GT75 (*TaGT75-4*)	Presumably involved in glucuronoarabinoxylan (GAX) biosynthesis, although specific functions are yet undefined. *TaGT43-4* and *TaGT47-13* are, respectively, orthologous to Arabidopsis *IRX14* AND *IRX10*	(Zeng *et al.*, 2010)
TaXAT2; OsXAT2, *OsXAT3*	Wheat, Rice	GT61	Arabinosylation of the xylan backbones of GAX	(Anders *et al.*, 2012)
OsIRX9, *OsIRX9L*, *OsIRX14*	Rice	GT8	Presumably involved in the synthesis and elongation of the xylan backbone of GAX	(Chiniquy *et al.*, 2013)
OsXAX1	Rice	GT61	Putatively involved in the β-(1,2) xylosyl substitution of α-(1,3) arabinosyl residues of GAX	(Chiniquy *et al.*, 2012)
GRMZM2G100143, *GRMZM2G059825*	Maize	GT47	Presumably involved in the synthesis of GAX in secondary cell walls. *GRMZM2G100143* and *GRMZM2G059825* are homologues to *IRX10* and *IRX10-L*	(Bosch *et al.*, 2011)

and the formation of acetyl-based fermentation inhibitors during maize cellulosic ethanol conversion. Contradictorily, an alternative strategy to enhance the enzymatic accessibility of cell wall polysaccharides would entail increasing the abundance of 'favorable' side-chain substitutions in the backbones of hemicelluloses. This approach is grounded on the assumption that highly branched xylan polymers have a reduced adsorption-affinity to cellulose and improved water solubility (Kabel *et al.*, 2007). More recently, Torres *et al.* (2013) demonstrated that the compounded effect of reduced cell wall lignin and high GAX arabinose-to-xylose ratio significantly improved the enzymatic conversion efficiency of mildly pretreated maize stem materials. Clearly, insights as to how GAX substitution patterns affect the strength and recalcitrance of the cell wall under different processing conditions are still necessary. However, it appears that maize harbors extensive genetic variation in the degree and (presumably) distribution of GAX substitution patterns (Torres *et al.*, 2013), thus opening the possibility to tailor maize cell wall hemicelluloses to the dynamic demands of the industry.

Lignin

The genetic and metabolic mechanisms that lead to the formation of lignin have been extensively studied and are well understood. For recent compendiums describing the structure, biosynthesis and biological function of this complex biopolymer, readers should refer to the work of Zhong & Ye (2009), Bonawitz and Chapple (2010), Vanholme *et al.* (2010) and Courtial *et al.* (2013b).

In the cell wall, lignin and other phenolic aromatics chemically cross-link to each other and to hemicellulose to produce an unyielding matrix that cohesively links and masks cell wall polysaccharides (Carpita, 1996). Evidence suggests that lignin reduces the effectiveness of enzymatic saccharification processes by adsorbing and nonproductively binding to hydrolytic enzymes (Berlin *et al.*, 2006; Nakagame *et al.*, 2010) and by

physically shielding cellulose microfibrils from enzymatic attack (Selig *et al*., 2007). As a consequence, efforts looking to reduce the inherent recalcitrance of bioenergy feedstocks have focused on understanding how variations in lignin content, composition and structure can improve cell wall degradability.

