Targets and tools for optimizing lignocellulosic biomass quality of miscanthus

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Tim van der Weijde

Thesis

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Contents

Summary

Miscanthus is a perennial energy grass characterized by a high productivity and resource-use efficiency, making it an ideal biomass feedstock for the production of cellulosic biofuels and a wide range of other biobased value-chains. However, the large-scale commercialization of converting biomass into cellulosic biofuel is hindered by our inability to efficiently deconstruct the plant cell wall. The plant cell wall is a complex and dynamic structure and its components are extensively cross-linked into an unyielding matrix. The production of biofuel depends on the extraction, hydrolysis and fermentation of cell wall polysaccharides, which currently requires energetically and chemically intensive processing operations that negatively affect the economic viability and sustainability of the industry. To address this challenge it is envisioned that the bioenergy feedstocks can be compositionally tailored to increase the accessibility and extractability of cell wall polysaccharides, which would allow a more efficient conversion of biomass into biofuel under milder processing conditions.

Extensive phenotypic and genetic diversity in cell wall composition and conversion efficiency was observed in different miscanthus species, including M. sinensis, M. sacchariflorus and interspecific hybrids between these two species. In multiple experiments a twofold increase in the release of fermentable sugars was observed in 'high quality' accessions compared to 'low quality' accessions. The exhaustive characterization of eight highly diverse M. sinensis genotypes revealed novel and distinct breeding targets for different bioenergy conversion routes. The key traits that contributed favourably to the conversion efficiency of biomass into biofuel were a high content of hemicellulosic polysaccharides, extensive cross-linking of hemicellulosic polysaccharides (revealed by a high content of trans-ferulic acids and a high ratio of arabinose-to-xylose), a low lignin content and extensive incorporation of para-coumaric acid into the lignin polymer.

Lignin is widely recognized as one of the key factors conveying recalcitrance against enzymatic deconstruction of the cell wall. The incorporation of para-coumaric acid into the lignin polymer is hypothesized to make lignin more easily degradable during alkaline pretreatment, one of the most widely applied processing methods that is used to pretreat biomass prior to enzymatic hydrolysis. Previous studies have shown that reducing lignin content is often implicated in reduced resistance of plants to lodging. We hypothesize that extensively cross-linked hemicellulosic polysaccharides may fulfil a similar function in supporting cell wall structural rigidity and increasing the content of hemicellulosic polysaccharides may be a way to reduce lignin content without adversely affecting cell wall rigidity. This strategy can be used to improve biomass quality for biobased applications, as hemicellulosic polysaccharides are more easily degradable during industrial processing than lignin. Furthermore, hemicellulosic polysaccharides adhere to cellulose, which negatively affects the level of cellulose crystallinity. Crystalline cellulose is harder to degrade than its more amorphous form. Therefore the reduction of cellulose crystallinity is another

mechanism through which increasing the content of hemicellulosic polysaccharides positively contributes to cell wall degradability. These results provided new insights into the traits that may be targeted to improve the quality of lignocellulose feedstocks.

However, evaluation of complex biochemical traits for selection purposes is hindered by the fact that their accurate quantification is a costly, lengthy and laborious procedure. To overcome these limitations an accurate and high-throughput method was developed based on near-infrared spectroscopy. Through extensive calibration we developed accurate prediction models for a wide range of biomass quality characteristics, which may be readily implemented as a phenotyping tool for selection purposes.

Additionally, progress through breeding may substantially be improved by marker-assisted selection, which will reduce the need for the evaluation of genotype performance in multi-year field trials. To this end, a biparental M. sinensis mapping population of 186 individuals was developed and genotyped using a genotyping-by-sequencing approach. A total of 564 short-sequence markers were used to construct a new M. sinensis genetic map. Cell wall composition and conversion efficiency were observed to be highly heritable and quantitatively inherited properties. This is the first genetic study in miscanthus to map quantitative trait loci (QTLs) for biomass quality properties and is a first step towards the application of marker-assisted selection for biomass quality properties.

Through the evaluation of a diverse set of miscanthus genotypes in multiple locations we demonstrated that in addition to genotypic variation, growing conditions may have a substantial influence on cell wall composition and conversion efficiency. While further research is needed to identify which specific environmental parameters are responsible for the observed effects, these results clearly indicate that the environmental influence on biomass quality needs to be taken into account in order to match genotype, location and end-use of miscanthus as a lignocellulose feedstock. Moreover, significant genotype-by-environment interaction effects were observed for cell wall composition and conversion efficiency, indicating variation in environmental sensitivity across genotypes. Although the magnitude of the genotypic differences was small in comparison to genotype and environmental main effects, this affected the ranking of accession across environments. Stability analysis indicated some stable accessions performed relatively across diverse locations.

In addition to trialing miscanthus in diverse locations, we also evaluated miscanthus biomass quality under drought conditions for a number of reasons: 1) drought stress is linked to a differential expression of cell wall biosynthesis genes, 2) incidence of drought events is increasing due to climate change, 3) irrigation is likely to be uneconomical during the cultivation of miscanthus and 4) miscanthus has many characteristics that make it a crop with a good potential for cultivation on marginal soils, where abiotic stresses such as drought may prevail. Drought stress was shown to result in a large reduction in cell wall and

cellulose content and a substantial increase in hemicellulosic polysaccharides and cellulose conversion rates. We hypothesized that the reduction in cellulose content was due to an increase in the production of osmolytes, which are well-known for their role in plant protection against drought. The results indicated that drought stress had a positive effect on the cell wall degradability of miscanthus biomass.

Overall the compendium of knowledge generated within the framework of this thesis provided insights into the variation in biomass quality properties in miscanthus, increased our understanding of the molecular, genetic and environmental factors influencing its conversion efficiency into biofuel and provided tools to exploit these factors to expand the use of miscanthus as a lignocellulose feedstock.

Chapter 1

General Introduction

1. Cellulosic biofuel; a unique, renewable transportation fuel

Energy is essential to our modern society, yet we are facing the near depletion of our principal energy carrier, fossil fuel, without any alternative applicable on large scales ready at hand (Wyman, 2008). Due to the growing world population and the exponential industrialization of upcoming economies, such as India and China, the worldwide fossil fuel consumption is rapidly growing, with the demand bound to surpass the global production in the foreseeable future (Sorrell *et al.*, 2010). These developments are affecting politics and economies worldwide, as oil prices are fluctuating and the oil import dependency of countries lacking fossil reserves is creating political unease. Equally concerning are the negative environmental effects of unprecedented rates of greenhouse gas emissions by the combustion of fossil fuels, which are becoming increasingly evident and drive governmental research investments in alternative energy options. While a range of renewable energy alternatives are being developed, liquid biofuels are the only form of renewable energy with the potential for large-scale displacement of fossil fuels as liquid energy carrier for the transportation sector, which incidentally is responsible for approximately one-third of all greenhouse gas emissions (Wyman, 1999, Wyman, 2008). With the depletion of fossil reserves, the increasing global fossil fuel consumption and the environmental concerns associated with the large scale use of fossil fuel, there is no doubt as to the importance and urgency of the development of renewable biofuels.

At present, the production of renewable transportation fuel is dominated by the production of first-generation bioethanol from corn grains and sugarcane juice and biodiesel from soybean, palm and rapeseed oils. Although these first-generation biofuels are economically successful technologies, they fail to reduce net greenhouse gas emissions, due to the high energy inputs required during the cultivation of these food crops (Hill, 2007, Tilman *et al.*, 2009). Moreover the large scale use of food products and quality farm land for the production of biofuels has raised ethical and socio-economic concerns and has already been associated with increasing global food prices (Zilberman *et al.*, 2013).

In contrast, second-generation biofuels refers to biofuels derived from lignocellulosic biomass, which has no or very limited use as food product. Worldwide lignocellulosic biomass represents the most abundant and renewable carbon resource. Large amounts of lignocellulosic biomass are already available in the form of municipal organic waste and agricultural and forestry residues, which are currently largely underutilized and can serve as a low-cost biomass feedstock for the production of cellulosic ethanol (Kim & Dale, 2004, Perlack *et al.*, 2005). In addition, biomass can be produced using second-generation energy crops that are capable of producing high biomass yields under low-input cultivation conditions and may even thrive on marginal soils (Anderson *et al.*, 2008, Lewandowski *et al.*, 2003b, Quinn *et al.*, 2015, van der Weijde *et al.*, 2013). A unique advantage of cellulosic ethanol produced from such crops is the reduction in greenhouse gas emissions,

which results from 1) low agricultural inputs, due to the minimal demand for inputs of agrochemicals during cultivation, 2) the full use of biomass, by extraction of fermentable sugars for bioethanol production and use of the remaining residue for the production of energy to power the processing plant, and 3) recycling of CO_2, as energy crops re-fixate the carbon dioxide that is released into the atmosphere by the combustion of cellulosic ethanol and biomass residues (Lynd, 1996) (Figure 1). Hence, the use of renewable biomass feedstocks for the production of cellulosic ethanol offers environmental benefits and simultaneously contributes to energy security (Farrell *et al.*, 2006b, Wyman, 2007).

2. Lignocellulose conversion technology

Plant biomass consists largely of carbon-rich cell wall material, which is mainly composed of cellulose, hemicellulosic polysaccharides and lignin. Both cellulosic and hemicellulosic polysaccharides can be processed into cellulosic ethanol. The production process starts with mixing the biomass into an aqueous slurry, which is heated and chemically pretreated to disrupt the intact cell walls. This improves the accessibility of cell wall polysaccharides to hydrolytic enzymes in the next step; enzymatic saccharification. During enzymatic saccharification the exposed polysaccharides are hydrolyzed into their monosaccharide constituents, which are subsequently fermented into bioethanol (Chundawat *et al.*, 2011, Hamelinck *et al.*, 2005, Lynd, 1996, Mosier *et al.*, 2005) (Figure 1). Since the 1980's significant technological advances have been made that reduced processing costs through improvements in chemical pretreatment technologies and engineering of enzyme cocktails with enhanced hydrolytic capability to increase the deconstruction of cellulosic and hemicellulosic polysaccharides into fermentable sugars (Alvira *et al.*, 2010, Chen *et al.*, 2012, Himmel *et al.*, 2007, Kumar *et al.*, 2009, Mosier *et al.*, 2005, Wyman, 1999, Wyman, 2007, Wyman *et al.*, 2005, Yang & Wyman, 2008, Zhang *et al.*, 2006, Zheng *et al.*, 2009). Moreover fermentation efficiency has been improved by genetic engineering of yeast and bacteria strains that are capable of fermenting both pentose and hexose sugars and are increasingly insensitive to end-product inhibition and to the toxic compounds often released from biomass during pretreatment (Balat, 2011, Kim *et al.*, 2010, Pereira *et al.*, 2014, Saha, 2003, Wyman, 1999).

These technological advances have brought the cellulosic ethanol industry to a point where production costs are becoming competitive for blending with gasoline (Lynd, 1996). In 2015 the three largest cellulosic ethanol plants so far became operational, bringing the total to six operational plants worldwide with a total production capacity of over 430 million liters of ethanol per year (Dale, 2015, RFA, 2016). Other large scale plants are likely to commence production in the next few years and there are already demonstration plants of various sizes in China, Denmark, Finland, Germany, Japan, Russia, Spain, Thailand and the US (Dale,

2015). These developments show that a global cellulosic ethanol industry is beginning to develop and that the stage is set for large scale displacement of fossil fuel by cellulosic ethanol produced from renewable biomass feedstocks.

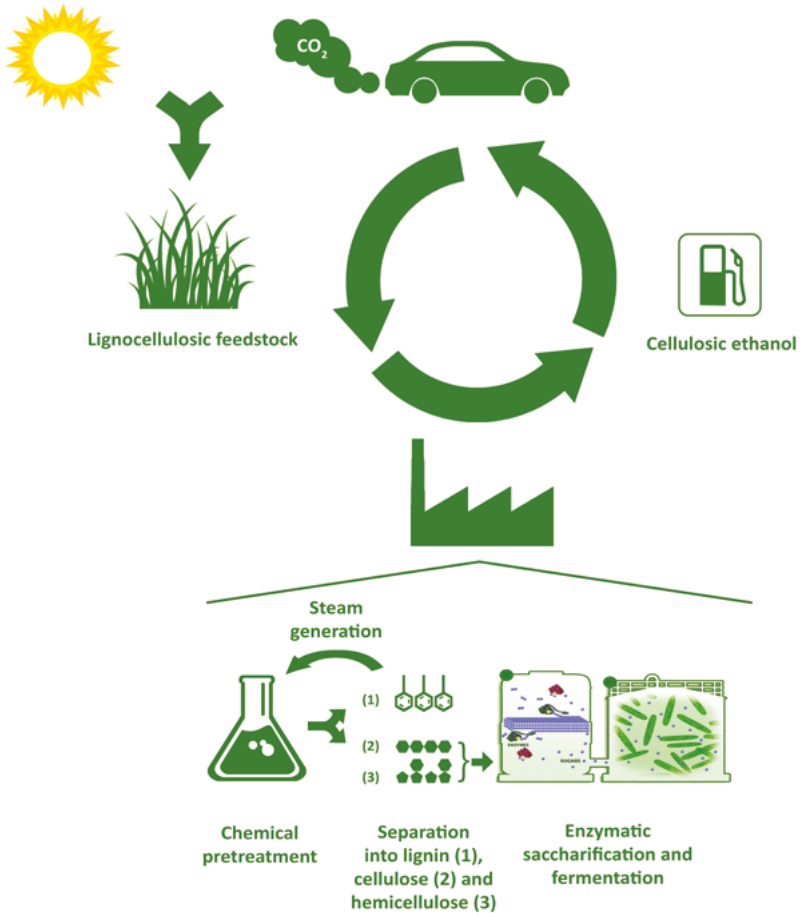


Figure 1. Overview of the carbon cycle and production process of cellulosic ethanol.

3. Genetic improvement of biomass quality; the key to unlocking the potential of cellulosic ethanol

While great progress has been achieved in biomass conversion technology, further reductions in processing costs are needed to make cellulosic ethanol competitive as a pure transportation fuel independent of government subsidies and mandates (Lynd, 1996, Wyman, 2007). The genetic improvement of lignocellulose feedstocks is envisioned to further reduce the production costs of cellulosic ethanol. The rationale behind this anticipation

is that the efficient fractionation of biomass into fermentable sugars is highly dependent on the quality of the feedstock and can be improved through the genetic improvement of energy crops (Himmel *et al.*, 2007, Himmel & Picataggio, 2008, Wyman, 2007). The reason is that the plant cell wall has evolved into a complex and dynamic network of cross-linked components that provides the plant with structural rigidity and protection against enzymatic deconstruction by plant pathogens (Himmel *et al.*, 2007, Zhao *et al.*, 2012). However, vast inter and intra-specific genetic variation exists in the cell wall composition and ultrastructure of promising lignocellulose feedstocks (Byrt *et al.*, 2011, Karp & Shield, 2008, van der Weijde *et al.*, 2013). Key targets for genetic improvement are to increase the content of polysaccharides and the accessibility of these polysaccharides to hydrolytic enzymes during enzymatic saccharification. These traits are the basis of what in this thesis is collectively referred to as 'biomass quality'. Until now very limited attention has been given to the role of biomass quality on the techno-economic performance of cellulosic ethanol production. At present highly stringent chemical pretreatment conditions are employed in order to achieve near-complete hydrolysis of cell wall polysaccharides. With improvements in biomass quality the severity of chemical pretreatments can be reduced, which can substantially reduce the production costs of cellulosic ethanol (Himmel *et al.*, 2007, Torres *et al.*, 2016, Torres *et al.*, 2013, Wyman, 2007).

The genetic improvement in biomass quality of second-generation energy crops is challenging, as in contrast to most food crops, which have been domesticated hundreds to thousands of years ago, these energy crops have only recently attracted interest and are virtually undomesticated (Sang, 2011). Moreover, improving biomass quality is a not a simple endeavor as we do not yet fully understand the complex structure of lignocellulose, nor how this affects the conversion efficiency of biomass into fermentable sugars (Himmel *et al.*, 2007, Zhao *et al.*, 2012). To make an already challenging task even more complex, there are limitations to the extent to which lignocellulose ultrastructure can be manipulated without affecting plant fitness. This has been clearly demonstrated by the example of '*brown-midrib'* mutants in maize and sorghum, in which the mutant phenotype is associated with increased cell wall digestibility, but also decreased resistance to lodging (Casler *et al.*, 2002, Pedersen *et al.*, 2005). We are now challenged to find ways to manipulate lignocellulose structure to improve its conversion efficiency into cellulosic ethanol without adversely affecting plant fitness (Himmel *et al.*, 2007, Zhao *et al.*, 2012).

4. Understanding and screening of lignocellulose conversion efficiency

Although our understanding of the factors that constitute lignocellulose conversion efficiency is far from complete, several structural features of the cell wall have been identified that limit the enzymatic hydrolysis of cell wall polysaccharides. Lignin is one of the key cell wall components limiting the conversion of biomass into biofuel. It cross-links to

hemicellulosic polysaccharides to form a highly impermeable matrix that imparts strength to the plant cell wall and shields cellulose - the main source of fermentable sugars - from chemical and enzymatic hydrolysis (Grabber, 2005, Grabber *et al.*, 2004, Himmel *et al.*, 2007, Himmel & Picataggio, 2008, Zhao *et al.*, 2012) (Figure 2). In addition, it negatively influences enzymatic saccharification by irreversibly adsorbing hydrolytic enzymes, which renders these ineffective (Jørgensen *et al.*, 2007, Zhao *et al.*, 2012).

Another key factor implicated to negatively affect conversion efficiency is the level of cellulose crystallinity (Hall *et al.*, 2010, Himmel *et al.*, 2007, Xu *et al.*, 2012). Cellulose is a homopolymer of β-(1-4)-linked glucose units that occurs in the cell wall in crystalline and amorphous form, with the crystalline form being less accessible to hydrolytic enzymes (Hall *et al.*, 2010). The amorphous form occurs as a result of cross-linking of cellulose with the network of hemicellulosic polysaccharides surrounding the cellulose fibers, which is why the content of hemicellulosic polysaccharides is negatively correlated with cellulose crystallinity (Xu *et al.*, 2012). There are a range of other lignocellulose features that are possibly implicated, such as the monomeric composition of hemicellulosic polysaccharides, the amount of side chains on the hemicellulose backbone, the level of acetylation and feruloyation of hemicellulosic polysaccharides, the degree of cellulose polymerization, the ratio between different lignin subunits incorporated into the lignin polymer, the incorporation of cell wall proteins into the cell wall matrix and the number of ferulate bridges between hemicellulosic polysaccharides and lignin (Zhao *et al.*, 2012).

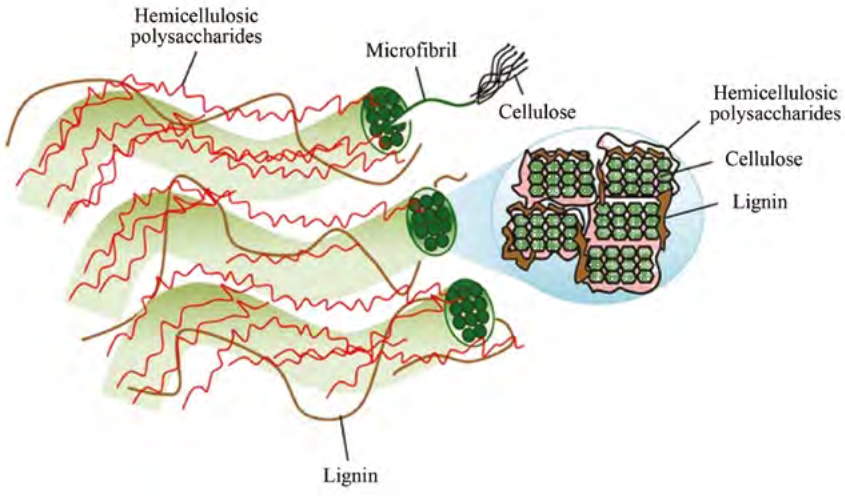


Figure 2. Schematic overview of the molecular structure of the plant cell wall.

Studying the factors involved in lignocellulose conversion efficiency is challenging because of the structural complexity and diversity of lignocellulose and the correlations between different cell wall components (Zhao *et al.*, 2012). Moreover, a wide variety of pretreatment

technologies have been developed, that act on different cell wall components to increase lignocellulose degradability (Mosier *et al.*, 2005). Different studies often use distinctive pretreatment methods, which may lead to different conclusions as to which factors play a role in conversion efficiency.

Screening lignocellulose feedstocks for differences in conversion efficiency is typically done by mimicking on a small scale the industrial process of cellulosic ethanol production up to the point of quantifying the yields of fermentable sugars per unit of biomass. Several small scale, high-throughput and automated laboratory systems have been developed to enable screening large numbers of samples (Decker *et al.*, 2009, Lindedam *et al.*, 2014, Santoro *et al.*, 2010, Selig *et al.*, 2010, Studer *et al.*, 2010). After collection of a sufficient amount of biochemical data and multivariate statistical analysis of the data, conversion efficiency can even be accurately predicted from easily obtained near-infrared spectra (Huang *et al.*, 2012, Payne & Wolfrum, 2015, Vogel *et al.*, 2011), which is a key technique used in this thesis.

An important concept to consider in screening differences in lignocellulose conversion efficiency is the relation of pretreatment severity, which is a combined severity measure of pretreatment temperature, chemical load, duration and biomass solids loading (Pedersen & Meyer, 2010), and the impact of pretreatment on conversion efficiency. At high pretreatment severity near-complete conversion of cell wall polysaccharides can be achieved, regardless of the composition or quality of the feedstock. When screening lignocellulosic feedstocks for differences in hydrolysis yields using highly severe pretreatments, feedstocks will be mainly discriminated on total content of cell wall polysaccharides. However, screening at lower pretreatment severities exposes genetic differences in conversion efficiency and may help to unravel the biochemical factors that are involved (Torres *et al.*, 2013). Efficient screening methods for conversion efficiency and increasing our understanding of the underlying factors involved is fundamental to the genetic improvement of biomass quality in lignocellulose feedstocks.

5. Miscanthus – one of the most promising lignocellulose feedstocks for biofuel production

5.1. Miscanthus: a highly productive biomass crop

Several second-generation energy crops have potential as a lignocellulose feedstock for biofuel production, but one of the strongest contenders is miscanthus. Miscanthus is a highly productive perennial rhizomatous grass that normally grows 2-4 meters tall (Anderson *et al.*, 2011), but can reach heights of up to 7 meters (Chen & Renvoize, 2005) (Figure 3). After a yield-building phase which typically lasts 2-4 years after establishment (depending on the

genotype and environment), miscanthus has a productive lifespan of 15-25 years, during which high annual biomass yields are maintained (Lewandowski *et al.*, 2003b{Gauder, 2012 #217, Zub & Brancourt-Hulmel, 2010).

Figure 3. Miscanthus fields at Wageningen UR

Miscanthus owes its high productivity in part to its efficient C4 photosynthesis system (Furbank, 1998, Lewandowski *et al.*, 2000). Due to their photorespiration-suppressing modifications, C4 plants have a higher potential efficiency of converting solar energy to biomass than C3 plants (Ehleringer & Cerling, 2002, Zhu *et al.*, 2008). However, usually C4 plants are restricted by an impairment of photosynthetic capacity at low temperatures, giving C3 plants an advantage in temperate and C4 plants in tropical climates (Byrt *et al.*, 2011, Long, 1983, Long *et al.*, 2001). One of the particular advantageous properties of miscanthus compared to other C4 grasses, is its relatively high cold tolerance and maintenance of photosynthetic activity at temperatures as low as 10°C (Dohleman & Long, 2009, Jones & Walsh, 2001, Naidu & Long, 2004). These properties allow for early spring emergence and subsequently a comparably longer growth season than many other crops.

M. × giganteus is currently the only species of the genus *Miscanthus* that is commercially exploited for biomass production in Europe and the United states and its yield and yield potential has been investigated in many field trials across diverse environments. Non-irrigated yields are reported to range from 15 – 25 t dm ha-1 yr-1, while maximum yields of up to 50 t dm ha-1 yr-1 were obtained under irrigated conditions (Clifton-brown *et al.*, 2004, Lewandowski *et al.*, 2000, Zub & Brancourt-Hulmel, 2010). Moreover, in a quantitative review combining the results of close to a hundred field trials across Europe and the United States an average biomass yield of 22 t dm ha-1 yr-1 was reported (Heaton *et al.*, 2004a). The annual growth cycle of miscanthus is presented in **Figure 4**.

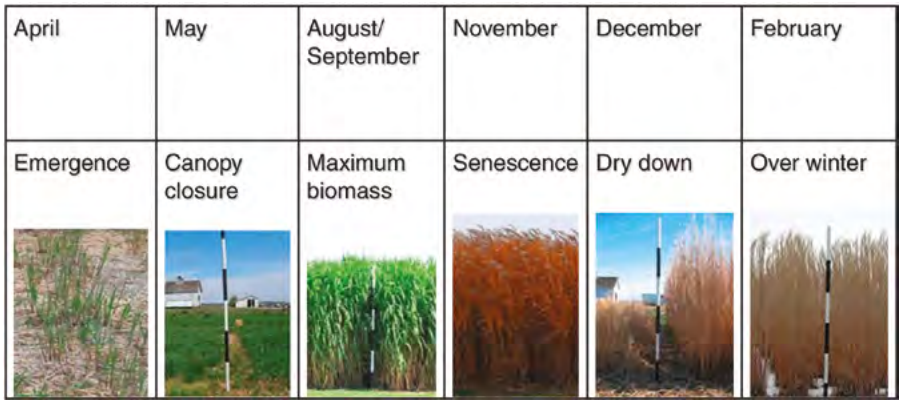
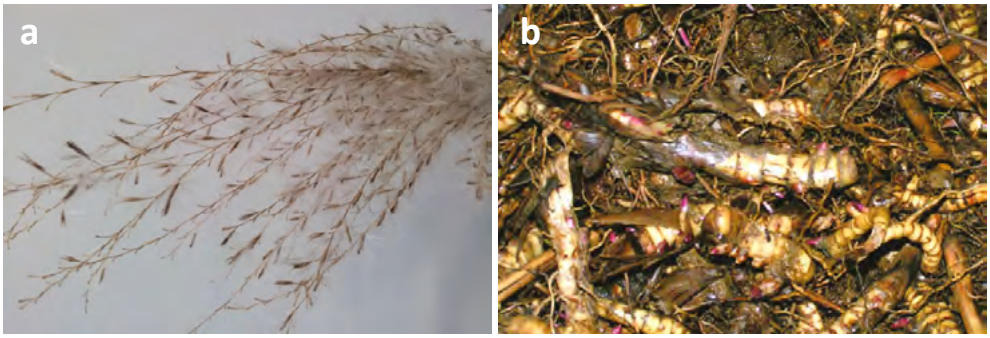


Figure 4. Annual growth cycle of a M. × giganteus stand (Source: (Heaton et al., 2010)).

5.2. Environmental benefits of biomass production using miscanthus

The production of miscanthus – and in general all perennial rhizomatous grasses - as a biomass feedstock offers several environmental benefits, in addition to reducing greenhouse gas emissions through displacement of fossil fuels (Lewandowski *et al.*, 2003b). Unlike annual crops, soil tillage in perennial grasses is limited to the year in which they are established. In combination with the formation of extensive root systems, the ecological benefits of long term cultivation without tillage include a reduced risk of soil erosion and an increase in soil carbon content (Blanco-Canqui, 2010, Lewandowski *et al.*, 2003b, Smeets *et al.*, 2009). Moreover, miscanthus stands provide a habitat for wildlife during winter as miscanthus is commonly harvested in spring, which allows the crop to dry naturally on the field and complete its senescence (Lewandowski *et al.*, 2003b).

Miscanthus cultivation also has environmental benefits associated with a low demand for agricultural inputs compared to many other crops. It is characterized by a high nutrient-use efficiency owing to its C4 photosynthesis system and its ability to translocate minerals to its rhizomes at the end of the growth season (Heaton *et al.*, 2010, Long *et al.*, 2001, Sage & Zhu, 2011, van der Weijde *et al.*, 2013, Zub & Brancourt-Hulmel, 2010) (Figure 5a). The recycling of nutrients through a rhizomatous growth habit also reduces the amount of nutrients removed from the soil by harvesting the above-ground biomass and the consequential need to replace those removed nutrients through fertilization (Heggenstaller *et al.*, 2009, Lewandowski *et al.*, 2003b). Miscanthus cultivation also requires less irrigation than many other crops, as it is characterized by a high water-use efficiency owing to its C4 photosynthesis system and because it forms extensive root systems allowing it to reach deep soil water layers (Beale *et al.*, 1999, Byrt *et al.*, 2011, Ghannoum *et al.*, 2011, Sage & Zhu, 2011, Smeets *et al.*, 2009, van der Weijde *et al.*, 2013).



Figures 5a-b. Miscanthus has a sexual and an asexual reproductive system. Typical miscanthus panicle containing seeds (a), i.e. sexual reproduction and typical miscanthus rhizome structure (b), i.e. asexual reproduction.

Moreover, during winter miscanthus plants shed most of their leaves providing natural mulch that limits the growth of weeds in between miscanthus plants (Figure 6). In combination with the fast canopy development of miscanthus, this allows the use of herbicides to be limited to the first two years of establishment (Christian & Haase, 2001, Heaton *et al.*, 2004b). In addition, cultivation of miscanthus is likely to require limited use of pesticides as few natural pests are currently known (Blanco-Canqui, 2010, Dohleman *et al.*, 2010, Lewandowski *et al.*, 2000, Lewandowski *et al.*, 2003b). All these properties associated with low-input cultivation minimize the environmental burden of agricultural inputs per unit of biomass produced.

Furthermore, there are also examples of innovative applications of using miscanthus that offer substantial environmental benefits, for example miscanthus established in a buffer strip around intensively cultivated fields to prevent nutrient runoff into nearby water bodies (Börjesson, 1999) or the potential use of miscanthus as a phytoremediation tool to reclaim soils contaminated with heavy metals (Ezaki *et al.*, 2008, Wanat *et al.*, 2013).

5.3. Genetic variation in the miscanthus gene pool

The genus *Miscanthus s.s.* (*sensu stricto*, in the strict sense) comprises approximately 10-12 species native to regions of eastern Asia, the Himalayas and the Pacific Islands, which frequently hybridize in regions where overlap exists in the occurrence of species (Clifton-Brown *et al.*, 2008, Hodkinson *et al.*, 2015). The species considered to have the highest potential for biomass production in temperature climates are *M. sacchariflorus*, *M. sinensis* and interspecific hybrids between these two species (Jones & Walsh, 2001) (Figure 7). Ploidy levels vary amongst species, although all species are characterized by a basic chromosome number of 19 (Adati & Shiotani, 1962, Clifton-Brown *et al.*, 2008, Lo *et al.*, 1978, Sacks *et al.*, 2013). Miscanthus species originating from China, the primary center of diversity, are nearly always diploid (*2n = 2x = 38*) and include *M. sinensis* and *M. sacchariflorus* (Sacks *et al.*, 2013, Sun *et al.*, 2010). Species with allopolyploid genome constitutions occur regularly

in the secondary centers of diversity Japan and the Korean peninsula (Sacks *et al.*, 2013). An allotetraploid species (*2n = 4x = 76*) that is most accurately named *M. ogiformis*, but is often erroneously referred to as tetraploid *M. sacchariflorus*, occurs in Japan and has a genome homologous to *M. sinensis* and a genome homologous to *M. sacchariflorus* (Sacks *et al.*, 2013). This allotetraploid species readily hybridizes with *M. sinensis*, resulting in triploid hybrid species (*2n = 3x = 57*) (Nishiwaki *et al.*, 2011, Sacks *et al.*, 2013). One remarkably productive genotype of this triploid species was imported into Europe by the Danish botanist Aksel Olson in 1935 and is now known as *M. × giganteus* (Heaton *et al.*, 2010, Lewandowski *et al.*, 2000, Sacks *et al.*, 2013).

Figure 6. Miscanthus drops most of its leafs during winter, which form a natural mulch preventing weed emergence in early spring.

Most of the cultivation of miscanthus in Europe is currently based on this single clone, which has several disadvantages. First of all, *M. × giganteus* is sterile and does not produce viable seeds due to its triploid genomic constitution (Greef & Deuter, 1993). As a result, the cultivated material has an extremely limited genetic variability and its propagation is restricted to vegetative propagation methods, either through in vitro culture or by rhizome splitting, which results in high costs associated with establishment of new miscanthus trials (Christian *et al.*, 2005, Clifton-Brown *et al.*, 2008, Greef *et al.*, 1997, Heaton *et al.*, 2010, Lewandowski, 1998). Another principal limitation of *M. × giganteus* is its poor overwintering and establishment at some cold northern European sites (Clifton-Brown & Lewandowski, 2000, Farrell *et al.*, 2006a, Lewandowski *et al.*, 2000).

To be able to extend the geographical adaptation of miscanthus and advance miscanthus for bioenergy applications it is crucial to broaden the genetic base of cultivated miscanthus in Europe (Clifton-Brown *et al.*, 2008, Heaton *et al.*, 2010, Lewandowski, 1998). Great and largely untapped genetic variation exists for genetic improvement of miscanthus, as obligate outcrossing through self-incompatibility and adaptation to diverse habitats (from agricultural grass lands to dry grassland and even wet, saline, and polluted land) have resulted in great genetic diversity within and among natural miscanthus populations (Clifton-Brown *et al.*, 2008, Hodkinson *et al.*, 2015, Sacks *et al.*, 2013, Yan *et al.*, 2012). *M. sinensis* is the most broadly distributed species of miscanthus and is of particular interest for breeding widely adapted miscanthus varieties for temperate climates (Jørgensen & Muhs, 2001, Sacks *et al.*, 2013, Stewart *et al.*, 2009). Other key advantages of *M. sinensis* include its diploid genomic constitution and ability to produce fertile seeds. The large numbers of flowers per plant and seeds per flower allow for great multiplication rates, which means a great cost reduction in propagation of plant material can be realized compared to the vegetative methods used for propagation of *M. × giganteus* (Christian *et al.*, 2005, Lewandowski *et al.*, 2000)(Figure 5b).

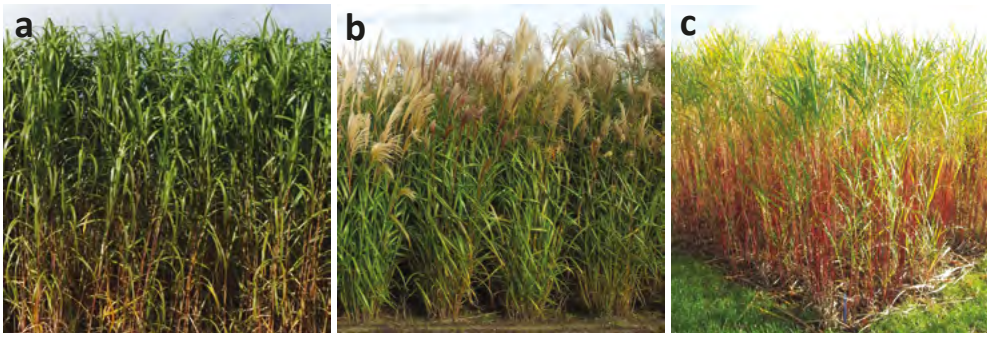


Figure 7. Typical plant morphology of different species of miscanthus: M. × giganteus (a), M. sinensis (b) and M. sacchariflorus (c).

Extensive phenotypic and genotypic variation in miscanthus has been reported for biomass yield (Atienza *et al.*, 2003, Clifton-Brown *et al.*, 2001, Gauder *et al.*, 2012, Robson *et al.*, 2013, Yan *et al.*, 2012, Zhao *et al.*, 2013, Zub & Brancourt-Hulmel, 2010), establishment (Jeżowski *et al.*, 2011, Jørgensen *et al.*, 2003, Yan *et al.*, 2012), biomass partitioning (Kaack *et al.*, 2003, Stewart *et al.*, 2009), morphological characteristics (Kaack *et al.*, 2003, Zhao *et al.*, 2013), flowering phenology (Jensen *et al.*, 2011, Jensen *et al.*, 2008, Zhao *et al.*, 2013), seed weight and seed germination (Christian *et al.*, 2005, Dwiyanti *et al.*, 2014), senescence (Robson *et al.*, 2012), tolerance to abiotic stresses such as drought (Clifton-Brown *et al.*, 2002), low temperature (Farrell *et al.*, 2006a) and frost (Zub *et al.*, 2012), contents of elemental minerals and combustion quality (Clifton-Brown & Lewandowski, 2002, Iqbal & Lewandowski, 2014, Jørgensen, 1997, Jørgensen *et al.*, 2003, Lewandowski *et al.*, 2003a) and cell wall compositional properties (Allison *et al.*, 2011, da Costa *et al.*, 2014, Qin *et al.*, 2012, van der Weijde *et al.*, 2016, Zhao *et al.*, 2014). Of particular interest

for improving biomass quality for biofuel production is the variation reported in cell wall composition, variation in miscanthus in cellulose content reported to range from 26 - 49%, in hemicellulosic polysaccharides from 25 - 43% and in lignin from 5 - 21% of stem dry matter (Allison *et al.*, 2011, Qin *et al.*, 2012, Zhao *et al.*, 2014).

5.4. Breeding of miscanthus

The exploitation of genetic variability through breeding is envisioned to lead to the development of new miscanthus varieties that perform well in diverse environments, are amenable to seed-propagation and are compositionally tailored for efficient bioconversion into cellulosic ethanol and other biobased products. However, breeding of miscanthus is in its infancy compared to food crop species (Clifton-Brown *et al.*, 2008). A breeding program was initiated in Japan in the 1950's to improve miscanthus as a fodder crop. In China breeding activities have focussed mainly on *M. sacchariflorus* and *M. lutarioriparia* for the paper industry. Breeding efforts to improve miscanthus as an energy crop started in Europe in the early 1990's at Tinplant (Magdeburg, Germany) (Clifton-Brown *et al.*, 2008). There are four different approaches in miscanthus breeding: 1) genetic modification to introduce new variability into the existing germplasm, 2) the development of highly productive allotriploid hybrids similar to *M. × giganteus*, 3) genetic improvement of *M. sinensis* through classical population improvement to produce synthetic cultivars or through pairwise crosses to produce intraspecific hybrids (Sacks *et al.*, 2013, Figure 8).

Progress in conventional breeding methods in perennial crops like miscanthus is slow compared to annual crops, due to the need to evaluate genotype performance in multi-year field trials. Miscanthus typically matures in three years and early morphometric selection – linking the phenotype of young plants to mature ones – has been proven unreliable (Arnoult *et al.*, 2015, Clifton-Brown *et al.*, 2008). The breeding cycle for miscanthus would be comparable to perennial ryegrass and ranges from 10 – 15 years (Clifton-Brown *et al.*, 2008). Therefore, the application of marker-assisted selection could substantially increase the efficiency of breeding miscanthus, as selections then could be done on marker-phenotype at the seedling stage instead of mature crops evaluated in multi-year field trials. Genetic resources such as biparental and association mapping populations have to be developed and evaluated to determine the genetic basis of desirable traits. Genetic maps form the basis for finding marker-trait associations, but their construction in miscanthus is complicated by its large genome size (varying from 4.5 – 7 pg, depending on the species (Rayburn *et al.*, 2009) and the high levels of heterozygosity that are the result of its obligate outcrossing nature (Głowacka, 2011, Hodkinson *et al.*, 2015). Nonetheless, several genetic maps are published to date (Atienza *et al.*, 2002, Kim *et al.*, 2012, Liu *et al.*, 2015, Ma *et al.*, 2012, Swaminathan *et al.*, 2012), which provide valuable resources for genetic studies to find markers that are related to traits of interest that can be exploited for marker-assisted selection in the foreseeable future.

