

Sweet Sorghum and *Miscanthus*: Two potential dedicated Bioenergy Crops in China

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Abstract

Among the potential non-food energy crops, the sugar-rich C4 grass sweet sorghum and the biomass-rich *Miscanthus* are increasingly considered as two leading candidates. Here, we outline the biological traits of these energy crops for large-scale production in China. We also review recent progress on understanding of plant cell wall composition and wall polymer features of both plant species from large populations that affect both biomass enzymatic digestibility and ethanol conversion rates under various pretreatment conditions. We finally propose genetic approaches to enhance biomass production, enzymatic digestibility and sugar-ethanol conversion efficiency of the energy crops.

Key words: sweet sorghum, *Miscanthus*, bioenergy crops, biofuels, plant cell wall, biomass saccharification, ethanol conversion.

1. INTRODUCTION

Bioenergy is regarded as a sustainable alternative to fossil energy supply (Chen and Peng 2013; Cotton *et al.* 2013). According to recent reports, global bioethanol production has risen gradually each year up to 88 billion liters in 2013 (Global Renewable Fuels Alliance, 2014).

As the second largest energy consumer globally, China has launched several non-fossil energy developing plans, including ‘The 11th Five-Year Plan’ for Energy Development Planning of China (National Development and Reform Commission 2007a), and The Medium- and Long-Term Developmental Plan for Renewable Energy in China (National Development and Reform Commission 2007b).

To reach the goals outlined in these plans, the selection of bioenergy crops is an important priority to meet the need of biomass production. In general, bioenergy crops can be classified as starch-producing crops, sugar-producing crops and lignocellulose-rich crops for bioethanol production, as well as oilseed crops for biodiesel (Li *et al.* 2010). Starch or sugar-based bioethanol and edible-oil-derived biodiesel may, however, impose challenges for food security if produced on a large scale in China. Nevertheless, conversion of lignocellulosic residues from food crops is a potential alternative (Xie and Peng 2011). Despite those approximately 0.7–0.9 billion tons of crop residues are produced each year, almost half of the residues are burnt to ash or directly discarded around the field (Chen *et al.* 2009). In addition, approximately 0.1 billion hectares of marginal lands not

suitable for food crops can be applied to grow energy crops to meet the large demand of biomass feedstock in China (Yan *et al.* 2008). We argue that sweet sorghum and *Miscanthus* should be considered as major candidates of non-food energy crops for marginal lands.

2. BIOLOGICAL CHARACTERISTICS OF SWEET SORGHUM AND *MISCANTHUS*

With the advances of biorefinery technologies of converting biomass into biofuels, efforts have been made to grow dedicated biomass crops in China. Sweet sorghum and *Miscanthus*, which are respectively originated in Africa and East Asia, are the candidate crops with extremely high biomass yields. Moreover, as these two species are evolutionary related, research advances in each of the crops will expedite improvement in the other crops (Van der Weijde, *et al* 2013).

Sweet sorghum grows rapidly (a life-cycle is around 120–150 days), and has high biomass yield (6.0-7.5 tons dry mass ha⁻¹ year⁻¹). Sweet sorghum is, furthermore, highly water-usage efficient, and needs typically only one third of water compared to sugarcane and half of that of corn (Li, *et al* 2013b). It is also drought, salt and cold tolerant as compared to conventional bioenergy crops (e.g., sugarcane and corn). As sweet sorghum can adapt to various environments with low fertilizer requirements, it is extensively grown globally, and is particularly well suited for agriculture in the north of China (Li and Chan-Halbrendt 2009; Xie and Su 2012). Li *et al* (2014b) and Wu *et al* (2015) examined over 200 sweet sorghum germplasm accessions stored in National Plant Germplasm System of China. These accessions were collected from across the world and displayed clear differences in agronomic trait, such as plant height, stem diameter, pitch numbers, lodging resistance, soluble sugar levels and seed yield. From such germplasm collections, it

may therefore be possible to find dedicated sweet sorghum accessions that are rich in soluble sugars and that have high digestible lignocellulosic bagasse suitable for bioenergy purposes (Byrt *et al.* 2011; Zegada-Lizarazu and Monti 2012; Li *et al.* 2014b).