Currently, the most accepted notion is that reductions in cell wall lignin concentration can contribute positively to the ease with which cell walls are deconstructed. Supporting this claim are studies on the conversion efficiency of the *brown midrib* mutants of maize (*bm*) and sorghum (*bmr*; Barrière *et al*., 2004; Vermerris *et al*., 2007; Saballos *et al*., 2008; Barrière *et al*., 2013) and other species exhibiting genetically engineered reductions in lignin content (Chen & Dixon, 2007; Fu *et al*., 2011; Jung *et al*., 2012). Modifying lignin composition with respect to its monomeric constituents has also been coined a promising approach for enhancing biomass degradability. In maize and other bioenergy grasses, perturbations in monolignol metabolism favoring lower syringyl/guaiacyl ratios have been associated to reductions in biomass recalcitrance (Saballos *et al*., 2008; Fu *et al*., 2011; Fornalé *et al*., 2012; Jung *et al*., 2012). However, as favorable changes in monolignol ratios are often accompanied by reductions in lignin content (Chen & Dixon, 2007; Saballos *et al*., 2008; Fornalé *et al*., 2012; Jung *et al*., 2012), it is still difficult to ascertain whether monolignol balance truly affects degradability properties (Grabber *et al*., 2009). More recently, the concept of redesigning lignin *in planta* has gained momentum (Eudes *et al*., 2012; Vanholme *et al*., 2012; Zhang *et al*., 2012). Fundamentally, this novel strategy exploits the combinatorial plasticity of the lignin polymerization process, which allows for the incorporation of 'un-conventional' monolignols into the lignin polymer (Vanholme *et al*., 2010, 2012). This strategy ultimately allows for the creation of crops with customized lignin polymers displaying enhanced solubility, extractability, and chemical valorization. As an example, Eudes *et al.* (2012) induced the expression of a hydroxycinnamoyl-CoA hydratase-lyase from *Pseudomonas fluorescens* in Arabidopsis, to divert the metabolism of regular C_6C_3 monolignols in favor of atypical C_6C_1 aromatics, naturally present in lignin in trace amounts. Compared with wild-type controls, engineered lines showed a higher incorporation of the atypical aromatic in lignin, and a concomitant reduction in the degree of lignin polymerization. The engineered lines also displayed improved enzymatic saccharification efficiency following thermochemical pretreatment. (Eudes *et al*., 2012).

In maize, classical breeding approaches have proven successful in the targeted modification of lignin for improved cell wall degradability properties. Extensive surveys of experimental populations and mutant panels have revealed the vast extent of lignin variation and enzymatic digestibility properties available in forage maize (Argillier *et al*., 1996; Fontaine *et al*., 2003a; Marita *et al*., 2003; Méchin *et al*., 2005; Barrière *et al*., 2009, 2010, 2013) and have served as platforms for the identification of quantitative trait loci (QTL) underlying maize lignification characteristics relevant to cellulosic ethanol production (Méchin *et al*., 2001; Cardinal *et al*., 2003; Barrière *et al*., 2008, 2009; Thomas *et al*., 2010; Courtial *et al*., 2013a). More recently, Lorenzana *et al.* (2010) and Torres *et al.* (2013) have demonstrated the strong negative correlation ($r > -0.65$) that exists between maize cell wall lignin content and enzymatic conversion efficiency after dilute-acid pretreatment. From these studies, it has become apparent that variation in lignin content leading to improved bioconversion efficiency is highly heritable, making it possible to select and advance dedicated maize feedstocks that can improve the efficiency and economics of biomass-to-ethanol conversion technologies.

Genetic engineering has also been explored as a viable strategy for the modification of lignin content and composition in maize. Piquemal *et al.* (2002) and He *et al.* (2003) used an antisense/gene approach to independently produce transgenic lines with reduced Caffeic-acid O-methyltransferase activity, thereby mimicking the naturally occurring *bm3* phenotype. In both studies, the resulting transgenics displayed significant reductions in whole-plant lignin content as well as improved *in vitro* enzymatic digestibility. More recently, Fornalé *et al.* (2012) used RNA interference to produce engineered lines with reduced cinnamyl/alcohol/dehydrogenase activity; and one was selected for extensive characterization. Although the selected transgenic displayed a slight reduction in lignin content and improved cell wall digestibility in leaf midribs, its stems showed no change in lignin accumulation or improved enzymatic digestibility relative to the wild-type control (Fornalé *et al*., 2012). These results ultimately strengthen the notion that a systematic understanding of lignin biosynthesis is elemental if we seek to maximize the beneficial effects, and avoid the detrimental consequences, of engineered perturbations in lignin metabolic fluxes. Extensive evidence suggests that targeted alterations in lignin properties are often accompanied by compensatory mechanisms that can either restore the original phenotype or reduce the phenotypic effect of a profound metabolic alteration (Barrière *et al*., 2003; Fontaine *et al*., 2003a; Shi *et al*., 2006; Guillaumie *et al*., 2007a; Vermerris *et al*., 2007; Barrière *et al*., 2009). To illustrate this, when the *bm3* gene was introgressed into different genetic backgrounds, the resulting lines exhibited clear differences in lignin content and overall digestibility (Gentinetta *et al*., 1990; Barrière *et al*., 2003; Shi *et al*.,

2006). Accordingly, effective lignin engineering strategies need to consider the effects of pathway cross-talk mechanisms, spatial expression, and allelic redundancy to achieve desired results.