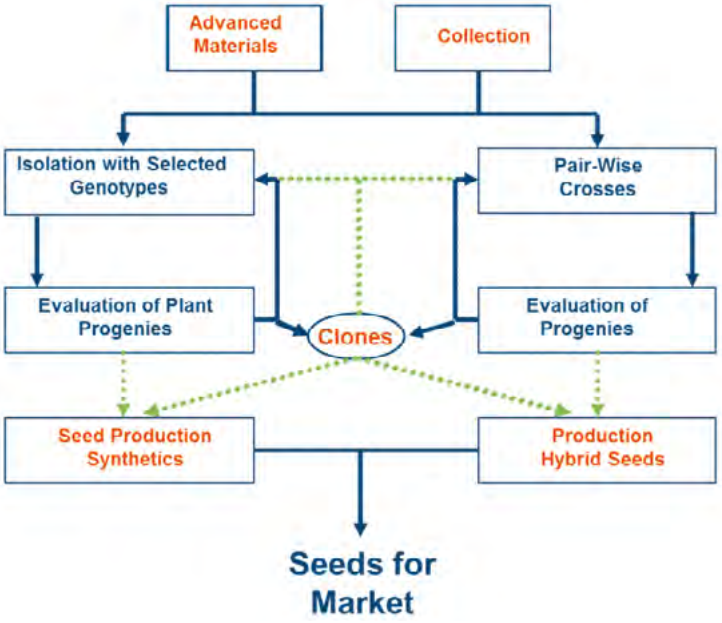


Figure 8. Overview of miscanthus breeding program aimed at the production of synthetic cultivars and inter- and intraspecific hybrids (Figure provided by dr.ir. O. Dolstra).

6. Objectives and structure of this thesis

Although miscanthus has great potential as a biomass feedstock for the biorefinery industry, genetic improvement of the crop is needed to enable its large scale introduction in Europe as a lignocellulose feedstock for the production of cellulosic ethanol and other biobased products. A key target for its genetic improvement is biomass quality. Improvements in biomass quality are envisioned to reduce the processing costs of cellulosic ethanol to make it competitive to fossil fuel and include increasing the contents of fermentable cell wall polysaccharides and improving the conversion efficiency of these polysaccharides by hydrolytic enzymes during industrial processing.

In view of these prospects, the main objectives of this thesis are:

1) to explore the genetic diversity in cell wall composition and conversion efficiency in miscanthus,
2) to explore which cell wall properties are impacting conversion efficiency and identify key targets for genetic improvement,
3) to assess how these cell wall properties are affected by environmental factors including abiotic stresses and
4) to identify genomic regions associated to traits of interest in order to enable an acceleration of genetic improvement of miscanthus through marker-assisted selection. These objectives are addressed in the following chapters:

Chapter 2 reviews the potential of C4 grasses in the biofuel industry. Although this thesis is focused on miscanthus, it is considered highly unlikely that any single species can fulfill all the needs and requirements of a global cellulosic ethanol industry. The genetic improvement of miscanthus, as a virtually undomesticated crop, can profit substantially from progress and results obtained in related C4 grasses, including maize, sorghum, switchgrass and sugarcane, and vice versa.

In **chapter 3** a comparison is made among different methods for the quantification of lignin, one of the primary components negatively influencing lignocellulose conversion efficiency. It is hypothesized that the reason why various methods result in highly different estimates of lignin content is that different lignin fractions are extracted and that these different lignin fractions may differentially affect conversion efficiency. A recommendation is given as to which lignin quantification method is the most applicable in screening lignocellulose recalcitrance.

In **chapter 4** the variation in biomass quality for different bioenergy applications, including combustion, anaerobic digestion and fermentation, is evaluated in 8 *M. sinensis* accessions that are highly contrasting in cell wall properties. Cell wall composition was analyzed in-depth in an attempt to identify which cell wall properties are key targets for improving biomass quality for various end-uses.

Chapter 5 describes the stability of cell wall composition and conversion efficiency properties of 15 *M. sacchariflorus*, *M. sinensis* and interspecific hybrid accessions across six diverse environments for the first three years following establishment. Accurate near-infrared prediction models were developed for the high-throughput analysis of biomass quality characters enabling processing of the large numbers of samples to be analyzed in the experiment.

In **chapter 6** these prediction models were extended and used for the analysis of biomass quality properties in a biparental mapping population. A new genetic map was created using a genotyping-by-sequencing approach and used to identify for the first time marker-trait associations for biomass quality characters relevant to the conversion of lignocellulose into cellulosic ethanol.

In order to grow miscanthus under low-input conditions or on marginal soils tolerance to drought is an important trait. **Chapter 7** is the first report on the implications of drought stress on the growth and the industrial quality of miscanthus biomass for biofuel production. This is a relevant subject of study, as modifications in cell wall biosynthesis and cell wall structure are among the primary responses of plants to sustain growth under conditions with reduced water potential.

Chapter 8 serves as a general discussion of how the results obtained in this thesis may contribute to the genetic improvement of miscanthus as a lignocellulose feedstock for the production of cellulosic ethanol and places the obtained results in the context of related scientific literature.

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Chapter 2

Review: The potential of C4 grasses for the production of cellulosic ethanol

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Abstract

With the advent of biorefinery technologies enabling plant biomass to be processed into biofuel, many researchers set out to study and improve candidate biomass crops. Many of these candidates are C4 grasses, characterized by a high productivity and resource use efficiency. In this review the potential of five C4 grasses as lignocellulose feedstock for biofuel production is discussed. These include three important field crops - maize, sugarcane and sorghum - and two undomesticated perennial energy grasses - miscanthus and switchgrass. Although all these grasses are high yielding, they produce different products. While miscanthus and switchgrass are exploited exclusively for lignocellulosic biomass, maize, sorghum and sugarcane are dual-purpose crops. It is unlikely that all the prerequisites for the sustainable and economic production of biomass for a global cellulosic biofuel industry will be fulfilled by a single crop. High and stable yields of lignocellulose are required in diverse environments worldwide, to sustain a year-round production of biofuel. A high resource use efficiency is indispensable to allow cultivation with minimal inputs of nutrients and water and the exploitation of marginal soils for biomass production. Finally, the lignocellulose composition of the feedstock should be optimized to allow its efficient conversion into biofuel and other by-products. Breeding for these objectives should encompass diverse crops, to meet the demands of local biorefineries and provide adaptability to different environments. Collectively, these C4 grasses are likely to play a central role in the supply of lignocellulose for the cellulosic ethanol industry. Moreover, as these species are evolutionary closely related, advances in each of these crops will expedite improvements in the other crops. This review aims to provide an overview of their potential, prospects and research needs as lignocellulose feedstocks for the commercial production of biofuel.

1. From biomass to biofuel

The growing global consumption of finite fossil fuel resources and the negative climatic consequences thereof are currently driving a search for renewable alternatives that bring the promise of energy security and sustainability (Charles et al., 2007). The successful replacement of oil as industrial raw material will depend largely on biomass processing techniques, as energy from biomass, in contrast to nuclear, wind, water and photovoltaic energy, can be stored as a liquid energy carrier in the form of biofuels (Perlack et al., 2005; Wyman, 2007; Karp and Halford, 2010). As such, it is currently the only alternative amenable to replace fossil fuels to support mobility on large scales (Wyman, 2008).

The production of biofuel from plant carbohydrates depends on the solar energy stored in plant biomass in the form of soluble sugars, starch and structural polysaccharides through photosynthesis. At the moment, the main pathway to convert these carbohydrates into biofuel is through biochemical extraction and fermentation to produce bioethanol (Balat, 2011). Structural polysaccharides constitute the bulk of all plant biomass, since they are the intrinsic components of the plant cell wall, and are by far the most abundant carbohydrates. However, currently most bioethanol is produced from soluble sugars and starch, as they are more easily processed into biofuel than cell wall polysaccharides (Naik et al., 2010). The plant cell wall, in particular the secondary cell wall, is a rigid, protective structure that confers stability and resistance to degradation. This is due to its main constituents – the structural polysaccharides cellulose and hemicellulose and the phenolic polymer lignin – and their interlinking into an unyielding matrix (Himmel et al., 2007; Zhao et al., 2012). Bioethanol production from the cell wall fraction of plant biomass, referred to as lignocellulose, requires a pretreatment to loosen the structure of the cell wall. Combinations of heat, pressure and chemicals are applied to disrupt the crosslinks between the main cell wall constituents and to improve the exposure of the polysaccharides to the enzymatic hydrolysis (Mosier et al., 2005; Zheng et al., 2009). Hydrolysis is required to disassemble cellulose and hemicellulose into their monomeric sugar constituents, which can subsequently be fermented into bioethanol (Balat, 2011). Structural polysaccharides represent the most abundant carbon resource for large-scale biofuel production, with no or very limited use in food and feed applications (Farrell et al., 2006; Wyman, 2007; Balat et al., 2008).

There is extensive interest in cellulosic ethanol, since biomass is considered a low-cost feedstock, which is available in massive quantities and can often be locally produced. A comparative study of gasoline and cellulosic ethanol with respect to net energy and net greenhouse gas emissions showed cellulosic ethanol to have 94% lower greenhouse gas emissions (Schmer et al., 2008). Hence, cellulosic ethanol can contribute to an environmentally sustainable supply of energy and simultaneously bring the promise of energy security (Farrell et al., 2006; Wyman, 2007). This has promoted major private and public investments in several demonstration and pilot scale cellulosic ethanol plants. Although some

of these facilities are operational, none of them are producing cellulosic ethanol at a true commercial scale to date. This is evidenced by the fact that the combined cellulosic ethanol production of such facilities in the United States, once estimated to exceed 750 million liters by 2012 (Coyle, 2010), is currently still stuck at a mere 30 million liters per year (RFA, 2012).

The difficulties that impede scaling up cellulosic ethanol production to a commercial level include infrastructural challenges and high capital and operating costs (Richard, 2010). Infrastructural challenges arise from the difficulties associated with the low density of biomass feedstocks. The costs of transport and storage of large volumes of biomass are high compared to those for fossil energy carriers, with a much higher energy density. Depending on the location of the ethanol plant, feedstock costs are estimated to account for ~38% of the plant's operating costs (Gnansounou and Dauriat, 2010). The larger the facility, the more complicated and expensive transportation may become, as hauling distance increases with increasing biomass supply demands. However, the smaller the facility, the longer it takes to get a positive return on investment in capital costs for the setup of a specialized plant, equipped with pretreatment reactors and saccharification and fermentation tanks made of non-corroding materials (Richard, 2010). The major hurdle with respect to the cost competitiveness of lignocellulose conversion technologies is the high input of energy and chemicals required to extract and hydrolyze cell wall carbohydrates (Wyman, 2007). The pretreatment procedure may account for up to 25% of the total processing expenses, due to the stringent processing conditions required to make the cell wall carbohydrates sufficiently accessible to enzymatic hydrolysis (Gnansounou and Dauriat, 2010).

To increase the profitability of biomass conversion platforms it is vital that a low cost lignocellulose feedstock is exploited. Hence, it is envisioned that agricultural, municipal and forestry biomass residues are the main substrate of the first cellulosic biorefineries, as they represent a widely available, low-cost feedstock. In the United States projections have been made to estimate the amount of biomass supply that will be potentially available by 2030 (Perlack et al., 2005). Of the projected total of 1366 million dry tonnes of biomass, 621 million tonnes are agricultural residues generated from 157 million hectares of arable land. To provide the additional supply, high yielding biomass crops are envisioned that are optimized for biofuel purposes. Probably, their cultivation will be in part limited to marginal lands, in order to minimize competition with food and feed production. Therefore optimization of these crops to low input conditions is desirable, as are breeding efforts to improve tolerance to drought and nutrient use efficiency, since irrigation and fertilization are both costly and unfavorable from a sustainability perspective.

In addition, biomass quality is seen as very important breeding objective. In this context, biomass quality pertains to the amenability of the lignocellulose feedstock for industrial conversion into bioethanol. Realizing the potential of cellulosic ethanol, but also the agronomical and physiological requirements that future bioenergy cropping systems must comply to, many researchers set out to identify, investigate and enhance candidate biomass

crops. Effectively, their main objectives are to 1) maximize the supply of lignocellulose in a sustainable and cost-effective way, and 2) improve the conversion efficiency of lignocellulosic biomass into ethanol. The development of biobased dedicated crops is envisioned to substantially reduce the production costs of cellulosic ethanol and thus contribute to the establishment of a viable cellulosic ethanol industry.

2. C4 grasses as lignocellulosic feedstocks

2.1. The benefits of C4 photosynthesis

One of the most important factors in the selection of energy crops is their high yield potential for biomass production. A high efficiency of CO_2 fixation into biomass is therefore of chief importance for energy crops, although biomass yield is determined by a number of other factors as well. The efficiency of CO_2 fixation is primarily determined by the type of photosynthesis found in a plant species.

The predominant form of photosynthesis amongst terrestrial plants is the C3 type of photosynthesis, in which CO_2 is fixated by ribulose biphosphate carboxylase oxygenase (Rubisco) (Ehleringer and Cerling, 2002). The efficiency of carbon fixation by Rubisco, however, is often compromised, as the enzyme has a dual role and may bind O_2 instead of CO_2 as a substrate, especially at higher temperatures and low atmospheric CO_2 conditions (Sage et al., 2012). This oxygenase reaction eventually leads to the production of CO_2 in a process known as photorespiration (Ehleringer and Cerling, 2002).

C4 photosynthesis is a morphological and biochemical modification of C3 photosynthesis in which Rubisco oxygenase activity is reduced due to a CO_2 concentrating mechanism that involves phospho*enol*pyruvate (PEP) carboxylase (Ehleringer and Cerling, 2002).

Due to their photorespiration-suppressing modifications, C4 plants have a higher potential efficiency of converting solar energy to biomass (Ehleringer and Cerling, 2002; Zhu et al., 2008), evidenced by the fact that 11 out of the 12 most productive plant species on Earth are C4 species (Furbank, 1998). In addition, the C4 mechanism is intrinsically linked to 1.3 – 4 times higher nitrogen use efficiency (NUE) and water use efficiency (WUE) (Sage and Zhu, 2011), owing to a respective reduction in leaf protein content and stomatal conductance (Taylor et al., 2010; Byrt et al., 2011; Ghannoum et al., 2011; Sage and Zhu, 2011). The former arises from a reduction in the amount of photosynthetic proteins required for optimal photosynthesis (Ghannoum et al., 2011). The higher WUE is associated with a faster fixation of CO_2 by the O_2-insensitive PEP carboxylase. Therefore the time stomata are required to be open for the uptake of CO_2 is shorter, leading to a reduction of leaf water evaporation (Byrt et al., 2011).

C4 photosynthesis is considered an advantageous characteristics for biomass crops, especially considering that most future climate scenarios predict an increase in dry and saline areas and erratic rainfall, conditions in which the advantages of C4 photosynthesis over the C3 type are even more apparent (Byrt et al., 2011). However, in colder regions of the world C3 plants may outperform C4 species as bioenergy crops (Carroll and Somerville, 2009). For the sake of being complete, a third type of photosynthesis exists, the crassulacean acid metabolism (CAM), which is employed by cactuses and succulents. These species are not deemed primary candidates for biomass production (Vermerris, 2008), although they may be productive in some extreme environments unsuitable for other species (Youngs and Somerville, 2012).

2.2. Promising C4 grasses for the industry

Many of the plant species that generate high yields of biomass with minimal inputs are C4 grasses. C4 plants dominate hot, open, arid environments around the world. The vegetation in these environments consists mainly of grasses and thus it is not surprising that about half of the world's grass species use C4 photosynthesis (Sage et al., 1999). Economically important food crops such as maize (*Zea mays* L. ssp. *mays*) and sugarcane (*Saccharum spp*.) are C4 grasses (Figure 1). These crops are important sources of biomass with well-established production chains that can supply large amounts of agricultural residues. Maize is an annual crop mainly cultivated for its grain or silage as a source of food, feed and in recent decades for the production of first generation bioethanol (Bennetzen, 2009). It is the largest crop worldwide in terms of total acreage (FAOSTAT, 2011). Sugarcane, a large perennial grass that can reach heights of over 5 meters, is cultivated primarily for its ability to accumulate sucrose in its stems, which is our predominant source of sugar (Tew and Cobill, 2008). It is the largest crop worldwide in terms of tonnes produced (FAOSTAT, 2011) and is exploited on a large scale in Brazil for sucrose-based bioethanol production (Waclawovsky et al., 2010).

Two of the currently leading dedicated biomass crops – miscanthus (*Miscanthus spp*) and switchgrass (*Panicum virgatum* L.) – are also C4 grasses (Lewandowski et al., 2003b). Both are rhizomatous perennials that typically reach heights of 2-4 meters and tend to give high biomass yields annually. Miscanthus is a genus comprising 15 species native to regions of eastern Asia, the Himalayas, the Pacific Islands and Africa (Clayton et al., 2002). The species are closely related to sugarcane (Figure 1, Clayton et al., 2002; Heaton et al., 2010). The research on bioenergy crops in Europa has been focused on miscanthus (Lewandowski et al., 2000; Heaton et al., 2008b). Due to its high yield potential, *M. × giganteus* is currently the main commercially exploited species of this genus for biomass purposes. Switchgrass is a versatile grass species native to North-America, with two major ecotypes: the lowland and the upland type (Sanderson et al., 1996; Casler and Monti, 2012). Due to its origin and prevalence in this region, the majority of research on biomass cropping systems in the Unit-

ed States has been focused on this crop (Heaton et al., 2008b; Parrish et al., 2012). Sorghum (*Sorghum bicolor* (L.) Moench) is another important C4 grass, as it is the fifth most produced cereal crop worldwide (Saballos, 2008; FAOSTAT, 2011). It is cultivated for its grain, sugar-rich stem juice and/or forage biomass depending on the type of sorghum (grain sorghum, sweet sorghum or forage sorghum) and is gaining increasing research interest as an annual bioenergy crop (Rooney et al., 2007; Saballos, 2008).

Each of these grasses has its strengths and prospects with respect to their use and development as lignocellulose feedstock. In part this is due to the fact that to sustain a large scale biomass supply, a wide range of environments is to be exploited – including marginal soils – and in part this is due to the diverse requirements that are posed to bioenergy cropping systems in terms of biomass quality. Different species are expected to be the best choice of feedstock for biomass production in different environments, as a species productivity is not constant from site to site and the local climate or soil type may provide an advantage or disadvantage from crop to crop. Hence, the efficient and large scale production of biomass across diverse environments will require a number of lignocellulosic feedstocks, each with a pallet of cultivars, so that a biomass cropping system can be chosen by growers that is optimally adapted to the production environment and processing methodology.

In the following sections the potential of these important C4 grasses – maize, sugarcane, miscanthus, switchgrass and sorghum – in relation to their use as feedstocks for the generation of cellulosic ethanol is discussed.

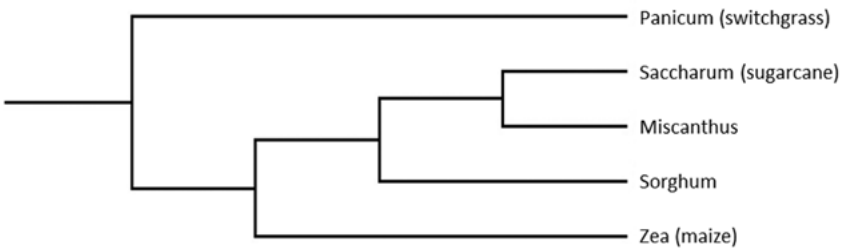

Figure 1. Phylogenetic tree depicting the relationships between the C4 grasses maize, miscanthus, sorghum, sugarcane and switchgrass. Adopted from Lawrence and Walbot (2007).

3. Biomass supply; yield and resource use

The future of cellulosic fuels will be determined by our ability to produce large volumes of inexpensive feedstocks without threatening food security or the environment. The combined supply of lignocellulose from organic residues and bioenergy dedicated cropping systems is envisioned to sustain a renewable supply of cellulosic biofuel and other biocommodities. For each of the designated C4 grasses their lignocellulose yield potential and resource use is discussed and summarized in Table 1.

3.1. Lignocellulose as a co-product

Of the grasses considered here, maize, sorghum and sugarcane are all contributing to the supply of lignocellulose in the form of agricultural or processing residues. An attempt is made to approach their average residue yield per hectare and their total supply of residue considering their global area harvested.

Maize is primarily cultivated to produce grain, in which case the leaf and stem fractions of the plant (referred to as stover) are available as lignocellulosic residue. A much smaller fraction of maize is cultivated for silage, in which case the whole plant is used and no residue is produced. In the world an area of over 170 million hectares of agricultural land is used for growing maize, with an average grain yield of 5.2 t DM ha^{-1} yr^{-1} (FAOSTAT, 2011). Based on the widely used assumption that the stover to grain ration is 1:1 in maize (Kim and Dale, 2004), the average stover yield is estimated on 5.2 t DM ha^{-1} yr^{-1}. Assuming these numbers the potential worldwide biomass supply from maize cultivation adds up to 884 million tonnes. However, the removal of this type of crop residues (normally left in the field) is a delicate issue, as it may increase soil erosion, deplete soil carbon and nutrient reserves and ultimately reduce future crop productivity (Graham et al., 2007). The amount of residue that can safely be removed without jeopardizing soil fertility depends on the cultivation practice (especially tillage regime), and crop yield. With current yield estimates and current rotation and tillage practices in the US ~30 % of the stover may be removed taking into account such considerations (Perlack et al., 2005; Graham et al., 2007).

For sugarcane an estimated total harvested area of almost 25.5 million hectares is reported, with an average yield of 70.5 tonnes ha^{-1} yr^{-1} (fresh cane yield) (FAOSTAT, 2011). Assuming a moisture content of 76% and a bagasse to dry matter ratio of 0.6:1 as in reported by Kim and Dale (2004) the global average bagasse yield per hectare is calculated to be 11.0 t DM yr^{-1}. An additional supply of lignocellulose comes from the field residue (leaves, immature stalks and dead tissue), which is estimated on 65% of the dry stalk yield (Waclawovsky et al., 2010) producing 11.9 t DM ha^{-1} yr^{-1}. So even though sugarcane is produced on only one seventh of the land used for growing maize, the total biomass supply from sugarcane cultivation is about two-thirds that of maize, adding up to 584 million tonnes. Taking into account that about 50% of the field residue is commonly left in the field to reduce soil erosion and depletion (Ferreira-Leitão et al., 2010), the available lignocellulosic biomass yield is reduced from 22.9 to 16.95 t DM ha^{-1} yr^{-1}.

The global area on which sorghum is cultivated is approximately 35 million hectares, with a global average grain yield of 1.5 t DM ha^{-1} yr^{-1} (FAOSTAT, 2011). With a residue/grain ratio of 1.3 (Kim and Dale, 2004) the stover yield from grain sorghums is on average 1.95 dry tonnes per hectare. The total potentially available supply is thus estimated to be 68 million dry tonnes. However, with the same correction applied to sorghum as to applied, to avoid soil erosion and nutrient depletion, the sustainably harvestable lignocellulose yield of sorghum

is also ~30% of the stover yield. Another type of sorghum, sweet sorghum, accumulates sugar in its stalks similar to sugar cane and is starting to gain momentum for syrup production (sweet sorghum types) in subtropical regions. These types give fresh yields ranging from 20 – 120 t per hectare (Saballos, 2008). They produce lignocellulose in the form of bagasse and field residue, with average residue yields reported to be 5.8 and 13.9 t DM ha^{-1} yr^{-1}, for bagasse and field residue respectively (Blümmel et al., 2009).

Annually, maize, sugarcane and sorghum can thus globally provide around 1500 million tonnes of lignocellulosic biomass from agricultural residues, from which over 700 million tonnes can be harvested sustainably. Greater productivities are likely with further advances in breeding and production technologies (Perlack et al., 2005). For all these crops dual-purpose breeding, addressing both grain/sugar and residue yield and quality will likely set off when the cellulosic ethanol industry adds value to the residue and if these breeding objectives can be advanced simultaneously. Together, agricultural residues from these (and other) crops will make a significant contribution to our global demand for cellulosic feedstocks, and will also play a crucial role in our effective transition towards the production of advanced biofuels (Schubert, 2006; Huber and Dale, 2009).

3.2. Lignocellulose as primary product

Miscanthus and switchgrass are amongst the species with the highest potential as dedicated biomass cropping systems. They are characterized by high dry matter yields and low cultivation inputs and have several advantages as biomass crops due to their perennial lifestyle. There is often considerable variation in biomass yields reported within each species, due to diverse ecological, climatic and cultivation conditions. In addition, side-by-side yield trials with multiple species at various locations and over several years are rare and may fail to assess yields at each species' respective optimum conditions. Therefore we focus on their average biomass yields as reported in literature. However, in order to appraise their yield potential also the most extreme yields observed to our knowledge are reported.

Miscanthus and switchgrass, being herbaceous perennial species, form extensive root systems and have the ability to store nutrient and carbohydrates in rhizomes at the end of the growing season. This supports early shoot emergence and growth in spring (Youngs and Somerville, 2012). Moreover, mature stands of such crops only have to make minor investments into root biomass compared to annual crops. Hence, these grasses, once successfully established, are renowned for their high yield potential (Lewandowski et al., 2003b). In a quantitative review of biomass yields reported for both crops in Europe and the US, miscanthus showed significantly higher biomass yields than switchgrass, with an average of 22 t DM ha^{-1} yr^{-1} from 97 observations, compared to 10 t DM ha^{-1} yr^{-1} from 77 observations, respectively (Heaton et al., 2004).

Only a few trials were set up to assess the yield potential of different miscanthus species, in which *M. × giganteus* often gave the highest autumn yields (Zub and Brancourt-Hulmel, 2010). In a single trial examining the peak yield of *M. × giganteus* under fully irrigated, non-limiting conditions of N, P and K, a yield of 50 t DM ha^{-1} yr^{-1} was reported in central France (Tayot et al., 1994). A more recent trial in Illinois (US), surprisingly with minimal agricultural inputs, even reported a peak biomass yield of 60.8 t DM ha^{-1} yr^{-1} (Heaton et al., 2008a), the highest recorded yield of this species to our knowledge. However, in many studies harvest is delayed until winter or even spring and no information on peak biomass yield is collected. Late harvest may reduce yields by on average 33% (Clifton-brown et al., 2004) and in the worst case by up to 50% compared to peak yields (Lewandowski et al., 2000). Nevertheless, it is a common practice in miscanthus to allow the crops to senescence, in order to let the above ground biomass dry on the field, and to allow translocation of nutrients to the rhizomes. In general, delayed harvest has a positive influence on biomass quality by reducing water and nutrient content and reduces the removal of nutrients from the system at harvest (Lewandowski et al., 2003a). One of the highest dry matter yields recorded after complete plant senescence was 44.1 t DM ha^{-1} yr^{-1}, again in the Illinois field trial of (Heaton et al., 2008a).

The maximum yield reported for switchgrass was observed in a United States trial spanning 10 years and several states, in which different switchgrass varieties and harvesting methods were evaluated. In this trial the variety 'Alamo' attained a yield of 34.6 t DM ha^{-1} yr^{-1} at a field location in Alabama using a system with two cuts; one harvest around flowering time and another in early spring (McLaughlin and Adams Kszos, 2005). To our knowledge the highest switchgrass peak biomass yield from a single cut trial was recorded to be 26.0 t DM ha^{-1} $yr^{-1}$with the locally adapted variety 'Cave-in-Rock' in Illinois (Heaton et al., 2008a). Losses in this species associated with late winter harvest are substantially less than in miscanthus (Heaton et al., 2004).

These yield estimates in all probability represent a baseline, since only limited efforts have been invested in the optimization of crop management and genetics. Although the average yield performance of these species is already impressive, the reported averages are still far less than half of the highest yields reported. This is indicative of the large yield improvements that could be realized in these grasses through breeding efforts, enabling them to advance the production of cellulosic ethanol through the reduction of feedstock costs.

Next to their potential as dual-purpose crops, maize, sorghum and sugarcane are also envisioned to have potential for the production of lignocellulose as primary product. Specific types are available with reduced grain or sugar yield and increased fiber production, such as forage sorghum hybrids (Venuto and Kindiger, 2008), temperate × tropical maize hybrids (White et al., 2011) and energy canes (Tew and Cobill, 2008), which all have the prospect of high biomass yields. However, at the moment their total lignocellulosic residue supply is the most important driver for the interest in these crops for cellulosic ethanol.

3.3. Inputs of nutrients and water

A high yield potential is a principal requirement for a biomass cropping system. Additionally, those yields are most preferably attained with minimal costs and agricultural inputs, such as fertilizer and irrigation. Moreover, the effects on soil fertility are a relevant issue, since nutrients are inevitably removed from the field at every harvest (Lal, 2005). Nonetheless, the implications of biomass cropping systems on nutrient fluctuations have so far received very limited attention.

Although C4 grasses have an intrinsic advantage over C3 species in terms of water and nutrient use efficiency (Taylor et al., 2010; Byrt et al., 2011; Ghannoum et al., 2011; Sage and Zhu, 2011), considerable differences exist amongst C4 species in their efficiency of biomass production per unit of available resource (Byrt et al., 2011). Crops cultivated for grain production, i.e. maize and grain sorghum, are generally considered to extract more nutrients from the soil, due to the high mineral and protein content of grains (Hons et al., 1986; Shewry and Halford, 2002). Perennials attain higher nutrient use efficiencies than annual crops due to their ability to recycle nutrients to the roots from one growing season to the next (Lewandowski et al., 2003b; Heggenstaller et al., 2009). Consequently, the cultivation of annual crops generally leads to a higher loss of nutrients upon removal of the above ground biomass, as no nutrients are recycled (Byrt et al., 2011). In addition, the extensive root systems of perennials and the reduced tillage compared to annual crop cultivation increase soil carbon content over time, can capture dissolved nitrogen and protect soils against wind erosion (Lewandowski et al., 2003b; Blanco-Canqui, 2010; Dohleman et al., 2010). In the case of miscanthus, nutrients are also returned to the soil through leaf fall prior to the winter harvest (Beale and Long, 1997; Lewandowski et al., 2000). In combination with the early canopy development of established stands, the resulting mulch also aids to prevent the emergence of weeds and reduces the need for herbicides after the establishment phase (Christian and Haase, 2001).

Table 1 provides an overview of recommended fertilization rates and water requirements for the grasses considered in this review. To be able to compare fertilizer/water requirements between these crops it is important to consider the dry matter yield per hectare. To do so, nutrient extraction rates per hectare and minimal annual water requirements are divided by the average dry matter yields reported in sections “Lignocellulose as a co-product” and “Lignocellulose as a primary product”. Note that only lignocellulose yields are considered, making the comparison for maize and sugarcane somewhat unfair, since fertilizer requirements are developed for grain/sugar plantations. In the table also nutrient extraction rates per kg dry matter yield and per hectare are given, to effectively show the effect of harvesting lignocellulose in each crop on the depletion of nutrients from the soil. These extraction rates are based on the nutrient content in the harvested biomass.

Maize is characterized by an inefficient uptake of nutrients, indicated by the fact that the recovery of applied fertilizer nitrogen is only 37% (Cassman et al., 2002). The replacement of extracted or lost nutrients through fertilization is one of the main production costs in maize cultivation (Berenguer et al., 2009; Subedi and Ma, 2009), although fertilization rates may differ considerably, due to differences in expected yield, local soil conditions and rainfall/irrigation levels (Shapiro et al., 2008).

Sorghum is considered more efficient in its nutrient use than maize, mainly due to its large fibrous root system (Saballos, 2008). It shows only a limited yield response to fertilizer application in medium- to high-fertility soils, and is virtually non-responsive to P applications. Hence, fertilization recommendations for sorghum are lower than for maize (Saballos, 2008). Grain and sweet sorghum varieties display similar quantities of nutrient removal as maize, but produce 25% to 50% higher biomass yields (Slaton et al., 2004; Propheter and Staggenborg, 2010; Propheter et al., 2010).

The high cane yields of sugarcane are often associated with substantial fertilizer applications (Wiedenfeld, 2000; Thorburn et al., 2011). Recommended applications of N can be as high as 300 kg N ha^{-1} (Roy et al., 2006). Nutrient removal varies considerably, due to environmental differences, different cultivation practices, and large yield differences.

In a comparative study on the nitrogen dynamics in switchgrass and miscanthus, no significant difference between the two species was found (Heaton et al., 2009). In both species mineral contents were high during the growing season, but decreased to minimal levels during plant senescence (Heaton et al., 2009). In a study simulating the impact of a change from unmanaged grassland to switchgrass, it was found that the content of soil organic carbon increased only when adequate N fertilizer was applied (Chamberlain et al., 2011). In most soils P and K levels are adequate for switchgrass (Sanderson et al., 2012). The fertilizer requirements of miscanthus are still under debate (Cadoux et al., 2012). In several experiments miscanthus was shown to give a very limited or no response to N fertilizer applications (Danalatos et al., 2007; Christian et al., 2008; Cadoux et al., 2012). Hence, for example, Christian et al. (2008) recommend no N application at all. This does not hold true for P and K, of which recommended applications are 7 and 100 kg ha^{-1} respectively. Davis et al. (2010) hypothesized that miscanthus is capable of nitrogen fixation, explaining the lack of response to N applications in these studies. In a review on the nutrient requirements of miscanthus, Cadoux et al. (2012) reported no need for N fertilization during the establishment phase of the crop, when yields are expected to be low, but recommend fertilization rates based on typical nutrient extraction levels.

Maize is shown to be the most demanding crop in terms of fertilizer demands, whereas switchgrass and miscanthus are shown to have the lowest requirements for fertilization (Table 1). For sorghum and sugarcane similar fertilizer applications are recommended, although sugarcane produces much higher average yields. In addition to fertilization rates,

an important parameter is the quantity of nutrients removed from the field at harvest, as this will have an adverse effect on soil fertility levels in the long term if these nutrients are not replaced. It can be deduced from the table that total nutrient (N, P and K) removal by sorghum and sugarcane are the highest in weight per kg biomass. The lowest quantities of nutrients removed from the field per kg crop are reported for miscanthus and switchgrass. From these figures it can also be deduced that there are large discrepancies in the data between recommended fertilization rates and nutrient removal per hectare, especially for sugarcane. If fertilization recommendations are higher, this may be the result of inefficient uptake of nutrients and/or leaching.

The large-scale production of biomass for biofuel may also have considerable implications on available water resources (Stone et al., 2010). Dedicated bioenergy cropping systems, therefore most likely will have limited possibilities for irrigation and have to rely on rainfall and soil water availability to sustain crop productivity. Therefore, a high water use efficiency (WUE) is considered a key trait of biomass crops. In general WUE is defined as the dry matter production / loss of soil water (g/kg). However, water loss is not only due to transpiration, but also due to non-biological factors such as soil evaporation. Unfortunately, only a few long-term studies were carried out in these crops that took all these factors into consideration. A further difficulty with the comparison of different studies is the need to normalize findings for differences in the vapor pressure deficit between the inner and outer leaf space (Beale et al., 1999; Jørgensen and Schelde, 2001).

Maize, with a WUE (dimensionless) of 0.0027 is reported to be less efficient than sorghum and miscanthus, with estimates of 0.0038 and 0.0075, respectively (Beale et al., 1999; Long et al., 2001). In another study, miscanthus and switchgrass were reported to have a similar and slightly higher WUE than maize (VanLoocke et al., 2012). The water use efficiency of sugarcane and maize expressed as kg DM ha^{-1} mm^{-1} evapotranspiration where reported to be 17-33 and 7-21, respectively (Berndes, 2002).

Even though WUE may be high in miscanthus and switchgrass, these crops still utilize large quantities of water for optimal crop production. In order to achieve yields of 30 t ha^{-1} with miscanthus, over 500 mm water is required (Long et al., 2001) and even though it is relatively tolerant to drought, miscanthus shows a strong yield response to irrigation at sites with insufficient soil water availability (Price et al., 2004; Cosentino et al., 2007). For switchgrass, economically feasible production is reported to be confined to regions with at least 450 mm annual rainfall (Bouton, 2008). Maize requires roughly 600 mm for optimal production and sugarcane as much as 1300 – 1600 mm (Al-Amoodi et al., 2004). Sorghum appears to be the least demanding crop, with a water requirement of 320 – 400 mm (Saballos, 2008).

To compare water use between these crops in a similar way as above for nutrient use, biomass yields have to be taken into account. A crop may require more water, but produce much higher yields than another crop. Therefore, water requirements are reported taking

the average yields into consideration and expressed as water requirement per kg DM yield (Table 1). These calculations show considerable differences between the crops, with sorghum and maize displaying the highest water requirements per kg lignocellulose produced. Of course, a large part of the water is used in these crops for the grain part of the plant. From the perennial species, miscanthus is shown to produce the highest lignocellulose yield per unit of water.

Table 1. Average lignocellulose yields and fertilizer and water requirements per hectare and per kg DM yield of important C4 grasses.

Lignocellulose as a co-product of dual purpose crops	Crop	Average lignocellulose yield[a] t DM ha^{-1} yr^{-1}	Total available[a] (worldwide) × 10^6 t DM yr^{-1}	General fertilizer recommendation[b], kg ha^{-1} yr^{-1}			Total nutrient removal[c] kg per hectare			Nutrient removal[d], g per kg DM yield			Water requirement for optimal production[e], mm yr^{-1}	Water required[d], mm yr^{-1} per kg DM yield
				N	P	K	N	P	K	N	P	K		
	Maize	5.2	884	200	100	100	37.5	4	57.5	7.20	0.73	11.02	>600	>115
	Sugarcane	22.9	584	90	67	67	118	31.5	391	10.02	2.67	33.13	>1300	>57
	Sorghum	1.95	68	90	67	67	28	5.5	30.5	14.47	2.78	15.72	>320	>164

Lignocellulose as a primary product	Crop	Average lignocellulose yield[a] t DM ha^{-1} yr^{-1}	Yield potential[a] t DM ha^{-1} yr^{-1}	General fertilizer recommendation[b], kg ha^{-1} yr^{-1}			Total nutrient removal[c] kg per hectare			Nutrient removal[d], g per kg DM yield			Water requirement for optimal production[e], mm yr^{-1}	Water required[d], mm yr^{-1} per kg DM yield
				N	P	K	N	P	K	N	P	K		
	Miscanthus	22.5	60.8	0	7	100	110	10	157.5	4.9	0.45	7.0	>500	>22
	Switchgrass	10	34.6	67	45	45	34	6.5	82.5	3.42	0.63	8.26	>450	>45

[a] = Average yields, total available lignocellulose and yield potentials as reported in sections 3.1 and 3.2.

[b] = Recommendations for medium fertility soils by University of Georgia's Cooperative Extension Service, except recommendation for miscanthus, which is based on Christian et al. (2008).

[c] = Data calculated using the average lignocellulose yields per hectare in USDA's Crop Nutrient Tool (USDA-NRCS, 2013), except the values for miscanthus, which are based on Cadoux et al. (2012).

[d] = Calculated by taking into account average lignocellulose yield.

[e] = Data based on Al-Amoodi et al. (2004) for maize and sugarcane, on Saballos (2008) for sorghum, on Long et al. (2001) for miscanthus and Bouton (2008) for switchgrass.

4. Biomass quality

Next to addressing the issues wrelated to the supply of lignocellulose, another important consideration is the efficiency of converting biomass into bioenergy. The challenge of effectively fractionating lignocellulosic feedstocks into fermentable sugars lies within the com-

positional nature of the plant cell wall. The cell walls of grasses have distinct differences in the balance between the main cell wall constituents (Table 2), even though all commelinoid monocots, including the C4 grasses discussed in this review, share some distinct features in cell wall architecture, as described comprehensively by Carpita (1996), Cosgrove (2005) and Vogel (2008). 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. 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. Variation in cell wall compositions of promising C4 energy grasses[a]

Lignocellulose feedstock	Cellulose	Hemicellulose	Lignin	Reference
Maize (stover)	~27-40%	~25-34%	~9-15%	(Lorenz et al., 2009b; Templeton et al., 2009; Wolfrum et al., 2009; Lorenzana et al., 2010; Jung and Bernardo, 2012)
Switchgrass	~28-37%	~25-34%	~9-13%	(Sladden et al., 1991; Vogel et al., 2011)
Sorghum (stover)	~21-45%	~11-28%	~9-20%	(Rooney et al., 2007; Murray et al., 2008; Shiringani et al., 2010; Stefaniak et al., 2012)
Sugarcane (bagasse)	~35-45%	~25-32%	~16-25%	(Canilha et al., 2011; Masarin et al., 2011)
Miscanthus	~28-49%	~24-32%	~15-28%	(Hodgson et al., 2010; Zhang et al., 2012)

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

From an economic perspective, feedstocks with the highest combined content of cellulose and hemicellulose (holocellulose) are likely to be favored by the industry, since techno-economic evaluations and comparative studies of ethanol biorefineries showed that the holocellulose content of feedstocks was directly proportional with ethanol yields under optimal processing conditions (Ruth and Thomas, 2003; Aden and Foust, 2009; Huang et al., 2009). This explains why the predominant strategy in energy grass breeding is to increase the overall abundance of holocellulose in the plant cell wall. As crops like miscanthus, sugarcane and sorghum have in potential a very high holocellulose content on a dry matter basis (~75%), in addition to high biomass yields, they are expected to dominate the future cellulosic ethanol market.

