Opinion

Maize and sorghum: genetic resources for bioenergy grasses

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The highly photosynthetic-efficient C4 grasses, such as switchgrass (*Panicum virgatum*), Miscanthus (*Miscanthus* × *giganteus*), sorghum (*Sorghum bicolor*) and maize (*Zea mays*), are expected to provide abundant and sustainable resources of lignocellulosic biomass for the production of biofuels. A deeper understanding of the synthesis, deposition and hydrolysis of the distinctive cell walls of grasses is crucial to gain genetic control of traits that contribute to biomass yield and quality. With a century of genetic investigations and breeding success, recently completed genome sequences, well-characterized cell wall compositions, and a close evolutionary relationship with future bioenergy perennial grasses, we propose that maize and sorghum are key model systems for gene discovery relating to biomass yield and quality in the bioenergy grasses.

Grasses and the bioenergy economy

Bioenergy derived directly from plants, or their use as feedstocks for fermentation by microbes, is making an ever-increasing contribution to the diversifying energy portfolio, with positive impacts for energy security, mitigation of environmental consequences of elevated greenhouse gases, and in stimulation of rural economies. In this issue of *Trends in Plant Science*, Joshua Yuan and colleagues [1] summarize the background and scientific, social and economic issues that need to be addressed before home-grown energy can become an agricultural reality. Sucrose and glucose from sugarcane (*Saccharum officinarum*) fermented to ethanol already provide Brazil with energy independence from petroleum fuels [2]. In the USA, production of ethanol from corn grain is a mature industry, but animal feed supplies and food products compete heavily for this substrate. Lignocellulosic biomass consists principally of cell walls, harvested from dedicated bioenergy crops or from dried crop residues, such as sugarcane 'bagasse', or maize (*Zea mays*) and sorghum (*Sorghum bicolor*) 'stover'. Lignocellulosic biomass provides a rich source of solar energy trapped as carbohydrate in a broad range of plants unrestricted by climate or geographic location, the conversion of which to biofuel would not impact the price of cereal commodities [3,4]. Perennial grasses, such as switchgrass and Miscanthus, are considered to be superior potential feedstocks because of their C4 photosynthesis and long growing season, their ability to sequester nutrients in rhizomes at the end of the growing season, and their high water-use efficiency [5–7]. The US Department of Energy's 'Billion-Ton' study [3] shows that crop residues, a large portion from maize stover, could make a substantial contribution to our liquid fuel needs. However, implementation of bioenergy crops in the agricultural landscape must be sustainable and cost-effective, and have a small agronomic footprint on land devoted to food and feed crops. Cell wall polysaccharides are hydrolyzed to glucose and other sugars, which are then metabolized by microbes to produce biofuels. A general consensus is emerging that the recalcitrance of lignified cell walls to hydrolysis by enzymes is a key obstacle to overcome that could be mitigated either by identifying more efficient cell wall-degrading enzymes, by expressing them recombinantly in transgenic plants, or by modifying biomass composition, 'biomass quality', to facilitate degradation [4–10]. However, an equally important goal is to maximize the amount of carbohydrate biomass per hectare to reduce the energy and economic costs of production and transport to refineries.

Maize and sorghum as genetic models for the improvement of C4 bioenergy grasses

Arabidopsis thaliana has served a pioneer role as a genetic model for plant growth and development. However, grass species diverged from dicot species early on in the evolution of flowering plants, which is evident in their evolution of distinct cell wall compositions and complement of cell wall-related genes, highlighting the need for additional genetic models within the grasses. Rice (*Oryza sativa*), with its compact and sequenced genome, is a suitable reference model for grass cell wall biology, and genetic tools for rice, such as insertional mutant lines and ease of transformation, are becoming comparable to those of Arabidopsis [11]. Soon, Brachypodium (*Brachypodium distachyon*), another small-genome grass, will be sequenced, adding value as a comparative model that is easily grown in the laboratory. The suitability of both rice and Brachypodium is diminished primarily because of their C3 photosynthesis and distant evolutionary relationships to the bioenergy grasses. Genetic resources for switchgrass are emerging [12,13], but there is only sketchy information on the genetic origin of *Miscanthus* × *giganteus*.