Deconstructing the matrix: the role of ferulate cross-links

In grasses, GAX molecules cross-link to each other via esterified diferulic bridges and to lignin via ferulic/diferulic ether bonds (Grabber *et al.*, 2000, 2004), thereby forming a matrix that encases the cellulosic skeleton of the plant cell wall. It is commonly understood that both diferulate cross-linking between xylans and ferulate cross-linking of xylans to lignin occur at the plant cell wall via oxidative coupling reactions, essentially devoid of enzymatic control (Grabber *et al.*, 1995; Ralph *et al.*, 1995; Grabber *et al.*, 2000). By contrast, ferulates are expectedly esterified to the arabinosyl residues of GAX through an enzymatically driven process occurring at the Golgi (Grabber *et al.*, 2004). To date, however, none of the genes involved in this process have been identified.

Unambiguous evidence from cell wall mimetic studies has demonstrated that both, xylan-to-xylan ferulate bridging (Grabber *et al.*, 1998a) and ferulate-to-lignin cross-links (Grabber *et al.*, 1998b, 2009) limit the enzymatic depolymerization of cell wall polysaccharides. Understandably, strategies that could reduce the incidence of ferulate cross-links in the cell wall have the potential to improve cell wall degradability properties relevant to cellulosic ethanol production. For instance, numerous studies in maize have revealed the extent of genetic variation potentially available in cell wall ferulate content, as well as its negative relationship with cell wall digestibility properties (Argillier *et al.*, 1996; Fontaine *et al.*, 2003b; Barrière *et al.*, 2008; Barros-Rios *et al.*, 2012). Similarly, Jung & Phillips (2010) have identified a putative maize mutation – seedling ferulate ester – which has been shown to reduce the content of etherified and esterified ferulates in the cell wall and increase cell wall digestibility, without affecting plant growth and yield. And while highly promising, the influence of ferulate cross-linking on degradability properties needs to be analyzed within the context of cellulosic ethanol production systems, considering that the physical, thermochemical, and enzymatic mechanisms underlying cell wall degradation processes in animal rumen and biomass-to-ethanol conversion platforms are not strictly similar (Jung & Bernardo, 2012; Torres *et al.*, 2013).

Transcription factors

Transcription factors regulate the quantitative, spatial and temporal expression of gene networks and define the differentiation of plant tissues, organs and other architectural features. Within the same organism, plant cell walls can vary greatly in their compositional and structural constitution among functionally divergent cell types (Zhong & Ye, 2007). The elucidation of the regulatory mechanisms that control cell wall differentiation will facilitate the tailoring of biomass yield and quality traits in a more coordinated and targeted fashion (Petersen *et al.*, 2012; Yang *et al.*, 2013).

In the last decade, numerous studies in Arabidopsis (as well as other species) have uncovered a vast array of key transcriptional regulators involved in secondary cell wall biosynthesis and differentiation. From these studies, it has become apparent that members of the *NAC* (e.g., *NST1*, *NST2*, *VND6*, and *VND7*) protein family act as master regulators of secondary cell wall deposition (Mitsuda *et al.*, 2007; Zhong & Ye, 2007) with different members displaying cell type specific expression patterns (Kubo *et al.*, 2005; Zhong *et al.*, 2006, 2008; Yamaguchi *et al.*, 2010). These master regulators appear to control downstream transcriptional cascades, which in turn activate cell wall lignin and carbohydrate biosynthetic pathways (Zhong *et al.*, 2008). In fact, *MYB* transcription factors have been highlighted as targets of *NAC* master regulators and have been shown to directly or indirectly activate cell wall deposition processes (Zhong *et al.*, 2008, 2010). For instance, while Arabidopsis *MYB*46 and *MYB*83 appear to globally regulate secondary cell wall deposition (Zhong *et al.*, 2007; Mccarthy *et al.*, 2009; Zhong & Ye, 2012), *MYB*58, *MYB*63, and *MYB*85 have been shown to specifically regulate lignin biosynthesis (Zhong *et al.*, 2008; Zhou *et al.*, 2009). Much work is needed, however, before we entirely comprehend the complex transcriptional network governing cell wall deposition processes. In particular, the identification of novel modulators and downstream targets of *NAC* master regulators and a better understanding of their spatial regulation in specific cell types will prove beneficial to the development of effective cell wall engineering strategies. In maize, advances in functional genomics are rapidly unraveling the identity of NAC and MYB transcription factors presumably involved in cell wall biosynthesis and differentiation (Fornalé *et al.*, 2006, 2010; Bosch *et al.*, 2011; Zhong *et al.*, 2011).