Miscanthus is also a typical C4 plant that grows rapidly with low fertilizer requirement and high tolerance/resistance to drought, salt and cold conditions. It has wide geographic distributions and high biomass yields ranged from 37.5 to 60.8 tons dry mass ha⁻¹ year⁻¹. For instance, the natural distribution of *M. sinensis* in China is 100.45~127.55 °E, 18.34~43.70 °N, Alt. -12~1900m across 23 provinces (Table 1). So far, eleven species of *Miscanthus* have been identified (Jakobet *et al.* 2009), and over 1400 natural *Miscanthus* accessions, including four different species (*Miscanthus sacchariflorus*, *Miscanthus lutarioriparius*, *Miscanthus sinensis*, and *Miscanthus floridulus*) have been collected in China (Xie and Peng 2011).

Regardless of the relatively low soluble sugars in the stalks compared with sweet sorghum, *Miscanthus* is considered as a leading lignocellulosic bioenergy crop in China, and across the world (Lewandowski *et al.* 2003; Angelini *et al.* 2009; Xie and Peng 2011). While *Miscanthus* is mainly exploited for lignocellulosic biomass, sweet sorghum, maize, and sugarcane are dual-purpose crops for foods and biofuels (Table 1).

3. BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC RESIDUES OF SWEET SORGHUM AND *MISCANTHUS*

Various technologies have been applied to enhance biomass enzymatic saccharification and ethanol conversion efficiency. Sweet sorghum contains approximately 160-180 g L⁻¹ fermentable sugars, including sucrose, glucose and fructose, in the stalk juice (Laopaiboon *et al.* 2009), which can be

readily converted into ethanol by yeast fermentation (Sipos *et al.* 2009; Ratnavathi *et al.* 2010). It is also an ideal substrate for fuel gas production, such as hydrogen, by biomass gasification (Antonopoulou *et al.* 2008). A two-step membrane separation process has been developed to increase sugar concentrations and thus ethanol productivity from the stalk juice (Sasaki *et al.* 2014). The remaining bagasse of sweet sorghum is lignocellulose-rich which can also be processed to ethanol. To enhance the enzymatic digestibility of sweet sorghum bagasse, various pretreatment methods have been examined. Dilute NaOH solution autoclaving and H₂O₂ immersing pretreatment significantly increased cellulose hydrolysis yield, total sugar yield and ethanol concentration by approximately 6-, 10- and 20-folds, respectively, compared with the control (Cao *et al.* 2012). Integrating hydrothermal pretreatment and alkaline post-treatment significantly increased the saccharification ratio of sweet sorghum bagasse (Sun *et al.* 2015). Steam-pretreatment also resulted in efficient enzymatic hydrolysis of bagasse and conversion of 85 to 90% of the bagasse into ethanol (Sipos *et al.* 2009). Integration of solid-state fermentation technology and alkaline pretreatment has been shown to be a cost-effective process for the production of the ethanol from the sweet sorghum bagasse (Li *et al.* 2013b). In addition, sweet sorghum stalk has been examined as the feedstock for methane (Matsakas *et al.* 2014) and hydrogen production (Antonopoulou *et al.* 2008). It has also been used for heat production (Sipos *et al.* 2009). Sweet sorghum produces grains at a yield of about 2.2-4.5 tons dry mass ha⁻¹ year⁻¹, which can be used as food as well as the feedstock for bioethanol and pigment production (Gao *et al.* 2010).

