Genetics and Bioenergy Potential of Forage Maize: Deconstructing the cell wall

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Chapter 1

General Introduction

Towards a sustainable bio-based economy: from *gasoline* to "*grassoline*"

As we enter the third millennium, the societal and environmental consequences of our incommensurate reliance on finite fossil fuels have finally reached political agendas worldwide. Climate change, energy supply insecurity and growing geo-political tensions over dwindling fossil reserves are the predominant forces driving a transition towards sustainable energy alternatives [1-5]. 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 green-house gas (GHG) emissions [4, 5]. This poses a serious predicament since mature renewable energy alternatives (i.e. solar, wind and geo-thermal) convey the exclusive production of heat or electricity [2, 3, 6].

By consequence, as society departs from its oil-reliant economy, researchers, governments and private investors worldwide have grown increasingly resolute on renewable fuels derived from plant lignocellulose – that is, the inedible fraction of plants (Box 1). Plant lignocellulose represents the world's greatest repository of renewable energy amenable to conversion into liquid fuels [2, 3, 6, 7]. Essentially, biomass-to-fuel conversion technologies rely on the release of solar energy captured in the form of chemical bonds in plant cell wall polymers (Figure 1).

Box 1. Biofuels done right

At present, the global market for renewable-fuels is dominated by "first-generation" ethanol and biodiesel derived from five major food crops: maize, sugarcane, soybean, oil palm and rapeseed [8]. Powered by blending mandates, tax incentives and subsidies, first-generation biofuel production is without doubt a profitable business [9, 10]. To illustrate, in 2006, maize-grain ethanol in the U.S. was produced at approximately $ 0.25/L, but was sold at $ 0.90/L [10]. However, despite their commercial success and rising popularity, first-generation biofuels do not fulfil their intended role of displacing significant quantities of fossil fuels, nor do they greatly reduce GHG emissions [1, 8, 11-14]. More importantly, the intensive utilization of food crops for biofuel production forecast a precarious competition for agricultural soils culminating in increased global food prices and detrimental changes in global land-use [8, 11, 12, 15, 16].

Ultimately, for biofuels to make a beneficial impact in society, these will need to be derived from feedstocks produced with much lower life-cycle GHG emissions than fossil fuels and minimal competition with food production [11]. Experts have thus

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developed a strategic vision wherein competitive quantities of hydrous fuels are produced sustainably from lignocellulosic biomass. After all, optimistic projections estimate that the global supply of lignocellulose has an energy content equivalent which exceeds our current consumption of 30 billion barrels of oil per year [2]. The "billion-ton" biofuel vision therefore recommends a balanced utilization of lignocellulosic feedstocks derived from soils not currently used for agriculture, as well as sustainably harvested crop and forestry residues [11]. The sustainable and environmentally sensitive realization of this goal will rely on advances in the productivity and intrinsic processing quality of next-generation bioenergy crops.
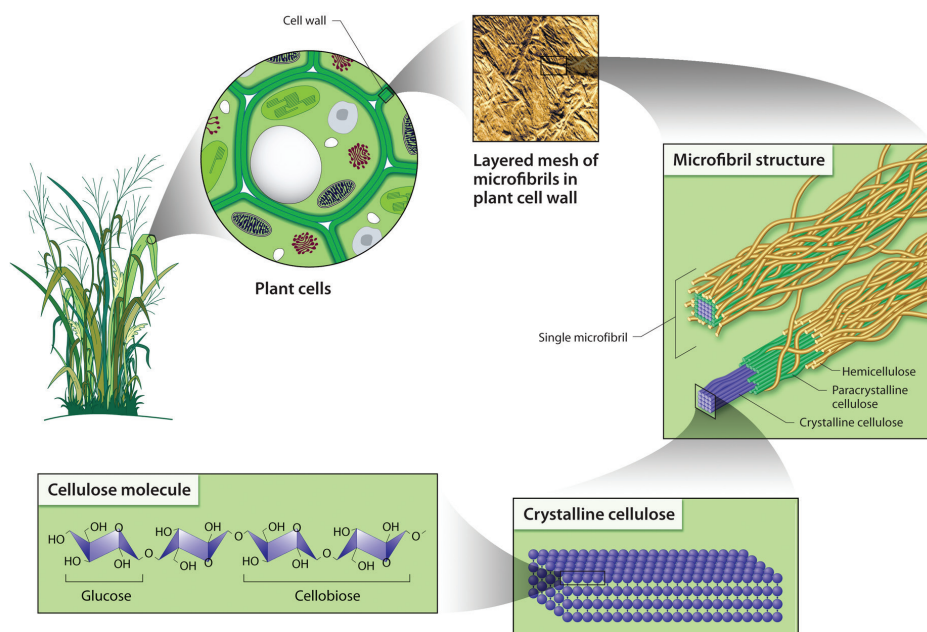
Figure 1 The structure of plant lignocellulose. Lignocellulose, arguably the most abundant renewable substrate on earth, is composed of three major constituents - cellulose, hemicellulose and lignin; all of which combine to produce the microfibrils that make-up the plant cell wall [17-19]. Cellulose is a beta(1–4)-linked chain of glucose molecules; hemicellulose is constituted by diverse 5- and 6-carbon sugars (i.e. arabinose, galactose, glucose, mannose and xylose); and lignin is composed of three major phenolic components (i.e. p-coumaryl, coniferyl and sinapyl alcohol) [18]. The solar energy collected by plants via photosynthesis is principally stored within the sugars of cellulosic and hemicellulosic polymers. (Source: Office of Biological and Environmental Research of the U.S. Department of Energy Office of Science. science.energy.gov/ber/)

Biomass-derived transportation fuels (henceforth referred to as "cellulosic fuels") can be generated through thermochemical or biochemical pathways [2, 3, 7, 20]. Thermochemical routes involve the deconstruction of plant biomass at high temperatures (>400 °C; either through pyrolysis or gasification); procedures which ultimately lead to the production of mixtures of simple organic compounds which can be transformed into hydrous fuels through chemical refinement [2, 3, 7]. Major drawbacks to these technologies reside in the need for thermochemical processing plants that require massive capital investments, convey high maintenance and operating costs and require insurmountable amounts of lignocellulose to run profitably [2, 7]. Huber and Dale [2] have estimated, for instance, that a "Syngas-FTS" plant valued at $ 2.0 billion would need to consume around 5,000 tons of biomass per day, for a period of 30 years, before start-up investments can be recovered. Alternatively, biomass can be deconstructed into monomeric sugars using a combination of thermochemical pretreatments and enzymatic hydrolysis [3, 7, 21, 22]. Through microbial fermentation, these sugars can be then converted into ethanol, butanol or other hydrocarbons, which can either be used as fuels or as precursors for the production of bio-based polymers or other bio-commodities (Figure 2) [3, 22]. Cellulosic ethanol refineries exhibit low start-up costs compared to thermochemical refineries and can potentially operate on a much smaller scale, thereby enabling the decentralization of fuel production [2, 3, 7]. These inherent attributes make cellulosic ethanol a more sustainable industrial option and a globally applicable technology.

Bioenergy experts cannot predict (or agree on) which conversion route(s) will prevail in the industry, nor can they accurately indicate when these platforms will reach economic viability. However, cellulosic ethanol production via biochemical pathways is currently the most commercially represented technology in the sector, and has therefore been selected as the reference technology in this thesis [5, 20, 23].

The plant cell wall is an unyielding energy lock

Despite important revamps in funding and unrelenting governmental support, cellulosic ethanol is yet to transcend the demonstration plant and achieve wide-scale commercialization [24-26]. Currently, the production of cellulosic ethanol is far from cost effective and experts agree that its commercial future depends on innovations that can increase the industry's productivity while simultaneously reducing processing and operational costs [2, 5, 23, 27]. A careful examination reveals that thermochemical pretreatment is the most expensive processing operation, followed in line by enzymatic hydrolysis and the production of cellulolytic enzymes [5, 22, 23]. In other words, the three costliest operations in the industry (accounting for nearly two-thirds of ethanol production costs) are those necessary for deconstructing plant biomass into fermentable sugars [5, 23].
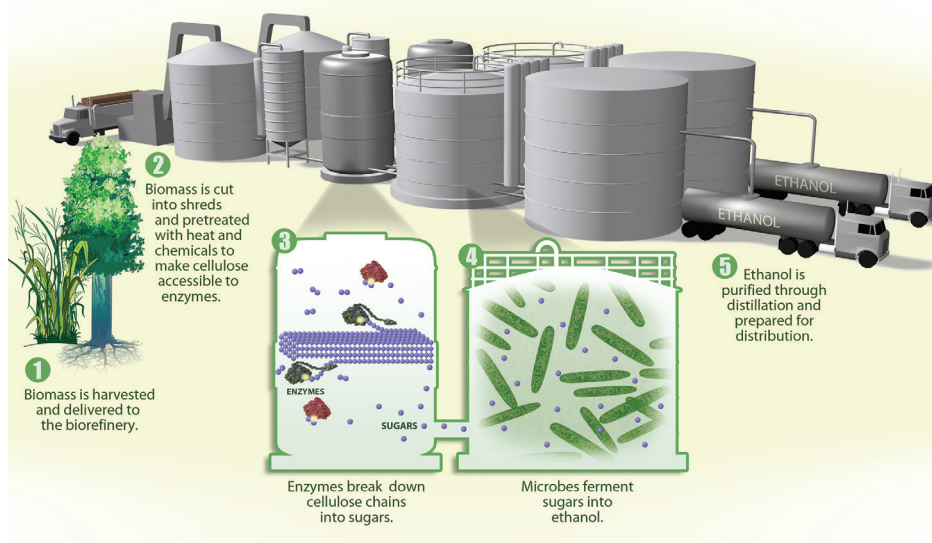
Figure 2 Schematic representation of a cellulosic ethanol refinery. The production of cellulosic ethanol via biochemical pathways starts with the deconstruction of the polysaccharide fraction (cellulose and hemicellulose) of plant lignocellulose by physical and chemical pre-treatment; followed by enzymatic depolymerization by exposure to enzymes from biomass-degrading organisms. Enzymatically released sugars are subsequently converted into fuels by fermentative microorganisms. (Source: Office of Biological and Environmental Research of the U.S. Department of Energy Office of Science. science.energy.gov/ber/)

Ultimately, the challenge of effectively fractionating lignocellulosic feedstocks into sustainable fuels resides within the compositional nature of the plant cell wall; the principal constituent of plant biomass (Figure 1). The plant cell wall is a complex bio-composite composed of cellulose, hemicellulose and lignin, as well as other minor aromatic compounds, pectins and structural proteins [18, 19, 28, 29]. This biological matrix delineates the physical characteristics of individual cells (i.e. shape and size) and ultimately determines plant morphology, size and fitness [30, 31]. Extensive evidence also indicates that its composition and structure greatly influence the effective conversion of plant biomass into bio-commodities [32-38]; regardless of the technological deconstruction route. After all, this biomaterial has evolved to stubbornly resist biological and chemical breakdown as it plays a crucial role in various plant growth and developmental processes, including the protection of the plant cell from biotic and abiotic stress [29, 31].

Until now, techno-economic evaluations of cellulosic fuel refineries have minimized the role of lignocellulosic feedstocks to cost, productivity and availability consider-

ations [39-45]. These comparative assessments indirectly propose that the economic and environmental performance of the industry can be solely improved through innovations in biomass-process engineering or advances in the productivity (per unit of land) of biomass species. Notwithstanding, in the last decade, numerous studies examining the extent of natural and induced variation in cell wall composition across diverse bioenergy crops have demonstrated that feedstocks with divergent chemical constitutions respond differentially to the combined operations of pretreatment and enzymatic hydrolysis [35, 46-51]. These findings have invariably opened prospects for genetically altering the chemical composition and structure of the plant cell wall to render biomass conversion processes less resource-intensive and expensive. From a theoretical standpoint, these developments could change our conceptual vision of cellulosic fuel refineries if cutbacks in biomass transportation, processing and fermentation processing costs eventually lead to reductions in the size and throughput of processing plants [5, 23]. By consequence, within the domain of cellulosic fuel research, important efforts are being (or need to be) devoted towards the development of advanced lignocellulosic crops that meet the demands of the industry [2, 3, 5, 6, 23, 28, 52-54]. In the long run, the ultimate challenge of up-coming bioenergy crop breeding programs will lie on identifying and modifying key cell wall compositional features that can reduce lignocellulose recalcitrance without compromising breeding efforts for increased yields or plant performance in the field.

C4 grasses are imperative to the development of a sustainable cellulosic fuel industry

The commercial viability of the cellulosic fuel industry will be primarily determined by our ability to produce large volumes of inexpensive feedstocks without threatening food security or the environment [11, 12, 15]. Experts have thus envisioned that a combined supply of lignocellulose from agricultural residues and biomass-dedicated cropping systems can sustainably match the demands of the bio-based industry (Box 1).

Given these provisions, grasses displaying C4 photosynthesis have been coined the most promising candidates for the industrial production of lignocellulosic biomass [6]. Relative to plant species with C3 photosynthesis, C4 grasses generally exhibit markedly improved biomass productivities; owing predominantly to their inherent photorespiration-suppressing mechanism [55-57]. Moreover, because they necessitate lower concentrations of photosynthetic proteins for optimal growth and also exhibit reduced stomatal conductance (leading to reduced leaf perspiration), C4 grasses display higher nitrogen (NUE) and water use efficiencies (WUE) [57-59]. By consequence, C4 grasses dominate hot, open and arid environments worldwide,

and offer the most realistic outlooks for the industrial production of biomass under low-input agricultural regimes.

Presently, some of the most advanced and promising bioenergy species are C4 grasses [53]. On the one hand, the economically important food crops, maize (*Zea mays* L. ssp. *mays*), sugarcane (*Saccharum* spp.) and sorghum [*Sorghum bicolor* (L.) Moench], have well-established production and distribution chains which can potentially supply vast amounts of lignocellulose in the form of agricultural residues [3, 28, 60]. On the other hand, the rhizomatous perennials miscanthus (*Miscanthus* spp) and switchgrass (*Panicum virgatum* L.) constitute promising biomass-dedicated crops exhibiting incredible productivities [3, 28, 53], even on marginal soils (Table 1).

Table 1. Average yields of lignocellulosic biomass, fertilizer use and nutrient removal, and water requirements per kg DM yield of important C4 grasses.

Crop	Biomass Yield $t\ ha^{-1}\ yr^{-1}$	Fertilizer Use $kg\ ha^{-1}\ yr^{-1}$			Nutrient Removal $kg\ ha^{-1}$			Water required, $mm\ yr^{-1}$ /kg DM
		N	P	K	N	P	K	
Maize [a]	5.2	200	100	100	37.5	4	57.5	>115
Sugarcane	22.9	90	67	67	160	43	546	>57
Sorghum [a]	1.95	90	67	67	28	5.5	30.5	>164
Miscanthus	22.5	0	7	100	110	10	157.5	>22
Switchgrass	10	67	45	45	34	6.5	82.5	>45

[a] Average stover yields (i.e. not including ear and grain) based on the widely used assumption that the stover to grain ration is 1:1 for maize and 1.3:1 for sorghum [61]

Table adapted and modified from van der Weijde [6].

Evidently, each of these species has its strengths and prospects with respect to their use and development for production in diverse environments and geographical locations [53]. The success of C4 grasses in the cellulosic and bio-based industries will therefore rely on the production of superior cultivars that increase the profitability and competitiveness of the industry while sustainably meeting projected market volumes. Common breeding objectives, regardless of species or cropping system, include increasing stem biomass yields, improving climatic adaptation and biotic-stress hardiness [6, 25, 52]. Equally important, and as discussed earlier, since

lignocellulose recalcitrance constitutes the single-most critical barrier towards the efficient conversion of plant biomass into added-value products, improving the processing amenability of C4 grasses crops is of utmost relevance to the industry.

In essence, the cell walls of C4 grasses share distinct architectural features common to all commelinoid monocots; and have been described comprehensively by Carpita [18], Cosgrove [19], and Vogel [17]. Notwithstanding, in each species, vast intra-specific genetic variation exists in cell wall composition, polymeric ultra-structure, physical architecture and (presumably) the weight ratio of primary to secondary cell walls (Table 2). The extent of inter- and intra-specific variation found in these species ultimately indicates opportunities for the development of feedstocks with cell wall characteristics better suited to the demands of the cellulosic ethanol industry.

Table 2. Observed variation in cell wall composition of promising C4 energy grasses

Crop	Cellulose	Hemicellulose	Lignin	Reference
Maize (stover)	~27-40%	~25-34%	~9-15%	[48, 62-65]
Switchgrass	~28-37%	~25-34%	~9-13%	[66, 67]
Sorghum (stover)	~21-45%	~11-28%	~9-20%	[68-71]
Sugarcane (bagasse)	~35-45%	~25-32%	~16-25%	[72, 73]
Miscanthus	~28-49%	~24-32%	~15-28%	[74, 75]

[a] Cell wall polymeric values are expressed as a weight percentage of dry matter

Maize is an outstanding model for cell wall and bioenergy research

At present, the experience, resources and breeding tools available for relevant C4 bioenergy grasses differ strongly. For instance, whereas miscanthus and switchgrass have barely been domesticated, maize represents the "testing fuel" of the first commercial-scale cellulosic fuel refineries [3, 7, 31, 39, 41, 42, 76]. Notwithstanding, the close evolutionary relationships (Figure 3) and common agronomic characteristics shared by these crops promise great possibilities to expedite their adaptation to the demands of a growing bio-based industry. Particularly, inter-species translational cell wall research is expected to become rewarding for the development of lignocellulosic feedstocks with reduced recalcitrance and improved processing amenability.
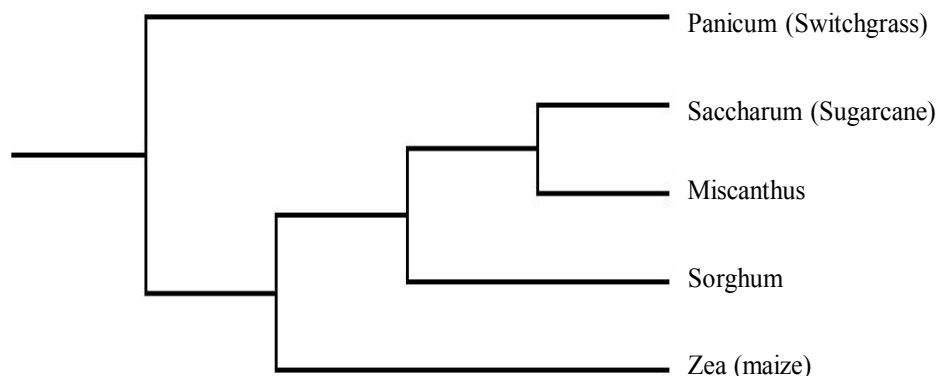
Figure 3 Phylogenetic tree depicting the evolutionary relationships between the C4 grasses maize, miscanthus, sorghum, sugarcane and switchgrass. Adapted from Lawrence and Walbot [77].

In this respect, maize is well ahead of the field [28]. For decades, cell wall depolymerization processes have been evaluated using forage maize germplasm, and it has been speculated that some of the mechanisms hindering rumen enzymatic processes also affect the efficacy of biomass-to-ethanol conversion technologies [3, 62]. Forage maize genetic resources are therefore ideal for assessing the extent to which different cell wall components, as well as their interactions, have an impact on the efficiency of biomass-to-ethanol conversion technologies. Accordingly, extensive surveys of forage maize experimental populations and mutant panels have already hinted at the vast extent of variation in cell wall constitution and enzymatic degradability properties potentially concealed in maize [31, 62, 78-87]. Also noteworthy, these studies have served as platforms for the identification of quantitative trait loci (QTL) and candidate genes underlying cell wall variation potentially relevant to cellulosic fuel production [81, 82, 86, 88, 89].

Undoubtedly, with an unrivalled wealth of agronomic and genomic resources, maize is an excellent model for studying complex cell wall characteristics and optimizing crop improvement strategies in C4 bioenergy grasses [28]. Owing to its global relevance as an agricultural and industrial staple, maize geneticists and breeders worldwide benefit from an impressive infrastructure of genotyping platforms, genomic annotations, expression analyses repositories and powerful experimental populations [90] (Box 2). In addition, the maize genome is now publically available [91] and numerous re-sequencing projects have extended our knowledge on the evolution, diversity, and complex heterotic nature of this crop species [92-95]. Also, relative to C4 perennials, the annual and diploid nature of maize implies shorter growing

cycles and often simpler genetics [28]. Certainly, quantitative genetic and genomic studies in maize will serve as the basis for assessing the extent of genetic diversity and inferring inheritance patterns controlling cell wall composition, structure and degradability properties for bioenergy production. The combination of genome sequencing with other "omics" strategies is still in its early stages in C4 grasses, but the use of comparative genetics coupled with transcriptomic and proteomic analyses will be an important tool to expedite our understanding of cell wall biosynthesis processes in other C4 grasses.

Box 2. Resources for genetic cell wall studies in maize

Genetic mapping and screening in maize is facilitated by an extensive array of dedicated genetic resources. A natural outcrosser, maize is remarkably diverse, with most of its desirable traits yet to be utilized [96]. This unexploited diversity has been preserved in gene bank collections worldwide in the form of elite inbred lines, native land races and wild relatives [96]. Public and private endeavors have avidly exploited this variation for the development of powerful recombinant inbred line (RIL) populations and association mapping panels [90]; some of which can be of great value for unraveling bioenergy-related traits. Likewise, the existence of dedicated transposable-element systems has facilitated the production of maize mutagenized populations [3, 97]. Gene-tagging through transposon insertional mutagenesis, in combination with high-throughput genomic/phenomic platforms, have the potential to simplify the generation, discovery and cloning of interesting cell wall mutants.

More recently, in vivo production of doubled haploid (DH) populations has been widely adopted in maize research and breeding programs; primarily because this technology enables the development of completely homozygous lines in less than half the time traditionally required for the production of RILs [98-101]. In essence, heterozygous source germplasm is first derived from crosses between two or multiple elite inbred parents with desired characteristics. Subsequently, maternal haploidy is induced by pollination of the source germplasm with a haploid inducer genotype. Putative haploid seeds are identified via morphological markers and are treated with colchicine to artificially double their chromosomes and produce DH plants. Confirmed DH lines are then self-pollinated to produce seed for maintenance and testing.

In this thesis, a maize population of doubled haploids (DHs), property of Limagrain Nederland B.V., was used to study the genetic diversity and architecture of cell wall

traits relevant to cellulosic fuel production. The source germplasm for this population was derived from a cross between two proprietary elite inbred lines; both highly contrasting in forage quality and ruminal cell wall digestibility. By consequence, this DH population captures decades of concerted breeding efforts seeking to concentrate the vast extent of diversity in cell wall traits potentially concealed in Northern-European maize.

Objectives and scope of this thesis

The development of dedicated bioenergy crops is envisioned to substantially reduce the production costs of cellulosic ethanol and contribute to the establishment of an economically viable and sustainable cellulosic fuel industry. In view of these prospects, the central objective of this thesis was to elucidate and dissect the biochemical and genetic mechanisms controlling maize cell wall characteristics relevant to the development of bioenergy feedstocks with improved processing amenability.

Correspondingly, this investigation i) yields insights into the extent of natural genetic diversity for cell wall characteristics potentially concealed in bioenergy grasses; ii) uncovers novel breeding targets aimed at increasing the bioconversion potential of lignocellulosic crops; and iii) evaluates the technical feasibility of exploiting natural variation in cell wall degradability for the production of superior bio-based cultivars. In parallel, this thesis also addresses and evaluates how, and under which circumstances, the development of bioenergy feedstocks with reduced lignocellulose recalcitrance can improve the commercial and environmental performance of the cellulosic fuel industry. To this end, this thesis has been methodologically structured as follows:

Chapter 2 reviews the prospects and benefits of advancing maize as a model system and second-generation lignocellulosic feedstock for ethanol production. Given that lignocellulose recalcitrance represents a critical barrier to efficient cellulosic fuel production, a comprehensive synopsis of current knowledge on the maize cell wall and promising genetic strategies for its modification was warranted. In addition, an overview of the state-of-art of genomic and phenotyping strategies available for bioenergy crop research and breeding has been provided.

In **Chapter 3** we have uncovered key compositional features of maize cell walls influencing the enzymatic conversion of biomass into fermentable sugars across pro-

cessing conditions of increasing energetic and chemical severity. This investigation was established to analyze the extent to which cellulosic feedstocks with tailored cell wall compositions can help reduce the chemical and energetic intensity of pretreatments used in the industry and improve the productivity of biomass-to-ethanol conversion technologies.

Through the exhaustive characterization of a forage maize doubled haploid (DH) population, we have also investigated the degree of heritable diversity in cell wall composition, polymeric ultrastructure and bioconversion potential available in maize. Additionally, the complex genetic architecture of complex cell wall characteristics was dissected via the analysis and identification of quantitative trait loci (QTL). These results, which are presented in **Chapter 4**, provide insights into the technical prospects and breeding strategies that can be used for the optimization of lignocellulosic biomass for the production of bioenergy and other bio-commodities.

In **Chapter 5** we have investigated whether complex cell wall bioconversion traits constitute accessible and reliable selection criterion for incorporation in modern maize breeding programs. In this regard, we have focused on exploring the heritability and environmental stability of complex cell wall characteristics at the inbred level and in hybrid combinations. An important focus of this study was to determine whether preliminary selection at the inbred level would expectedly lead to successful hybrid selection; thereby minimizing the need for recurrent test-crossing procedures and evaluations.

In **Chapter 6** we have proposed a conceptual framework incorporating the economic and environmental benefits of advancing lignocellulosic crops with reduced enzymatic recalcitrance and improved processing amenability. To this end, the productivity of biomass-to-ethanol conversion systems was explored using cultivars with varying degrees of cell wall digestibility and under different processing scenarios. A focus on the relationship between biomass yield and processing quality has been warranted, as general convention wrongly dictates that yield penalties are a common consequence of breeding efforts leading to reduced lignocellulose recalcitrance.

Finally, in **Chapter 7**, knowledge generated in this investigation was used to evaluate the technical feasibility, conceptual bottlenecks and commercial prospects of breeding activities seeking to advance bioenergy crops which require lower energetic and chemical inputs for their effective deconstruction into cellulosic fuels. In this respect, technical recommendations are also provided to guarantee the operational success of pioneering programs seeking to advance the next generation of C4 bioenergy grasses.

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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, 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: Maize, lignocellulose, bioethanol, cell wall, genes, molecular tools, breeding

Introduction

As we enter the 3rd 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 [1-3]. 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 green-house gas (GHG) emissions [3]. 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 [4, 5].

Cellulosic ethanol essentially derives from lignocellulose, arguably the most abundant renewable carbon substrate on earth [2, 4, 6]. Since lignocellulose production requires less agricultural and energetic inputs relative to first-generation bioenergy feedstocks, once industry logistics and processing technologies have matured, cellulosic ethanol could outperform gasoline and starch-based ethanol as the transportation fuel with lowest GHG emissions and greatest net-energetic outputs [6-9]. Despite extensive revamps in funding and unrelenting governmental support, cellulosic ethanol is yet to transcend the demonstration plant and achieve wide-scale commercialization [10-12]. With the first commercial endeavors underway [10, 11, 13], progress in the commercialization of cellulosic ethanol could be conditioned by the instability of oil-prices, market incentives and governmental policies [13, 14]. 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) [1, 2, 13]. Nevertheless, cellulosic ethanol production via biochemical pathways is currently the most commercially represented technology in the sector [3, 13], 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 [13].

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 [3]. To circumvent this problem, thermochemical pretreatments are typically employed to increase the accessibility of biomass polysaccharides to hydrolytic enzymes [15]. 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 [3, 15].

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 [1, 3]. 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 [2, 3, 16, 17].

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 [1, 2, 7]. 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 (Figure 1), broad geographic adaptation, superior carbon sequestration and efficient nutrient utilization [16, 18]. 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 [7, 19, 20]. 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 [18]. Breeding objectives include increasing biomass yields and yield sta-
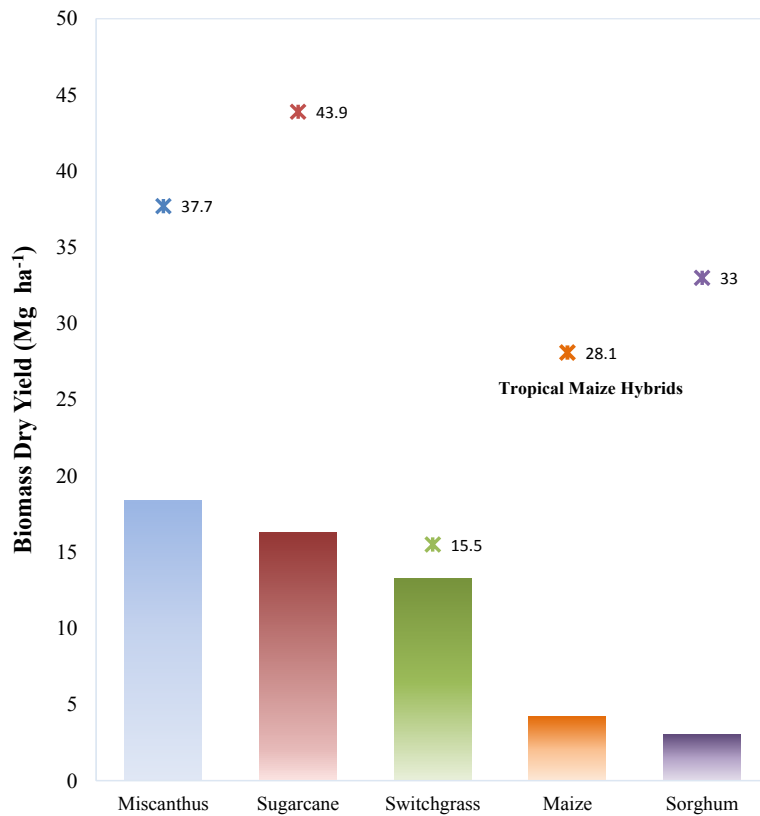
Figure 1. Mean and potential annual dry biomass yields (Mg ha^{-1}) for relevant C4 energy grasses. For Miscanthus and Switchgrass, delayed harvest yields (after winter) are reported. Colored asterisks correspond to highest reported yields in literature, except for sugarcane, for which highest reported yields were extracted from FAOSTAT [27]. Mean and maximum yield values were calculated or extracted from [28, 29] for Miscanthus, [27] for Sugarcane, [23, 29] for Switchgrass, [27, 30] for maize and [23, 27] 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.

bility under low-input agricultural systems; enhancing pest and disease resistance; and modifying biomass composition for improved industrial processing [16, 18].

With the first cellulosic ethanol commercial plants on the way [13], a reliable and abundant feedstock is a pressing necessity [21]. 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 [2, 4, 17, 18, 22, 23]. 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 40-65% of all maize agricul-

tural residues can be sustainably harvested for advanced fuel production [24-26]. 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 [2, 18]. Furthermore, implementing the technology required for cellulosic fuel production entails significant capital investments and financial risks [1, 4, 5]. 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 [2, 4, 22]. By doing so, nascent enterprises will reduce financial burdens by benefitting from the commercially-effective maize-farming, processing and transportation infrastructure [22, 23].

In the future, grower's acceptance of bioenergy perennials will also impact the prevalence of maize as a lignocellulosic feedstock [22]. This perspective takes into consideration the high costs and financial risks associated with the set-up 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 [21-23]. 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 [22, 23, 30, 31]. 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 (Figure 1). These temperate x 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 [30], and up to 21.3 Mg ha^{-1} annual dry biomass without supplemental N fertilization [31]. Because TTM hybrids can also accumulate high amounts of soluble sugars in their stems ($\sim$50% more when compared to commercial hybrids), these can expectedly yield comparable amounts of ethanol ($\sim$8000 L ha^{-1}) per hectare under no supplemental N fertilization as commercial grain hybrids supplemented with N ($\sim$10500 L ha^{-1}) [30]. Although preliminary in nature, these results demonstrate the potential behind breeding endeavours 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 [23, 30]. 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 [32-35].

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 [36]. A natural outcrosser, maize is remarkably diverse, with most of its desirable traits yet to be utilized [32, 37, 38]. This unexploited diversity has been preserved in gene bank collections at numerous international research centers and are publically available upon request. In addition, public and private efforts like the Latin American Maize Project (LAMP) [39], the Germplasm Enhancement of Maize (GEM) project [40], 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 [17].

Cellulose

Improving the relative content and industrial quality of cellulose is a pivotal strategy towards 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 [41, 42].

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 [43, 44]. Based on sequence orthology, these genes presumably encode the catalytic subunits of the maize Cellulose Synthase Complex (CSC) [43-45]. In accordance with the functional specialization of *CesA* isoforms in Arabidopsis [46-48], rice [49] and barley [50], expression studies reveal that at least three specific maize *CesAs* (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 [44, 45].

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 [51]. In maize, a gene orthologous to the Arabidopsis *Cobra-Like4* isoform was cloned from the *brittle stalk-2* (*bk-2*) mutant [52, 53]; a naturally-occurring phenotype characterized by stalks which break easily under mechanical pressure. Although *bk-2* exhibits reduced cellulose deposition in the secondary cell walls of sclerenchyma fibers [52, 53], Sindhu *et al.* [53] have proposed that *Bk-2* is not directly involved in the synthesis of cellulose, but instead participates in the orientation and patterning of both, cellulose and lignin, in the secondary cell wall. 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 proteins. 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 vigour 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.* [54] 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 [54, 55].

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 [56-58]. In addition, because hemicellulose binds to cellulose microfibrils and threads them via cross-links with lignin [59, 60], 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 [60, 61], 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 multi-gene complex highly homologous to the *CesA* family. Richmond and Somerville [62, 63] originally ascribed *Csl* gene products a processive glycosyltransferase (GT) function after observing that all *Csl* proteins possess a conserved domain defining their ability to catalyse the characteristic β-linkage common to cell wall polysaccharides. Thus far, expression studies suggest that primary wall xyloglucans [64], (gluco)mannans [65, 66] and grass-specific mixed linkage glucans [67] 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) [68-72]. 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 non-cellulosic polysaccharide in monocots. In maize, Bosch *et al.* [68] 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 towards characterizing enzymes mediating GX and GAX branching reactions. Recent breakthroughs include the identification of the Reduced Wall Acetylation (*RWA*) [87]my of½ and Glucuronic Acid Substitution of Xylan (*GUX*) [81] genes from Arabidopsis, as well as the Xylan Arabinosyltransferase

Table 1. Genes involved in cell wall xylan biosynthesis

Gene	Species	GT Sub-Class	Presumed Function	Reference
IRX8, FRA8, FH8, PARVUS	Arabidopsis	GT47 (Fra-8, FH8) GT8 (IRX-8, Parvus)	Synthesis of a unique β-d-Xyl-(1 → 2)-α-l-Rha-(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.	[73-76]
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.	[77-80]
GUX-1, GUX-2	Arabidopsis	GT8	Addition of glucuronic acid and 4-O-methylglucuronic acid side branches on GX backbone.	[81, 82]
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.	[83]
TaXAT2; OsXAT2, OsXAT3	Wheat, Rice	GT61	Arabinosylation of the xylan backbones of GAX.	[84]
OsIRX9, OsIRX-9L, OsIRX14	Rice	GT8	Presumably involved in the synthesis and elongation of the xylan backbone of GAX.	[85]
OsXAX1	Rice	GT61	Putatively involved in the β-(1,2) xylosyl substitution of α-(1,3) arabinosyl residues of GAX.	[86]
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.	[68]

(*XAT*) genes from rice and wheat [84] and the Xylosyl Arabinosyl Substitution of Xylan (*XAX*) gene from rice [86]. Exciting new evidence would also suggest that homologous side-group 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 [82]. It is yet to be determined, however, whether GXs differ in the proportion, length and distribution of substitution patterns [82], 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 [81, 88, 89]. In maize, the high substitution frequency of GAX has proven detrimental to the enzymatic conversion of cell wall polysaccharides following dilute-acid pretreatment [88, 89]. Based on the work of Van Eylen *et al.* [88] and Appeldoorn *et al.* [89], 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 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 [90]. More recently, Torres *et al.* [17] 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 harbours extensive genetic variation in the degree and (presumably) distribution of GAX substitution patterns [17]; 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 *et al.* [91], Bonawitz and Chapple [92],

Vanholme *et al.* [93] and Courtial *et al.* [94].

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 [60]. Evidence suggests that lignin reduces the effectiveness of enzymatic saccharification processes by adsorbing and non-productively binding to hydrolytic enzymes [95, 96] and by physically shielding cellulose microfibrils from enzymatic attack [97]. 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*) [2, 98-100] and other species exhibiting genetically-engineered reductions in lignin content [101-103]. 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 [98, 102-104]. However, since favorable changes in monolignol ratios are often accompanied by reductions in lignin content [98, 101, 103, 104], it is still difficult to ascertain whether monolignol balance truly affects degradability properties [105]. More recently, the concept of redesigning lignin *in planta* has gained momentum [106-108]. 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 [93, 108]. 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 (HCHL) from *Pseudomonas fluorescens* in Arabidopsis, in order 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 to 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. [106].

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 lig-

nin variation and enzymatic digestibility properties available in forage maize [100, 109-114] and have served as platforms for the identification of quantitative trait loci (QTL) underlying maize lignification characteristics relevant to cellulosic ethanol production [114-119]. More recently, Lorenzana *et al.* [120] and Torres *et al.* [17] 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.* [121] and He *et al. [122]* used an antisense-gene approach to independently produce transgenic lines with reduced Caffeic-acid O-methyltransferase (COMT) 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.* [104] used RNA interference (RNAi) to produce engineered lines with reduced Cinnamyl-alcohol-dehydrogenase (CAD) 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 [104]. 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 which can either restore the original phenotype or reduce the phenotypic effect of a profound metabolic alteration [2, 110, 114, 123-125]. 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 [124-126]. 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 [127, 128]; 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 [128-130]. By contrast, ferulates are expectedly esterified to the arabinosyl residues of GAX through an enzymatically driven process occurring at the Golgi [127]. 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 [131] and ferulate-to-lignin cross-links [132, 133] 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 [109, 118, 134, 135]. Similarly, Jung and Phillips (2010) have identified a putative maize mutation – seedling ferulate ester (*sfe*)- 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 [17, 136].

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 [137]. 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 [138, 139].

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 [137, 140]; with different members displaying cell type specific expression patterns [141-144]. These master regulators appear to control downstream transcriptional cascades, which in turn activate cell wall lignin and carbohydrate biosynthetic pathways [143]. 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 [143, 145]. For instance, while Arabidopsis *MYB*46 and *MYB*83 appear to globally regulate secondary cell wall deposition [146-148], *MYB*58, *MYB*63 and *MYB*85 have been shown to specifically regulate lignin biosynthesis [143, 149]. 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. Similarly, *MYB* transcription factors are warranted further research, especially when considering their versatile role (e.g. *MYB* factors have been shown to act as repressors of cell wall biosynthetic processes) in the control of cell wall biosynthetic mechanisms [150]. 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 [68, 151-153].

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 [106, 138, 139]. Noteworthy, Yang *et al.* [139] "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 (APFL) 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 [154-158]. Notwithstanding, phenotyping tools which 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) [2, 159, 160]. In these systems, a core set of samples is exhaustively analyzed using conventional chemical assays in order to build calibration models which 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 doesn't 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 [2, 33, 36]. 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 [118, 120, 161-164].