Interestingly, despite gaps in our understanding of cell wall regulatory processes, several studies have demonstrated successful approaches to alter cell wall biosynthesis through controlled modulations of transcription factors (Eudes *et al.*, 2012; Petersen *et al.*, 2012; Yang *et al.*, 2013). Noteworthy, Yang *et al.* (2013) 'rewired' the secondary cell wall deposition network of Arabidopsis using a sophisticated approach that enabled for simultaneous cell wall alterations in specific tissues. Firstly, *c4h* lignin-

deficient mutants were transformed with a functional *C4H* variant containing the vessel-specific promoter of *VND6* (pVND6), which allowed for the targeted recovery of cell wall lignification in stem vessels. The resulting pVND6::C4H lines were then transformed with an engineered construct of *NST1* coupled to the promoter sequence of *IRX8* (pIRX8); itself known to be a downstream target of *NST1*. By doing so, an artificial positive feedback loop was created whereby the expression of *NST1*, a master regulator of secondary wall formation in stem fibers, was specifically enhanced in tissues undergoing secondary wall deposition. Ultimately, pVND6::*CH4*-pIRX8::*NST1* lines showed wild-type vigor and growth, functional stem vessels, and increased cell wall deposition in fiber cells without over lignification. Also, the double-transgenics displayed higher fermentable sugar release relative to wild-type following pretreatment and enzymatic saccharification. Clearly, advances in our understanding of cell wall regulatory mechanisms have expanded our potential to precisely engineer biomass yield and quality characteristics, while circumventing the detrimental effects on yield and vigor commonly associated with transgenic approaches targeting cell wall metabolic fluxes.

Advancing energy maize: tools and concepts

Phenotyping tools

The greatest challenge in 'bioenergy crop' research and breeding programs is the screening of thousands of genetic variants to evaluate, map, and select traits that enhance the conversion potential of biomass into liquid fuels. Currently, numerous analytical platforms are in place for the exhaustive analysis of cell wall compositional and conversion efficiency parameters (Table 2). This comprehensive toolkit ranges from simple enzymatic assays to evaluate the saccharification efficiency of lignocellulosic substrates, to state-of-art chromatographic tools used to pinpoint the compositional diversity and ultrastructure of cell wall polymers. With the advent of highly precise weighing and liquid-handling robotic workstations, standard compositional quantification methods and bioconversion assays have been successfully down-scaled and automated to accommodate high-throughput analyses (Chundawat *et al*., 2008; Gomez *et al*., 2010; Santoro *et al*., 2010; Selig *et al*., 2010; Studer *et al*., 2010). Notwithstanding, phenotyping tools that provide additional layers of information, like imaging techniques used to study the effects of pretreatments on biomass substrates or methods, which allow for the quantitative partitioning of biomass fibers (i.e., ratio of rind to pith in maize internodes), are yet to be adapted into automated systems.