However, current biomass-to-ethanol conversion systems are not optimal and concerns exist as to whether these technologies are universally transferable between different lignocellulosic feedstocks. Leading technologies have almost exclusively been optimized using maize stover and (more recently) switchgrass (Wyman et al., 2005; Elander et al., 2009; Garlock et al., 2011; Tao et al., 2011), with little information available on their applicability in other C4 feedstocks. So far, most efforts to improve biomass-to-ethanol conversion systems have not taken into consideration the impact of biomass composition (Gregg and Saddler, 1996; Kim et al., 2011). Yet, biomass composition may have a large impact on conversion efficiency as, for instance, Kim et al. (2011) demonstrated with compositionally different eco-

types of switchgrass, using the industry's leading pretreatment systems (AFEX, dilute sulphuric acid, liquid hot water, lime, and soaking in aqueous ammonia). Strikingly, the ecotype with the highest cellulose content on a dry matter basis was the worst performer under all test conditions. These results highlight the difficulties of developing universally applicable conversion technologies for different biomass types and indicate the practical limitations of breeding solely for increased levels of cell wall polysaccharides.

Consequently, a second approach to optimize feedstock composition focusses on reducing the natural resistance (biomass recalcitrance) of plant cell walls to enzymatic deconstruction. Significant efforts have been devoted towards understanding and dissecting the biochemical and genetic mechanisms affecting the depolymerization of cell wall polysaccharides. A considerable wealth of studies has documented the extent of natural – and induced – variation of promising C4 grasses with respect to their processing amenability under a diverse array of conversion technologies (Vermerris et al., 2007; Saballos et al., 2008; Dien et al., 2009; Lorenzana et al., 2010; Chuck et al., 2011; Fu et al., 2011a; Fu et al., 2011b; Kim et al., 2011; Lygin et al., 2011; Masarin et al., 2011; Saathoff et al., 2011; Sarath et al., 2011; Xu et al., 2011; Fornalé et al., 2012; Jung and Bernardo, 2012; Jung et al., 2012; Park et al., 2012; Vandenbrink et al., 2012; Yee et al., 2012; Zhang et al., 2012; Torres et al., 2013). Some relevant research highlights are summarized in Table 3.

The majority of studies aiming at the reduction of biomass recalcitrance in C4 grasses has focused on exploring the effect of lignin content on conversion efficiency. Indeed, reductions in cell wall lignin content often led to improved enzymatic digestibility, as shown in studies with brown midrib mutants in maize and sorghum (Vermerris et al., 2007; Saballos et al., 2008; Dien et al., 2009; Sattler et al., 2010; Wu et al., 2011; Sattler et al., 2012); as well as in studies with transgenes that down-regulate monolignol biosynthesis genes in maize (Park et al., 2012), sugarcane (Jung et al., 2012) and switchgrass (Fu et al., 2011a; Fu et al., 2011b; Saathoff et al., 2011; Yee et al., 2012). In addition to reductions in lignin content, alterations in the ratio between the main constituents of lignin have been found to affect recalcitrance. For instance, a lower S/G ratio – two of the main subunits of lignin –, can reduce biomass recalcitrance in C4 grasses, as demonstrated both in natural mutants (Vermerris et al., 2007; Saballos et al., 2008; Sattler et al., 2012) and using transgenic approaches (Fornalé et al., 2012; Jung et al., 2012).

However, lignin content and composition are not the sole factors explaining variation in the conversion efficiency of lignocellulose feedstocks. Several studies on biomass recalcitrance have investigated the impact of differences in the composition and structure of cell wall polysaccharides, and the interactions between polysaccharides and other cell wall components. These demonstrated how cell wall characteristics other than lignin – including the degree of cell wall porosity, cellulose crystallinity, polysaccharide accessible surface area and the protective sheathing of cellulose by hemicellulose – can also contribute to the natu-

ral resistance of plant biomass to enzymatic degradation (Mosier et al., 2005; Himmel et al., 2007; Jeoh et al., 2007; Gross and Chu, 2010; Zhang et al., 2012; Zhao et al., 2012).

Furthermore, fundamental cell wall studies in Arabidopsis and other model crops have contributed considerably to the understanding of the synthesis of cellulose and hemicellulose. Consequently, strategies to develop novel genotypes, with reduced recalcitrance, through targeted modifications of cell wall biosynthesis genes are beginning to gain momentum. For instance, alterations in the cellulose synthesis machinery – or its accessory complexes –, may lead to modifications in the structure of cellulose microfibrils, with, for example, reduced crystallinity, a lower degree of polymerization and/or a higher degree of porosity. Recently, Vandenbrink et al. (2012) demonstrated a large variation in cellulose crystallinity within a diverse association mapping panel in sorghum, and reaffirmed that genotypes with lower cellulose crystallinity exhibit higher enzymatic hydrolysis rates, as has been reported for pure microcrystalline cellulose samples (Bansal et al., 2010) and ground miscanthus powder (Yoshida et al., 2008). In addition, recent studies that uncovered the function of genes and enzymes in the synthesis and substitution patterns of hemicelluloses provide novel opportunities for the modification of the structural and functional characteristics of hemicellulose (Mortimer et al., 2010; Anders et al., 2012). A possible strategy to improve the processing efficiency of feedstocks aims at a reduction of the number of side-chain substitutions in hemicelluloses, which shield the xylan-backbone from enzymatic hydrolysis (Mortimer et al., 2010).

Despite crucial advances in our understanding of the synthesis and structural properties of the plant cell wall, much still remains to be explored before effective, targeted manipulation of cell wall properties can be fully exploited for the creation of biomass feedstocks optimally suited to bioconversion. Many different pretreatment types exist - and new technologies are developed continuously -, that target different components of biomass recalcitrance. Hence, they will require different compositional features of feedstocks for optimal effectiveness. To address this, more research in the area of pretreatment and enzymatic hydrolysis of lignocellulose, as well as research on the intricacies of the cell wall synthesis machinery and on the available genetic variation in cell wall properties within biofuel crops is needed. In particular, quantitative genetic studies and systems biology approaches are anticipated to aid in the understanding of the synergistic and antagonistic interplay of cell wall components and their effect on biomass recalcitrance. The findings from such studies will enable plant breeders to design effective breeding programs and facilitate the development of energy C4 grasses optimized to increase the efficiency of bioconversion technologies.

5. Genetic improvement

The C4 grass species discussed in this review are all expected to play an important role as bioenergy crop in the emerging cellulosic ethanol industry. Their success as biomass crop is not only dependent on their biomass yield, efficiency in using resources during cultivation and level of biomass recalcitrance (and other cell wall properties), but also on their amenability for improvements through breeding efforts. In the following sections the improvement of these crops through plant breeding are discussed, with emphasis on crop-specific differences in breeding strategy, selection criteria and tools for breeding and the currently available insights with respect to the genetics of relevant traits.

5.1. Variety concept

Ploidy level and genome architecture are important factors in the design of a breeding program and determine to a large extent the type of variety to be developed. The variety concept therefore is species-specific and apart from commercial considerations it takes into account matters such as mating system, seed production issues, ploidy level and inheritance of traits. Annual crops are generally fertile and are propagated by seeds. If possible, breeding in annual crops aims to generate hybrid varieties to benefit from hybrid vigor (heterosis). Perennial crops are quite often polyploids, with an unbalanced genome constitution causing sterility due to meiotic irregularities. Polyploids tend to be vigorous crops due to a high degree of heterozygosity and gene redundancy.

Polyploidy, however, also complicates genetic studies and the inheritance of traits (Comai, 2005). The mapping and genetic studies of polyploid genomes has to deal with complex levels of allele recombination, especially when chromosome pairing in meiosis is not merely restricted to homologous chromosomes. In addition, as a result of polyploidy sequencing becomes more difficult due to the large genome sizes and within-genome similarities. Another important consideration is a crop's mode of reproduction, which is a key determinant to which breeding systems can effectively be used to improve a species (Allard, 1960).

Both cultivated maize and cultivated sorghum are diploid species ($2n = 2x = 20$) with a basic chromosome number of 10, although other ploidy levels exist in annual and perennial wild relatives in the genus sorghum (Acquaah, 2012b). Maize is predominantly a cross-pollinator (95%) with male and female inflorescences. The former produces pollen which are dispersed by wind (Acquaah, 2012a). Self-pollinations, however can be done by hand for breeding and research purposes (de Leon and Coors, 2008). The inflorescence of sorghum, in contrast, has separate male and female organs and self-pollination is the main mode of reproduction, with degrees of outcrossing ranging from 5 – 30% (Saballos, 2008).

Table 3. Summary of relevant reports on the variation in conversion efficiency in C4 grass species

Crop	Genetic Nature	Conversion Technology	Summary of Results	Reference
Maize	Experimental Mapping Population – Hybrid testcrosses of 223 recombinant inbred lines from the IBM collection.	Mild dilute-acid pretreatment followed by hydrolysis with commercial enzyme cocktails.	Variation within population for cell wall glucose release after mild pretreatment and enzymatic saccharification ranged from ~48-56%. Glucose conversion efficiency was strongly correlated to lignin content (r = -0.74).	(Lorenzana et al., 2010)
Sugarcane	Transgenic lines with RNAi-induced down-regulation of caffeic acid O-methyltransferase (*COMT*).	Mild dilute-acid pretreatment followed by hydrolysis with commercial enzyme cocktails.	Maximum reduction in lignin content in transgenic lines compared to controls of 13.7% and a maximum increase in fermentable glucose yield of 35% (after pretreatment and enzymatic hydrolysis).	(Jung et al., 2012)
Switchgrass	Two sets of genotypes obtained by divergent selection for ruminant digestibility.	Various intensities of dilute-acid pretreatments followed by simultaneous saccharification and fermentation (SSF).	A 40% difference in ethanol yield (after dilute-acid pretreatment followed by SSF) between the two genotypes with the largest contrast in lignin content.	(Sarath et al., 2011)
Switchgrass	Transgenic lines with RNAi-induced down-regulation of caffeic acid O-methyltransferase (*COMT*).	Various intensities of dilute-acid pretreatments followed by SSF.	Maximum reduction in lignin content in transgenic lines compared to controls of ~15% and a maximum increase in ethanol yield of 38% (after severe pretreatment [0.5% H_2SO_4, 180 ^{0}C] followed by SSF).	(Fu et al., 2011a; Fu et al., 2011b)
Sorghum	Collection of brown-midrib (*bmr*) mutant collection and their corresponding wild-types.	Mild dilute-acid pretreatment followed by hydrolysis with commercial enzyme cocktails.	Glucose conversion after thermo-chemical processing and enzymatic hydrolysis across a set of 5 *bmr* mutants and their corresponding counterparts ranged from 59-77%. The maximum increase in glucose fermentable yields (relative to wild-type) was of 21%.	(Saballos et al., 2008)

The perennial grasses discussed here are all wind-pollinated outcrossing species and are characterized by more complex genetics (Vogel and Pedersen, 2010). Switchgrass is highly self-incompatible and possesses a chromosome number of 9, but with varying somatic chromosome numbers and ploidy levels (*2n = 2x =18* to *2n = 12x = 108*). Amongst lowland ecotypes tetraploids predominate, whereas amongst upland ecotypes octoploids are more abundant (Bouton, 2008). In miscanthus, ploidy levels vary amongst species in the genus, with the three species with the highest potential for biomass production, *M. × giganteus* being a triploid (*2n = 3x = 57*), *M. sinensis* a diploid (*2n = 2x = 38*) and *M. sacchariflorus* a tetraploid (*2n = 4x = 76*) (Heaton et al., 2010). Recently, Kim et al. (2012) reported *M. sacchariflorus* accession from Japan to be typically tetraploid, whereas accessions from China were reported to be typically diploid. *M. × giganteus* is a sterile hybrid, but the other two species are obligate outcrossers due to self-incompatibility (Heaton et al., 2010). All three species are characterized by a basic chromosome number of 19 (Clifton-Brown et al., 2008). Sugarcane is predominantly cross-pollinating, but selfing is possible by covering the inflorescences with bags (OGTR, 2008). The genus *Saccharum* displays a large variation in chromosome number and ploidy levels. The three most important species in the genus used to make modern cultivars are *S. officinarum* (*2n = 70-140*), *S. spontaneum* (*2n = 36-128*) and *S. robustum* (*2n = 60-200*) (D'Hont, 2005; Scortecci et al., 2012). D'Hont (2005) identified a basic chromosome number of 10 for *S. officinarum* and *S. robustum* and a basic chromosome number of 8 for *S. spontaneum.* The genetics of sugarcane and its trait inheritance are very complex, since it is a hybrid of different species and displays both autopolyploid and allopolyploid types of inheritance (OGTR, 2008).

The predicted genome sizes of the C4 grasses vary widely, the smallest being sorghum (1.21pg), followed by switchgrass (1.88pg) and maize (2.73pg) (Bennett and Leitch, 2010). For *Saccharum officinarum* and *Saccharum spontaneum*, genome sizes are predicted to be 3.37pg and 4.71pg, respectively (Bennett and Leitch, 2010). The genome size estimations of *M. × giganteus* and its two progenitors species, *M. sacchariflorus* and *M. sinensis* are 7.0pg, 4.5pg and 5.5pg, respectively (Rayburn et al., 2009).

5.2. Genetic resources and breeding tools

There are many differences in the experience, resources and techniques available for each of the crops, giving certain crops distinct advantages over others. Miscanthus and switchgrass have barely been domesticated (Jakob et al., 2009), whereas maize is arguably the most domesticated of all field crops, unable to survive as a wild plant (Acquaah, 2012a).

Maize and sorghum have several advantages over the other crops with respect to their improvement as lignocellulose feedstocks. The complete genomes of maize and sorghum have been released (Paterson et al., 2009; Schnable et al., 2009), while for some of the other grasses sequencing projects are still in progress, such as for *M. sinensis* and switch-

grass by the U.S. DOE Joint Genome Institute (JGI, www.jgi.doe.gov/genome-projects). In addition there is a wealth of genomic tools available, especially in maize (genetic markers, genome annotations, quantitative trait loci's (QTL's), extensive expressed sequence tag (EST) libraries, well-mapped populations, large collections of mutants) that can be used to study and enhance biomass quality traits. Their diploid nature makes maize and sorghum easier to study than (allo)polyploid crops, and since they are both C4 grasses and have a close evolutionary relationship to the other crops, they are most likely better models to this group of biofuel crops than other model plant species as Arabidopsis, rice or Brachypodium (Carpita and McCann, 2008). Hence, the knowledge that will be acquired on the synthesis, deposition and recalcitrance of the cell wall in maize or sorghum can most likely be utilized to improve biomass quality of the other C4 grasses. Sorghum shares a high level of co-linearity with the genomes of miscanthus (Kim et al., 2012; Ma et al., 2012; Swaminathan et al., 2012) and sugarcane (Wang et al., 2010), which makes the sorghum genome ideal as a template for comparative genomic studies with these species. In addition, the use of comparative genetics coupled with transcriptomic and proteomic analyses will be an important tool to expedite the genome assembly of closely related C4 grasses. Transcriptome datasets are valuable sources of information to monitor gene expression during different growth stages and biotic or abiotic stress responses. Such datasets can also compensate for the lack of genome sequence information, in those grasses in which sequence information is still unavailable. For example, a large sugarcane EST database is publicly accessible (sucest-fun.org) (Vettore et al., 2003). The combination of genome sequencing with other "omics" strategies is still in its early stages in C4 grasses, but is expected to be a successful strategy for studying cell wall biosynthesis.

Maize and sorghum are the two crops discussed in this review that are annual species, which is likely to positively affect the speed with which these crops can be advanced in breeding programs. Genetic improvement is generally faster in annual crops than in perennials, due to the relatively shorter selection-cycle. All the grasses discussed here, with the exception of sorghum, are outcrossing species. This has the advantage compared to self-pollinating crops that they are amenable to heterosis breeding, in particular when the production of inbred lines is possible by repeated selfings as in maize. An improvement of this technique, nowadays frequently used in maize breeding, is the development of doubled-haploid lines, which are completely homozygous as a result of artificial or spontaneous chromosome doubling of induced haploids (Maluszynski, 2003; Tang et al., 2006). This is a major advantage for hybrid breeding and genetic studies (Forster and Thomas, 2010).

The availability of genetically diverse and advanced germplasm is key to the success of breeding programs. Breeding efforts to improve bioenergy crops can initially take advantage of the knowledge and technologies developed in food and forage breeding programs (Jakob et al., 2009). In maize, sorghum and sugarcane breeding programs have a long history and although these programs mainly target the increase of grain/sugar yield and har-

vest index, improvements in traits such as disease and lodging resistance affecting yield stability are also useful for their use as dual-purpose crops (Jakob et al., 2009). In forage breeding programs, such as are established in maize, switchgrass and sorghum, the main aim is to improve the total yield of biomass as well as its digestibility. Due to the similarities between enzymatic deconstruction of lignocellulosic biomass in the rumen of cattle and in cellulosic ethanol platforms, crops optimized for forage quality parameters may prove extremely valuable germplasm sources for optimizing biomass quality (Weimer et al., 2005; Dhugga, 2007; Anderson and Akin, 2008; Dien et al., 2009; Lorenz et al., 2009a; Anderson et al., 2010; Sarath et al., 2011).

To expedite the genetic improvement of C4 grass species as lignocellulosic feedstocks molecular breeding technologies are being considered (Jakob et al., 2009; Takahashi and Takamizo, 2012). Genetic engineering with the help of transformation technologies continues to be a topic of debate, especially in Europe, but public acceptance of genetically modified (GM) crops for dedicated biofuel purposes might be higher than for food and feed commodities. However, transformation technologies are relatively much more developed in dicots than in monocots. Thus for most of these grasses, the exception being maize, major progress is required in the development and optimization of transformation protocols. Reviews on the status of transformation of sorghum (Howe et al., 2006; Girijashankar and Swathisree, 2009), switchgrass (Somleva et al., 2002; Conger, 2003; Bouton, 2007; Burris et al., 2009; Xi et al., 2009; Saathoff et al., 2011), miscanthus (Wang et al., 2011; Paterson et al., 2013) and sugarcane (Santosa et al., 2004; Hotta et al., 2010) provide further information. However, transgenic approaches are regarded with great caution in dedicated bioenergy crops as well, as they are mostly outcrossing perennial grasses (Wang and Brummer, 2012). To address the risk of unwanted transmission of transgenes through pollen-mediated gene flow, there are, however, various strategies for gene confinement in perennial biofuel feedstocks (Kausch et al., 2009).

While considerable differences are described between the designated C4 grasses that may affect their improvement as lignocellulose feedstock, the fact that these crops are evolutionary closely related provides great opportunities for the exchange of acquired knowledge between them. Several online services have been developed to facilitate this exchange of information, e.g. GRASSIUS, a platform integrating information on transcription factors and their target genes in grasses (www.grassius.org) (Yilmaz et al., 2009), GRAMENE, a comparative genome mapping database for grasses (www.gramene.org) (Ware et al., 2002; Liang et al., 2008) and CSGRqtl, a comparative quantitative trait locus database for Saccharinae grasses (http://helos.pgml.uga.edu/qtl/) (Zhang et al., 2013). Hence, advances in each of these crops may expedite research progress in the other crops, with maize and sorghum being anticipated to serve as models in the study of cell wall recalcitrance.

6. Prospects and research needs

The group of C4 grasses regarded in this paper has a great potential for the sustainable, large scale production of lignocellulose to support a cellulosic fuel industry. The supply of biomass from different sources and niches will prove to be indispensable, as different growing conditions and refinery technologies require different types of lignocellulose feedstocks. These grasses are likely to represent different sources of biomass supply as they have distinct prospects and potential roles in the future supply chain of lignocellulose, which stipulates the importance of research into the genomics, genetics and breeding of this group of promising grasses.

Globally, the cultivation of maize, sugarcane and grain sorghum can sustainably provide around 1500 million tonnes of lignocellulosic agricultural residues per year. Greater yields are likely with advances in breeding and production technologies (Perlack et al., 2005), especially when the lignocellulose fraction becomes an important product and dual-purpose breeding sets off. Common plant breeding research needs in such crops for advancing the use of agricultural residues for cellulosic ethanol production focus on (1) increasing the yield of harvestable biomass without jeopardizing food/feed production, (2) exploring the effect of the use of crop residues on soil quality and (3) improving the biomass quality of the residue for bioprocessing. To make the conversion of agricultural residues economically attractive, it is critical that advances are made in biomass quality, in addition to technological improvements in the refinery processes. Maize and sorghum are the crops will most likely serve as models in the research on biomass quality improvement, due the presence of the required expertise, genetic resources, proper breeding tools and the availability of their genome sequences. Together, agricultural crop residues can make a significant contribution to our global supply of lignocellulose for biofuel production (Schubert, 2006; Huber and Dale, 2009).

As the industry matures, dedicated energy crops that can be cultivated with limited agricultural resources and grown on surplus cropland and on degraded or marginal soils are needed. Under these provisions, fast-growing perennial C4 grasses have been coined as the most promising candidates for the industrial production of lignocellulosic biomass (Hill, 2007; Carroll and Somerville, 2009). Switchgrass and miscanthus are commercially attractive because of their high biomass yields, broad geographic adaptation, climatic hardiness, efficient nutrient use and nitrogen fixation capacities (Sanderson et al., 1996; Sanderson et al., 2006; Hill, 2007; Yuan et al., 2008; Tilman et al., 2009; Heaton et al., 2010). Since their cultivation is expected to require low mineral-nutrient inputs and pesticides, these crops are also expected to have high net energy gains and major environmental benefits. As breeding in these crops is still in its infancy, there is most likely ample room for improvement.

The success of C4 grasses in the cellulosic ethanol industry will 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 and cell wall polysaccharide content, as well as reducing the recalcitrance of biomass to industrial processing. Cellulosic grasses, particularly those destined to marginal soils, will be required to combine improved resource use efficiency (water and nutrients), broad climatic adaptation and biotic-stress hardiness.

Although, the above mentioned targets are universal, the advances in breeding programs are different for each species and the initial research focus will be species specific to ensure an important role for each of the C4 grasses in the future cellulosic ethanol industry.

Box 1.
A key factor that will most likely influence the economics of the cellulosic ethanol industry is the production of different bio-commodities in addition to ethanol, utilizing the diversity of compounds present in biomass. Several high-value chemicals can be produced, some of which may in fact provide greater economic returns than ethanol. However, the value of such commodities is determined to a large extent by market-demand and their value may be reduced when the industry grows to a larger scale. To our knowledge, no research has been conducted to compare different C4 grasses for the production of such bio-products. Reviews on the different products and production routes can be found in – amongst others – (Gallezot, 2007; FitzPatrick et al., 2010; Deutschmann and Dekker, 2012).

6.1. The case of maize

As the largest crop worldwide in terms of total acreage (FAOSTAT, 2011), maize is expected to play an essential role in the development and wide-scale commercialization of cellulosic fuels (Schubert, 2006; Vermerris, 2009). This requires the breeding of maize as a dual-purpose crop, displaying optimal grain yield and quality characteristics, as well as high stem-biomass yield and improved processing amenability. Lewis et al. (2010) demonstrated that grain yield, agronomic fitness and stover quality were not mutually antagonistic breeding targets; and concluded that current maize breeding programs could incorporate stover traits interesting to the cellulosic ethanol industry without having to resort to exotic germplasm. With a wealth of agronomic and genomic resources, the possibilities of advancing maize as a dual crop with desirable biomass quality characteristics and a high stover yield are plentiful (Carpita and McCann, 2008). Due to the availability of such resources, its use as a forage crop and its widespread cultivation, producing tons of lignocellulosic residues, maize is most likely the

best model crop in the research on biomass quality. The primary research goal in maize bioenergy research lies thus in the dissection and understanding of biomass recalcitrance and the targeted manipulation of cell wall composition. In addition, recent research endeavors are also investigating the potential of maize as a dedicated biomass crop with the development of temperate × tropical maize varieties that produce much higher biomass yields, much lower grain yield and accumulate sugar in the stems (White et al., 2011; Dweikat et al., 2012).

6.2. The case of sorghum

Sorghum is a unique species, in which both grain-types, sugar-types and biomass-types exist (Rooney et al., 2007; Saballos, 2008; Serna-Saldívar et al., 2012). Together with the availability of its genome sequence, this opens up opportunities for sorghum to become a model crop for research on the production of both first and second generation biofuels (Olson et al., 2012). The highest lignocellulose yield potential in sorghum exists in forage sorghums (Vermerris and Saballos, 2012). They may provide a good alternative to perennial cropping systems, as they can provide similar dry matter yields, while offering the advantage of an annual growth cycle with respect to the choice of new planting material and the possibility to make changes in the crop rotation system in use.

Sweet sorghums types are also of interest, in particular in areas where sugarcane is already being produced, as the same equipment and processing facilities can be used. They may provide several advantages over sugarcane in terms of resource use efficiency, abiotic stress tolerance and due to its annual nature and simpler genetics.

Enhancing sorghum as a bioenergy crop can be accomplished through a combination of genetics, agronomic practice and processing technology. A particular research objective in sorghum is to increase the germination of seeds at low temperatures, and the ability of seedlings to withstand low temperatures; these cold-tolerance traits will enable earlier planting and therefore extend the growing season, potentially giving rise to higher biomass yields.

6.3. The case of miscanthus

Miscanthus has a high potential for biomass production over a wide range of climates. However, the triploid hybrid *Miscanthus* × *giganteus* is currently the only commercially grown species in the genus. This hybrid, a vegetatively propagated clone, is sterile and lacks genetic variation. It is crucial to broaden the genetic base of the germplasm to be able to extend its geographical adaptation and advance miscanthus for bioconversion and biomaterial applications and as a precaution against potential future pest outbreaks (Clifton-Brown et al., 2008; Heaton et al., 2010). In addition, being reliant on vegetative propagation, either through tissue culture or through rhizome division, the generation and handling of the planting material of this sterile clone leads to high establishment costs (Christian et al., 2005).

To broaden the genetic variation, attempts are made to resynthesize this interspecific hybrid by making new crosses between its parental species and by searching for more natural hybrids. However, breeding goals are difficult to meet, if the end products of the breeding process are sterile. A way out of this impasse is a focus of the breeding efforts on the development of seed-propagated varieties in genetically stable and fertile species, such as *M. sinensis*. This is economically attractive as this will most likely lower the costs of planting material considerably, result in a better establishment and speed up the development of miscanthus as dedicated biomass crop. The self-incompatibility system in miscanthus allows breeders to fix heterosis in the form of hybrid varieties. Alternatively, the creation of hexapoid *M. x giganteus* may also provide opportunities for the production of fertile germplasm (Yu et al., 2009).

6.4. The case of sugarcane

Sugarcane is one of the most efficient crops in collecting solar energy and converting it into chemical energy (Tew and Cobill, 2008) and as the largest crop worldwide in terms of tonnes produced (FAOSTAT, 2011) its potential as a biomass feedstock widely acknowledged. In sugarcane, breeding efforts have focused on increasing the yield of stem juice volume and stem juice sugar content. However, as stem yield is positively correlated to stem juice yield, lignocellulose yield has to some extent been indirectly advanced (Singels et al., 2005). Current breeding efforts take several directions: breeding solely for sugar content, breeding for its use as a dual crop (energy cane type I) and breeding solely for biomass yield (energy cane type II) (Tew and Cobill, 2008). These energy canes are being generated possessing a higher percentage of alleles from the high fibre, low sucrose species *S. spontaneum* (Ming et al., 2010). Moreover, Inman-Bamber et al. (2011) disproved that these types can attain high biomass yields because of a low sucrose content, rejecting the widespread hypothesized feedback inhibition of sucrose content on the efficiency of photo assimilation. Hence, increases in biomass yield through breeding and selection doesn't necessarily come at the expense of sucrose content.

Another challenge in sugarcane breeding is its envisioned geographic expansion to more temperate environments. Advances in sugarcane genetics are challenged by its large autopolyploid genome, organized into variable numbers of chromosomes. Hence, the construction of genetic maps and the identification of molecular markers for the targeted traits will play an important role to improve selection of sugarcane varieties and to speed up the breeding process.

6.5. The case of switchgrass

Switchgrass is adapted to a wide range of climates and efforts to enhance switchgrass for bioenergy purposes benefit from a history of forage breeding (Mitchell et al., 2008). Since switchgrass currently falls behind most of the other crops in terms of lignocellulose yields (section 3.2), productivity is the single most important objective in switchgrass. Due to the major investments in switchgrass research in the U.S. and due to the extensive variation present in the species there is a lot of potential for improvement in this versatile crop.

Significant heritable variation has been shown to exist in biomass yield and related traits (Taliaferro, 2002; McLaughlin et al., 2006; Boe and Lee, 2007). Yield improvements may be achieved in a number of ways. Tiller density and mass per phytomer were shown to have large direct effects on biomass yields in a path analysis, and may have potential as indirect selection criteria for enhancing biomass production in switchgrass (Boe and Beck, 2008). There is a large potential for yield increase through heterosis in upland × lowland crosses, producing hybrid cultivars with up to 40% yield increase compared to the parental lines (Mitchell et al., 2008; Vogel et al., 2010).

As switchgrass seedlings grow slowly in comparison to locally adapted C3 weeds (Parrish and Fike, 2005), earlier emergence and seedling vigor are also deemed important traits to the success of switchgrass as bioenergy crop. Issues with seed dormancy and germination are partly alleviated with seed treatment methods, such as cold storage for 24 months (Haynes et al., 1997), but still remain targets for improvement. Most research is focused on seed size, quality and seedling growth, to enable more successful establishment (Boe, 2003; McLaughlin and Adams Kszos, 2005; Bouton, 2008; Vogel et al., 2010).

7. Final Remarks

The exploitation of organic residues for the production of cellulosic ethanol may finally become a commercially viable technology, now that research efforts are increasingly devoted to the understanding and improvement of biomass quality. Equally significant is the progress that has been made in the identification and development of dedicated lignocellulose feedstocks. However, we are still far from the ideal of high yielding, resource efficient and stress-tolerant crops that can be sustainably cultivated in diverse environments and produces lignocellulose with a favorable balance of carbohydrates and a low level of recalcitrance. It is important to stress here that it is highly unlikely that a single crop will be able to attend this wide variety of agronomical and physiological requirements. The C4 grasses discussed in this review are envisioned to be the key players in the future supply of lignocellulose, due to their productivity under diverse ecological conditions and because they include both dual-purpose and biomass dedicated crops. Still, their evolutionary relationship and common characteristics may open ways to speed up research progress, for instance

through comparative genomics and the exchange of acquired knowledge and resources. As a group C4 grasses are amongst the most promising plants for biofuel production, containing highly productive, resource-use efficient species, harboring great genetic diversity. Maize, miscanthus, sorghum, sugarcane and switchgrass will all play a central role in the future biomass supply chain for the production of biofuel and other byproducts, and their improvement as lignocellulose feedstock will contribute to the commercial success of the cellulosic ethanol industry.

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Chapter 3

Impact of different lignin fractions on saccharification efficiency in diverse species of the bioenergy crop miscanthus

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Abstract

Lignin is a key factor limiting saccharification of lignocellulosic feedstocks. In this comparative study various lignin methods – including acetyl bromide lignin (ABL), acid detergent lignin (ADL), Klason lignin (KL) and modified ADL and KL determination methods - were evaluated for their potential to assess saccharification efficiency. Six diverse accessions of the bioenergy crop miscanthus were used for this analysis, which included accessions of *M. sinensis*, *M. sacchariflorus* and hybrid species. Accessions showed large variation in lignin content. Lignin estimates were different between methods, but (highly) correlated to each other ($0.54 \leq r \leq 0.94$). The strength of negative correlations to saccharification efficiency following either alkaline or dilute acid pretreatment differed between lignin estimates. The strongest and most consistent correlations ($-0.48 \leq r \leq -0.85$) were obtained with a modified Klason lignin method. This method is suitable for high throughput analysis and was the most effective in detecting differences in lignin content ($p < 0.001$) between accessions.

1. Introduction

Biomass is an abundant source of carbon that can be used for the production of biofuels. This carbon is an important basic element of the different plant components including the cell walls, which are mainly composed of the structural polysaccharide cellulose, hemicellulosic polysaccharides and the aromatic polymer lignin (Carpita, 1996, Doblin *et al.*, 2010, Harris & Stone, 2008, Rose, 2003). The conversion of biomass into biofuel depends on the enzymatic saccharification of structural polysaccharides into their monosaccharide building blocks, which can be subsequently fermented into bioethanol.

Lignin is one of the key components limiting the conversion of biomass into biofuel. It cross-links to hemicellulosic polysaccharides to form a highly impermeable matrix that imparts strength to the plant cell wall and shields cellulose - the main source of fermentable sugars - from chemical and enzymatic hydrolysis (Grabber, 2005, Grabber *et al.*, 2004, Himmel & Picataggio, 2008, Zhao *et al.*, 2012). In addition, it impedes the efficiency of enzymatic saccharification by irreversibly adsorbing hydrolytic enzymes, which renders them ineffective (Jørgensen *et al.*, 2007, Zhao *et al.*, 2012). As lignin is one of the most important barriers in the conversion of biomass into biofuels, reducing lignin content (or altering its composition) in bioenergy crops is critical to reduce processing costs and increase the cost-competitiveness of cellulosic biofuels (Simmons *et al.*, 2010, Wyman, 2007).

Lignin is chemically described as a heteropolymer of phenylpropanoids, primarily p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, although a variety of other compounds are incorporated in smaller quantities (Grabber *et al.*, 2004, Ralph *et al.*, 2004, Vanholme *et al.*, 2010). Polymerization of these subunits occurs in the cell wall via oxidative radicalization reactions that lead to a large number of different coupling products and bonds, some of which are difficult to break (Davin *et al.*, 2008, Frei, 2013, Vanholme *et al.*, 2010). Similar radical coupling reactions incorporate ferulate monomers and dimers into lignin, which, via diferulate cross-linking, give rise to cross-links between lignin and feruloylated hemicellulose side-chains that anchor lignin onto the cell wall polysaccharides (Carpita, 1996). These chemical characteristics and its extensive cross-linking to hemicellulose render lignin an intractable component. Lignin is hydrophobic due to its aromatic nature and has a high tendency to self-associate (Davin *et al.*, 2008, Hatfield & Fukushima, 2005). As a result, the isolation, structural characterization and quantification of the complete native lignin polymer is challenging (Davin *et al.*, 2008).

Nonetheless, a number of different methods for determining lignin content in biomass samples have been developed over the past decades, primarily for their application in paper making and animal nutrition. These methods can be grouped into two distinct categories, gravimetric methods and spectrophotometric methods (Frei, 2013, Hatfield & Fukushima, 2005, Vermerris & Nicholson, 2006). Gravimetric methods are the most widely used in both research and industry. The most common and one of the oldest methods to quantify lignin

content is the Klason method, developed in the early 1900's by Klason. It is based on the hydrolysis of cell wall polysaccharides using sulphuric acid and gravimetric determination of the acid insoluble residue as Klason lignin (KL) (Browning, 1967, Vermerris & Nicholson, 2006). To deal with concerns related to the contamination of KL with protein, Van Soest proposed to include an acid detergent extraction step to remove proteins (and some other components) prior to polysaccharide hydrolysis, which was particularly useful for forage samples containing high protein concentrations (Goering & Van Soest, 1970, Van Soest, 1963, Van Soest, 1967). This lignin quantification method – measuring acid detergent lignin (ADL) - is widely used for evaluating feed quality in forage grasses. It is adopted in a sequential fiber analysis protocol that is based on the sequential solubilization of cell wall fractions using neutral detergent, acid detergent and sulphuric acid solutions (Goering & Van Soest, 1970, Van Soest, 1963, Van Soest, 1967). While this method is widely employed for evaluating the nutritive value of forages, it may underestimate lignin concentration, as a fraction of the lignin is solubilized during the detergent and acid digestion reactions (Hatfield *et al.*, 1994, Kondo *et al.*, 1987).

Spectrophotometric methods are based on the solubilization of lignin from the cell wall and subsequent determination of its specific absorbance at certain wavelengths. To enable the solubilization of lignin this polymer must be derivatized, which is most commonly accomplished using acetyl bromide, leading to the determination of acetyl bromide lignin (ABL) (Browning, 1967, Hatfield & Fukushima, 2005, Johnson *et al.*, 1961). These spectrophotometric lignin determination lignin methods may suffer from interference of light absorption by other biomass components at the same wavelengths, leading to an overestimation of lignin content (Hatfield & Fukushima, 2005). Furthermore, these methods require a well-defined lignin standard to calibrate the estimation of lignin concentration from optical density measurements (Fukushima & Hatfield, 2001, Hatfield & Fukushima, 2005).

None of the currently available lignin methods is considered a standard unambiguous method for determining lignin content, as concerns exist for each of these lignin methods related to their under or overestimation of lignin content (Davin *et al.*, 2008, Hatfield & Fukushima, 2005). More problematically, while all these methods are widely used, large discrepancies are reported in the different estimates of lignin content between them (Brinkmann *et al.*, 2002, Fukushima & Hatfield, 2004, Goff *et al.*, 2012, Hatfield *et al.*, 1994, Jung *et al.*, 1999, Lacerda *et al.*, 2006, Moreira-Vilar *et al.*, 2014, Takahashi *et al.*, 2004). In a recent study, Fukushima and Hatfield compared the performance of some lignin methods on a number of different plant samples and reported on average a twofold difference in lignin content between the ABL method and the ADL method (Fukushima & Hatfield, 2004). In specific plant samples, however, even more than a fourfold difference between the two methods was reported, emphasizing that differences between lignin concentrations obtained using different methods are not systematic and directly convertible, but rather dependent on

the sample being analyzed. If the ratio between different lignin measurements is variable between samples, this suggests that different lignin methods are measuring different fractions of the native lignin polymer and that these fractions are variable between samples.

Recently, Moreira-Vilar and co-workers, compared a number of different lignin methods and concluded that the ABL method outperformed the other methods, primarily because it gave the highest estimates of lignin content (Moreira-Vilar *et al.*, 2014). However, considering that lignin is usually quantified because of its effect on the efficiency of a certain process, for example paper production, ruminant digestion and saccharification for biofuel production, selecting the most appropriate lignin method may depend on which estimate of lignin content has the highest predictive ability of biomass quality for a certain application. Some recent studies, for example, evaluated different lignin methods in forage samples for their predictive ability of digestibility (Fukushima & Hatfield, 2004, Goff *et al.*, 2012, Jung *et al.*, 1999). Fukushima and Hatfield concluded from their comparative study that ABL provided stronger and more consistent negative correlations to digestibility characters than the other methods (Fukushima & Hatfield, 2004).

Discrepancies in the utilization of lignin content (measured through different methodologies) as an indicator of biomass quality could result from the fact that lignin extraction procedures act differently on lignin types with different monomeric composition or that possess different types and numbers of crosslinks between lignin and other cell wall components. Although such factors might underpin differences in recalcitrance between different lignin fractions, first it must be established if different lignin fractions have different implications for saccharification of miscanthus biomass.