The advantages of maize and sorghum as genetic models are numerous: for maize, Lawrence and Walbot [14] cite its close evolutionary relationship with future bioenergy perennial grasses, its C4 photosynthesis, a historical depth of genetic knowledge and a rapidly growing resource of genetic tools. Insights from comparative

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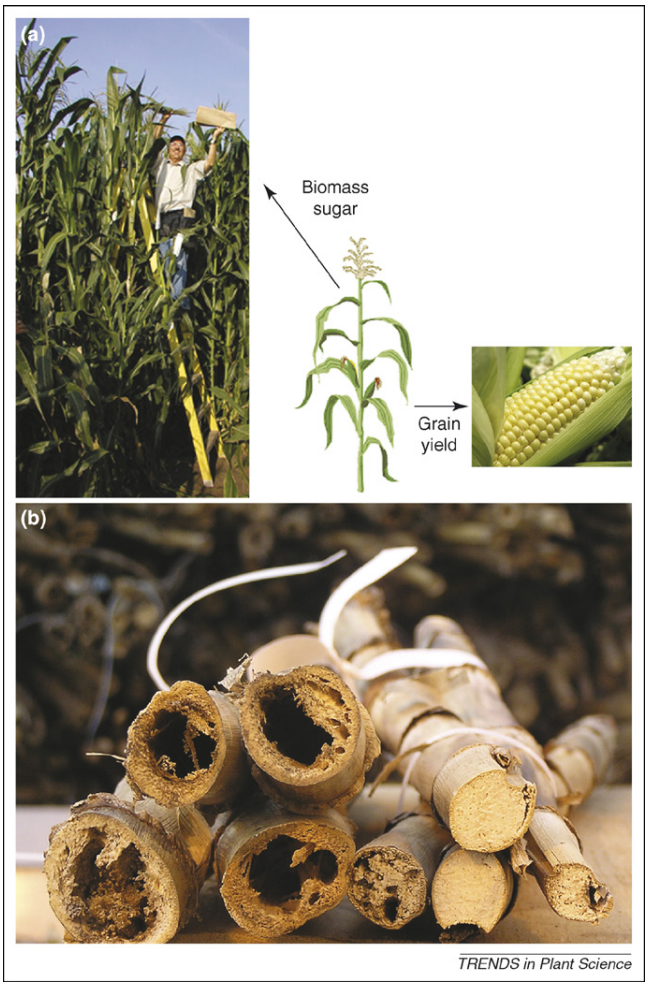

Figure 1. **(a)** Maize exhibits a remarkable genetic diversity, resulting in an enormous range of plant architectures. Maize has been selected for millennia for enhancement of the size and quantity of grains. Only recently have researchers begun to mine maize genetic diversity for bigger plants with high levels of sugar or lignocellulosic biomass that fit diverse biomes. Tracing the genetic basis of trait diversity is facilitated by a complete genome sequence and recombinant inbred lines (RILs), which enable the underlying genes to be fine-mapped in a few generations. Transgression segregation in the Intermated B73 × Mo17 RILs is remarkable, with differences in height ranging from one to nearly three meters, and the dimensions in stem diameter and density mentioned vary by at least threefold (B. Penning *et al.*, unpublished). **(b)** Although a hallmark of grasses is the appearance of hollow stems at maturity, RILs have been discovered that have pith that is undegraded at maturity. Traits such as this one increase stalk density, contributing to biomass yield. Photograph of tropical maize courtesy of Dr Mike Blanco, USDA-ARS, Ames, IA, USA (http://www.ars.usda.gov/is/AR/archive/jul07/corn0707.htm).

studies of grass genome sequences can provide knowledge of how C4 metabolism arose, how C4 grasses partition carbon into sugar stores versus cell wall mass, and the genetic basis of several physiological and architectural features, such as tillering, canopy formation, stalk reserve retention, perennialization, and nutrient- and water-use efficiency. Vermerris *et al.* [15] and Sarath *et al.* [13] make the same case for sorghum, citing the success of mutant screens, transformation potential, and the plant breeding capabilities that have already led to commercial cultivars with enhanced properties. Both maize and sorghum genome sequences have been completed this year [16,17].