More efficient and economical alternatives to robotic platforms have also been proposed; the most promising of which are based on spectroscopic methods, such as near-infrared (NIR), Fourier-transformed infrared (FT-IR) and pyrolysis molecular beam mass spectroscopy (Py-MBMS; Labbe *et al*., 2005; Vermerris *et al*., 2007; Philip Ye *et al*., 2008). In these systems, a core set of samples is exhaustively analyzed using conventional chemical assays to build calibration models that can link compositional information to specific spectral variants. Once the model has been established, the biochemical properties of unknown samples can be predicted based on their spectral fingerprint. Although these screening tools convey considerable capital investments, their principle advantage is that spectral acquisition is fast, simple and does not require chemical consumables. For maize, NIRS is routinely employed in a commercial setting for the assessment of complex forage quality traits including the analysis of cell wall digestibility properties (Lauer *et al*., 2001; Vermerris *et al*., 2007; Lewis *et al*., 2010). Within the scope of biomass research for cellulosic ethanol, several reports have demonstrated the successful application of NIRS for the prediction of polysaccharide, neutral sugar, lignin and ferulate content, as well as bioconversion efficiency (Barrière *et al*., 2008; Lorenz *et al*., 2009; Wolfrum & Lorenz, 2009; Jung & Phillips, 2010; Lorenzana *et al*., 2010; Chavigneau *et al*., 2012).

Genomic and molecular tools

Because of its global relevance as an agricultural and industrial staple, maize remains at the forefront of fundamental developments in molecular and genomic technologies. Currently, maize geneticists and breeders worldwide benefit from an extensive infrastructure of genotyping platforms, expression analyses repositories and powerful experimental populations. In addition, a draft sequence of the maize genome is now available (Schnable *et al*., 2009), and numerous resequencing projects have updated our knowledge on the evolution, diversity, and complex heterotic nature of this crop species (Gore *et al*., 2009; Lu *et al*., 2009; Lai *et al*., 2010; Chia *et al*., 2012; Wallace *et al*., 2014). Complemented by powerful data-mining resources (e.g., POPcorn, MaizeGDB, Panzea), marker discovery, and gene annotation in maize are advancing rapidly.

Classical linkage analysis will prove integral to the identification of QTL influencing complex biomass accumulation and cell wall architectural traits. Lorenzana *et al*. (2010), for instance, surveyed the testcrosses of 223 recombinant inbred lines from the IBM population (Lee *et al*., 2002) for variation in different biomass characteristics, including conversion efficiency after dilute acid pretreatment. Despite the appreciably lim-

Table 2 High-throughput techniques available for the analysis of cell wall traits relevant to cellulosic ethanol production

Analysis	Technique	Used for determining	Description	References
Cell wall composition	Chromatography	Polysaccharide monomeric composition, lignin content	Cell wall polysaccharides are extracted and digested through a two-stage acid hydrolysis. Released monosaccharides are quantified via HPLC. Klasson lignin estimates are obtained gravimetrically	(Demartini *et al.*, 2011)
	Analytical Pyrolysis	Polysaccharide content, lignin content, lignin monomeric composition	Lignocellulosic samples are pyrolyzed and the resulting fragments are analyzed via GC/MS or MB/MS	(Fontaine *et al.*, 2003a; Sykes *et al.*, 2009; Studer *et al.*, 2011)
	Spectroscopy	Polysaccharide content, lignin content, lignin monomeric composition	Chemical composition is predicted through an array of spectroscopic platforms (NIR, FT-IR, Raman, NMR) based on calibration models linking compositional information to specific spectral patterns	(Lupoi *et al.*, 2013)
Cell wall ultrastructure	Chromatography, Electrophoresis	Hemicellulose and pectin degree of polymerization and degree of substitution	Cell wall polysaccharides are chemically extracted and digested using selective hydrolytic enzymes. The resulting oligosaccharides are separated and quantified via chromatographic (HPLC) or electrophoretic (PACE, CE) techniques, and/or identified through mass spectrometry (MALDI, NMR)	(Lerouxel *et al.*, 2002; Obel *et al.*, 2009; Persson *et al.*, 2010)
	Spectroscopy	Lignin content, monomeric composition and linkage analysis	Lignin is isolated from the cell wall and analyzed using one- and two-dimensional NMR spectroscopy	(Foston *et al.*, 2012; Lupoi *et al.*, 2013)
	Immuno-profiling	Hemicellulose, pectin and glycoprotein degree of polymerization and degree of substitution	Cell wall polysaccharides are chemically extracted and/or digested using selective hydrolytic enzymes. The resulting fractions are fixed onto microarrays (CoMPP) or ELISA microplates (Glycome Profiling) and probed using mAbs and CBMs with specificity for polysaccharide epitopes	(Moller *et al.*, 2007; Knox, 2008; Duceppe *et al.*, 2012; Pattathil *et al.*, 2012; Demartini *et al.*, 2013)
Cell wall recalcitrance	Enzymatic	Bioconversion efficiency of lignocellulosic substrates	The NREL LAP-009 bioconversion assay has been automated and downscaled to via robotic platforms. To accommodate an accessory pretreatment step, the most sophisticated systems rely on stackable 96-well metallic reactor plates that can withstand the chemical loads, pressure, and high temperatures used in industry	(Chundawat *et al.*, 2008; Gomez *et al.*, 2010; Santoro *et al.*, 2010; Selig *et al.*, 2010; Studer *et al.*, 2010)