Despite the implications of lignin as a major recalcitrance factor in bioenergy conversion technologies, to our knowledge no studies have been performed to compare the different lignin methods for their predictive ability of saccharification efficiency in potential bioenergy feedstocks. Consequently, it is possible that the limited resources available for compositional analysis of biomass feedstocks for fuel production are spent on lignin analysis using a method that is not optimal for this evaluation. Similarly, selection of genotypes with reduced lignin content in a bioenergy crop breeding program might be more effective in improving saccharification efficiency using a certain lignin determination method.

In this manuscript various lignin methods, including ABL, KL and ADL, are compared for their applicability to assess the potential of bioenergy feedstocks. To this end, their predictive ability of saccharification efficiency and their ability to discriminate between genotypes are evaluated in a diverse set of accessions of the important bioenergy crop miscanthus.

2. Materials and methods

2.1. Plant materials

Six different miscanthus accessions, belonging to three different miscanthus species, were used in this study; two accessions of *M. sinensis,* including the commercial cultivar *'Goliath'*, two of *M. sacchariflorus*, including the commercial cultivar '*Robustus*', and two clones derived from crosses between the two species, including the commercially-used clone known as *M. x giganteus* (Supplementary Table S1). The accessions were grown in Wageningen, the Netherlands, in a field trial with a randomized block design with three replications. The field trial was established in May 2012. The planting material used to establish the trial was produced clonally by *in vitro* propagation, except for one accession (OPM-13), which consisted of seed-derived plants resulting from a cross between two *M. sinensis* parents. A total of 49 plantlets were planted per plot with a density of two plants m^{-1}, resulting in a plot size of 25 m^2. To avoid influence of a potential border effect, only the inner nine plants within plots were harvested for analysis in March 2013. After harvesting, the plant shoots were stripped from leaves and the remaining stems were chopped and air dried at 60°C for 72 hours. The dried stem material was ground using a hammer mill with a 1-mm screen.

Compositional analysis

Ground stem samples from the field trial were used for compositional analysis. An overview of compositional characteristics determined in this study is provided in Supplementary Table S2. All samples were measured in quadrupole.

Cell wall carbohydrate content

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents of stem dry matter were determined according to protocols developed by Ankom Technology (ANKOM Technology Corporation, Fairpoint, NY), which are essentially based on the work of Goering and Van Soest (Goering & Van Soest, 1970, Van Soest, 1963, Van Soest, 1967). Detergent fiber contents were subsequently used to obtain estimates for the contents of cell wall (CW), cellulose (Cel) and hemicellulosic polysaccharides (Hem) in stem dry matter, as described in Supplementary Table S2.

Acid detergent lignin

The residue of each ADF analysis was used for the determination of ADL, according to the protocol developed by Ankom Technology (ANKOM Technology Corporation, Fairpoint, NY), which is based on the work of Goering and Van Soest (Goering & Van Soest, 1970, Van Soest & Wine, 1967, Van Soest, 1963, Van Soest, 1967). It comprised a three hour hydrolysis

in 72% H_2SO_4 in 1L laboratory bottles that were placed horizontally on an orbital shaker set at 160 RPM. After hydrolysis, the samples were extensively washed with deionized water and dried for 12 hours at 103°C. The remaining sample residue is considered as acid detergent lignin and was gravimetrically determined. In parallel, an alternative method was also tested, in which the ADL determination was performed on sample material that was sequentially subjected to both a neutral and an acid detergent extraction treatment. Sequential determinations of NDF, ADF and ADL fastens the procedure and results in a purer ADL fraction, from which xylans and pectins have been removed (Goering & Van Soest, 1970, Hatfield & Fukushima, 2005). The lignin content determined in this way is henceforth referred to in this study as ADLseq.

Acetyl bromide lignin

The residual material from each of the four NDF determinations per sample were pooled and used as basis for determining ABL following the method described by Fukushima and Kerley (Fukushima & Kerley, 2011). A single ABL determination comprised precise weighing of 20-25 mg of NDF material into a 2-ml Eppendorf tube. The sample was then digested using 1.5 ml 25% (v/v) acetyl bromide in acetic acid for 2 hours at 50°C and constant shaking (800 rpm). After digestion of the sample, tubes were centrifuged for 5 minutes at 13.000 rpm. A 15 µl aliquot of the solution was then added to an Eppendorf tube containing 200 µl of 0.3M NaOH and 685 µl of acetic acid. Finally, 100 µl 0.5M hydroxylamine hydrochloride was added and after exactly 30 minutes the optical density of the solution was measured in duplicate at 280 nm against a blank containing all chemical reagents, but no sample material. Acetyl bromide lignin concentration (mg/ml) was then calculated using a regression equation (1):

$$\mathrm{ABL}\left(\%\mathrm{cw}\right)=\frac{\left(\mathrm{A}-0.0009\right)\times \mathrm{DF}}{17.78\times Sample}\times 100\% \qquad (1)$$

where *A* = average optical density reading of the two measurements; *0.0009* = mean intercept value of the regression equation as determined by Fukushima and Kerley (Fukushima & Kerley, 2011); *DF* = dilution factor, 100x; *17.78* = miscanthus specific extinction coefficient as determined by Lygin et al. (Lygin *et al.*, 2011) and *Sample* = the amount of NDF material in mg.

Klason lignin

The same pooled NDF residues used as starting material for the ABL analyses were used for the determination of KL. This was done according to the Laboratory Analytical Procedure "Determination of Structural Carbohydrates and Lignin in Biomass", a two-step acid hydrolysis method developed by the National Renewable Energy Laboratory (NREL) (Sluiter *et al.*, 2012). The procedure started with the hydrolysis of 300 mg of NDF material in 3 ml 72% H_2SO_4 in a 100 ml glass pressure tube (Ace Glass Inc., Vineland, NJ) for 1 hour at 30°C

with constant shaking (160 rpm). After one hour the acid concentration was diluted to 4% by adding 84 ml deionized water, after which samples were hydrolyzed by autoclaving the tubes at 121°C for 1 hour. After cooling down, the samples were vacuum-filtered using glass filtering crucibles (30 ml, P4, Klaus Hofman, Staudt, Germany). The residue was dried for 12 hours at 103 °C and weighed for the determination of Klason lignin. A separate experiment was conducted to test the feasibility of analyzing smaller biomass samples, with the aim of increasing the throughput of the KL method. In this modified KL method, the sample and reagent quantities were reduced 10-fold, while all other process steps remained the same. The Klason lignin results of this down-scaled experiment are reported as ds-KL.

Acid soluble lignin

The filtrate obtained from the vacuum-filtration step of the Klason lignin determination was captured and purified using 0.45 µm filters to quantify acid soluble lignin (ASL). ASL was determined spectrophotometrically at 205 nm using quartz cuvettes. ASL concentrations were calculated using equation (2) (Lin & Dence, 1992):

$$\mathrm{ASL}\left(\%\mathrm{cw}\right) = \frac{\mathrm{A} \times \mathrm{V} \times \mathrm{DF}}{\mathrm{K} \times \mathit{Sample}} \times 100\% \qquad (2)$$

where *A* = absorption value; *V* = hydrolyzate volume; *DF* = dilution factor, 20x; *K* = absorptivity constant, 110 L/g/cm as determined by Xu et al. (Xu *et al.*, 2012) and *Sample* = the amount of NDF material in mg.

Neutral sugar contents

The filtrate obtained from the vacuum-filtration step of the Klason lignin determination was captured and purified using 0.45 µm filters to quantify the amount of neutral sugars released from cell wall samples. Two different dilutions were made, one for determining the content of glucose and xylose (dilution factor 50) and another for determining the arabinose content (dilution factor 10). Neutral sugar contents were determined by high performance anion exchange chromatography (HPAEC) analysis on a Dionex system equipped with a CarboPac PA1 column and a pulsed amperometric detector (Dionex, Sunnydale, CA). The ratio of arabinose to xylose was also determined, which constitutes an estimate of the degree of hemicellulose substitution (DHS) (Torres *et al.*, 2014).

2.2. Saccharification efficiency

Separate analyses of ground stem samples were performed for the characterization of saccharification efficiency. Saccharification reactions were carried out using three 500 mg subsamples per stem sample. All samples were briefly treated with α-amylase and repeatedly washed with deionized water (3x, 5 minutes, ~60°C) in order to remove all

interfering stem soluble sugars. The remaining biomass was then subjected to either an alkaline pretreatment or a dilute acid (DA) pretreatment. Alkaline pretreatments were carried out in 50 ml plastic centrifuge tubes with 15 ml 2% NaOH at 50°C with constant shaking (160 RPM) for 2 hours in an incubator shaker (Innova 42, New Brunswick Scientific, Enfield, CT). Dilute acid pretreatments were carried out in custom-built stainless steel reactors, essentially as described by Torres *et al.* (2013). Briefly, pretreatment comprised hydrolysis in 15 ml of 0.17% (w/v) H_2SO_4 at 140°C for 30 minutes in a temperature controlled oil bath. After 30 minutes the reactions were quickly quenched by submerging the reactors in a cold water bath.

The conditions chosen for pretreatment were fairly mild. In this study the objective of the pretreatment was not to maximize sugar yields but to use conditions that better discriminate genotypic differences in the release of sugars following the combined operations of pretreatment and enzymatic saccharification. The severity (log M_0) of the pretreatment was 1.78 for the sodium hydroxide pretreatment and 1.99 for the dilute sulphuric acid pretreatment, as calculated by the following equation (3) (Pedersen & Meyer, 2010):

$$\log \mathrm{M}_0 = \log\left(\mathrm{t} \times C^n \times \exp\frac{T-100}{14.75} \right) \quad (3)$$

where *t* = reaction time; *C* = concentration of chemical catalyst (%w/v); *n* = empirically determined constant fitted to be 0.849 and 3.90 for sodium hydroxide and sulfuric acid, respectively, (Pedersen & Meyer, 2010, Silverstein *et al.*, 2007) and *T* = reaction temperature in °C.

Pretreated samples were then washed to neutral pH with deionized water (2x, 5 minutes, 50°C) and with 0.1 M sodium citrate buffer (pH 4.6, 5 minutes, 50°C). Saccharification reactions were subsequently carried out according to the NREL Laboratory Analytical Procedure "Enzymatic saccharification of lignocellulosic biomass" (Selig *et al.*, 2008). Pretreated samples were hydrolyzed for 48 hours with 300 µl of the commercial enzyme cocktail Accellerase 1500 (DuPont Industrial Biosciences, Leiden, NL) supplemented with 15 µl endo-1,4-β-xylanase M1 (Megazyme, Bray, IE) in an incubator shaker (Innova 42, New Brunswick Scientific, Enfield, CT) set at 50°C and constant shaking (160 RPM). These enzymes combined have the following specific activities: endoglucanase 2200-2800 CMC U/g, beta-glucosidase 450-775 pNPG U/g and endoxylanase 230 U/mg. Reactions were carried out in 44 ml 0.1 M sodium citrate buffer (pH 4.6), containing 0.4 ml 2% sodium azide to prevent microbial contamination.

The release of sugars during dilute acid pretreatment and enzymatic saccharification reactions was analyzed by HPAEC as described previously for neutral sugars. Saccharification efficiency was assessed by the respective percentages of glucose and xylose released from the biomass samples by the combined actions of pretreatment and enzymatic saccharification (Supplementary Table S2).

2.3. Statistical analysis

General analyses of variance (ANOVA) were performed for all traits taking into account the randomized block design of the field trial using the following equation (4):

$$Y_{ij} = \mu + \beta_1 X_i + \beta_2 X_j + \text{error} \quad (4)$$

where Y_{ij} is the response variable, μ is the overall mean, $\beta_1 X_i$ is the contribution of the genotype and $\beta_2 X_j$ is the block effect.

Multiple comparisons analysis were performed to distinguish significant (p<0.05) genotypic differences using Fisher's protected least significant difference (LSD) test on genotype means. Correlation analysis was performed to identify the significance (p<0.05), strength and direction of interrelationship between sample characteristics using Pearson's correlation coefficients. All statistical analyses were performed using Genstat for Windows, 14th edition software package (VSN International, Hemel Hempstead, UK).

3. Results and discussion

3.1. Characterization of cell wall carbohydrate content and composition of six diverse miscanthus accessions

Ground miscanthus stem samples were analyzed for cell wall compositional characteristics. The results are presented in Table 1 as accession means for neutral and acid detergent fiber, structural carbohydrate and neutral sugar contents. As expected for miscanthus harvested at complete senescence, low moisture and high cell wall contents were observed (Heaton *et al.*, 2010, Hodgson *et al.*, 2010, Robson *et al.*, 2012). On average, approximately 85% of the dry biomass consisted of cell wall material, ~46% consisted of cellulose and ~31% consisted of hemicellulosic polysaccharides. Within the panel of miscanthus accessions, large variation was present in the contents of cellulose, ranging from ~43 to ~48.5%, and hemicellulosic polysaccharides, ranging from ~27 to ~34%. Glucose and xylose were the most abundant monosaccharides and accounted for almost 70% of the cell wall material. A minor fraction of the monosaccharides consisted of arabinose, which made up on average less than 2% of the cell wall material. Between the species of miscanthus, the *M. sacchariflorus* accessions showed the highest contents of cellulose, glucose and xylose, whereas the *M. sinensis* accessions had the highest contents of hemicellulosic polysaccharides and arabinose. Hybrid accessions were intermediate for cellulose content, but had the lowest fraction of hemicellulosic polysaccharides. OPM-4 was the accession that had the highest amount of glucose potentially available for saccharification reactions, with both the highest cell wall content (87.06%) and the highest cell wall glucose content (43.96%).

3.2. Large differences observed between the various lignin methods

The lignin contents of miscanthus stem samples were evaluated using different lignin methods. The comparative study showed large differences in lignin content between the three most commonly used methods, ABL, KL and ADL (Figure 1). The highest lignin contents were obtained by the ABL method, ranging from ~16-22%. Values obtained by the KL method were slightly lower and ranged from ~13-20%. Considerably lower estimates were obtained by the ADL method, which ranged from ~7-14%. Such striking differences were anticipated and are consistent with the results of previously published comparisons of these lignin methods (Fukushima & Hatfield, 2004, Goff *et al.*, 2012, Hatfield *et al.*, 1994, Jung *et al.*, 1999, Jung *et al.*, 1997, Moreira-Vilar *et al.*, 2014). The most likely explanation for the low lignin concentrations obtained by the ADL method is the loss of lignin during the acid detergent extraction of the method (Hatfield *et al.*, 1994, Jung *et al.*, 1999).

The mean values for each accession for the different lignin methods are reported in Table 2. The overall means over accessions were 18.66% for ABL, 16.61% for KL and 9.87% for ADL. Despite such differences in lignin estimates between the methods, all lignin methods, except the ASL method, consistently identified OPM-9 (*M. x giganteus*) as the accession with the highest lignin content and OPM-2 as the accession with the lowest lignin content. Lignin concentrations also displayed a similar trend between species, with the hybrid accessions generally having higher lignin contents than *M. sinensis* and with *M. sacchariflorus* accessions generally having the lowest lignin contents (Table 2).

In addition to the three most commonly used lignin measurements, three additional lignin measurements were obtained on the same samples: a modified (sequential) ADL, a modified (down-scaled) Klason lignin and acid soluble lignin (ASL) measurement. Both modified versions of the ADL and the KL protocol resulted in lower lignin estimates compared to their respective reference methods (Figure 1, Table 2). The sequential variant of the ADL method resulted in an overall mean lignin content over accessions of 6.30%, which was ~3.5% lower than that of the conventional ADL method. The additional neutral detergent extraction step in the modified ADL method thus resulted in a loss of lignin compared to the reference protocol. For the modification of the Klason method an overall mean of 14.48% was found, which was ~2.1% lower than the reference (Table 2). The reason why the down-scaled protocol resulted in a lower estimate of lignin remains unclear, as biomass samples in both protocols receive the same treatment. However, similar observations were made by Ibáñez *et al.* (2014) upon down-scaling the Klason method, so it is unlikely to be due to a technical error (Ibáñez & Bauer, 2014). The third additional measurement was the quantification of ASL in the hydrolyzate resulting from the sulphuric acid hydrolysis reactions in the Klason method. For all samples the acid soluble fraction of the lignin was small compared to the amount of acid insoluble lignin. Accession means ranged from 3.23% to 3.85%, with an overall mean over accessions of 3.69%.

Table 1. Accession means for various stem fiber and fiber composition characteristics

	Accession *						
Trait	**OPM-2**	**OPM-4**	**OPM-5**	**OPM-9**	**OPM-11**	**OPM-13**	**Average**
	Fiber (%dm)						
NDF	86.1 ^{b}	87.06 ^{b}	84.71 ^{a}	86.46 ^{b}	84.3 ^{a}	86.3 ^{b}	85.82
ADF	54.59 ^{b}	57.22 ^{c}	55.34 ^{b}	59.21 ^{d}	50.63 ^{a}	51.94 ^{a}	54.82
Cel	47.98 ^{c}	48.58 ^{c}	45.72 ^{b}	48.5 ^{c}	43.01 ^{a}	44.33 ^{ab}	46.35
Hem	31.5 ^{c}	29.83 ^{b}	29.37 ^{b}	27.25 ^{a}	33.67 ^{d}	34.35 ^{d}	31.00
	Fiber composition (%cw)						
Glu	42.55 ^{b}	43.96 ^{c}	41.59 ^{b}	42.79 ^{bc}	39.47 ^{a}	39.48 ^{a}	41.64
Xyl	28.13 ^{c}	27.68 ^{c}	25.14 ^{a}	24.57 ^{a}	26.04 ^{ab}	26.9 ^{bc}	26.41
Ara	1.47 ^{b}	1.23 ^{a}	1.61 ^{b}	1.46 ^{b}	2.21 ^{c}	2.05 ^{c}	1.67
DHS	5.25 ^{b}	4.43 ^{a}	6.42 ^{c}	5.93 ^{c}	8.52 ^{e}	7.64 ^{d}	6.37

NDF = Neutral detergent fiber, ADF = Acid detergent fiber, Cel = Cellulose, Hem = Hemicellulosic polysaccharides, Glu = Glucose, Xyl = Xylose, Ara = Arabinose, DHS = Degree of hemicellulose substitution (Ara/Xyl)
* Accession means having no common suffix letter for a given lignin determination method differ significantly ($p < 0.05$) from each other.

Large variation was present among the accessions in lignin estimates derived from the different lignin determination methods and all methods were able to uncover statistically significant differences among them (Tables 2 and 3). The most significant differences ($p<0.001$) among accessions were found using the ADL method and the modified KL method (Table 3). Both methods also resulted in a large range in the performance of accessions (4.71% for ADL and 4.56% for ds_KL). The other methods resulted in smaller ranges of variation in lignin estimates (<4.07) and lower discriminative abilities among accessions ($0.002 \leq p \leq 0.018$), making them less suitable for screening small differences between accessions.

Correlation analysis was used to investigate the interrelationships between the different lignin estimates (Figure 2). This analysis showed that despite the large differences in the estimation of lignin between different lignin determination methods, the estimates of lignin content were correlated ($0.54 \leq r \leq 0.94$). Most notably, ADL and KL were more strongly correlated to each other (r=0.87) than they were to ABL (r=0.63 and r=0.54, respectively). A reason for this might be the similarities in the way these lignin estimates are determined. ADL and KL concentrations are both determined by weighing the remaining residue after acid-catalyzed hydrolysis of the cell wall polysaccharides, whereas ABL concentration is determined through spectrophotometric quantification of solubilized lignin.

In addition, the correlation analysis showed that the lignin estimates obtained by the modified Klason and modified acid detergent protocols tested in this study were highly correlated ($r=0.87$ and $r=0.91$, respectively) to lignin estimates obtained by their corresponding reference methods. Furthermore, ASL showed remarkably few correlations to the other lignin methods, undermining the plausible assumption that an increase in acid

soluble lignin would lead to a decrease in acid insoluble lignin. Such an inverse correlation was only found between ASL and ADL, and not for example between ASL and KL. This would suggest that these two traits can be investigated or selected for independently.

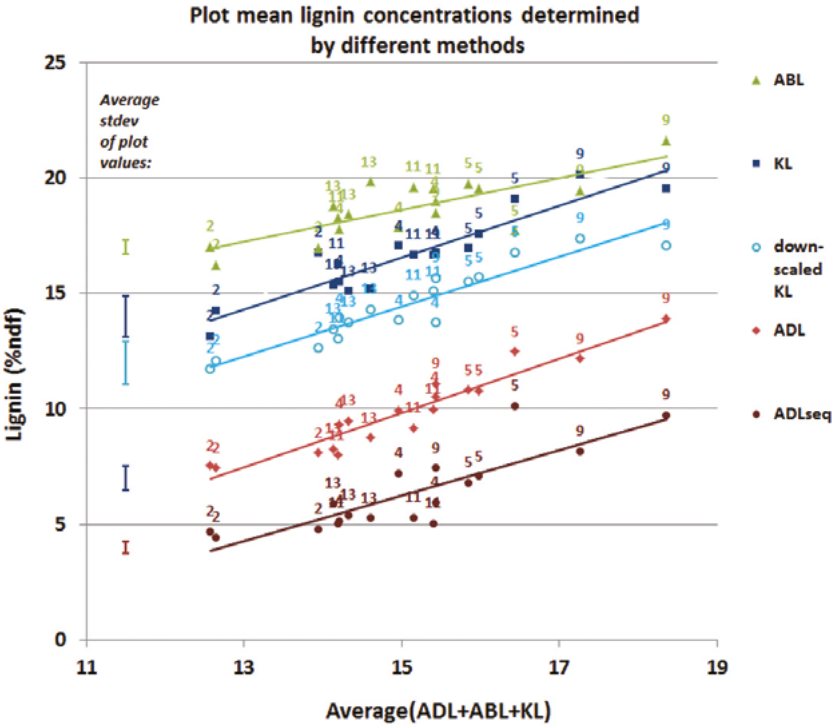

Figure 1. Lignin content in miscanthus stem samples as determined by the acetyl bromide lignin (ABL), the acid detergent lignin (ADL) and the Klason lignin (KL) methods, as well as modified versions of the ADL and the KL method. Plotted lines are regression lines of the plot lignin contents on the average plot lignin content as determined by ADL, ABL and KL.

3.3. Extensive variation among accessions in the release of fermentable sugars upon mild alkaline or dilute acid pretreatment and enzymatic saccharification

In this comparative study, two pretreatment protocols were evaluated for determining the saccharification efficiency of ground stem samples. Samples were subjected to an alkaline pretreatment with NaOH and a dilute acid pretreatment with sulphuric acid. Saccharification efficiency was determined by the total release of fermentable sugars during pretreatment and subsequent enzymatic saccharification (Table 4). Glucose yields were similar between pretreatment methods, with average total glucose yields of 64.96% for NaOH pretreated materials and 64.43% for DA pretreated materials. Xylose yields, in contrast, were not similar between the two methods, and average total xylose yields were on average almost 13% higher for NaOH pretreated materials (49.44%) than for DA pretreated materials (36.82%).

These results show that at similar pretreatment severity, the enzymatic hydrolysis of xylose upon alkaline pretreatment (thus following partial removal of lignin) is more efficient than the combined chemical and enzymatic hydrolysis of xylose using sulfuric acid pretreatment.

At the alkaline pretreatment conditions evaluated in this study approximately 50% of the lignin is likely to be removed, as predicted by a formula developed to estimate lignin removal during NaOH pretreatment (Silverstein *et al.*, 2007). During dilute acid pretreatment, however, the solubilization of lignin is only minimal (Yang & Wyman, 2008). Such a difference between pretreatment types in the amount of lignin remaining in pretreated sample, is likely to affect the efficiency of enzymatic saccharification as lignin can irreversibly adsorb hydrolytic enzymes (Jørgensen *et al.*, 2007, Zhao *et al.*, 2012). In addition, since lignin and hemicellulosic polysaccharides are extensively cross-linked by ferulate bridges (Carpita, 1996, Grabber *et al.*, 2004, Hatfield *et al.*, 1999), the large difference in the amount of lignin that remains in the pretreated sample can possibly cause a difference in the release of xylose between the two pretreatments.

Table 2. Accession mean lignin concentrations (% cw) in stems of six miscanthus accessions as determined by various lignin determination methods

Lignin estimate	Accession *						
	OPM-2	**OPM-4**	**OPM-5**	**OPM-9**	**OPM-11**	**OPM-13**	**Average**
ABL	16.75 ^{a}	18.21 ^{bc}	19.01 ^{bc}	19.86 ^{c}	19.14 ^{bc}	19.01 ^{bc}	18.66
ADL	7.68 ^{a}	9.92 ^{bc}	11.36 ^{cd}	12.39 ^{d}	9.05 ^{ab}	8.83 ^{ab}	9.87
KL	14.73 ^{a}	16.45 ^{ab}	17.89 ^{bc}	18.80 ^{c}	16.55 ^{ab}	15.22 ^{a}	16.61
ASL	3.47 ^{ab}	3.23 ^{a}	3.40 ^{ab}	3.29 ^{a}	3.85 ^{cb}	3.80 ^{b}	3.42
ADLseq	4.63 ^{a}	6.09 ^{a}	8.00 ^{b}	8.44 ^{b}	5.11 ^{a}	5.52 ^{a}	6.30
ds_KL	12.15 ^{a}	13.83 ^{b}	16.00 ^{c}	16.71 ^{c}	14.36 ^{b}	13.84 ^{b}	14.48

ABL = Acetyl bromide lignin, ADL = Acid detergent lignin, KL = Klason lignin, ASL = Acid soluble lignin, ADLseq = Sequentially determined ADL, ds_KL = downscaled determination of KL

* Accession means having no common suffix letter for a given lignin determination method differ significantly ($p < 0.05$) from each other.

Both the alkaline and the dilute acid pretreatment resulted in significant differences among accessions for total glucose release ($p = 0.007$) (Tables 3 and 4). Significant differences among accessions for total xylose release, however, were only obtained using the alkaline pretreatment ($p = 0.009$) and not using the dilute acid pretreatment ($p = 0.073$), which can be attributed to the higher residual error in the data from the acid pretreated samples. However, both the alkaline and the dilute acid pretreatment consistently identified OPM-2 as the accession with the highest glucose (73% for alkaline and 84% for dilute acid) and xylose yields (55% for alkaline and 41% for dilute acid).

Even though mild pretreatment conditions were used, fairly good sugar release rates were obtained using this set of accessions. More importantly, however, a large variation in the performance of accessions at these mild conditions was observed. Especially for

total glucose release from dilute acid pretreated materials, for which the best performing accession (OPM-2) yielded 33% more glucose than the worst performing accession (OPM-9). This exemplifies the importance of feedstock optimization on process performance.

Table 3. ANOVA derived statistics describing the variation in lignin content and saccharification efficiency among and within six miscanthus accessions for various lignin determination methods and pretreatment types. Saccharification efficiency was measured as the percentage of monosaccharides released from stem samples upon NaOH or dilute acid (DA) pretreatments and subsequent enzymatic saccharification.

Trait	Range	$LSD_{(0.05)}$	MS residual	F ratio	Probability
Lignin					
ABL	3.11	1.33	0.53	6.51	0.006
ADL	4.71	1.63	0.80	11.33	<0.001
KL	4.07	1.87	1.06	6.75	0.005
ASL	1.18	0.35	0.04	4.25	0.018
ADLseq	3.81	1.67	0.84	8.81	0.002
ds_KL	4.56	1.39	0.58	14.05	<0.001
Saccharification efficiency					
NaOH Glucose release %	16.16	7.23	15.77	6.18	0.007
NaOH Xylose release %	11.64	5.49	9.112	5.79	0.009
DA Glucose release %	33.07	14.66	64.95	6.32	0.007
DA Xylose release %	9.67	6.77	13.86	2.87	0.073

ABL = Acetyl bromide lignin, ADL = Acid detergent lignin, KL = Klason lignin, ASL = Acid soluble lignin, ADLseq = Sequentially determined ADL, ds_KL = downscaled determination of KL

The two protocols were equally effective at screening differences in glucose release among accessions (*p = 0.009)*, despite the larger range in the performance of accessions in glucose release upon acid pretreatment. This was mainly due to the larger residual error for glucose release using the acid pretreatment. As accessions could not be discriminated for xylose release using acid pretreatment, we conclude that in this study the alkaline pretreatment was overall more effective in screening variation in saccharification efficiency than the acid pretreatment. However, the type of pretreatment used for future screening purposes will largely depend on which pretreatment option will prevail in industry.

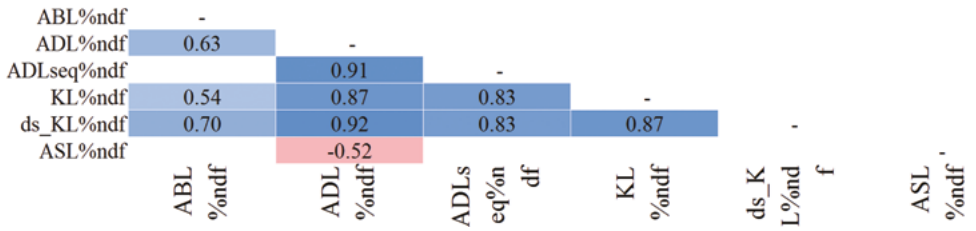

Figure 2. Correlations among lignin estimates obtained using different lignin determination methods. Only Pearson correlation coefficients that differed significantly from zero (*p>0.05*) are reported.

3.4. The modified Klason lignin method showed the highest potential for predicting saccharification efficiency

Saccharification characters are generally strongly and negatively impacted by lignin content (Figure 3). All different estimates of lignin content were significantly and negatively correlated to total glucose yields. Not all lignin estimates were, however, significantly correlated to xylose release after NaOH pretreatment. The only significant correlations to xylose yields of NaOH pretreated samples were found for ABL (r=-0.56) and ds-KL (r=-0.48). In contrast, correlations between glucose release and lignin concentrations were slightly stronger for NaOH pretreated (*r=-0.74* to *-0.85*) than for DA pretreated samples (*r=-0.63* to *-0.84*). These differences are most likely related to the different modes of action of the two pretreatment types. A major part of the lignin is likely to be removed upon NaOH pretreatment and fraction of the lignin that remains in the sample apparently doesn't affect hydrolysis of hemicellulosic polysaccharides to a large extent. Also in other studies it was shown that glucose release is more negatively affected by residual lignin in the pretreated sample than xylose release (Chen *et al.*, 2009).

Table 4. Accession means for the saccharification efficiency of six miscanthus accessions upon alkaline or dilute acid pretreatment. Saccharification efficiency is expressed as the percentage of glucose and xylose that is released by the pretreatment and subsequent enzymatic saccharification.

Pretreatment type	Monosaccharide release (%)	Accession *												Average
		OPM-2		**OPM-4**		**OPM-5**		**OPM-9**		**OPM-11**		**OPM-13**		
NaOH	Glucose	73.08	c	66.63	bc	60.10	ab	56.92	a	65.94	bc	67.08	bc	64.96
	Xylose	54.99	d	53.37	cd	48.87	bc	48.40	abc	43.35	a	47.66	ab	49.44
Dilute sulphuric acid	Glucose	84.45	c	62.40	ab	55.45	ab	51.39	a	69.61	b	63.27	ab	64.43
	Xylose	41.02	n.s.	39.93	n.s.	33.93	n.s.	31.35	n.s.	37.72	n.s.	36.95	n.s.	36.82

* Accession means having no common suffix letter for a given lignin determination method differ significantly ($p < 0.05$) from each other.

Although all methods thus provided significant correlations to saccharification characters, there are differences observable when comparing the performance of the different lignin methods to predict the saccharification efficiency. First of all it should be concluded that correlation patterns between saccharification efficiency and lignin content differed depending on the pretreatment method. The largest impact on sugar yields upon NaOH pretreatment was found for ds-KL. For DA pretreated samples the largest impact on sugar yields were found for ADL. However, ds-KL also had a strong correlation to both xylose and glucose yields for DA pretreated samples. It was remarkable that the modified KL method performed considerably better than the reference as evaluated by the negative correlations with the different saccharification efficiency parameters. Apart from the fact that the smaller scale might be more suitable for this analysis, the higher correlation can also be due to a smaller technical error as more samples can be analyzed simultaneously.

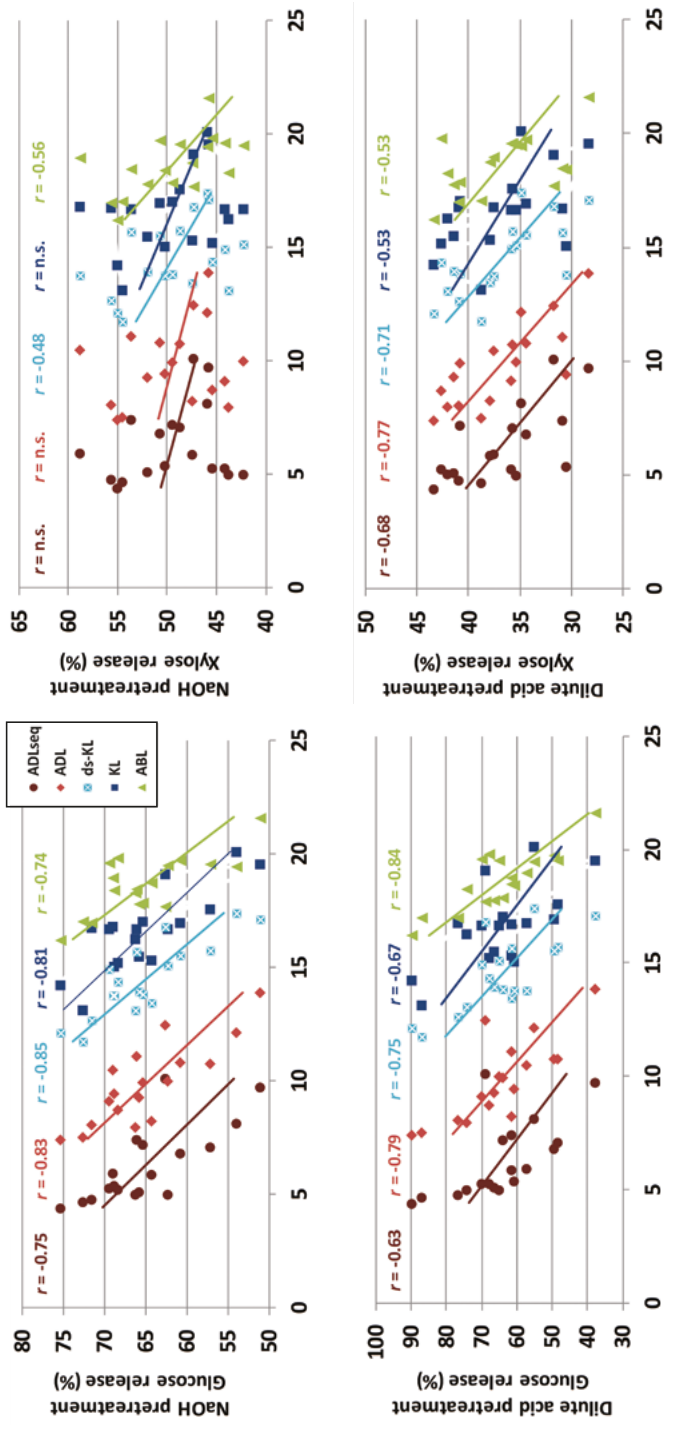

Figure 3. Associations between saccharification characters and lignin estimates obtained using five different lignin determination methods. Regression lines and Pearson correlation coefficients are shown to indicate the strength and direction of trait correlations.

The modified variant of the ADL method performed worse than the reference, as negative associations to saccharification characters were less strong for ADLseq than for ADL. Therefor this method is not recommended, although sequential extraction of NDF, ADF and ADL on the same bag provided a considerable reduction in time and labor.

Considering all lignin determination methods evaluated in this study, the strongest overall correlations to saccharification characters were found using the modified KL method. Lignin estimates obtained using this method were moderately correlated to xylose release (*r=-0.48*) when using NaOH pretreatment, but showed strong correlations to all other saccharification characters (*r=-0.71* to *-0.85*). Hence the lignin estimates obtained using this method had the highest predictive value for sample saccharification efficiency. Furthermore, the down-scaled Klason method can provide a large advantage over the unmodified Klason protocol, as it allows for more high-throughput analysis. This is especially true when additional modifications – such as disposable glass filters - are employed as proposed by Ibáñez and Bauer (Ibáñez & Bauer, 2014). The modified Klason method furthermore has the additional advantage that it provides a reaction hydrolysate in which acid soluble lignin and neutral sugars can be determined. The latter is a considerable advantage as neutral sugar determination is essential to be able to express the release of fermentable sugars as a percentage of total available cell wall glucose and xylose. This method thus provides a complete analysis of biomass quality.

4. Conclusions

Large differences were observed between the various lignin methods. Despite that different lignin estimates were generally highly correlated to each other, they exhibited different correlation patterns to saccharification efficiency characters. The largest overall impact on saccharification yields was found for ds-KL. This modification of the KL method furthermore showed a large potential to discriminate differences among accessions and resulted in a reduction of time, labor and technical error compared to the reference method. As it also allows for the parallel determination of neutral sugars and ASL it provides a complete analysis of biomass quality.

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Supplementary data

Supplementary Table S1. Accession, species and propagation information of the six miscanthus accessions used in this study.

Accession	Species	Plants	Cultivar
OPM-2	*M. sacchariflorus*	In vitro	-
OPM-4	*M. sacchariflorus*	In vitro	*Robustus*
OPM-5	Hybrid	In vitro	-
OPM-9	Hybrid	In vitro	*M. x giganteus*
OPM-11	*M. sinensis*	In vitro	*Goliath*
OPM-13	*M. sinensis*	Seed	-

Supplementary Table S2. Abbreviations and descriptions of sample characteristics analyzed in this study

Characteristic	**Unit**	**Description**
NDF	% DM	Neutral detergent fiber content in stem dry matter
ADF	% DM	Acid detergent fiber content in stem dry matter
CW	% DM	Stem cell wall content; is equal to NDF
Cel	% DM	Stem cellulose content; is equal to the difference between ADF and ADL
Hem	% DM	Stem hemicellulosic polysaccharides content; is equal to the difference between NDF and ADF
ADL	% CW	Cell wall acid detergent lignin content
ADLseq	% CW	Cell wall acid detergent lignin content determined on sample subjected to sequential NDF and ADF extractions
ABL	% CW	Cell wall acetyl bromide lignin content
KL	% CW	Cell wall Klason lignin content
ds-KL	% CW	Cell wall Klason lignin content determined using 10x down-scaled Klason method
ASL	% CW	Cell wall acid soluble lignin content
Glu	% CW	Cell wall glucose content
Xyl	% CW	Cell wall xylose content
Ara	% CW	Cell wall arabinose content
DHS	%	Ratio of arabinose to xylose
Glucose Release	%	Percentage of total cell wall glucose released after pretreatment and enzymatic saccharification
Xylose Release	%	Percentage of total cell wall xylose released after pretreatment and enzymatic saccharification

Chapter 4

Evaluation of *Miscanthus sinensis* biomass quality as feedstock for conversion into different bioenergy products

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Abstract

Miscanthus is a promising fiber crop with high potential for sustainable biomass production for a biobased economy. The effect of biomass composition on the processing efficiency of miscanthus biomass for different biorefinery value chains was evaluated, including combustion, anaerobic digestion and enzymatic saccharification for the production of bioethanol. Biomass quality and composition was analyzed in detail using stem and leaf fractions of summer (July) and winter (March) harvested biomass of eight compositionally diverse *Miscanthus sinensis* genotypes. Genotype performance in tests for enzymatic saccharification, anaerobic digestion and combustion differed extensively. The variation between the best and the worst performing genotype was 18% for biogas yield (ml/g dm) and 42% for saccharification efficiency (glucose release as %dm). The ash content of the best performing genotype was 62% lower than that of the genotype with the highest ash content and showed a considerably high ash melting temperature during combustion. Variation between genotypes in biomass quality for the different thermochemical bioconversion processes was shown to be strongly correlated to differences in biomass composition. The most important traits that contributed favorably to biogas yields and saccharification efficiency were a high content of *trans*-ferulic acid, a high ratio of *para*-coumaric acid to lignin and a low lignin content. Additionally, a high content of hemicellulosic polysaccharides positively affected saccharification efficiency. Low contents of ash and inorganic elements positively affect biomass quality for combustion and low potassium and chloride contents contributed to a higher ash melting temperature. These results demonstrate the potential for optimizing and exploiting *M. sinensis* as a multi-purpose lignocellulosic feedstock, particularly for bioenergy applications.