We add an essential element to the list of reasons why maize and sorghum are advantageous models – an existing knowledge base of the cell wall composition and architecture and of the thousands of gene products required for assembly of the unique walls of grasses. A thorough understanding of how these gene products function in the synthesis and architectural construction of the cell wall will enable design of the optimal bioenergy crop plant. Identification of key cross-links in wall architecture will suggest improvements to methods of deconstruction through bioprocess engineering. In our opinion, maize and sorghum comprise essential models to provide the knowledge base of gene functions for translation to future energy crops. After all, classic breeding strategies for maize have been responsible for most of the almost ninefold (~1.24 Mg/hectare to nearly 11.12 Mg/hectare) increases in grain yield since the advent of hybrid maize [9]. The wealth of genomic resources and tools for both of these species can be put to immediate use to make similar advances in biomass yield and quality (Figure 1). The high degree of genetic synteny among grass genomes should facilitate the translation of gene-function discovery in maize and sorghum to more genetically recalcitrant grass species.

Flowering plants make two distinct types of walls

All plant cells are surrounded by primary cell walls, but a few cell types deposit thick secondary walls. Flowering plants make two fundamental types of primary walls (Box 1). All dicots and most monocots make a Type I cell wall in which cellulose microfibrils of the expanding wall are tethered by xyloglucans embedded in a gel of pectin and cross-linked with structural proteins at cessation of growth [18,19]. Potential forest biomass crops, such as poplar (*Populus* ssp.), willow (*Salix* ssp.), and eucalyptus (*Eucalyptus* ssp.), produce thick secondary walls of cellulose, glucuronoxylans, and lignin in fibers and vascular elements of the xylem. Poplar is the first tree species to have its genome sequenced [20], and is a genetic model for all bioenergy hardwoods.

Grasses are members of the commelinoid monocots that make a Type II cell wall (Box 1). There are two major polysaccharide structural networks in the walls of grass species: the cellulose network coated and tethered by glucuronoarabinoxylans (GAX), and mixed-linkage (1→3),(1→4)-β-D-glucans embedded in an acidic polysaccharide network of highly-substituted GAXs and some pectins [19,21]. Another distinction of the walls of grasses is the cross-linking phenylpropanoid networks in the primary walls that are deposited as cells mature during development. These phenylpropanoid networks include several patterns of ester, ether and phenyl–phenyl linkages initiated from arabinosyl residues of the GAX [22–24]. The Type II secondary walls are composites of cellulose and relatively unsubstituted GAX interconnected with a network of acidic phenylpropanoids and lignin (Box 1).

Our characterization of cell wall composition of Type I and Type II cell walls is based upon average cell wall compositions. However, cell wall polysaccharides also vary in their proportions among different cell types, which are likely to vary in their recalcitrance to degradation by microbial enzymes. The networks of genes that build particular kinds of cell wall architectures need to

Box 1. A cell wall primer

Flowering plants make two distinctive types of primary cell walls. The basic physical structure is the same: strata of cellulose microfibrils are coated with cross-linking glycans, some of which extend and interconnect neighboring microfibrils. This fundamental framework is embedded in a matrix of acidic polysaccharides, which serves as residence for numerous proteins and enzymes that function in wall assembly and degradation. The distinctions between the two types of walls are the materials used in their construction.

All dicot and about a half of the monocot plants make a Type I cell wall (Figure Ia). Xyloglucan is the principal cross-linking glycan of the Type I wall: its (1→4)-β-D-glucan backbone is typically substituted with three contiguous xylose units, and several other sugars, such as arabinose, galactose and fucose, are added to certain xylose units in a species- and cell-specific manner [43,44]. Type I-specific glucuronoarabinoxylans (GAXs), with most arabinosyl units on the *O*-2 position of the xylan backbone, and glucomannans are also found in lesser abundance. The Type I wall is rich in uronic acid-rich pectins of two kinds, homogalacturonans and rhamnogalacturonan I [18,19]. Several neutral sugar-containing side-chains of β-galactans, α-arabinans, and type I arabinogalactans are typically attached at the *O*-4 position of the rhamnose units. The extent and type of side-group attachment varies in a cell- and developmental stage-specific manner [18]. At the end of cell growth, the Type I wall is cross-linked with several types of structural proteins, such as the hydroxyproline-, proline- and glycine-rich proteins [19].