ited degree of variation available in the population (e.g., lignin content on cell wall basis ranged from 20.3 to 21.9% across the experimental panel), the authors uncovered 152 small effect QTLs for a variety of cell wall and cellulosic ethanol-relevant characters. Knowledge obtained from linkage studies should also be complemented with findings from a wealth of forage maize studies elucidating crucial QTLs for cell-wall digestibility, lignin content, and lignin composition. Also, the advent of high-throughput single nucleotide polymorphism genotyping platforms, sophisticated biometric models and high-resolution mapping panels (including the powerful Nested Association Mapping Panel of maize) will expectedly expedite genomewide association studies for biomass yield and quality characteristics (Riedelsheimer *et al.*, 2012; Windhausen *et al.*, 2012; Massman *et al.*, 2013; Wallace *et al.*, 2014).

Functional genomics will also contribute immensely to our understanding of the genetic and biochemical mechanisms governing the construction of the plant cell

wall. In maize, expression studies using diverse developmental models have led to the identification, annotation, and functional classification of numerous genes involved in cell wall biosynthesis (Shi *et al*., 2006; Guillaumie *et al*., 2007a,b; Penning *et al*., 2009; Bosch *et al*., 2011). Expression analyses of the elongating maize internode have proven particularly appealing, as these have provided a developmental snapshot for the deposition of the highly recalcitrant secondary cell wall. Forward- and reverse-genetic assessments of mutagenized maize populations are also powerful tools for identifying and underpinning the function of cell wall genes. In particular, gene tagging through transposon insertional mutagenesis, in combination with high-throughput genomic/phenomic platforms, has simplified the generation, discovery and cloning of cell wall mutants. Within the framework of the Cell Wall Genomics project (http://cellwall.genomics.purdue.edu), Vermerris *et al.* (2007) have conceptualized the use of NIR and Py-BMS platforms to identify novel cell wall mutants from the Uniform*Mu* population. Using the same mutant collection, Penning *et al*. (2009) have shown the versatility of next-generation sequencing for the identification of mutants in specific cell wall genes, with the goal of better understanding their role in cell wall metabolic processes. Without doubt, the wealth of dedicated genomic resources currently available for maize make it an outstanding model organism for understanding complex biomass characteristics and defining the path for breeders looking to improve this crop for a bio-based economy.

Transgenic approaches

Conventional bioengineering strategies have been extensively used for the production of novel phenotypes with improved biomass characteristics. Knock-out, antisense construct and RNA-interference technologies have been the de facto routes for studying the effects of targeted alterations in cell wall metabolic fluxes and regulatory networks.

More recently, protein engineering and heterologous expression systems have broadened the horizons of energy crop bioengineering (Table 3). Heterologous

Table 3 Examples of studies using heterologous expression systems for the improvement of bioenergy crops