Unlike sweet sorghum, *Miscanthus* is a dedicated lignocellulosic crop. Field trials in Europe during the last 15 years with the sterile, triploid hybrid

Miscanthus × giganteus (Clifton-brown *et al.* 2004; Heaton *et al.* 2004) have produced annual harvestable yields that range from 10 to 40 tons dry matter (DM) ha⁻¹ year⁻¹; more than double that of switchgrass. One ton of *Miscanthus* could produce up to 80 gallons of cellulosic ethanol (Lewandowskiet *et al.* 2000). Compared with maize (*Zea mays*), *Miscanthus* (*Miscanthus × giganteus*) is almost 60% more productive (Dohleman and Long 2014). An almost complete digestion (95%) was achieved by employing a two-stage method (alkaline peroxide and electrolyzed water). This was a better yield than the use of 1% H₂SO₄ pretreatment (200 °C, 8 min) (Wang *et al.* 2010). Various chemical and physical pretreatments have also been applied to enhance biomass enzymatic digestibility and ethanol production from *Miscanthus* (Zhang *et al.* 2013; Li *et al.* 2014c). However, harsher pretreatment conditions are required in *Miscanthus* than that of sweet sorghum bagasse, probably due to its distinct biomass recalcitrance.

4. LIGNOCELLULOSIC FEATURES AFFECTING BIOMASS SACCHARIFICATION OF SWEET SORGHUM AND *MISCANTHUS*

In principal, conversion of biomass into ethanol involves three major steps: physical and chemical pretreatments to deconstruct the cell wall, subsequent enzymatic hydrolysis leading to the release of soluble sugars, and yeast fermentation of the sugars for ethanol production (Ragauskas *et al.* 2006). However, the recalcitrance of the plant cell walls to enzymatic hydrolysis is crucial for this conversion process. Genetic modifications of plant cell wall composition and wall polymer features have been proposed as a promising solution to reduce the recalcitrance. Therefore, it becomes essential to identify the key factors of the plant cell wall that determine the efficiency of

enzymatic saccharification of various biomass feedstocks (Reddy and Yang 2005), particularly sweet sorghum and *Miscanthus*.

Cellulose consists of β -1, 4-glucans and makes up about 30% of the dry mass of primary cell walls, and up to 40% of the secondary cell walls, depending on tissue and plant species (Fry 1988; Arioli *et al.* 1998). There are two major cellulose features that are critical for efficient enzymatic digestibility in sweet sorghum, *Miscanthus* and other plants: cellulosic crystallinity (CrI) and the degree of polymerization (DP) of the cellulose microfibrils (Zhang *et al.* 2013; Wu *et al.* 2013; Jia *et al.* 2014; Li *et al.* 2014c; Huang *et al.* 2015). As crystalline cellulose is less accessible to cellulases than amorphous cellulose, the cellulose crystallinity negatively affects the biomass enzymatic digestibility. This scenario holds for all examined plant species, including sweet sorghum and *Miscanthus* (Zhang *et al.* 2013; Wu *et al.* 2013; Li *et al.* 2014b, c). Recently, it was reported that the DP of cellulose also affects the biomass saccharification negatively in sweet sorghum, *Miscanthus* and other plants (Yang *et al.* 2011; Zhang *et al.* 2013; Wu *et al.* 2013; Li *et al.* 2014c). This is likely due to the fact that reduced cellulose DP increases cellulose chain-reducing ends and therefore reduces cellulose crystallinity (Pan *et al.* 2008; Zhang and Lynd 2004). In addition, the mole number (MN) of cellulose is an important parameter that influences biomass enzymatic digestibility. The cellulose MN can be determined by dividing cellulose content per unit length by mole weight of cellulose (Kokubo *et al.* 1991). In *Miscanthus*, the MN correlates negatively with biomass enzymatic digestion after pretreatments with NaOH and H₂SO₄ (Zhang *et al.* 2013).

Hemicelluloses are a class of heterogeneous polysaccharides with various hexose and pentose units. In grasses, xylans are the major hemicelluloses