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

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

Analysis	Technique	Used for determining	Description	Reference
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.	[187]
	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.	[110, 188, 189]
	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.	[190]
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).	[191-193]
	Spectroscopy	Lignin content, monomeric composition and linkage analysis	Lignin is isolated from the cell wall and analyzed using one- and two-dimensional NMR spectroscopy.	[190, 194]
	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 micro-plates (Glycome-Profiling) and probed using mAbs and CBMs with specificity for polysaccharide epitopes.	[195-199]
Cell wall recalcitrance	Enzymatic	Bioconversion efficiency of lignocellulosic substrates.	The NREL LAP-009 bioconversion assay has been automated and down-scaled to via robotic platforms. To accommodate an accessory pretreatment step, the most sophisticated systems rely on stackable 96-well metallic reactor plates which can withstand the chemical loads, pressure and high-temperatures used in industry.	[154-158]

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 [165] and numerous re-sequencing projects have updated our knowledge on the evolution, diversity, and complex heterotic nature of this crop species [166-170]. 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 quantitative trait loci (QTL) influencing complex biomass accumulation and cell wall architectural traits. Lorenzana *et al.* [120], for instance, surveyed the testcrosses of 223 recombinant inbred lines (RILS) from the IBM population [171] for variation in different biomass characteristics, including conversion efficiency after dilute-acid pretreatment. Despite the appreciably limited 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 SNP genotyping platforms, sophisticated biometric models and high-resolution mapping panels (including the powerful Nested Association Mapping Panel of maize) will expectedly expedite genome-wide association studies for biomass yield and quality characteristics [170, 172-174].

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 [45, 68, 123, 124, 175]. 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.* [2] 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.* [45] 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 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 [106, 107, 176]. 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.* [177] engineered a cell wall degrading xylanase containing a thermoregulated intein sequence which could self-splice and restore the catalytic activity of the enzyme at high temperatures. When subjected to mild thermochemical pretreatment (55 ^{0}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.* [177] 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

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

Species	Target polymer	Approach	Results	Reference
Arabidopsis	Lignin	Expression of a *Pseudomonas fluorescens* hydroxycinnamoyl-CoA hydratase-lyase (HCHL).	The overproduction of atypical C_6C_1 monolignols leads to the formation of side-chain truncated lignin with a lower degree of polymerization. Transformed lines displayed improved lignin extractability and enzymatic conversion after mild pretreatment.	[106]
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.	[107]
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.	[176]
Maize	Cellulose	Expression of a thermo-stable 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.	[200]
Maize	Hemicellulose	Expression of an engineered cell wall degrading xylanase containing a thermo-regulated 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.	[177]

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 (DH) production technologies, has led to the conceptualization of "next-generation" breeding platforms with the potential to accelerate maize cultivar development and commercial release [170, 172]. 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 techno-economic 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, analysis 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.* [17] 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 techno-economic analyses, however, neither technology has a clear competitive environmental or commercial advantage in the industry [178-180]. 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 towards 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 [181, 182]. Understandably, bio-based maize will ideally encompass dual-purpose hybrids combining both, optimal grain yield and high stover productivity [2, 18]. Simultaneously improving grain and stover yields is a feasible undertaking [33, 34, 36, 161], but maize production will also be constrained by the urgencies of modern agriculture [181, 182]. In this regard, ongoing endeavors have achieved major accomplishments in uncovering and exploiting novel genetic diversity for climate-related stresses and sustainable production un-

der lower agricultural inputs [35, 183]. 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 [8, 9, 35, 179, 180, 183]. The diversification of maize into an energy-dedicated species should be examined with caution; however, as socio-economic 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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Effect of maize biomass composition on the optimization of dilute-acid pretreatments and enzymatic saccharification

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Abstract

At the core of cellulosic ethanol research are innovations leading to reductions in the chemical and energetic stringency of thermochemical pretreatments and enzymatic saccharification. In this study, key compositional features of maize cell walls influencing the enzymatic conversion of biomass into fermentable sugars were identified. Stem samples from eight contrasting genotypes were subjected to a series of thermal dilute-acid pretreatments of increasing severity and evaluated for glucose release after enzymatic saccharification. The biochemically diverse set of genotypes displayed significant differences in glucose yields at all processing conditions evaluated. The results revealed that mechanisms controlling biomass conversion efficiency vary in relation to pretreatment severity. At highly severe pretreatments, cellulose conversion efficiency was primarily influenced by the inherent efficacy of the thermochemical process, and maximum glucose yields were obtained from cellulosic feedstocks harboring the highest cellulose contents per dry gram of biomass. When mild dilute-acid pretreatments were applied, however, maximum bioconversion efficiency and glucose yields were observed for genotypes combining high stem cellulose contents, reduced cell wall lignin and highly substituted hemicelluloses. For the best performing genotype, glucose yields under sub-optimal processing regimes were only 10% lower than the genotype-set mean at the most stringent processing conditions evaluated, while furfural production was reduced by approximately 95%. Our results ultimately established that cellulosic feedstocks with tailored cell wall compositions can help reduce the chemical and energetic intensity of pretreatments used in the industry and improve the commercial and environmental performance of biomass-to-ethanol conversion technologies.

Keywords: Maize, lignocellulose, cell wall composition, pretreatment, severity index

Introduction

With a promising suite of environmental benefits and economic opportunities, the conversion of cellulosic biomass into ethanol represents a viable alternative to fossil-based transportation fuels. Notwithstanding, despite important revamps in funding and legislative support [1], cellulosic ethanol is yet to achieve wide-scale commercialization. Experts ultimately agree that the commercial future of cellulosic ethanol is pending on innovations that can increase the industry's productivity while simultaneously lowering capital and operating costs [2,3].

With efforts to meet this challenge underway, research has been prioritized towards advancing and improving the technoeconomic efficiency of biomass processing technologies. Innovations leading to reductions in the chemical and energetic stringency of thermochemical pretreatments are at the core of cellulosic ethanol research and are expected to yield the greatest economic benefits [2-5]. Accordingly, biotechnological endeavors aimed at increasing the yields of enzymatic hydrolysis and fermentation processing steps are seen with great anticipation [2,6-8]. Critical advances in the field include, amongst others, the development of "consolidated bioprocessing" (CBP) strategies [9-11] and the production of microorganisms capable of co-fermenting C5 and C6 monosaccharides [12-14].

Less recognition has been given to the influence lignocellulosic feedstocks exert on the processing efficiency and economics of biomass-to-ethanol conversion technologies. Assessments of the technical and economic feasibility of ethanol biorefineries have minimized the role of lignocellulosic feedstocks to cost and availability considerations [15-17]. Nevertheless, studies examining the extent of natural and induced variation in cell wall composition across diverse bioenergy crops have demonstrated that feedstocks with divergent chemical constitutions respond differentially to the combined operations of pretreatment and enzymatic hydrolysis [18-26]. These studies have exclusively focused on understanding how reductions in cell wall lignin content and alterations in its monomeric composition lead to improved enzymatic conversion. To our understanding, however, there have been no reports associating genetic variation in other major cell wall polymers with variation in the enzymatic degradability of cellulosic feedstocks pretreated with the industry's leading technologies (AFEX, dilute sulphuric acid, liquid hot water, lime, and soaking in aqueous ammonia). Additionally, no study has yet evaluated the extent to which specific cell wall compositional profiles, combining the synergistic effects of variation in multiple cell wall characters, can influence the selection of processing conditions towards more sustainable and cost-efficient alternatives.

Forage maize is an attractive model for studying the influence of cell wall composition on the enzymatic conversion efficiency of lignocellulosic feedstocks. For

decades, cell wall depolymerization processes in animal rumen have been evaluated using forage maize germplasm, and it has been speculated that some of the mechanisms hindering rumen enzymatic processes will also affect the efficacy of biomass-to-ethanol conversion technologies [27,28]. Previous studies have also demonstrated that cell wall enzymatic digestibility is a dynamic trait governed by the complex interactions of different cell wall components, including lignin [29,30]. With abundant genetic variation in cell wall composition and cell wall digestibility characters, forage maize genetic resources are ideal for assessing the extent to which different cell wall components, as well as their interactions, have an impact on the efficiency of biomass-to-ethanol conversion technologies.

In this study we identify key compositional features of maize cell walls influencing the enzymatic conversion of biomass into fermentable sugars, with the aim of demonstrating that cellulosic feedstocks with tailored cell wall compositions can help reduce the chemical and energetic intensity of pretreatments used in the industry. To this effect, stem fractions of the stover from eight genetically distinct maize lines were subjected to a series of thermal dilute-acid pretreatments and were evaluated for carbohydrate release upon enzymatic saccharification. The genotypes used in this study originate from an experimental population of forage maize doubled haploids (DH) harboring extensive levels of genetic variation in stem fiber and cell wall components. Moreover, thermal-dilute acid pretreatments were selected for this study as these represent the most widely accessible and cost-effective technologies in the industry [3].

Materials and methods

Plant material

A maize population of doubled haploids (DHs) was generated by Limagrain Nederland B.V. (Rilland, Netherlands) following a cross between two proprietary inbred lines (referred to as P1 and P2) highly contrasting in forage quality and cell wall digestibility traits. A total of 230 DH genotypes and their parental inbred lines were sown in replicate, at Wouw, The Netherlands, in adjacent completely randomized blocks during the summer of 2009. Entries were planted in two-row plots with a length of 2.5 m and an inter-row distance of 0.75 m at a density of 10 plants m^{-1}. Tissue samples from this trial were analyzed for rumen liquor cell wall digestibility (data not shown). Eight DH genotypes representing the diversity of this larger population were selected for this study and kindly provided by Limagrain Nederland B.V. These eight DH genotypes are referred to as Lim-001 through Lim-008. For each plot, stalks of 10 randomly selected plants were harvested at a 10 cm stubble height just prior to silage maturity (approximately 7 weeks after the population's mean silking period). At this physiological stage, differences between genotypes in stem cell wall composition and digestibility were expected to be largely genetic [31-33]. Due to the intensive workload, however, replicate blocks were harvested separately on consecutive days. The collected biomass feedstocks were chopped and air dried at 70 ^{0}C for 48 hours, and were subsequently ground through a 1-mm screen using a hammer mill. Feedstock samples used in this study (8 in total) were produced by pooling, per genotype, the milled material collected from the two replicate experimental plots as to minimize random variation due to environment and processing.

Compositional analysis

Stem detergent fiber composition

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) components were determined by means of the ANKOM filter bag method (ANKOM Technology Corporation, Fairpoint, NY), which essentially derives from the work of Goering and Van Soest [34]. All analyses were performed in duplicate and were carried out using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY). Stem cellulose (Cel), hemicellulose (Hem) and acid-insoluble lignin (ADL) contents were derived from detergent fiber data as described in Table 1.

Cell wall composition

Cell wall content (CW) –defined in this study as water un-extractable solids– was gravimetrically determined as the percentage of biomass residue remaining af-

ter sequentially de-starching samples (1.5 hrs, ~100 °C) with 0.5 mL heat-stable α-amylase (ANKOM Technology Corporation, Fairpoint, NY) and repeatedly washing them with hot water (5x, ~70 °C). The enzyme load provided ~18,000 liquefon units (LU) per gram of dry biomass All extractions were performed using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY).

Cell wall neutral sugar components were determined by gas chromatography essentially as described by Englyst and Cummings [35]. Briefly, lyophilized water un-extractable solids were first treated with 72% sulphuric acid (1 hr, 30 °C), followed by a second hydrolysis process with 4% sulphuric acid (3 hrs, 100 °C). Released neutral sugars were then reduced with 1.5% sodium tetrahydroborate solution and derivatized to their respective alditol isoforms using acetic anhydride as the acetylating agent, and N-methylimidazole as a reaction catalyst. The derivatized products were quantified on an Agilent 7890A Gas Chromatography System (Agilent Technologies, Santa Clara, CA) using a DB-250 column (Agilent Technologies, Santa Clara, CA).

Cell wall p-coumaric (pCA) and ferulic acid (FA) esters were determined by high-pressure liquid chromatography (HPLC). Essentially, water un-extractable solids (50 mg) were incubated in 5 mL 2M NaOH at 39 °C for 24 hrs in the dark. After incubation, the mixture was centrifuged (3500 RPM, 5 min) and the resulting supernatant was collected and acidified to pH 2.0 using concentrated HCl. Subsequently, cell wall phenolics were extracted from the acidified sample (5 mL) using ethyl acetate (2x, 5 mL). The ethyl acetate extract was then evaporated and the remnants were re-suspended in 80% aqueous methanol. Identification and quantification of phenolic acid esters was performed using a Waters HPLC-PDA system (Waters Associates, Milford, MA) equipped with a HyPurity C_{18} (3μm, 150mm x 3mm) column (Thermo Electron Corporation, Bellefonte, PA).

Bioconversion efficiency

Pretreatment setup

Thermal dilute-acid pretreatments of increasing severity were performed in duplicate on all ground maize stalk samples (Table 2). For all processing parameters, the 'combined severity' factor $logR'_0$ was defined as:

$$logR'_0 = \log(t \cdot \exp[(T_H - 100)/14.75]) - pH_{out}$$

where t is the reaction time in minutes, T_H is the hydrolysis temperature in °C and pH_{out} is the pH of pretreatment liquors after thermochemical processing [36,37]. The combined severity factor $logR'_0$ describes the intensity of dilute-acid pretreatments in relation to their effect on xylan solubilization and lignin alteration [37,38]. By

Table 1. Description of quality traits measured on stem material of eight maize doubled haploid lines

Trait	Unit	Description
CW	g Kg^{-1} DM	Stem cell wall content; determined as the water un-extractable fraction
Cel	g Kg^{-1} DM	Stem cellulose content; determined as the difference between ADF and ADL
Hem	g Kg^{-1} DM	Stem hemicellulose content; determined as the difference between NDF and ADF
ADL	g Kg^{-1} DM	Stem acid insoluble lignin content; determined as ADL
Ara	g Kg^{-1} CW	Cell wall arabinose content
Xyl	g Kg^{-1} CW	Cell wall xylose content
Man	g Kg^{-1} CW	Cell wall mannose content
Gal	g Kg^{-1} CW	Cell wall galactose content
Glu	g Kg^{-1} CW	Cell wall glucose content
Lig	g Kg^{-1} CW	Cell wall lignin content; determined as the ratio between ADL and NDF
pCA	g Kg^{-1} CW	Cell wall esterified p-coumaric acid content
FA	g Kg^{-1} CW	Cell wall esterified ferulic acid content
CWD	% NDF	In-vitro cell wall digestibility; determined as the difference in NDF content before and after sample incubation in rumen liquor for 48 hours relative to NDF content prior to incubation.
Glu-Rel	g Kg^{-1} DM	Amount of glucose released from one gram of dry biomass after pretreatment and enzymatic saccharification.
Glu-Con	% CW Glucose	Percentage of total cell wall glucose released after pretreatment and enzymatic saccharification.
Xyl-Rel	g Kg^{-1} DM	Amount of xylose released into pretreatment liquors from one gram of dry biomass after thermochemical processing.
Xyl-Con	% CW Xylose	Percentage of total cell wall xylose hydrolyzed during pretreatment.
FUR	mg L^{-1}	Concentration of furfural accumulated in pretreatment liquors after thermochemical processing.
HMF	mg L^{-1}	Concentration of 5-(hydroxymethyl)furfural accumulated in pretreatment liquors after thermochemical processing.

collectively combining the conditions of reaction time, temperature and the effect of acid catalyst into a single parameter, this index facilitates the comparison of yield data from pretreatment strategies employing different reaction conditions.

Table 2. Thermochemical parameters used for the pretreatment of stem material of eight maize doubled haploid lines

Temperature	Duration	Acid Loading[¥]	Solids Loading[§]	pH_{out}	Combined Severity Factor ($\log R'_0$)
No Pretreatment	----	-----	----	----	----
120 °C	20 min.	0.67%	30 %	1.32	0.57
140 °C	20 min.	0.17%	30 %	1.82	0.66
150 °C	20 min.	0.17%	30 %	1.81	0.95
140 °C	20 min.	1.33%	30 %	1.15	1.33
175 °C	10 min.	0.17%	30 %	1.79	1.42
180 °C	10 min.	0.17%	30 %	1.81	1.64

¥- 98% H_2SO_4 (w/v%)

§ Pretreatment-slurry solids to liquid ratio (w/v%)

Reactions were carried out using 25 mL custom built stainless steel high-pressure reactors equipped with a K-type thermocouple and a 12 cm stainless steel thermocouple probe. Biomass samples (500 mg) were contained inside heat/acid resistant nylon filter bags (ANKOM Technology Corporation, Fairpoint, NY) which allowed for easy biomass transfer while preventing biomass losses during processing reactions. Prior to thermochemical treatment, samples were briefly de-starched with α-amylase and repeatedly washed with deionized water (3x, ~50 °C) in order to remove all interfering stem soluble sugars.

During pretreatments, two separately controlled oil baths were employed; the first one -set at 180 °C- was used to rapidly heat up reactors, while the second bath was used to control reactions at the desired temperature. Depending on the conditions, target temperatures were typically reached between 3-5 minutes. To maintain the temperature within +/- 1.0 °C of the target temperature, reactors were either manually hoisted from the oil bath or re-submerged in the higher-temperature oil bath when necessary. After the desired treatment time, reactions were rapidly quenched by plunging the reactors in an ice-water bath. Pretreatment liquors were collected for further chemical analyses, and biomass samples were washed with abundant distilled water.

Analysis of pretreatment liquors

After thermal dilute-acid pretreatment, liquors were filtered through a 0.45 μm syringe filter. Monomeric xylose release was analyzed using a Dionex High Pressure Liquid Chromatography system (Dionex, Sunnyvale, CA) equipped with a CarboPac Pa100 column (Dionex, Sunnyvale, CA). Furfural (FUR) and 5-(hydroxymethyl)furfural (HMF) concentrations were analyzed using a Waters HPLC-PDA (Waters Associates, Milford, MA) equipped with an Altima HP C_{18} (5μm) column (Alltech, Deerfield, IL).

Enzymatic hydrolysis

Enzymatic saccharification efficiency traits were analyzed by means of the National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedure-009 assay [39] after dilute acid/high temperature pretreatment. Briefly, pretreated samples contained within nylon filter bags were treated with 250 μL of an Accelerase 1500 cellulolytic enzyme cocktail (Genencor B.V., Leiden, NL) in 40 mL 0.1 M citrate buffer. The enzyme load provided 50 filter paper units (FPU) of cellulase per gram cellulose. Samples were then incubated at 50 °C in an Innova 42 air incubator (New Brunswick Scientific, Enfield, CT) at 200 RPM for 24 hrs. Enzymatic saccharification liquors were analyzed for glucose content using a Boehringer Mannheim D-Glucose kit (Boehringer Mannheim, Indianapolis, IN, USA). The colorimetric assay was adapted to a 96 micro-titer plate format, and spectrophotometric reads were made using a Bio-Rad 550 Micro-plate Reader (Bio-Rad, Richmond, CA). For all samples, glucose content was expressed as both, the amount of glucose released from one gram of dry biomass (Glu-Rel) and the percentage of total cell wall glucose released after enzymatic saccharification (Glu-Con) (Table 1).

Statistical analyses

General analysis of variance (ANOVA) was used to determine the significance of sample differences in stem fiber and cell wall components, as well as bioconversion parameters. For bioconversion parameters, the statistical significance of the variation observed across the set of genotypes was estimated separately for each of the 7 processing conditions evaluated, and variance analysis was also performed to test for the plausible effect of genotype-pretreatment interactions. Pearson correlations between bioconversion parameters and stem fiber and cell wall components were also independently determined for each pretreatment condition analyzed. Where applicable, linear regression analyses was performed to evaluate the relationship between bioconversion parameters and feedstock compositional characters. All statistical analyses were performed using the GenStat for *Windows* 14th Edition Software Package (VSN International, Hemel Hempstead, UK).

Results and Discussion

Maize doubled haploid (DH) genotypes harbor great levels of diversity in feedstock composition

Stem fiber and cell wall components of the evaluated DH genotypes are presented in Table 3. Biochemical analyses were performed with great precision (relative standard deviation [RSD%] < 5.0%) and observed feedstock compositions were well within the range of previously reported values [23,40,41].

Statistically significant differences between genotypes were detected for all stem fiber and cell wall components evaluated, except for cell wall glucose content (Glu). Accordingly, ranges (min-max) were high for most statistically contrasting traits. Among all components evaluated, however, lignin content on a dry matter (ADL) and cell wall (Lig) basis displayed the highest levels of variation; with range values respectively representing ∼94% and ∼91% of the corresponding trait mean. This result was expected as the evaluated genotypes were selected to widely contrast for rumen liquor cell wall digestibility (CWD). In fact, regression analysis reveals that Lig explained 96% of observed variation for CWD across the genotype set (*data not shown*).

Principle components analysis discloses, nevertheless, that the compositional diversity of the DH-set was not restricted to genotypic variation in lignin characters (Figure 1). The first component (PC1), which summarized ∼45.5% of observed compositional variance, was dominated by both, lignin characters (ADL, Lig) and C5 sugar components. The second component (PC2) explained ∼35.5% of observed phenotypic variation and separated genotypes on their stem cell wall and polysaccharide contents (CW, NDF, Cel, Hem). Despite a limited degree of variation in cell wall xylose content (Xyl), considerable genotypic differences in arabinose (Ara), galactose (Gal) and mannose (Man) might imply a rich level of diversity in hemicellulosic substitution and structural patterns. PC1 allowed for the classification of the DH-set into high- and low-digestibility groups; and thus suggests a plausible association between hemicellulose monomeric composition and cell wall enzymatic recalcitrance.

Incremental pretreatment severity leads to improved cell wall conversion efficiency

Bioconversion trends observed across pretreatments of increasing severity were well in accordance with benchmark observations [38,36]. For every pretreatment condition evaluated, bioconversion performance was calculated as the mean performance of the DH-set within a given thermochemical environment (Table S1, Figure 2). Increasing the energetic and chemical stringency of pretreatments led to improved fermentable glucose release after enzymatic saccharification. Maximum

Table 3. In-vitro cell wall digestibility and stem fiber and cell wall composition for eight maize doubled haploid lines

Trait	Sample Mean								Descriptive Statistics			
	Lim-001	Lim-002	Lim-003	Lim-004	Lim-005	Lim-006	Lim-007	Lim-008	Average[φ]	S.E.M.	Range (min-max)	RSD%
CWD %	40.8	49.7	38.3	50.8	63.9	51.9	43.8	50.8	48.5***	1.4	25.60	3.4%
Digestibility												
Stem Bulk-Fiber Composition												
CW (g kg^{-1} DM)	600.0	524.6	505.8	525.8	621.1	463.2	521.2	673.0	554.3***	7.4	209.80	1.8 %
Cel (g kg^{-1} DM)	288.8	246.7	235.3	250.3	280.9	210.8	248.6	316.2	259.7***	1.8	105.40	0.9 %
Hem (g kg^{-1} DM)	219.6	206.1	166.2	205.3	283.5	187.6	194.2	283.2	218.2***	6.4	117.30	3.1 %
ADL[¥] (g kg^{-1} DM)	38.7	22.6	31.6	20.6	15.1	18.8	27.7	30.2	25.7***	1.3	23.60	5.9 %
Stem Cell Wall Composition												
Ara (g kg^{-1} CW)	32.4	37.2	31.5	37.2	39.9	43.6	34.1	37.1	36.6**	0.3	12.10	0.7 %
Xyl (g kg^{-1} CW)	211.6	224.6	200.7	208.1	215.5	240.4	214.8	221.2	217.1***	1.8	39.70	0.9 %
Man (g kg^{-1} CW)	3.2	2.9	3.1	3.8	4.5	3.7	3.0	3.9	3.5***	0.1	1.60	3.9 %
Gal (g kg^{-1} CW)	7.5	9.6	8.8	11.3	12.1	11.5	10.1	10.0	10.1***	0.2	4.60	1.6 %
Gluc (g kg^{-1} CW)	384.8	393.5	382.1	399.0	389.7	407.1	391.4	396.5	393.0	5.1	25.00	1.4 %
Lig (g kg^{-1} CW)	70.7	47.4	73.0	43.3	26.1	45.1	58.9	48.0	51.6**	2.7	46.89	5.9 %
pCA (g kg^{-1} CW)	25.1	20.5	24.2	19.2	23.5	18.7	25.0	17.9	21.8***	0.6	7.27	2.9 %
FA (g kg^{-1} CW)	6.5	7.3	6.7	6.3	7.1	6.9	6.2	6.5	6.7***	0.2	1.03	3.98%

¥- Stem lignin content quantified as acid detergent lignin (ADL), not corrected for ash content.

φ- Significance of sample differences at p<0.05 (*), p<0.01 (**) and p<0.001 (***)
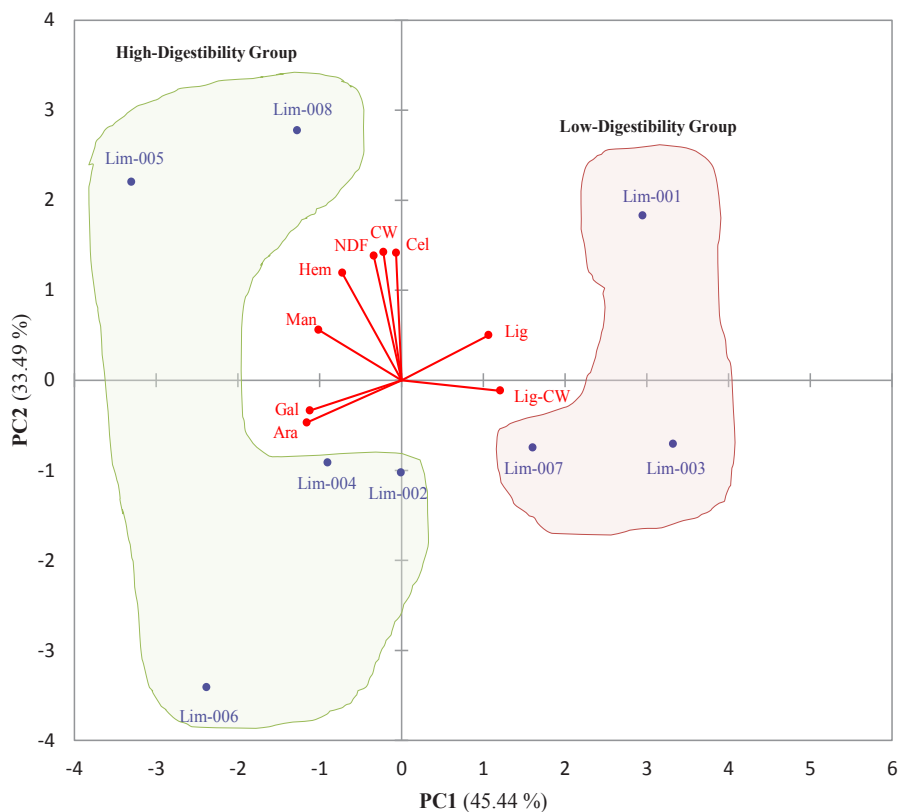
Figure 1. Principle components biplot displaying the classification of eight DH genotypes based on stem fiber and cell wall components. Red vectors summarize the correlation between relevant feed-stock compositional characters and the corresponding principal component. The contribution of the C5 sugars arabinose (Ara), galactose (Gal) and mannose (Man) to PC1 might reflect a possible association between hemicellulose monomeric composition and cell wall enzymatic recalcitrance.

cellulose conversion efficiency rates (Glu-Con ~90.0%) and glucose yields (Glu-Rel ~200.0 g Kg^{-1} DM) were attained at the most intensive thermochemical condition (LogR'$_0$ 1.64). The underlying objective of dilute-acid pretreatments is to effectively solubilize hemicelluloses from the cell wall matrix as to maximize cellulose exposure and accessibility to hydrolytic enzymes [37]. Correspondingly, total xylose conversion efficiency (Xyl-Con) and monomeric xylose recovery in pretreatment liquors (Xyl-Rel) were also positively influenced by increments in pretreatment severity.

As anticipated, furfural (FUR) production increased exponentially with increased thermochemical severity. The sharp accumulation of furfural at high severity pretreatments has been amply evidenced in previous research [38], and serves as explanation for the sudden decay in xylose yields observed at the most intensive

pretreatment condition (LogR'$_0$ 1.64). Lloyd and Wyman [36] demonstrated that at highly stringent thermochemical environments, xylan solubilization and depolymerization rates lead to a fast decay in the concentration of xylan oligomers, favoring the production of monomeric xylose [42]. Under these conditions, any further increase in thermochemical severity limits monomeric xylose recovery as the latter degrades into furfural at almost exponential rates [38,42].

Bioconversion trends presented in this section exemplify a scenario which underscores the effect of cellulosic feedstock composition on the global performance of biomass conversion processes. This analysis supports the widely accepted view that maximum cellulose conversion rates and competitive glucose yields can only be achieved through highly stringent and uneconomic pretreatments. In the next sections we demonstrate, however, that feedstocks with specific compositional profiles can influence the selection of processing conditions towards more sustainable and cost-efficient alternatives.
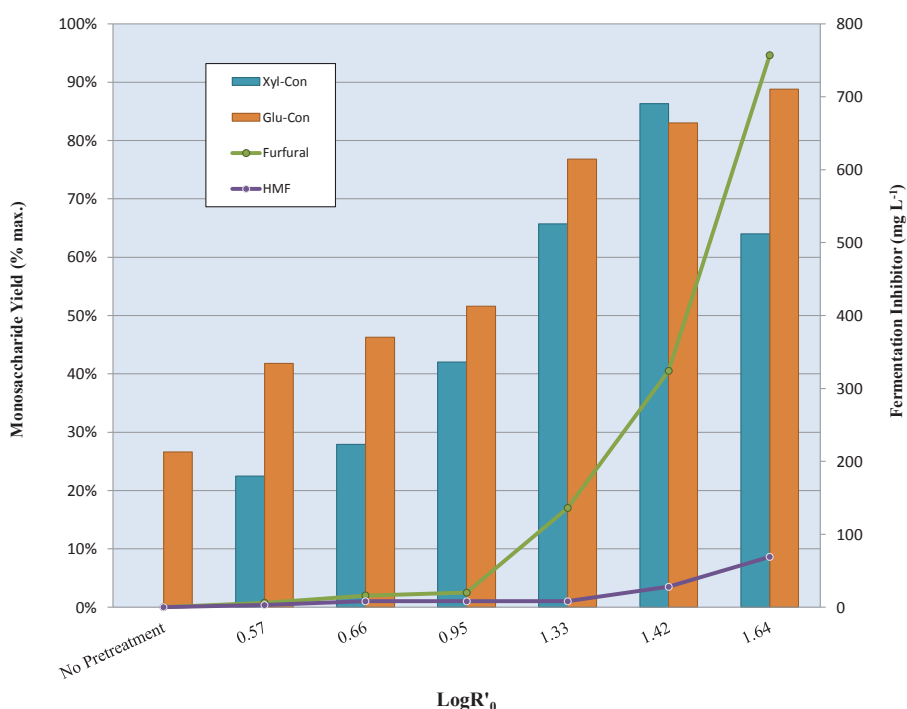
Figure 2. Carbohydrate conversion trends (Glu-Con and Xyl-Con) across dilute-acid pretreatments of increasing severity. Per pretreatment, bioconversion performance was calculated as the mean performance of all DH-genotypes analyzed within a given thermochemical environment. Increasing pretreatment severity leads to improved cell wall carbohydrate conversion and fermentable glucose yields. At high severity pretreatments (LogR'$_0$>0.95), the production of furfural displays an exponential-like accumulation rate.

The effect of feedstock composition on bioconversion efficiency is dependent on pretreatment severity

From the onset of this investigation, we hypothesized that the eight doubled haploid (DH) genotypes would contrast in their response to enzymatic conversion after dilute-acid pretreatment. The underlying premise was that their highly divergent stem fiber and cell wall compositions could be associated to an inherent differential response in bioconversion potential. As expected, at every pretreatment condition analyzed, stem samples of the eight DH genotypes showed statistically significant differences for all bioconversion parameters evaluated. The only exception found was for furfural (FUR), for which genotypic differences could not be detected at highly stringent pretreatments ($LogR'_0 \geq 1.42$). Summary statistics detailing the ample range in variation for bioconversion characters, categorized across all processing regimes evaluated, are presented in Table S2. The extent and pattern of sample-to-sample differences, however, are best evidenced when plotting the conversion efficiency of each genotype against the array of evaluated pretreatment conditions (Figure 3). Figure 3a, for example, depicts the differential performance in fermentable glucose release (Glu-Rel) for all DH genotypes across the pretreatment series, and clearly demonstrates how specific genotypes consistently outperform (Lim-005 and Lim-008) or underperform (Lim-003 and Lim-007) the rest. Similar variation patterns were also observed for cellulose conversion efficiency (Glu-Con) (Figure 3b).

Significant genotype-pretreatment interactions were also uncovered for Glu-Rel and Glu-Con. This finding explains that the extent of sample-to-sample variation for these parameters fluctuated across different processing regimes, and led to either the amplification or waning of differences between genotypes at specific pretreatment conditions. Increasing the intensity of dilute-acid pretreatments led to the progressive amplification of genotypic differences in Glu-Con at mild pretreatments ($logR'_0 < 0.95$). Figure 3b clearly demonstrates the progressive increase in response range between the two most contrasting feedstocks (Lim-005 and Lim-001), and also depicts how escalating thermochemical conditions widen the discerning response of genotypes to enzymatic saccharification. At high severity pretreatments ($logR'_0 > 0.95$), however, differences in response between genotypes sharply decayed and ultimately led to significant genotype cross-over events. In fact, at the most intensive pretreatment condition ($logR'_0$ 1.64), maize genotypes consistently displaying the lowest conversion efficiencies (i.e. Lim-001 and Lim-003) finally matched or outperformed the most degradable genotypes (Lim-005, Lim-004).

Whereas the mechanisms governing genotype-pretreatment interactions at mild severities are less understood, the inherent mode of action of thermal dilute-acid pretreatments provides an explanation for the trends observed at higher severities. At
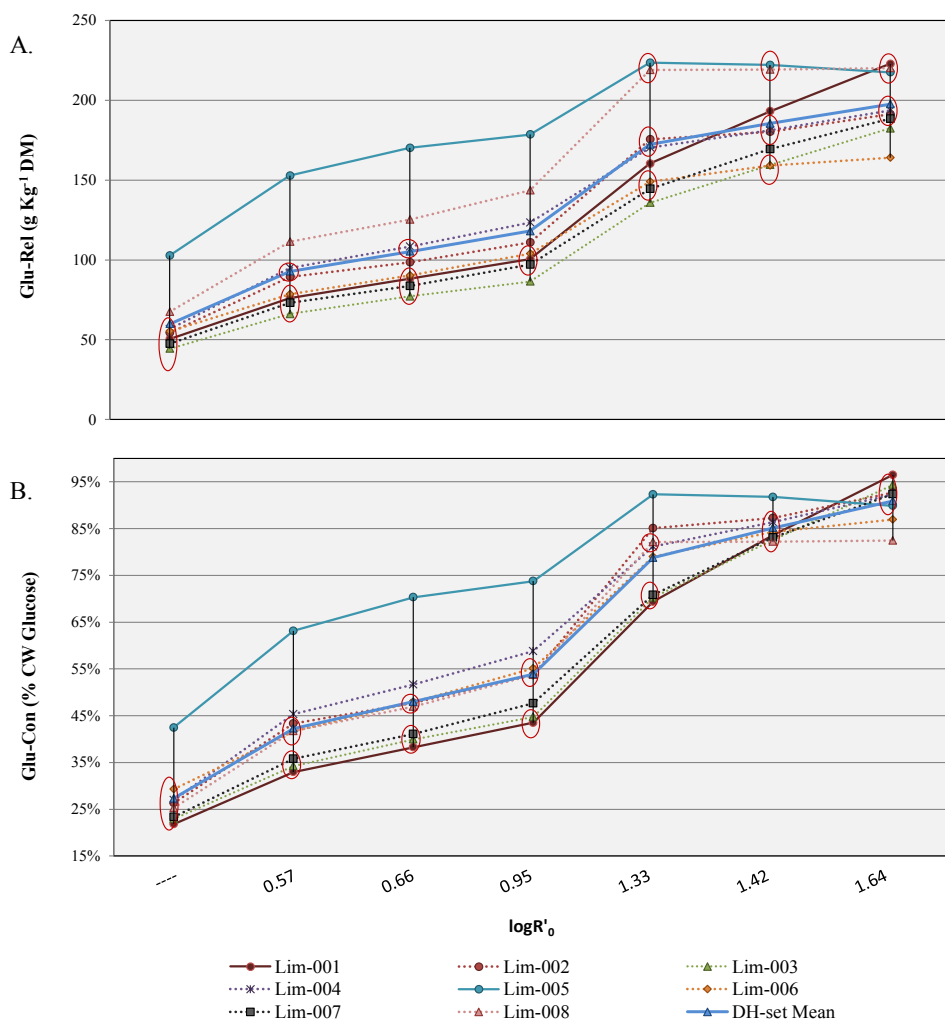
Figure 3. Individual genotype conversion performance across pretreatments of increasing severity for (a) Glu-Rel and (b) Glu-Con. Encircled data points are not statistically different from each other at $p \leq 0.05$. The "DH-Set Mean Performance" represents the mean performance of all DH-genotypes analyzed within a given thermochemical environment. Glu-Rel is the amount of glucose released from one gram of dry biomass after pretreatment and enzymatic saccharification. Glu-Con is the percentage of total cell wall glucose released after pretreatment and enzymatic saccharification.

high severity pretreatments ($logR'_0 > 1.33$), xylan depolymerization and solubilization rates are substrate independent [42-44] and presumably lead to near-maximum xylan conversion (and lignin disruption) regardless of input feedstock. This implies that at high-severity regimes, the efficient conversion of cellulose into fermentable glucose (Glu-Con) is primarily determined by the inherent efficacy of the thermo-

chemical process in rendering cellulose accessible to enzymatic hydrolysis. Under these provisions, near-maximum yields in fermentable glucose release (Glu-Rel) are also expected irrespective of the compositional nature of the input feedstock. This would help explain genotype-pretreatment interactions observed for Glu-Rel, where at high severity pretreatments ($logR'_0 > 1.33$), the most recalcitrant genotypes (Lim-001, Lim-003 and Lim-007) began to match or outrank the rest (Figure 3a).