Species	Target polymer	Approach	Results	References
Arabidopsis	Lignin	Expression of a *Pseudomonas fluorescens* hydroxycinnamoyl-CoA hydratase-lyase	The overproduction of atypical C_6C_1 monolignols leads to the formation of sidechain truncated lignin with a lower degree of polymerization. Transformed lines displayed improved lignin extractability and enzymatic conversion after mild pretreatment	(Eudes *et al*., 2012)
Arabidopsis	Lignin	Expression of *Clarkia breweri* monolignol 4-O-methyltransferase (MOMT) engineered via iterative saturation mutagenesis	In engineered lines, MOMT etherifies the para-hydroxyl group of lignin monomeric precursors, necessary for oxidative cross-coupling. Engineered lines showed a marked reduction in lignin content, the accumulation of novel phenolic esters and improved digestibility	(Zhang *et al*., 2012)
Poplar	Lignin	Expression of a *Petroselinum crispum* tyrosine-rich glyco-peptide and targeted accumulation in the cell wall	Engineered lines displayed normal levels of lignification and increased saccharification after pretreatment with proteases. The incorporation of the glyco-peptide into the lignin polymer is yet to be confirmed	(Liang *et al*., 2008)
Maize	Cellulose	Expression of a thermostable endocellulase E1 (Cel5A) from *Acidothermus cellulolyticus*	Relative to wild-type, engineered lines displayed improved digestibility after mild pretreatment. The exact mechanism through which the enzyme affects cell wall recalcitrance is yet to be uncovered	(Brunecky *et al*., 2011)
Maize	Hemicellulose	Expression of an engineered cell wall degrading xylanase containing a thermoregulated intein sequence	Transformed lines were able to produce their own xylanase and release up to 60% cell wall glucose after enzymatic hydrolysis following mild thermochemical pretreatment	(Shen *et al*., 2012)

gene transfer has been pursued as a means to redesign cell wall polymers *in planta*; proving particularly successful in the creation of de novo lignin configurations exhibiting higher solubility and extractability (Liang *et al.*, 2008; Eudes *et al.*, 2012; Zhang *et al.*, 2012). Also, the expression of microbial cellulases and other exogenous cell wall modifying enzymes has proven a viable strategy for the production of lignocellulosic crops with the ability to guide their own 'self-digestion'. Noteworthy, Shen *et al.* (2012) engineered a cell wall degrading xylanase containing a thermoregulated intein sequence that could self-splice and restore the catalytic activity of the enzyme at high temperatures. When subjected to mild thermochemical pretreatment (55 °C), maize lines transformed with the engineered enzyme were able to produce their own xylanase and release up to 60% cell wall glucose after enzymatic hydrolysis. Moreover, because the xylanase only becomes active after thermochemical treatment, the transgenics showed normal seed development, fertility, and biomass accumulation. Along other exemplary works, Shen *et al.* (2012) demonstrate that it is fundamentally possible to control the accumulation and timely expression of exogenous CWD enzymes *in planta* and circumvent the repercussions on plant health commonly associated with heterologous gene expression.

The way forward

Genetic engineering has an immense appeal for the production of efficient bioenergy crops, especially when considering that promising perennial species either have complex genomes, difficult reproductive patterns or limited genetic variation for relevant cell wall characteristics. Notwithstanding, we are just beginning to learn about the intricate regulation of cell wall biosynthetic processes and we are still far from fully comprehending how targeted perturbations in cell wall metabolic fluxes will affect plant vigor and agronomic fitness. Also worthy of consideration, while public acceptance of genetically modified (GM) crops for bioenergy purposes might be higher than for GM food and feed commodities, unyielding governmental regulations (particularly in Europe) can stall, delay or discourage the deployment of GM energy grasses.

To circumvent the technical challenges and political issues related to GM technologies, we believe that advancing maize for the cellulosic ethanol industry can be effectively achieved by harnessing the standing variation available in commercial germplasm through modern selection tools. The convergence of classical selection schemes with inexpensive genotyping, advanced biometric models and double-haploid production technologies, has led to the conceptualization of 'next-generation' breeding platforms with the potential to accelerate maize cultivar development and commercial release (Riedelsheimer *et al.*, 2012; Wallace *et al.*, 2014). In addition, the advent of high-throughput bioconversion assays and cell wall phenotyping technologies can expedite selection for complex biomass and cell wall characters without the need for an in-depth understanding of cell wall genetic mechanisms. Cell wall functional genomic and classical linkage studies should by no means be underestimated, however, as they will still constitute the fundamental base upon which to guide biomass breeding programs. We should also remember that the unexploited variation concealed within exotic germplasm offers great opportunities for the transformation of maize into a biomass- or energy-dedicated feedstock and modern selection tools are opening avenues for the rapid incorporation of rare alleles into elite material.