The Poales and related commelinoid monocots make Type II cell walls [19,21] (Figure Ib). The GAXs are the major cross-linking glycans of the cellulose microfibrils, but to hydrogen-bond to the surface of cellulose microfibrils and cross-link them, the majority of the arabinosyl side-group substitutions at the *O*-3 position of the xylose unit must be removed [45]. The xylosyl units of GAX can be substituted with acetyl groups at the *O*-2 and *O*-3 position [46]; the acetyl content of some ryegrasses has been reported to approach 10% of the mole% of xylose in the wall [47]. During growth, a (1→3),(1→4)-β-D-glucan appears at the onset of cell expansion and is largely hydrolyzed as cell expansion concludes [48,49]. By sequential chemical extraction and enzymatic digestion of specific polysaccharides, and imaging of the resulting cell wall architectural modifications in maize coleoptile cells, the GAXs occupy most of the volume between cellulose microfibrils, whereas the β-glucans tightly coat the microfibrils [50]. Other cross-linking glycans include a glucomannan and a grass-specific xyloglucan. The GAXs are largely cross-linked by the phenylpropanoid network. Chlorite oxidation of the aromatic residues extracts little material from the grass cell wall but renders the GAXs of the interstitial space between β-glucan-coated microfibrils easily extracted by as little as 0.1 M NaOH [51]. Figure Ia and b is modified from Refs [18,19].

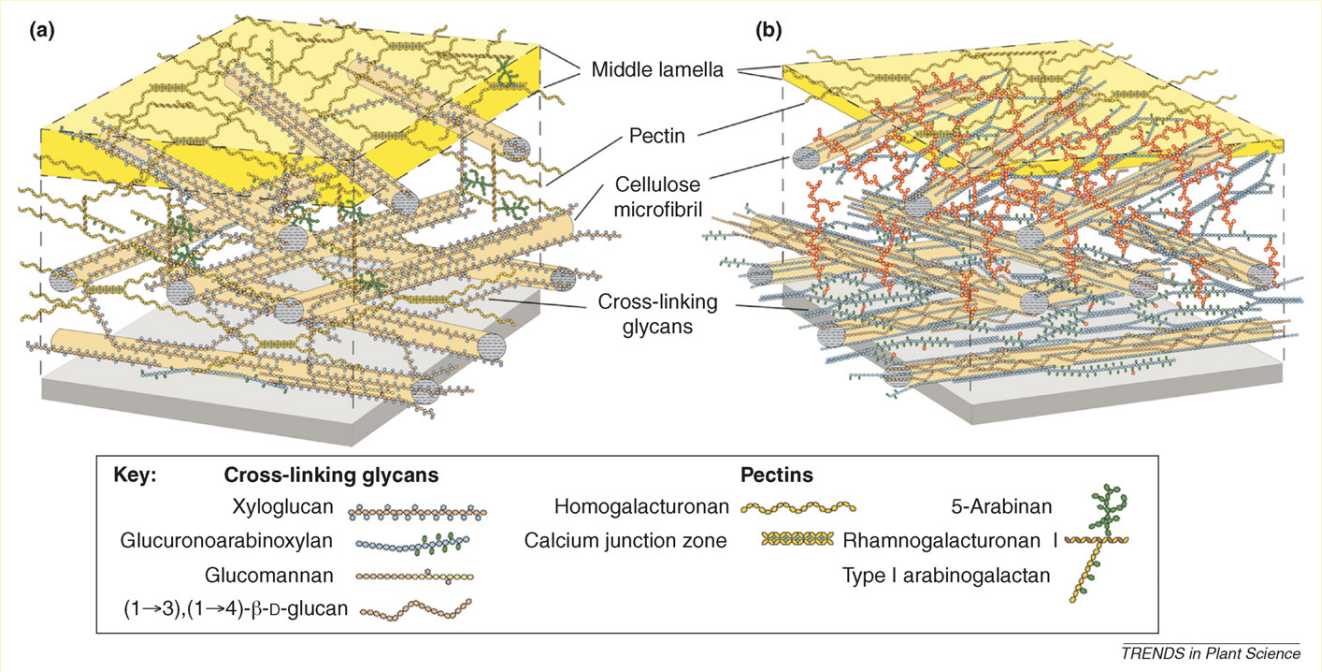

Figure I. The remarkable phenotypic diversity of maize.

be identified to gain genetic control of the proportions of useful cells in biomass feedstocks.