Genotype-pretreatment interactions observed in this study ultimately demonstrate that the mechanisms controlling bioconversion efficiency at high severity pretreatments are different from those controlling bioconversion efficiency at mild pretreatments. At mild pretreatments, where only partial deconstruction of the cell wall occurs, biomass conversion efficiency (measured either as Glu-Con or Glu-Rel) appears to be primarily influenced by cell wall compositional features controlling the extent of enzymatic recalcitrance of the input feedstock (discussed in the next section). Remarkably, even under suboptimal pretreatment conditions, some genotypes (Lim-005, Lim-008) achieved industrially acceptable glucose yields while significantly reducing the accumulation of carbohydrate degradation products (FUR, HMF). To illustrate the extent of gain in performance that can be achieved if distinctions are made between feedstock origin and composition, Figure 3a schematically compares the conversion performance of the overall best (Lim-005) and worst genotypes (Lim-003) for Glu-Rel against the mean performance of the DH-set. At $logR'_0$ 0.95, Glu-Rel was ~180 g Kg^{-1} DM for Lim-005; a value just 10% lower from the DH-set mean (~198 g Kg^{-1} DM) at the most stringent pretreatment condition ($logR'_0$ 1.64). Moreover, the production of furfural (FUR) for Lim-005 at $logR'_0$ 0.95 was almost 95% lower than the DH-set mean at $logR'_0$ 1.64. Furfural (FUR) and 5-(hydroxymethyl)furfural (HMF) are considered to limit the efficiency of fermenting microorganisms in downstream processes, and reductions in their production during dilute-acid pretreatments are expected to greatly improved the environmental performance and yields of the conversion process.

Overall, these results demonstrate that industrial goals to reduce the energetic and chemical stringency of pretreatments can also be achieved through the selection of cellulosic feedstocks optimal to a given set of thermochemical parameters. More recently, diverse studies have demonstrated that differences in bioconversion efficiency are in part heritable [23,28,40], Torres *et al.*, unpublished results], thus enabling the possibility to select and advance dedicated cellulosic feedstocks that improve the efficiency and economics of biomass-to-ethanol conversion technologies. Uncovering cell wall compositional features leading to maximum bioconversion efficiency at mild processing regimes is therefore of utmost importance.

Key compositional features of maize cell walls influencing enzymatic conversion efficiency

The main objective of this study was to identify key compositional features of maize cell walls influencing the enzymatic conversion of biomass into fermentable sugars. To this effect, relationships between bioconversion parameters (Glu-Con and Glu-Rel) and feedstock fiber and cell wall components were studied with the help of correlation analyses.

At mild pretreatments ($logR'_0 < 0.95$), Glu-Rel was consistently and strongly correlated ($r > 0.80$) to cell wall digestibility (CWD), and was negatively associated ($r > -0.80$) to cell wall lignin content (Lig). This finding confirms the notion that enzymatic saccharification efficiency after suboptimal pretreatment is predominantly determined by cell wall compositional features governing cell wall enzymatic recalcitrance. At high severity pretreatments ($logR'_0 > 1.33$), however, the statically strong associations between Glu-Rel, CWD and Lig gradually disappeared, and were in turn replaced by moderate-to-high correlations ($r > 0.75$) with stem cellulose (Cel) and cell wall (CW) content (Figure 4). At highly intensive thermochemical conditions, cellulose conversion efficiency (both as Glu-Con and Glu-Rel) appears to be primarily influenced by the inherent efficacy of the thermochemical process. Congruently, feedstock differences in Glu-Rel performance at high severity pretreatments were determined by genotypic differences in the availability of cellulose per gram (Cel) of input feedstock. At $logR'_0$ 1.64, overall performance rankings in Glu-Rel favored genotypes with higher stem cellulose concentrations (Lim-001, Lim-005, Lim-008).

From an economic perspective, the efficiency of cellulosic ethanol conversion systems is primarily conditioned by yields of fermentable glucose (Glu-Rel) achieved during enzymatic saccharification. If high severity dilute-acid pretreatments are to be favored by the industry, then maximum economic productivities are anticipated when using cellulosic feedstocks harboring the highest cellulose contents per dry gram of biomass [15,16]. Notwithstanding, the cellulosic ethanol industry is actively seeking to reduce the chemical and energetic stringency of thermochemical pretreatments in an attempt to improve the industry's commercial and environmental performance. Uncovering stem fiber and cell wall components responsible for differences in cellulose conversion efficiency (Glu-Con) at mild pretreatments will facilitate the selection of bioenergy dedicated feedstocks that can maximize fermentable carbohydrate yields (Glu-Con) under more sustainable and cost-efficient processing alternatives.

Lignin content on a dry matter (ADL) and cell wall bases (Lig) were strongly, but negatively, correlated to Glu-Con ($r > -0.80$) at all mild pretreatments analyzed ($logR'_0 < 0.95$) (Figure 5). Lignin has been long known to limit the efficiency of en-
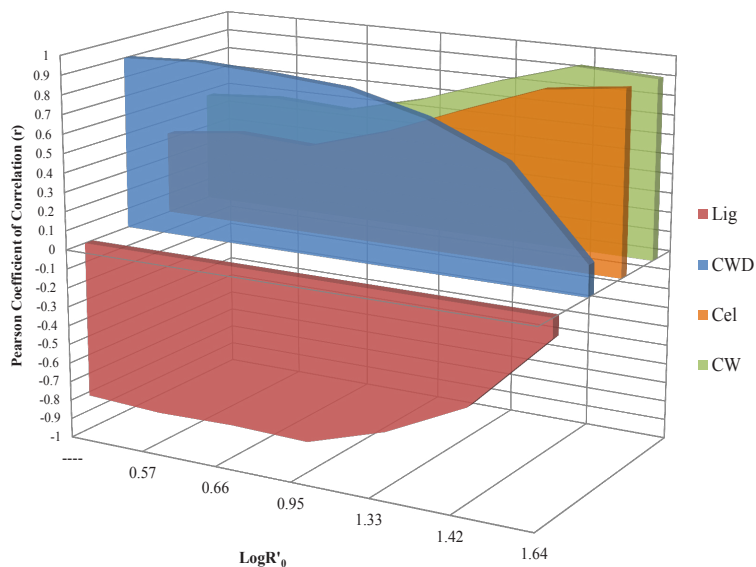
Figure 4. Progression of correlation patterns between relevant maize compositional features and Glu-Rel across dilute-acid pretreatments of increasing severity. Correlations are statistically significant at r≥0.7. At mild processing regimes (LogR'$_0$<0.95), strong associations between Glu-Rel and Lig imply that enzymatic saccharification efficiency is predominantly determined by the extent of cell wall enzymatic-recalcitrance.
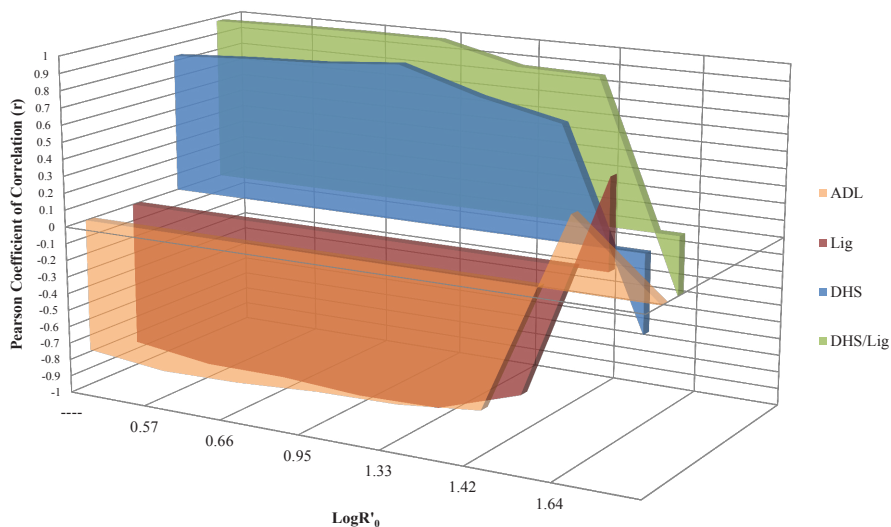
Figure 5. Progression of correlation patterns between relevant maize compositional features and Glu-Con across dilute-acid pretreatments of increasing severity. Correlations are statistically significant at r≥0.7. As pretreatment severity increases, the relationships between Glu-Con and cell wall compositional features gradually disappear. At high severity pretreatments (logR'$_0$>1.33), cell wall recalcitrance is maximally overcome by the inherent efficacy of the thermochemical process.

zymatic conversion processes either through the mechanical sheathing of cell wall polysaccharides or through the irreversible binding of hydrolytic enzymes [45]. In grasses, several studies have also demonstrated that alterations in lignin monomeric composition, favoring lower syringyl/guaiacyl ratios, can lead to significant reductions in biomass recalcitrance. In mature cell walls, p-coumaric acid (pCA) is predominantly esterified to syringyl moieties, and its concentration can serve as a marker for lignin monomeric composition. In this study, however, a direct relationship between pCA and Glu-Con could not be established, making it impossible to confirm whether lignin monomeric composition exerts an influence on enzymatic saccharification efficiency.

Correlation analysis could not confirm significant associations between specific C5 sugar components and Glu-Con. Notwithstanding, based on the fact that arabinose (Ara), galactose (Gal) and mannose (Man) contributed significantly to the compositional diversity of the DH-set, we decided to investigate a potential relationship between Glu-Con and hemicellulosic substitution patterns. The degree of hemicellulose substitution (DHS) was thus derived as the ratio between the sum of Ara, Gal and Man over cell wall xylose content (Xyl). Strong positive correlations ($r > 0.85$) were detected for Glu-Con and DHS at mild processing regimes ($\log R'_0 < 1.33$) (Figure 5). The exact mechanism through which the level of xylan substitution may affect cellulose conversion processes has not been extensively studied. An interesting hypothesis explains that less densely substituted xylan oligomers have a higher affinity towards cellulose and may in fact re-adhere to the polysaccharide matrix during mild dilute-acid pretreatments [46]. In this study, DHS was also positively correlated ($r > 0.80$) to xylose conversion efficiency (Xyl-Con) (*data not shown*); a finding that would suggest that the effective hydrolysis of hemicelluloses during mild pretreatments is hindered by the re-adsorption of released xylan oligomers back into the cell wall matrix. It is important to mention, however, that DHS was also negatively correlated ($r > -0.89$) with ADL and Lig, suggesting that the correlation between xylan substitution dynamics and biomass recalcitrance is the effect of an indirect relationship between lignification cross-linking patterns and hemicellulose side-chain concentration. Remarkably, the ratio DHS/Lig, which summarizes the relationship of these two variables into a novel parameter, displayed the highest correlations ($r > 0.97$) with Glu-Con at mild processing regimes (Figure 5).

Bioconversion efficiency is a complex trait

Until recently, the majority of studies analyzing the potential for improving the processing amenability of bioenergy crops have concentrated on cell wall lignification patterns. In this study we demonstrate, nonetheless, that biomass conversion efficiency is a highly complex trait, controlled not only by the balance and synergistic

action of multiple cell wall components, but also by the inherent effectiveness of the conversion process. To illustrate this, regression analysis was used to evaluate the contribution of relevant cell wall components (as determined from correlation analyses) on the extent of variation in fermentable glucose yields (Glu-Rel) observed across three highly contrasting processing regimes (Figure 6).
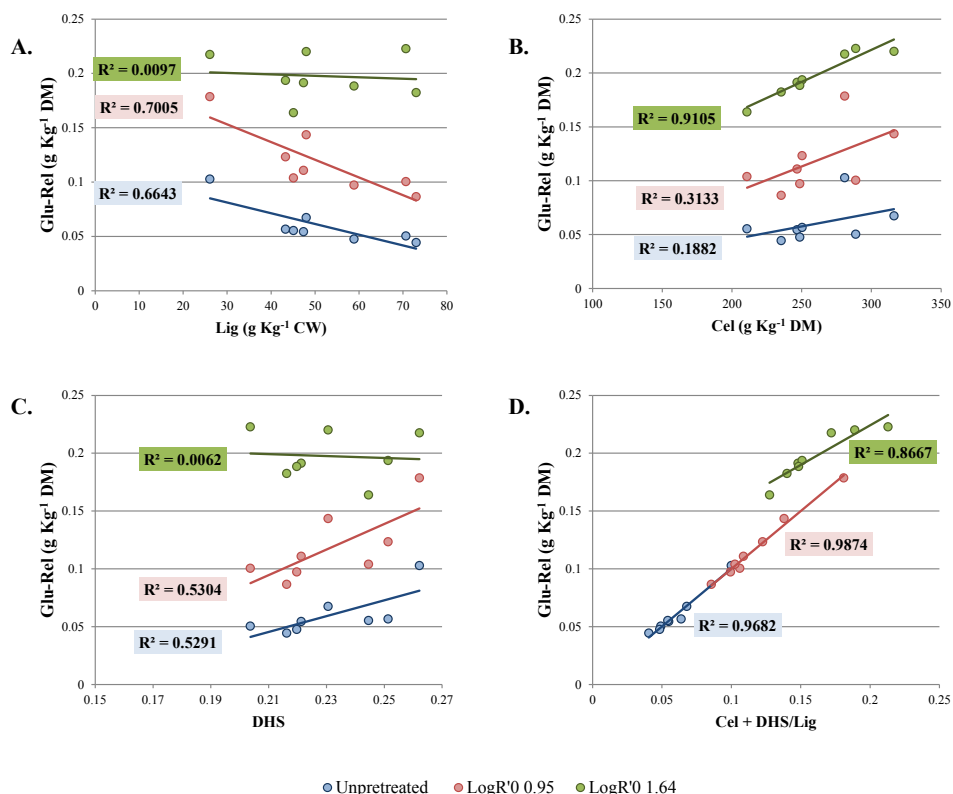
Figure 6. Linear regression of relevant stem fiber and cell wall components on Glu-Rel, across three highly contrasting processing regimes (No-pretreatment, LogR'$_0$ 0.95 and LogR'$_0$ 1.64). a) Lig; b) Cel; c) DHS, d) Multiple-Linear Regression including Lig, Cel and DHS.

Individually, none of the compositional characters analyzed could fully explain observed Glu-Rel values. At mild processing regimes, cell wall lignin content (Lig) was the strongest predictor for Glu-Rel performance (Figure 6b). This finding supports the previous observations that feedstocks with reduced cell wall lignin exhibit improved enzymatic digestibilities and higher carbohydrate conversion rates. Remarkably, Lim-006, which ranked favorably for Lig, was highly outranked in Glu-Rel per-

formance by genotypes with similar or higher lignin contents (Lim-002, Lim-004, Lim-008) (Figure 3a). Despite the lack of significant correlations between Cel and Glu-Rel at mild pretreatments (Figure 4), we hypothesized that the poor yields of Lim-006 were related to its low stem cellulose content.

Consequently, a regression model combining the synergistic effects of multiple stem fiber and cell wall components was conceived. The model, which used Cel and the ratio DHS/Lig as explanatory variables, assumed that Glu-Rel was a function of the amount of cellulose available during the reaction and its susceptibility to enzymatic hydrolysis. Whereas extensive evidence suggests that lignin acts as an enzymatic inhibitor during the saccharification process [45], we propose that the degree of substitution of hemicelluloses (DHS) directly impacts dilute-acid pretreatment effectiveness under suboptimal conditions ($logR'_0 < 0.95$). Our results suggest that feedstocks with highly substituted hemicelluloses exhibit higher xylan solubilization rates and lower oligomeric xylan reabsorption to cellulose during thermochemical processing (see section 3.2), thereby improving the accessibility of cellulose to enzymatic hydrolysis.

The formulated model could explain over 95% of observed variance in Glu-Rel at mild processing regimes (Figure 6d). This result ultimately confirms that the interaction of multiple compositional features determine the degree of processing amenability of cellulosic feedstocks. Above all, however, our results re-enforce previous observation that feedstocks accumulating multiple beneficial compositional features lead to the greatest gains in performance under specific processing regimes. In this study, Lim-005, which consistently yielded the highest concentrations of fermentable glucose across all processing conditions evaluated, was not only characterized for having the lowest cell wall lignin content within the DH-set; it also displayed one of the highest stem cellulose concentrations and the highest degree of hemicellulose substitution.

Conclusions

The results of this investigation demonstrate that maize cellulosic feedstocks with highly divergent biochemical constitutions respond differentially to the combined operations of dilute-acid pretreatment and enzymatic saccharification. We reveal, nevertheless, that biomass conversion efficiency is a complex trait, controlled not only by the balance and synergistic action of multiple cell wall components, but also by the inherent effectiveness of the conversion process. At highly severe pretreatments, cellulose conversion efficiency was primarily influenced by the inherent efficacy of the thermochemical process, and maximum glucose yields were obtained from cellulosic feedstocks harboring the highest cellulose contents per dry gram of biomass. When favoring mild dilute-acid pretreatments, however, maximum bioconversion efficiency and glucose yields were observed for genotypes combining high stem cellulose contents, reduced cell wall lignin and highly substituted hemicelluloses.

Our results re-enforce the notion that cellulosic feedstock composition can exert great influence on the efficiency of biomass-to-ethanol conversion platforms, and should be an essential parameter when investigating their technoeconomic feasibility. In this study, we reveal how industrially competitive glucose yields were obtained from genotypes with favorable compositional profiles under more sustainable and cost-efficient processing alternatives. Clearly, the selection and use of cellulosic feedstocks that best match the processing conditions used in the industry will undoubtedly aid in reaching industrial goals aimed at improving the commercial and environmental performance of cellulosic ethanol.

Table S1. Mean bioconversion performance characteristics of 8 stem samples of maize genotypes contrasting for cell wall quality traits after pretreatments increasing in severity

Pretreatment	Pretreatment Liquor Analysis				Enzymatic Saccharification Liquor Analysis	
	Xyl-Rel	Xyl-Con	Fur	HMF	Glu-Rel	Glu-Con
	$g\,Kg^{-1}\,DM$	% CW Xylose	$mg\,L^{-1}$	$mg\,L^{-1}$	$g\,Kg^{-1}\,DM$	% CW Glucose
No Pretreatment	0.0	0.0%	0.0	0.0	59.9	26.6%
120 °C - 20 min – 0.67%	29.1	24.3%	5.7	3.4	94.0	41.8%
140 °C - 20 min – 0.17%	43.1	36.2%	16.4	8.7	104.1	46.3%
150 °C - 20 min – 0.17%	53.1	44.3%	19.9	8.4	116.2	51.6%
140 °C - 20 min – 1.33%	83.4	69.4%	136.3	7.6	172.3	76.8%
175 °C - 10 min – 0.17%	107.6	89.8%	324.1	28.4	185.5	83.0%
180 °C - 10 min – 0.17%	81.2	67.6%	757.3	69.3	198.1	88.8%

Table S2. Sample-to-sample variation statistics for a diverse range of bioconversion traits determined after pretreatments with increasing severity

Pretreatment	Pretreatment Liquor Analysis				Enzymatic Saccharification Liquor Analysis	
	Xyl-Rel	Xyl-Con	Fur	HMF	Glu-Rel	Glu-Con
	$g\,Kg^{-1}\,DM$	% CW Xylose	$mg\,L^{-1}$	$mg\,L^{-1}$	$g\,Kg^{-1}\,DM$	% CW Glucose
No Pretreatment	-----	-----	-----	-----	44.4 - 102.8	21.8 - 42.5
120 °C - 20 min – 0.67%	21.2 - 42.0	18.8 - 31.4	4.5 - 7.5	2.6 - 4.4	66.2 - 152.8	33.0 - 63.1
140 °C - 20 min – 0.17%	32.7 - 54.0	25.8 – 40.3	12.5 – 22.8	7.3 – 9.6	77.2 - 170.1	38.3 - 70.3
150 °C - 20 min – 0.17%	45.9 - 67.2	37.3 - 51.3	16.9 – 24.9	5.7 – 11.2	86.5 - 178.6	42.2 - 73.8
140 °C - 20 min – 1.33%	72.7 – 100.8	64.5 - 75.3	120.7 – 157.6	6.6 - 9.7	135.8 - 223.5	69.4 - 92.3
175 °C - 10 min – 0.17%	89.9 - 124.7	82.3 - 94.2	260.4 – 382.9	24.5 – 35.0	159.0 - 222.1	82.2 - 91.8
180 °C - 10 min – 0.17%	66.5 – 108.7	54.7 - 81.2	703.6 – 863.5	59.2 – 81.4	163.9 - 227.5	82.5 - 98.5

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Cell wall diversity in forage maize: genetic complexity and bioenergy potential

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Abstract

Genetic studies are ideal platforms for assessing the extent of genetic diversity, inferring the genetic architecture and evaluating complex trait inter-relations for cell wall compositional and bioconversion traits relevant to bioenergy applications. Through the exhaustive characterization of a forage maize doubled haploid (DH) population, we have revealed the vast degree of highly heritable (h^2>~65%) diversity in cell wall composition, polymeric ultrastructure and bioconversion potential available within this model grass species. In addition to variation in lignin content, extensive genotypic diversity was found for the concentration and composition of hemicelluloses; the latter found to exert an influence on the recalcitrancy of maize cell walls. Our results also demonstrate that forage maize harbors considerable variation for the release of cell wall glucose following pretreatment and enzymatic saccharification. In fact, the extent of variability observed for bioconversion efficiency (nearly 30% between population extremes) greatly exceeded ranges reported in previous studies. In our population, genotypic diversity for cell wall composition and quality was found to be controlled by 52 quantitative trait loci (QTLs). Noteworthy, from 8 QTLs regulating bioconversion properties, 5 were previously unidentified and warrant further investigation. Ultimately, our results substantiate forage maize germplasm as a valid genetic resource for advancing cell wall degradability traits in bioenergy maize breeding programs. However, since useful variation for cell wall traits is defined by QTLs with "minor" effects (R^2=~10%), cultivar development for bio-based applications will rely on advanced marker-assisted selection procedures centered on detecting and increasing the frequency of favorable QTL alleles in elite germplasm.

Keywords: Maize, cell wall composition, QTL, biofuel, pretreatment, saccharification

Introduction

Despite gaining prominence in scientific spheres and political agendas worldwide, the production of cellulosic ethanol from plant biomass is yet to achieve an economic stronghold in the renewable-energy sector [1, 2]. Presently, the greatest challenge to its wide-scale commercialization is technical [3]. During the production of cellulosic ethanol, the polysaccharide fraction of plant biomass is enzymatically deconstructed into monomeric sugars which are later fermented into hydrous fuel. Notwithstanding, plants have evolved to resist enzymatic breakdown and a thermo-chemical pretreatment is typically employed to increase the accessibility of biomass polysaccharides to hydrolytic enzymes [4]. This accessory procedure maximizes fermentable sugar yields, but greatly reduces the energetic, environmental and economic performance of the industry [3-5].

To reduce ethanol production costs, research in the field has prioritized advances in the techno-economic efficiency of biomass processing technologies [3, 4, 6, 7]. A second, but equally valid strategy entails the development of advanced bioenergy feedstocks with improved processing amenability [3, 5, 8, 9]. At its core, this approach requires an in-depth understanding of the composition, structure and synthesis of the plant cell wall; the principle constituent of lignocellulosic biomass. The plant cell wall is a complex biocomposite stemming from the functional interaction of cellulose, hemicellulose and lignin, as well as other minor aromatic compounds, pectins and structural proteins [10-12]. This biological matrix delineates the physical characteristics of individual cells (i.e. shape and size) and ultimately determines plant morphology, size and fitness [5, 10]. Extensive evidence also suggests, however, that the composition and structure of the plant cell wall greatly influence the effective fractionation of plant biomass into fermentable sugars [8, 13-19]. Ultimately, the challenge of bioenergy crop breeding programs lies on identifying and modifying key cell wall compositional features that can reduce lignocellulose recalcitrance without sacrificing plant vigor or its suitability for cultivation.

Warranted to become the first large-scale lignocellulosic feedstock, maize is also an excellent model for studying complex cell wall characteristics and optimizing crop improvement strategies in bioenergy grasses (i.e. miscanthus, sugarcane, switchgrass, etc.) [5, 12, 20, 21]. In particular, quantitative genetic studies in maize are ideal platforms for assessing the extent of genetic diversity and inferring inheritance patterns controlling cell wall composition, structure and degradability properties for bioenergy production. To date, however, few studies have described the underlying genetic basis and extent of heritable variation in maize cell wall traits relevant to the cellulosic ethanol industry [17, 22, 23]. And while highly valuable, these studies have been limited to the evaluation of grain-dedicated germplasm presumably

displaying moderate genetic variation for cell wall composition and degradability traits.

Contrary to grain-dedicated variants, genetic improvement in forage maize has been historically focused towards advancing biomass yield and stover ruminal digestibility [23, 24]. In fact, forage maize potentially conceals an unexploited wealth of genetic variation for cell wall compositional characters of beneficial value for bio-based industrial applications [8, 23, 25-32]. In this respect, forage maize constitutes an attractive genetic resource for investigating how cell wall composition can be harnessed to improve the enzymatic depolymerization of plant biomass for cellulosic ethanol production. In this study, our objectives were to i) quantify the extent of heritable variation for cell wall composition and degradability traits potentially available in forage maize, and to ii) dissect the interrelationships and underlying genetic architecture of cell wall characteristics relevant to cellulosic ethanol production.

Materials and methods

Plant material

A maize population of doubled haploids (DHs) was generated by Limagrain Nederland B.V. (Rilland, Netherlands) following a cross between their flint proprietary inbred lines Lim-531 and Lim-789 (henceforth referred to as P1 and P2); both highly contrasting in forage quality and cell wall digestibility traits [8]. A total of 230 DH genotypes and their parental inbred lines were sown in replicate, at Wouw, The Netherlands, in adjacent completely randomized blocks during the summer of 2009. Genotypes were planted in two-row plots with a length of 2.5 m and an inter-row distance of 0.75 m at a density of 10 plants m^{-1}. For each plot, stalks of 10 randomly selected plants were harvested at a 10 cm stubble height just prior to silage maturity (approximately 7 weeks after the population's mean silking period). At this physiological stage, differences between genotypes in stem cell wall composition and digestibility were expected to be largely genetic [33-35]. Due to the intensive workload, replicate blocks were harvested separately on consecutive days. Collected biomass feedstocks were chopped and air dried at 70 ^{0}C for 48 hours, and were subsequently ground through a 1-mm screen using a hammer mill.

Compositional analysis

Stem detergent fiber composition

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) components were determined by means of the ANKOM filter bag method (ANKOM Technology Corporation, Fairpoint, NY), which essentially derives from the work of Goering and Van Soest [36]. All analyses were performed in duplicate and were carried out using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY). Stem cellulose (Cel), hemicellulose (Hem) and acid-insoluble lignin (ADL) contents were derived from detergent fiber data as described in Table 1.

Cell wall composition

Water un-extractable solids – used for cell wall compositional analyses- were prepared by sequentially de-starching biomass feedstocks (1.5 hrs, ~100 °C) with 0.5 mL heat-stable α-amylase (ANKOM Technology Corporation, Fairpoint, NY) and repeatedly washing them with hot water (5x, ~70 °C). The enzyme load provided ~18,000 liquefon units (LU) per gram of dry biomass. All extractions were performed using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY).

Cell wall neutral sugar components were determined by gas chromatography essen-

Table 1. Description of cell wall compositional and bioconversion characters analyzed within the framework of this study

Trait	Unit	Description
CW	g Kg^{-1} DM	Stem cell wall content; determined as neutral detergent fiber (NDF)
Cel	g Kg^{-1} DM	Stem cellulose content; determined as the difference between acid detergent fiber (ADF) and acid insoluble lignin (ADL)
Hem	g Kg^{-1} DM	Stem hemicellulose content; determined as the difference between NDF and ADF
Lig	g Kg^{-1} DM	Stem acid insoluble lignin content; determined as ADL
Cel/CW	g Kg^{-1} CW	Stem cellulose content relative to cell wall content (CW)
Hem/CW	g Kg^{-1} CW	Stem hemicellulose content relative to cell wall content (CW)
Lig/CW	g Kg^{-1} CW	Stem acid insoluble lignin content relative to cell wall content (CW)
Gluc	g Kg^{-1} CW	Cell wall glucose content
Xyl	g Kg^{-1} CW	Cell wall xylose content
Ara	g Kg^{-1} CW	Cell wall arabinose content
Gal	g Kg^{-1} CW	Cell wall galactose content
pCa	g Kg^{-1} CW	Cell wall esterified p-coumaric acid content
DHS	%	Degree of hemicellulose substitution, expressed as the ratio of cell wall arabinose to cell wall xylose (Ara/Xyl)
CWD	% CW	In-vitro cell wall digestibility; determined as the difference in NDF content before and after sample incubation in rumen liquor for 48 hours relative to NDF content prior to incubation.
Gluc-Rel	g Kg^{-1} DM	Amount of glucose released from one gram of dry biomass after pretreatment and enzymatic saccharification.
Gluc-Con	% CW Glucose	Percentage of total cell wall glucose released after pretreatment and enzymatic saccharification.

tially as described by Englyst and Cummings [37]. Briefly, lyophilized water un-extractable solids were first treated with 72% sulphuric acid (1 hr, 30 °C), followed by a second hydrolysis process with 1% sulphuric acid (3 hrs, 100 °C). Released neutral sugars were then reduced with 1.5% sodium tetrahydroborate solution and derivatized to their respective alditol isoforms using acetic anhydride as the acetylating agent, and N-methylimidazole as a reaction catalyst. The derivatized products were quantified on an Agilent 7890A Gas Chromatography System (Agilent Technologies, Santa Clara, CA) using a DB-250 column (Agilent Technologies, Santa Clara, CA).

Cell wall p-coumarate (pCA) esters were determined by high-pressure liquid chromatography (HPLC). In essence, water un-extractable solids (50 mg) were incubated in 5 mL 2M NaOH at 39 °C for 24 hrs in the dark. After incubation, the mixture was centrifuged (3500 RPM, 5 min) and the resulting supernatant was collected and acidified to pH 2.0 using concentrated HCl. Subsequently, cell wall phenolics were extracted from the acidified sample (5 mL) using ethyl acetate (2x, 5 mL). The ethyl acetate extract was then evaporated and the remnants were re-suspended in 80% aqueous methanol. Identification and quantification of p-coumaric esters was performed using a Waters HPLC-PDA system (Waters Associates, Milford, MA) equipped with a HyPurity C_{18} (3μm, 150mm x 3mm) column (Thermo Electron Corporation, Bellefonte, PA).

Bioconversion efficiency

Thermochemical pretreatment and enzymatic conversion efficiency

Biomass samples (500 mg) were pretreated at a 30% solids loading in 0.17% (w/v) sulfuric acid for 30 min at 140°C, essentially as described by Torres *et al.* [8]. Enzymatic saccharification of pretreated samples was performed following a modified version of the Laboratory Analytical Procedure-009 [38] from the National Renewable Energy Laboratory (NREL). In essence, pretreated samples were treated with 250 μL of an Accelerase 1500 cellulolytic enzyme cocktail (Genencor B.V., Leiden, NL) in 40 mL 0.1 M citrate buffer. The enzyme load provided 50 filter paper units (FPU) of cellulase per gram cellulose. Samples were subsequently incubated at 50 °C in an Innova 42 air incubator (New Brunswick Scientific, Enfield, CT) at 200 RPM for 24 hrs. Enzymatic saccharification liquors were analyzed for glucose content using a Boehringer Mannheim D-Glucose kit (Boehringer Mannheim, Indianapolis, IN, USA). The colorimetric assay was adapted to a 96 micro-titer plate format, and spectrophotometric reads were made using a Bio-Rad 550 Micro-plate Reader (Bio-Rad, Richmond, CA). For all samples, glucose content was expressed as both, the amount of glucose released from one gram of dry biomass (Glu-Rel) and the percentage of total cell wall glucose released upon enzymatic saccharification (Glu-Con) (Table 1).

Cell wall digestibility

In-vitro cell wall digestibility was determined as the difference in neutral detergent fiber (NDF) content before and after sample incubation in rumen liquor for 48 hrs; expressed as a proportion of NDF content prior to incubation. In-vitro rumen assays were performed in duplicate by BLGG Agroxpertus B.V. (Wageningen, The Netherlands). In this study, *in-vitro* cell wall digestibility was used as a comparative marker for lignocellulose degradability.

Statistical Analyses

General analysis of variance (ANOVA) was used to determine the significance of genotypic differences in stem fiber and cell wall components, as well as bioconversion parameters. From these analyses, estimates of genotypic and phenotypic variances were used to calculate trait narrow sense heritability (h^2) estimates. Coefficients of variation over genotype means (CV_G) were also calculated for all evaluated traits and were used as standardized measures of genotypic variation.

Inter-relationships between cell wall compositional and degradability traits were analyzed by means of Pearson correlations. In addition, path analysis was employed to estimate the direct and indirect effects of cell wall compositional features on bioconversion traits. Path analysis is an extension of linear regression analysis which allows for the disambiguation of associations between sets of interrelated dependent and response variables [39]. For every dependent variable, a path coefficient (*p*) –provided as a standardized partial regression coefficient- indicates its direct contribution in the model. All statistical analyses were performed using the GenStat for Windows 14th Edition Software Package (VSN International, Hemel Hempstead, UK).

Molecular mapping and QTL analyses

DNA isolation and genotyping for the entire population were performed by Limagrain France S.A. using a proprietary SNP array. A total of 684 non-redundant SNP markers and 163 DH genotypes were used for linkage map construction and QTL mapping. Linkage analysis was performed using JoinMap 4.1 (Kyazma, Wageningen, NL). A total of 10 unambiguous linkage groups were produced with a minimum LOD score of 3.0. SNP markers of public domain were used to anchor the generated linkage map to the publically available IBM high resolution genetic map [39], making it possible to identify chromosomes and delimit chromosomal bin positions.

QTL mapping was carried out using the Restricted MQM mapping function of MapQTL 6.0 (Kyazma, Wageningen, NL). Cofactor selection was performed by se-

lecting an initial set of markers in the vicinity of identified QTLs and testing them for significance with the automatic cofactor selection option provided in the software. For any given trait, no more than 4 cofactors were selected for QTL analysis. To declare the presence of a QTL, 10,000 permutations were performed for each trait to determine genome-wide significance at $\alpha=0.05$. For all traits analyzed, a LOD threshold of 3.0 was sufficient to detect a significant QTL.

Results

Maize cell wall compositional diversity

We have evaluated a forage maize DH population for a wide selection of biomass compositional and bioconversion characters. For all traits analyzed, parental values, population statistics and narrow-sense heritability estimates (h^2) are presented in Table 2. Overall, the DH progeny displayed considerable genetic variation ($p<0.05$) for all stem fiber and cell wall compositional characters; especially for phenolic (Lig, Lig/CW, pCa) and hemicellulosic (Hem/CW, Ara, DHS) traits. Phenotypic observations were highly reproducible under our field conditions and trait means were consistent with values reported by others [17, 31, 40]. Correspondingly, narrow-sense heritability estimates (h^2) were high (66%< h^2< 89%), except for Glu and Xyl (h^2 = 24% and 28%, respectively).

Table 2. Statistics of the DH population for maize biomass compositional and bioconversion characters relevant to cellulosic ethanol production.

| Trait | Parents | | | | Population | | |
	P1	P2	Mean	Range	LSD (0.05)	CV$_G$	Heritability (h^2)
CW $_{(g\,Kg^{-1}\,DM)}$	426	456	458	367 - 615	26	10%	92%
Cel $_{(g\,Kg^{-1}\,DM)}$	252	262	265	200 - 366	20	11%	89%
Hem $_{(g\,Kg^{-1}\,DM)}$	153	162	165	121 - 252	16	12%	82%
Lig $_{(g\,Kg^{-1}\,DM)}$	21.0	32.3	27.8	14.5 - 45.3	4.2	19%	84%
Cel/CW $_{(g\,Kg^{-1}\,CW)}$	593	574	578	518 - 651	26	4%	58%
Hem/CW $_{(g\,Kg^{-1}\,CW)}$	358	355	361	274 - 454	30	7%	61%
Lig/CW $_{(g\,Kg^{-1}\,CW)}$	49.5	71.0	60.7	27.6 - 91.7	8.9	17%	82%
Glu $_{(g\,Kg^{-1}\,CW)}$	466	454	463	425 - 516	27	4%	28%
Xyl $_{(g\,Kg^{-1}\,CW)}$	291	276	270	231 - 306	20	4%	24%
Ara $_{(g\,Kg^{-1}\,CW)}$	45.8	36.6	40.5	31.6 - 51.2	4.3	10%	72%
Gal $_{(g\,Kg^{-1}\,CW)}$	14.2	11.9	12.5	7.6 - 19.4	1.9	14%	68%
pCa $_{(g\,Kg^{-1}\,CW)}$	25.0	32.6	27.5	20.5 – 33.0	2.3	10%	80%
DHS $_{(Ara/Xyl\%)}$	15.7	13.3	15.0	11.7 – 19.0	1.6	10%	71%
CWD $_{(\%\,CW)}$	54	46	51	38.5 - 65.2	4	8%	82%
Glu-Rel $_{(g\,Kg^{-1}\,DM)}$	99.9	78.7	91.2	68.7 - 150.7	10.5	13%	80%
Glu-Con $_{(\%\,CW\,Glucose)}$	50	37	43	31.8 - 61.6	4	10%	73%

Frequency distributions across all traits were reasonably uniform (Figure 1), although exceptional extremes were not uncommon. In all cases, segregation patterns were transgressive, with trait ranges (min-max) consistently exceeding parental values and typically accounting for over 50% of the population mean. For several traits, however, the extent of phenotypic transgression outside parental boundaries was moderate in at least one direction (e.g. Lig, Lig/CW, Xyl and pCa), as parental performance (P1, P2) leaned closer to progeny extremes (Table 2, Figure 1). In other words, for these traits, genetic gains beyond parental values are expected to be modest within the population.
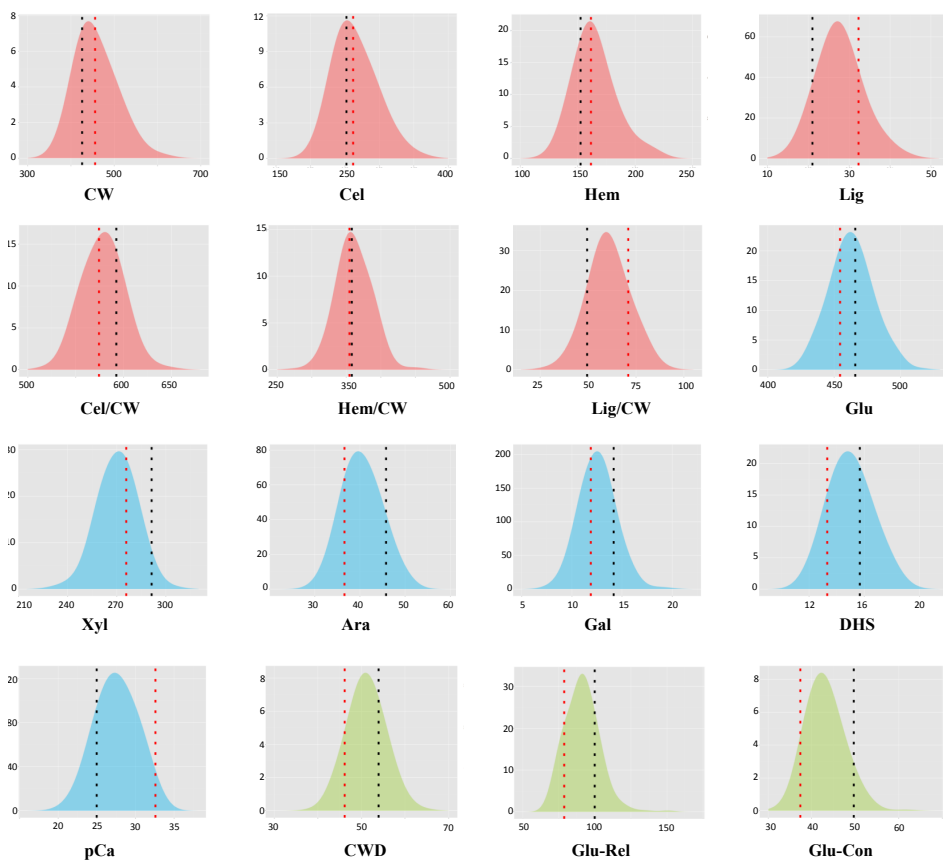
Figure 1. Frequency distribution for all biomass compositional and bioconversion traits analyzed on a forage maize DH population. Dotted arrows show the performance of Parent 1 (P1, black) and Parent 2 (P2, red) relative to the population.