1. Introduction

Miscanthus is a promising fiber crop with high potential for sustainable biomass production in temperate climates (Heaton *et al.*, 2010). It is a perennial C4 grass characterized by high annual biomass yields and a high resource-use efficiency (Heaton *et al.*, 2004, Heaton *et al.*, 2008, Lewandowski *et al.*, 2003b, Long *et al.*, 2001, van der Weijde *et al.*, 2013). Given its potential as a high yielding, low-input lignocellulosic feedstock, there is growing interest in the use of miscanthus biomass for a plethora of applications, in particular the production of bioenergy and biofuels (Brosse *et al.*, 2012). Applications of lignocellulosic biomass are manifold and three important bioenergy conversion routes include direct combustion, anaerobic digestion to produce biomethane and enzymatic saccharification and fermentation to produce bioethanol. The chemical composition and structure of cell walls play an important role in biomass quality for each of the aforementioned processes. Therefore, optimization of chemical composition and physical structure are envisioned to improve the process efficiency, which will subsequently contribute to the feasibility and economic success of bioenergy conversion technologies (Torres *et al.*, 2016, Wyman, 2007).

There are different options to optimize and improve the biomass quality to facilitate the respective thermochemical conversion processes. Improved biomass quality can be achieved through breeding for quality traits. Currently, biomass quality is an important breeding objective in bioenergy crops such as miscanthus (Hodgson *et al.*, 2010, van der Weijde *et al.*, 2013). Another option is to improve biomass quality through 'on field quality management practices' such as fertilization and harvest time (Iqbal & Lewandowski, 2014, Lewandowski *et al.*, 2003a, Lewandowski & Kicherer, 1997, Lewandowski & Heinz, 2003). However, intrinsic differences between the distinct conversion routes result in route-specific requirements on biomass quality, either because they target other plant components or use another process to convert them into products. Lignin, for example, negatively affects the efficiency of biological conversion routes, such as fermentation or anaerobic digestion (Jørgensen *et al.*, 2007, Wyman, 2007, Zhao *et al.*, 2012), but has a favorable influence on the heating value of biomass for direct combustion (Lewandowski & Kicherer, 1997). Furthermore, for most bioconversion processes the route-specific biomass quality requirements are not yet clearly defined due to a number of reasons. First of all, most bioconversion processes are not yet mature technologies and still need to be optimized. A second reason is that we do not yet fully understand the complex structure of lignocellulose and how it affects different bioconversion processes. The final reason is that biomass recalcitrance factors have evolved over a long time to protect the plant against environmental threats and we are now challenged to find ways to manipulate biomass recalcitrance without adversely affecting plant performance (Himmel *et al.*, 2007, Zhao *et al.*, 2012).

Improving biomass quality in miscanthus through plant breeding is plausible, as the genus *Miscanthus* harbors extensive genetic diversity that may be exploited for the development

of new varieties (e.g. Clifton-Brown *et al.*, 2008, Heaton *et al.*, 2010). *M. sinensis* is one of the most promising species of miscanthus for biomass production in different environments, as it naturally occurs over a large geographical range in terms of latitude, longitude and altitude (Clifton-Brown *et al.*, 2008, Farrell *et al.*, 2006, Lewandowski *et al.*, 2000). Moreover, extensive variation in cell wall composition has been reported in *M. sinensis*, with cellulose content ranging from ~26 – 47%, hemicellulose content from ~25 – 43% and lignin content from ~5 – 15% of dry matter (Allison *et al.*, 2011, Qin *et al.*, 2012, Zhao *et al.*, 2014). Due to this extensive variation, the development of varieties with optimized biomass quality seems to be promising for various bioconversion routes.

The aim of this study was to understand how variation in lignocellulose composition affects the efficiency of different bioconversion processes and to explore the potential of miscanthus as a multi-purpose crop that can be bred for a variety of different biobased applications. In this study a diverse set of eight *M. sinensis* genotypes was selected from the miscanthus breeding program of Wageningen University and evaluated to gain insight in their potential for different biobased applications, including combustion, anaerobic digestion and enzymatic saccharification for ethanol production. The chemical composition of the stem and leaf fractions of biomass of these genotypes harvested in summer and winter was investigated to get insight in the effects of harvest time and genotype on traits considered to be relevant to the different bioenergy conversion technologies. The aim was to demonstrate the potential options for the use of miscanthus biomass as a feedstock for generating different types of bioenergy and to further define the selection criteria that will allow breeders to develop new varieties that are compositionally tailored to different value chains.

2. Materials and methods

2.1. Plant materials

Eight *M. sinensis* genotypes with a diverse cell wall composition profile were selected from the miscanthus breeding program of Wageningen University and used to establish a replicated field trial on a sandy soil in Wageningen, The Netherlands, in June 2013. A more detailed description and background information of the evaluated genotypes is given in supplementary table S1. The genotypes were propagated *in vitro* to generate enough plantlets for setting up the trial. The trial was managed without irrigation, fertilization, pest or weed control. The field trial had a design with four randomized blocks of eight plots. Plots had a size of 9 m^2 and contained 16 plants. All plots were surrounded by two rows with medium-sized *M. sinensis* plants to minimize possible border effects. Plant spacing between and within rows was 75 cm. In the establishment year the trial was harvested (March 2014), but no samples were taken for analysis. After the establishment year, two different harvest regimes were imposed on the trial: two of the four blocks were randomly assigned to be

subjected to a double-cut harvest regime and the other two blocks were subjected to a single-cut harvest regime. The single-cut harvesting regime involved a cut in March 2015, referred to as 'winter cut'. The double-cut harvesting regime involved a green cut in mid-July 2014, referred to as 'summer cut' and a harvest of the regrowth in March 2015, referred to as 'regrowth cut', which coincided with the winter cut of the single-cut harvesting regime. At the time of the summer cut, genotypes OPM-42, 49 and 87 were already flowering, whereas the other genotypes were still in the vegetative phase.

For each of the 3 cuts, the leaf, stem and total dry matter yield were determined per plot after chopping the samples into ~2 cm chips and subsequent air drying at 60°C for 72 hours in a forced-air oven. The leaf fraction consisted only of leaf blades, with leaf sheets remaining in the stem fraction. The samples from the summer and the winter cut were subsequently used for laboratory analysis: the separated leaf and stem fractions were used for biochemical analysis of the different cell wall components in both tissues, while a subsample in which stems and leaves were kept together was used for biomass quality assessment, including analyses of biogas yield, saccharification efficiency and combustion quality. All samples were ground using a hammer mill with a 1-mm screen.

2.2. Cell wall polymer composition

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin contents (ADL) of stem dry matter were determined according to protocols developed by Ankom Technology (ANKOM Technology Corporation, Fairpoint, NY), which are essentially based on the work of Van Soest and coworkers (Goering & Van Soest, 1970, Van Soest, 1967). Neutral and acid detergent extractions were performed using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY, USA). Acid detergent lignin was determined after 3-hour hydrolysis of the ADF residue in 72% H_2SO_4 with continuous shaking. All fiber analyses were performed in triplicate. The weight fractions of detergent fiber residues in dry matter were subsequently used as estimate for the content of cell wall in dry matter (NDF%dm) and to obtain the contents of cellulose ([ADF%dm-ADL%dm]/NDF%dm × 100%), hemicellulosic polysaccharides ([NDF%dm-ADF%dm]/NDF%dm × 100%) and lignin (ADL%dm/NDF%dm × 100%) relative to the cell wall content.

2.3. Cell wall monosaccharide composition

The residual NDF material of the replicated fiber analyses was pooled per sample and used as a basis for determination of neutral sugar contents as described previously by van der Weijde *et al*. (2016). Measurements were performed in 3 replications. Briefly, 30 mg of NDF material was hydrolyzed in 0.3 ml 72% H_2SO_4 in a 10 ml glass pressure tube for one hour at 30°C with constant shaking (160 rpm). After one hour the acid concentration was diluted to 4% by adding 8.4 ml deionized water, after which samples were hydrolyzed for three hours

in a heating block set at 100°C with a rotation speed of 160 rpm. After cooling down, the samples were centrifuged and a subsample of the supernatant was purified using a 0.45 µm filter. Contents of glucose (Glu), xylose (Xyl), arabinose (Ara) and galactose (Gal) in the purified supernatant were determined by high performance anion exchange chromatography (HPAEC) analysis on a Dionex system equipped with a CarboPac PA1 column and a pulsed amperometric detector (Dionex, Sunnydale, CA). The degree of hemicellulose substitution (DHS) is the weight ratio of arabinose to xylose expressed as a percentage.

Monosaccharide acetylation

The amount of acetyl-groups on monosaccharides was estimated by quantifying acetic acid in the undiluted, purified neutral sugar hydrolysate using an acetate dehydrogenase assay kit (Megazyme International Ireland Ltd., Bray, Ireland) adapted to a 96-wells microplate format. The increase in sample absorbance at 340 nm following enzymatic dehydrogenase reactions was quantified using a Bio-Rad Microplate reader (Bio-Rad, Richmond, CA, USA).Acetic acid concentration in the sample was calculated from the increase in sample absorbance by interpolation from a six point standard curve of acetic acid (Megazyme International Ireland Ltd., Bray, Ireland). The degree of hemicellulose acetylation (DHA) is the dry weight of acetic acid expressed as a percentage of the dry weight of xylose on a sample basis.

Hydroxycinnamic acids

Hydroxycinnamic acids, specifically *p*-coumaric acid (*p*CA) and *trans*-ferulic acid (TFA) were quantified after extraction as described previously (Buanafina *et al.*, 2006). Briefly, an Eppendorf tube was filled with 10 mg NDF material of the samples and for the reference tests with 10 mg cellulose (Cellulose type 101, Sigma-Aldrich, Diegem, Belgium). The latter are also spiked with 100 µg TFA (Sigma-Aldrich, Diegem, Belgium) and *p*CA (Sigma-Aldrich, Diegem, Belgium). The tubes were subsequently incubated overnight in 750 µl 2M NaOH at 25°C and under constant shaking. Trimethoxycinnamic acid (TMCA, Sigma-Aldrich, Diegem, Belgium) was added as internal standard and the pH of all samples was adjusted to 2 with HCL. A liquid-liquid extraction with diethyl ether was performed twice, after which the residue was dried for 1 hour at 40 °C and resuspended in 1 ml 5% acetonitrile (MeCN) and vortexed for 15 sec. Subsequently samples were 10 times diluted with 5% MeCN and stored at -20°C before analysis. For each sample 10 µl was injected into a liquid chromatography-high resolution mass spectrometry (LC-HRMS) system. Chromatographic separation was performed with an Acquity Ultra Performance system (Waters, Milford, MA, USA) using a Waters BEH Shield C18 column (2.1 x 150 mm, 1.7 µm) held at 40°C and equipped with an Shield C18 VanGuard pre-column (Waters, Milford, MA, USA). The mobile phase consisted of H_2O + 0.1% TFA (solvent A) and MeCN + 0.1% TFA (solvent B) at a flow rate of 0.35 ml/min. Gradient separation was performed as follows: linear increase from 5% to 50% B in 30 min, subsequent linear increase to 100% B in 1 min, held at 100% B for 6 min, followed by immediate decrease to 5% B and

finally re-equilibration at 5% B for 5 min. Mass spectrometric detection and quantification were performed using a Synapt G2-S high resolution mass spectrometer (Waters, Milford, MA, USA) acquiring full scan HRMS data (50 to 1,200 Da) in resolution mode negative (20,000 FWHM). Source temperature and desolvation temperature were set 120 °C and 500 °C, respectively. Prior to analysis, the HRMS was calibrated (50-1,200 Da) using a sodium formate solution. During analysis, leucine-enkephalin (200 pg/µl) was constantly infused as lock mass. Data were analysed using the MassLynx software version 4.1 (Waters, Milford, MA, USA). The ratio of *p*CA to ADL (*p*CA/ADL) was calculated by expressing the dry weight of *p*CA as a percentage of the dry weight of ADL on a sample basis. Similarly, the ratio of TFA to xylose (DHF, for degree of hemicellulose feruloylation) was calculated by expressing the dry weight of TFA as a percentage of the dry weight of xylose on a sample basis.

2.4. Contents of ash and inorganic elements

Dried biomass samples were analyzed in the laboratory for N, Na, K, Ca, Mg, P, Cl, Si, and ash content. Analysis of N was carried out following the Dumus principle using a Vario Macro cube (Elementaranalysensysteme GmbH, Hanau, Germany). For determination of Na, K and Ca, 500 mg dried biomass samples were dissolved in 8 ml HNO_3 (65%), to which 4 ml H_2O_2 was added to remove color. Samples were then digested in a microwave (Ethos.Lab, MLS GmbH, Leutkirch, Germany) at 120-180ºC and a pressure of 24.16 bar for 40 minutes. Digested samples were then filtered through Whatman filter paper and contents of P, K, Mg, Ca and Na in the extracts were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES, Vista Pro, Varian Inc., Palo Alto, California, US). For determination of Cl, extractions were performed with hot deionized water and treated with a clarifying agent (Carrez I, containing 15 g $K_4Fe(CN)_6.3H_2O$ in 100 ml deionized water and Carrez II, containing 30 g $Zn(CH_6COO)_2.2H_20$ in 100 ml deionized water). The extracts were measured by ion chromatography (ICS 2000, Dionex Corporation, Sunnyvale, CA, USA). For the determination of Si content, samples were digested with HNO_3 and HF and measured with help of using ICP-OES (Vista Pro, Varian, Palo Alto, CA, USA). Ash content was quantified gravimetrically after 4 hour incineration in an electric muffle furnace at 550 °C.

Table 1. Ash fusion classes and ash fusion temperature along with microscopic observations (source: Tonn et al., 2012).

Ash-fusion classes	Microscopic observations
1) Loosening	Particles are arranged in loose layers, spatula can move through without any resistance, shiny surfaces with tiny molten vesicles
2) Partially sintered	Particles start becoming compact through strong adhesive forces, still easy to disintegrate, produces crispy sound when spatula passes through, larger molten vesicles on the surface
3) Highly sintered	Difficult to disintegrate, most of the area covered with larger molten vesicles. Organogenic material also visible in some parts
4) Molten	Particles are completely molten, manual disintegration is not possible, no organogenic material visible

2.5. Ash melting behavior during combustion

To assess the ash melting behavior during combustion process, the method was adopted from Tonn *et al*., (2012). Briefly, 100 mg ash samples were transferred to ceramic combustion boats (Lab Logistics group GmbH, Meckenheim, Germany) and subjected to four different combustion temperature treatments (800 °C, 900 °C, 1000 °C and 1100 °C) for two hours in an electric muffle furnace. The electric muffle furnace was heated at an average rate of 10 °C min^{-1} until the required heating temperature was achieved. After two hours, the combustion boats were transferred into an exicator to allow them to cool down before microscopic analysis. Each sample was analyzed under a stereo microscope (Zeiss Stemi 2000-C, Carl Zeiss AG, Oberkochen, Germany) at magnifications up to 40 × and classified into one out of four ash-fusion classes (AFC) (Table 1) as described by Tonn *et al.* (2012).

2.6. Biogas yield upon anaerobic digestion

The substrate-specific biogas (SSBY) and methane yields (SSMY) were measured in a biogas batch test under mesophilic conditions at 39°C according to VDI guideline 4630. The biogas batch method was described by Kiesel & Lewandowski (2016) and certified after KTBL and VDLUFA inter-laboratory comparison test 2014. The fermentation period was 35 days. Four replicates of each sample were analyzed. A maize standard was analyzed alongside the miscanthus samples to monitor the activity of the inoculum. The inoculum originated from the fermenter of a commercial mesophilic biogas plant which uses the following substrates: maize silage, grass silage, cereal whole crop silage, liquid and solid cattle manure and small quantities of horse manure. The inoculum was sieved and diluted to 4% (w/w) dry matter with deionized water. Various macro- and micronutrients were added as described by Angelidaki *et al.* (2009). Afterwards the inoculum was incubated at 39 °C under anaerobic conditions for 6 days.

For the biogas batch analysis, 200 mg miscanthus samples were transferred into a 100 ml fermentation flask, 30 g inoculum was added and the gas-containing headspace was flushed with nitrogen to attain anaerobic conditions. The fermentation flasks were closed gastight by a butyl rubber stopper and an aluminium cap. The pressure increase in the fermentation flasks was measured by puncturing the butyl rubber stopper with a cannula attached to a HND-P pressure meter (Kobold Messring GmbH, Hofheim, Germany). The biogas production was calculated as dry gas (water vapor pressure was considered) from the pressure increase and was standardized to 0 °C and 1013 hPa using equations (1) and (2). Equation (1) was used for the first measurement and considers the pressure increase due to warming from laboratory temperature to 39 °C and the water vapor partial pressure. Equation (2) was used for the subsequent 17 measurements, which were taken on regular basis.

$$V_{biogas} = V_{HS} * T_S / T_F * \left(\left(P_{A1} + P_{F1} \right) - \left(P_{A0} * T_F / T_{Lab} \right) - P_{WP} \right) / P_S \quad (1)$$

where V_{biogas} = volume of produced biogas, V_{HS} = volume gas containing headspace, T_S = standard temperature of 273.15 ^{0}K (= 0°C), T_F = fermentation temperature of 312.15 ^{0}K (= 39 C), P_{A1} = ambient pressure at first measurement, P_{F1} = overpressure in fermentation flasks at first measurement, P_{A0} = ambient pressure at sealing of the fermentation flasks (batch test start), T_{Lab} = laboratory temperature at sealing of the fermentation flasks (batch test start), P_{WP} = water vapor partial pressure at 39 C, P_S = standard pressure (1013 hPa)

$$V_{biogas} = V_{HS} * T_S / T_F * \left(\left(P_{An} + P_{Fn} \right) - \left(P_{A(n-1)} + P_{F(n-1)} \right) \right) / P_S \quad (2)$$

where P_{An} = ambient pressure at the actual measurement, P_{Fn} = overpressure in fermentation flask at the actual measurement, $P_{A(n-1)}$ = ambient pressure at the previous time-point, $P_{F(n-1)}$ = overpressure in the fermentation flasks at the previous time-point.

During the course of the biogas batch test it was occasionally necessary to remove the produced biogas from the fermentation flasks. The overpressure in the fermentation flasks was removed using a gastight syringe once it had reached an approximate value of 500 mbar. The biogas was transferred to a gastight storage flask where it was kept until the end of the batch test. After each gas collection, the remaining overpressure in the fermentation flasks was allowed to level off to ambient pressure by injecting a blank cannula. For the subsequent measurement, $P_{F(n-1)}$ was then set to zero in equation (2). At the end of the batch test, the remaining biogas in the headspace of the fermentation flasks was removed by active extraction with a syringe and also transferred into the storage flask. An aliquot of the collected biogas was used for analyzing the methane content using gas chromatography (GC-2014, Shimadzu, Kyoto, Japan). The gas chromatograph was equipped with a thermal conductivity detector and the detection temperature was set to 120 °C. Two columns (HayeSep and Molsieve) were used for separation, with system temperature set at 50 °C and argon as carrier gas. The gas samples were injected using a Combi-xt PAL auto-sampler (CTC Analytics AG, Zwingen, Germany).

2.7. Saccharification efficiency for bioethanol production

Saccharification reactions were carried out as described previously by van der Weijde *et al.*, (2016). Briefly, 500 mg biomass samples were briefly treated with α-amylase and repeatedly washed with deionized water (3x, 5 minutes, ~60°C) in order to remove all interfering soluble sugars. Remaining biomass was subjected to alkaline pretreatment with 15 ml 2% NaOH at 50°C and constant shaking (160 rpm) for 2 hours in a shaker incubator (Innova 42, New Brunswick Scientific, Enfield, CT, USA). Pretreated samples were then washed to neutral pH with deionized water (2x, 5 minutes, 50°C) and with 0.1 M sodium citrate buffer (pH 4.6, 5 minutes, 50°C).

Saccharification reactions were subsequently carried out according to the NREL Laboratory Analytical Procedure “Enzymatic saccharification of lignocellulosic biomass” (Selig *et al.*,

2008). Pretreated samples were hydrolyzed for 48 hours with 300 µl of the commercial enzyme cocktail Accellerase 1500 (DuPont Industrial Biosciences, Leiden, The Netherlands) supplemented with 15 µl endo-1,4-β-xylanase M1 (Megazyme, Bray, IE, USA) in a shaker incubator (Innova 42, New Brunswick Scientific, Enfield, CT, USA) set at 50°C and constant shaking (160 RPM). These enzymes combined have the following specific activities: endoglucanase 2200-2800 CMC U/g, beta-glucosidase 450-775 pNPG U/g and endoxylanase 230 U/mg. Reactions were carried out in 44 ml 0.1 M sodium citrate buffer (pH 4.6), containing 0.4 ml 2% sodium azide to prevent microbial contamination.

Enzymatic saccharification liquors were analyzed for glucose and xylose content by HPAEC as described previously for neutral sugars. The potential of a genotype for bioethanol production was assessed by expressing the total fermentable sugar yield in two ways. The first is the absolute yield of glucose and xylose as a percentage of dry matter (glucose release %dm and xylose release %dm). The second way is to express the yield of glucose and xylose as a percentage of the respective total available cell wall glucan (glucose conversion %) and xylan (xylose conversion %), as measures of saccharification efficiency.

2.8. Statistical analyses

General analyses of variance (ANOVA) were performed to determine the significance of genotype differences (p<0.05) in compositional traits and quantitative route-specific quality parameters. Friedman's non-parametric ANOVA was performed to determine the significance of genotype differences in ash fusion classes. Variance analyses were performed following the standard procedure of a mixed effect model with a random genetic effect and a fixed block effect, following the model (3):

$$Y_{ij} = \mu + G_{ij} + B_j + e_{ij} \quad (3)$$

where Y_{ij} is the response variable, µ is the grand mean, G_{ij} is the genotype effect, B_j is the block effect and e_{ij} is the residual error.

Correlation analyses were performed to identify the significance, strength and direction of interrelationships between traits using Pearson's correlation coefficients. Multiple linear regression analyses were performed for the development of simple regression equations for biogas yield and saccharification efficiency. All statistical analyses were performed using Genstat for Windows, 18th edition software package (VSN International, Hemel Hempstead, UK).

3. Results

3.1. Large differences in field performance between genotypes and harvest regimes

The field performance of the eight miscanthus genotypes was evaluated by assessing dry stem, leaf and total biomass yields of the genotypes from a single- and a double-cut harvest regime (Table 2). Biomass yields from the double-cut harvest regime were significantly lower than from the single-cut harvest regime. Averaged over all genotypes the summer cut yielded 1803 kg dm ha^{-1}, the regrowth cut yielded an additional 630 kg dm ha^{-1}. The winter cut, however, yielded on average 6314 kg dm ha^{-1}. The highest yielding genotype (OPM-69) in the winter cut had an average total biomass yield as high as 10583 kg dm ha^{-1}. Furthermore, roughly 60% of the summer cut and roughly 45% of the regrowth cut consisted of stem material, whereas the biomass of the winter cut consisted almost completely of stem material. The genotypic variation for dry biomass yield and stem fraction of total yield, respectively, as realized during the first whole growing season was extensive (Table 2).

3.2. Genotypes show highly diverse cell wall composition

The summer cut and winter cut biomass samples of the eight miscanthus genotypes were analyzed biochemically (Tables 3 and 4, respectively). The tables show the mean performance of each genotype for a wide set of stem biomass and cell wall traits, such as the content, chemical composition and structural complexity of various cell wall polymers. Significant differences between genotypes were found for nearly all cell wall components. Stem samples of the winter cut were analyzed in most detail, as they represent the largest weight fraction of all the harvested biomass.

In the summer cut, approximately 82% of the stem dry matter consisted of cell wall material, which increased to approximately 92% in the winter cut (Tables 3 and 4). In the winter cut very little variation in stem cell wall content existed and genotypes were not found to be significantly different from each other (Table 4). The composition of the cell wall material also differed markedly between the summer and winter cut samples, with the summer cut samples generally being lower in cellulose and lignin contents, but higher in contents of hemicelluloses. In both cuts particularly large genotypic variation was found for Hem and ADL. Hemicellulose content in stem cell walls varied amongst genotypes ~31 to 41% in the summer cut and from ~29 to 37% in the winter cut. For stem cell wall lignin content variation amongst genotypes ranged from ~6 to 10% in the summer cut and from ~8 to 13% in the winter cut (Tables 3 and 4).

In both cuts also large variation was found in the neutral sugar composition of the stem cell wall material, particularly for arabinose and galactose, which are sugars that are present in side chains on the xylan backbone of grass hemicelluloses (Tables 3 and 4). For the winter

Table 2. Means of a diverse set of eight M. sinensis accessions for total dry matter yield and the weight distribution of total dry matter among stem and leaf fractions evaluated in a single cut and double cut harvest regime following the first complete growing season.

			Accession										
Harvest regime	Trait	Unit	OPM 42	OPM 48	OPM 49	OPM 65	OPM 69	OPM 73	OPM 77	OPM 87	Average	Range	F-prob.
Double-cut	Yield	kg dm ha^{-1}	747	490	423	614	994	1099	661	568	700	675	<.001
(Summer cut)	Stem	%	60	53	63	52	70	66	58	58	60	18	<.001
	Leaf	%	40	47	37	48	30	34	42	42	40	18	<.001
Double-cut	Yield	kg dm ha^{-1}	1866	2649	1102	1664	2206	2427	1873	2329	2015	1548	0.006
(Regrowth cut)	Stem	%	44	54	56	43	46	55	70	44	52	27	0.022
	Leaf	%	56	46	44	57	54	45	30	56	48	27	0.022
Single-cut	Yield	kg dm ha^{-1}	5788	5948	4975	5422	11759	7925	7494	6809	7015	6783	0.005
(Winter cut)	Stem	%	93	96	92	97	94	91	94	88	93	9	0.056
	Leaf	%	7	4	8	3	6	9	6	12	7	9	0.056

Table 3. Means of a diverse set of eight M. sinensis genotypes for stem biomass and cell wall components of the summer cut

					Genotype						
Trait	Unit	OPM 42	OPM 48	OPM 49	OPM 65	OPM 69	OPM 73	OPM 77	OPM 87	Average	Range	F-prob.
NDF	%dm	83.92	83.10	80.81	80.00	80.33	82.06	83.54	81.47	81.90	3.91	0.005
ADF	%dm	49.22	50.73	48.44	47.51	55.39	51.34	50.72	48.76	50.26	7.88	0.003
CEL	%cw	51.11	54.85	53.51	53.40	58.84	55.63	53.82	52.45	54.20	7.73	<.001
HEM	%cw	41.34	38.95	40.06	40.63	31.04	37.43	39.29	40.14	38.61	10.31	<.001
ADL	%cw	7.55	6.20	6.42	5.97	10.12	6.94	6.89	7.41	7.19	4.15	0.003
Glu	%cw	51.82	52.29	53.93	53.00	53.37	55.26	51.69	51.33	52.84	3.93	0.195
Xyl	%cw	32.02	31.30	33.07	30.84	27.54	30.24	31.62	30.89	30.94	5.53	0.005
Ara	%cw	3.43	3.52	3.78	3.36	2.57	2.73	3.44	3.28	3.26	1.21	0.002
Gal	%cw	0.38	0.27	0.33	0.31	0.24	0.20	0.37	0.23	0.29	0.19	<.001

* NDF=neutral detergent fiber, ADF=Acid detergent fiber, Cel=Cellulose, Hem=Hemicellulose, ADL=Acid detergent lignin, Glu=Glucose, Xyl=Xylose, Ara=Arabinose.

Table 4. Means of a diverse set of eight M. sinensis genotypes for stem biomass and cell wall components of the winter cut

		Genotype										
Trait	Unit	OPM 42	OPM 48	OPM 49	OPM 65	OPM 69	OPM 73	OPM 77	OPM 87	Average	Range	F-prob.
NDF	% dm	92.01	90.51	90.91	91.56	93.12	91.18	90.83	91.77	91.49	2.62	0.193
ADF	% dm	58.39	58.61	59.20	57.68	66.55	61.75	58.83	59.39	60.05	8.87	0.002
CEL	% cw	53.99	55.71	56.44	55.03	58.31	58.87	55.12	54.35	55.98	4.88	0.006
HEM	% cw	36.53	35.24	34.88	37.01	28.54	32.28	35.23	35.29	34.38	8.47	<.001
ADL	% cw	9.47	9.05	8.68	7.96	13.15	8.86	9.65	10.36	9.65	5.19	<.001
PCA	µg/mg cw	14.98	16.48	16.16	15.45	15.15	13.75	15.96	15.51	15.43	2.74	<.001
PCA/ADL	% ADL	15.81	18.21	18.62	19.40	11.53	15.52	16.56	14.97	16.33	7.87	<.001
Glu	% cw	46.20	46.33	45.32	45.30	48.30	49.09	45.03	46.65	46.53	4.07	0.040
Xyl	% cw	32.06	30.66	30.49	31.13	26.28	30.64	31.35	31.42	30.50	5.77	<.001
Ara	% cw	3.18	3.21	3.08	3.00	2.41	2.44	3.11	2.81	2.91	0.80	0.001
Gal	% cw	0.38	0.25	0.26	0.28	0.22	0.16	0.34	0.23	0.26	0.22	<.001
TFA	µg/mg cw	4.97	5.60	5.98	5.68	3.70	5.03	5.65	4.98	5.20	2.28	<.001
Acetic acid	µg / mg cw	0.28	0.27	0.26	0.25	0.25	0.26	0.25	0.28	0.26	0.03	0.069
DHS	% Xyl	9.94	10.46	10.10	9.65	9.18	7.98	9.93	8.94	9.52	2.48	0.016
DHA	% Xyl	0.09	0.09	0.09	0.08	0.10	0.08	0.08	0.09	0.09	0.02	0.062
DHF	% Xyl	1.43	1.65	1.78	1.67	1.31	1.50	1.64	1.45	1.55	0.47	<.001

NDF=neutral detergent fiber, ADF=Acid detergent fiber, Cel=Cellulose, Hem=Hemicellulose, ADL=Acid detergent lignin, PCA=para-coumaric acid, Glu=Glucose, Xyl=Xylose, Ara=Arabinose, TFA=trans-ferulic acid, DHS=Degree of hemicellulose substitution (ratio of arabinose to xylose), DHA=Degree of hemicellulose acetylation (ratio of acetic acid to xylose), DHF=Degree of hemicellulose feruloyation (ratio of TFA to xylose).

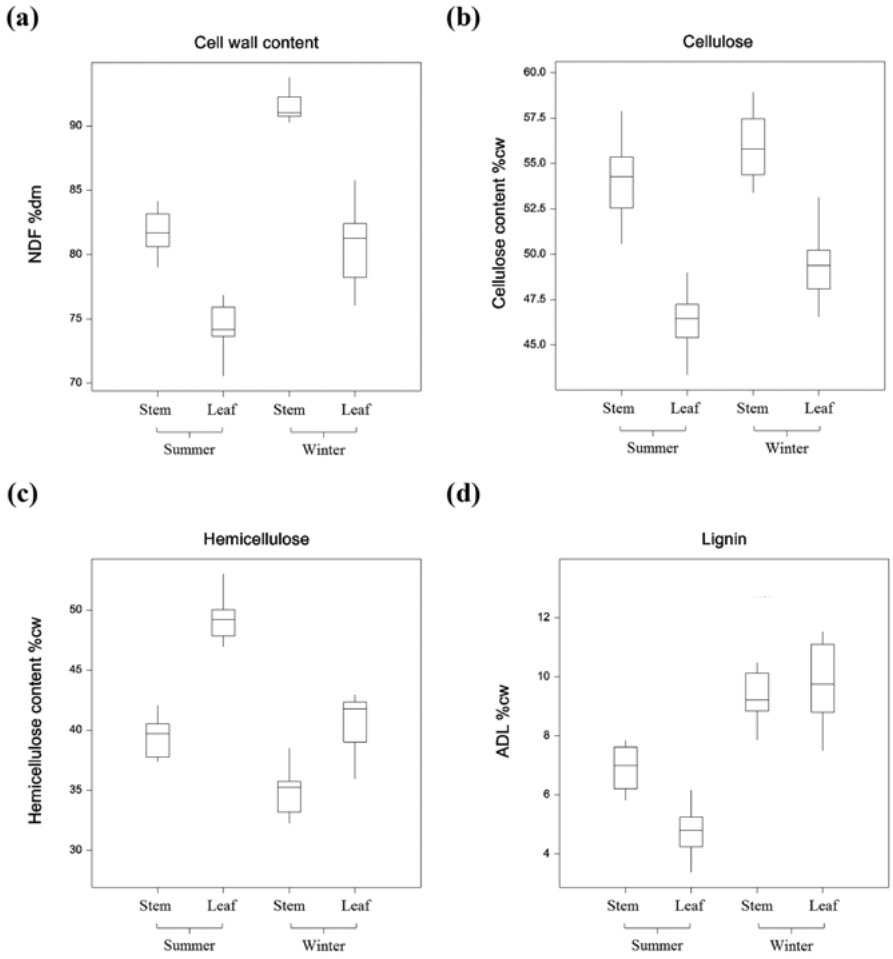
cut stem samples, additional measurements were done to investigate minor components of the cell wall matrix, such as hydroxycinnamic acids (TFA and pCA) and acetic acid. The ratios of arabinose to xylose (DHS), TFA to xylose (DHF), acetic acid to xylose (DHA) and pCA to ADL were investigated, as these provide indications of the complexity and level of substitutions/ side-groups on xylose and lignin residues. Significant genotypic differences were found for all these ratios, with the exception of DHA, indicating that genetic variation for these trait ratios is available in the species (Table 4).

Leaf samples of the summer and winter cuts were also analyzed biochemically and the results are summarized in boxplots that display the variation in cell wall, cellulose, hemicellulose and lignin contents, respectively (Figures 1a-d). Compared to the stem samples, leaf samples generally contained lower contents of NDF and cellulose, but higher contents of hemicellulosic polysaccharides. In the summer cut samples, stem tissues were higher in lignin content than leaf tissues, while in the winter cut samples the opposite trend was observed (Figure 1d).

3.3. Large variation in genotype performance in different bioenergy conversion processes

The potential as feedstock of eight *M. sinensis* genotypes for three different types of bioenergy conversion processes, i.e. anaerobic digestion, enzymatic saccharification and combustion, was evaluated in this study. Genotype means of specific quality characteristics relevant to the different types of bioenergy conversion route are presented in Tables 5 and 6, for biomass samples harvested in the summer and winter cut, respectively. Genotypes showed significant differences for many specific quality traits relating to the different bioenergy applications. Anaerobic digestion of samples from the summer cut resulted in higher biogas yields compared to biomass samples from the winter cut, with genotypes means for substrate specific biogas yields ranging from 539 to 591 ml/g dry matter for the summer cut and 441 to 520 ml/g dry matter for the winter cut. Methane content in the produced biogas was approximately 52%, regardless of the time of harvest. The highest biogas yields where achieved by OPM-65 in the summer cut and OPM-73 in the winter cut, while in both cuts OPM-69 consistently had the lowest biogas yields.

To assess the quality of the biomass samples from the summer and the winter cut for fermentation of structural sugars into bioethanol, the samples were pretreated and incubated with a commercial enzyme cocktail to study the yield and efficiency of the release of fermentable sugars. Significant differences amongst genotypes were found for glucose release and glucose conversion in both harvests and for xylose release in the green cut, but not for xylose conversion (Tables 5 and 6), despite large differences between genotypes in hemicellulose content (Tables 3 and 4). Similar to the results for biogas yield, higher sugar release and saccharification efficiency were found using the biomass samples of the summer cut compared to the samples of the winter cut. Variation amongst genotypes in glucose conversion was extremely large, especially for biomass samples from the winter cut, ranging from 33% (for OPM-69) to 50% (for OPM-65).



Figures 1a-d. Boxplots depicting variation in the cell wall content (a), cellulose (b), hemicellulose (c) and lignin (d) contents of miscanthus stem and leaf fractions of eight M. sinensis accessions harvested in a summer cut (July) or a winter cut (March).

For combustion quality, ash content is an important biomass quality determinant. The average ash content of the samples was 1.54% of dry matter in biomass from the winter cut compared to 3.28% of dry matter in biomass from the summer cut, when the plants had not yet senesced. As a result of the lower ash content the quality of the biomass samples for combustion was higher in the winter cut than in the summer cut. Significant differences between genotypes for ash content were only found for biomass samples from the summer cut. Genotypes also showed significant differences in the contents of silicon and potassium in the summer cut and chloride and potassium in the winter cut. Furthermore, microscopic observations of ash melting behavior at different combustion temperatures were performed to make a classification of the genotypes into different ash fusion classes. Although samples could be assigned to distinct classes at each of the different temperatures, the classification for none of the tested temperatures has proven to lead to significant differences amongst genotypes (Tables 5 and 6).

Table 5. Mean performance of biomass of eight M. sinensis genotypes from a summer cut for quality traits relevant for specific bioenergy conversion routes

	Genotype										
Route-specific quality characteristics	OPM 42	OPM 48	OPM 49	OPM 65	OPM 69	OPM 73	OPM 77	OPM 87	Average	Range	F-prob.
Anaerobic digestion											
SSBY (ml/g dm)*	562.90	572.87	575.40	591.78	538.84	572.20	561.01	560.33	566.92	52.94	<.001
SSMY (ml/g dm)*	290.30	296.50	296.64	305.34	278.19	293.57	288.39	290.37	292.41	27.15	<.001
Methane (% SSBY)	52.13	52.25	52.11	52.13	52.18	51.94	52.02	52.30	52.13	0.36	0.203
Relative quality rating¥	-	+	+	++	--	+	-	-			
Fermentation											
Glucose release(%dm)	23.70	24.17	26.25	25.84	25.64	24.96	25.55	25.43	25.19	2.55	0.018
Xylose release (%dm)	6.70	6.85	7.28	7.27	7.71	7.25	7.09	6.82	7.12	1.01	0.175
Glucose conversion (%)	62.96	64.82	66.62	67.59	64.92	64.17	67.95	66.38	65.68	4.99	0.014
Xylose conversion (%)	30.84	32.24	32.48	32.68	33.13	30.95	32.65	31.38	32.04	2.30	0.409
Relative quality rating¥	-	++	+	++	--	+	-	-			
Combustion											
Ash (%dm)	2.59	5.05	3.27	3.38	3.42	2.38	3.00	3.13	3.28	2.67	0.006
Silicon (Si) (%dm)	0.31	0.44	0.26	0.33	0.29	0.18	0.26	0.34	0.30	0.26	0.046
Chloride (Cl) (%dm)	0.14	0.24	0.18	0.15	0.19	0.12	0.16	0.14	0.16	0.12	0.065
Potassium (K) (%dm)	0.84	1.90	1.28	1.15	1.42	0.95	1.13	1.17	1.23	1.07	<.001
Calcium (Ca) (%dm)	0.18	0.21	0.15	0.16	0.09	0.28	0.18	0.18	0.18	0.19	0.670
AFC-800°C	1.00	3.00	3.00	1.00	1.75	1.00	1.50	1.75	1.75	2.00	0.066§
AFC-900°C	2.00	3.25	4.25	2.00	3.00	1.50	2.00	3.75	2.72	2.75	0.130§
AFC-1000°C	3.50	4.25	4.50	4.50	3.50	2.00	4.50	4.25	3.88	2.50	0.088§
AFC-1100°C	5.00	4.50	5.00	5.00	4.50	3.75	5.00	5.00	4.72	1.25	0.195§
Relative quality rating¥	+	--	-	-	-	++	+	+			

*SSBY = substrate-specific biogas yield, SSMY = substrate-specific methane yield

¥Rating based on ranking genotypes by SSBY for anaerobic digestion, by glucose yield for fermentation and by HHV for combustion route. Rank 1 scored '++', rank 2-4 scored '+', rank 5-7 scored '-' and rank 8 scored '--'.

§P-value using chi-square approximation resulting from Friedman's nonparametric ANOVA test.