and are commonly substituted with α -L-arabinofuranosyl units on the C2- and/or C3-position (Girio *et al.* 2010; Scheller and Ulvskov 2010). Hemicelluloses are generally believed to provide cross-linking interactions with cellulose and lignin, which strengthens the cell wall and possibly function as molecular spacers for cellulose microfibrils. Using large numbers of *Miscanthus* accessions with diverse cell wall compositions, hemicelluloses were found to be a predominant factor that positively determines biomass enzymatic digestibility after pretreatments with NaOH and H₂SO₄ by reducing cellulose crystallinity (Xu *et al.* 2012). Furthermore, a higher degree of arabinose substitution (reverse xylose/arabinose, Xyl/Ara) of xylans positively influenced biomass digestibility in *Miscanthus* (Li *et al.* 2013a). Here, hemicelluloses with high arabinose levels correlated negatively with cellulose crystallinity and enhanced both plant lodging resistance and biomass enzymatic digestibility in rice (Li *et al.* 2014a). In sweet sorghum and wheat, a high arabinose substitution degree of non-KOH-extractable hemicelluloses can also enhance biomass enzymatic digestibility by reducing cellulose crystallinity (Wu *et al.* 2013; Li *et al.* 2014b).

Lignin is a stable and complex polymer consisting of three major phenylpropane units: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Sun *et al.* 2013). As lignin is associated with other wall polymers via ester- and ether-linked bonds, it acts as barriers that hinder enzyme penetration to access cellulose surfaces (Achyuthan *et al.* 2010). Due to its structural diversity and heterogeneity, lignin has multiple roles in biomass enzymatic digestions. For example, increased S/G ratios negatively affect digestibility of *Miscanthus* biomass, whereas increased H/G ratios positively affects saccharification of rice and wheat biomass (Xu *et al.* 2012; Wu *et al.* 2013;

Jia *et al.* 2014; Li *et al.* 2014a). Although lignin did not appear to influence cellulose crystallinity in sweet sorghum, high levels of lignin G-monomers had a negative impact on biomass digestion, and the release of G-monomers from the biomass significantly inhibited yeast fermentation (Li *et al.* 2014b). In *Miscanthus*, the minor wall-networks between monolignols and interlinked-phenolics predominantly affects biomass digestibility (Li *et al.* 2014c), and mild alkali-pretreatment effectively extracts guaiacyl-rich lignin for high lignocellulose digestibility coupled with largely diminishing yeast fermentation inhibitors (Li *et al.* 2014c). In addition, lignin extraction enhances biomass enzymatic saccharification in hemicelluloses-rich *Miscanthus* species under various alkali and acid pretreatments (Si *et al.* 2015).

In conclusion, reduced cellulose crystallinity/DP and increased arabinose substitution degree of xylans positively influence biomass enzymatic saccharification under various pretreatments in both sweet sorghum and *Miscanthus*, whereas high levels of G-monomers and low S/G ratios of lignin negatively affect biomass digestibility, respectively (Table 2). This suggests that optimizing certain wall characteristics will make sweet sorghum and *Miscanthus* more suitable as the feedstock for liquid biofuel production.

5. BIOTECHNOLOGY FOR SWEET SORGHUM AND *MISCANTHUS* BIOENERGY BREEDING

As large populations of natural germplasm accessions of sweet sorghum and *Miscanthus* have exhibited a diverse cell wall composition and biomass saccharification, it may be appropriate to screen for high biomass digestibility for biofuel production. However, traditional screening approaches are labor-intensive, time-consuming and expensive as it includes

chemical analyses of plant cell wall compositions and estimates of total sugar yields released via enzymatic hydrolysis (Roberts *et al.* 2011; Li *et al.* 2014b). Recently, near infrared spectroscopy has been used for high-throughput screening of sweet sorghum and *Miscanthus* accessions (Huang *et al.* 2012; Wu *et al.* 2015). Using 199 *Miscanthus* accessions, seven optimal models were identified with high determination coefficient for biomass enzymatic digestibility upon various physical (heat) and chemical (1% NaOH, 1% H₂SO₄) pretreatments (Huang *et al.* 2012). In addition, a total of 123 sweet sorghum accessions and 50 mutants were examined for stalk soluble sugars, bagasse enzymatic saccharification and wall polymer features. From these measurements, calibration equations were generated that can effectively determine the relationships between stalk soluble sugars, bagasse enzymatic saccharification and cell wall polymers (Wu *et al.* 2015).