Conclusions

The economic impact of maize cell wall modifications

Over the last decade, diverse studies have demonstrated that bioenergy crops diverging in cell wall constitution respond differentially to the combined operations of pretreatment and enzymatic hydrolysis. These findings have invariably led to the recognition that the processing efficiency and environmental performance of biomass-to-ethanol conversion systems can be greatly improved through the adequate selection of biomass substrates. Remarkably, most technoeconomic assessments of the feasibility of cellulosic ethanol refineries appear to disregard this evidence, and only a handful of projective studies support the notion that the economics of the industry could be improved through the utilization of biomass feedstocks with enhanced processing amenability.

Notwithstanding, analyses of genetic variants in maize, switchgrass, poplar and sugarcane, have indicated that reductions in the chemical, enzymatic, and energetic stringency of biomass-to-ethanol conversions systems can be achieved through the utilization of genotypes displaying highly degradable cell walls. In fact, Torres *et al.* (2013) has even demonstrated that industrially competitive saccharification yields at milder processing conditions are accompanied by a 95% reduction in the production of toxic inhibitors that can affect fermentation efficiencies and down-stream process economics. And while the extent of these beneficial effects are yet to be confirmed on large-scale trials, it becomes clear that most comparative analyses of the economic and environmental performance of ethanol refineries are underestimating the impact of biomass composition on the overall efficiency of the industry.

Beyond cellulosic ethanol and the plant cell wall

In addition to biochemical pathways, thermochemical routes are also regarded frontrunners for the production of cellulosic biofuels. Based on comparative life-cycle and technoeconomic analyses, however, neither technology has a clear competitive environmental or commercial advantage in the industry (Wright & Brown, 2007a, b; Anex *et al*., 2010). Irrespective of the uncertainty over which conversion route(s) will ultimately prevail, the successful deployment of maize as a lignocellulosic substrate will adhere to the same incontrovertible principles.

To begin with, the plant cell wall will indubitably remain a central focus of bio-based maize breeding endeavors. Extensive evidence has demonstrated the influence biomass composition exerts on the economic, environmental, and technical efficiency of biomass-to-fuel conversion systems. And while cell wall 'ideotypes' will be largely determined by the conversion route (e.g., higher lignin content is favored by fast-pyrolysis conversion routes), all knowledge pertaining the maize cell wall (i.e., biosynthesis, phenotyping tools, and genomic approaches for modification) can be universally extrapolated toward the selection of specific cell wall compositional profiles that can best match the conversion system.

Notwithstanding, maize breeding for improved agronomic and environmental efficiency will also have great implications for the industry and cannot be disregarded. Being a central pillar to global food security, maize demand for human and animal nutrition will greatly expand by 2050 (Long & Ort, 2010; Tilman *et al*., 2011). Understandably, bio-based maize will ideally encompass dual-purpose hybrids combining both, optimal grain yield and high stover productivity (Vermerris *et al*., 2007; Weijde *et al*., 2013). Simultaneously improving grain and stover yields is a feasible undertaking (Lauer *et al*., 2001; Lorenz *et al*., 2009; Lewis *et al*., 2010; Lorenz *et al*., 2010), but maize production will also be constrained by the urgencies of modern agriculture (Long & Ort, 2010; Tilman *et al*., 2011). In this regard, ongoing endeavors have achieved major accomplishments in uncovering and exploiting novel genetic diversity for climate-related stresses and sustainable production under lower agricultural inputs (Cairns *et al*., 2012; Edmeades, 2013). Ultimately, the incorporation of agronomic 'hardiness' in dual-purpose hybrids will improve the economics and environmental performance of the industry (regardless of the conversion route) by lowering the GHG footprint of maize production, offsetting the conversion of virgin agricultural soils and reducing farm-to-plant transportation distances (Wright & Brown, 2007a,b; Wang *et al*., 2011; Cairns *et al*., 2012; Wang *et al*., 2012; Edmeades, 2013). The diversification of maize into an energy-dedicated species should be examined with caution; however, as socioeconomic and environmental concerns are likely to arise if energy-dedicated maize is to replace grain maize production. To avoid a food-over-fuel debacle, biomass-dedicated maize will only make sense if it can be produced on marginal soils and compete with the high yields, agronomic hardiness and soil-recovery properties displayed by other promising bioenergy grasses.

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