Table 6. Mean performance of biomass of eight M. sinensis genotypes from a winter cut for quality traits relevant for specific bioenergy conversion routes

	Genotype										
Route-specific quality characteristics	OPM 42	OPM 48	OPM 49	OPM 65	OPM 69	OPM 73	OPM 77	OPM 87	Average	Range	F-prob.
Anaerobic digestion											
SSBY (ml/g dm)*	457.89	500.75	502.08	507.83	441.15	520.08	473.11	462.90	483.22	78.93	0.01
SSMY (ml/g dm)*	236.61	256.40	258.59	260.77	228.70	266.57	244.52	238.11	248.78	37.86	0.018
Methane (% SSBY)	52.27	51.94	52.13	52.02	52.39	51.95	52.27	52.12	52.14	0.46	0.013
Relative quality rating¥	-	+	+	+	--	++	-	-			
Fermentation											
Glucose release (%dm)	18.63	18.66	19.68	20.78	14.64	18.25	18.67	17.00	18.29	6.14	0.007
Xylose release (%dm)	8.41	7.74	7.88	8.25	5.78	7.37	8.12	7.66	7.65	2.62	0.005
Glucose conversion (%)	43.83	44.48	47.74	50.11	32.55	40.77	45.65	39.77	43.11	17.57	0.003
Xylose conversion (%)	29.29	28.59	29.12	29.62	24.35	27.22	29.31	27.44	28.12	5.27	0.077
Relative quality rating¥	+	-	+	++	--	-	+	-			
Combustion											
Ash (%dm)	1.67	1.77	1.54	1.09	1.64	1.45	1.62	1.56	1.54	0.68	0.358
Silicon (Si) (%dm)	0.37	0.35	0.36	0.30	0.36	0.34	0.35	0.37	0.35	0.07	0.993
Chloride (Cl) (%dm)	0.02	0.06	0.00	0.01	0.05	0.03	0.03	0.02	0.03	0.06	0.002
Potassium (K) (%dm)	0.07	0.28	0.06	0.04	0.18	0.10	0.11	0.04	0.11	0.24	0.001
Calcium (Ca) (%dm)	0.10	0.10	0.11	0.08	0.08	0.09	0.10	0.11	0.10	0.03	0.531
AFC-800°C	1.00	2.75	1.50	1.00	1.50	1.00	1.75	1.25	1.47	1.75	0.076§
AFC-900°C	1.50	3.25	1.50	1.25	2.00	1.75	2.00	1.50	1.84	2.00	0.076§
AFC-1000°C	1.75	4.25	2.00	2.00	2.25	2.25	2.50	2.25	2.41	2.50	0.254§
AFC-1100°C	2.25	5.00	2.50	2.25	2.50	2.75	3.00	2.75	2.88	2.75	0.277§
Relative quality rating¥	-	--	+	++	-	+	-	+			

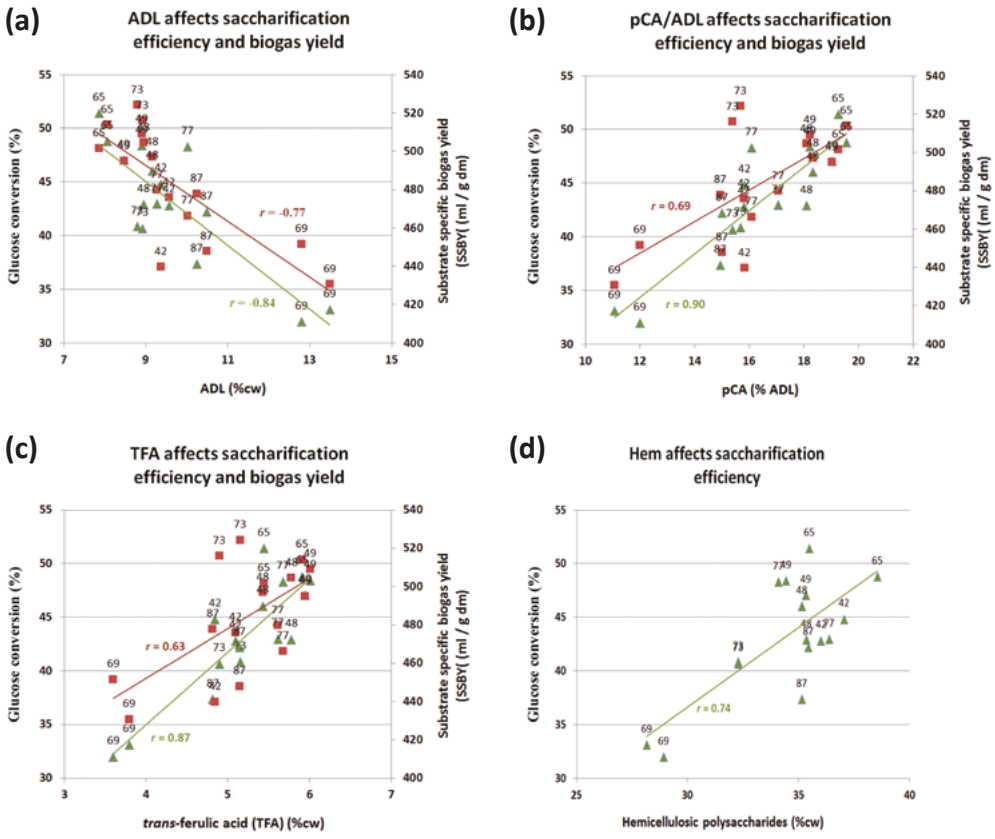
*SSBY = substrate-specific biogas yield, SSMY = substrate-specific methane yield

¥Rating based on ranking genotypes by SSBY for anaerobic digestion, by glucose yield for fermentation and by ash content for combustion route. Rank 1 scored '++', rank 2-4 scored '+', rank 5-7 scored '-' and rank 8 scored '--'.

§P-value using chi-square approximation resulting from Friedman's nonparametric ANOVA test.

Influence of biomass composition on genotype performance in different types of bioenergy conversion processes

The interrelations between compositional characteristics and specific quality traits for the different bioconversion processes were assessed using correlation analysis. Some of the most important correlations were highlighted in Figures 2a-d, while the full correlation matrix is presented in Figure 3. Similarities were found in the traits affecting the efficiency of enzymatic saccharification and anaerobic digestion. Both were negatively correlated to ADL, and positively correlated to *p*CA/ADL and TFA (Figures 2a-c). Additionally, both traits were negatively correlated to NDF and positively correlated to DHF (Figure 3). No significant correlations were found between biogas yield and saccharification efficiency, but a weak correlation was found between biogas yield and glucose release. Some cell wall compositional traits were not correlated to biogas yield, but did show correlations to the release and yield of glucose and xylose. Such correlations included positive correlations with Hem, Xyl, Ara and Gal, and negative correlations with ADF, Cel, and Glu (Figures 2d and 3).



Figures 2a-d. The effects of cell wall compositional traits ADL (a), pCA/ADL (b), TFA (c) and Hem (d) on saccharification efficiency and biogas yield in stem samples of the winter cut. Saccharification efficiency was plotted as glucose conversion as a percentage of total cell wall glucan (green triangles) and biogas yield was plotted as substrate specific biogas yield expressed in ml/g dm (red squares). Number-labels represent accession numbers (OPM).

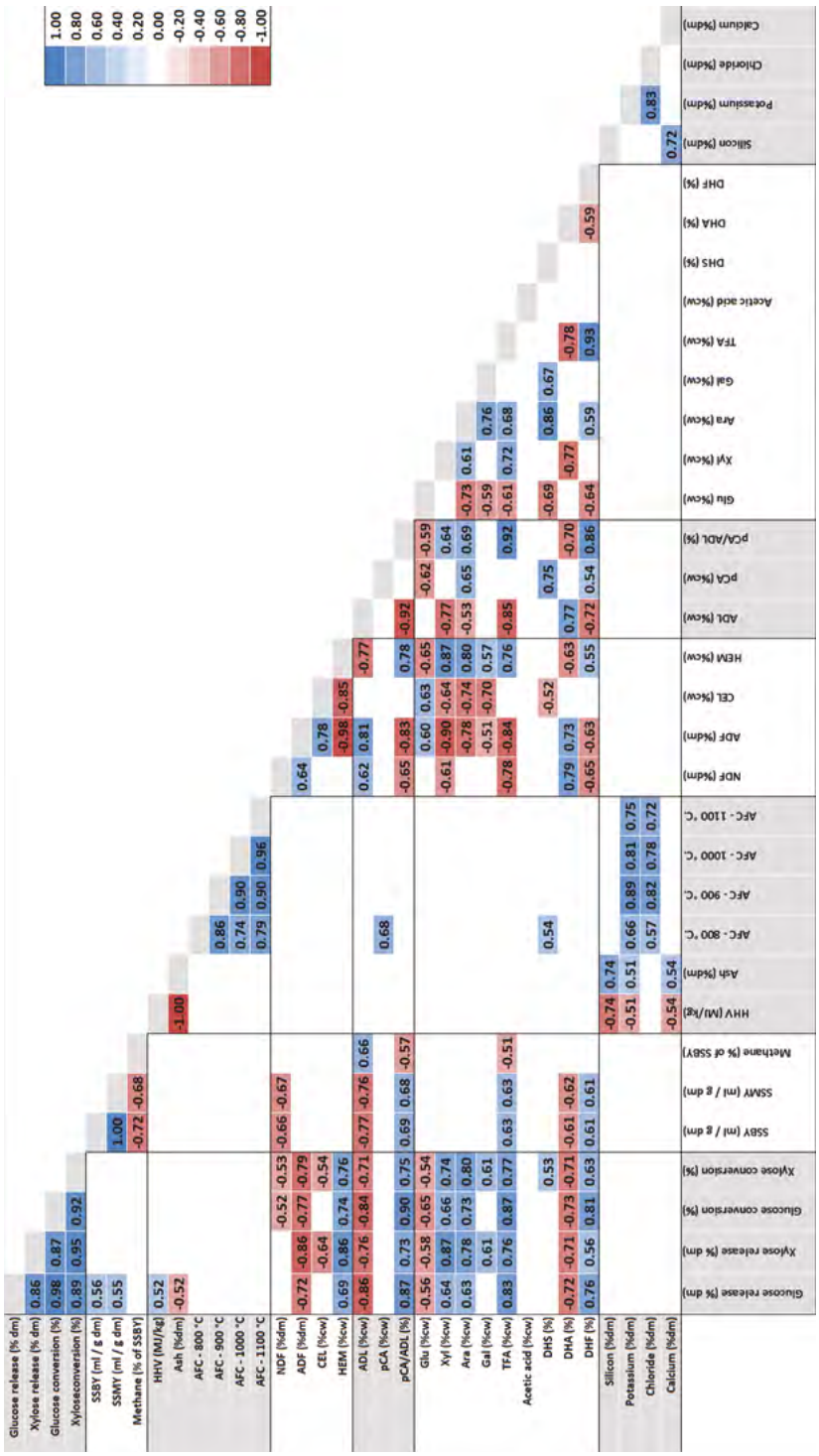


Figure 3. Heat map depicting the extent and the direction of correlations among biomass compositional and biomass quality traits. Only Pearson correlation coefficients that differed significantly from zero (p>0.05) are reported. Blue values indicate positive correlation coefficients and red values indicate negative correlation coefficients.

Multiple regression analysis was performed to develop regression models for glucose conversion and biogas yield based on cell wall compositional characteristics. A simple regression model was found for glucose conversion including only two traits, *p*CA/ADL and galactose, which cumulatively explained 83.2% of the variation for glucose conversion amongst these genotypes. Two simple regression models were found for SSBY, one which included ADL and galactose, and a second which included *p*CA/ADL and arabinose. Both models were able to account for 83.4% of the variation for SSBY amongst these genotypes.

Only two cell wall compositional characteristics were found to be correlated to combustion specific quality traits, *i.e. p*CA content (r = 0.68) and DHS (r = 0.54), which both showed a positive correlation to the classification of samples to ash fusion classes at a combustion temperature of 800°C (Figure 3). However, inorganic elements silicon, potassium and calcium were strongly positively correlated to ash formation during combustion. Moreover, potassium and chloride were shown to be significantly correlated to classification of the genotypes in different ash fusion classes at all tested combustion temperatures (Table 7).

Table 7. Impact of elemental composition on ash formation and ash melting behavior during combustion assessed using correlation analysis. Only Pearson correlation coefficients that differed significantly from zero (*p>0.05*) are reported.

Combustion specific quality traits	Silicon (% dm)	Potassium (% dm)	Chloride (% dm)	Calcium (% dm)
Ash (% dm)	0.74	0.51		0.54
AFC – 800 °C		0.66	0.57	
AFC – 900 °C		0.89	0.82	
AFC – 1000 °C		0.81	0.78	
AFC – 1100 °C		0.75	0.72	

4. Discussion

4.1. Large genetic diversity in biomass composition and quality

The extensive genetic diversity in cell wall compositional traits found in the eight *M. sinensis* genotypes analyzed in this study indicate that there is a large potential in this species for the improvement of biomass quality for different applications. Particularly large variation between genotypes was found for the contents of Hem and ADL, which are the key factors determining lignocellulose recalcitrance (Torres *et al.*, 2014, van der Weijde *et al.*, 2016, Xu *et al.*, 2012). Additionally, significant genotypic variation was found for specific traits that relate to the degree of crosslinking between hemicelluloses or between hemicelluloses and lignin, and more specifically to the degree of substitution of the xylan backbone of hemicellulosic polysaccharides by arabinose (DHS), the degree of xylan acetylation (DHA) and feruloyation (DHF), and the ratio of *para*-coumaric acid to lignin (*p*CA/ADL). This is an

important observation, as there is strong evidence that cell wall crosslinks play important roles in cell wall degradability (e.g. De Souza *et al.*, 2015, Grabber *et al.*, 2004, Hatfield *et al.*, 1999, Torres *et al.*, 2014).

The performance of the genotypes in different bioenergy conversion processes was evaluated. These tests showed significant genotypic differences for many specific quality traits relating to anaerobic digestion and enzymatic saccharification. This finding indicates that considerable improvements in the techno-economic efficiency of bioconversion processes can be achieved by selecting a more suitable feedstock, as for example suggested for maize stover (Torres *et al.*, 2016). For enzymatic saccharification of winter harvested biomass, for example, the best performing genotype released 42% more glucose and 45% more xylose per gram dry matter than the worst performing genotype (Table 6). Similarly, for anaerobic digestion the best performing genotype achieved 18% higher biogas yield than the worst performing genotype (Table 6). These findings indicate that major improvements in final product yield are possible, which will probably have a favorable effect on process economics. Also processing conditions may become less severe with a more suitable feedstock. The mild pretreatment reactions in saccharification experiments were not only chosen because they are optimal for the detection of genotypic differences in conversion efficiency, but also because they give information on the potential to reduce the severity of pretreatment conditions, while maintaining high yields of fermentable sugars. Savings with respect to energy and chemical consumption can be realized in this way, which will be a major cost reduction for the production of bioethanol.

Similarly, significant genotypic variation in contents of ash and inorganic elements were found, which can be exploited to improve the techno-economic performance of biomass combustion processes. Ash and certain inorganic elements are known to cause corrosion, slagging and fouling of the combustion chamber, thereby decreasing the quality of the biomass for combustion. Good combustion quality pertains to low ash content and a high ash melting point. Considerable genotypic variation in potassium and chlorine contents was found (Tables 5 and 6). This is in agreement with the large genotypic variation for elemental composition reported for *M. sinensis* (Atienza *et al.*, 2003a, Atienza *et al.*, 2003b). The classification of genotypes in ash fusion classes showed that the ashes of some genotypes (OPM-49 and OPM-65) were still only partly sintered at a combustion temperature of 1000 °C, whereas ashes of another genotype (OPM-48) at the same temperature were already completely molten (Tables 5 and 6). OPM-65 was shown to have a 62% lower ash content and was consistently classified in a lower ash fusion class during combustion than OPM-48, which is indicative of a higher ash melting point. For many important biochemical components and biomass quality traits significant genotypic differences were found in this diverse set of *M. sinensis* genotypes that can potentially be exploited to optimize the feedstock for different applications.

4.2. Improving bioconversion efficiency by optimization of biomass composition

To show that genotype performance in bioconversion processes can be improved by optimizing biomass composition, correlation analyses were performed between compositional traits and biomass quality characteristics. It was shown that the efficiency of anaerobic digestion and saccharification are affected by biomass composition in a similar way. Lignin content had a negative impact on both conversion technologies, as anticipated and as is well established in literature (Akin, 2008, Campbell & Sederoff, 1996, Dandikas *et al.*, 2014, van der Weijde *et al.*, 2016). A high content of hemicellulosic polysaccharides was furthermore shown to be favorable for saccharification efficiency (r = 0.74, Figures 2d and 3).

Hemicellulosic polysaccharides and lignin both provide structural rigidity to the cell wall and are often negatively correlated (in this study r = -0.77, Figure 3) (Qin *et al.*, 2012, Torres *et al.*, 2014, van der Weijde *et al.*, 2016). Reductions in lignin content may be compensated for by an increase in hemicellulosic polysaccharides, as well as in hemicellulose-hemicellulose and hemicellulose – lignin crosslinks, so that lowering lignin content not necessarily leads to concomitant detrimental reductions in plant cell wall rigidity and associated negative effects on plant fitness. The accompanying changes in the cell wall matrix, however, while still imparting strength to the cell wall, might make the cell wall less recalcitrant to biological conversion processes, such as anaerobic digestion or enzymatic saccharification. This theory is supported by the fact that hemicelluloses are often found to be positively associated to cell wall degradability and saccharification efficiency (Li *et al.*, 2013, Torres *et al.*, 2013, Xu *et al.*, 2012).

Detailed profiling of the samples for minor cell wall components, such as acetic acid, *trans*-ferulic acid and *para*-coumaric acid, as well as hemicellulose monomeric constitution was also proven to be important for understanding the effects of composition on biomass quality. The content of *trans*-ferulic acid was found to have a strong positive effect on the efficiency of both anaerobic digestion and enzymatic saccharification (Figure 2c). In literature, ferulate content is often considered to be negatively associated with cell wall degradability, as it is a key component that mediates crosslinks between hemicelluloses and lignin (Grabber, 2005, Hatfield *et al.*, 1999, Yu *et al.*, 2005) and because feruloylated arabinose side-chains of hemicelluloses are implicated as an initiation/nucleation site for lignin polymerization and deposition (Ralph *et al.*, 1995). However, it has also been reported that lignins that extensively incorporate hydroxycinnamic esters can be easily depolymerized using alkaline pretreatments (Ralph, 2010), which may help to explain the positive associations found in this study. Moreover, TFA content had a strong negative correlation (r = -0.85) to lignin content. Therefore, TFA content may be indirectly positively associated to biogas yield and saccharification efficiency.

In addition, ratios between the different cell wall components were also found to be important, such as the ratio of *p*CA to ADL and the ratio of arabinose to xylose (DHS), which both positively affected biogas yield and saccharification efficiency (Figures 2b and 3). The positive effect of a higher ratio of arabinose to xylose is implicated to be due to a reduction in cellulose crystallinity associated with increase hemicellulose-cellulose crosslinking (Li *et al.*, 2013, Xu *et al.*, 2012). *p*CA is a phenolic compound that is ester-bound mainly to the S-subunit of the lignin polymer. A higher ratio of *p*CA to ADL might thus reflect a higher fraction of the lignin polymer to be comprised by the S-subunit. A higher S/G ratio is in literature sometimes associated with a higher saccharification efficiency (Li *et al.*, 2010, Studer *et al.*, 2011), especially with no or mild pretreatment (Chen & Dixon, 2007). It is also suggested that acylation of lignin with *p*CA impairs the copolymerizing of ferulates with monolignols (Grabber, 2005), which may also contribute to increased cell wall degradability. A high content of TFA, a high ratio of *p*CA to ADL and a low content of lignin are thus potentially interesting breeding targets for miscanthus for improving biomass quality for both saccharification and anaerobic digestion.

Although anaerobic digestion and enzymatic saccharification shared similar correlation patterns to compositional characteristics, the strength of these correlations was higher for saccharification efficiency, which indicates that this conversion process was more dependent on cell wall composition than biogas production. Moreover, biogas yield and saccharification efficiency were not significantly correlated to each other, suggesting that there are biomass quality traits that influence these conversion processes differently. One such trait was found to be Hem, which positively contributed to saccharification efficiency (r = 0.74, Figures 2d and 3), but not to biogas yield. Torres *et al*. (2014), showed that digestibility in rumen liquid (an anaerobic digestion process) and saccharification efficiency have many communalities, but a critical difference was that degree of hemicellulose substitution was relevant for saccharification efficiency, but not a major determinant for rumen-liquid digestibility; a digestion process that resembles the process of anaerobic digestion for biogas production. This is also shown by the fact that the relative quality rating of the genotypes differed for anaerobic digestion and saccharification processes, with the best genotype for biogas production (OPM-73) being one of the worst for saccharification (Table 4). However, there were also genotypes that performed well in both platforms (for example, OPM-65), which indicates that it might be possible to improve biomass quality for both anaerobic digestion and enzymatic saccharification simultaneously.

For both conversion routes it was clear that the summer cut had a better biomass quality than the winter cut, which is partly explained by the fact that lignin contents in the summer cut were much lower than in the winter cut. Lignin is mainly deposited after plant cells stop growing, when cell walls no longer need to accommodate cell expansion and become rigidified by lignification (da Costa *et al.*, 2014, Lam *et al.*, 2013). Other factors that contributed to the higher conversion efficiencies of biomass of the summer cut are

the facts that the relative weight ratio of leaves to total biomass was higher in the summer cut (Table 2), and that leaves were shown to have lower lignin contents in the summer cut then stem fractions (Figure 1d). Despite higher conversion efficiencies, summer harvesting of miscanthus was shown to have a considerable and negative impact on total annual harvestable biomass yields, as the accumulated yield of the summer cut and the regrowth cut achieved only ±40% of the yield achieved in the winter cut (Table 2). Like for the genotypes evaluated in this study, a low tolerance to early green cuttings in July and August was also reported for *M. x giganteus*. However, a green harvest in October was shown to have less detrimental effects on crop yield, while beneficially affecting biomass quality for biogas production compared to winter harvesting (Kiesel & Lewandowski, 2016).

Combustion efficiency is known to be heavily dependent on the elemental composition of the feedstock, as such elements form ash in the combustion chamber, can be corrosive and cause slagging and fouling (Atienza *et al.*, 2003a, Atienza *et al.*, 2003b, Lewandowski & Kicherer, 1997). Not surprisingly, contents of inorganic elements and ash were much lower in samples from the winter cut than from the summer cut, as these elements are translocated into the roots during winter and removed from the plant by leaf shed (Lewandowski *et al.*, 2003a, Lewandowski & Heinz, 2003). In addition, due to natural drying on the field during winter, the dried stems and leafs are more easily fractured by wind, which facilitates the leaching of inorganic elements in periods of rain. The low ash contents in samples of biomass from the winter cut compared to the corresponding samples from the summer cut favorable affect combustion quality (Tables 3 and 4). Moreover, it is known that lignin has a higher caloric value than cellulose and hemicellulose (Lewandowski & Kicherer, 1997) and samples harvested from the winter cut were shown to have higher lignin contents (Tables 3 and 4, Figure 1d). Ash melting behavior could also be optimized. It was shown that potassium and chlorine were associated with lowering the ash melting point and that low contents of these elements positively affect combustion quality (Table 7). The relative quality rating of genotypes for combustion quality differed for some genotypes from that for biogas or for saccharification, but notably there were as well genotypes that performed well in all conversion platforms, such as OPM-49 and OPM-65. However, these were not the highest yielding genotypes. The highest yielding genotype (OPM-69) on the other hand unfortunately tended to slag and had higher contents of Cl and K, resulting in a low quality for combustion. These results show that it is possible to optimize biomass quality for different utilization options simultaneously and develop multi-purpose genotypes, but that several quality traits need to be cross-bred. Extensive genetic variation for many biomass quality traits was found in the eight *M. sinensis* genotypes evaluated in this study, but it is likely that the full extent of variation for these traits within the species is even broader. The exploitation of such variation through breeding will greatly accelerate the realization of biomass derived energy and fuel production, as well as many other biobased applications, generating many market options for the use of miscanthus biomass.

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Supplementary data

Supplementary Table S1. Information on genotype backgrounds

Genotype	Species	Accession information	Plant dry weight (g/plant)*	Stem dry weight (g/plant)*	Stem %*	Plant height without inflorescence (cm)*	Heading date (Julian days)*
OPM 42	*M. sinensis*	Parent Biomis mapping population (F1.7)	704.7	479.6	68.0	210	190
OPM 48	*M. sinensis*	Individual from EMI project (EMI-7)	627.6	485.8	77.0	230	*
OPM 49	*M. sinensis*	Parent of SunLIBB mapping population	*	*	*	*	*
OPM 65	*M. sinensis*	New accession	927.5	705.3	76.4	255	221
OPM 69#	*M. sinensis*	New accession	1444.6	1357.5	94.0	330	*
OPM 73	*M. sinensis*	New accession	1268.6	913.7	72.0	225	214
OPM 77	*M. sinensis*	Female parent is individual from Biomis	849.8	688.0	81.1	255	216
OPM 87	*M. sinensis*	Female parent is individual from Biomis	973.3	760.9	78.4	250	235

Performance of common genotypes as determined in an older field trial at Wageningen University: A trial with a completely-randomized block design with single row plots of 4 vegetatively propagated plants per genotype established in 2010. * missing values # OPM 69 has morphologically great similarities with Miscanthus x gigantheus.

Chapter 5

Stability of cell wall composition and saccharification efficiency in *Miscanthus* across diverse environments

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Abstract

To investigate the potential effects of differences between growth locations on the cell wall composition and saccharification efficiency of the bioenergy crop miscanthus, a diverse set of fifteen accessions were evaluated in six locations across Europe for the first three years following establishment. High-throughput quantification of cellulose, hemicellulose and lignin contents, as well as cellulose and hemicellulose conversion rates was achieved by combining near-infrared reflectance spectroscopy (NIRS) and biochemical analysis. Prediction models were developed and found to predict biomass quality characteristics with high accuracy. Location significantly affected biomass quality characteristics in all three cultivation years, but location differences decreased towards the third year as the plants reached maturity in all locations and the effect of location-dependent differences in rate of establishment reduced. In all locations extensive variation in accession performance was observed for quality traits. The performance of the different accessions in the second and third cultivation year was strongly correlated, while accession performance in the first cultivation year did not correlate with performance in later years. Significant genotype-by-environment (G×E) interactions were observed for most traits, revealing differences in between accessions in environmental sensitivity. Stability analysis of accession performance for calculated ethanol yields suggested that selection for good and stable performance is a viable approach. Environmental influence on biomass quality is substantial and should be taken into account in order to match genotype, location and end-use of miscanthus as a lignocellulose feedstock.

1. Introduction

To expedite the utilization of renewable plant biomass as an alternative to fossil fuel it is necessary to develop high yielding biomass crops producing biomass of high quality in different environments (van der Weijde et al., 2013). Several second-generation energy crops have potential as a lignocellulose feedstock for biofuel production, but one of the strongest contenders is miscanthus (Heaton et al., 2010). Miscanthus is a highly productive perennial grass with a high nutrient-use efficiency, owing to its highly efficient C4 photosynthesis system and ability to translocate minerals to underground rhizomes at the end of the cultivation year (Heaton et al., 2010). The genus *Miscanthus* comprises approximately 15 different species of which *M. sinensis*, *M. sacchariflorus* and their interspecific hybrids are considered to have the highest potential for biomass production (Jones and Walsh, 2001). These miscanthus species harbor great genetic diversity and occur naturally over a large geographical range in East Asia (Clifton-Brown et al., 2008). As a result miscanthus displays a wide adaptation to different soils types and climates, which may allow its exploitation as a second generation biofuel feedstock across a broad range of environments.

However, the potential of a lignocellulose feedstock for the production of biofuel is also highly determined by the quality of the biomass for biofuel production. Lignocellulosic biomass is mainly composed of cellulose, hemicellulosic polysaccharides and lignin (Doblin et al., 2010). The content of polysaccharides determines how much fermentable sugars are theoretically available at a maximum conversion rate of 100%. The content of lignin, on the other hand, is one of the main factors that limit the extraction of fermentable sugars from the cell wall (Chundawat et al., 2011). Lignin is a complex aromatic polymer that crosslinks to hemicellulosic polysaccharides, forming a highly impermeable matrix that imparts strength to the plant cell wall and shields cell wall polysaccharides against chemical and enzymatic hydrolysis (Himmel and Picataggio, 2008; Chundawat et al., 2011). Cell wall compositional characteristics are therefore considered important quality criteria for biofuel feedstocks and the development of improved varieties with increased polysaccharide, reduced lignin content and increased saccharification efficiency is seen as crucial to reduce the production costs of cellulosic biofuels (Wyman, 2007; Torres et al., 2016; van der Weijde et al., 2016a).

There is ample scope for the development of such varieties through breeding as extensive genetic variation for cell wall composition is found in miscanthus, with contents of cellulose ranging from ~26 – 51%, hemicellulosic polysaccharides from ~25 – 43% and lignin from ~5 – 15% of dry matter (Allison et al., 2011; Qin et al., 2012; Zhao et al., 2014). Cell wall compositional characteristics, however, are complex polygenic traits and are commonly affected by environmental as well as genetic determinants. Cell wall biosynthesis, particularly lignin deposition, is spatially and temporally regulated during the development of the plant and like any other complex metabolic pathway it can be reprogrammed in response to environmental signals (Boerjan et al., 2003; Pauly and Keegstra, 2010). The effect of

environment on miscanthus cell wall composition was first demonstrated by Hodgson and coworkers, who studied the extent of genotypic and environmentally derived variation in cell wall composition in a study at five field trial locations (Hodgson et al., 2010). They concluded that overall the degree of observed genotypic variation in cell wall composition indicated a high potential for breeding for biomass quality characteristics, but also stressed the significance of environmentally induced differences in cell wall composition.

In this study we investigated in-depth how differences between growth locations affect biomass quality in miscanthus. To this end we studied the cell wall composition and saccharification efficiency of a set of 15 accessions across different locations and cultivation years. The test comprised 4 *M. sacchariflorus*, 5 *M. sinensis* and 6 hybrid accessions, which were evaluated for three years in six locations across Europe: Aberysthwyth (UK), Adana (TR), Potash (UA), Moscow (RU), Stuttgart (DE) and Wageningen (NL). This is the first study on genotype-by-environment interactions for biomass quality in miscanthus and these insights are important for the development of new varieties and the growth of these across different environments.

2. Material and Methods

2.1. Plant materials

Fifteen miscanthus accessions, belonging to three different miscanthus species, were used in this study; five accessions of *M. sinensis,* including the commercial cultivar *'Goliath'*, four of *M. sacchariflorus*, including the commercial cultivar '*Robustus*', and six hybrid accessions derived from crosses between *M. sinensis* and *M. sacchariflorus*, including the commercially-used clone '*M. × giganteus'* (Table 1). The accessions were tested in a multi-location trial with six locations (Table 2): Aberysthwyth (UK), Adana (TR), Potash (UA), Moscow (RU), Stuttgart (DE) and Wageningen (NL). The trials were established using a completely randomized block design with three replications per accession between April and May 2012. The planting materials used to establish the trials were clones produced by *in vitro* propagation (OPM 1-11) or seed-derived plantlets (OPM 12-15). A total of 49 plantlets were planted per plot with a density of two plants per m^2, resulting in a plot size of 25 m^2. Field trials were managed without irrigation, except for the trial in Adana, in which irrigation had to be applied on several occasions to ensure plant survival. All trials were fertilized once, prior to establishment of the trials, with a single application of 44 kg P ha^{-1} and 110 kg K ha^{-1}. The trials were harvested between January and April for three consecutive years after establishment of the trials (2012 – 2015). To minimize potential border effects, for each plot only the inner nine plants were harvested, bundled and processed further. Each bundle of biomass was weighed and subsequently a subsample from every bundle was

drawn randomly for determination of moisture content. Moisture content was determined after chopping and drying of the subsamples in a forced-air oven at 60°C for 72 hours and used for the calculation of dry matter yields per plot. A second subsample of shoots was randomly drawn from each bundle and stripped from leaves. The remaining stem material was chopped and dried in a forced-air oven at 60°C for 72 hours and used for the calculation of stem dry matter yields per plot. Subsequently, the dried stem material was ground using a hammer mill with a 1-mm screen and used for biomass quality analyses (n = 810 [3 years × 6 locations × 15 accessions × 3 blocks]).

2.2. Fiber analyses

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid-detergent lignin contents (ADL) of stem dry matter were determined according to protocols developed by Ankom Technology (ANKOM Technology Corporation, Fairpoint, NY), which are essentially based on the work of Goering and Van Soest (Van Soest, 1967; Goering and Van Soest, 1970). NDF and ADF fractions are the residues remaining after refluxing the samples in neutral or acid detergent solutions, respectively, using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY). Acid detergent lignin was determined after 3-hour hydrolysis of the ADF residue in 72% H_2SO_4 with continuous shaking. All analyses were performed in triplicate and fiber fractions were expressed in gram per kg dry matter.

2.3. Determination of saccharification efficiency

Saccharification efficiency of the samples was assessed by the conversion of cellulose into glucose and hemicelluloses into xylose using a mild alkaline pretreatment and enzymatic saccharification reaction, essentially as described by (van der Weijde et al., 2016b). Reactions were carried out in triplicate using 500 mg subsamples per stem or leaf sample. All subsamples were incubated for 13 minutes with α-amylase (thermostable α-amylase, ANKOM Technology Corporation, Fairpoint, NY), followed by three five minute incubations with warm deionized water (~60°C) in order to remove interfering soluble sugars. The remaining biomass was then subjected to a mild alkaline pretreatment, carried out in 50 ml plastic centrifuge tubes with 15 ml 2% NaOH at 50°C with constant shaking (160 RPM) for two hours in an incubator shaker (Innova 42, New Brunswick Scientific, Enfield, CT). In this study the objective of the pretreatment was not to maximize cellulose conversion but to treat samples to better allow discrimination of genotypic differences in cellulose conversion efficiency. Pretreated samples were washed to neutral pH with deionized water (2x, 5 minutes, 50°C) and with 0.1 M sodium citrate buffer (pH 4.6, 5 minutes, 50°C).

Saccharification reactions were subsequently carried out according to the NREL Laboratory Analytical Procedure “Enzymatic saccharification of lignocellulosic biomass” (Selig et al., 2008). Pretreated samples were hydrolyzed for 48 hours with 300 µl (25.80 mg of enzyme)

of the commercial enzyme cocktail Accellerase 1500 (DuPont Industrial Biosciences, Leiden, NL) supplemented with 15 µl (0.12 mg of enzyme) endo-1,4-β-xylanase M1 (EC 3.2.1.8, Megazyme International Ireland, Bray, IE) in an incubator shaker (Innova 42, New Brunswick Scientific, Enfield, CT) set at 50°C and constant shaking (160 RPM). This enzyme mixture has the following reported specific activities: endoglucanase 2200-2800 CMC U/g, beta-glucosidase 450-775 pNPG U/g and endoxylanase 230 U/mg. Reactions were carried out in 44 ml 0.1 M sodium citrate buffer (pH 4.6), containing 1.3 ml of a 1% benzoate solution for the prevention of microbial contamination.

Glucose and xylose contents in the enzymatic saccharification liquors were determined using enzyme-linked D-glucose (R-Biopharm, Darmstadt, DE) and D-xylose (Megazyme International Ireland, Bray, IE) assay kits. These assays were adapted to a 96-well microplate format and the increases in sample absorption following enzyme-mediated conversion reactions were spectrophotometrically determined at 340 nm using a Bio-Rad Microplate Reader (Bio-Rad, Richmond, CA, USA). Spectrophotometric determination of each sample was done in duplicate and all absorbance measurements were corrected using blanks, containing demineralized water instead of sample solution. Glucose and xylose release was determined by calculating the glucose and xylose content, respectively, in the saccharification liquor from absorbance measurements using equation (1).

$$\text{Glucose / xylose release } (\text{mg}) = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times df \times \Delta Abs \quad (1)$$

where *V* = final well volume (3.02 ml for glucose and 2.97 ml for xylose measurement); *MW* = molecular weight of glucose (180.16 g/mol for glucose and 150.13 for xylose); ε = the molar extinction coefficient of NADPH or NADH for glucose and xylose measurements, respectively (6.3 L × mol^{-1} × cm^{-1}); *d* = light path-length (=1.016 cm); *v* = sample volume (0.1 ml); *df* = dilution factor (10 for glucose and 5 for xylose measurement); and ΔAbs = increase in sample absorbance, corrected for the increase in blank absorbance. Cellulose/hemicellulose conversion rates were calculated from the release of glucose/xylose relative to the content of cellulose/hemicellulose, respectively, as detailed in equations 2 and 3.

$$\text{Hemicellulose conversion } (\%) = \frac{\text{Xylose release}(\text{mg})}{HC \times 1.136} \times 100\% \quad (2)$$

$$\text{Cellulose conversion } (\%) = \frac{\text{Glucose release}(\text{mg})}{CC \times 1.111} \times 100\% \quad (3)$$

where *CC* = cellulose content (in mg) in the sample, calculated as described below, *1.111* = the mass conversion factor that converts cellulose to equivalent glucose (the molecular weight ratio of 180.16 to 162.16 g/mol for glucose and anhydro-glucose) (Dien, 2010), *HC* = hemicellulose content (in mg) in the sample, calculated as described below, *1.136* = the mass conversion factor that converts xylan to equivalent xylose (the molecular weight ratio of 150.13 to 132.12 g/mol for xylose and anhydro-xylose) (Dien, 2010). Calculated ethanol

yield (CEY, g / kg dm) was calculated by considering full conversion of all the released glucose and xylose into ethanol.

Table 1. Accession, species and propagation information of the fifteen miscanthus accessions used in this study.

Genotype	Species	Plants
OPM 1	*M. sacchariflorus*	In vitro
OPM 2	*M. sacchariflorus*	In vitro
OPM 3	*M. sacchariflorus*	In vitro
OPM 4	*M. sacchariflorus 'Robustus'*	In vitro
OPM 5	*Hybrid*	In vitro
OPM 6	*Hybrid*	In vitro
OPM 7	*Hybrid*	In vitro
OPM 8	*Hybrid*	In vitro
OPM 9	*Hybrid 'M. × giganteus'*	In vitro
OPM 10	*M. sinensis*	In vitro
OPM 11	*M. sinensis 'Goliath'*	In vitro
OPM 12	*M. sinensis*	Seed
OPM 13	*M. sinensis*	Seed
OPM 14	*M. sinensis*	Seed
OPM 15	*Hybrid*	Seed

Table 2. Location characteristics and long term annual and growth season (approximated April - September) temperature and rainfall for the six trial locations.

				Air Temperature*, °C		Rainfall*, mm	
Location name	Latitude	Longitude	Altitude (m)	Annual	April to Sept	Annual	April to Sept
Aberystwyth (UK)	52.43	-4.01	39	9.7	13.8	1038	401
Adana (TR)	37	35	27	19.0	26.1	575	75
Moscow (RU)	55	37	140	4.1	14.8	644	347
Potash (UA)	48.89	30.44	237	8.9	18.5	537	300
Stuttgart (DE)	48.74	8.93	463	9.8	16.4	725	379
Wageningen (NL)	51.59	5.39	10	10.3	15.8	826	376

*Climate data for Adana: 2000-2011, for Stuttgart: 1988-1999, for Potash: 2003-2012, for Wageningen: 2002-2012, for Aberystwyth: 1954-2000 and for Moscow: 1881-1980

2.4. Analysis of miscanthus biomass using near infrared spectroscopy (NIRS)

Multivariate prediction models based on near-infrared (NIR) spectral data were developed to allow high-throughput prediction of biomass quality traits. Near-infrared absorbance spectra of stem and leaf samples were obtained using a Foss DS2500 near-infrared spectrometer (Foss, Hillerød, Denmark). Averaged spectra were obtained consisting of 8 consecutive

scans from 400 nm to 2500 nm using an interval of 2 nm using ISI-Scan software (Foss, Hillerød, Denmark). Obtained spectra were further processed by weighted multiplicative scatter correction and mathematical derivatization and smoothing treatments using in WinISI 4.9 statistical software (Foss, Hillerød, Denmark). These statistical transformations of spectra help to minimize effects resulting from light scatter and differences in particle size. Parameters for derivatization and smoothing were set at 2-6-4-1, in which the first number of this mathematical procedure refers to order of derivatization, the second number to the gap in the data-points over which the derivation is applied and the third and fourth number refers to the number of data-points used in the smoothing of the first and second derivative.