Amongst all cell wall components, Hem, Lig/CW and Lig displayed the highest levels of genetic diversity ($CV_G > 12\%$). Maximal differences between DH lines for these traits accounted for 80%, 106% and 111% of the corresponding population mean. Substantial variation was also observed ($CV_G \sim 10\%$) for DHS and pCa. By contrast, Cel/CW, Glu and Xyl displayed low levels of genotypic variability ($CV_G=4\%$; in all cases). As anticipated, in addition to biomass compositional diversity, extensive heritable variation among lines was also found for bioconversion characters. Maximal differences between genotypes for CWD, Gluc-Rel and Glu-Con accounted for 50%, 80% and 90% of their corresponding trait mean.

Finally, principle components analysis reveals that biomass compositional diversity across the DH population could be largely resolved through two composite variables (Figure 2). The first component (PC 1), which summarized 37% of all variation among DH lines was dominated by lignin (Lig, Lig/CW, pCa) and hemicellulosic (Hem, Ara, Gal, DHS) characters. The second component (PC 2), accounting for 21% of observed variation, resolved genotypes based on differences in stem cell wall and polysaccharide accumulation characteristics (CW, Cel, Hem).

Inter-relationships between stem, cell wall and bioconversion characters

Correlation patterns between biomass compositional characters varied broadly and offered insightful perspectives on the plasticity of the maize cell wall (Figure 3). On a dry matter basis, stem fiber parameters (CW, Cel, Hem, Lig) displayed moderate-to-strong positive relations amongst each other ($r > \sim 0.45$); except between Hem and Lig. On a cell wall basis, Cel/CW displayed a strong negative association ($r = -0.90$) to Hem/CW, and also correlated negatively ($r \sim -0.35$) to Ara, Gal and DHS. All hemicellulosic characters (Hem/CW, Ara, Gal and DHS) correlated positively and strongly between each other ($0.60 \leq r \leq 0.90$), but associated negatively ($-0.27 \leq r \leq -0.56$) to CW, Cel, Lig, Lig/CW and pCa. By contrast, Lig/CW showed a positive correlation with pCa ($r = 0.34$), but its associations with cellulosic traits (Cel, Cel/CW and Glu) were not significant.

Correlation analyses were also performed to dissect the underlying relationships between stem bioconversion properties and biomass compositional characters. CWD and Glu-Con displayed remarkably similar correlation patterns; both associating positively and strongly ($0.50 \leq r \leq 0.65$) with hemicellulosic characters (Hem/CW, Ara, Gal and DHS), and negatively ($-0.30 \geq r \geq 0.65$) to Lig, Lig/CW, Cel/CW and pCa. Glu-Rel also exhibited a negative association ($r < -0.53$) to Lig/CW, but correlated positively ($r > 0.60$) with CW, Cel and Hem.
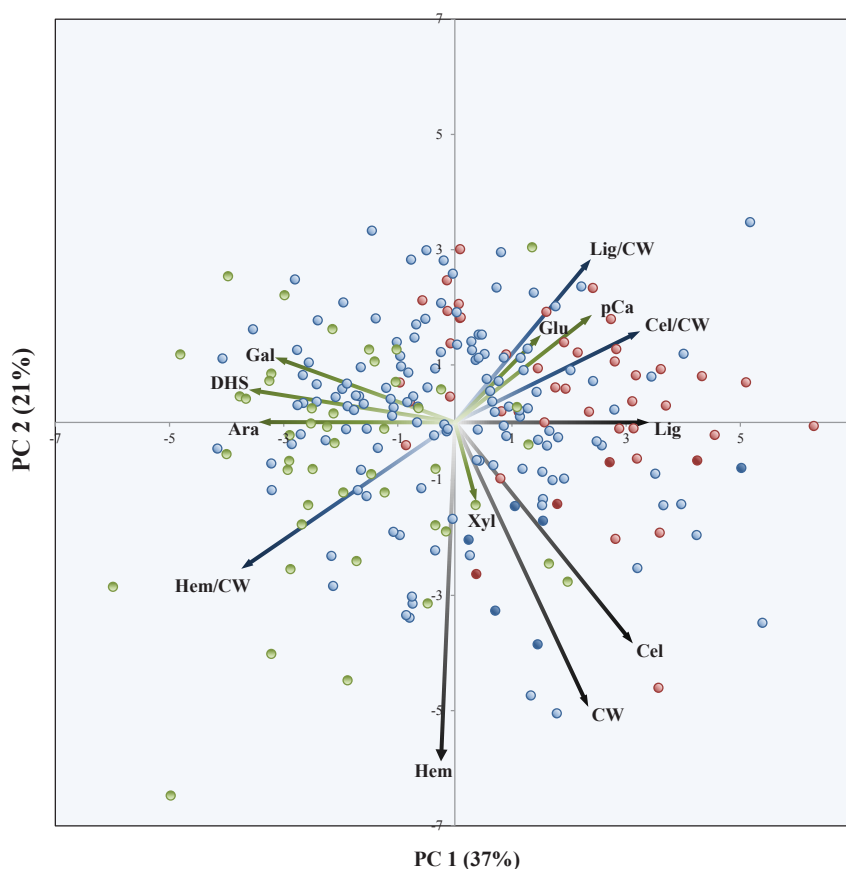
Figure 2. Principle components biplot displaying the compositional diversity of a forage maize DH population for stem fiber and cell wall traits relevant to the cellulosic ethanol industry. Length and direction of a vector are indicative of the contribution of a biomass compositional character to the corresponding principal component.

Path analysis was employed to determine the precise influence of specific biomass components on bioconversion properties, after accounting for the interrelatedness between all compositional characters correlating to a given bioconversion trait (Figure 4, Figure 5). Congruent with observed phenotypic correlations, the best-fitting models for CWD and Glu-Con shared a similar set of component variables (i.e. Lig/CW, pCa and DHS) and could explain, respectively, 64% and 58% of observed variation among DH lines. Path coefficients (*p*) reveal, however, that the influence of each explanatory variable diverges for both bioconversion traits. Whereas for CWD, Lig/CW is the strongest regressor; for Glu-Con, both Lig/CW and DHS display prominent effects. For Glu-Rel, two models (each accounting for over 70% of observed variation) were selected; one incorporating CW and CWD as explanatory variables and a

second one incorporating CW, Lig/CW, DHS, Glu and pCa (Figure 5). In both cases, the most prominent regressor was CW, but the strong influence exerted by all other variables clearly demonstrates that the release of glucose following pretreatment and enzymatic conversion is also dependent on compositional characters influencing "degradability" properties.

	Glu-Rel	Glu-Con	Cel-Con	CW	Cel	Hem	Lig	Cel/CW	Hem/CW	Lig/CW	Glu	Xyl	Ara	Gal	pCa	DHS	
	0.5	0.7	0.7	-0.1	-0.2	0.3	-0.7	-0.3	0.6	-0.7	0.1	0	0.5	0.4	-0.5	0.5	CWD
		0.6	0.6	0.7	0.6	0.7	-0.1	0.1	0.2	-0.5	0.2	0.1	0.2	0	-0.2	0.2	Glu-Rel
			0.9	-0.2	-0.2	0.2	-0.6	-0.3	0.5	-0.6	-0.2	-0.1	0.6	0.4	-0.4	0.6	Glu-Con
				-0.2	-0.3	0.2	-0.6	-0.4	0.6	-0.6	0	-0.1	0.6	0.5	-0.4	0.7	Cel-Con
					0.9	0.8	0.4	0.2	-0.1	-0.1	0	0.2	-0.3	-0.4	0.1	-0.4	CW
						0.6	0.5	0.5	-0.4	0	0.2	0.2	-0.3	-0.5	0.2	-0.4	Cel
							0	-0.4	0.5	-0.4	-0.3	0.1	0	-0.1	-0.2	0	Hem
								0.3	-0.6	0.8	0	0	-0.5	-0.4	0.3	-0.5	Lig
									-0.9	0.2	0.6	0.2	-0.3	-0.4	0.3	-0.4	Cel/CW
										-0.6	-0.5	-0.1	0.4	0.4	-0.3	0.5	Hem/CW
											0	-0.1	-0.4	-0.2	0.3	-0.3	Lig/CW
												0.1	0	-0.1	0.1	-0.1	Glu
													0.2	0.1	-0.1	-0.2	Xyl
														0.7	-0.4	0.9	Ara
															-0.3	0.6	Gal
																-0.4	pCa

Scale legend: 1, 0.8, 0.6, 0.4, 0.2, 0, -0.2, -0.4, -0.6, -0.8, -1

Figure 3. Heat-map displaying the extent and direction of correlations (r) -over genotypic means- between maize compositional characters and cell wall bioconversion properties. Correlations were statistically significant at $r \geq 0.25$ and $r \leq -0.25$. Blue colors signal positive correlations and red colors indicate negative associations.
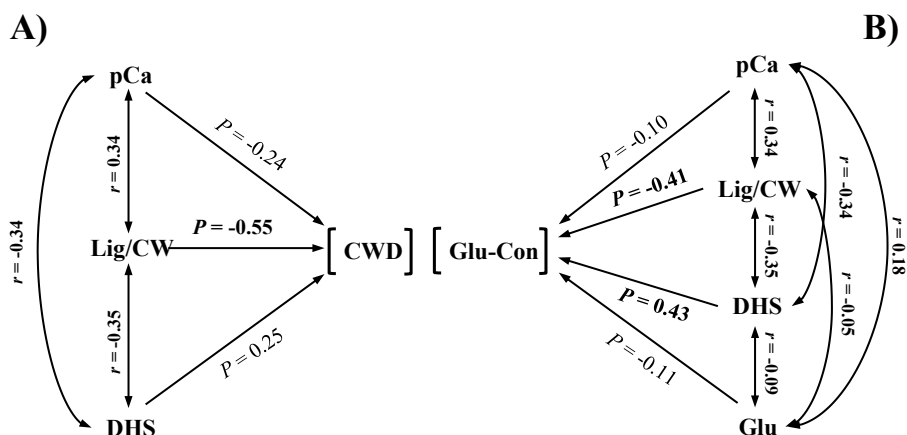
Figure 4. Schematic representation of correlation patterns and path coefficients between biomass compositional characters and (A) CWD, or (B) Gluc-Con. Double-arrowed lines indicate mutual associations as measured by Pearson correlation coefficients (r), and single-arrowed lines represent direct influences as measured by path coefficients (p).
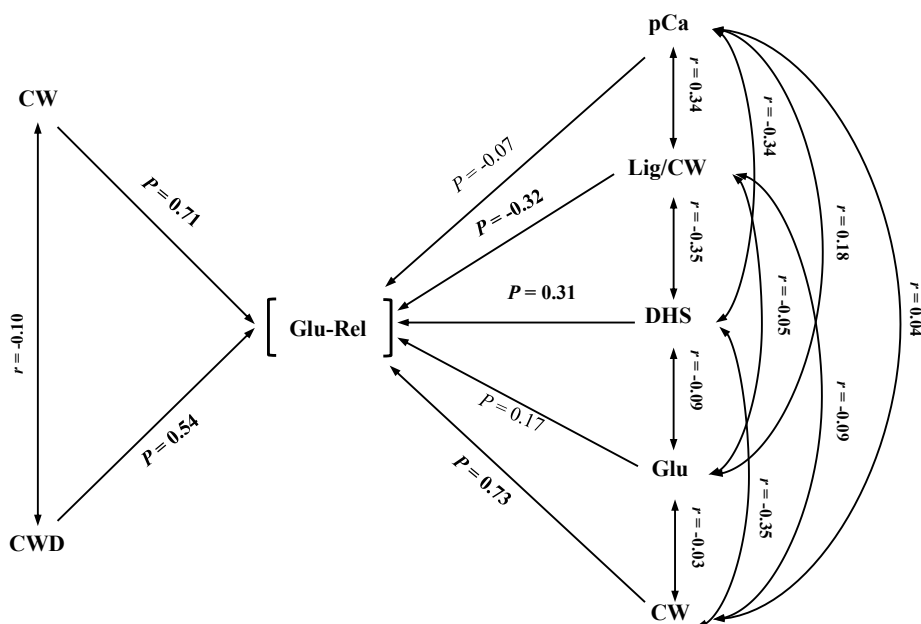
Figure 5. Schematic representation of correlation patterns and path coefficients for biomass compositional characters and Gluc-Rel. Two best-fitting models were produced; the first one including CW and CWD as explanatory variables (left-hand side), and a second model including CW, Lig/CW, DHS, pCa and Glu (right-hand side). Double-arrowed lines indicate mutual associations as measured by Pearson correlation coefficients (r), and single-arrowed lines represent direct influences as measured by path coefficients (p). The models accounted for 71% and 75% of variance observed across the population, respectively.

Quantitative trait loci (QTL) mapping

QTL analysis was performed to elucidate the genetic architecture of cell wall characteristics relevant to cellulosic fuel production. A total of 52 QTLs were detected for biomass compositional and bioconversion characters across 8 chromosomes (Table 3). Overall, between two to seven QTLs were identified for all traits evaluated; except for Glu and Gal, for which no significant QTLs were detected (Figure 6). On average, the 1-LOD and 2-LOD support intervals for all identified QTLs spanned 11.4 and 23.2 cM. QTL explained variances (R^2) ranged from 4.2% to 15.9%, but in the majority of cases accounted for around 10% of variation observed among DH lines. For all traits analyzed, both parents contributed favorable alleles to the mapping population; except for pCa, Glu-Rel and Glu-Con, for which all favorable alleles originated from Parent 1 (P1; Table 3).

At 12 chromosomal regions, the 2-LOD support confidence intervals of QTLs regulating different cell wall characteristics overlapped (Figure 7). In general, multi-trait QTL co-localizations were in good agreement with observed correlation patterns (Figure 3). For instance, QTLs for Lig/CW and CWD co-localized on Chromosomes 1 and 4, thereby underpinning the strong negative association (r>-0.71) found between these two characters. Other interesting multi-trait co-localizations include the positional coincidence of QTLs for lignin (Lig, Lig/CW) and hemicellulose (Hem, Hem/CW, Ara, DHS) on Chromosomes 1, 3, 4 and 10; the clustering of QTLs for Glu-Con with lignin (Lig, Lig/CW) and hemicellulose-related (Ara, DHS) QTLs on Chromosomes 1 (two separate regions) and 7; and the co-localization of QTLs for Glu-Rel and cell wall degradability traits (CWD and Glu-Con) on Chromosomes 1, 4 and 5.

With relevance to bioenergy maize breeding endeavors, 13 QTLs contributing to variation for cell wall degradability and bioconversion traits (i.e. CWD, Glu-Rel, Glu-Con) have been identified; 5 of which were exclusive to our mapping population (Table S1). From the latter, one QTL corresponded to CWD (QTL# 41), and two each were identified for Glu-Rel (QTL# 47 and 48) and Glu-Con (QTL# 49 and 51). Noteworthy, these novel QTLs co-localized (based on bin positions) with QTLs for related traits detected in other studies (Table S1). Essentially, the QTL for CWD (QTL#41) co-localized with a QTL detected for saccharification efficiency and all QTLs for Glu-Rel (QTLs#47 and 48) and Glu-Con (QTLs# 49 and 51) coincided with QTLs identified for ruminal cell wall digestibility features.

Table 3. Parameters for putative quantitative trait loci (QTL) influencing cell wall composition and bioconversion properties in a maize DH population

QTL#	Trait	Chr.	Bin	Position (cM)	1-LOD Support Interval (cM)	2-LOD Support Interval (cM)	LOD	Additive Value (P1)	R^2 (%)
1	CW (g kg-1 DM)	4	4.05	55	48-59	44-64	4.0	-14.8	9.5
2		4	4.09	121	116-124	106-129	4.8	15.8	11.3
3		5	5.09	2	0-6	0-8	4.5	-14.8	9.5
4	Cel (g kg-1 DM)	4	4.04	51	47-61	44-66	4.8	-10.3	10.4
5		4	4.09	121	112-129	101-151	3.3	8.3	7.2
6		5	5.09	2	0-6	0-12	3.4	-8.1	6.7
7		6	6.07	98	91-110	89-113	4.9	9.8	9.6
8	Hem (g kg-1 DM)	4	4.09	122	113-114	103-127	5.4	7.2	12.9
9		5	5.09	2	0-7	0-10	3.4	-5.6	7.9
10	Lig (g kg-1 DM)	1	1.05	103	96-107	90-110	3.6	-1.3	5.4
11		1	1.06	120	113-125	110-132	3.9	-1.3	5.8
12		3	3.04	54	53-56	48-59	7.6	-1.9	11.9
13		4	4.06	72	63-75	59-79	5.0	-1.6	7.6
14		5	5.06	50	46-56	45-60	7.3	-1.9	11.3
15		9	9.02	59	57-63	45-72	3.5	1.3	5.2
16		10	10.06	39	30-43	23-47	3.1	1.2	4.5
17	Cel/CW (g kg-1 CW)	4	4.05	59	55-70	46-79	4.1	-6.2	8.3
18		6	6.07	111	93-114	85-114	3.0	5.4	6.4
19	Hem/CW (g kg-1 CW)	3	3.03	41	33-47	29-57	4.4	7.5	8.9
20		4	4.05	64	56-72	49-81	5.4	9.1	11.1
21		6	6.07	99	85-104	81-114	3.8	-7.5	8.3
22	Lig/CW (g kg-1 CW)	1	1.11	185	181-187	176-192	4.6	2.9	6.6
23		3	3.04	57	54-59	34-69	5.6	-3.0	4.9
24		4	4.08	110	106-112	104-113	10.9	-4.5	15.9
25		5	5.05	54	49-62	48-70	9.3	-4.2	13
26		7	7.05	106	100-115	89-119	3.2	-2.2	4.2

Table 3. Parameters for putative quantitative trait loci (QTL) influencing cell wall composition and bioconversion properties in a maize DH population

QTL#	Trait	Chr.	Bin	Position (cM)	1-LOD Support Interval (cM)	2-LOD Support Interval (cM)	LOD	Additive Value (P1)	R² (%)
27	Xyl (g Kg-1 CW)	4	4.09	128	124-139	117-148	3.2	3.1	7.6
28		5	5.07	21	20-27	19-33	4.9	-3.9	12.1
29	Ara (g Kg-1 CW)	1	1.03	50	42-55	35-57	3.3	1.1	6.7
30		1	1.05	105	100-110	96-116	5.0	1.3	9.7
31		3	3.03	43	39-48	36-49	5.4	1.4	10.7
32		4	4.06	79	60-85	54-91	3.0	1.0	5.8
33		10	10.06	36	31-40	26-43	3.1	-1.1	6
34	pCa (g Kg-1 CW)	1	1.05	97	94-100	92-108	5.4	-0.9	12.3
35		3	3.09	150	146-150	140-150	3.6	-0.7	7.2
36	DHS (Ara/Xyl%)	1	1.03	74	59-85	42-85	4.2	0.5	8.8
37		1	1.05	102	98-107	96-110	5.8	0.5	11.9
38		3	3.03	42	30-49	26-50	3.5	0.4	6.4
39		10	10.06	36	30-41	27-47	4.4	-0.4	8.2
40	CWD (% CW)	1	1.05	97	93-108	86-110	5.4	1.5	11.4
41		1	1.06	120	117-123	111-124	5.6	1.5	11.8
42		1	1.11	185	180-188	178-193	5.8	-1.5	12.3
43		4	4.08	111	103-121	91-123	6.5	1.5	11.1
44		10	10.06	37	31-41	25-43	5.2	-1.3	8.7
45		10	10.02	78	73-82	68-87	3.6	-1.1	6.1
46	Glu-Rel (g Kg-1 DM)	1	1.03	72	68-90	49-100	3.1	3.3	6.5
47		4	4.08	110	105-117	102-123	6.1	4.7	13.5
48		5	5.05	70	68-74	56-94	3.8	3.7	8.2
49	Glu-Con (% CW Glucose)	1	1.03	72	69-76	63-87	3.2	1.2	7.3
50		1	1.06	112	110-121	96-123	4.4	1.3	9.8
51		5	5.04	87	84-94	80-98	5.8	1.5	12.5
52		7	7.05	106	99-110	93-117	3.2	1.1	6.6
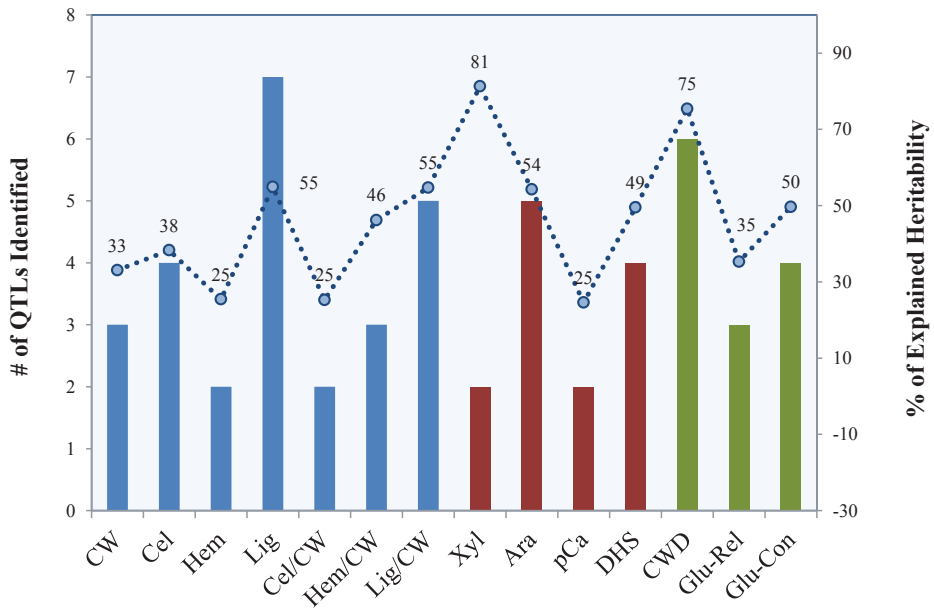
Figure 6. Survey of QTLs identified for biomass compositional and bioconversion characters in a forage maize DH population. Colored bars indicate total number of QTLs identified per trait. Circular points refer to the cumulative explained variance of all identified QTLs for a given trait, as a proportion of observed heritable variation (h^2).
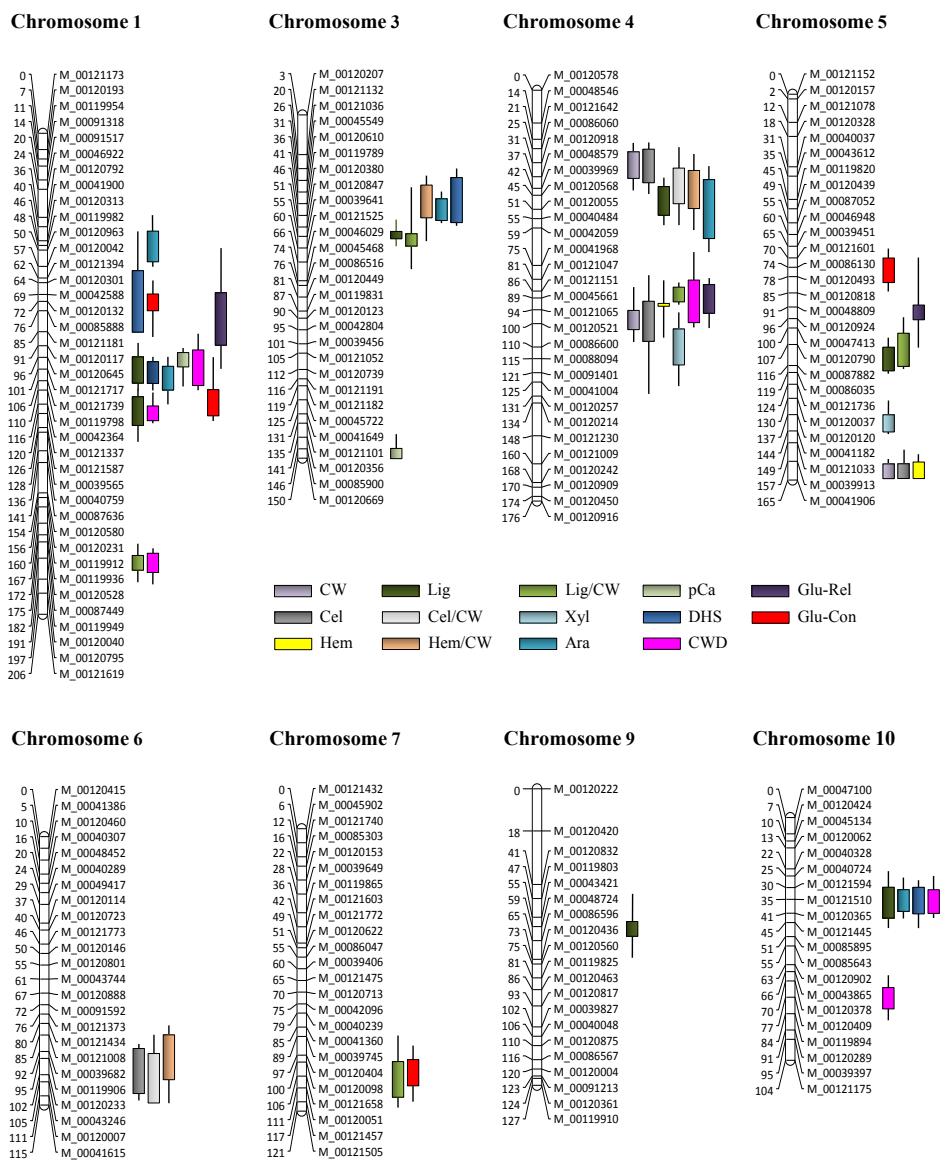
Figure 7. Distribution of QTLs identified for biomass compositional and bioconversion characters across 8 chromosomes of a forage maize DH population. For every QTL, colored boxes indicate 1-LOD support intervals while extension bars delimit 2-LOD support intervals

Discussion

The maize cell wall is a highly diverse and malleable biocomposite

Through the exhaustive characterization of a forage-maize doubled haploid (DH) population, we have revealed the vast degree of genetic diversity in cell wall concentration, composition and polymeric ultra-structure potentially available within this model grass species. In principle, our findings suggest that the maize cell wall can sustain pronounced levels of variation in its phenolic and hemicellulosic fractions, but not for its cellulosic core (Table 2).

Across the population, vast genotypic differences for lignin and p-coumarate ester concentrations were highly representative of forage maize germplasm [23, 25-29, 32, 35, 41-43]. Notwithstanding, abundant genetic variation was also observed for the content and monomeric constitution of non-cellulosic polysaccharides. Specifically, considerable differences in the absolute ratio of arabinose to xylose (DHS) might be implicative of the great diversity in glucoronoarabinoxylan (GAX) substitution patterns concealed within the population. To our understanding, the breadth of variation in maize hemicellulose compositional diversity described herein has not been reported by others. For instance, whereas the range of variation for arabinose content (Ara) spanned from 31.6-51.2 g Kg^{-1} CW across our population, in a related study, maximal differences for cell wall arabinose content were only 31.8-37.0 g Kg^{-1} CW [17].

In this study, reductions in cell wall lignin concentration were generally compensated by increments in the content of hemicellulosic polysaccharides, and vice-versa. Alongside the extant diversity reported for hemicellulosic traits, this inverse inter-relation suggests that hemicellulosic polymers can play a crucial role in maintaining the structural integrity of the plant cell wall. After all, GAX molecules cross-link to each other and to lignin via ferulate bonds, and constitute an integral element of the hydrophobic matrix that encases and protects the cellulosic fraction from enzymatic degradation [44-46]. More recently, several studies have revealed the existence of orthologous glycosyl transferases capable of producing unique xylan branching patterns in Arabidopsis [47, 48]; a finding that validates the considerable degree of genotypic differences in glucoronoarabinoxylan (GAX) substitution patterns (DHS) detected for our population. Presumably, xylans varying in their degree (and patterning) of glycosylation will exhibit unique molecular affinities, biological functions and industrial properties [8, 44-46, 48, 49]. Moreover, it should also be noted that xylan-to-xylan ferulate bridging [50] and ferulate-to-lignin cross-links [51, 52] have been shown to influence the biochemical and structural properties of the cell wall. Certainly, future research should encompass an assessment of how cell wall integrity and lignification patterns are mechanically affected by variation in hemicellulose

content, ultra-structure and degree of glycosylation/feurylation.

Ultimately, even when its construction follows a seemingly predisposed architecture, the plethora of cell wall compositional profiles found within our DH population reveals that the maize cell wall is a highly malleable biocomposite (Table 2, Figure 2). Since adverse inter-relationships between cell wall traits (e.g. phenolic and hemicellulosic content) were not necessarily strict (r>-0.56), possibilities exist to generate cell wall compositional profiles tailored to industrial needs. In particular, the extent of genetic variation found in this species for lignin-related traits (Lig/CW, pCa) and hemicellulose monomeric complexity opens new avenues for exploiting cell wall degradability traits in maize.

Inheritance patterns of cell wall polymeric profiles

Population studies are excellent tools for inferring the genetic architecture of complex cell wall characteristics in lignocellulosic crops. Previous genetic assessments of the maize cell wall have adamantly demonstrated that natural variation in cell wall content and composition is quantitatively inherited and putatively ascribed to the segregation of multiple genetic loci with minor additive effects [17, 22, 27, 32]. Consistent with these observations, all cell wall characters evaluated in this study displayed polygenic inheritance patterns (Figure 1).

Accordingly, we have identified numerous quantitative trait loci (QTLs) for relevant cell wall compositional characters; most of which individually explained around 10% of observed variation among DH lines (Table 4). These results were foreseeable, given that the construction of the maize cell wall potentially requires the synergistic action of over 1,000 genes [12, 53]; all presumably subject to intra-allelic, epistatic and environmental interactions [54, 55]. In segregating populations, this level of genetic complexity precludes the prevalence of "major" QTLs and hinders the possibility of identifying genes with exceptionally small effects [22, 54, 56, 57]. In related studies, only few QTLs with "large" R^2 values (R^2 > 15%) have been identified for cell wall composition or cell wall degradability traits. Moreover, it has been demonstrated that estimated QTL effects detected in single-cross mapping studies are commonly biased by factors such as population size, sampling independence and genetic background [56]. Given these provisions, it seems highly plausible that that the majority of QTLs underlying useful variation for cell wall characteristics have "minor" effects and are hard to detect even under favorable experimental conditions (e.g. large population size, multiple-replications). Notwithstanding, in this study, the combined genetic action of all identified QTLs for numerous cell wall traits (Lig, Lig/CW, Hem/CW, Xyl, Ara, DHS) could account for a large proportion (~50% - 80%) of observed heritable variation (Figure 6). For the purpose of bioenergy crop

breeding programs, this observation ultimately strengthens the prospects of breeding efforts and selection procedures (i.e. genomic selection or marker-assisted selection) centered on detecting and increasing the frequency of favorable QTL alleles with "small-to-moderate" effects in elite maize germplasm.

In this study, we have also identified several chromosomal regions where QTLs for different cell wall characteristics overlapped. In general, multi-trait QTL co-localizations were consistent with trait correlation patterns (Figure 3); the latter widely perceived to arise from the underlying action of closely-linked genes or pleiotropic gene action. Courtial *et al.* [42] have proposed that the simplest explanation for the positional coincidence of QTLs controlling different cell wall traits would entail the clustering of tightly-linked genes involved in independent cell wall biosynthetic routes. The authors base this assumption on studies demonstrating that QTLs with large effects and lengthy support-intervals can be fractioned into independent QTLs [58]. The presence of pleiotropic gene action influencing multiple cell wall components should not be ruled out, however [42]. For instance, in our population, the co-localization of QTLs for cell wall concentration (CW) and cell wall polysaccharide accumulation patterns (Cel, Cel/CW, Hem, Hem/CW) on Chromosomes 4, 5 and 6 could be indicative of transcription factors acting as master regulators of secondary cell wall deposition. Similarly, the positional coincidence of QTLs controlling lignin (Lig, Lig/CW) and hemicellulosic (Hem, Hem/CW, Ara, DHS) traits further stimulates a discussion as to how the deposition of these cell wall polymers is modulated, and whether such control is regulated by antagonistic gene pleiotropy. Thus far, however, the mechanisms through which specific chromosomal regions regulate multiple cell wall characteristics remains uncertain and speculative, but rapid advances in sequencing technologies and map densification strategies will expectedly open avenues for improving our understanding of these genetic phenomena [58].

Regardless of the inherent complexity governing cell wall genetics, cell wall trait heritabilities reported in this study were generally high (h^2 >0.65). Numerous genetic assessments of the maize cell wall have also reported moderate-to-high heritability estimates for cell wall traits in *per se* and test-cross examinations of recombinant inbred populations across multiple environments [17, 22, 26, 27, 32]. Collectively, these results demonstrate that effective genetic gain for cell wall compositional features is attainable through breeding and selection. Expectedly, the convergence of classical selection schemes with inexpensive genotyping, advanced biometric models, high-throughput cell wall phenotyping and double haploid (DH) production technologies can accelerate maize cultivar development and commercial release for bio-based applications [20, 54, 59, 60]. The extent of genetic gain potentially achievable for cell wall compositional characters, however, will be largely determined by the incidence and frequency of favorable alleles (influencing relevant bioconversion

properties) concealed within the program's germplasm. In this study, for example, transgressive segregation beyond parental performance for all lignin related traits (Lig, Lig/CW) was intermediate, even when the population exhibited an impressive range of variation for lignin content. This was mostly due to the fact that positive and negative alleles for lignin traits had been differentially fixed in the parents of our mapping population. Remarkably, while this further demonstrates that divergent selection for cell wall polymeric profiles is possible, it also illustrates that efficient alleles for relevant cell wall characteristics can be concealed in unexpected genetic resources. After all, even after cycles of intensive divergent selection, the parent (P2) selected for unfavorable lignin-related traits could still contribute positive alleles to the DH progeny.

Implications for breeding for the cellulosic ethanol industry

The greatest challenge for the cellulosic ethanol industry lies on reducing the chemical and energetic stringency of thermochemical pretreatments, with the aim of improving the industry's commercial and environmental performance [3]. Consequently, the selection of dedicated feedstocks that can maximize fermentable carbohydrate yields under more sustainable and cost-effective processing alternatives constitutes a major goal in bioenergy-crop breeding endeavors. Given these provisions, one of the pivotal objectives of this study was to infer the breeding potential and dissect the underlying molecular and biochemical mechanisms regulating maize cell wall degradability characters relevant to cellulosic ethanol production.

Our results demonstrate that forage maize harbors a considerable degree of heritable variation (h^2 = ~0.80) for the release of cell wall glucose following pretreatment and enzymatic conversion (Glu-Rel, Glu-Con). In fact, the extent of genetic variability observed in this study for degradability traits (approximately ~30% between population extremes for Glu-Con) greatly exceeded ranges reported for grain maize germplasm [17, 22]. As an example, following a seemingly comparable bioconversion protocol, which also included a mild dilute-acid pretreatment, Lorenzana *et al.* [17] reported a maximal difference of ~8% for cell wall glucose release across the IBM population. In the past, forage maize genetic variants have been shown to display a considerable degree of genetic variability for biomass yield, cell wall composition and cell wall degradability properties in ruminal digestion systems [5, 8, 23, 25, 26, 28, 29, 32, 34, 35, 42, 43, 61]. Our results complement these findings and empirically substantiate forage maize germplasm as a valid genetic resource for advancing cell wall degradability traits in bioenergy maize breeding programs.

Moreover, we also reinforce the notion of the plant cell wall as the basis for the genetic improvement of lignocellulose degradability. In this study, a large fraction

(~60-75%) of the variation for bioconversion traits (CWD, Glu-Con, Glu-Rel) observed across the DH population could be ascribed to genetic differences in cell wall concentration and composition. Highest fermentable glucose yields (Glu-Rel) following pretreatment and enzymatic saccharification were obtained for genotypes combining both, a high content of cell wall glucose (CW, Cel) per unit of biomass and improved cell wall degradability (CWD, Glu-Con). Correspondingly, enhanced lignocellulose degradability (CWD, Glu-Con) could be explained by reductions in cell wall lignin content (Lig/CW) and a concomitant increase in the ratio of cell wall arabinose to xylose (DHS).

In the breeding industry, the dissection of highly complex industrial traits into their underlying components offers practical, technical and commercial advantages. Specifically, trait dissection leads to the identification of phenotypic attributes which can be measured with greater precision and at a lower cost; but most importantly, these component traits typically display simpler inheritance patterns and provide additional information regarding the genetic mechanisms controlling highly polygenic traits [55]. Given the multiplicity of biomass-to-ethanol conversion routes currently under investigation, it would appear impractical to propose a maize cell wall ideotype that could meet all the demands of the growing cellulosic ethanol industry. Nevertheless, we have demonstrated that lignin content (Lig, Lig/CW) and hemicellulose composition (DHS) are highly variable, highly heritable and easily quantifiable traits exerting a pivotal influence on the physical integrity of the plant cell wall (Figure 4). Ultimately, since lignin and hemicellulosic traits crucially impact the efficient deconstruction and utilization of plant biomass under a variety of biochemical [8, 13-19] and thermochemical fuel conversion routes [62, 63], these represent relevant breeding targets for improving the conversion efficiency of lignocellulosic biomass in bioenergy maize breeding programs. In this regard, all QTLs for lignin content (Lig, Lig/CW) and hemicellulose monomeric complexity (DHS) identified in our mapping study are warranted further investigation for their potential use in "marker-assisted" breeding strategies. After all, the vast majority of these co-localized with favorable QTLs controlling related biochemical traits and/or bioconversion properties (Figure 7 and Table S1). Under the same assumption, all QTLs contributing to variation for bioconversion characters (CWD, Glu-Rel and Glu-Con), particularly those which have not been reported earlier (QTLs # 41, 47, 48, 49 and 51; refer to Table S1), could prove useful in marker-assisted selection schemes.