Characterizing the genes for wall biogenesis in grasses

Genetic improvement of cell wall composition and architecture is a goal for two reasons: cell walls constrain cell size and shape and so have a significant role in plant growth, impacting biomass yield, and cell walls are recalcitrant to degradation by microbes to release sugars for fermentation, impacting biomass quality. Plants devote ~10% of their genome, ~2500 genes, to construction and dynamic rearrangement of their cell walls during growth [25–27]. We have annotated, and assembled into gene families, ~1200 cell wall-related genes into six stages of wall biogenesis consisting of substrate generation, polysaccharide synthesis, membrane trafficking, assembling and turnover, secondary wall formation, and signaling (http://cellwall.genomics.purdue.edu). Using *Arabidopsis* and rice as backbone annotated sequences, we are assembling the comparable gene families of maize and sorghum. What emerges from this study is that the differences in Type I and Type II wall compositions are reflected in the structure of these gene families. For example, the Carbohydrate-Active Enzyme (CAZy) database (http://www.cazy.org/) has assembled hundreds of plant genes into 40 families of glycosyl transferases and 34 families of glycosyl hydrolases. All genes of angiosperms are represented in the same families, but few families have similar numbers

of members when comparing *Arabidopsis* with the grasses. We observed differences in gene number of one group of family members in species with Type I or Type II walls, in the number of gene family members, and in the presence or absence of new family groups in species with one cell wall type compared with the other. Dicot species have a much larger proportion of pectins (30% for Type I walls versus 5% for grasses), so it is perhaps not surprising that *Arabidopsis* has many more genes for pectin metabolism than do rice and maize. By contrast, the composition of phenylpropanoid-rich Type II walls of rice and maize is consistent with much larger families of genes whose products function in monolignol biosynthesis (see http://cellwall.genomics.purdue.edu). We estimate that at least a third of cell wall-related genes of grasses could have no, or few, orthologs in *Arabidopsis*, making genetic functional analyses in a grass model system essential.

Mining the diversity of C4 grasses

Genetic screens for mutants that affect cell wall composition and architecture, either directly or indirectly, provide unbiased ways to identify biomass-relevant quality traits, including those resulting from mutations in cell wall-related genes. The *brown mid-rib* mutants of sorghum are an excellent example of how a defect in lignin structure, which improves forage digestibility by ruminants, can also enhance yields of glucose in screens using commercial cellulases [15]. The Uniform*Mu* population was generated by introgressing Robertson's *Mutator*, an insertional mutational element, into established maize inbreds W22 and B73 [28,29]. Both flank-based random sequencing of the Uniform*Mu* populations and reverse-genetic approaches to identify sites of Mu insertions has already augmented the set of mutants in candidate cell-wall genes of interest by several hundred (http://currant.hos.ufl.edu/mutail/). Additional insertional DNA resources, such as Activator [30] and RescueMu [31], are well established, and TILLING (Targeting Induced Local Lesions IN Genomes) in maize [32] provides a means of generating rich allelic series.

Screens of the Uniform*Mu* population by Near Infrared (NIR) spectroscopy identified unusual spectra indicative of putative mutants with altered cell wall composition or architecture [15]. Several dozen NIR 'spectrotypes' were identified: only six displayed visible phenotypes, such as alterations in leaf texture and architecture; the vast majority were indistinguishable from the W22 control in field conditions. Pyrolysis-mass spectrometry, a high-throughput method that gives hexose, pentose, and phenolic compounds derived from lignin and hydroxycinnamic acids [33], confirmed an altered lignin-carbohydrate cell wall composition for a subset of the NIR mutants [15] (http://cellwall.genomics.purdue.edu).