Miscanthus is a natural hybrid, and has more than 20 species originating from East-Asia. The triploid hybrid *Miscanthus* × *giganteus* ($2n = 3x = 57$), diploid *M. sinensis* ($2n = 2x = 38$) and tetraploid *M. sacchariflorus* ($2n = 4x = 76$) are currently considered as the most promising varieties for bioethanol production (Zub and Brancourt-Hulmel 2010). In sweet sorghum, heterosis has yielded new hybrids with high stalk sugar yield (Pfeiffer *et al.* 2010). In addition, EMS mutations could be used to produce elite lines of biomass traits of sweet sorghum (Wu *et al.* 2015).

Although conventional breeding methods play an important role in developing new crop cultivars, biotechnological tools are becoming faster and more precise, and allow for specific design of crops for target characteristics. Molecular markers, such as simple sequence repeats, have increased our ability to characterize genetic diversity (Li *et al.* 2010). Moreover, rapid development of sequencing technologies and bioinformatic

tools have made the first whole genome sequences for a grain sorghum, BTx623 (Paterson *et al.* 2009), and the re-sequencing of two sweet and one grain sorghum inbred lines (Zheng *et al.* 2011), possible. These studies have resulted in the identification of nearly 1,500 genes that differ in terms of sugar and starch metabolism, lignin and coumarin biosynthesis, nucleic acid metabolism, stress responses and DNA damage repair between sweet and grain sorghum. In addition, a large quantity of SNPs, indels, PAVs and CNVs were identified, which should be used for comparative genomics and crop breeding in sorghum to improve sugar- and biofuel-associated traits (Zheng *et al.* 2011). As an example, expression profiling of one sucrose synthase, two sucrose phosphate synthases and a vacuolar invertase gene revealed that they are less highly expressed in sweet sorghum as compared to grain sorghum. Furthermore, differential expression of sugar metabolizing enzymes and sucrose transporters in sweet and grain sorghum suggests transcriptional regulation of sugar accumulation (Qazi *et al.* 2012). It is, however, important to note that certain developmental differences may also lay as ground for some of these differences. Nevertheless, over-expression of a sucrose-sucrose fructosyl transferase in sugarcane led to sugar accumulation in the parenchyma cells vacuoles of mature stalk (Arruda, 2011).

Several candidate genes have been reported to reduce cellulosic crystallinity and/or to increase arabinose substitution degrees and/or altering the lignin constitution, which impacted on biomass saccharification in rice. These genes include *OsGH9*, *OsXAT*, *OsGT61*, *OsIRX*, *OsCCRI*, *OsCCOMT*, *Os4CL* (Xie *et al.* 2013; Guo *et al.* 2014; Li *et al.* 2014a; Wang *et al.* 2014, Feng *et al.* 2013). Hence, targeting of the gene orthologs of

these in sweet sorghum and *Miscanthus* may modify lignocellulose features for high biomass saccharification.

Recently, bombardment of transgene containing gold particles has been used to successfully transform *Miscanthus* (Wang *et al.* 2011), and *Agrobacterium*-mediated transformation has also been attempted in both *Miscanthus* (Hwang *et al.* 2014) and sorghum (Gao *et al.* 2005a, b; Zhao *et al.* 2000). However, these approaches have yielded low transformation efficiency in *Miscanthus*, and many factors that affect the transformation efficiency have been established (Hyoung *et al.* 2010; Liu *et al.* 2013). Sorghum is difficult to transform, but Zhao *et al.* (2000) found that the embryo source plays a very important role in the transformation efficiency with an average frequency of 2.1% after co-cultivation of immature embryos with *Agrobacterium* carrying a super-binary vector. Gao *et al.* (2005a) used Green Fluorescent Protein (GFP) screening to assess the stable transformation efficiency of sorghum plants *Agrobacterium*-mediated transformation protocol. In addition, Gao *et al.* (2005b) used the *Escherichia coli* phosphomannose isomerase gene, *pmi*, as a selectable marker gene and generated 167 transgenic plants with transformation frequencies around 3%. Subsequently, Akhok Kumar Shrawat *et al.* (2006) concluded a general scheme for *Agrobacterium* transformation of cereals. These results are promising for targeting of specific genes in the future.