Finally, a cautionary conclusion must be extrapolated from the collective of our observations. While this and numerous other studies have demonstrated the inter-relationship between cell wall composition and degradability traits, much remains to be learned with regards to the latter's biochemical and genetic underpinnings. On the one hand, the fact that genetic variation for degradability characters (i.e. CWD,

Glu-Con, Glu-Rel) cannot be fully ascribed to variation in cell wall composition (Figures 4 and 5) suggests that the occurrence of obviated or unexplored stem-related parameters should be given further consideration. For instance, variation in the ratio of pith to rind tissue, the structural distribution of lignin in stem tissues and the degree of stem vascularization represent, amongst others, putative determinants of lignocellulose recalcitrance. Moreover, the underlying genetic basis of QTLs regulating variation for cell wall convertibility characters remains largely unresolved. Courtial *et al.* [42] have highlighted the need to re-examine the genetic determinants influencing maize biofuel potential, based on the fact that QTLs for cell wall digestibility traits do not regularly co-localize with candidate genes encoding structural enzymes of cell wall biosynthetic routes. In the same context, much remains to be learned with respect to the genetic action of chromosomal regions controlling multiple cell wall characteristics. In particular, this information will prove fundamental to plant breeders seeking to introgress genomic regions (QTLs) with favorable effects for cell wall degradability characters, but detrimental to agronomic fitness or yield properties. In the long run, a precise definition of breeding targets and a clear understanding of their underlying genetic mechanisms are essential to maximize the efficacy of bioenergy breeding endeavors in maize. Namely, this will facilitate the identification of the most promising factors for improving bioconversion efficiency and directing the identification of useful QTL variants that can be used in marker-assisted selection programs.

Conclusions

The results of this investigation reveal that forage maize conceals an unexploited wealth of heritable variation for cell wall compositional characters of beneficial value for bioenergy applications. Noteworthy, in addition to variation in lignin content, extensive genotypic diversity was also found for the concentration and composition of hemicelluloses; the latter playing a prominent role in the recalcitrancy of the maize cell wall. In correspondence with these findings, our DH population also displayed the highest levels of genotypic variation thus far reported in literature for the release of cell wall glucose following pretreatment and enzymatic conversion (Glu-Con). Certainly, these results reinforce forage maize germplasm as a valid genetic resource for advancing cell wall degradability traits in bioenergy maize breeding programs.

Ultimately, the extent of malleable and highly heritable genetic diversity for maize cell wall traits found in this study validates classical breeding strategies as a means towards the optimization of lignocellulosic biomass for the production of bioenergy and other bio-commodities. In fact, since adverse inter-relationships between cell wall traits are not necessarily strict, possibilities exist to generate cell wall compositional profiles better tailored to specific industrial needs. Notwithstanding, since useful variation for cell wall traits is potentially defined by QTLs with "minor" effects, maize cultivar development for bio-based applications will rely on marker-assisted selection procedures (i.e. genomic selection or marker-assisted selection) centered on detecting and increasing the frequency of favorable QTL alleles in elite germplasm. In this regard, all QTLs identified in this study, especially the 5 novel QTLs detected for bioconversion traits, warrant further investigation.

Table S1. Survey of co-localizations between QTLs identified in this study and those reported elsewhere in literature

QTL#	Trait	1-LOD Bin Span	Polysaccharides	Lignin	Ferulates	Digestibility	Saccharification	Yield	References
1	CW (g Kg-1 DM)	4.03 - 4.05	✓	✓	✓	✓	-	✓	[17, 26, 64-66]
2		4.08 - 4.09	✓	✓	✓	✓	-	-	[17, 26, 28, 42, 58, 65]
3		5.08 - 5.09	-	✓	-	✓	-	-	[42, 58, 65]
4	Cel (g Kg-1 DM)	4.03 - 4.05	✓	✓	✓	✓	-	✓	[17, 22, 26, 28, 64, 66]
5		4.08 - 4.09	✓	✓	✓	✓	-	-	[17, 26, 28, 42, 58, 65]
6		5.08 - 5.09	-	✓	-	✓	-	-	[42, 58, 65]
7		6.07	✓	✓	✓	✓	-	-	[17, 42, 58, 66]
8	Hem (g Kg-1 DM)	4.08 - 4.09	✓	✓	✓	✓	-	-	[17, 26, 28, 42, 58, 65]
9		5.08 - 5.09	-	✓	-	✓	-	-	[42, 58, 65]
10	Lig (g Kg-1 DM)	1.04 - 1.05	✓	✓	✓	✓	-	✓	[17, 22, 26, 28, 43, 66]
11		1.06 - 1.07	-	✓	-	-	✓	-	[17, 26]
12		3.04	✓	✓	-	-	-	✓	[17, 28, 65]
13		4.05 - 4.06	-	-	✓	-	-	✓	[17, 22]
14		5.05 - 5.06	-	✓	✓	-	-	-	[26, 64]
15		9.01 - 9.03	✓	✓	-	✓	-	-	[17, 42, 58, 66]
16		10.05 - 10.06	✓	-	-	✓	-	-	[17, 65, 66]
17	Cel/CW (g Kg-1 CW)	4.05 - 4.06	✓	-	-	-	-	✓	[17, 65]
18		6.07 - 6.08	✓	✓	✓	✓	-	-	[17, 42, 58, 64, 66]
19	Hem/CW (g Kg-1 CW)	3.02 - 3.03	✓	-	-	-	-	-	[64]
20		4.05 - 4.06	✓	-	-	-	-	✓	[17, 65]
21		6.06 - 6.07	✓	✓	✓	✓	-	-	[17, 42, 58, 66]
22	Lig/CW (g Kg-1 CW)	1.11	✓	✓	-	✓	-	✓	[17, 22, 28, 64-66]
23		3.04	✓	✓	✓	✓	-	✓	[26, 28, 42, 58, 65, 66]
24		4.07 - 4.08	✓	-	-	✓	-	✓	[17, 22, 32, 43, 65, 66]
25		5.05 - 5.06	-	✓	✓	✓	-	-	[26, 64, 66]
26		7.05 - 7.06	✓	-	-	✓	-	-	[17]

Table S1. Survey of co-localizations between QTLs identified in this study and those reported elsewhere in literature

QTL#	Trait	1-LOD Bin Span	Polysaccharides	Lignin	Ferulates	Digestibility	Saccharification	Yield	References
27	Xyl (g Kg-1 CW)	4.09	✓	✓	-	✓	-	-	[17, 42, 58]
28		5.07	✓	-	-	-	-	-	[22]
29	Ara (g Kg-1 CW)	1.02 - 1.03	✓	✓	✓	-	-	-	[17, 26, 64, 66]
30		1.05	✓	✓	✓	✓	-	✓	[17, 22, 26, 28, 43, 66]
31		3.02- 03	✓	-	-	-	-	-	[64]
32		4.05 - 4.06	-	-	✓	-	-	✓	[17, 22, 65]
33		10.05 - 10.06	✓	-	-	✓	-	-	[17, 65, 66]
34	pCa (g Kg-1 CW)	1.04 - 1.05	✓	✓	-	✓	-	✓	[17, 22, 26, 28, 64, 66]
35		3.09	✓	-	✓	-	-	-	[17]
36	DHS (Ara/Xyl%)	1.03 - 1.04	✓	-	✓	✓	✓	-	[17, 32, 64, 66]
37		1.05	✓	✓	✓	✓	-	✓	[17, 22, 26, 28, 43, 66]
38		3.02 - 04	✓	-	-	✓	-	-	[64, 66]
39		10.05 - 10.06	✓	-	-	✓	-	-	[17, 65, 66]
40	CWD (% CW)	1.04 - 1.05	✓	✓	✓	✓	-	✓	[17, 22, 26, 28, 43, 64, 66]
41		1.06	-	✓	-	-	✓	-	[17, 26]
42		1.10 - 1.11	✓	✓	-	✓	-	✓	[17, 22, 28, 64-66]
43		4.07 - 4.09	✓	✓	✓	✓	-	✓	[17, 22, 26, 28, 32, 43, 58, 65, 66]
44		10.05 - 10.06	✓	-	-	✓	-	-	[17, 65, 66]
45		10.02 - 10.03	✓	✓	✓	✓	-	-	[22, 26, 66]
46	Glu-Rel (g Kg-1 DM)	1.03 - 1.04	✓	-	✓	✓	✓	-	[17, 32, 64, 66]
47		4.07 - 4.08	✓	✓	✓	✓	-	✓	[17, 22, 26, 28, 32, 43, 65, 66]
48		5.05	-	-	-	✓	-	-	[66]
49	Glu-Con (% CW Glucose)	1.03	✓	-	✓	✓	-	-	[17, 32, 66]
50		1.05 - 1.06	-	✓	-	-	✓	-	[17, 26]
51		5.03 - 5.04	-	✓	-	✓	-	✓	[28, 65]
52		7.05	✓	-	-	-	✓	-	[17]

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Chapter 5

Extent of genotypic variation for maize cell wall bioconversion traits across environments and among hybrid combinations

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Abstract

The imminent utilization of maize stover as a substrate for bioenergy production demands the development of dual-purpose hybrid varieties combining both, optimal grain yield and improved biomass processing amenability. In this study, our objectives were to assess the environmental stability and hybrid inheritance patterns of genetic variation for maize cell wall degradability characteristics. To this end, a panel of maize double haploid (DH) lines and their corresponding test-cross (TC) offspring were tested under different locations (primarily in the Netherlands) and characterized for a variety of cell wall compositional and bioconversion features relevant to cellulosic fuel production. Overall, the DH and TC sets displayed extensive genotypic diversity in cell wall composition, polymeric ultrastructure and bioconversion characteristics. Trait heritabilities were generally high ($h^2 > \sim 0.60$); essentially implying that systematic differences between genotypes remained constant across divergent environmental conditions. Moreover, correlations between the performance of DH lines and related TC hybrids were significant and favorable for most investigated traits. Strong associations ($r > \sim 0.50$) were especially prominent for cell wall lignin content, degree of substitution of cell wall glucuronoarabinoxylans and cell wall convertibility following pretreatment and enzymatic hydrolysis. Conclusively, complex cell wall bioconversion traits constitute accessible and reliable selection criteria for incorporation in modern breeding programs seeking to advance bio-based maize hybrid varieties. The high heritability and environmental stability of these traits guarantee high selection efficacy during the development of superior DH/inbred material; and their predominantly additive nature prescribe that preliminary selection at the inbred level will guarantee similar correlated genetic gains in hybrid breeding.

Keywords: Maize, cell wall composition, biofuel, heritability, environment, hybrid

Introduction

As the most important crop worldwide in relation to global acreage [1], maize is envisioned to play an essential role in the wide-scale realization and commercialization of cellulosic fuel technologies [2-4]. In fact, with an unrivalled production and distribution infrastructure, and nearly 1300 million tons of dry stover available annually, maize is warranted to become the first large-scale lignocellulosic crop in the industry [3-5]. Conceivably, the intensive utilization of maize stover as a substrate for bioenergy production will create a demand for dual-purpose hybrid varieties combining both, optimal grain yield and improved stover quality [3, 4].

In this context, a pivotal objective for breeding "bioenergy" maize is improving complex cell wall characteristics influencing the industrial quality of its biomass [3, 5, 6]. Numerous studies have demonstrated that bioenergy crops diverging in cell wall constitution exert a differential influence on the technical efficacy of biomass-to-fuel conversion platforms [7-12]. These investigations have invariably led to the recognition that the economic and environmental performance of the cellulosic fuel industry can be improved through the selection of biomass substrates which require lower energetic and chemical inputs for their deconstruction [3, 6].

With a wealth of dedicated agronomic and genomic resources, advancing dual-purpose maize with improved biomass-processing amenability is a realistic prospect [3]. Extensive evidence has demonstrated that maize conceals a considerable degree of genetic variation for cell wall compositional traits of beneficial value for bio-based industrial applications [10, 13-16]. These results suggest that favorable genetic gains for complex cell wall characteristics are attainable by exploiting available germplasm resources through classical breeding and selection. Despite these promising projections, nevertheless, much remains to be investigated in relation to the environmental stability and hybrid combinatorial patterns of cell wall composition and bioconversion traits relevant to cellulosic fuel production. Certainly, this information will be deemed essential when designing selection strategies that maximize the efficacy of bio-based maize breeding endeavors.

This study was concerned with two distinct, yet inter-related objectives. The first one was to assess whether heritable variation (at the inbred level) for maize cell wall composition and degradability characteristics relevant to cellulosic fuel production remains stable across contrasting environments. The second one was to investigate how this variation, especially in relation to bioconversion traits, is inherited and expressed in hybrid combinations. Collectively, these analyses would yield insights into the technical feasibility of exploiting standing variation for complex maize cell wall characteristics at the inbred level for the production of superior hybrid cultivars with reduced lignocellulose recalcitrance and improved processing amenabil-

ity. To this end, a panel of maize double haploid (DH) lines and their corresponding test-cross offspring were tested under different locations (primarily in the Netherlands) and characterized for a variety of cell wall compositional and bioconversion features relevant to cellulosic fuel production via dilute-acid hydrolysis and enzymatic saccharification.

Materials and methods

Plant material

A maize population of doubled haploids (DHs), property of Limagrain Nederland B.V. (Rilland, The Netherlands), was grown in 2009 at Wouw, The Netherlands, and was characterized for variation in cell wall composition and degradability traits relevant to cellulosic fuel production (Chapter 4). The experimental population, consisting of 230 genotypes, was developed from the cross between proprietary inbred lines Lim-531 and Lim-789; both highly differing in ruminal cell wall digestibility. From this trial, a panel of 34 DH genotypes (henceforth referred to as the DH-set) was selected to evenly represent the range of variation in cell wall bioconversion traits observed across the DH population. In parallel, these 34 lines were crossed to a Limagrain proprietary tester to produce a corresponding set of test-cross (TC) hybrids (henceforth referred to as the TC-set). The tester line was selected because of its favorable combining ability effects for cell wall digestibility traits in commercial test-cross procedures.

Field evaluations

The DH-set was employed to study the extent and stability of heritable variation for maize cell wall composition and bioconversion traits across contrasting environments. DH experiments were conducted during the summer of 2013 at three distinct locations: Steenbergen (The Netherlands), Wageningen (The Netherlands) and Greven (Germany). Trials were sown in replicate in adjacent randomized blocks. Genotypes were planted in two-row plots with a length of 1.5 m and an inter-row distance of 0.77 m at a density of 10 plants m^{-1}. Per plot, stalks of 8 randomly selected plants were harvested at a 10 cm stubble height prior to silage maturity (between 6-8 weeks after the population's mean silking period). Due to logistic impediments, however, test locations had to be harvested on separate dates (Table 1). Collected biomass feedstocks were chopped and air dried at 70 ^{0}C for 48 hours, and were subsequently ground through a 1-mm screen using a hammer mill.

The TC-set was used to investigate how genetic variation for maize cell wall traits is inherited and expressed in hybrid combinations. TC experiments were also conducted during the summer of 2013, but were sown in Eindhoven and Wouw (both in The Netherlands). The experimental design, harvesting methodology and sample processing conditions for the TC experiments were identical to those prescribed for the DH trials; with the only difference being that TC trials were harvested at silage maturity (Table 1).

Compositional analyses

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) components were determined through the ANKOM filter bag method (AN-KOM Technology Corporation, Fairpoint, NY), which fundamentally derives from the work of Goering and Van Soest [17]. All analyses were performed in duplicate and were carried out using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY). The proportion of cellulose (Cel/CW), hemicellulose (Hem/CW) and acid-insoluble lignin (Lig/CW) contents on a cell wall basis were derived from detergent fiber data as described in Table 2.

The degree of substitution of cell wall glucuronoarabinoxylans (DHS), measured as the ratio of cell wall arabinose-to-xylose, was derived from the analysis of cell wall neutral sugar components; the latter determined by gas chromatography essentially as described by Englyst and Cummings [18]. Briefly, lyophilized water un-extractable solids were first treated with 72% sulphuric acid (1 hr, 30 °C), followed by a second hydrolysis process with 4% sulphuric acid (3 hrs, 100 °C). Released neutral sugars were then derivatized to their respective alditol isoforms and quantified on an Agilent 7890A Gas Chromatography System (Agilent Technologies, Santa Clara, CA) using a DB-250 column (Agilent Technologies, Santa Clara, CA).

Bioconversion efficiency

Bioconversion efficiency following mild dilute-acid pretreatment and enzymatic hydrolysis was determined as detailed by Torres *et al.* [10]. In essence, biomass samples (500 mg) were pretreated at a 30% solids loading in 0.17% (w/v) sulfuric acid for 30 min at 140°C. Subsequently, pretreated samples were treated with 250 µL of an Accelerase 1500 cellulolytic enzyme cocktail (Genencor B.V., Leiden, NL) in 40 mL 0.1 M citrate buffer. The enzyme load provided 50 filter paper units (FPU) of cellulase per gram cellulose. Samples were subsequently incubated at 50 °C in an Innova 42 air incubator (New Brunswick Scientific, Enfield, CT) at 200 RPM for 24 hrs. Saccharification liquors were analyzed for glucose concentration using a Boehringer Mannheim D-Glucose kit (Boehringer Mannheim, Indianapolis, IN, USA). The colorimetric assay was adapted to a 96 micro-titer plate format, and spectrophotometric reads were made using a Bio-Rad 550 Micro-plate Reader (Bio-Rad, Richmond, CA). For all samples, glucose content was expressed as both, the amount of glucose released from one gram of dry biomass (Glu-Rel) and the percentage of total cell wall glucose released upon enzymatic saccharification (Glu-Con) (Table 2).

Statistical analyses

Independent analyses of variance (ANOVA) were used to determine the significance

Table 1. Planting and climatic information for DH and TC experiments conducted across different locations in the Netherlands and Germany

	Location	Year	Sowing Date	Harvest Date	Temperature[a]	Relative Humidity[b]
DH-Experiments	Wouw	2009	May 5	September 10	15.5 °C	NA
	Greven	2013	April 27	September 9	NA	NA
	Steenbergen	2013	May 2	October 10	16.7 °C	77 %
	Wageningen	2013	May 12	September 30	15.8 °C	73%
TC-Experiments	Eindhoven	2013	April 26	October 1	16.4 °C	76 %
	Wouw	2013	May 1	October 10	16.3 °C	78%

[a] Mean daily temperature over growing season (°C)
[b] Mean relative air humidity over growing season (%)
NA = information not available

Table 2. Description of cell wall compositional and bioconversion characters analyzed within the framework of this study

Trait	Unit	Description
CW	g Kg⁻¹ DM	Stem cell wall content; determined as neutral detergent fiber (NDF)
Lig	g Kg⁻¹ DM	Stem acid insoluble lignin content; determined as ADL
Cel/CW	g Kg⁻¹ CW	Stem cellulose content (ADF-ADL) relative to cell wall content (CW)
Hem/CW	g Kg⁻¹ CW	Stem hemicellulose content (NDF-ADF) relative to cell wall content (CW)
Lig/CW	g Kg⁻¹ CW	Stem acid insoluble lignin content (ADL) relative to cell wall content (CW)
DHS	Ara/Xyl%	Degree of hemicellulose substitution, expressed as the ratio of cell wall arabinose to cell wall xylose (Ara/Xyl)
Glu-Rel	g Kg⁻¹ DM	Amount of glucose released from one gram of dry biomass after pretreatment and enzymatic saccharification.
Glu-Con	% CW Glucose	Percentage of total cell wall glucose released after pretreatment and enzymatic saccharification.

of genotypic differences in stem fiber and cell wall components, as well as bioconversion parameters in DH and TC experiments. From these analyses, estimates of genotypic and phenotypic variances were used to calculate trait heritability (h^2) estimates. Coefficients of genetic variation over genotype means (CV_G) were also calculated for all evaluated traits and were used as standardized measures of genotypic variation. Inter-relationships between cell wall compositional and degradability traits were analyzed by means of Pearson correlations.

Results and Discussion

Phenotypic variation for complex cell wall characteristics is highly heritable and stable across environments

In this study, a panel of inter-related maize doubled-haploid (DH) genotypes was investigated across multiple locations for variation in cell wall composition and bio-conversion properties relevant to cellulosic fuel production. Overall, the influence of location was highly significant ($p<0.001$) for all investigated cell wall parameters, but the extent and pattern of fluctuations in cell wall compositional means across environments varied considerably from trait to trait (Figure 1). To illustrate, average glucose conversion efficiency for Greven (Glu-Con= ~49%) was significantly higher than Glu-Con means reported for all other locations. In fact, at Steenbergen, Wageningen and Wouw, the DH-set displayed lower but fairly similar mean enzymatic convertibility rates (Glu-Con= ~41%). By contrast, DH-set average values for lignin content (Lig) displayed broader variation across tested environments; this time, Greven ranked lowest (Lig=12 g Kg^{-1} DM), but Steenbergen (Lig=32 g Kg^{-1} DM) exhibited markedly higher values than Wageningen (Lig=23 g Kg^{-1} DM) and Wouw (Lig=27 g Kg^{-1} DM).

Differences in cell wall compositional profiles across environments can often be ascribed to management and harvesting practices leading to inter-location differences in plant maturity [19, 20]. The significantly lower lignin contents (Lig, Lig/CW) and improved conversion efficiencies (Glu-Con) reported for Greven relative to all other locations could be explained by the fact that harvesting occurred earlier at this site (Table 1). Accordingly, as the maize plant matures, changes in the compositional balance of stem cell wall polymers lead to an increased concentration of phenolic components and a concomitant decrease in cell wall degradability properties [21, 22]. Incidentally, seasonal and spatial variation in "environmental" conditions has also been shown to alter maize lignocellulose constitution and quality [20, 23, 24]. Relevant factors affecting cell wall characteristics include temperature, light intensity and water availability. Given the substantial agro-climatic contrasts observed across our trials (Table 1), it seems plausible that systematic variation for cell wall polymeric profiles across locations is also a reflection of constitutive adaptations to divergent environmental conditions.

Regardless of the extent of environmental influences, highly significant (p<0.001) genotypic differences were detected for all evaluated parameters. Means, descriptive statistics and narrow-sense heritability estimates (h^2) for the DH-set are summarized in Table 3. As expected, among cell wall components, variation was highest for Lig and Lig/CW (CV$_G$ = 16%), but also for DHS (CV$_G$ = 8%). These observations reinforce the notion that natural diversity in the biochemical composition of the maize

cell wall and its physical properties is primarily ascribed to variation in the balance, monomeric make-up and ultra-structure of non-cellulosic cell wall polymers [10, 25-29]. In this context and concurrent with previous studies [10, 30], correlation analyses confirm that the extent of enzymatic depolymerization (Glu-Con) of maize biomass is strongly, and negatively associated ($r > -0.75$) to the concentration of cell wall phenolics (Lig, Lig/CW), and positively impacted ($r = 0.85$) by increments in the degree of substitution (DHS) of cell wall glucuronoarabinoxylans (Figure 2). Correspondingly, across the DH-set, genotypic differences for cell wall bioconversion traits (Glu-Rel, Glu-Con) were likewise prominent ($CV_G = 9\%$; in both cases). In particular, the maximal difference for Glu-Con across lines was approximately 23 percentage units; a finding that reiterates forage maize as a promising genetic resource for advancing complex biomass degradability properties in bioenergy-maize breeding programs [10, 11].
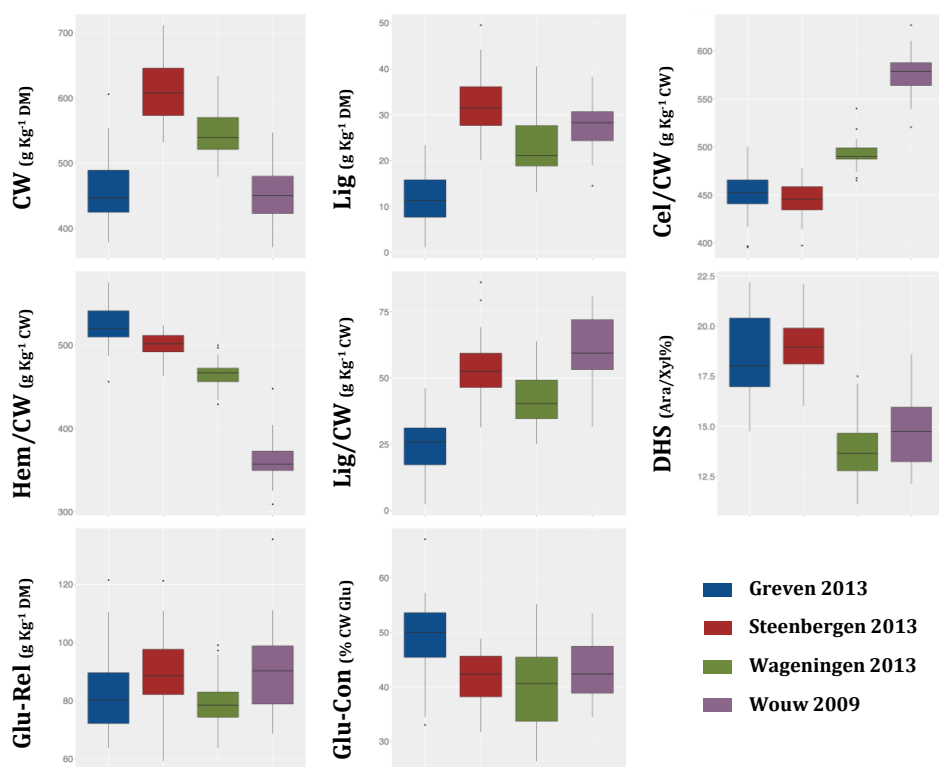

Figure 1. Box-plots summarizing the extent of variation of a panel of DH genotypes (34 in total) for diverse cell wall characteristics relevant to cellulosic fuel production. For every box-plot, horizontal solid lines represent DH-set medians, boxes represent the interquartile range and bars indicate extremes.

Table 3. DH-set means, ranges and heritabilities (h^2) for stem cell wall composition and bioconversion properties relevant to cellulosic fuel production

Trait	Mean	Range	S.E.M.	CV_G	Heritability (h^2)
CW $_{(g\,Kg^{-1}\,DM)}$	517	442 - 605	35	7%	77%
Lig $_{(g\,Kg^{-1}\,DM)}$	23.4	13.1 – 37.5	3.6	16%	88%
Cel/CW $_{(g\,Kg^{-1}\,CW)}$	492	471 – 530	18	4%	57%
Hem/CW $_{(g\,Kg^{-1}\,CW)}$	463	428 – 502	18	4%	70%
Lig/CW $_{(g\,Kg^{-1}\,CW)}$	44.9	23.8 – 68.5	7.0	16%	88%
DHS $_{(Ara/Xyl\%)}$	16.6	14.0 – 19.3	1.3	8%	78%
Glu-Rel $_{(g\,Kg^{-1}\,DM)}$	86	69 – 107	4	9%	89%
Glu-Con $_{(\%\,CW\,Glucose)}$	43.9	32.7 – 55.2	7.8	9%	87%

Equally noteworthy, narrow-sense heritabilities (estimated across test locations) were high ($h^2 > {\sim}0.75$) for the majority of investigated traits (Table 3), including the highly polygenic bioconversion characters Glu-Rel and Glu-Con. Essentially, these results imply that differences between DH lines are systematic and remained constant across divergent environmental conditions. This observation is in agreement with prior findings emphasizing on the highly-heritable nature of maize cell wall phenolic characteristics and ruminal cell wall digestibility properties across multi-location and multi-year trials [13, 30-34]. In fact, Dolstra et al. [31] had alluded that effective genetic gains for highly variable cell wall digestibility properties were theoretically possible without recurring to intensive replicated testing.

High heritabilities for quantitative cell wall traits observed here and elsewhere are the product of two factors: broad genetic diversity and the stability of genotypic differences across contrasting environments. In our investigation, pooling of samples did not allow estimation of G*E effects per se, but the stability in the span of cell wall trait ranges (Figure 1) and the low frequency of genotype-rank cross-over events across environments (*data not* shown) indicate that the magnitude of G*E interactions was limited. Presumably, extensive heritable variation for the biochemical makeup and biological functionality of the maize cell wall is the result of adaptation to a wide range of agro-climatic conditions and end-uses. Contradictorily, despite being a highly complex and polygenic process (similar to "yield"), maize cell wall biosynthesis appears to adhere intractably to a pre-determined genetic blueprint (unlike "yield") and appears rather impervious to genotype-by-environment (G*E) interaction effects [19, 25, 35-37]. It would appear as if maize cell wall construction (at the individual/genotype level) is under the control of a highly robust genetic system which constrains cell wall phenotypic plasticity, probably because a functional cell wall is crucial to plant fitness.
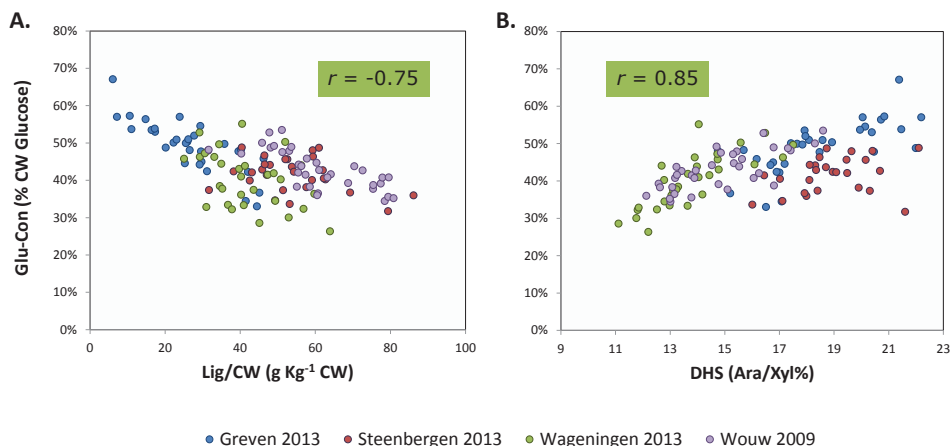
Figure 2. Trait inter-relations between A) Glu-Con and Lig and B) Glu-Con and DHS across a diverse set of DH lines. Colored dots indicate the mean performance of DH lines at specific locations. Pearson correlation coefficients (*r*) were calculated using genotypic means across all investigated locations.

Notwithstanding, and as previously noted, seasonal and agro-climatic variation will induce changes in whole-plant cell wall composition [19]; but the effects of these external influences appear seemingly systematic across genotypes (Figure 1). Ultimately, coordinated alterations in whole-plant cell wall polymeric profiles at diverse environments could indicate the presence of conserved mechanisms in the way maize adapts cell wall formation across stem tissues in response to environmental changes (e.g. alterations in the concentration and composition of stem vascular bundles). Certainly, this field warrants further investigation, especially when considering that a promising prospect of C4 bioenergy grasses (i.e. miscanthus, sugarcane, sorghum, etc.) demands their production under low-input and marginal agricultural scenarios [4].

Doubled haploids and related hybrids display similar patterns cell wall architecture and degradability properties

Test-cross (TC) hybrids derived from our selection of DH genotypes were investigated across two locations; Eindhoven and Wouw. Trait means, ranges and broad-sense heritability estimates (h^2) for all investigated parameters are summarized in Table 4.

Significant genotypic differences (p<0.05) were observed across the TC-set for all investigated cell wall characteristics, except for Hem/CW. As anticipated, ranges (min-max) for cell wall components and bioconversion traits in the TC-set were substantially lower than corresponding values observed for the DH-set [38]. This held

especially true for Hem/CW and Glu-Con, for which maximal differences across genotypes in the TC-set were reduced by nearly 50%. Regardless of these noticeable reductions, variation among hybrids for most cell wall characteristics remained high. Once again, Lig, Lig/CW and DHS displayed the highest levels of genotypic variation (CV_G > ~7%). Likewise, Glu-Con was highly variable across the TC-set (CV_G = 9%), and we speculate that maximal differences (~11.0 %) could have been even larger, if the DH panel had included the population extremes. As was the case for the DH-set, cell wall trait broad-sense heritabilities were generally high (~0.60 <h^2<~0.80); thus reiterating our previous asseverations regarding the environmental stability of genetic variation for complex cell wall characteristics.

Table 4. TC-set means, ranges and heritabilities (h^2) for stem cell wall composition and bioconversion properties relevant to cellulosic fuel production

Trait	Mean	Range	S.E.M.	CV_G	Heritability (h^2)
CW $_{(g Kg}$ $^{-1}$ $_{DM)}$	652	599 – 713	17	5%	68%
Lig $_{(g Kg}$ $^{-1}$ $_{DM)}$	32.6	18.4 – 44.8	3.2	14%	75%
Cel/CW $_{(g Kg}$ $^{-1}$ $_{CW)}$	573	552 – 598	8	2%	54%
Hem/CW $_{(g Kg}$ $^{-1}$ $_{CW)}$	393	375 – 411	7	3%	52%
Lig/CW $_{(g Kg}$ $^{-1}$ $_{CW)}$	49.9	30.3 – 64.3	5.0	14%	68%
DHS $_{(Ara/Xyl\%)}$	12.2	10.6 – 14.7	0.4	7%	70%
Glu-Rel $_{(g Kg}$ $^{-1}$ $_{DM)}$	91	80 – 106	4	7%	59%
Glu-Con $_{(\% CW Glucose)}$	30.9	26.2 – 37.7	2.0	9%	77%

We have also detected systematic differences between DH lines and their TC offspring in the content and polymeric balance of cell wall polymers (Table 5). Specifically, hybrid genotypes displayed a greater accumulation of cell wall material (CW) in stem tissues and exhibited a higher proportion of lignin (Lig, Lig/CW) and cellulose (Cel/CW) in their cell walls. Presumably, these structural and constitutional adaptations would lead to improved stalk mechanical-strength; the latter deemed necessary to sustain the increased growth rates and yields typical of hybrid maize [39, 40]. These compositional contrasts, however, did not appreciably alter the prevalent inter-relations that exist between cell wall compositional characters and biomass enzymatic convertibility (Figure 3). Expectedly, given their higher concentration in lignin content (Lig, Lig/CW) and reduced DHS, Glu-Con values were on average lower for TC genotypes than for DH lines (Table 5). Furthermore, while a significant association between Glu-Con and CW was not detected in the DH panel, a negative relation (r = -0.56) between these two traits was detected at the TC level (Figure 3). By extension, we presume that the lower enzymatic convertibility (Glu-Con) observed for TC genotypes can also be attributed to the increased thickness of

their secondary cell walls. In this study, bioconversion assays for the DH- and TC-set, employed identical processing conditions, including the same concentration of sulfuric acid per gram of dry biomass (5% w/w) during thermochemical pretreatment. Therefore, because TC genotypes display a higher content of cell wall per dry gram of biomass, the concentration of acid in relation to cell wall content was lower for TC lines, thereby rendering the conversion process less effective [10]. Ultimately, these results reinforce the notion that the efficient deconstruction of biomass (under cellulosic fuel conversion platforms) is greatly conditioned by its biochemical composition, especially under suboptimal processing regimes. Therefore, conclusions and projections regarding the efficiency of biomass-to-ethanol conversion systems should be constructed based on models that closely emulate conditions used in the industry. In this regard, since the lignocellulosic composition of hybrid maize differs from that of DH/inbred maize, different sets of analytical conditions should be employed to explore their bioconversion potential.

Table 5. Contrasts between DH lines and TC offspring in stem cell wall composition and bioconversion properties

Trait	CW	Lig	Cel/CW	Hem/CW	Lig/CW	DHS	Glu-Rel	Glu-Con
DH Lines	517	23.4	492	463	44.9	16.6	86	44%
TC Lines	652	32.6	573	393	49.9	12.2	91	31%
t-test probability [a]	***	***	***	***	*	***	*	***

[a] Significance of differences between DH and TC lines determined at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***); NS indicates non-significant differences.

Test-cross performance for relevant cell wall characteristics can be predicted at the DH level

In our study, correlations between the performance of DH lines and related hybrids were significant and favorable for most investigated traits (Table 6). Strong associations ($r > \sim 0.6$) were especially prominent for Lig, Lig/CW and Glu-Con. Extensive evidence has demonstrated that genotypic differences for complex cell wall characteristics, which define or describe qualitative properties of maize lignocellulosic biomass (i.e. Lig, Lig/CW, DHS and Glu-Con), are independent of developmental variation [29, 31, 41]. Therefore, the correlated performance of DH lines and their corresponding TC offspring substantiates the notion that variation for complex cell wall characteristics is quantitatively inherited and predominantly additive. Only for CW, the observed positive association between DH and TC performances can be partially attributed to earliness effects.
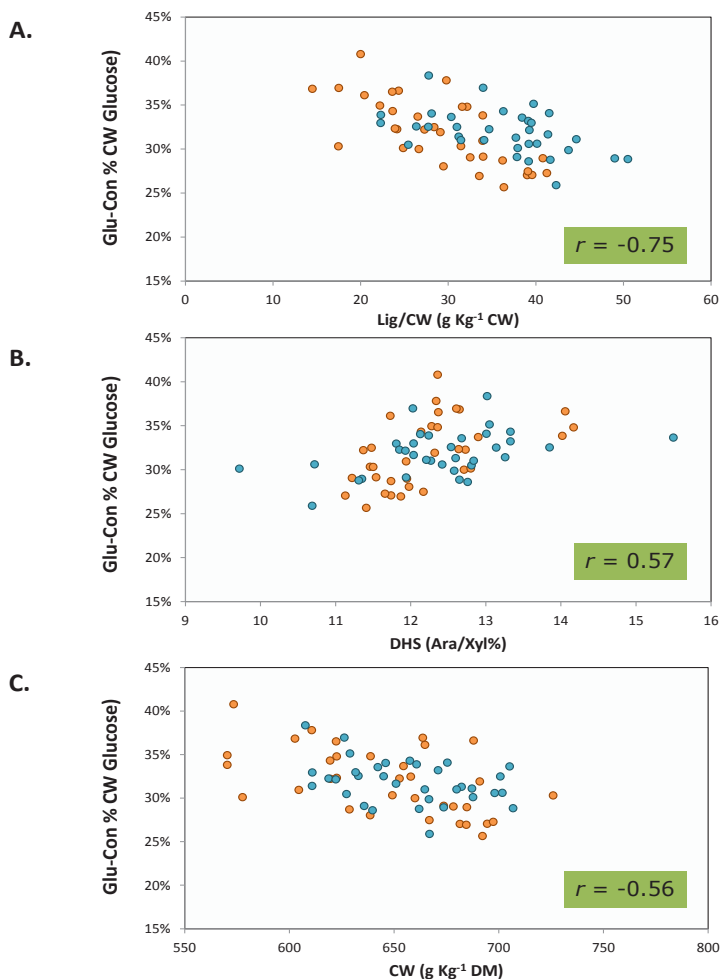
Figure 3. Trait inter-relations between Glu-Con and A) Lig, B) DHS and C) CW across a contrasting set TC genotypes. Orange colored dots indicate the mean performance of TC lines at Eindhoven and blue colored dots indicate the performance of TC at Wouw. Pearson correlation coefficients (*r*) were estimated on the basis of genotypic means across all investigated locations.

Table 6. Correlation analyses between the mean performance of TC and DH lines with respect to stem cell wall composition and bioconversion characters

	CW	Lig	Cel/CW	Hem/CW	Lig/CW	DHS	Glu-Rel	Glu-Con
	0.51	0.65	0.15	0.21	0.59	0.52	0.42	0.60
p value [a]	**	***	NS	NS	***	**	*	***

[a] Correlations are significant at p>0.05 (*), p>0.01 (**) or p>0.001 (***); NS indicates non-significant correlations

Understandably, the lack of strict proportionality (r<1) between DH per se and hybrid values is a consequence of the fact that genotypic means for DH and TC genotypes cannot be determined with complete accuracy (h^2<100%). In addition, discrepancies between expected and realized hybrid performance might be partially attributed to the occurrence of non-additive gene action (e.g. dominance effects at heterozygous loci) in specific allelic combinations. Previous investigations have demonstrated, nevertheless, that for complex cell wall characteristics, genetic variation due to additive effects is generally more important than variation attributed to non-additive gene action [19, 25, 35-37].