For the creation of prediction models a calibration set of 250 samples was selected from the complete set of samples (n = 810): 110 samples of the first cultivation year, 80 samples of the second cultivation year and 60 samples of the third cultivation year, all selected at random or for being identified by the software as spectral outliers. The biochemical reference data and near-infrared spectra of the calibration samples were used for the development and cross-validation of prediction models using WinISI version 4.9 (Foss, Hillerød, Denmark). The prediction equations were generated using modified partial least squares regression analyses (Shenk and Westerhaus, 1991). The optimal number of principal components used for development of the prediction models was manually determined to be 8. Inclusion of more factors hardly improved the prediction models as determined by validation and increases the risk of 'over-fitting' of the data. The prediction models were validated using the squared Pearson coefficient of correlation (r^2) between predicted and biochemical data and by evaluating for these samples the standard error of cross-validation (SECV) for each of the traits (Table 3). The prediction models were subsequently used to determine NDF, ADF, ADL, cellulose conversion and hemicellulose conversion for all 810 stem samples. The predicted fiber fractions were used to calculate the concentrations (in g/kg dm) of cell wall (NDF) cellulose (CEL, equals ADF - ADL), hemicellulosic polysaccharides (HEM, equals NDF - ADF) and acid-detergent lignin (LIG, equals ADL) in stem dry matter.

Table 3. Summary of cross-validation statistics of mPLS models used for the prediction of biomass quality traits from NIRS spectral data

		Chemical analysis			NIRS prediction				
Constituent	**Samples**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	r^{2*}	**SECV**[§]
NDF (g/kg dm)	246	85.04	71.55	92.69	85.04	71.28	92.35	0.99	0.88
ADF (g/kg dm)	243	54.96	38.43	68.55	54.97	39.40	68.47	0.99	1.13
ADL (g/kg dm)	239	9.22	4.88	14.45	9.20	5.26	14.42	0.88	0.79
Cellulose conversion (%)	237	29.89	8.17	52.10	30.21	13.14	46.81	0.92	3.22
Hemicellulose conversion (%)	243	12.43	5.84	22.20	12.34	6.70	20.27	0.82	2.06

[*] r^2 = coefficient of determination. [§]SECV = Standard error of cross-validation

2.5. Statistical analyses

General analyses of variance (ANOVA) were performed to determine the significance of accession differences, locations, cultivation years and their interactions (p<0.05) on cell wall composition and saccharification efficiency. Variance analyses were performed following the standard procedure of a mixed effect model with a random genetic effect, a fixed location effect, a random year effect and a fixed block effect, following the model (4):

$$R_{ijkr} = \mu + G_i + L_j + Y_k + B_r(L_j Y_k) + GL_{ij} + GY_{ik} + LY_{jk} + GLY_{ijk} + e_{ijkr} \quad (4)$$

where $\underline{R}_{ijkr}$ is the response variable, μ is the grand mean, G_i is the genotype effect, L_j is the location effect, Y_k is the year effect, B_r $(L_j$ $Y_k)$ is the block effect, GL_{ij} is the genotype-by-location interaction, GY_{ik} is the genotype-by-year interaction, LY_{jk} is the location-by-year interaction, GLY_{ijk} is the genotype-by-location-by year interaction and e_{ijkr} is the residual error. To study the potential of early selection correlation analyses were performed on accession means to identify the significance (p<0.05) of correlations between traits across cultivation years using Pearson's correlation coefficients. In addition a Finlay Wilkinson stability analysis was performed using the data of the third cultivation year (5) (Finlay and Wilkinson, 1963; Malosetti et al., 2013):

$$R_{ij} = \mu + G_i + \beta_i \times L_j + e_{ij} \quad (5)$$

where $\underline{R}_{ij}$ is the response variable, μ is the grand mean, G_i is the genotype effect, β_i is the regression coefficient of accession i for environment j (environmental sensitivity), L_j is a measure of environmental quality determined by the mean performance of accessions for CEY in environment j and e_{ij} is the residual error. Accession means per location for the third cultivation year were also used to fit a GGE model by singular value decomposition of environment-centered genotype by location data (6) (Malosetti et al., 2013):

$$R_{ij} = \mu + L_j + \sum_{k=1}^{K} \beta_{ik} \times L_{jk} + e_{ij} \quad (6)$$

where accession performance is explained by K multiplicative terms (k = 1…K), each formed by the product of environmental sensitivity (β_{ik}) of accession i and environmental score (L_{jk}). A GGE biplot was constructed in which accession performance (accounting for both genotype main effect and genotype-by-location interaction) across environments is visualized in a scatter plot of accession and location scores for the first two principal components (Yan and Kang, 2002; Malosetti et al., 2013). Correlation analyses were performed to identify the significance, strength and direction of interrelationships between morphological and quality traits using Pearson's correlation coefficients. All statistical analyses were performed using Genstat for Windows, 18th edition software package (VSN International, Hemel Hempstead, UK).

3. Results and discussion

3.1. Impact of genotype, location and cultivation year on biomass quality

Analyses of variance revealed that cell wall composition and saccharification efficiency differed significantly between accessions and that all these traits were strongly affected by both trial location and cultivation year (Tables 4 and 5). Genotypic variation in biomass quality across the locations for three cultivation years is depicted in Figures 1-2. Biomass composition in the first cultivation year differed considerably from that in the second and third, with substantially lower overall cell wall (NDF), cellulose (CEL) and lignin (LIG) contents and substantially higher contents of hemicellulosic polysaccharides (HEM) in the first year. For cultivation years 1, 2 and 3 mean NDF contents were ~829, ~860 and ~876 g/kg dm, respectively. Similarly, mean CEL contents were ~422, ~474 and ~485 g/kg dm and LIG contents were ~85, ~93 and ~99 g/kg dm, respectively. Mean HEM contents decreased from ~322 in the first, to ~293 in the second and ~291 g/kg dm in the third year (Figure 1). Saccharification efficiency also differed substantially between cultivation years (Table 5) and was much higher in the first year than in the second or third year (Figure 2). Mean cellulose conversion reduced from ~38% in the first year to ~27% in the second and ~22% in the third year. Similarly, mean hemicellulose conversion reduced from ~14% in the first, to ~11 in the second and ~10% in the third year. These changes in biomass composition and quality culminated in substantial reductions in mean calculated ethanol yields (CEY) from ~117 in the first, to 91 in the second and 77 g/kg dm in the third cultivation year (Figure 2).

As miscanthus is a perennial crop, differences in biomass quality between the cultivation years are likely to be explained to large extent by location-dependent differences in rate of establishment. Differences in rate of establishment are likely to occur, as at northern latitudes miscanthus tends to mature slower than at latitudes closer to the equator (Lewandowski et al., 2000; Clifton-Brown et al., 2001). Such differences will become less pronounced as stands in all locations reach full maturity, which may explain why location differences for most traits are smaller in year 3 than in year 1 (Figures 1-2). However year-to-year variation in environmental factors may also play a role. The year-to-year variation in accession performance for calculated ethanol yield per location is depicted in Figure 3. A low similarity (r^2<0.32) in accession performance between the first and the third cultivation year was observed for all locations except for Adana (r^2 = 0.45). However, for all locations accession performance in CEY in the second cultivation year correlated well with that in the third cultivation year (r^2 = 0.42 - 0.83). Since for all locations three year old miscanthus stands represent mature, well-established stands (Lewandowski et al., 2003; Gauder et al., 2012), this means that performance at full maturity can be estimated with reasonable accuracy from accession performance after two cultivation years. In contrast, selection for CEY based on performance after only one cultivation year is not recommended, due to its low predictive value of performance at full maturity.

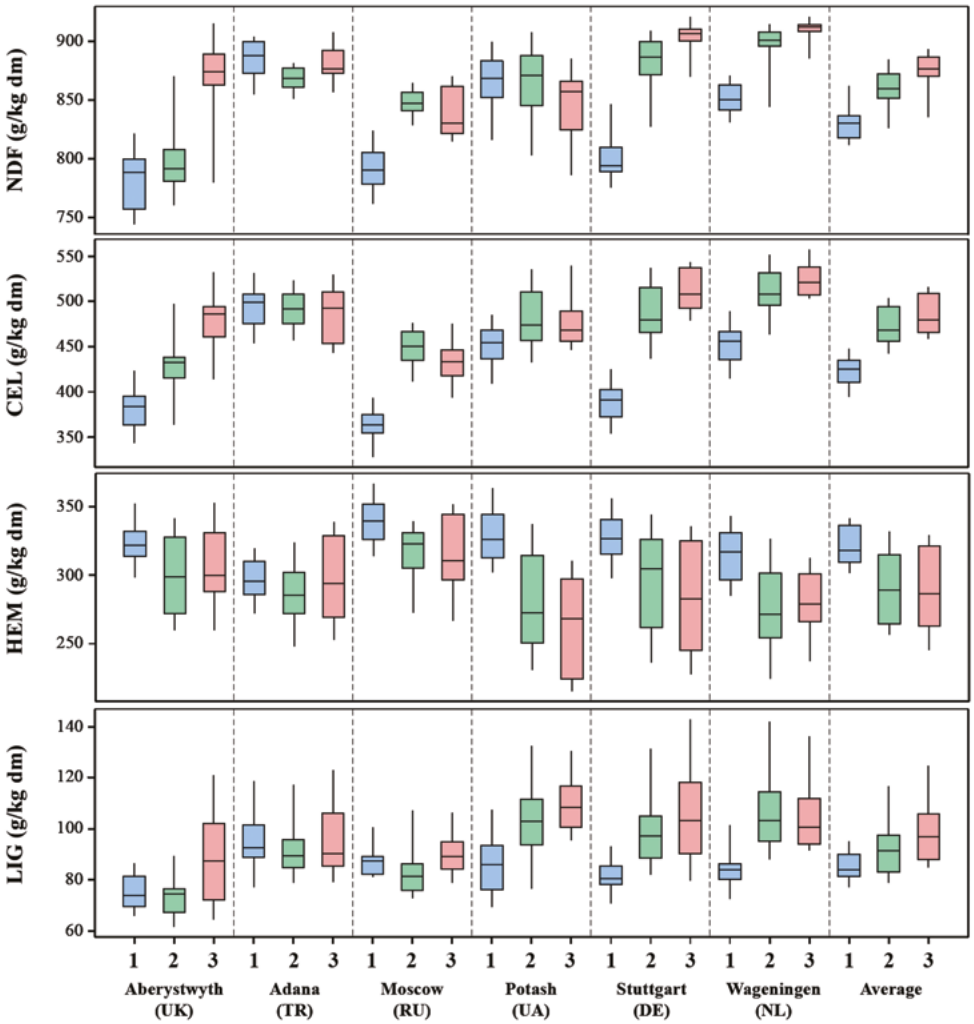

Figure 1. Variation in genotype means of fifteen miscanthus accessions for cell wall composition characteristics in six growth locations and three cultivation years (2012-2013, 2013-2014 and 2014-2015).

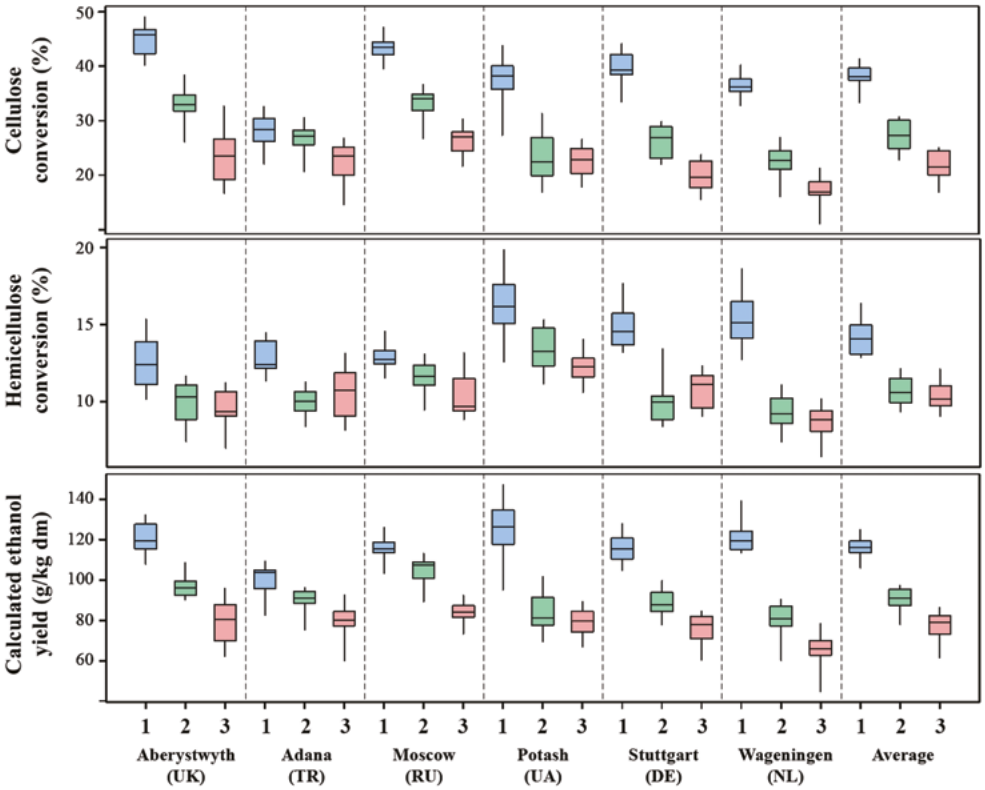

Figure 2. Variation in genotype means of fifteen miscanthus accessions for conversion efficiency characteristics in six growth locations and three cultivation years (2012-2013, 2013-2014 and 2014-2015).

For more in-depth evaluation of location differences in biomass quality, the material from the third cultivation year was examined (Tables 6 and 7), which is assumed to represent mature, well-established miscanthus stands in all locations. Biomass composition varied extensively across locations, with mean NDF content ranging from 840 - 910 g/kg dm, CEL content from 434 - 524 g/kg dm, HEM content from 262 - 316 g/kg dm and ADL content from 89 - 109 g/kg dm (Table 6). The highest NDF and CEL contents were observed in Wageningen, while the lowest were observed in Moscow. These two locations were found to be the most contrasting of the evaluated locations regarding cell wall composition. Locations also differed extensively in saccharification efficiency. Mean cellulose conversion ranged from 17.3 - 26.4% across locations, with the lowest rate observed in Wageningen and the highest in Moscow. Likewise, mean hemicellulose conversion ranged from 8.7 - 12.3%, with the lowest rate observed in Wageningen and the highest in Potash. Calculated ethanol yields ranged from 65.6 - 83.5 g/kg dm across locations, with the highest yields for Moscow and the lowest for Wageningen (Table 6). The ethanol yields reported in this study are relatively low compared to industrial standards, because very mild pretreatment conditions were chosen in this study to better allow the evaluation of genotypic differences in saccharification efficiency.

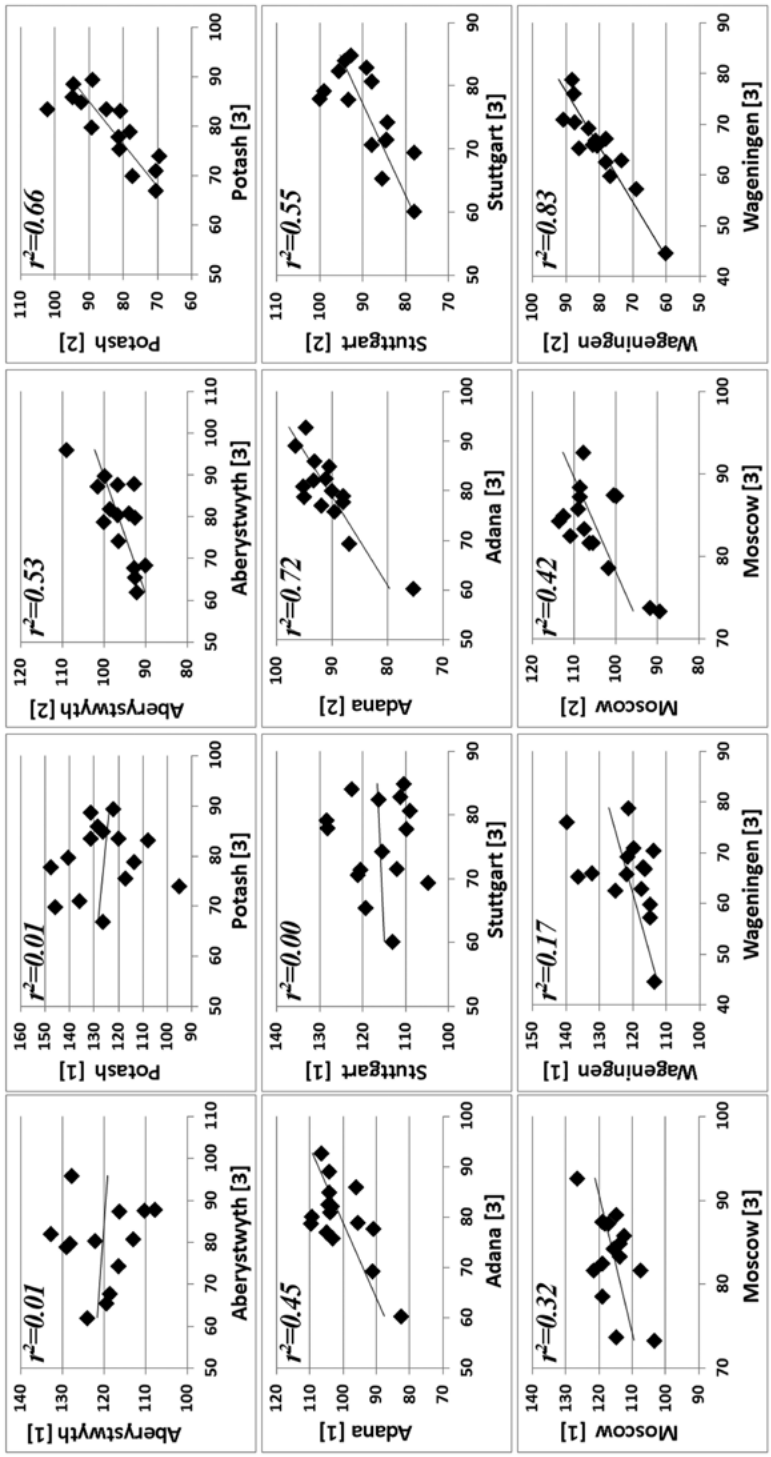

Figure 3. Scatter plot matrix of calculated ethanol yields (g/kg dm) of the first [1] and the second [2] cultivation year of 15 miscanthus accessions in six locations compared to that of the third cultivation year [3].

Table 4. Analyses of variance for cell wall composition of 15 miscanthus genotypes grown in six locations and evaluated for three cultivation years.

Source of variation*	Degrees of freedom	NDF (g/kg dm)		CEL (g/kg dm)		HEM (g/kg dm)		LIG (g/kg dm)	
		Mean squares	*F prob.*	*Mean squares*	*F prob.*	*Mean squares*	*F prob.*	*Mean squares*	*F prob.*
L	5	104619.6	<.0001	145509.5	<.0001	22834.8	<.0001	8375.0	<.0001
Residual[1]	12	489.8		835.1		992.9		196.1	
G	14	9644.3	<.0001	18230.8	<.0001	28602.2	<.0001	5027.7	<.0001
Y	2	150768.8	<.0001	309417.8	<.0001	84714	<.0001	13962.3	<.0001
GL	70	1308.7	0.0002	1312.6	<.0001	697.5	<.0001	143.1	0.0904
GY	28	960.2	0.0632	1139.5	0.0059	1548.2	<.0001	465.4	<.0001
LY	10	37187.4	<.0001	31550	<.0001	6469.7	<.0001	2283.3	<.0001
GLY	138	637.2	<.0001	579.9	<.0001	297.5	0.000	109.2	<.0001
Residual[2]	500	242.8		308		184.6		50.3	

*G = Genotype, L = Location, Y = Year, GL = Genotype-by-location interaction, GY = Genotype-by-year interaction, LY = Location-by-year interaction, GLY = Genotype-by-location-by-year interaction, Residual[1] = Residual block stratum, Residual[2] = Residual block*units stratum

Table 5. Analyses of variance for conversion efficiency and calculated ethanol yield (CEY) of 15 miscanthus genotypes grown in six locations and evaluated for three cultivation years (2012-2013, 2013-2014 and 2014-2015).

Source of variation*	Degrees of freedom	CelCon (%)		HemCon (%)		CEY (g/kg dm)	
		Mean squares	*F prob.*	*Mean squares*	*F prob.*	*Mean squares*	*F prob.*
L	5	2071.2	<.0001	184.6	<.0001	3171.3	<.0001
Residual[1]	12	23.8		2.5		84.2	
G	14	283.2	<.0001	51.1	<.0001	3171.3	<.0001
Y	2	18801.3	<.0001	1151.8	<.0001	84.2	<.0001
GL	70	21.1	0.0003	3.1	0.0639	141.1	0.0099
GY	28	26.2	0.0002	2.5	0.3834	205.2	0.0007
LY	10	508.0	<.0001	46.1	<.0001	2836.9	<.0001
GLY	138	10.7	<.0001	2.3	<.0001	88.4	<.0001
Residual[2]	500	4.8		1.1		25.1	

*G = Genotype, L = Location, Y = Year, GL = Genotype-by-location interaction, GY = Genotype-by-year interaction, LY = Location-by-year interaction, GLY = Genotype-by-location-by-year interaction, Residual[1] = Residual block stratum, Residual[2] = Residual block*units stratum

Locations also varied in the extent of variation amongst accessions in cell wall composition and conversion efficiency (Table 6). The coefficient of trait variation (CV_t) across locations ranged from 0.9 – 3.7% for NDF, 3.4 – 6.4% for cellulose, 8.7 -13.5% for hemicellulosic polysaccharides and 9.2 – 18.4% for lignin (Table 6). This showed that differences in variation in accession performance across locations were the largest for lignin content. Locations also differed with respect to variation in accession performance for conversion rates, with a CV_t ranging from 10.1 – 18.3% for cellulose conversion and 8.0 – 14.8% for hemicellulose conversion. For four out of seven evaluated traits the largest variation in accession performance was observed in Aberystwyth.

Not only the extent of variation in accession performance differed between locations, also the ranking of genotypes varied for all traits, as indicated by the statistical significance of genotype-by-environment interactions observed for all evaluated traits, except for lignin and hemicellulose conversion efficiency (Tables 4 and 5). When variance was analyzed on third cultivation year data only, statistically significant genotype-by-environment interactions were also observed for lignin and hemicellulose conversion efficiency (Supplementary Tables S1 and S2). This is the first report on genotype-by-location interactions for cell wall components and saccharification efficiency in miscanthus. Such interactions have important implications for the set-up of selection experiments, as they implicate that the relative ranking of accessions is dependent on the environment.

3.2. Stability of genotype performance

For all evaluated traits mean accession performance and variation in accession performance across locations is displayed in Table 7. These results show that the genotypes are differentially sensitive to location differences for different traits. For example, the largest variation in cellulose content across locations was observed for OPM 12, while the largest variation in contents of hemicellulosic polysaccharides and lignin was observed for OPM 2. Similarly, OPM 9 displayed the largest variation for NDF and CEY, while OPM 10 and OPM 3, respectively displayed the largest variation in CelCon and HemCon. As accession performance differed across locations, a Finlay Wilkinson stability analysis was performed on CEY data of the third cultivation year, to estimate the environmental sensitivity of accessions for this trait (Finlay and Wilkinson, 1963) (Table 8). The higher the sensitivity estimate, the more sensitive an accession is to the 'quality' of the growth location for the evaluated trait. The environmental quality in this analysis refers to deviation of mean accession performance in that location from the mean accession performance over all evaluated locations. Accession performance of OPM 1 was found to be the least sensitive (sensitivity 0.54) and OPM 9 the most sensitive (sensitivity 1.50) to environmental quality (Table 8). The static stability parameter of each accession was also calculated, which is a measure of the variance in accession performance across locations (Becker and Leon, 1988).

A smaller static stability means smaller variation in accession performance across locations. Accession performance of OPM 1 was the most stable (static stability 30) and OPM 10 the least stable (static stability 119) across environments (Table 7). The superiority coefficient is used to identify accessions that perform relatively well in all test locations and accounts for both mean performance and stability (Lin and Binns, 1988). OPM 6 ranked first in overall performance across environments, while OPM 9 ranked last (Table 8).

A useful tool to visualize the variation in accession performance across locations is the GGE biplot (Figure 3) (Yan et al., 2000; Yan and Kang, 2002; Malosetti et al., 2013). The origin of the plot represents the average performance of accessions across the environments, the length of environment vectors is proportional to the genetic variance within environments and the angle between vectors is proportional to the correlation between environments (Yan and Kang, 2002; Malosetti et al., 2013). The first two principal components visualized in the biplot explained 91.28% of the variation (Figure 4). The angle between the vector for Potash and the vector for Aberystwyth is almost 90 degrees, indicating that there is virtually no correlation in accession performance between these two locations. The perpendicular projection of accessions on the environment vectors approximates accession performance per environment, showing that OPM 2 performed the best in Aberystwyth, while OPM 6 performed the best in all other trial locations. OPM 9 performed the worst in all locations.

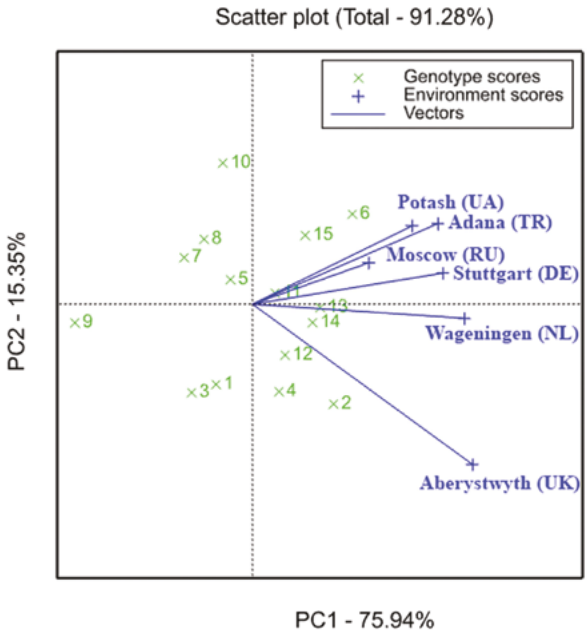

Figure 4. GGL biplot of variation in genotype performance in calculated ethanol yield (g / kg dm) across six locations in the third cultivation year (2014-2015). Numbers represent genotype OPM codes.

Along with the previous observation that OPM-6 had the highest superiority coefficient and the highest mean performance across locations (Table 8), this shows that accession performance of OPM-6 for CEY was relatively insensitive to differences between locations and was superior in 5 out of 6 trial locations. The selection of stable accessions to counter the effects of genotype-by-location interactions is a viable approach if, like is the case here, the performance of the stable accession is not much lower compared to adapted accessions. However, the stable and superior accession OPM-6 did perform relatively poor in Aberystwyth compared to OPM-2, but still had average performance among all accessions.

Table 6. Summary table of average, range and least significant differences for biomass quality traits of 15 genotypes evaluated in six locations (cultivation year 3, 2014-2015)

		Location								
Trait	Statistic	Aberystwyth (UK)	Adana (TR)	Moscow (RU)	Potash (UA)	Stuttgart (DE)	Wageningen (NL)	Mean	Range	LSD
NDF	**Average**	**871.8**	**881.0**	**839.5**	**847.1**	**904.3**	**909.9**	875.6	70.4	6.5
(g/kg dm)	Range	135.1	49.9	54.8	98.8	50.7	34.9	70.7		
	CV_t (%)[§]	3.7	1.5	2.5	3.6	1.3	0.9	2.3		
	LSD[¥]	40.9	32.8	18.0	24.8	10.9	9.0			
Cellulose	**Average**	**478.1**	**487.3**	**433.7**	**476.1**	**513.2**	**524.4**	485.5	90.7	7.3
(g/kg dm)	Range	117.4	86.7	81.5	92.4	64.3	53.4	82.6		
	CV_t (%)	6.4	6.3	6.0	5.8	4.5	3.4	5.4		
	LSD	43.7	34.7	19.2	24.9	16.3	19.4			
Hemicellulose	**Average**	**305.3**	**298.6**	**315.6**	**262.3**	**284.1**	**280.7**	291.1	53.3	5.5
(g/kg dm)	Range	93.0	85.3	84.0	94.7	107.7	74.9	89.9		
	CV_t (%)	8.8	10.5	8.8	13.5	13.8	8.7	10.7		
	LSD	27.4	19.9	18.0	15.0	17.3	21.9			
Lignin	**Average**	**88.5**	**95.0**	**90.2**	**108.8**	**107.0**	**104.8**	99.0	20.3	3.1
(g/kg dm)	Range	56.0	43.4	27.3	34.7	63.1	44.6	44.9		
	CV_t (%)	18.3	13.2	9.2	9.7	18.4	12.2	13.5		
	LSD	19.8	9.7	6.8	7.2	11.6	11.4			
Cellulose	**Average**	**23.3**	**22.5**	**26.4**	**22.7**	**20.0**	**17.3**	22.0	9.0	0.9
conversion	Range	16.2	12.3	8.7	8.9	8.3	10.4	10.8		
(%)	CV_t (%)	18.3	15.0	10.1	12.3	14.0	14.1	14.2		
	LSD	19.8	3.6	2.0	3.0	2.0	2.2			
Hemicellulose	**Average**	**9.6**	**10.5**	**10.4**	**12.3**	**10.8**	**8.7**	10.4	3.6	0.4
conversion	Range	4.3	5.0	4.3	3.5	3.3	3.8	4.0		
(%)	CV_t (%)	11.9	14.8	12.7	8.0	10.3	11.0	11.5		
	LSD	2.2	1.7	1.6	1.6	2.0	1.4			
CEY[*]	**Average**	**79.3**	**79.8**	**83.5**	**79.5**	**75.5**	**65.6**	77.2	17.9	2.0
(g/kg dm)	Range	34.0	32.5	19.3	22.5	24.7	34.2	27.9		
	CV_t (%)	12.5	9.8	6.3	8.7	9.7	12.3	9.9		
	LSD	13.4	8.3	4.9	7.7	6.5	6.1			

[*]CEY = Calculated ethanol yield. [§]CV_t = Coefficient of trait variation (standard deviation over genotype means / location mean × 100%). [¥]LSD = least-significant difference (0.05).

Table 7. Mean and variation in accession performance of fifteen miscanthus accessions over six trial locations (cultivation year 3, 2014 - 2015)

	NDF (g/kg dm)		CEL (g/kg dm)		HEM (g/kg dm)		LIG (g/kg dm)		CelCon %		HemCon %		CEY (g/kg dm)	
Genotype	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
OPM-1	893.0	47.0	516.1	63.5	259.2	75.1	117.7	30.7	19.6	6.0	10.7	3.5	73.0	15.0
OPM-2	835.6	106.0	470.2	96.1	269.4	83.6	96.1	51.8	25.1	12.1	11.5	2.6	84.1	19.9
OPM-3	878.5	103.3	511.1	109.5	252.3	60.9	115.1	38.1	19.3	8.7	11.1	5.3	71.5	19.0
OPM-4	876.7	92.4	509.9	87.4	260.5	81.2	106.4	32.2	21.5	9.6	11.9	4.3	79.5	24.0
OPM-5	892.7	67.2	512.0	66.1	282.5	48.0	98.1	23.1	19.9	6.4	10.8	3.7	75.2	16.7
OPM-6	859.8	80.9	478.9	81.0	286.6	37.3	94.3	12.8	24.6	6.5	12.1	4.4	86.6	13.9
OPM-7	888.9	46.2	479.6	67.4	311.3	65.0	98.0	19.1	20.2	8.4	9.2	5.2	71.1	21.7
OPM-8	876.9	89.5	480.8	100.5	291.7	32.2	104.4	20.2	20.8	10.4	10.1	2.8	73.1	18.7
OPM-9	869.3	120.3	499.1	91.4	245.6	41.4	124.7	36.6	16.8	11.4	10.1	4.2	61.3	29.1
OPM-10	889.0	76.8	505.5	109.9	286.4	61.7	97.2	20.1	20.5	12.4	10.6	4.3	75.4	27.7
OPM-11	878.7	54.0	461.2	83.1	329.2	51.5	88.3	28.5	23.7	8.8	9.2	4.6	79.2	16.7
OPM-12	873.0	88.2	463.7	127.1	322.5	53.7	86.8	24.4	24.4	11.3	9.0	2.6	80.0	20.6
OPM-13	873.1	86.3	459.2	107.8	328.7	45.0	85.1	24.6	25.2	10.9	9.7	2.8	83.3	17.0
OPM-14	880.1	61.2	472.2	91.2	322.5	36.9	85.4	27.3	24.2	8.3	9.7	3.2	82.5	17.7
OPM-15	868.7	86.3	462.4	107.6	318.3	68.0	88.0	24.4	24.8	11.4	9.8	3.3	82.2	19.8

3.3. Implications for the use of miscanthus as a lignocellulose feedstock

A consistent supply of biomass of predictable composition is a crucial factor for the success of lignocellulose biorefineries (Perlack et al., 2005). The large extent of observed genotypic variation in cell wall composition and saccharification efficiency observed in this study indicates potential for the selection of miscanthus accessions with favorable biomass quality characteristics. However, in addition to genetic factors also environmental factors substantially affect cell wall composition and conversion efficiency. From a breeding perspective a large environmental influence on the trait of interest is undesirable, as the environmentally derived part of the phenotypic variation is hard to control. This is especially problematic if the effect is unpredictable due to unknown and/or fluctuating environmental stimuli. To increase our understanding of which environmental stimuli are the cause of the observed environmental differences in mean cell wall composition and conversion efficiency values further research is needed in which a much broader range of environments is evaluated. In this way the most suitable production environment can be identified given certain biomass quality criteria posed by the end-user.

Table 8. Environmental sensitivity and genotype stability and superiority scores for calculated ethanol yield (g/kg dm) of 15 miscanthus genotypes evaluated across six locations (cultivation year 3, 2014-2015).

Genotype	Mean CEY	Environmental sensitivity *	Static stability¥	Superiority coefficient§	Superiority rank‡
OPM 1	73.05	0.54	29.65	132.10	11
OPM 2	84.05	0.66	52.48	17.40	2
OPM 3	71.51	0.93	55.73	161.90	13
OPM 4	79.53	1.13	76.25	54.10	7
OPM 5	75.23	0.93	43.31	103.70	9
OPM 6	86.64	0.78	34.05	16.30	1
OPM 7	71.13	1.21	65.50	174.00	14
OPM 8	73.13	0.99	49.76	145.50	12
OPM 9	61.29	1.50	93.61	405.00	15
OPM 10	75.43	1.48	118.93	131.50	10
OPM 11	79.25	0.94	38.76	56.00	8
OPM 12	80.03	1.03	53.48	45.10	6
OPM 13	83.25	0.92	40.40	22.00	3
OPM 14	82.52	0.85	40.66	28.70	4
OPM 15	82.24	1.04	50.60	34.80	5

*Environmental sensitivity = the slope of the regression line of the fitted Finlay Wilkinson (FW) model. ¥Static stability = the variance around the genotypic mean across environments. §Superiority coefficient = the mean square distance between genotype performance and maximum observed performance in each environment. ‡Superiority rank = Genotype ranking based on superiority coefficient.

The influence of environmental factors on biomass quality characters can also be reduced through the development of genotypes with a stable performance. Across locations large differences were observed in the range of variation between accessions, which is indicative of variation in environmental sensitivity across accessions. However, such breeding efforts are complicated by the presence of genotype-by-environment interactions, leading to differences in the ranking of accessions across locations. The fact that accession ranking varied across locations implicates that accession performance in one location might not be predictive of its performance at another location. One approach for breeders to minimize the effects of G×L effects on biomass quality is the selection of stable accessions that perform relatively well. This was shown to be a viable strategy for CEY, as a stable and superior accession (OPM-6) had the best performance in 5 out of 6 locations and average performance in the remaining trial location.

Miscanthus is a perennial crop that matures in approximately 3 years. Variation in accession performance differed substantially between cultivation years, showing that accession performance is plant age dependent. It was also observed that locational differences in plant establishment rates confounded with environmental effects during the establishment phase of the crop. Accession performance at full maturity could be predicted with reasonable accuracy based on performance in the second cultivation year. Selection for biomass quality in miscanthus through breeding should take into account these effects of environmental factors on accession performance in order to match genotype, location and end-use of miscanthus as a lignocellulose feedstock.

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Supplementary data

Supplementary Table S1. Analyses of variance for cell wall composition of 15 miscanthus genotypes grown in six locations (cultivation year 3, 2014-2015)

Source of variation*	Degrees of freedom	NDF (g/kg dm)		CEL (g/kg dm)		HEM (g/kg dm)		LIG (g/kg dm)	
		Mean squares	*F prob.*	*Mean squares*	*F prob.*	*Mean squares*	*F prob.*	*Mean squares*	*F prob.*
L	5	37557.1	<.001	46260.2	<.001	16794.5	<.001	3627.5	<.001
Residual[1]	12	504.1		802.4		572.2		122.3	
G	14	3797.6	<.001	8102.4	<.001	15565.7	<.001	2755.2	<.001
GL	70	907.8	<.001	896.9	<.001	388	<.001	134.7	<.001
Residual[2]	162	226.1		276.7		145		48.9	

*G = Genotype, L = Location, Y = Year, GL = Genotype-by-location interaction, GY = Genotype-by-year interaction, LY = Location-by-year interaction, GLY = Genotype-by-location-by-year interaction, Residual[1] = Residual block stratum, Residual[2] = Residual block*units stratum

Supplementary Table S2. Analyses of variance for conversion efficiency characters and calculated ethanol yield (CEY) of 15 miscanthus genotypes grown in six locations (cultivation year 3, 2014-2015)

Source of variation	Degrees of freedom	CelCon (%)		HemCon (%)		CEY (g/kg dm)	
		Mean squares	*F prob.*	*Mean squares*	*F prob.*	*Mean squares*	*F prob.*
L	5	427.9	<.001	66.4	<.001	1743.5	<.001
Residual[1]	12	9.0		1.7		23.8	
G	14	126.9	<.001	17.2	<.001	777.8	<.001
GL	70	11.2	<.001	1.7	0.011	56.1	<.001
Residual[2]	162	3.8		1.0		23.8	

*G = Genotype, L = Location, Y = Year, GL = Genotype-by-location interaction, GY = Genotype-by-year interaction, LY = Location-by-year interaction, GLY = Genotype-by-location-by-year interaction, Residual[1] = Residual block stratum, Residual[2] = Residual block*units stratum

Chapter 6

Dissecting genetic complexity of miscanthus cell wall composition and biomass quality for biofuels

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Abstract

Miscanthus sinensis is a high yielding perennial grass species with great potential as a bioenergy feedstock. One of the challenges that currently impede commercial cellulosic biofuel production is the difficulty to efficiently convert lignocellulosic biomass into biofuel. The development of feedstocks with better biomass quality will improve conversion efficiency and the sustainability of the value-chain. Progress in the genetic improvement of biomass quality may be substantially expedited by the development of markers associated to quality traits, which can be used in a marker-assisted selection program. To this end, a mapping population was developed by crossing two parents of contrasting cell wall composition. The performance of 182 F1 offspring individuals along with the parents was evaluated in a field trial with a randomized block design with three replicates. Plants were phenotyped for cell wall composition and conversion efficiency characters in the second and third growth season after establishment. A new SNP-based genetic map for *M. sinensis* was build using a genotyping-by-sequencing (GBS) approach, which resulted in 464 short-sequence uniparental markers that formed 16 linkage groups in the male map and 17 linkage groups in the female map. A total of 73 QTLs for a variety of biomass quality characteristics were identified, twelve of which were detected in both growth seasons. Seventeen QTLs were directly associated to different conversion efficiency characters, for which the corresponding QTLs cumulatively explained 9 - 64% of observed heritable variation. Marker sequences were aligned to the sorghum reference genome to facilitate cross-species comparisons. Analyses revealed that for some traits previously identified QTLs in sorghum occurred in homologous regions on the same chromosome. These results are a first step towards the development of marker-assisted selection programs in miscanthus to improve biomass quality for the use of miscanthus as feedstock for biofuel production.