6. CONCLUSION

Among many bioenergy crops, sweet sorghum and *Miscanthus* have been regarded as the two leading feedstock candidates, largely due to their high biomass yields and excellent biological characteristics. Over the past years, various new technologies of biomass pretreatments have been applied

in sweet sorghum and *Miscanthus* to enhance biomass enzymatic digestibility and to reduce ethanol conversion cost by yeast fermentation. Genetic modifications that affect the plant cell wall have been proposed as holding great promise to overcome biomass recalcitrance by reducing cellulose crystallinity, increasing arabinose substitution degree of xylans, or altering the relative proportions of the three monolignols in lignin in sweet sorghum and *Miscanthus*. Furthermore, screening of large populations of natural germplasm accessions and cell wall mutants is an alternative approach to identify new lines with improved saccharification rates. Molecular breeding will be a powerful approach to develop new varieties for bioenergy production in sweet sorghum and *Miscanthus*.

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Table 1 . Information on Sweet sorghum and *Miscanthus* distribution and current biofuel application in China

Crop	Origin region	Subspecies	Distribution	Biomass yield (t DM ha ⁻¹ yr ⁻¹) ^a	Advantages	Application	Inhibitors for application	References
Sweet sorghum	Africa	<i>S. bicolor</i> subsp. <i>Bicolor</i> ; <i>S. bicolor</i> subsp. <i>Drummondii</i> (Steud.) de Wet; <i>S. Bicolor</i> subsp. <i>Arudinaceu.</i> (Desv.) de Wer et Harlan	74,36~122.30°E, 18.10~50.20°N, Alt. 24-3000m, more than 10 provinces	6.0-7.5	Well adaptation to environments; Rapid growth; High biomass production; Rich soluble sugars; dual-purpose crop	Juice-derived ethanol, jiggery, syrup; Biomass-based ethanol, animal feed, organic manure; Grain-based pigment industry	Sugar content; Cell wall recalcitrance;	Zhang et al., 2012; Zheng et al., 2011; Laopaiboon et al., 2009; Paterson et al., 2009 Li et al., 2014b; Wu et al., 2015
<i>Miscanthus</i>	East Asia	<i>Miscanthus sacchariflorus</i> ; <i>Miscanthus lutarioriparius</i> ; <i>Miscanthus sinensis</i> ; <i>Miscanthus floridulus</i>	100.45~127.55°E, 18.34~43.70°N, Alt. -12~1900m, including 23 provinces	37.5-60.8	High yielding; Environmentally friendly; Easy to grow; Low agricultural inputs; Long lifespan; Non-food crop	Green heat and electricity; High quality paper pulp; Bioplastic composite; Bioethanol or diesel fuel	Cell wall recalcitrance; High economic input; Lack of raw material	Lewandowski et al., 2000; Zhou et al., 2012; Huang et al., 2012; Zub et al., 2010; Yu et al., 2014; Slavov et al., 2013; Himmel et al., 2007

Table 2. Effects of cell wall composition and polymer features on biomass saccharification in sweet sorghum and *Miscanthus*

Plant species	Cell wall polymers	Cell wall composition (%dry matter)	Polymer features	Impacts on biomass saccharification	References
Sweet sorghum	Cellulose	27-37	CrI ^a , DP ^b	Negative	Yang et al., 2011; Li et al., 2013b;
	Hemicellulose	29-33	Reverse Xyl/Ara ^c	Positive	Zhang et al., 2013; Li et al., 2014b;
	Lignin	17-20	G ^d , S/G ^e	Negative	Wang et al., 2014
<i>Miscanthus</i>	Cellulose	28-49	CrI, DP, MN ^f	Negative	Xu et al., 2012;
	Hemicellulose	24-32	Reverse Xyl/Ara	Positive	Van et al., 2013; Li et al., 2014c
	Lignin	15-28	S/G	Negative	

^aCrI, Crystalline index;

^bDP, Degree of polymerization of crystalline cellulose;

^cReverse Xyl/Ara, Degree of arabinose substitution of xylans;

^dG, Guaiacyl;

^eS/G, Syringyl/ Guaiacyl ratio;

^fMN, The mole number of cellulose.