Ultimately, our observations suggest that preliminary selection for improved biomass composition and bioconversion properties at the DH level will guarantee similar correlated genetic gains in hybrid breeding. Moreover, because biomass processing amenability (Glu-Rel, Glu-Con) is intrinsically defined by the chemical constitution of plant cell walls, hybrid performance for bioconversion traits could be theoretically predicted by models which account for genetic variation in multiple cell wall characteristics. Correspondingly, we have idealized a regression model to forecast Glu-Con values at the hybrid level based on the performance of DH lines for CW, Lig and DHS; all shown to impact the extent of maize biomass convertibility (Figure 3). Notably, predicted values correlated strongly (r = 0.78) with realized means (Figure 4) and effectively surpassed the predictive accuracy (r = 0.60) of the DH-TC correlated response for Glu-Con. From a commercial standpoint, the ability to predict hybrid performance based on the productivity of parental lines offers important practical, technical and economic advantages. On the one side, effective selection at the DH/inbred level will prove highly advantageous if fewer resources are devoted to factorial test-cross procedures and evaluations. On the other hand, the dissection of complex biomass quality traits may lead to the identification of phenotypic attributes which can be measured with greater precision and at a lower cost. Generally, these component traits display simpler inheritance patterns and their targeted selection can yield greater genetic gains with respect to highly complex cell wall quality traits.

A.

B.

Glu-Con (TC) = 39.1% + 0.08%*(DHS/Lig) - 25.9%*CW
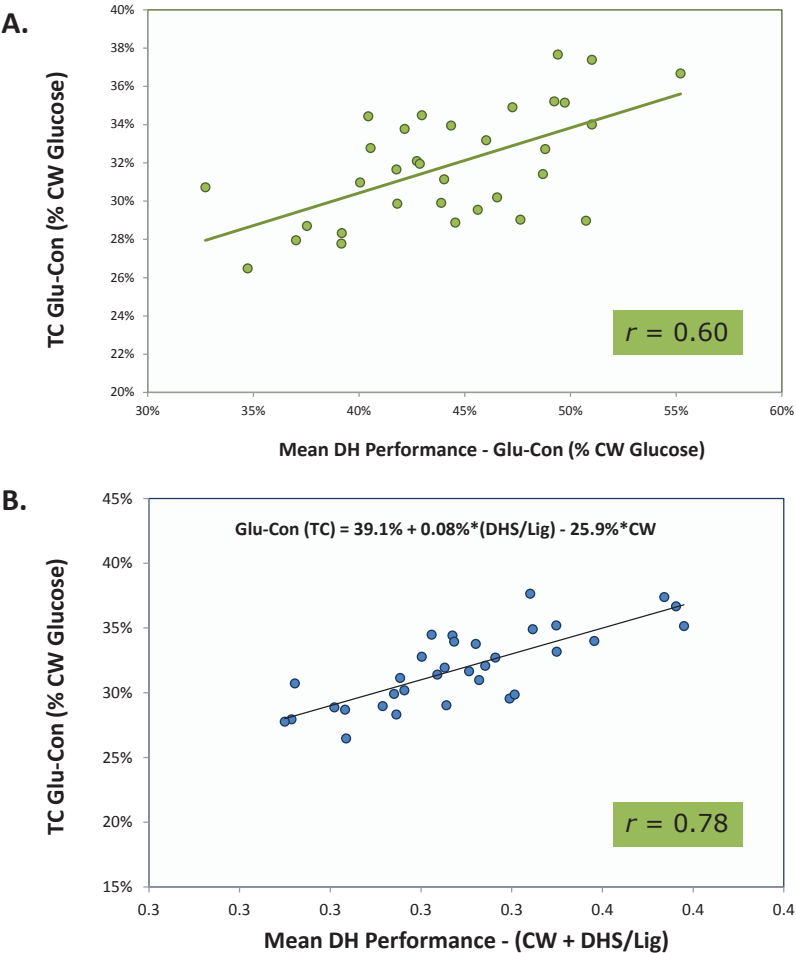
Figure 4. Comparison of predictive value for the performance of Glu-Con at the hybrid level based on: A) direct correlation between DH *per se* and realized hybrid values, and B) correlation between DH *per se* and hybrid values estimated through multiple linear regression. The regression model predicted Glu-Con performance at the hybrid level based ON DH performance for the highly variable parameters, Lig and DHS; both shown to impact the extent of maize biomass convertibility. The model is presented in panel **B**.

Conclusions

The results of this investigation prescribe positive prospects and practical advantages for the development of bioenergy maize cultivars with improved cell wall characteristics. In particular, the high heritability and environmentally stability of cell wall compositional and degradability properties guarantee high selection efficacy during the development of superior DH/inbred material, and predispose that multi-environment testing will only be necessary at advanced stages of bioenergy-maize breeding programs. Moreover, because genetic variation for complex cell wall characteristics is predominantly additive, preliminary selection at the inbred level will expectedly lead to successful hybrid selection; thereby minimizing the need for recurrent test-crossing procedures and evaluations. Notwithstanding, because inbred and hybrid maize exhibit seemingly distinct cell wall compositional profiles, careful consideration is required when determining optimal analytical parameters for evaluating their bioconversion potential.

Cell wall bioconversion traits (Glu-Con, Glu-Rel) constitute accessible and reliable selection criteria which can be incorporated in modern breeding programs seeking to develop advanced bio-based hybrid varieties. And while the convergence of classical selection schemes with advanced marker-assisted selection strategies (e.g. genomic selection) can accelerate maize cultivar development for bioenergy applications, maximal genetic gains are expected from breeding programs focusing on preselected germplasm harboring substantial levels of favorable genotypic variation for relevant target traits. In this respect, we advocate the screening of elite forage maize germplasm known to display substantial amounts of genetic variability for biomass yield, cell wall composition and cell wall degradability properties relevant to cellulosic fuel production.

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Chapter 6

Bioenergy feedstocks with improved digestibility can enhance the commercial and environmental performance of cellulosic fuels

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Abstract

Despite the recognition that feedstock composition influences biomass conversion efficiency, limited information exists as to how bioenergy crops with reduced recalcitrance can improve the economics of cellulosic fuel conversion platforms. Consequently, we have compared the bioenergy potential –estimated as total production of lignocellulosic glucose per hectare (TGP) - of maize cultivars contrasting for cell wall digestibility, across processing conditions of increasing thermochemical severity. Overall, systematic gains in cell wall degradability can lead to significant advances in the productivity (TGP) of cellulosic fuel biorefineries under suboptimal processing; only if gains in digestibility are not accompanied by substantial yield penalties. Under mild processing (conveying a 50% reduction in acid consumption and a 30°C reduction in processing temperature relative to the harshest processing regime), maximum TGP (2.9 t ha^{-1}) for the overall-best cultivar was only 80% of the highest TGP achieved within the framework of this investigation under the most stringent processing condition ($\sim$3.7 t ha^{-1}). If breeding would allow for the combination of the best characteristics available in the entry panel, then maximum TGP at suboptimal conditions ($\sim$3.7 t ha^{-1}) would match the highest realizable yields. Conceptually this demonstrates that the advance of superior bioenergy cultivars (surpassing the performance of modern elite material) would allow us to improve the productivity of currently available biomass-to-fuel conversion systems using more cost-effective and sustainable systems. We also speculate that diminished acid and energy consumption during thermochemical pretreatment could lead to cutbacks in the throughput, size, and capital costs of cellulosic fuel biorefineries and facilitate decentralization of renewable fuel production.

Keywords: Maize, cell wall digestibility, biomass yield, technoeconomic, refinery, pretreatment

Introduction

Within the domain of cellulosic fuel research, major efforts have been devoted towards the development of advanced lignocellulosic crops designed to meet the demands of the industry. In essence, plant breeders have been faced with the challenge of identifying highly-productive biomass varieties which can be produced inexpensively, sustainably and in abundant quantities [1,2]. Moreover, since lignocellulose recalcitrance constitutes the single-most critical barrier towards the efficient conversion of plant biomass into added-value products [3-5], improving the processing amenability of lignocellulosic crops remains a pivotal goal of bioenergy breeding endeavors [6,1,7].

Our understanding of the composition, structure and biosynthesis of the plant cell wall has notably expanded in the last decade. This knowledge has enabled the development of breeding strategies targeting the modification of key cell wall compositional features that can reduce the inherent recalcitrance of lignocellulosic substrates [7,8,1]. In fact, extensive evidence has been provided to demonstrate that it is possible to advance bioenergy crops requiring lower energetic and chemical inputs for their effective fractionation into fermentable monosaccharides [9-14,6,15-17].

Despite the prevalent notion that biomass composition can exert a determinant influence on cellulosic fuel conversion efficiency, limited information exists as to how bioenergy crops with reduced lignocellulose recalcitrance can improve the economics and environmental performance of the industry. To date, techno-economic evaluations of cellulosic fuel refineries have minimized the role of biomass feedstocks to cost, productivity or availability considerations [18-24]. These comparative analyses unfairly propose that the profitability and sustainability of the cellulosic fuel industry can only be attained through innovations in process engineering or advances in the yielding capacity of lignocellulosic species. They also erroneously suggest that breeding for increased cell wall digestibility can prove detrimental to the industry, since major alterations in cell wall composition will presumably lead to concomitant reductions in biomass yield.

With an ongoing debate as to whether bioenergy crop breeding endeavors should only focus on improving agronomic performance, the main objective of this study was to develop a conceptual framework which demonstrates how, and under which circumstances, the development of bioenergy feedstocks with improved processing amenability can improve the commercial and environmental performance of the cellulosic fuel industry. To this end, we have analyzed the bioenergy potential -in relation to yield of fermentable monosaccharides and fermentation inhibitors- of a set of forage maize commercial cultivars contrasting for ruminal cell wall digestibility. A focus on the relationship between biomass yield and processing quality has

been warranted, as general convention dictates that yield penalties are a common consequence of breeding efforts leading to reduced lignocellulose recalcitrance. In addition, evaluations were performed across a range of processing conditions of increasing thermochemical severity in order to study whether improvements in biomass processing quality can factually facilitate the advance of more cost-effective and sustainable conversion platforms. Consequently, our study has focused on the production of cellulosic ethanol derived via dilute-acid pretreatment and enzymatic hydrolysis as the latter constitutes the most advanced and commercially represented platform in the industry [3,25].

Materials and methods

Plant materials and field trials

A set of 23 maize hybrids were selected for this investigation (Table 1). Of these, 18 corresponded to forage-dedicated commercial cultivars bred primarily for Northern-European markets. These cultivars were selected to be diverse for ruminal cell wall digestibility and overall biomass productivity. The panel also included 5 experimental hybrids (derived from 5 of the 18 commercial cultivars mentioned earlier) carrying either the brown-midrib *3* (*bm3*) or the cinnamyl alcohol dehydrogenase-2 deficient (*cad2*) mutation.

Entries were evaluated in replicated trials (in adjacent completely randomized blocks) at three different locations in The Netherlands (Biddinghuizen, Eindhoven, and Wouw) during the Summer of 2012. Unfortunately, due to unfavorable climatic conditions in that year, the complete panel was only successfully grown at Eindhoven. The trial at Biddinghuizen included all 18 commercial cultivars, and the trial at Wouw included all experimental mutants. Genotypes were planted in two-row plots with a length of 2.5 m and an inter-row distance of 0.75 m at a density of 10 plants m^{-1}. For each plot, stalks of 10 randomly selected plants were harvested at a 10 cm stubble height just prior to silage maturity (approximately 7 weeks after the population's mean silking period). At this physiological stage, differences between genotypes in stem cell wall composition and digestibility were expected to be largely genetic [26-28]. Due to the intensive workload, locations were harvested on separate days. Collected biomass feedstocks were chopped and air dried at 70 ^{0}C for 48 hours, and were subsequently ground through a 1-mm screen using a hammer mill. Compositional analyses were performed on ground samples on a per-plot basis. However, for bioconversion analyses, feedstock samples were produced by pooling, per genotype, the milled material collected from all experimental plots as to minimize random variation due to environment and processing (as would happen in the industry).

Compositional analysis

All biomass compositional analyses, with the exception of the degree of substitution of cell wall glucuronoarabinoxylans (DHS) and cell wall glucose concentration (Glu), were estimated using near infrared reflectance spectroscopy (NIRS) at Limagrain Nederland B.V. Briefly, ground stover samples were scanned using a FOSS NIRS DS 2500 system (Foss, Hillerod, Denmark) and biochemical predictions were realized using calibration equations developed at INRA Lusignan [29]. This calibration is specific for the analysis of maize stem forage quality traits (including detergent fiber components) and ruminal cell wall digestibility parameters. A detailed description

Table 1. Digestibility rating of Northern-European maize silage cultivars and experimental mutant counterparts of five cultivars

Accession	DINAG [a] (%)	Digestibility Class
2-04R00015	36.6	Excellent
210P106790	34.1	Excellent
210RI13169	33.8	Excellent
210XX08399	30.7	Excellent
AASTAR	34.1	Excellent
ATRIUM	28.1	Good
FANTASTIC	24.8	Good
FORMULA	24.3	Good
LG30218	25.0	Good
BALTIMORE	28.4	Good
BANGUY	29.3	Good
AMBROSINI	15.7	Poor
LG30217	19.9	Poor
LG30216	14.2	Poor
RICARDINIO	18.8	Poor
GROSSO	17.2	Poor
SECURA	15.9	Poor
SUPERBE	15.2	Poor
BALTIMORE-*bm3*	38.8	Cell Wall Mutant
BANGUY-*bm3*	35.0	Cell Wall Mutant
ATRIUM-*cad2*	33.2	Cell Wall Mutant
LG30218-*cad2*	34.3	Cell Wall Mutant
LG30216-*cad2*	26.0	Cell Wall Mutant
Mean	26.7	
S.E.M	1.5	

[a] DINAG: In-vitro ruminal cell wall digestibility; determined as the difference in NDF content before and after sample incubation in rumen liquor for 48 hours relative to NDF content prior to incubation.

of all traits evaluated is presented in Table 2 and calibration statistics are presented in Table S1.

The degree of substitution of cell wall glucuronoarabinoxylans (DHS), measured as the ratio of cell wall arabinose-to-xylose, was derived from the analysis of cell wall neutral sugar components; the latter determined by gas chromatography essentially as described by Englyst and Cummings [30]. Briefly, lyophilized water un-extractable solids were first treated with 72% sulphuric acid (1 hr, 30 °C), followed by a second hydrolysis process with 4% sulphuric acid (3 hrs, 100 °C). Released neutral

Table 2. Description of quality traits measured on stem material of 23 maize silage hybrids diverging in cell wall digestibility

Trait	Unit	Description
CW	g Kg^{-1} DM	Stem cell wall content; determined as neutral detergent fiber (NDF)
Cel	g Kg^{-1} DM	Stem cellulose content; determined as the difference between acid detergent fiber (ADF) and acid insoluble lignin (ADL)
Lig	g Kg^{-1} DM	Stem acid insoluble lignin content; determined as ADL
Cel/CW	g Kg^{-1} CW	Stem cellulose content relative to cell wall content (CW)
Hem/CW	g Kg^{-1} CW	Stem hemicellulose content relative to cell wall content (CW)
Lig/CW	g Kg^{-1} CW	Stem acid insoluble lignin content relative to cell wall content (CW)
pCa I	g Kg^{-1} CW	Esterified p-coumaric acid released after alkaline hydrolysis of the cell wall at 25°C
pCa II	g Kg^{-1} CW	Total p-coumaric acid released after alkaline hydrolysis of the cell wall at 170°C
FA I	g Kg^{-1} CW	Esterified ferulic acid released after alkaline hydrolysis of the cell wall at 25°C
FA II	g Kg^{-1} CW	Total ferulic acid released after alkaline hydrolysis of the cell wall at 170°C
Di-FA I	g Kg^{-1} CW	Esterified di-ferulic acid released after alkaline hydrolysis of the cell wall at 25°C
Di-FA II	g Kg^{-1} CW	Total di-ferulic acid released after alkaline hydrolysis of the cell wall at 170°C
DHS	%	Degree of hemicellulose substitution, expressed as the ratio of cell wall arabinose to cell wall xylose (Ara/Xyl)
H	g Kg^{-1} CW	H lignin content estimated as 4-p-Hydroxybenzaldehyde released following nitrobenzene oxidation of the cell wall at 170°C
G	g Kg^{-1} CW	G lignin content estimated as vanillin released following nitrobenzene oxidation of the cell wall at 170°C
S	g Kg^{-1} CW	S lignin content estimated as syringaldehyde released following nitrobenzene oxidation of the cell wall at 170°C
Gluc-Sol	g Kg^{-1} DM	Amount of glucose released from one gram of dry biomass into pretreatment liquors following thermochemical processing.
Gluc-Rel	g Kg^{-1} DM	Amount of glucose released from one gram of dry biomass after pretreatment and enzymatic saccharification.
Gluc-Con	% CW Glucose	Percentage of total cell wall glucose released after pretreatment and enzymatic saccharification.
DINAG	%$_{NDF}$	In-vitro cell wall digestibility; determined as the difference in NDF content before and after sample incubation in rumen liquor for 48 hours relative to NDF content prior to incubation.

sugars were then derivatized to their respective alditol isoforms and quantified on an Agilent 7890A Gas Chromatography System (Agilent Technologies, Santa Clara, CA) using a DB-250 column (Agilent Technologies, Santa Clara, CA).

Bioconversion efficiency

Thermochemical pretreatment and enzymatic conversion efficiency

Thermal dilute-acid pretreatments of increasing severity were performed in triplicate on all ground maize stalk samples (Table 3). Reactions were carried out using 25 mL custom built stainless steel high-pressure reactors equipped with a K-type thermocouple and a 12 cm stainless steel thermocouple probe. Biomass samples (500 mg) were contained inside heat/acid resistant nylon filter bags (ANKOM Technology Corporation, Fairpoint, NY) which allowed for easy biomass transfer while preventing biomass losses during processing reactions. During pretreatments, two separately controlled oil baths were employed; the first one -set at 180 °C- was used to rapidly heat up reactors, while the second bath was used to control reactions at the desired temperature. Depending on the conditions, target temperatures were typically reached between 3-5 minutes. To maintain the temperature within +/- 1.0 °C of the target temperature, reactors were either manually hoisted from the oil bath or re-submerged in the higher-temperature oil bath when necessary. After the desired treatment time, reactions were rapidly quenched by plunging the reactors in an ice-water bath. Pretreatment liquors were collected for further chemical analyses, and biomass samples were used for enzymatic saccharification analyses.

Table 3. Thermochemical parameters used for the pretreatment of stem material of 23 maize silage hybrids diverging in cell wall digestibility

Processing Severity	Temperature	Duration	Acid Loading[¥]	Solids Loading[§]
Low	150 °C	30 min.	0.07%	3.3 %
Low-Mid	150 °C	20 min.	0.17%	3.3 %
Mid-High	175 °C	10 min.	0.17%	3.3 %
High	180 °C	10 min.	0..34%	3.3 %

[¥] 98% H_2SO_4 (w/v%)

[§] Pretreatment-slurry solids to liquid ratio (w/v%)

Analysis of pretreatment liquors

After thermal dilute-acid pretreatment, pretreatment liquors were filtered through a 0.45 μm syringe filter. Monomeric glucose release (Glu-Sol) was analyzed using a Dionex High Pressure Liquid Chromatography system (Dionex, Sunnyvale, CA) equipped with a CarboPac Pa100 column (Dionex, Sunnyvale, CA). Furfural (FUR) and 5-(hydroxymethyl)furfural (HMF) concentrations were analyzed using a Waters HPLC-PDA (Waters Associates, Milford, MA) equipped with an Altima HP C18 (5μm) column (Alltech, Deerfield, IL).

Enzymatic hydrolysis

Enzymatic saccharification efficiency traits were analyzed by means of the National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedure-009 assay [31] after dilute acid/high temperature pretreatment. Briefly, pretreated samples contained within nylon filter bags were treated with 250 μL of an Accelerase 1500 cellulolytic enzyme cocktail (Genencor B.V., Leiden, NL) in 40 mL 0.1 M citrate buffer. The enzyme load provided 50 filter paper units (FPU) of cellulase per gram cellulose. Samples were then incubated at 50 °C in an Innova 42 air incubator (New Brunswick Scientific, Enfield, CT) at 200 RPM for 24 hrs. Enzymatic saccharification liquors were analyzed for glucose content using a Boehringer Mannheim D-Glucose kit (Boehringer Mannheim, Indianapolis, IN, USA). The colorimetric assay was adapted to a 96 micro-titer plate format, and spectrophotometric reads were made using a Bio-Rad 550 Micro-plate Reader (Bio-Rad, Richmond, CA). For all samples, glucose content was expressed as both, the amount of glucose released from one gram of dry biomass (Glu-Rel) and the percentage of total cell wall glucose released after enzymatic saccharification (Glu-Con) (Table 2).

Statistical Analyses

General analysis of variance (ANOVA) was used to determine the significance of entry differences in stem fiber and cell wall components, as well as bioconversion parameters. For bioconversion parameters, the statistical significance of the variation observed across the set of genotypes was also estimated separately for each of the 4 processing conditions evaluated. Pearson correlations between bioconversion parameters and stem fiber and cell wall components were also independently determined for each pretreatment condition analyzed. All statistical analyses were performed using the GenStat for Windows 14th Edition Software Package (VSN International, Hemel Hempstead, UK).

Results and Discussion

Commercialized forage maize displays substantial diversity in cell wall composition and biomass degradability characters

Entries evaluated in this study comprised forage maize cultivars primarily bred for Northern-European markets. Overall, the panel displayed a broad range of variation for ruminal cell wall digestibility (DINAG) as maximal differences between entries amounted to nearly 25 percentage units (Table 1). Henceforth, all commercial cultivars were classified based on their DINAG ratings as either having "Excellent," "Good" or "Poor" cell wall digestibility. The counterparts of 5 proprietary hybrids carrying either the bm3 or cad2 mutation were catalogued as "Cell Wall Mutants."

Highly significant (p<0.001) differences were detected between entries for all investigated cell wall components (Table S2). Accordingly, clear distinctions could be made between the cell wall polymeric profiles of the four distinct cultivar classes (Figure 1). Multivariate analysis reveals that compositional diversity observed across entries could be primarily ascribed to variation in the phenolic and hemicellulosic fractions of their cell walls (PC 1 = 68%). A direct comparison between the "Excellent" and "Poor" classifications confirms that selection in the past for enhanced ruminal digestibility has favored cell walls with reduced lignin content (Lig/CW) and increased hemicellulose concentration (Hem/CW) [32-34,27,35-38]. In addition to these responses, highly digestible cultivars were found to have cell walls with a higher concentration of di-ferulic esters (Di-FA I, Di-FA II), as well as an increased ratio of cell wall arabinose to xylose (DHS); the latter presumed to be indicative of the degree of side-chain glycosylation of glucoronoarabinoxylan (GAX) molecules. In conjunction, higher Di-FA I, Di-FA II and DHS would imply an increased incidence of hemicellulose-to-hemicellulose cross-linking in highly-digestible accessions [39-41]. In our view, maize cell walls with reduced lignin content can restructure their hydrophobic cell-wall matrix by increasing the concentration and rate of cross-linking of GAX molecules to maintain the physical integrity of the cell wall. Incidentally, highly-branched GAX polymers (deemed necessary for a greater extent of cross-linking) exhibit reduced adsorption-affinity to cellulose and improved water-solubility, and have been shown to significantly improve the enzymatic depolymerization of maize cell walls [15].

Cell wall mutants also displayed good-to-excellent cell wall digestibility (Table 1), but in the components bi-plot these did not allocate with the other cultivar classes, nor did they form their own specific group (Figure 1). Presumably, the latter reflects the contrasting genetic effects of the *bm3* and *cad2* mutations (Table 4). Relative to their original hybrid, *bm3* mutants presented prominent reductions (~29%) in lignin content, but also displayed statistically significant decrements in the concen-
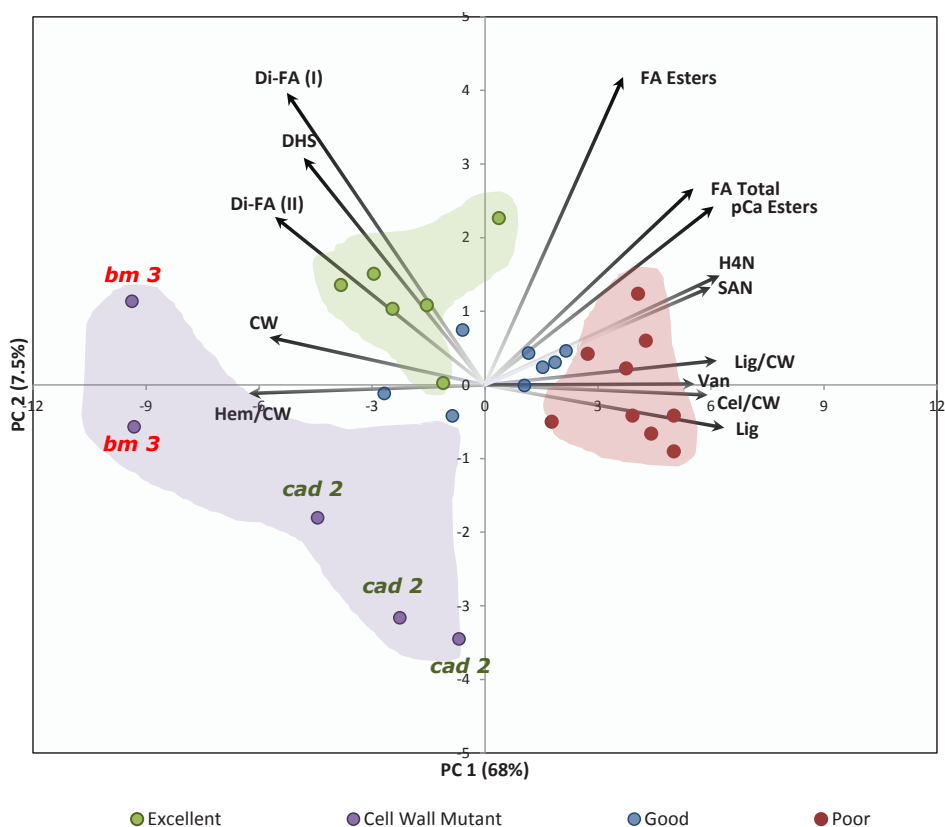
Figure 1. Principle components biplot displaying the classification of a panel of forage maize cultivars primarily bred for Northern-European markets based on stem fiber and cell wall components. Cultivars were classified based on their DINAG ratings as either having "Excellent" (Green), "Good" (Blue) or "Poor" (Red) cell wall digestibility. The 5 proprietary hybrids carrying either the *bm3* or *cad2* mutations were catalogued as "Cell Wall Mutants" (Purple). Black vectors summarize the correlation between relevant feedstock compositional characters and the corresponding principal component.

tration of total ferulic acids (~2%), p-coumaric acids (~31%) and syringyl residues (~56%). The *cad2* mutants displayed similar modification patterns in their cell wall phenolic profile, but there were clear differences with respect to *bm3* mutants as to the components which were more profoundly affected (Table 4). Specifically, *cad2* mutants presented less prominent reductions in lignin content (~17%) and syringyl units (~30%), but displayed a superior decrease in the concentration of total ferulic acids (~8%). Seemingly, while improvements in the cell wall digestibility of *bm3* mutants can be ascribed to reductions in lignin content; higher digestibility in *cad2* mutants appeared to be a product of both, a decrease in lignin concentration and marked reductions in the extent of ferulate-mediated crosslinking between lignin polymers and (possibly) between lignin and hemicellulose.

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Ultimately, targeted reductions in lignin content will potentially remain a pivotal goal of efforts seeking to reduce the enzymatic recalcitrance of maize biomass, but our results confirm that improved cell wall digestibility can be attained through other mechanistic alterations of the plant cell wall. In this regard, Torres *et al.* [15] have shown that the accumulation of multiple beneficial compositional features will expectedly lead to the greatest gains in cell wall enzymatic convertibility in processing for cellulosic fuel. Therefore, the underlying genetic and biochemical foundations controlling the content, composition and cross-linking of non-cellulosic cell wall polymers warrant further investigation, as these open unexplored avenues for the development of novel cell wall polymeric profiles with interesting projections for bio-based applications.

Highly digestible cultivars display improved fermentable glucose yields upon pretreatment and enzymatic saccharification

In this study, the four pre-determined cultivar classes showed statistically significant ($p < 0.05$) differences for bioconversion efficiency (Glu-Con) under nearly all examined pre-treatment conditions; with the only exception ensuing at the harshest processing severity (Table 5). The converged performance of all cultivar groups at highly-stringent regimes was anticipated, given that under such conditions biomass conversion efficiency is primarily determined by the efficacy of the thermochemical process.

Table 4. Comparison of cell wall compositional profiles for 5 commercial maize cultivars and their corresponding cell wall mutant counterpart

	CW	Cel	Lig	Cel/CW	Hem/CW	Lig/CW	pCa I	pCa II	FA I	FA II	Di-FA I	Di-FA II	DHS	H	G	S
BALTIMORE	630	378	63	583	318	99	26.9	25.0	7.57	10.0	0.04	0.12	0.12	3.7	16.3	12.2
BALTIMORE-*bm3*	625	339	41	55.5	377	66	17.4	15.8	7.57	10.0	0.09	0.19	0.14	2.4	6.9	9.6
BANGUY	657	387	64	572	331	97	24.1	22.6	7.20	9.85	0.06	0.17	0.13	3.3	14.1	11.6
BANGUY-*bm3*	638	353	46	560	368	72	17.8	16.7	7.44	9.58	0.10	0.18	0.13	2.6	6.5	10.0
ATRIUM	682	409	68	587	312	100	27.1	25.0	8.31	10.70	0.04	0.11	0.11	38.0	15.1	11.5
ATRIUM-*cad2*	690	399	61	573	340	88	19.7	17.9	7.57	9.73	0.04	0.11	0.11	2.8	11.6	10.2
LG30218	672	410	72	590	304	106	29.5	27.3	8.16	10.70	0.04	0.11	0.11	4.2	16.8	12.7
LG30218-*cad2*	684	388	58	563	353	85	17.9	16.5	7.43	9.59	0.06	0.15	0.12	2.6	10.7	10.1
LG30216	704	435	81	594	292	114	32.1	30.0	7.83	10.71	0.01	0.08	0.10	4.8	18.2	15.1
LG30216-*cad2*	678	397	64	580	326	94	23.0	21.1	7.52	9.89	0.03	0.08	0.10	3.4	13.1	12.4
S.E.M.	9	8	2	5	6	2	0.9	0.8	0.08	0.11	0.01	0.01	0.01	0.1	0.5	0.3

Table 5. Conversion performance of four distinct cultivar classes for Glu-Con (%) across pretreatments of increasing severity

Digestibility Rating	Pretreatment Severity			
	Low	Low-Mid	Mid-High	High
Cell Wall Mutant	31%	60%	79%	88%
Excellent	31%	58%	78%	89%
Good	30%	49%	68%	86%
Poor	25%	45%	65%	84%
F probability [a]	**	***	**	NS

[a] Significance of differences between digestibility classes determined at p<0.05 (*), p<0.01(**) and p<0.001(***); NS indicates non-significant differences.

Figure 2A depicts the performance of the four entry classes for Glu-Con across the complete pretreatment series, and demonstrates that the "Excellent" and "Cell Wall Mutant" classifications consistently outperformed classes with lower cell wall digestibility. From the onset of this investigation, we hypothesized that entries exhibiting improved forage quality would also display higher enzymatic convertibility upon thermochemical processing. This assumption was endorsed by observations demonstrating that ruminal and industrially-driven cell wall depolymerization processes share similar underlying biochemical mechanisms [15,6,13,12,42]. Congruent with these asseverations, under sub-optimal processing, Glu-Con correlated negatively ($r < -0.50$) with all lignin-related traits; but associated positively ($r > 0.4$) with characters defining the concentration, extent of glycosylation and degree of cross-linking of hemicelluloses (Figure 3).

Correspondingly, entries presenting improved enzymatic convertibility (both as DI-NAG or Glu-Con) typically displayed a higher absolute release of fermentable glucose upon enzymatic conversion (Table 6; Figure 2B). However, while higher bioconversion efficiency (Glu-Con) consistently led to superior productivities (Glu-Rel), a strictly proportional relationship between the two could not be established. To better illustrate, whereas the "Excellent" and "Cell Wall Mutant" classes of entries displayed similar bioconversion rates (Figure 2A), the latter outperformed the former for the absolute release of fermentable glucose (Glu-Rel) across the complete processing series (Figure 2B). Relative to the "Excellent" class, the class with "Cell Wall Mutant" entries exhibited a higher concentration of cellulose per gram of dry cell wall biomass (376 g Kg^{-1} CW > 358 g Kg^{-1} CW). Expectedly, since both classes showed similar levels of cell wall recalcitrance, the "Cell Wall Mutant" class exhibited higher glucose yields upon enzymatic conversion simply because it had cell walls with a superior concentration of cell wall glucose. Likewise, because all cultivar groups greatly outranked the "Excellent" class for cellulose content (~40.2 g Kg^{-1}

CW), these outperformed the latter at the most intensive processing regime where enzymatic convertibility reaches a near-maximum regardless of compositional differences between genotypes.

Finally, the amount of glucose released during pretreatment (Glu-Sol) is also an important source of fermentable monosaccharides in biomass-to-ethanol conversion systems. Across the complete processing series, the four distinct entry classes displayed significant differences ($p<0.05$) in the amounts of glucose in pretreatment liquors (Figure 2C). These sugars presumably originate from the soluble carbohydrate fraction of the maize stalk, as there was a strong correlation ($r>0.7$) between the latter and glucose concentration in pretreatment liquors. Unlike trends observed earlier, however, glucose yields in pretreatment liquors remained reasonably constant across pretreatments of increasing severity, and only exhibited a slight reduction at the highest processing intensity (Figure 2C).

The technical efficiency of cellulosic fuel refineries is influenced by feedstock processing amenability and crop productivity

Techno-economic and life-cycle assessments of cellulosic fuel refineries have demonstrated that plant size, commercial viability and environmental performance are primarily influenced by the extent of fermentable monosaccharides recovered per area of a feedstock crop [18-24]. This is calculated as the product of the crop's overall biomass productivity (t ha^{-1}) by the total amount of sugars (t t^{-1} DM) released via the conversion process (both in pretreatment and enzymatic saccharification liquors). Given that modeled scenarios ignore the effect of biomass composition on conversion efficiency; these analyses commonly reiterate that improvements in the productivity of cellulosic fuel refineries can be solely realized through increments in the yielding capacity of lignocellulosic feedstocks.

The panel of forage maize cultivars evaluated in this study exhibited highly significant differences ($p<0.001$) in whole-plant biomass productivity (i.e. ear and stover). The maximal contrast across entries for biomass yield was approximately 7 t ha^{-1}. A closer examination reveals, nevertheless, that differences in biomass yield among the three classes of commercial cultivars were reasonably minor; with the "Poor" index ranking highest ($\sim$21 t ha^{-1}), followed respectively by the "Excellent" ($\sim$20 t ha^{-1}) and "Good" ($\sim$19 t ha^{-1}) digestibility selections. By contrast, differences in total biomass yield between the "Cell Wall Mutant" class ($\sim$16 t ha^{-1}) and the average of all commercial cultivars ($\sim$20 t ha^{-1}) were considerably more pronounced. The markedly lower yields observed for mutant hybrid varieties were greatly anticipated as numerous studies have demonstrated the detrimental effects on plant fitness conveyed by the *bm3* and *cad2* mutations [43].
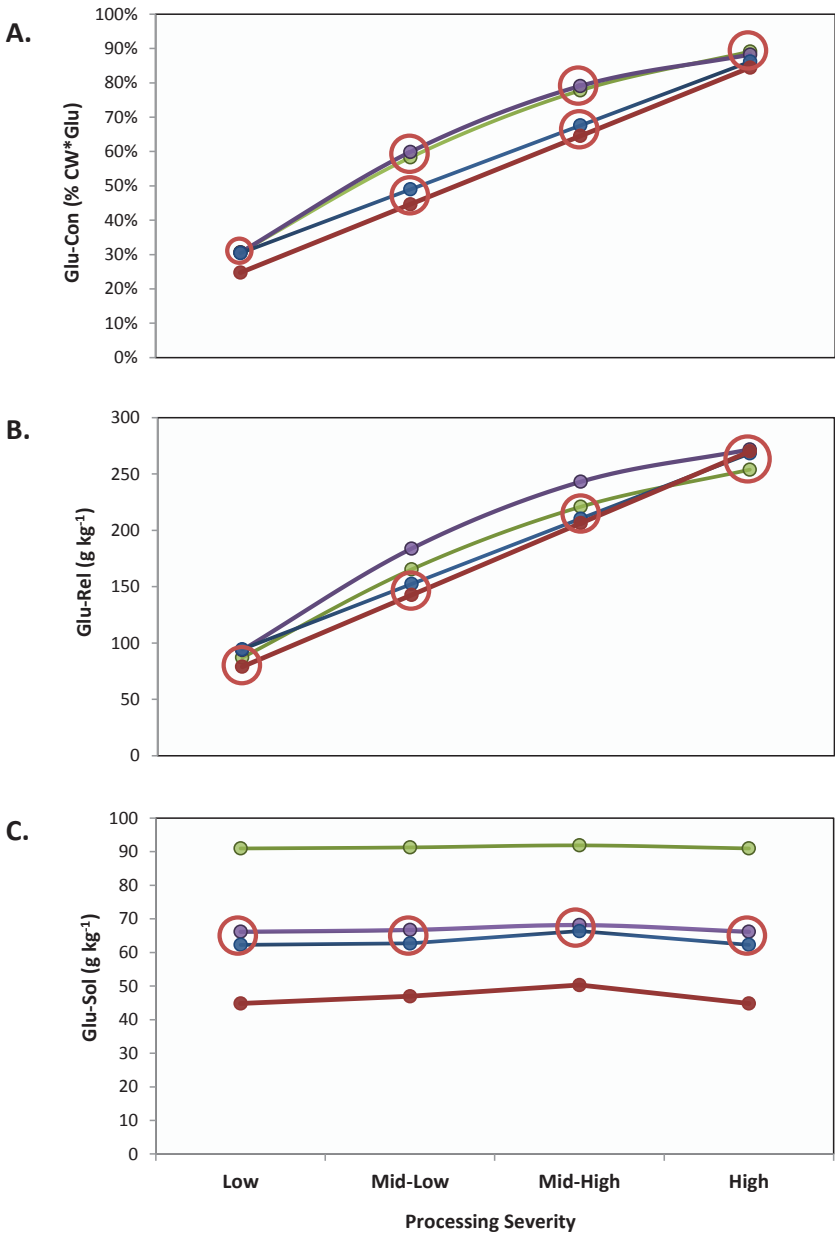
Figure 2. Conversion performance of four distinct cultivar indices (diverging in cell wall digestibility) across pretreatments of increasing severity for (A) Glu-Rel, (B) Glu-Con and (C) Glu-Sol. Encircled data points are not statistically different from each other at p≤0.05. Gluc-Rel is the amount of glucose released from one gram of dry biomass after pretreatment and enzymatic saccharification. Gluc-Con is the percentage of total cell wall glucose released after pretreatment and enzymatic saccharification. Glu-Sol is the absolute amount of glucose released from one gram of dry biomass into pretreatment liquors following thermochemical processing.
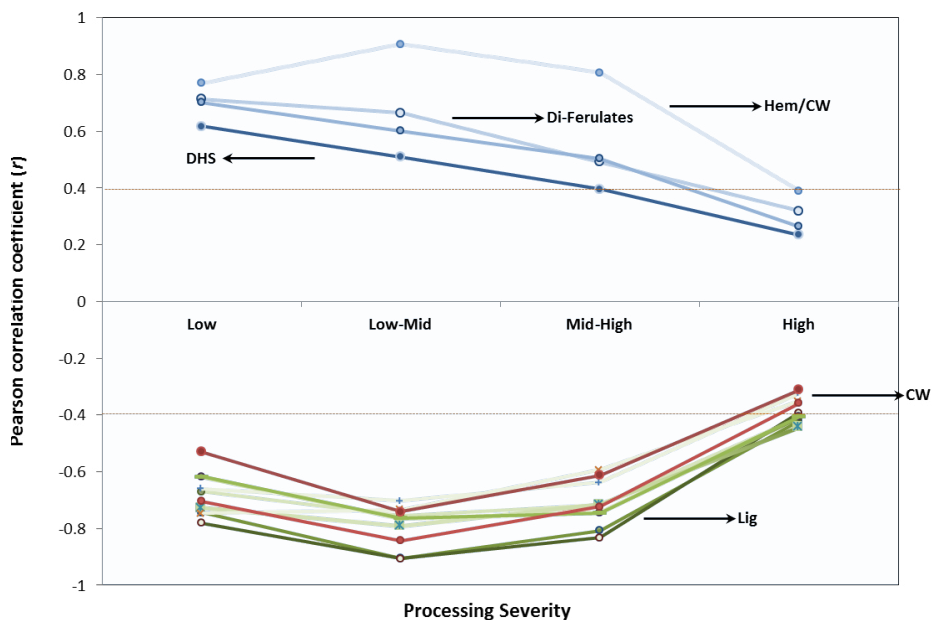
Figure 3. Progression of correlation patterns between relevant maize compositional features and Gluc-Con across dilute-acid pretreatments of increasing severity. Correlations are statistically significant at $r \geq 0.4$ or $r \leq -0.4$.