The natural diversity of a population can be exploited in breeding programs [5,7,9], but having well-mapped populations of recombinant inbred lines (RILs) from species with fully sequenced genomes allows rapid gene identification by association mapping or analysis of quantitative trait loci (QTL) of the genes relevant for biomass improvement [34]. The Intermated B73 × Mo17 (IBM) population of 264 recombinant inbred lines has been used to generate a high-density genetic linkage map [35] (http://www.maizegdb.org/ibm302scores.html). Already some surprises have emerged upon inspection of this diversity (Figure 1). More recently, the Nested Association Mapping (NAM) lines, 200 RILs each derived from crosses of B73 with 25 diverse inbreds, include many high-biomass tropical maize inbreds that capture a substantial amount of the existing genetic diversity of the species [36] (http://www.panzea.org). At least 300 additional landraces and inbreds capture the full range of genetic diversity of maize [37]. These rich genetic resources greatly facilitate the discovery of biomass-relevant genes, the orthologs of which can be identified in other bioenergy grasses.

Maize and sorghum as transitional bioenergy crops

Perennial grasses are deemed central to the development of dedicated bioenergy crops that are high-yielding in a sustainable and cost-effective way. The principal advantages frequently cited over annual grasses, such as maize and sorghum, are the low input of mineral nutrients required and the subsequent sustainability inherent in nutrient-sequestering plants.

However, a major factor in the agronomic success of the perennials that is rarely discussed is grower acceptance. All things being equal, farmers prefer the flexibility of an annual choice of crops. Switchgrass and Miscanthus require a dedication of land for their growth, and several years of establishment are required to obtain maximal yields of biomass. Genetic improvements in the perennials would have to be enormous to offset the down-time required for starting over with new plantings. Nutrient- and water-inputs calculated for maize are based on the high demand for grain development, but the minimal amounts required for vegetative growth only have not been established, and could well be lower than those required for maximal grain production.

Maize and sorghum have an astounding diversity of plant size and architecture, with tremendous potential to accumulate sugar in the stem or starch in grains (Figure 1). Mutants with traits of high-yield or digestibility have been identified, along with their underlying genes [15]. The maize *brachytic2* and sorghum *dw3* mutations add extra layers of cells that make the stalks pyramidal rather than cylindrical [38]. The rapid pace of gene discovery can be used for translational research in perennial grasses, but traits can also be stacked to create bioenergy maize and sorghum. Inclusion of bioenergy maize or sorghum in a crop rotation scheme with grain crops and legumes constitutes an alternative approach to sustainability that would allow the flexibility of annual crops while mitigating the dedicated land-use issue with respect to balancing fuel versus food and feed crops.

Conclusions

Advancing appropriate genetic model systems for perennial grasses is essential in the development of systems approaches to improve wall architectures and plant anatomies for the end-use of biofuel production. The genetic diversity of maize and sorghum provides many choices of target genes for modification, both in these transitional energy crops and in translation to future

perennial bioenergy grasses. The RILs and NAMs provide a rich resource to identify QTL and the underlying genes for useful traits, such as plant height, anatomy and architecture, density, mass, and leaf area. Many cell wall-related genes can also impact both accumulation of biomass and its degradability. Beyond the obvious modification of lignin content and architecture, increasing carbohydrate content could be accomplished by ectopic expression of transcription factors, such as the NAC- and Myb-domain-containing proteins that initiate secondary wall formation [39–42], or by coordinately up-regulating expression of the genes of the cellulose synthase complex. Upregulation of genes for mixed-linkage β-glucan synthesis would increase amounts of an easily hydrolysable polymer, and gene silencing of the endogenous endo- and exo-β-glucanase that degrades it during cell growth could allow the polymer to persist in the cell wall until harvest. Other modifications could include reducing the degree of xylan acetylation, through silencing of acetyl transferases or overexpression of acetyl esterases, to reduce the content of this fermentation-inhibiting acid. With 10% of the genome devoted to cell wall construction, the possibilities are numerous for directly modifying wall composition and architecture. Grass-specific genetic models will be indispensable for defining and assembling the gene networks of Type II cell wall biogenesis.

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