Table 6. Conversion performance of four distinct cultivar classes for Glu-Rel (g Kg^{-1} DM) across pretreatments of increasing severity

Digestibility Rating	Pretreatment Severity			
	Low	Low-Mid	Mid-High	High
Cell Wall Mutant	93	184	243	271
Excellent	87	165	220	254
Good	94	152	210	268
Poor	79	142	206	270
F probability [a]	*	***	**	NS

[a] Significance of differences between digestibility classes determined at p<0.05 (*), p<0.01(**) and p<0.001(***); NS indicates non-significant differences.

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The panel of forage maize cultivars evaluated in this study exhibited highly significant differences (p<0.001) in whole-plant biomass productivity (i.e. ear and stover). The maximal contrast across entries for biomass yield was approximately 7 t ha^{-1}. A closer examination reveals, nevertheless, that differences in biomass yield among the three classes of commercial cultivars were reasonably minor; with the "Poor" index ranking highest (~21 t ha^{-1}), followed respectively by the "Excellent" (~20 t ha^{-1}) and "Good" (~19 t ha^{-1}) digestibility selections. By contrast, differences in total biomass yield between the "Cell Wall Mutant" class (~16 t ha^{-1}) and the average of all commercial cultivars (~20 t ha^{-1}) were considerably more pronounced. The markedly lower yields observed for mutant hybrid varieties were greatly anticipated as numerous studies have demonstrated the detrimental effects on plant fitness conveyed by the *bm3* and *cad2* mutations [43].

In general, biomass productivity correlated negatively ($r \leq -0.6$) with cell wall degradability and bioconversion properties (DINAG, Glu-Con, Glu-Rel). From a commercial standpoint, this would tacitly imply that gains in productivity arising from the use of bioenergy feedstocks with improved processing amenability (Glu-Con, Glu-Rel) would be potentially offset by tradeoffs in biomass yield capacity. Consequently, to explore the dynamics of yield-by-quality relations, we have estimated total glucose productivity per hectare (TGP) for all examined entries across all evaluated conditions (Table S3). TGP was calculated as the sum of fermentable glucose recovered in pretreatment (Glu-Sol) and enzymatic saccharification liquors (Glu-Rel) times "lignocellulosic biomass productivity" on a hectare basis (t ha^{-1}). Since we only had at our disposition whole-plant biomass productivity values, "lignocellulosic biomass productivity" was estimated based on the rule-of-thumb assumption that the stover to grain ratio in maize is 1:1 [1]. Clearly, this assumption does not take into consideration that this ratio may vary among cultivars.

Overall, the four divergent cultivar classes exhibited statistically significant (p<0.05) differences in TGP across all evaluated processing conditions. The "Excellent" cultivar selection consistently and prominently outperformed all other cultivar indices; although at the most intensive processing regime, the aforementioned selection did not differ significantly from the "Poor" digestibility class (Figure 4). Under suboptimal processing, contrasts in TGP amongst the "Good," "Poor" and "Cell Wall Mutant" classifications were statistically non-significant. In principle, these results demonstrate that systematic gains in cell wall degradability (i.e. DINAG, Glu-Con and Glu-Rel) can lead to significant advances in the productivity (TGP) of cellulosic fuel biorefineries, but only given suboptimal processing scenarios. Moreover, this is only valid if genetic advances in cell wall degradability properties have not been counteracted by substantial biomass yield reductions. For instance, since the "Excellent" and "Poor" cultivar selections exhibited similar biomass productivities (~20 t ha^{-1}),

the competitive advantage in TGP displayed by the former can be directly attributed to its improved processing amenability (Glu-Con, Glu-Rel) and higher content of stalk soluble glucose. By contrast, the substantially enhanced bioconversion efficiencies displayed by mutant hybrid varieties (Figure 2B) were counterbalanced by their greatly inferior biomass productivities ($\sim$16 t ha^{-1}).

Contrary to the common opinion today, our results ultimately demonstrate that genetic gains in biomass degradability and processing quality do not necessarily come at the expense of substantial yield reductions. In fact, some of the highest ranked commercial cultivars for cell wall digestibility (i.e. 210P106790 and 210RI13169) were also among the highest yielding genotypes in the entire set ($\sim$21 t ha^{-1}). Furthermore, recent investigations have demonstrated that biomass quality, biomass productivity and grain yield are not mutually antagonistic breeding targets, and can in fact be improved independently [44-47,13,36,38,48,27]. The use of interesting mutations, however, needs further investigation as it is clear that their introgression in elite material can affect biomass productivity.
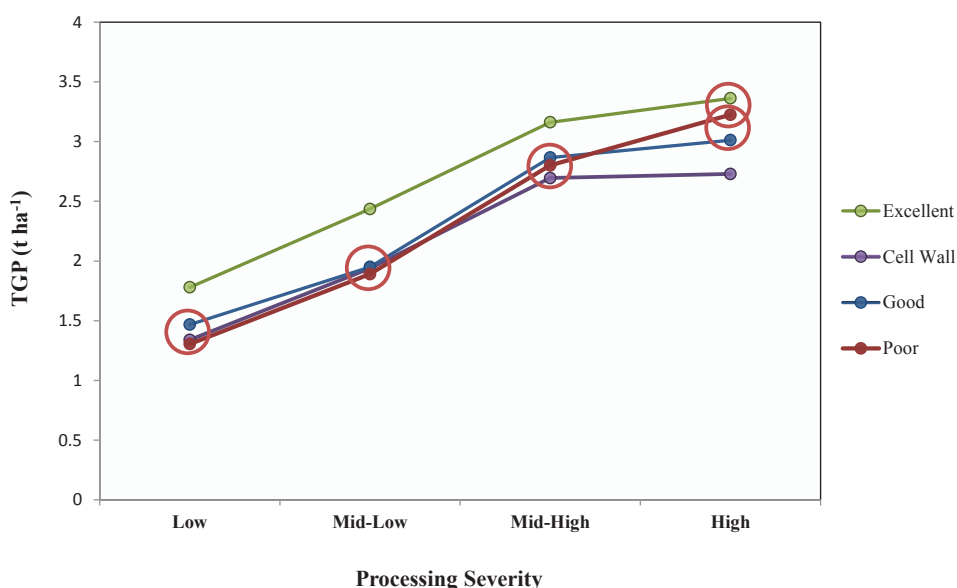

Figure 4. Performance of four distinct cultivar indices (diverging in cell wall digestibility) across pretreatments of increasing severity for total glucose productivity (TGP) per hectare. Encircled data points are not statistically different from each other at $p \leq 0.05$.

A conceptual framework advocating the advance of bioenergy crops with improved processing amenability

Given that the product value of cellulosic fuels will be primarily determined by the efficiency of the manufacturing process in relation to production costs, the ultimate goal of the cellulosic fuel industry resides on attaining maximum biomass conversion efficiency at the lowest conceivable processing intensity. Our conceptual vision explains that the realization of this commercial objective can be achieved through the development of plant feedstocks with improved biomass processing amenability. To accentuate this vision, we have evaluated the economic and environmental advantages that could stem from the wide-scale implementation of cellulosic fuel refineries operating under mild processing regimes. Our conceptual analysis is based on the Low-Mid processing scenario as it offers a combination of favorable technical advantages, including important reductions in processing stringency, high TGP and a significantly reduced production of fermentation inhibitors (Figure 5).
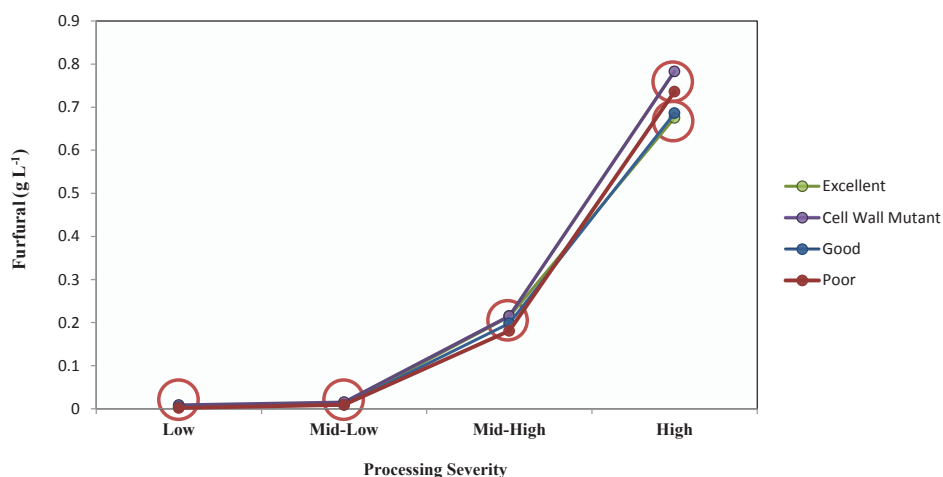

Figure 5. Performance of four distinct cultivar indices (diverging in cell wall digestibility) across pretreatments of increasing severity for furfural production. Encircled data points are not statistically different from each other at $p \leq 0.05$.

Presently, the costs of energetic and chemical utilities (including cellulase consumption) for a cellulosic ethanol refinery with a 250,000 t yr^{-1} processing capacity are at approximately 17 ¢ L^{-1} EtOH; wherein total ethanol production costs are set at 83 ¢ L^{-1} [49,50]. Relative to the most effective processing regime, the Low-Mid processing scenario conveyed a 50% reduction in sulfuric acid consumption and a reduction in pretreatment processing temperature of 30°C (i.e. from 180°C to 150°C). Important savings on chemical utilities are also expected from reductions in cellu-

lase consumption, as several investigations have indicated that bioenergy crops with reduced lignin content typically necessitate lower concentrations of cellulolytic enzymes for their complete and effective fractionation [10]. From a commercial standpoint, however, substantial improvements in the product value of cellulosic fuels are only possible if relevant manufacturing cost reductions are accompanied by gains in fermentable monosaccharide productivity (TGP). At the Low-Mid processing scenario, maximum TGP (2.9 t ha^{-1}; which corresponded to cultivar LG210P) was only ~80% of the highest TGP achieved within the framework of this investigation (~3.7 t ha^{-1}; Table S3). However, if breeding would allow for the combination of the best characteristics available in the entry panel (i.e. highest concentration of stem soluble glucose, hollocellulose content, enzymatic convertibility and biomass yields), then maximum TGP at Low-Mid conditions (~3.7 t ha^{-1}) would correspond to 100% of the highest realizable yields. Under these provisions, the product value of ethanol at Low-Mid processing regimes would logically outperform the current standard. Expectedly, further gains in TGP (beyond maximum realizable yields) at Low-Mid processing conditions are conceivable from genetic advances in biomass yielding capacity and lignocellulose degradability deriving from our improved understanding of the plant cell wall and the exploitation of previously neglected germplasm.

The most important, albeit less apparent benefits arising from the development of feedstocks with reduced lignocellulose recalcitrance correspond to possible tradeoffs in capital investments associated to improvements in pretreatment and downstream processing technologies. To begin with, since highly degradable feedstocks require lower sulfuric acid and temperature usage for their thermochemical fractionation, the industry could potentially move to less costly reactors with lower-corrosion and heat-deformation resistance [50]. Diminished acid consumption during pretreatment also conveys a diminution in the quantity of alkali usage and salt formation during slurry neutralization, as well as a markerd decrement in the formation of fermentation inhibitors (Figure 5). Ultimately, these technical benefits can lead to cutbacks in the throughput, size, and cost of downstream equipment (including waste treatment, slurry neutralization systems and slurry separation systems), or facilitate the integration of consolidated bioprocessing technologies; the latter expected to greatly reduce operational and capital costs [50,22,51,20].

The realization of an industry that operates at "suboptimal" processing conditions requires that monosaccharide yields recovered from a hectare of established feedstock compete or outperform current realizable productivities. The lingering question remains: how should the industry proceed? Certainly, the development of cellulosic fuel refineries with an improved economic viability and environmental footprint will require an integrative scientific approach. In our vision, the development of advanced lignocellulosic feedstocks for the industry will benefit from

parallel developments in enzyme and fermentation technologies which maximize the yield and conversion of all fermentable biomass components. In this regard, numerous studies have demonstrated that at mild thermochemical pretreatments, the complementation of cellulolytic cocktails with specialized xylan degrading enzymes greatly improves the release of monomeric xylose and enhances cellulose conversion [52-54]. Similarly, the derivation of pentoses into added-value ethanologens is seen by experts as a crucial step towards improving the productivity and product value of cellulosic fuels [55-57]. Breeders can simultaneously complement and potentiate these advances by creating cultivars with improved conversion efficiency, higher hemicellulose content and competitive biomass yields. Ultimately, because cellulosic refinery sizes are constrained by the poor performance figures on economics and efficiencies of current conversion technologies, improvements in productivity and cost performance deriving from the utilization of advanced bioenergy crops can lead to a re-conceptualization of plant size and geographical distribution. After all, the decentralization of cellulosic fuel production not only allows for the diversification of rural economies, but also improves the overall environmental performance of the industry.

Conclusions

In this investigation, we have developed a conceptual framework demonstrating how the development of bioenergy crops with reduced lignocellulose recalcitrance can provide an important economic boost for the cellulosic fuel industry. Overall, our results suggest that systematic changes in cell wall composition leading to improved cell wall digestibility are advantageous for cellulosic fuel production, especially if "suboptimal" processing regimes are favored for further development. We have demonstrated that if breeding would allow for the combination of the best characteristics available in modern germplasm (i.e. high biomass productivity, high hollocellulose content, and improved enzymatic convertibility of cell walls), it should be principally possible to surpass the productivity of currently available biomass-to-fuel conversion systems using more cost-effective and sustainable conversion platforms. The concerted development of advanced bioenergy feedstocks and sustainable processing technologies will prove fundamental to the wide-scale commercialization and decentralization of cellulosic fuel production.

Ultimately, while our projections are optimistic, these are based on empirical data obtained from lab-scale experiments which might not entirely reflect the chemical and energetic reality of the industry. For this reason, our results are bound to draw criticism, but they can also raise interest and debate as to how the cellulosic fuel industry as well as the breeding industry should proceed in the coming years. In this regard, a multidisciplinary approach that converges the strengths of plant, biotechnological and processing disciplines will be instrumental to the advanced of cellulosic fuels which maximize all, environmental, economic and societal benefits.

Table S1. Near infrared reflectance spectroscopy (NIRS) calibration statistics for maize stover cell wall composition traits

Trait	Unit	N [a]	Mean	Min.	Max.	R^2	SEC	SECV
CW	g Kg^{-1} DM	710	61.37	40.73	82.00	0.93	1.81	1.84
ADF [b]	g Kg^{-1} DM	357	36.16	20.12	52.19	0.94	1.34	1.39
Lig	g Kg^{-1} DM	337	3.96	0.58	7.35	0.59	0.73	0.76
pCa I	g Kg^{-1} CW	1563	15.60	1.45	21.72	0.87	1.23	1.26
pCa II	g Kg^{-1} CW	1494	11.54	2.24	20.83	0.88	1.09	1.13
FA I	g Kg^{-1} CW	1561	5.55	3.01	8.08	0.64	0.51	0.52
FA II	g Kg^{-1} CW	1499	6.90	4.21	9.60	0.66	0.53	0.54
Di-FA I	g Kg^{-1} CW	516	0.15	0.03	0.27	0.66	0.02	0.03
Di-FA II	g Kg^{-1} CW	514	0.33	0.11	0.54	0.61	0.04	0.05
H	g Kg^{-1} CW	1048	1.43	0.00	3.10	0.74	0.28	0.30
G	g Kg^{-1} CW	1051	6.38	1.30	11.46	0.60	1.07	1.11
S	g Kg^{-1} CW	1054	7.22	0.41	14.03	0.79	1.03	1.09

[a] N is the number of samples analyzed for development of calibration equation; R^2 is the coefficient of determination between laboratory analysis and NIRS prediction, SEC is the standard error of calibration and SECV is the standard error of cross-validation predictions.

[b] Acid detergent fiber.

Table S2. Comparison of cell wall compositional profiles for a panel of commercial silage maize cultivars and experimental mutant counterparts of five cultivars

	CW	Cel	Lig	Cel/CW	Hem/CW	Lig/CW	pCa I	pCa II	FA I	FA II	Di-FA I	Di-FA II	DHS	H	G	S
2-04R00015	613	363	54	338	574	88	25.9	24.4	7.67	9.73	0.08	0.16	0.13	3.4	15.3	10.7
210P106790	566	330	52	347	562	92	25.8	24.1	7.68	9.76	0.07	0.12	0.12	3.3	15.5	11.9
210RI13169	675	373	70	318	579	103	28.4	26.4	8.39	10.86	0.07	0.12	0.12	3.8	15.2	12.0
210XX08399	631	374	62	324	578	98	27.3	25.7	7.48	9.81	0.05	0.11	0.13	3.7	16.7	12.3
AASTAR	598	351	55	339	570	91	26.2	24.1	8.22	10.35	0.06	0.14	0.12	3.6	14.7	11.5
ATRIUM	682	409	68	587	312	100	27.1	25.0	8.31	10.70	0.04	0.11	0.11	3.8	15.1	11.5
FANTASTIC	693	401	79	305	582	113	29.8	28.1	7.92	10.72	0.05	0.12	0.12	4.2	16.8	13.4
FORMULA	632	411	64	324	575	101	26.6	24.9	7.97	10.26	0.06	0.13	0.12	3.5	15.4	11.2
LG30218	672	410	72	590	304	106	29.5	27.3	8.16	10.70	0.04	0.11	0.11	4.2	16.8	12.7
BALTIMORE	630	378	63	583	318	99	26.9	25.0	7.57	10.0	0.04	0.12	0.12	3.7	16.3	12.2
BANGUY	657	387	64	572	331	97	24.1	22.6	7.20	9.85	0.06	0.17	0.13	3.3	14.1	11.6
AMBROSINI	717	445	85	291	590	119	32.6	30.5	7.94	10.89	0.02	0.09	0.11	4.7	18.6	14.2
LG30217	678	419	75	283	598	119	28.9	27.1	7.81	10.38	0.04	0.13	0.12	3.9	17.1	12.1
LG30216	704	435	81	594	292	114	32.1	30.0	7.83	10.71	0.01	0.08	0.10	4.8	18.2	15.1
RICARDINIO	699	435	76	288	598	114	28.6	26.7	7.78	10.42	0.04	0.11	0.12	4.0	16.4	13.2
GROSSO	715	432	85	306	585	109	32.7	30.9	7.78	10.73	0.02	0.07	0.11	4.5	19.0	13.5
SECURA	705	435	78	293	597	110	32.6	30.4	8.48	11.31	0.05	0.10	0.11	4.5	16.4	12.9
SUPERBE	714	438	81	294	592	114	32.5	30.5	8.05	11.01	0.03	0.10	0.11	4.6	18.9	14.1
BALTIMORE-*bm3*	625	339	41	55.5	377	66	17.4	15.8	7.57	10.0	0.09	0.19	0.14	2.4	6.9	9.6
BANGUY-*bm3*	638	353	46	560	368	72	17.8	16.7	7.44	9.58	0.10	0.18	0.13	2.6	6.5	10.0
ATRIUM-*cad2*	690	399	61	573	340	88	19.7	17.9	7.57	9.73	0.04	0.11	0.11	2.8	11.6	10.2
LG30218-*cad2*	684	388	58	563	353	85	17.9	16.5	7.43	9.59	0.06	0.15	0.12	2.6	10.7	10.1
LG30216-*cad2*	678	397	64	580	326	94	23.0	21.1	7.52	9.89	0.03	0.08	0.10	3.4	13.1	12.4
S.E.M.	9	8	2	5	6	2	0.9	0.8	0.08	0.11	0.01	0.01	0.01	0.1	0.5	0.3

Table S3. Comparison of total glucose productivity (in t ha⁻¹) across pretreatments of increasing severity for a panel of commercial silage maize cultivars and experimental cell wall mutants

	TGP (t ha⁻¹)			
	Low	Low-Mid	Mid-High	High
2-04R00015	1.8	2.3	3.1	3.1
210P106790	2.1	2.9	3.6	3.7
210RI13169	1.6	2.2	2.8	3.4
210XX08399	1.6	2.3	3.1	3.2
AASTAR	1.7	2.5	3.1	3.4
ATRIUM	1.5	2.0	3.0	3.1
FANTASTIC	1.3	1.7	2.5	2.9
FORMULA	1.6	2.1	2.7	3.0
LG30218	1.3	1.9	3.0	3.0
BALTIMORE	1.6	2.2	3.2	3.4
BANGUY	1.5	1.8	2.8	2.9
AMBROSINI	1.2	1.8	2.6	3.2
LG30217	1.1	1.7	2.4	3.1
LG30216	1.4	2.2	3.2	3.5
RICARDINIO	1.4	1.8	2.8	3.3
GROSSO	1.5	2.0	3.0	3.2
SECURA	1.2	1.9	2.7	3.2
SUPERBE	1.2	1.8	2.9	3.2
BALTIMORE-*bm3*	1.7	2.3	3.0	2.8
BANGUY-*bm3*	1.4	1.9	2.5	2.5
ATRIUM-*cad2*	1.2	1.7	2.5	2.6
LG30218-*cad2*	1.3	1.9	2.8	2.9
LG30216-*cad2*	1.2	1.8	2.8	2.8

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Chapter 7

General Discussion

Introduction

Without doubt, the greatest impediment towards the wide-scale realization of renewable cellulosic fuels resides in our inability to effectively deconstruct plant biomass into added-value commodities [1-3]. Plant lignocellulose has evolved to resist chemical and enzymatic deconstruction, and its conversion into liquid fuels requires energetically stringent processes that render the industry economically and environmentally unviable at this point in time [1-5]. A crucial and promising strategy to address this challenge entails the development of advanced bioenergy crops which require lower energetic and chemical inputs for their effective deconstruction. At its core, this approach requires an in-depth understanding of the composition, synthesis and breeding amenability of the plant cell wall; the principal constituent of total plant dry biomass and generally the most recalcitrant fraction of the crop at physiological maturity [6, 7].

In this thesis, we have dissected and elucidated the biochemical and genetic mechanisms controlling cell wall characteristics relevant to the development of "dual-purpose" maize with improved processing quality for cellulosic ethanol production. A focus on maize was warranted, as it currently represents the *de facto* model system for translational research into the development and domestication of C4 bioenergy perennials with greater technical and economic prospects in emerging bioenergy applications [4, 7]. In the following sections, knowledge generated in this research is used to identify possible conceptual bottlenecks and to evaluate the technical feasibility and commercial relevance of breeding activities seeking to advance bioenergy grasses with cell wall polymeric profiles tailored to the demands of the cellulosic fuel industry.

Advancing bioenergy grasses with improved processing amenability is a realistic undertaking

Prospects for the development of "dual-purpose" maize

As the largest crop worldwide in terms of total acreage [8], maize is expected to play an essential role in the development and wide-scale commercialization of cellulosic fuel technologies [3, 7]. This requires the breeding of maize as a "dual-purpose" crop displaying optimal grain yield, high stover productivity and improved biomass processing quality [9].

Numerous studies have demonstrated that the parallel advance of grain yield and stover characteristics is a feasible undertaking [10-18]. For instance, historical surveys of maize productivity in the U.S. Corn Belt indicate that gains in grain yield over a 70-year span (1930-1990) have been accompanied by significant increments

(~0.7% yr^{-1}) in stover productivity [11, 12]. Likewise, Lewis and coworkers [13] have shown that grain yield, agronomic fitness and stover quality are not mutually antagonistic breeding targets. In fact, the authors reported favorable genotypic correlations (r = 0.36) between grain yield productivity and biomass conversion efficiency (*i.e.* cellulose convertibility following dilute-acid pretreatment and enzymatic saccharification). In our genetic assessment of a forage maize DH population (**Chapters 4 and 5**), correlations between stover productivity and relevant bioconversion parameters were slightly unfavorable (r = -0.1), but statistically insignificant. By contrast, in a panel of forage maize hybrids primarily bred for Northern-European markets (**Chapter 6**), whole-plant biomass productivity correlated negatively and strongly (r ≤ -0.6) with cell wall degradability properties. Notwithstanding, the panel also included commercial hybrids (*e.g.* LG210P1; DINAG = 34%; Yield = 21 t ha^{-1}) displaying outstanding ruminal cell wall digestibility, and whole-plant productivities similar to those of the highest yielding cultivars (*e.g.* GROSSO; DINAG = 17%; Yield = 22 t ha^{-1}).

Ultimately, the amenable nature of genetic variation underlying relevant cell wall characteristics prescribes that advancing biomass processing quality is theoretically simpler than improving grain or stover productivity [13]. Above all, cell wall degradability and bioconversion efficiency appear to be stable and highly heritable traits (h^2 > ~0.5) across different populations, and would therefore constitute excellent selection criteria for immediate use in modern maize breeding programs [14, 24, **Chapters 4 and 5**]. This asseveration is congruent with previous studies emphasizing on the highly heritable nature of maize ruminal cell wall digestibility properties across multi-location and multi-year trials [13, 16, 18-24]. In this regard, significant genetic gains for improved stover bioconversion can be expected through phenotypic selection, as the latter has proven fundamentally successful in the development of silage cultivars displaying highly divergent degrees of ruminal cell wall digestibility [10, 18, 21]. To illustrate, across the aforementioned panel of silage-dedicated maize hybrids, maximal variation for ruminal cell wall digestibility (DINAG) between entries was remarkable and amounted to nearly 23 percentage units (**Chapter 6**). Also relevant, because genetic variation for complex cell wall characteristics appears to be predominantly additive [24, **Chapters 4 and 5**], preliminary selection at the inbred level will lead to correlated genetic gains at the hybrid level; thereby minimizing the need for recurrent test-cross procedures and evaluations (**Chapter 5**).

Certainly, the convergence of classical selection schemes with inexpensive genotyping, advanced biometric models, high-throughput cell wall phenotyping and doubled haploid (DH) production technologies can accelerate development and commercial release of maize cultivars for bio-based applications [25-29]. However, to play a determinant role in the development and realization of sustainable and cost-effec-

tive cellulosic fuel processing technologies, novel dual maize cultivars will have to surpass the performance in lignocellulose processing quality and biomass yields of the best elite germplasm (**Chapter 6**). Conclusively, maximum genetic gains will be expected from bioenergy maize breeding programs focusing on genetic material with substantial levels of favorable variation for relevant biomass productivity and cell wall compositional characters (Box 1). This last observation requires emphatic attention, as several studies have demonstrated the stagnation of genetic gains in cell wall digestibility from breeding endeavors relying on modern germplasm with limited diversity for cell wall compositional and degradability traits [10, 21, 30].

Box 1. The unexplored genetic potential of cell wall degradability and biomass productivity traits in maize

Premature inferences describing the "restricted" genetic potential [13, 14, 24] for advancing biomass quality traits should be pondered with caution. In maize, the extent of genetic variation for biomass quality traits has been greatly undervalued and has therefore remained vastly commercially unexplored [10, 30]. The seed industry's prevalent focus on advancing grain yield and stress tolerance has led to the erosion of genetic variation for cell wall degradability and biomass productivity traits in elite (and intensively commercialized) germplasm [10, 30, 31]. Fortunately, this and numerous other studies have revealed the great extent of variation in biomass quality traits available in forage, exotic or "ancient" maize genetic resources [15, 19-21, 30, 32-34]. As an example, our results demonstrate that forage maize harbors almost twice as much heritable variation (maximal differences were approximately ~30% between population extremes) for the release of cell wall glucose following pretreatment and enzymatic conversion than conventional grain genetic resources (maximal range was 16% for the IBM population, Chapter 4) [14, 24]. Likewise, the rediscovery of useful silage mutations and the targeted production of novel cell wall mutants offer prospective opportunities to expand the genetic basis for cell wall degradability and biomass productivity traits for bioenergy maize [6, 15, 35, 36].

Relevant strategies and tools towards the optimization of biomass processing quality - insights from maize

Historically, breeding endeavors in maize have primarily focused on advancing grain yield and yield stability, and only a minority have specialized on exploiting useful biomass characteristics [10, 12]. In this regard, the wide-scale adoption of complex

biomass degradability and productivity traits in modern maize breeding programs will require a drastic revolution in *de rigueur* commercial operations. In view of this challenge, upcoming bioenergy crop breeding programs should implement the following strategies and tools to guarantee their operational success.

1. Breeding for improved processing quality requires a holistic understanding of the plant cell wall

From the onset of this investigation, we have identified the plant cell wall as the foundation for the genetic improvement of biomass degradability and quality characteristics. One of our pivotal discoveries was the recognition that the optimization of biomass processing quality requires an integral overview of the cell wall with respect to all of its constituent polymers, their interaction with each other and their impact on the ensuing processing technology (**Chapters 3, 4, 5 and 6**).

Until recently, relevant advances in our understanding of cell wall biosynthetic and deconstruction mechanisms have been realized by analyzing and exploring individual cell wall polymers; particularly lignin [37-45]. Incidentally, this has precluded an evaluation of how genetic variation in multiple cell wall components can be simultaneously exploited to produce unique cell wall polymeric profiles of beneficial value for bio-based applications. Since cell wall integrity is defined by the functional interaction of its constituent polymers [46], the mechanistic magnitude of targeted cell wall alterations will be influenced by the content and monomeric composition of untargeted cell wall components.

To exemplify this, in **Chapter 6** we have demonstrated that maize cultivars with highly digestible cell walls have been selected for lower lignin content, but also bred (presumably unknowingly) for increased hemicellulose concentration, a higher degree of glucuronoarabinoxylan (GAX) glycosylation and superior hemicellulose-to-hemicellulose diferulate-mediated cross-linking (Figure 1; **Chapter 6**). In our view, maize cell walls with reduced lignin content appear to restructure their hydrophobic matrix by increasing the concentration and rate of cross-linking of GAX molecules in an attempt to maintain the physical integrity of the cell wall. This would suggest that alleles favoring incremental GAX cross-linking have been fixed in maize breeding populations with increased ruminal digestibility as a correlated response to selection for optimal agronomic characteristics (i.e. improved dry matter yield, stover standability and resistance to stalk rot and corn borer). Correspondingly, we have hypothesized that increments in diferulate cross-links can prove beneficial to the production of dual-purpose maize, despite evidence demonstrating that hemicellulose-to-hemicellulose diferulate bridging impedes the enzymatic depolymerization of cell wall carbohydrates [47-49]. The aforementioned studies focused exclusively on the analysis of the effects of diferulate cross-linking in cell wall systems where

lignin content remained constant; a factor which precluded an in-depth analysis of how different combinatorial profiles of variation for these two component traits affect cell wall physical integrity and industrial quality

Along a similar line of thought, we have demonstrated that biomass conversion efficiency is a highly complex trait, dependent not only on the balance and synergy between multiple cell wall components, but also on the inherent effectiveness of the conversion process (**Chapters 3 and 6**). Concerning the production of cellulosic ethanol via the combined operations of dilute-acid pretreatment and enzymatic saccharification, our results revealed that the chemical mechanisms controlling biomass conversion efficiency vary in relation to pretreatment severity (Figure 3, **Chapter 3**; Figure 2, **Chapter 6**). At highly severe pretreatments (*i.e.* high temperatures and acid loads), cellulose conversion efficiency was primarily influenced by the inherent efficacy of thermochemical cell wall deconstruction, and maximum glucose yields were obtained from cellulosic feedstocks harboring the highest cellulose contents per dry gram of biomass. When mild dilute-acid pretreatments were applied, however, maximum bioconversion efficiency and glucose yields were observed for genotypes combining high stem cellulose contents, reduced cell wall lignin and highly substituted hemicelluloses. Logically, breeding for these two contrasting processing regimes entails distinct selection approaches.

Ultimately, maximum genetic gains in the development of biomass crops with reduced lignocellulose recalcitrance are expected from breeding programs that understand how the ensuing processing technology affects cell wall deconstruction at the molecular level. Such an understanding will prove fundamental towards the definition of an efficient selection strategy to improve cell wall deconstruction under mild processing conditions in order to develop more cost-effective and sustainable cellulosic fuel conversion technologies. Simultaneously, plant breeders should recognize how the cell wall needs to be constructed in order to improve biomass degradability without reducing plant agronomic performance. In this regard, the development of crops with improved cell wall degradability and high agronomic value necessitates a clearer understanding of how functional interactions between different cell wall components can be balanced to maintain primary cell wall functions in the field but provide ease of deconstruction in a processing reactor.

2. High-throughput biomass phenotyping platforms need to be simple and inexpensive

As any plant breeder knows, inexpensive, reliable and accurate phenotyping is fundamental to breeding progress. Incidentally, since the proposed ideotypes and breeding objectives for silage and bio-based maize exhibit numerous parallels [6, 14, 50], the wide-scale implementation of relevant biomass quality traits in modern maize breeding can be achieved by emulating routine phenotyping practices em-

ployed by the small, yet highly efficient forage maize breeding industry.

In commercial breeding of forage maize, on-site and laboratory near infrared spectroscopy (NIRS) methodologies have been routinely employed because of their low costs and outstanding through-puts [6, 12, 13, 24]. Currently, NIRS is utilized for the assessment of complex forage quality characteristics, including the analysis of diverse ruminal cell wall digestibility parameters. In cell wall research for cellulosic ethanol, several studies have also reported on the application of NIRS for the prediction of bulk polysaccharide and lignin content, cell wall neutral sugar composition and biomass conversion efficiency [15, 24, 51]. Notwithstanding, while highly practical, the wide-scale adoption of NIRS methodologies in commercial bioenergy crop breeding endeavors conveys two important caveats. Firstly, persisting difficulties exist to accurately predict highly complex compositional and degradability traits [6, 20, 24, 50]. Thus far, low-to-moderately reliable prediction accuracies have been reported for bioconversion efficiency parameters and cell wall monomeric constituents (i.e. ferulate derivatives, monolignols and neutral sugar components); all of which have been shown to influence the processing amenability of lignocellulosic biomass (**Chapter 2**). Secondly, NIRS predictions are accurate only for populations that have the same spectral characteristics as the training population used to develop NIRS prediction equations [28, 51]. By consequence, the reliability of NIRS predictions is subject to the effects of variation in environment and genetic background on the spectral and compositional characteristics of samples analyzed.

In the last decade, standard biomass compositional quantification methods and bioconversion assays have been successfully down-scaled and automated to accommodate high-throughput analyses via weighing and liquid-handling robotic platforms (Box 2). These analytical platforms have the power to accurately phenotype complex biomass characteristics; but their costs for implementation and operation greatly surpass those of NIRS technologies (**Chapter 2**). To illustrate, in a recent study, Massman *et al.* [28] claimed that the cost of phenotyping maize biomass for cell wall compositional characters and bioconversion efficiency was $153 per sample with wet chemistry, but less than $5 per sample with NIRS. For the time being, NIRS methodologies are the uncontested front-runners for wide-scale adoption in bio-based maize breeding programs, but up-coming high-throughput analytical platforms based on wet-chemistry will prove fundamental in the development of inexpensive marker-assisted selection strategies. These prospects are analyzed in the following section.

3. High-throughput genotyping and marker-assisted selection can expedite development of bioenergy crops

The exponential development of high-throughput sequencing and genotyping plat-

Box 2. High-throughput analyses of bioconversion traits via wet-chemistry

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. Biomass conversion efficiency is generally determined through a standardized saccharification assay (NREL LAP-009) which measures the amount of glucose released from a lignocellulosic substrate following enzymatic hydrolysis [52]. To better represent industrial conditions, conversion assays based on the NREL LAP-009 also include a thermochemical pretreatment emulating one of the industry's leading technologies (AFEX, dilute sulphuric acid, liquid hot water, lime, and soaking in aqueous ammonia). In its original format, the NREL LAP-009 could not be implemented on a commercial scale as it is labor-intensive, low-throughput, and requires copious amounts of substrate and chemicals. In the last decade, however, the development of flexible and highly-accurate weighing and liquid-handling robotic workstations has opened avenues to circumvent these limitations. By now, standard bioconversion assays have been successfully automated and down-scaled to accommodate high-throughput analyses [53-57]. The most sophisticated systems rely on stackable 96-well metallic reactor plates which can withstand the chemical loads, pressure and high-temperatures (> 150 0C) typical of pretreatments used in industrial scenarios [53, 56]. Thus far, automated robotic platforms have not been used for the large-scale analysis of maize germplasm, but successful use were reported for Arabidopsis [55], Miscanthus [58] and Populus [56, 59].

forms is rapidly shifting the research and commercial panorama of maize breeding endeavors [25, 60-63]. In relation to bioenergy crop research, the application of novel molecular-marker technologies in the analysis of variation in agronomic traits using advanced mapping populations (e.g. Intermated B73 x Mo17 (IBM); [64]) and diversity panels (e.g. Maize Nested Association Mapping panel (MNAM); [65]) offers important opportunities to gain a greater understanding of the genetic foundations controlling biomass quality and productivity traits.

Certainly, fine-mapping via high-density molecular linkage maps will help us resolve the identity of genes located in chromosomal hotspots controlling multiple cell wall characteristics and in genomic regions underlying negative inter-relations between useful cell wall variation and undesirable agronomic characteristics. Courtial *et al.* [66, 67] have proposed that the simplest explanation for the positional coincidence of quantitative trait loci (QTLs) controlling diverse cell wall traits would entail the

clustering of tightly-linked genes involved in independent cell wall biosynthetic routes. This preposition, which was founded on observations showing that QTLs with large effects can be fractioned into independent QTLs [67], would be further reinforced by the fact that adverse inter-relations between relevant cell wall traits are never strict [13, 24], and by studies demonstrating that alterations in cell wall composition do not necessarily come at the expense of grain or stover productivity [10-18]. Indeed, if pleiotropic effects minimally affect cell wall biosynthesis, then independent and targeted selection for specific cell wall and biomass productivity characters should be principally possible.

Understandably, projections highlighting the possibility to independently modify relevant cell wall components might be overoptimistic and therefore warrant further investigation. On the one hand, the use of high-resolution genetic maps have led to the understanding that relevant cell wall-related multi-trait QTLs are positioned in the vicinity of centromeres exhibiting low recombination rates [21]. On the other hand, mining for candidate genes located in genomic regions known to influence maize cell wall compositional and degradability properties typically highlight transcriptional regulators as the putative determinants of useful cell wall variation [21, 31, 66]. Given our largely unresolved understanding of the underlying genetic architecture of cell wall biosynthesis, the logical next step will be to further investigate relevant cell wall-related QTLs through association mapping and positional cloning studies. In particular, genome wide association studies in maize offer an unprecedented opportunity to accelerate the elucidation of the genetics of complex biomass characteristics relevant to cellulosic fuel production [68-71], and to identify useful rare alleles of genes controlling complex biomass characters without the need to resort to the construction and analysis of specialized mapping populations. Until this information can be translated into useful breeding tools, combined selection for superior cell wall degradability characters, higher biomass productivity and excellent agronomic performance is probably the most effective selection route in the near-future.

Ultimately, advanced comprehension of the genetics of maize cell wall biosynthesis can be employed in marker-assisted selection schemes for use in commercial breeding. Congruent with previous studies [21, 24], our investigation revealed that the concerted genetic action of all identified QTLs for any given cell wall-related trait could account for a large proportion (~50% - 80%) of observed heritable variation, but never for its entirety. Moreover, given that estimated QTL effects detected in single-cross mapping studies are commonly inflated, it seems very likely that the majority of QTLs underlying useful variation for cell wall characteristics have "minor" effects and are hard to detect even under favorable experimental conditions (e.g. large population size, multiple-replications) [72]. By consequence, since very

few QTLs with moderately large effects have been shown to influence complex cell wall characteristics, the most efficient method for marker-assisted breeding for enhanced processing amenability would entail the utilization of selection procedures centered on detecting and increasing the frequency of favorable QTL alleles with "small-to-moderate" effects in elite maize germplasm.

At present, experts are especially confident on genomic selection (GS) as it offers (at least hypothetically) higher prediction accuracies than traditional marker-assisted selection procedures based on the identification of QTLs with significant effects [25-28, 60]. GS in maize remains prospective [73], but its most promising feature prescribes the capability to reliably and accurately predict complex cell wall compositional phenotypes based on molecular marker data alone [11, 61, 69, 74]. For bioenergy applications, GS would therefore allow for the efficient selection of traits which are too expensive to evaluate via wet-chemistry in a commercial breeding program. The exhaustive characterization of genomic selection training sets via high-throughput cell wall phenotyping technologies based on wet-chemical analysis would certainly offer new dimensions in our capability to predict complex cell wall and biomass productivity characters. To this end, Massman *et al.* [28] explored how diverse biomass compositional and grain yield productivity characters respond to multiple cycles of GS in comparison to marker assisted recurrent selection (MARS). Selections were performed over testcrosses between the IBM recombinant inbred population and a Monsanto proprietary tester, and were based on two categories: i) a "Stover Index" which included biomass conversion efficiency and other cell wall compositional parameters, and ii) a "Yield + Stover Index" which gave equal weight to grain yield and stover quality. The results of this investigation demonstrated that after three cycles of recurrent selection, genetic gains with respect to the aforementioned indices were 14% and 50% higher with GS than for MARS. Notwithstanding, while these results are highly appealing, when analyzing realized gains on individual component traits, the authors did not emphasize (presumably avertedly) on the fact that genetic gains for bioconversion efficiency (i.e. cellulose convertibility following dilute-acid pretreatment and enzymatic saccharification) were similar upon GS and MARS, or were sometimes higher for the latter.

In my view, before the prospects of genomic selection become a reality (as it is still an unproven model), optimistic genetic gains in stover quality characteristics could already be accomplished through classical marker-assisted selection procedures. Currently, maize breeders have at their disposition a wide inventory of relevant cell wall-related QTLs derived from diverse mapping populations with divergent genetic backgrounds [14, 16, 20-22, 24, 66, 67, 75-79]. Notably, these investigations have demonstrated a considerable overlap in genomic areas involved in maize cell wall compositional and degradability traits which are of relevance to cellulosic fuel

production [21, 66, 79]. Correspondingly, in our investigation, all identified QTLs underlying bioconversion efficiency co-localized with cell wall degradability or cell wall compositional QTLs described in other experimental mapping studies (Table S1, **Chapter 4**). In particular, a QTL for bioconversion efficiency (Glu-Con) located at bin 5.03-5.04 appears highly promising, as this genomic region has been found to include QTLs for cell wall digestibility across different genetic backgrounds [21, 79]. Certainly, all these genomic regions are important in cell wall digestibility properties and should therefore be validated in commercial breeding populations or employed for the exploration of useful allelic variants in existing elite germplasm. Such endeavors would particularly benefit breeding programs attempting to introgress useful cell wall and biomass compositional variation into breeding germplasm traditionally bred for grain production.

The way forward: advancing C4 perennials with improved biomass quality

Fast growing C4 perennials, like miscanthus, switchgrass and sugarcane, are currently considered to be the most promising candidates for the industrial production of lignocellulosic biomass [9]. These species are particularly coveted for their high biomass productivity, broad geographic adaptation, superior carbon sequestration and efficient nutrient utilization (**Chapter 2**). With respect to bio-based industrial applications, the wide-scale implementation and commercial success of upcoming perennials will rely on the availability of superior cultivars that increase the competitiveness of the industry, while sustainably meeting projected market volumes [9]. Common breeding objectives, regardless of species or cropping system, include increasing stem biomass yields and reducing the recalcitrance of biomass to industrial processing.

Overall, our experience with maize indicates good prospects for the genetic advance of C4 perennials with improved cell wall degradability properties. These expectations are predominantly founded on observations highlighting the high heritability and malleability of cell wall compositional and degradability traits [13, 18, 24]. Indeed, if the genetic mechanisms underlying cell wall variation in C4 grasses are similar, the genetic advance of biomass quality and productivity characteristics in C4 perennials should also be possible through the exploitation of standing variation via phenotypic selection. By now, a considerable wealth of studies has documented the vast extent of genetic variation in promising C4 perennials with respect to cell wall composition and processing amenability under a diverse array of conversion technologies [45, 80-90]. Correspondingly, in a long-running breeding experiment, the group of Dr. Kenneth Vogel at the University of Nebraska has explored the viability of divergent selection in the development of switchgrass synthetic populations contrasting for *in vitro* dry matter ruminal cell wall digestibility (IVDMD) [45, 80,

90]. After six cycles of recurrent mass selection, the divergently selected populations displayed highly significant differences in cell wall compositional and degradability properties. Notably, when assessing ethanol yield production from hexoses following dilute-acid pretreatment and simultaneous enzymatic saccharification and fermentation, the high-IVDMD synthetic population displayed much higher productivities ($\sim$90 mg g^{-1}) than its low-IVDMD counterpart ($\sim$79 mg g^{-1}) [90]. Results of reiterating phenotypic selection as a valid tool for the advance of C4 perennials with improved processing quality have also been observed for sugarcane [86], but are yet to be reported for miscanthus. The latter, however, has been shown to possess extensive genetic variation for relevant cell wall characteristics among species within the genus, between geographically divergent populations within species and among genotypes within local populations [81-85].

Overall, the prospects to advance C4 perennials with cell wall characteristics tailored to the demands of the cellulosic fuel industry are plentiful. As in the case for maize, the incorporation of accurate and high-throughput screening tools and a holistic understanding of cell wall variation will be deemed necessary to maximize the effectiveness of C4 perennial breeding programs. Moreover, translational genomics represents an important route to accelerate the improvement of desirable traits in undomesticated bioenergy grasses [9]. Given their close evolutionary ties with maize, knowledge acquired on the synthesis, deposition and recalcitrance of the maize cell wall can facilitate the design of efficient marker-assisted selection or gene-transfer strategies aimed at improving the processing amenability of C4 perennial varieties. In particular, comparative genomics can help us to localize orthologs of maize QTLs and relevant cell wall genes involved in the control of useful degradability properties [4, 9]. In fact, advances in our understanding of the maize lignin biosynthesis machinery have already been successfully employed in the development of sugarcane and switchgrass transgenic lines with reduced lignin deposition and improved bioenergy potential [42, 91-93]. It is important to remember, however, that the perennial grasses discussed herein are all wind-pollinated outcrossing species characterized by complex intra-genus and intra-species ploidy levels and unbalanced genome constructions [9]. Therefore, further advances in the breeding of bioenergy-dedicated C4 perennials will strongly depend on the development of dedicated genetic and genomic resources in coming years. In this regard, the experience of the maize breeding industry in successfully translating research into innovation and commercial products can also help to shape the domestication and commercialization of promising C4 grasses.

Overcoming possible hindrances slowing the development of bioenergy grasses with improved biomass quality

The inherent heterogeneity of lignocellulosic biomass

Inferences regarding cell wall composition and degradability properties in biomass species have been traditionally resolved through the characterization of homogenized whole-stem or whole-stover materials. In fact, the terms "cell wall" and "biomass" composition are often, yet erroneously used as interchangeable technical elements. While conventional cell wall analytical methodologies offer numerous practical advantages (especially since fractioning biomass into components is a laborious task), their uncontested adoption might be restricting our capability to design better crops for the cellulosic fuel industry. As presumed in **Chapter 4**, because genetic variation for biomass degradability and bioconversion characters can never be fully explained by variation in "cell wall" composition [21, 24], the occurrence of obviated or unexplored stem anatomical parameters should be given further consideration.

A sizeable wealth of evidence has demonstrated that plant cell wall composition and architecture can vary substantially within the same organism [94-99]. These studies suggest that cell wall diversity is deemed essential to the functional differentiation of plant tissues and organs. Invariably, this has led to the recognition that lignocellulosic biomass is constituted by a contrasting array of heterogeneous components with divergent chemical and physical properties. As an example, Zeng *et al.* [98] have demonstrated that the pith, rind and leaf portions of maize stover greatly differ in their biochemical makeup and degree of enzymatic recalcitrance. In fact, following liquid hot-water pre-treatment and enzymatic hydrolysis, pith fractions exhibited significantly higher cellulose conversion rates than leaf or rind tissues (90%, 80% and 50% cellulose conversion efficiency, respectively).

New directions towards the optimization of biomass processing amenability in bioenergy grasses should encompass breeding models that recognize the inherent heterogeneity of lignocellulosic biomass. I especially advocate breeding strategies targeting both: the reorganization of stems in relation to their constitutive tissues and the concomitant modification of their cell wall composition. For instance, it is possible to envisage the development of bioenergy grasses with thicker stems containing favorable ratios of highly-digestible parenchymal to epidermal/sclerenchymal tissues, while simultaneously optimizing the cell wall constitution of the latter (*e.g.* by increasing lignin deposition or degree of cross-linking) to guarantee optimal nutrient and water uptake, stalk standability and pest resistance. Prospects for such endeavors seem reasonably feasible given recent advances. The combined use of advanced sequencing platforms and powerful mapping populations has yield-

ed preliminary insights into the complex genetic architecture and spatio-temporal transcriptional regulation of useful genetic variation for stem tissue development, organization and compositional differentiation [95, 97, 100]. For instance, in depth analyses of genetic expression profiles covering a wide array of tissues and developmental stages in maize have demonstrated how specific paralogs of genes involved in lignin biosynthesis display organ- and developmental-specific expression patterns [85]. Correspondingly, the group of Dr. Dominique Loque at the Joint Bioenergy Institute in the US has devised clever engineering strategies to control the accumulation and deposition of specific cell wall polymers in divergent stem tissues [101-103]. Through modulations of transcription factors, Yang *et al.* [104] successfully "rewired" the secondary cell wall deposition network of Arabidopsis allowing for the targeted lignification of stem vessels without incrementing lignin deposition in other tissues.

As reasoned earlier, nevertheless, the incorporation of such complex breeding objectives in commercial breeding operations will only be viable if accurate, high-throughput and inexpensive phenotyping platforms should become available. Promising technologies for the compositional analysis of tissue and cell wall compositional heterogeneity on a whole-plant basis have only recently began to emerge in literature [95, 96, 99, 105-108]. If amenable to automation, technologies combining the analysis of stem cross-sectional preparations with microspectroscopic methods (e.g. Raman, Fourier Infrared or Matrix-assisted laser desorption/ionization imaging spectroscopy) could revolutionize the way we analyse lignocellulosic biomass characteristics [107, 108]. For the foreseeable future, however, these tools are likely to be constrained to off-line laboratory operations (unlike NIRS) and their most appropriate implementation would be restricted to the characterization of training sets and mapping populations for the underpinning of dedicated genomic selection and marker- assisted selection programs.

Uncovering the "real" genetic basis of heritable variation for cell wall degradability traits

In plant breeding, knowledge of the genes controlling cell wall degradability and bioconversion properties is fundamental for the development of effective molecular-breeding tools and genetic engineering strategies. At present, genes involved in the synthesis of cellulose, hemicellulose and lignin have been identified (**Chapter 2**), but we are still unable to efficiently harness them for the production of superior bioenergy crops. Knock-out, antisense construct and RNA-interference technologies have been the *de facto* routes for the production of transgenic genotypes with targeted alterations in cell wall metabolic fluxes and regulatory networks. These endeavours, however, have been met with mixed results. On the one side, transgenic perturbations of known cell wall metabolic routes generally lead to the production of crops

with desired degradability characteristics, but also severe agronomic and structural deficiencies [37, 109-111]. On the other side, derived transformants do not always exhibit the desired cell wall compositional modification, or the change is minimal [112, 113]. Presumably, deleterious changes in specific cell wall structural genes can be suppressed by the regulation of interchangeable paralogs, or by compensatory mechanisms derived from changes in the deposition of other cell wall components.

Moreover, although numerous studies have uncovered quantitative trait loci (QTLs) regulating cell wall degradability and bioconversion variation across several grass species, these QTLs seldom co-localize with candidate genes encoding structural enzymes of cell wall biosynthetic routes [66]. Interestingly, the lack of co-localization of QTLs and known candidate genes appears to be the norm rather than the exception [69]. This phenomenon could be explained by the way in which candidate genes for cell wall biosynthesis have been discovered. Prior to the advent of high-throughput genotyping and transcriptomic platforms, cell wall genes have been identified via the characterization of mutants with extreme phenotypes. Evidently, this has led to the discovery of structural genes vital to cell wall biosynthesis, but not to the discovery of genes involved in the control of "useful" heritable variation. The fact that structural genes do not underlie QTLs regulating useful cell wall properties could indicate that relevant variation in natural populations is primarily delimited to the transcriptional regulation of metabolic networks, or post-translation regulatory mechanisms [114, 115].

Ultimately, the realization that known candidate genes do not regularly underlie natural variation for relevant cell wall characteristics calls for a re-examination of the genetic determinants influencing the biofuel potential of grasses. Until now, targeted alterations of known cell wall structural genes have not yielded the most effective results, and variation attained through mutation breeding is comparable with advances achieved through the exploitation of natural variation (**Chapter 6**). Certainly, the advent of advanced phenotyping and sequencing platforms will shed new light onto the true genetic determinants of cell wall variation. This knowledge is deemed fundamental for the development of effective molecular-breeding tools and cell wall genetic engineering strategies.

Beyond the physical boundaries of the plant cell wall

Despite the acknowledgement that natural variation for cell wall properties is amenable to breeding, it seems relevant to recognize that the plant cell wall has its physical limitations. The frailty to modification of this complex biocomposite resides in its intricate inter-relation with plant health and agronomic productivity. After all, the plant cell wall delineates the architectural characteristics of individual cells (*i.e.* shape and size) and fundamentally determines plant morphology, size and fitness [46].

Certainly, extreme disruptions in the cell wall's compositional balance are possible, but these are often accompanied by yield and productivity penalties. The flagship exemplification of this trend comes from the renowned maize *bm3* mutation which, depending on the genetic background, may lead to drastic improvements in cell wall digestibility, but concomitantly reduces biomass yields and resistance to lodging [116, 117]. These observations conclusively prescribe that cell wall integrity is determined by compositional thresholds which "should" not be surpassed. Evidently, such thresholds would restrict the extent of genetic progress (in relation to the optimization of plant cell walls) which can be achieved through the exploitation of native cell wall variation through classical breeding and selection.

At present, several research groups have began working on strategies to circumvent the natural limitations of the plant cell wall but we are still far from realizing the maximum potential in degradability properties concealed in bioenergy crops. As described in **Chapter 2**, these approaches are based on heterologous gene expression technologies facilitating the incorporation of exogenous cell wall degrading or modifying enzymes, mediating the introduction of unconventional cell wall polysaccharides or altogether reengineering conventional cell wall polymers. And while these transgenic schemes add a new dimension to plant cell wall breeding, these still need to be evaluated for agronomic and environmental suitability and societal acceptability (especially in Europe).

Conclusions

Nearly a decade ago, experts in the field of cellulosic fuel research concluded that the effective commercialization of cellulosic fuel technologies demanded the parallel development of both, effective processing platforms and improved bioenergy feedstocks [1, 3, 6]. In their vision, a multi-disciplinary approach was the key to successfully overcome the economic and environmental drawbacks of the cellulosic fuel industry. Remarkably, despite the implementation of wide-scale cooperative research networks integrating divergent scientific disciplines (*e.g.* Great Lakes Bioenergy Research Center, National Renewable Energy Laboratory, Joint Bioenergy Institute, etc.), the gap between the sciences of bioenergy feedstocks and processing technologies is still present. To date, plant breeders are faced with the challenge of designing crops without clear breeding targets, and processing engineers continue to underestimate the benefits that could arise from the utilization of feedstocks with tailored biomass composition (**Chapter 6**).

In part, the divide in communication between the above mentioned disciplines resides in the fact that the cellulosic fuel industry lies at the cross-roads of antagonistic technological developments and commercial interests. Indeed, the conversion of

biomass into transportation fuels (or other added-value bio-based commodities) can be achieved through a variety of technological routes, including advanced thermo-chemical technologies (e.g. Fischer-Tropsch synthesis, gasification or catalytic-pyrolysis). Based on comparative life-cycle and techno-economic analyses, however, none of the technologies under development has a clear competitive environmental or commercial advantage to the industry. Irrespective of the uncertainty over which conversion route(s) will ultimately prevail, the development of efficient bioenergy crops needs to adhere to the same incontrovertible principles.

Firstly, the plant cell wall will indubitably remain a central focus of bio-based crop breeding endeavours. 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. lower lignin content is favoured by biochemical conversion routes and higher lignin content is favoured by fast-pyrolysis conversion routes), all knowledge pertaining the cell wall (i.e. biosynthesis, phenotyping tools, and genomic approaches for modification) can be universally extrapolated towards the selection of specific cell wall compositional profiles that can best match the conversion system. For instance, although our investigation focused on the deconstruction of the maize cell wall via biochemical pathways (and more concretely, via dilute-acid and enzymatic hydrolysis), our results ultimately demonstrate that lignin content (Lig, Lig/CW) and hemicellulose composition (DHS) are highly variable, highly heritable and easily quantifiable traits exerting a pivotal influence on the physical integrity of the plant cell wall. Ultimately, since lignin and hemicellulosic traits crucially impact the efficient deconstruction and utilization of plant biomass under a variety of biochemical [24, 34, 38, 40, 42, 44, 45, 118] and thermochemical fuel conversion routes [119, 120], these represent relevant breeding goals for improving the conversion efficiency of lignocellulosic biomass in any bioenergy breeding program.

Secondly, breeding for improved agronomic and environmental efficiency will also have great implications for the industry and cannot be disregarded; after all, the production of energy-dedicated crops will also be constrained by the urgencies of modern agriculture[121, 122]. In this regard, ongoing endeavours have achieved major accomplishments in uncovering and exploiting novel genetic diversity for climate-related stresses and sustainable production under lower agricultural inputs [123, 124]. Ultimately, the improvement of agronomic "hardiness" of energy grasses will improve the economics and environmental performance of the industry (regardless of the conversion route) by lowering the GHG footprint of their production, offsetting the conversion of virgin agricultural soils and reducing farm-to-plant transportation distances [123-127]. Moreover, to avoid a food-over-fuel debacle,

cultivation of biomass-dedicated bioenergy grasses will make more sense if it can be done on marginal soils and these are able to meet the expectations with respect to yields, agronomic hardiness and soil-recovery properties.

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Summary

Despite gaining prominence in scientific spheres and political agendas worldwide, the production of biofuels from plant biomass is yet to achieve an economic stronghold in the renewable-energy sector. Plant lignocellulose has evolved to resist chemical and enzymatic deconstruction, and its conversion into liquid fuels requires energetically stringent processes that currently render the industry economically and environmentally unviable.

To address this challenge, experts have envisioned the development of advanced bioenergy crops which require lower energetic and chemical inputs for their effective fractionation. At its core, this approach requires an in-depth understanding of the composition, synthesis and breeding amenability of the plant cell wall; the principal constituent of total plant dry biomass and the most recalcitrant fraction of the crop at physiological maturity to deconstruction. To this end, the primaryaim of this thesis was to dissect and elucidate the biochemical and genetic factors controlling cell wall characteristics relevant to the development of bioenergy grasses with improved processing quality for cellulosic based fuel production. A focus on maize was warranted as it currently represents the de facto model system for bioenergy crop research; offering an unrivalled platform to underpin the complex genetic architecture of cell wall biosynthesis, develop advanced bioenergy-crop breeding strategies and translate cell wall research into innovations and commercial products.

This thesis exposed that the biomass-to-fuel conversion of crops is a highly complex trait dependent on both, the balance and synergy between multiple cell wall components, and the inherent effectiveness of the conversion technology. Concerning the production of cellulosic ethanol via the combined operations of dilute-acid pretreatment and enzymatic saccharification, our results revealed that the chemical mechanisms affecting biomass conversion efficiency depend on pretreatment severity. Whereas at harsh pretreatments biomass conversion efficiency was primarily influenced by the inherent efficacy of thermochemical cell wall deconstruction, at milder pretreatments, maximum fermentable glucose release was observed for maize genotypes exhibiting systematic cell wall changes leading to higher ruminal cell wall digestibility. These results confirmed that the selection and use of cellulosic feedstocks that best match the processing conditions used in the industry can aid in reaching industrial goals aimed at improving the commercial and environmental performance of cellulosic fuels.

In turn, the exhaustive characterization of a forage maize doubled haploid (DH) population demonstrated the vast degree of genetic diversity in maize cell wall composition and bioconversion potential amenable to breeding. Principally, these findings

suggest that natural diversity in the biochemical composition of the maize cell wall and its physical properties is primarily ascribed to variation in the balance, monomeric make-up, and extent of cross-linking of non-cellulosic cell wall polymers (i.e. lignin and hemicellulose). Indeed, correlation analyses confirmed that the extent of enzymatic depolymerization of maize biomass was strongly and negatively associated to the concentration of cell wall phenolics, but positively impacted by the degree of glucuronoarabinoxylan (GAX) glycosylation and extent of hemicellulose-to-hemicellulose cross-linking. Our results also showed that natural variation in cell wall content and composition is quantitatively inherited and putatively ascribed to the segregation of multiple genetic loci with minor additive effects. In our population, genotypic diversity for cell wall composition and quality was found to be controlled by 52 quantitative trait loci (QTLs). From eight QTLs regulating bioconversion properties, five were previously unidentified and warrant further investigation.

Despite the apparent complexity of cell wall genetics, however, the high heritability and environmentally stability of cell wall compositional and degradability properties guarantee high selection efficacy during the development of superior DH/inbred material, and predispose that multi-environment testing will only be necessary at advanced stages of bioenergy-maize breeding programs. Moreover, because genetic variation for complex cell wall characteristics appears to be predominantly additive, preliminary selection at the inbred level will expectedly lead to successful hybrid selection; thereby minimizing the need for recurrent test-crossing procedures and evaluations. In this regard, maize cell wall bioconversion efficiency constitutes an excellent selection criterion for immediate application in modern maize breeding programs.

Ultimately, the convergence of classical selection schemes with inexpensive genotyping, advanced biometric models, high-throughput cell wall phenotyping and doubled haploid (DH) production technologies can accelerate development and commercial release of maize cultivars for bioenergy applications. To play a determinant role in the development and realization of sustainable and cost-effective cellulosic fuel processing technologies, however, novel dual-purpose maize cultivars (i.e. delivering both, grain for feed or food and fiber materials for bioconversion) will have to surpass the performance in lignocellulose processing quality and biomass yields of the best elite germplasm. These prospects seem realistic as the parallel advance of grain yield and stover productivity and quality characteristics is a feasible undertaking. Conceptually, the advance of superior bioenergy cultivars (surpassing the performance of modern elite material) would allow us to make the currently available biomass-to-fuel conversion systems more cost-effective and sustainable, and may also have favorable consequences for the ideal size and geographical distribution of biofuel refineries.

Samenvatting

Ondanks het feit dat de productie van biobrandstoffen uit planten wereldwijd prominent op de wetenschappelijke en politieke agenda staat, moeten deze brandstoffen nog een positie verwerven in de groene energiesector. De celwanden van planten die gebruikt worden voor omzetting in biobrandstoffen hebben vanwege hun natuurlijke functie (verstevinging van de plant) een natuurlijke resistentie tegen chemische en enzymatische afbraak en het omzetten van de lignocellulose in vloeibare brandstof kost daarom momenteel zo veel energie dat dit vanuit economisch- en duurzaamheidsoogpunt niet rendabel is.

Dit probleem zou volgens experts opgelost kunnen worden door energiegewassen te ontwikkelen waarvan de celwandcomponenten effectief kunnen worden afgebroken met minder energie en chemische input. In de kern vraagt deze aanpak om fundamentele kennis van de samenstelling en synthese van de celwand van de plant en de mogelijkheden voor veredeling. De celwand is de belangrijkste component van de totale droge biomassa en vormt het meest lastige deel van een fysiologisch volgroeid gewas om af te breken. Het doel van het in dit proefschrift beschreven onderzoek was daarom het identificeren van de biochemische en genetische factoren die de degradatiekarakteristieken van de celwand bepalen. De focus van het onderzoek lag op maïs mede omdat dit gewas de facto als hét model systeem fungeert voor onderzoek aan energiegewassen. Het biedt een ongeëvenaard platform voor de studie van de complexe genetische architectuur van celwandbiosynthese en de ontwikkeling en innovatie van plantenveredelingstrategieën voor de ontwikkeling van energiegewassen en de ontwikkeling van nieuwe commercieel aantrekkelijke producten.

Dit proefschrift bevestigt en borduurt verder op het feit dat de ontwikkeling van nieuwe energiegewassen een complex proces is dat afhankelijk is van zowel de balans en synergie tussen verschillende componenten van de celwand, alsmede van de inherente effectiviteit van het industriële conversieproces. Betreffende de productie van bio-ethanol uit planten, via de gecombineerde aanpak met verdund zwavelzuur als voorbehandeling en enzymatische sacharificatie, laten de resultaten zien dat de chemische mechanismen die de conversie van biomassa beïnvloeden afhankelijk zijn van de sterkte van de voorbehandeling. Een zware voorbehandeling bleek primair de efficiëntie van de biomassaconversie te beïnvloeden door effectieve thermochemische celwandafbraak, terwijl een mildere voorbehandeling het meest effectief bleek bij het vrijmaken van de fermenteerbare glucose uit celwanden van maïslijnen met een goede pensverteerbaarheid (ruminale verteerbaarheid) van de celwanden. Deze resultaten bevestigden dat selectie en het gebruik van cellulose-houdende grondstoffen die zijn toegesneden op de verwerkingscondities in de industrie kunnen bijdragen tot realisatie van industriële doelen ten aanzien van commerciële pro-

ductie en het duurzame gebruik van bio- brandstoffen uit lignocellulose.

Tegelijkertijd heeft de grondige karakterisering van een populatie verdubbelde maïshaploïden (DHs) laten zien dat een groot deel van de genetische diversiteit in de celwandsamenstelling van maïs en de potentie voor bioconversie toegankelijk is voor verbetering door veredeling. Deze bevindingen suggereren dat de natuurlijke diversiteit in de biochemische samenstelling van de maïscelwand, en haar fysische eigenschappen, primair toegeschreven dienen te worden aan variatie in de balans, monomere opbouw en de onderlinge binding van de verschillende celwandpolymeren (bijvoorbeeld tussen lignine en hemicellulose). Correlatieanalyse bevestigde een sterke en negatieve relatie tussen enzymatische depolymerisatie van maïsbiomassa en de concentratie aan celwandfenolen, maar toonde ook een positieve relatie aan tussen de mate van glucurono-arabinoxylan (GAX) glycosylatie en de mate van onderlinge binding van hemicellulosepolymeren. Onze resultaten laten ook zien dat de natuurlijke variatie in celwandinhoud en -compositie erfelijk is en toegeschreven kan worden aan de uitsplitsing van meerdere genetische loci met beperkt effect. In onze populatie bleek genotypische diversiteit voor celwandcompositie en -kwaliteit voor de verschillende kwantitatieve bioconversie-eigenschappen te samen bepaald te worden door 52 loci (QTLs). Van acht QTLs die bioconversie-eigenschappen reguleren zijn er vijf nog niet eerder geïdentificeerd en vragen om verder onderzoek.

Ondanks de complexiteit van celwandgenetica, beloven de hoge mate van erfelijkheid en omgevingsstabiliteit van celwandcompositie en -afbreekbaarheid een hoge selectierespons bij het ontwikkelen van superieure DHs/inteeltlijnen en zijn additionele multi-locatietesten voor deze eigenschappen alleen noodzakelijk in gevorderde fasen van een veredelingsprogramma voor bio-energie bij maïs. Omdat de genetische variatie voor complexe celwandeigenschappen voornamelijk additief blijkt te zijn, is het bovendien te verwachten dat voorselectie op inteeltniveau tot versnelling van de ontwikkeling van hybride maïsrassen zal leiden; hierbij wordt de noodzaak voor herhaalde test-kruisingsprocedures en -evaluaties geminimaliseerd. De efficiëntie van maïscelwandbioconversie vormt daarom een uitstekend selectiecriterium voor directe toepassing in moderne maïsveredelingsprogramma's.

Tenslotte kan en zal de combinatie van klassieke selectieschema's met goedkope genotypering, geavanceerde biometrische modellen, high-throughput celwandfenotypering en gebruik van verdubbelde haploïden (DH) de ontwikkeling en commerciële toepassing van maïscultivars voor productie van bio-energie versnellen. Om een belangrijke rol te spelen in de ontwikkeling en realisatie van duurzame en kost-effectieve verwerkingstechnieken voor bio-brandstoffen zullen nieuwe maïsrassen nodig zijn met een dubbel gebruiksdoel die geschikt zijn voor zowel productie

van graan als biomassa als grondstof voor bio-brandstof en in productievermogen de bestaande rassen moeten overtreffen. Dit geldt zowel voor wat betreft de biomassa cq -celwandverwerkingskwaliteit als de totale oogst aan biomassa van graan en andere oogstbare plantdelen. De vooruitzichten zijn realistisch omdat veredeling met meervoudige doelen, zoals het verhogen van graanproductie, biomassaproductiviteit en het verbeteren van kwaliteitseigenschappen, simultaan kan plaats vinden. Conceptueel is het voordeel van superieure cultivars voor productie van biobrandstof dat ze ons in staat stellen om de huidige beschikbare systemen voor conversie van biomassa in brandstof meer kosten effectief en duurzaam te maken en dit laatste heeft mogelijk ook voordelen voor wat betreft de ideale schaalgrootte en geografische verspreiding van raffinaderijen voor de productie van biobrandstof .

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all of whom accompanied me from the beginning. Likewise, I was blessed with a family away from home...my Wageningen bros! Joao, Sebas, Ali, Luigi, Ale, Pavlos, Hanna, Mira, Adam, Andreu, all the Portuguese Andre's and Nelson: thank you so much for all the incredible dinners, gazillion beers, awesome bro trips and (above all) your unconditional friendship.

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<div align="center">

That being said...on to the next journey!

And to all my dear friends: "Gracias Totales!"

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<div align="center">

Quito, July 2014

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About the Author

Andres Francisco Torres was born in Quito, Ecuador, on April 24, 1985. Although he spent most of his youth in Venezuela and India, Andres returned to Ecuador to study at Universidad San Francisco de Quito, where he obtained his Bsc degree in Biotechnology. Following the successful acquisition of the HSP Huygens International Talent Scholarship, he then obtained his MSc degree in Plant Biotechnology at Wageningen University and Research Centre (WUR), The Netherlands. In 2010, Andres began his PhD studies at the Department of Plant Breeding WUR under the supervision of Dr. Oene Dolstra and Dr. Luisa Trindade. As of September 2014, Andres will return to his *alma-mater*, Universidad San Francisco de Quito, as Professor of Biotechnology.

List of Publications

AF Torres *et al.* 2014. "Cell wall diversity in forage maize: genetic complexity and bioenergy potential." BioEnergy Research (In Press)

LR Montes Osorio, **AF Torres** *et al.* 2014. "High level of molecular and phenotypic biodiversity in Jatropha curcas from Central America compared to Africa, Asia and South America." BMC Plant Biology 14:77 (doi:10.1186/1471-2229-14-77)

AF Torres *et al.* 2013. "Bioethanol from maize cell walls: genes, molecular tools and breeding prospects." GCB Bioenergy. (doi:10.1111/gcbb.12164)

AF Torres *et al.* 2013. "Effect of maize biomass composition on the optimization of dilute-acid pretreatments and enzymatic saccharification." BioEnergy Research (2013): 1-14.

T van der Weijde, **AF Torres** *et al.* 2013. "The potential of C4 grasses for cellulosic biofuel production." Frontiers in Plant Science 4:107 (doi:10.3389/fpls.2013.00107)

AF Torres *et al.* 2008. "Preliminary assessment of genetic diversity and phenetic relations for section Lasiocarpa by means of heterologous SSR markers." Crop Science 48.6 (2008): 2289-2297.

Experimental Plant Sciences

The Graduate School **EXPERIMENTAL PLANT SCIENCES**
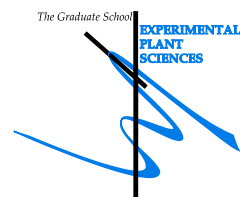
Issued to: **Andres Francisco Torres Salvador**

Date: **4 September 2014**

Group: **Plant Breeding, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
"Dissecting the genetics of maize stover"	Mar 02, 2010
► **Writing or rewriting a project proposal**	
"Optimization of maize cell wall composition and structure for improved conversion into biofuels"	Apr 13, 2010
► **Writing a review or book chapter**	
"Bioethanol from maize cell walls: genes, molecular tools and breeding prospects", GCB Bioenergy (2014), DOI: 10.1111/gcbb.12164.	Jun-Jul, 2010
► **MSc courses**	
► **Laboratory use of isotopes**	

<div align="right">Subtotal Start-up Phase   13.5 credits*</div>

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Student Day, Utrecht University	Jun 01, 2010
EPS PhD Student Day, University of Amsterdam	Nov 30, 2012
EPS PhD Student Day, Leiden University	Nov 29, 2013
► **EPS theme symposia**	
► EPS Theme 3 Symposium 'Metabolism and Adaptation, Leiden University	Feb 19, 2010
EPS Theme 4 Symposium 'Metabolism and Adaptation', University of Amsterdam	Dec 13, 2013
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 19-20, 2010
CCC (Carbohydrate Competence) Research Days 2010	Jun 10-11, 2010
CCC (Carbohydrate Competence) Open Days 2012	Apr 26-27, 2012
CCC (Carbohydrate Competence) Open Days 2013	Apr 16-17, 2013
► **Seminars (series), workshops and symposia**	
Plant Breeding Research Day 2012	Feb 02, 2012
Invited seminar Bruce Dale: 'Why we must (and how we can) have sustainable biofuels'	Mar 15, 2012
Invited seminar Rudy Rabbinge: 'Food security'	Oct 21, 2012
Invited seminar Andrew Sugden: 'Writing for high impact journals'	Feb 08, 2013
Invited seminar Young PSG & Young ESG: 'Make more business with your research'	Apr 23, 2013
Invited seminar Salvatore Ceccarelli: 'The efficiency of plant breeding'	Oct 16, 2013
► **Seminar plus**	
PhD Masterclass Bioenergy with Jason Hill	Mar 20, 2014
► **International symposia and congresses**	
SUNLIBB Meeting - Versailles, France	Sep 26-28, 2012

	LignoBiotech II Symposium - Fukuoka, Japan	Oct 14-17, 2012
	13th Cell Wall Meeting - Nantes, France	Jul 07-12, 2013
►	**Presentations**	
	Oral: WUR 92nd Dies Natalis	Mar 09, 2010
	Oral: CCC Research Days	Jun 10, 2010
	Oral: SUNLIBB Meeting - Versailles, France	Sep 27, 2012
	Poster: LignoBiotech II Symposium - Fukuoka, Japan	Oct 14-17, 2012
	Oral: PhD Course Bio-Energy Production from Crop Plants and Algae	Nov 23, 2012
	Poster 1: 13th Cell Wall Meeting - Nantes, France	Jul 07-12, 2013
	Poster 2: 13th Cell Wall Meeting - Nantes, France	Jul 07-12, 2013
►	**IAB interview**	
	Meeting with a member of the International Advisory Board	Nov 15, 2013
►	**Excursions**	

Subtotal Scientific Exposure   *16.4 credits**

3) In-Depth Studies		<u>*date*</u>
►	**EPS courses or other PhD courses**	
	VLAG course 'Systems Biology: Statistical analysis of ~omics data'	Dec 13-17, 2010
	EPS course 'Bio-energy Production from Crop Plants and Algae'	Nov 21-23, 2012
	Kyazma course 'Genetic Linkage Mapping'	Mar 20-22, 2013
	Kyazma course 'QTL Analysis'	Mar 25-27, 2013
	PE&RC course 'Introduction to R'	Jun 10-11, 2013
►	**Journal club**	
►	**Individual research training**	

Subtotal In-Depth Studies   *4.8 credits**

4) Personal development		<u>*date*</u>
►	**Skill training courses**	
	EPS 'ExPectations Career Day 2010'	Nov 19, 2010
	WGS course 'Communication with the Media and the General Public'	Nov-Dec,2012
	WGS course 'Techniques for Writing and Presenting a Scientific Paper'	Dec 11-14, 2013
	EPS course 'The Art of Presenting Science'	Apr-May 2013
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	

Subtotal Personal Development   *3.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**38,2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

* A credit represents a normative study load of 28 hours of study.