1. Introduction

Miscanthus is a perennial C4 grass capable of producing high biomass yields in temperate climates (Heaton et al., 2010). It is a crop characterized by high resource-use efficiency owing to its early spring emergence and long vegetative phase, as well as a rhizomatous growing habit, which allows recycling of nutrients between growing seasons (Long et al., 2001; Lewandowski et al., 2003; Heaton et al., 2008). These characteristics make miscanthus an interesting lignocellulose feedstock for the production of cellulosic biofuel (van der Weijde et al., 2013). So far, *M. × giganteus* is the only species of the genus *Miscanthus* that is commercially exploited for biomass production (Lewandowski et al., 2000; Clifton-Brown et al., 2008). *M. × giganteus* (*2n = 3x = 57*) is derived from a natural cross between a diploid *M. sinensis* (*2n = 2x = 38*) and a Japanese allotetraploid species (*2n = 4x = 76*) that is most accurately named *M. ogiformis*, but is often erroneously referred to as tetraploid *M. sacchariflorus* (Lafferty and Lelley, 1994; Sacks et al., 2013). Its success is mainly due to its high productivity. In a quantitative review of biomass yields of *M. × giganteus* across hundred diverse field trial locations, the calculated average dry matter yield was 22 t ha^{-1} yr^{-1} (Heaton et al., 2004). However, the genetic variation in this triploid clone is extremely limited due to its sterility, which poses risks upon large-scale cultivation and in addition significantly limits further progress through breeding (Clifton-Brown et al., 2008; Głowacka, 2011; Yan et al., 2012; Sacks et al., 2013; Hodkinson et al., 2015). In contrast, great and largely untapped genetic diversity is harbored within and among natural populations of *M. sacchariflorus* and *M. sinensis*, which have adapted to a wide range of geographical conditions (Clifton-Brown et al., 2008; Hodkinson et al., 2015).

One of the key challenges that currently impede the wide-scale commercialization of cellulosic ethanol production resides within our inability to efficiently deconstruct the plant cell wall to release its fermentable sugars. The development of feedstocks with better biomass quality are envisioned to contribute to the economic feasibility of cellulosic biofuel technologies (Wyman, 2007; Torres et al., 2013; van der Weijde et al., 2013; Torres et al., 2016). Lignocellulosic feedstocks are chiefly composed of cellulose, hemicellulosic polysaccharides and lignin (Pauly and Keegstra, 2010). High contents of cellulose and hemicellulosic polysaccharides are desirable, as these constituents can be hydrolyzed and subsequently fermented to produce biofuels. Lignin, on the other hand, cross-links to hemicellulosic polysaccharides and forms a highly impermeable and complex matrix that shields cell wall polysaccharides from degradation and impedes the extraction of fermentable sugars from the cell wall (Grabber et al., 2004; Grabber, 2005; Himmel et al., 2007; Zhao et al., 2012). Genotypic variation in cell wall composition has been reported in *M. sinensis* and *M. sacchariflorus*, providing ample scope for improving biomass quality in these species through breeding (Allison et al., 2011; Zhao et al., 2014).

Compared to annual crops, progress in breeding of perennials, such as miscanthus, is slowed-down by the need to evaluate genotype performance in multi-year field trials. Miscanthus typically matures in three years and selection at premature stage, particularly in the first year, has been proven to be unreliable (Arnoult et al., 2015). Therefore, the application of marker-assisted selection could substantially increase the efficiency of breeding miscanthus, as selections can be done at the seedling stage using marker data. Genetic maps form the basis for finding marker-trait associations, but their construction in miscanthus is complicated by the large genome size and the high levels of heterozygosity that are the result of its obligate outcrossing nature (Głowacka, 2011; Hodkinson et al., 2015). Nonetheless, a few genetic maps of miscanthus have been published to date (Atienza et al., 2002; Kim et al., 2012; Ma et al., 2012; Swaminathan et al., 2012; Liu et al., 2015).

So far three of these genetic maps have been used for the identification of quantitative trait loci (QTLs) for different traits of interest, but none of these studies focused on biomass quality for biofuel production. The randomized amplified polymorphic DNA (RAPD) marker-based map by Atienza *et al.* has been used for identification of QTLs associated with agronomic performance and combustion quality (Atienza et al., 2003a; Atienza et al., 2003b; Atienza et al., 2003c; d; e). The simple-sequence repeat (SSR) marker-based map by Swaminathan *et al.* was used for identification of QTLs associated with agronomic performance (Gifford et al., 2015). Later it was extended with simple nucleotide polymorphism (SNP) markers obtained from restriction site-associated DNA (RAD) sequencing and used for identification of QTLs for the zebra stripe phenotype that is desirable for the use of miscanthus as an ornamental grass (Liu et al., 2015). Currently no marker-trait associations have been reported in miscanthus for traits relating to cell wall composition or biomass quality for the production of cellulosic biofuel.

Here we report the construction of a new genetic map for *M. sinensis* using short-sequence markers obtained through a genotyping-by-sequencing (GBS) approach. The mapping population used for constructing the genetic map was created to segregate for biomass quality as it was derived from a cross between two parents that were chosen based on contrasting cell wall composition. The objectives of this study were (1) to detect QTLs for biomass composition and quality in miscanthus regarding its use as a lignocellulose feedstock for biofuel production and (2) to align marker sequences to the sorghum reference genome to facilitate cross-species comparisons.

2. Materials and methods

2.1. Mapping population

A mapping population of 182 F1 progeny generated from a cross between two *M. sinensis* genotypes contrasting for cell wall composition from the breeding program of Wageningen University and Research (WUR). The male parent, hereafter referred to as P1, was a genotype (H0227) that originates from the miscanthus collection of WUR, whereas the female parent, hereafter referred to as P2, was derived from a cross between two other genotypes from the BIOMIS mapping population (H0012 × H0163). Accession H0012 was a selected genotype from the BIOMIS population described by Atienza *et al.*, (2002). Both H0012 and H0163 (grandparents) were also included in the field trial and are hereafter referred to as G1-P2 (H0012) and G2-P2 (H0163), respectively. A random sample of seeds was sown in August 2011 in trays in a heated greenhouse; seedlings were subsequently potted and raised to give rise to vigorous plants by the end of the winter of 2011/2012. They were split by the end of May 2012 into four roughly equally sized clonal pieces (ramets). Three pieces of each genotype were immediately used to establish a field trial in May 2012; one spare piece per genotype was potted to replace possible fall-outs. The trial was located at an experimental site of WUR at Wageningen and had a randomized block design with the individual ramets used as experimental units. The ramets were planted in rows with a distance between and within rows of 75 cm. The trial was fully surrounded by two rows with medium-sized *M. sinensis* plants to minimize possible border effects. In the second and third growth season heading date was scored per plant. At the end of the second and third growth season (December 2013 and 2014) all plants were harvested separately, dried to a constant dry weight using ventilated air (dm% ~92%), and weighed. A random sample of each plant was subsequently taken, from which leaves and inflorescences were separated from the stem material. The stem fraction of each plant was then chopped into ~2 cm chips, and air-dried at 60°C for 72 hours in a forced-air oven. Stem samples (n = 184 genotypes × 3 replicates × 2 years = 1104) were ground using a hammer mill with a 1-mm screen and used for biochemical analysis.

2.2. Biomass quality analysis

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents of stem dry matter were determined by detergent fiber analysis using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY). Acid detergent lignin (ADL) contents were determined after 3-hour hydrolysis of the ADF residue in 72% H_2SO_4 with continuous shaking. All analyses were performed in triplicate and fiber fractions were expressed in gram per kg dry matter. Fiber fractions were used to calculate the concentrations (in g/kg dm) of cell wall (NDF), cellulose (CEL, equals ADF – ADL), hemicellulosic polysaccharides (HEM, equals

NDF – ADF) and acid detergent lignin (ADL) in stem dry matter. The residual NDF material of the replicated fiber analyses was pooled per sample and used as a basis for determination of neutral sugar contents and Klason lignin (KL) content as described previously (van der Weijde et al., 2016b). Briefly, 30 mg of NDF material was hydrolysed for 1 hour in 0.3 ml 72% H_2SO_4 after which the acid concentration was diluted to 4% and samples were autoclaved for 60 minutes at 121 °C. Autoclaved samples were cooled and centrifuged, after which the supernatant was used for determination of glucose (GLU), xylose (XYL) and arabinose (ARA) contents using high performance anion exchange chromatography (HPAEC) analysis on a Dionex system equipped with a CarboPac PA1 column and a pulsed amperometric detector (Dionex, Sunnydale, CA). The pellet remaining after centrifugation was vacuum-filtered through a pre-weighed glass fibre filter (AP25, Fischer Scientific, Loughborough, UK). The residue was dried overnight at 103°C and weighed for the determination of KL.

Separate analyses of ground stem samples were performed for the characterization of saccharification efficiency by two different methods. The first method is used for high-throughput, small-scale quantification of the rates of glucose release by enzymatic hydrolysis of hot-water pretreated samples, as described previously (Gomez et al., 2011). The release of glucose is expressed as nmol reducing sugar released per mg biomass per hour of digestion, which is hereafter referred to as saccharification rate (SacR). The second method is aimed at quantifying final yields of fermentable sugars using a highly controlled lab-scale alkaline pretreatment and enzymatic saccharification setup, as described by van der Weijde *et al.*, (2016a). The released amounts of glucose and xylose are expressed either (1) as a weight percentage of the amount of glucose and xylose present in the untreated sample as determined by neutral sugar analysis, hereafter referred to as glucose conversion (GC) and xylose conversion (XC) or (2) as a weight percentage of the amount of cellulose and hemicellulose present in the untreated sample as determined by fiber analysis, hereafter referred to as cellulose conversion (CC) and hemicellulose conversion (HC).

To allow high-throughput analysis of all biomass quality traits we used near-infrared spectroscopy (NIRS) technology. Multivariate prediction models that combined near-infrared (NIR) spectral data and biochemical data were developed for all traits except for SacR. Near-infrared absorbance spectra of stem samples were obtained using a Foss DS2500 near-infrared spectrometer (Foss, Hillerød, Denmark) and processed by weighted multiplicative scatter correction and mathematical derivatization and smoothing treatments (2,6,4,1) using WinISI 4.9 statistical software (Foss, Hillerød, Denmark). Different prediction models were developed for different traits, depending on the number of samples that could be biochemically analyzed and on the availability of existing data for creating robust prediction models (containing a range of miscanthus samples from different experiments) (Table 1). All models contained at least 140 samples from the first growing season of the mapping population. The quality of the prediction models was validated using the squared Pearson coefficient of correlation (r^2) between predicted and biochemical data and by

evaluating for these samples the standard error of cross-validation (SECV) for each of the traits (Table 1). Subsequently, the developed prediction models were used to determine biomass composition and conversion efficiency of all 1104 stem samples.

Table 1. Summary of calibration and cross-validation statistics of mPLS models used for the prediction of biomass quality traits

	Calibration		Cross-validation								
Constituent	# Samples	SECV	# Samples	Chemical analysis			NIRS prediction			*r2*	SEP
				Mean	Min	Max	Mean	Min	Max		
NDF (g/kg dm)	510	0.81	162	88.04	80.00	92.82	88.06	81.42	92.37	0.94	0.615
ADF (g/kg dm)	512	1.05	162	55.27	47.81	63.22	55.34	48.87	63.00	0.85	0.939
ADL (g/kg dm)	491	0.96	162	6.61	4.07	11.22	6.64	4.32	10.59	0.85	0.804
KL (%ndf)	116	0.78	135	13.89	11.05	17.59	13.88	11.76	16.06	0.62	0.95
GLU (%ndf)	120	1.87	135	39.16	33.30	47.56	44.78	39.39	50.56	0.51	2.26
XYL (%ndf)	111	1.07	135	19.82	16.78	24.12	22.56	19.59	25.03	0.46	1.18
ARA (%ndf)	125	0.19	135	1.87	1.16	2.59	2.16	1.63	2.79	0.77	0.19
CC (%)	413	3.07	158	40.14	28.33	52.94	40.38	29.90	46.31	0.73	3.26
HC (%)	408	0.39	158	22.20	17.87	27.03	21.97	19.98	23.40	0.37	1.60
GC (%)	115	4.73	135	56.84	42.42	74.89	56.44	45.82	73.11	0.53	5.32
XC (%)	114	3.14	135	43.15	33.92	54.85	43.02	33.40	52.10	0.50	3.48

SECV = standard error of cross-validation in the set of calibration samples, r^2= coefficient of determination between biochemical data and NIRS predicted data; SEP = standard error of prediction

2.3. Genotyping-by-sequencing

Genomic DNA from young leaf tissues was extracted following a CTAB based protocol (Tai and Tanksley, 1990). DNA concentration and quality was checked using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and standardized using a Qubit fluorometer (ThermoFisher Scientific). DNA integrity was also confirmed on 1% agarose gels. Libraries were prepared for GBS using the restriction endonuclease ApeKI (five-cutter) to digest the genomic DNA for complexity reduction. Each digested DNA sample was ligated to a set of uniquely barcoded sequencing adaptor pairs, following PCR amplification with adapter-specific primers, and amplicons between 300-500bp were extracted from an agarose gel and sequenced in four single lanes of Illumina HiSeq2000 using a 100bp paired-end protocol. DNA digestion, adapter ligation, library construction, and sequencing were carried out by the Beijing Genomics Institute (BGI), China.

The de-multiplexed sequence reads obtained from BGI were filtered by removing those reads that did not start with the 5'-CWCG-3' site pattern, typically resulting from ApeKI digestion, or that contained undefined ('N') nucleotides. Reads were right-trimmed to a length of 82 nucleotides and clustered in order to count the number copies per unique read sequence. Note that this clustering was not only done for each sample individually,

but also separately for the forward and reverse reads. Only unique reads that occurred at least four times were kept. Unique reads from all samples were jointly clustered using the RADSNP program (RADNPGTv1.1 package, BGI, China). Our initial approach to classify genotypes was to assign a genotypic score to the studied genotypes with a cluster size of at least five reads by applying a set of classification rules to separate clustered reads. The first classification rule was that if the genotype had a frequency of 0.8 or higher for the most abundant read in the cluster, this was considered to be present in homozygous condition. The second classification rule was applied when the two most abundant reads in a cluster both had frequencies of at least 0.2. The genotype was then classified to be heterozygous. If for a particular cluster neither rule 2 nor 3 held true, no genotypic assignment was given. Unfortunately this approach did not result in acceptable data for map construction, because the average cluster size was too to allow a proper genotypic classification due to insufficient sequencing depth. Therefore we refrained from this approach and focused on segregation analyses for single reads. The number of reads for each selected sequence was in this case the basis for genotypic classification using a dominant way of scoring. Genotypes with one or more reads were considered to be either homozygous dominant or heterozygous for this short-sequence marker, whereas the ones showing no reads were supposed to be homozygous recessives. A missing value was assigned to genotype-marker combinations when both the number of reads for this marker over genotypes as well as the average number reads over all markers for a genotype was low. This was done to prevent misclassification of genotypes.

2.4. Map construction

A genetic map was constructed following the two-way pseudo test-cross strategy (Grattapaglia and Sederoff, 1994), using the dominantly scored short-sequence markers. To this end, suitable markers were first filtered out of all available markers (49102) based on segregation ratio, with only uniparental single-dose markers, i.e. markers that segregated in a 1:1 ratio in the population, used for further analysis. A total of 1145 markers remained and were coded according to segregation type following the coding scheme for cross pollinated populations as used in JoinMap (Van Ooijen, 2006).

Male simplex × female nulliplex markers were classified as *lm×ll*, while male nulliplex × female simplex markers were classified as *nn×np*. Markers were imported into JoinMap 4.1 (Kyazma, Wageningen, Netherlands) and after elimination of segregation distorted markers and markers that had high similarity (>0.99) to other markers, a total of 1003 markers were used for linkage analyses. These markers were separated into linkage groups using JoinMap grouping analysis with a maximum recombination threshold of 0.25 and a minimum independence logarithm of odds (LOD) score of 2. Markers resolved into 33 linkage groups, 16 linkage groups for the male map and 17 linkage groups for the female map. Marker order within each linkage group was

then determined using Haldane's regression mapping algorithm in JoinMap with a maximum recombination threshold of 0.40 and a minimum independence logarithm of odds (LOD) score of 1. This procedure built a map by adding loci one by one, starting from the most informative pair of loci. Each locus was added at its best position according to a goodness-of-fit measure or removed again until all loci are handled two times. The male map spanned 2139.7 cM and consisted of 242 markers with a median inter-marker spacing of 8.0 cM. The female map spanned 2479.5 cM and consisted of 322 markers with a median inter-marker spacing of 6.7 cM.

2.5. Statistical analysis and QTL mapping

General analyses of variance (ANOVA) were performed to determine the significance of genotype differences ($p<0.05$) in the mapping population for cell wall composition and saccharification efficiency. Variance analyses were performed separately for both growing seasons, taking into account the randomized complete block design of the trial. Estimates of genotypic (σ_g^2) and residual (σ_e^2) variance were used to calculated narrow sense heritability (h^2) estimates following $h^2 = \sigma_g^2/(\sigma_g^2+\sigma_e^2)$. To visualize associations amongst traits, a principle component analysis was performed on genotype means for all traits evaluated in both growth seasons. Origin centered, normalized scores for the first two principle components were plotted in a principle-component biplot. All statistical analyses were performed using Genstat for Windows, 18th edition software package (VSN International, Hemel Hempstead, UK).

Quantitative trait locus analysis was performed with MapQTL 6.0 (Kyazma, Wageningen, Netherlands) using a maximum likelihood mixture model. An interval mapping approach was used with a step size of 1.0 cM. Significance of a QTL was called based on a LOD score higher than a genome-wide significance threshold based on 1000 permutations (Churchill and Doerge, 1994), which was determined to be 3.561 for the male and 3.655 for the female map. One-LOD and two-LOD support intervals were determined to show the uncertainty on the QTL position. The percentage of variance explained (PVE) by the QTL was calculated by 100 × ([residual variance with no QTL fitted – residual variance with QTL fitted] / population variance) (Van Ooijen, 2009).

3. Results and discussion

3.1. Genotypic variation for biomass quality traits

Significant heritable variation was observed in the mapping population for all stem biomass quality traits determined after the growth seasons 2 and 3 as shown by the population statistics and parental and grand-parental values summarized in Table 2. Cell wall material (NDF) comprised by far the largest fraction of the biomass and ranged from ~815 - 911 g/kg

dm in the second and from ~877 - 918 g/kg dm in the third growth season. The main cell wall components were CEL and HEM, with variation in the population in the second growth season ranging from ~446 - 527 and ~304 - 365 g/kg dm, respectively. In the third growth season plants had on average higher CEL and lower HEM contents compared to the second growth season and ranged from ~474 - 532 and ~282 - 345 g/kg dm, respectively. Particularly large variation in cell wall glucose content (GLU) was also found, ranging from ~35 - 50% of the cell wall fraction in the second and from ~21 - 39% in the third growth season.

Table 2. Descriptive statistics of the mapping population for biomass growth and quality characteristics relevant to the use of miscanthus for biofuel production.

	Growth	P1	G-P1	G-P2	Population statistics			
Trait	**season**	**(*H0227*)**	**(*H0012*)**	**(*H0163*)**	**Mean**	**Range**	**LSD**	**h^2**
NDF (g/kg dm)	1	*	899.8	838.7	880.0	815.3 - 911.3	16.5	0.63
	2	911.5	889.0	899.1	903.0	876.9 - 917.6	10.4	0.39
ADF (g/kg dm)	1	*	576.8	500.2	546.0	490.9 - 606.8	18.8	0.63
	2	584.7	585.8	593.1	594.0	549.4 - 628.7	19.7	0.40
CEL (g/kg dm)	1	*	506.2	453.8	482.0	446.5 - 527.2	15.3	0.62
	2	499.3	495.4	497.3	502.0	474.1 - 532.1	16.6	0.40
HEM (g/kg dm)	1	*	323.0	338.5	334.0	304.5 - 364.7	11.0	0.72
	2	326.8	303.1	306.0	309.0	282.1 - 347.7	14.2	0.55
ADL (g/kg dm)	1	*	70.7	46.4	62.0	42.0 - 81.8	6.5	0.73
	2	85.4	90.4	95.8	90.0	74.8 - 110.3	6.3	0.59
CEL/cw (% NDF)	1	*	56.2	54.1	55.1	51.4 - 57.9	1.1	0.72
	2	54.8	55.7	55.3	55.5	52.9 – 58.0	1.4	0.47
HEM/cw (% NDF)	1	*	35.9	40.4	38.0	33.4 - 42.5	1.4	0.68
	2	35.8	34.1	34.0	34.3	31.4 - 38.8	1.7	0.49
ADL/cw (% NDF)	1	*	7.8	5.5	7.0	4.9 - 9.2	0.7	0.72
	2	9.4	10.2	10.7	10.0	8.3 - 12.3	0.7	0.62
KL (% NDF)	1	*	13.5	14.0	13.7	12.1 - 15.2	0.5	0.72
	2	12.3	13.9	14.1	14.1	11.5 - 15.7	0.8	0.65
GLU (% NDF)	1	*	47.5	43.3	44.5	35.5 - 49.8	4.6	0.27
	2	19.1	33.6	28.6	29.8	20.6 - 39.2	6.1	0.33
XYL (% NDF)	1	*	23.6	20.9	22.8	20.6 - 25.1	0.9	0.59
	2	25.8	23.8	24.7	24.7	22.4 - 27.1	1.3	0.46
ARA (% NDF)	1	*	2.1	2.7	2.2	1.8 - 2.8	0.2	0.65
	2	2.0	2.2	1.9	2.0	1.7 - 2.5	0.2	0.56
CC (% CEL)	1	*	40.1	46.2	41.2	36.9 - 46.2	1.7	0.66
	2	34.7	36.0	33.2	25.3	30.2 - 39.1	3.1	0.35
HC (% Hem)	1	*	22.8	21.6	22.0	21.3 - 22.6	0.4	0.46
	2	20.6	21.7	21.1	13.7	20.0 - 22.1	1.5	0.31
GC (% GLU)	1	*	52.4	66.2	55.7	48.7 - 66.4	3.8	0.57
	2	50.8	55.9	47.6	42.0	29.6 – 72.0	8.4	0.47
XC (% XYL)	1	*	41.0	48.6	42.2	34.8 - 50.2	2.9	0.58
	2	33.9	39.8	32.5	29.6	18.7 - 51.9	6.5	0.45
SacR (nmol/mg/h)	1	18.1	14.4	21.3	18.1	10.7 - 24.3	3.7	0.53

LSD = least significant difference (p = 0.05), h^2 = heritability

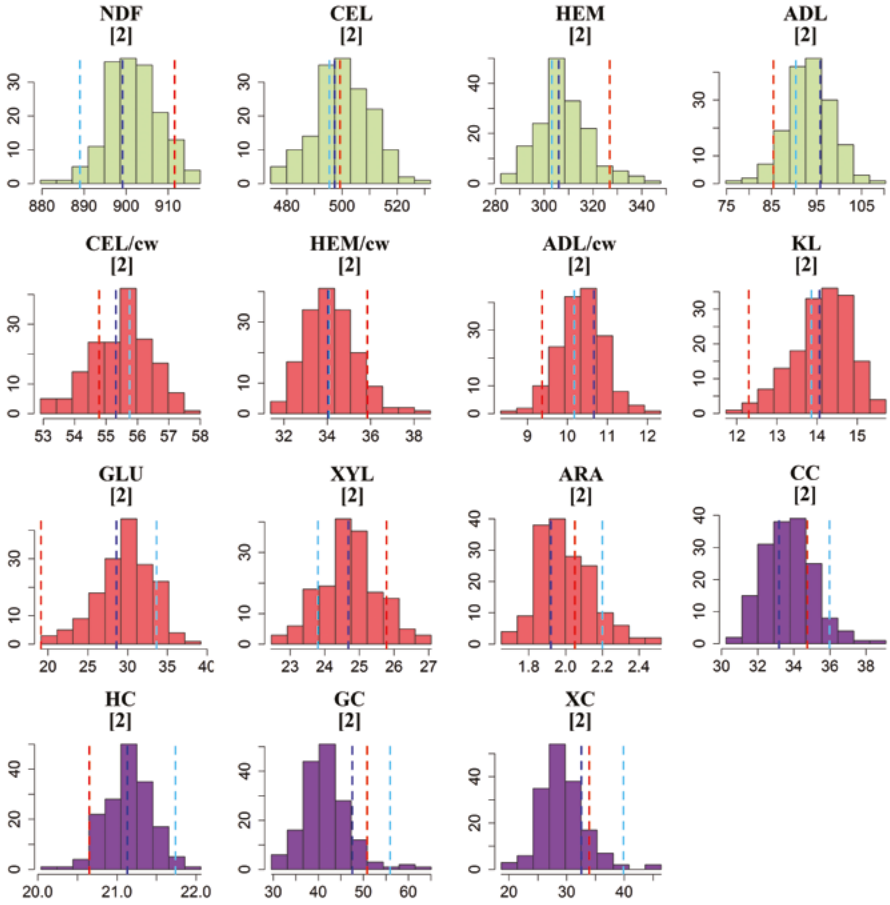

Figure 1. Histograms displaying the frequency distributions of genotype values for stem composition and conversion efficiency characters after the third growth season. Unit of the y-axis is the number of genotypes, while the unit of the x-axis depends on the unit of the plotted trait. Lines represent (grand)parental values, with red line depicting P1, the light-blue line depicting G1-P2 and the dark-blue line depicting G2-P2.

Variation in ADL ranged from ~42 - 82 g/kg dm in the second and from ~75 - 110 g/kg dm in the third growth season. ADL/cw and KL ranged from ~5 - 9% and ~12 - 15% of the cell wall in the second and from ~8 - 12% and 12 - 16% in the third growth season, respectively. Variation in lignin content is of particular interest for improving biomass quality of miscanthus, and variation in both ADL and KL was extensive. KL values are higher than ADL values, as during the quantification of ADL detergents are used that likely dissolve a fraction of the total lignin. However, KL values might overestimate lignin as it is more likely to be contaminated with protein (Hatfield et al., 1994; Hatfield and Fukushima, 2005). However, both methods provide valuable insights into biomass quality (van der Weijde et al., 2016b).

The mapping population also harbored extensive variation in conversion efficiency. Particularly for SacR, GC and XC considerable variation was observed among genotypes.

Variation in SacR ranged from ~11 - 24 nmol reducing sugars per mg biomass per hour. Variation in GC and XC ranged from ~49 - 66% and ~35 - 50%, respectively, in the second and from ~30 - 72% and ~19 - 52% in the third growth season. These ranges are congruent with the ranges observed in other highly diverse sets of miscanthus genotypes (Xu et al., 2012; Li et al., 2013; Zhao et al., 2014; van der Weijde et al., 2016a; van der Weijde et al., 2016b), indicating that variation in conversion efficiency in this population created by crossing two highly compositionally distinct parents is substantial. Conversion efficiency values in the third growth season were substantially lower than those found in the second, which is presumably associated with the increase in lignin content observed with increasing plant age (Table 2).

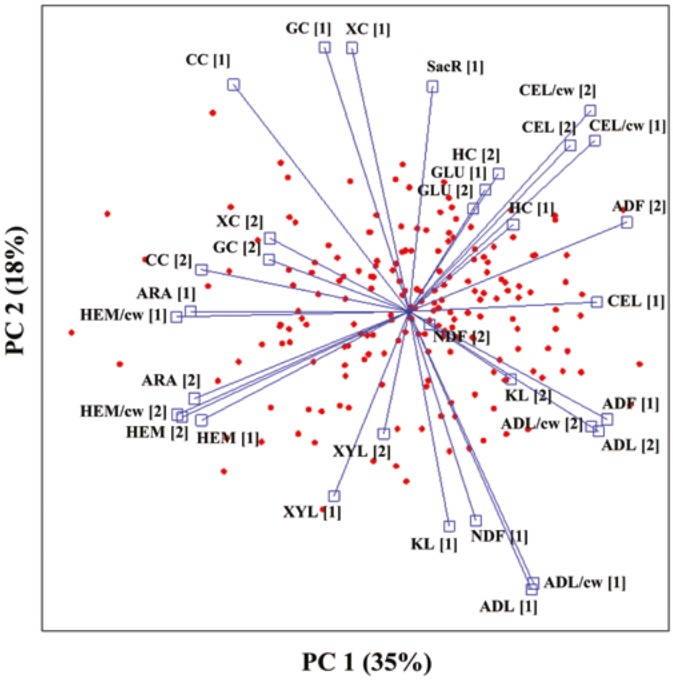

Figure 2. Principal component biplot displaying variation in cell wall composition and conversion efficiency harbored within the mapping population. Red dots are genotype mean scores. Trait names designated with '[1]' were scored after the second growth season of the population, while those designated with '[2]' were scored after the third growth season. Vectors represent traits, with the angle between a vectors and the principal component axis proportional to the contribution of the corresponding trait to those principal components. The length of vectors is proportional to the variance observed for the corresponding trait and the angle between vectors is proportional to the correlation among characters.

Genotype performance for most of the evaluated traits was highly reproducible across replicated blocks. As result, for most traits a high heritability ($h^2 > 0.5$) was observed, with the highest heritability observed for lignin measurements ($h^2 = 0.62$ - 0.72). For GLU a relatively low heritability was observed ($h^2 < 0.27$ - 0.33). The heritability estimates for compositional and conversion efficiency characters, including the low heritability observed for GLU, are

consistent with values observed by others in maize and sorghum mapping studies (Murray et al., 2008; Lorenzana et al., 2010; Torres et al., 2014). The low heritability of GLU can be the result of environmental effects, error in biochemical analyses and/or low NIRS prediction accuracy.

Frequency distributions of all traits evaluated in the third growth season were all reasonably uniform and showed continuous unimodal histograms (Figure 1). For all traits, with the exception of CEL, parental and grand-parental performance were contrasting and for most traits population variation extended beyond parental and grand-parental values in both directions. For KL and GLU the performance of P1 was very near the low-end population extreme, so genetic variation leading to concentrations lower than observed in P1 for those traits is not expected in this population.

Principle component analysis revealed that approximately 53% of the observed genotypic variation in biomass quality resolved into two composite variables (Figure 2). The first principle component summarized 35% of the observed genotypic variation and predominantly discriminated genotypes based on differences in contents of cellulosic and hemicellulosic polysaccharides. The second component, which summarized 18% of the observed variation, discriminated genotypes mostly based on differences in lignin and conversion efficiency characters. As the angle between vectors is representative of correlations between traits, from this plot it can be deduced that the different conversion efficiency characters are associated to different compositional features, with CC being positively affected by a high content of hemicellulosic polysaccharides, while SacR was positively affected by a high content of cellulosic polysaccharides. As expected, conversion characters were negatively associated to ADL and KL. These trait associations are consistent with other reports on miscanthus biomass composition and quality for biofuel production (Xu et al., 2012; van der Weijde et al., 2016a; van der Weijde et al., 2016b).

3.2. Synteny with Sorghum bicolor and coding of linkage groups

The DNA sequences of the mapped markers were aligned to the *Sorghum bicolor* (L.) Moench genome (version 'sbi1') from the Plant Genome Database using NCBI BLASTN (Altschul et al., 1990). Only hits with an identity score greater than 85% and an alignment length of at least 50 nucleotides were retained and used to label the miscanthus linkage groups according to which sorghum chromosome the markers in each linkage group mapped (Supplementary figure S1). Linkage groups of the female map were designated by the corresponding *Sorghum bicolor* chromosome numbers, followed by an 'a' or 'b', as well as linkage groups of the male map, but followed by a 'c' or 'd'. These suffixes are randomly appointed to the two homeologous miscanthus linkage groups of each map that are syntenic to each sorghum chromosome, as the genome of *M. sinensis* consists of two sub-genomes with a high level of synteny to the sorghum genome (Kim et al., 2012; Ma et al., 2012). In both the male and the female map there was one linkage group that aligned with two sorghum chromosomes; these groups were

designated '4b7b' and '4d7d'. The occurrence of this phenomenon in miscanthus has been reported previously and is ascribed to an ancient chromosome fusion or translocation event between two miscanthus chromosomes syntenic to sorghum chromosomes 4 and 7. This event explains why miscanthus has a basic chromosome number of 19 and not 20 (twice the basic chromosome number of sorghum) (Ma et al., 2012; Swaminathan et al., 2012).

Table 3. Observed QTLs for stem cell wall composition and conversion efficiency characters

QTL	Year	LG	Position (cM)	1-LOD support interval (cM)	2-LOD support interval (cM)	LOD	PVE
NDF1	2013	2c	0.0	0.0 - 4.0	0.0 - 8.7	5.77	13.9
NDF2	2014	3a	61.0	52.0 - 67.1	48.0 - 76.5	4.98	13.6
NDF2	2013	3a	64.1	51.0 - 67.1	33.3 - 70.6	3.96	9.5
NDF3	2014	3a	101.8	96.1 - 106.8	41.9 - 134.3	3.70	10.3
NDF4	2013	3b	190.1	188.9 - 195.1	186.9 - 213.0	4.33	10.4
NDF5	2013	3c	4.0	0.0 - 9.6	0.0 - 12.6	5.73	15.0
NDF6	2014	3d	40.1	36.1 - 55.3	20.3 - 58.3	4.00	10.3
NDF6	2013	3d	44.2	37.1 - 50.4	36.1 - 55.3	4.72	12.9
NDF7	2013	4c	81.1	71.4 - 95.1	67.4 - 102.1	3.77	11.0
NDF8	2014	4c	156.7	148.7 - 164.7	143.0 - 168.7	3.58	10.2
NDF9	2013	6c	98.8	86.8 - 104.0	80.8 - 131.0	3.59	10.1
ADF1	2013	3b	191.1	187.9 - 197.5	185.9 - 213.0	3.81	9.6
ADF2	2014	4b7b	40.7	31.9 - 48.7	25.8 - 66.6	3.74	9.3
CEL1	2013	6c	181.9	176.9 - 183.3	143.8 - 183.3	3.98	10.8
CEL/cw1	2014	6b	18.5	14.8 - 22.5	4.2 - 60.1	4.44	11.2
CEL/cw2	2013	6b	33.8	26.8 - 40.8	10.2 - 50.9	7.54	23.0
CEL/cw2	2014	6b	36.8	27.8 - 48.9	6.2 - 57.1	4.78	14.6
CEL/cw3	2014	6c	81.8	48.1 - 92.8	38.0 - 98.8	4.85	15.8
CEL/cw3	2013	6c	82.8	72.8 - 89.8	53.1 - 92.8	9.02	29.1
CEL/cw4	2014	6c	136.0	129.0 - 147.8	125.0 - 153.8	4.14	11.2
CEL/cw4	2013	6c	147.8	126.0 - 157.8	122.0 - 162.8	4.61	16.5
HEM1	2013	2c	2.0	0.0 - 6.7	0.0 - 8.7	3.78	10.3
HEM2	2013	6b	34.8	26.8 - 59.1	18.5 - 63.1	4.88	15.5
HEM2	2014	6b	48.9	28.8 - 59.1	18.5 - 63.1	4.16	10.7
HEM3	2014	6c	79.8	71.8 - 89.8	45.1 - 94.8	6.06	18.5
HEM3	2013	6c	81.8	71.8 - 89.8	52.1 - 92.8	7.81	25.6
HEM/cw1	2013	4b7b	39.7	31.9 - 42.5	27.8 - 46.7	4.40	11.6
HEM/cw2	2013	4d7d	55.8	52.5 - 58.8	49.5 - 61.8	3.64	9.3
HEM/cw3	2014	6c	79.8	52.1 - 89.8	36.0 - 95.8	5.11	16.1
HEM/cw3	2013	6c	74.8	52.1 - 85.8	45.1 - 90.8	4.61	12.7
ADL1	2013	3c	2.0	0.0 - 7.0	0.0 - 11.6	5.18	13.4
ADL2	2013	4c	74.4	66.4 - 95.1	62.4 - 103.1	3.87	10.8
ADL3	2013	4d7d	31.7	26.7 - 37.0	16.1 - 40.0	3.76	11.4
ADL4	2013	6c	116.7	109.8 - 132.0	106.8 - 138.8	3.81	10.2
ADL5	2013	8c	7.0	2.0 - 9.5	0.0 - 11.5	5.57	14.4
ADL6	2014	8c	28.8	25.6 - 33.8	20.6 - 36.8	5.25	13.4
ADL/cw1	2013	3c	2.0	0.0 - 7.0	0.0 - 13.6	4.45	11.6
ADL/cw2	2013	4d7d	31.7	26.7 - 37.0	9.0 - 40.0	3.79	11.4
ADL/cw3	2013	4d7d	55.5	50.5 - 60.8	47.5 - 63.8	3.78	9.9

ADL/cw4	2013	8c	6.0	2.0 - 9.5	0.0 - 12.5	5.51	14.8
ADL/cw5	2014	8c	28.8	24.6 - 33.8	20.6 - 35.8	5.61	14.2
KL1	2014	1b	71.7	68.2 - 74.7	57.2 - 102.9	5.23	12.8
KL2	2013	1b	87.9	80.9 - 94.9	77.9 - 98.9	6.45	21.0
KL2	2014	1b	87.9	79.9 - 96.9	56.2 - 106.5	4.84	16.3
KL3	2014	1c	77.8	72.1 - 84.8	58.5 - 114.0	3.69	9.9
KL4	2014	2a	0.0	0.0 - 2.0	0.0 - 4.0	4.66	11.4
KL5	2014	2a	49.1	45.1 - 54.9	44.1 - 56.9	5.98	16.7
KL6	2014	2d	102.3	95.3 - 105.3	81.3 - 118.1	5.58	15.0
KL7	2014	3a	63.0	52.0 - 67.1	46.0 - 69.1	4.07	10.0
KL8	2014	3d	25.3	18.3 - 33.3	10.6 - 60.5	4.30	14.6
KL9	2014	4c	157.7	150.7 - 163.7	147.7 - 165.7	5.57	15.0
KL10	2014	6d	76.0	66.0 - 87.9	62.0 - 120.3	3.87	10.3
KL11	2014	6d	100.8	94.8 - 112.3	62.0 - 120.3	3.92	12.8
GLU1	2013	5a	94.0	78.4 - 101.7	57.7 - 106.7	4.30	12.5
GLU2	2013	6b	36.8	25.8 - 55.1	13.8 - 58.1	4.01	12.5
GLU3	2014	6c	155.8	144.8 - 172.3	137.8 - END	4.44	15.1
XYL1	2013	1a	28.3	16.3 - 39.3	9.0 - 44.3	3.71	12.7
XYL2	2013	2c	0.0	0.0 - 6.7	0.0 - 8.7	3.76	9.2
XYL3	2013	6b	42.8	31.8 - 51.9	26.8 - 59.1	4.23	11.1
XYL4	2013	6c	83.8	71.8 - 93.8	60.1 - 99.8	3.79	14.2
ARA1	2014	2c	89.2	82.2 - 97.0	78.9 - 101.0	3.83	11.7
ARA2	2014	3d	27.3	19.3 - 40.1	4.6 - 58.3	3.68	12.5
ARA3	2014	3d	45.2	42.2 - 51.4	4.6 - 58.3	3.64	11.0
ARA4	2013	6b	33.8	24.8 - 43.8	13.8 - 63.1	4.61	14.6
ARA4	2014	6b	49.9	35.8 - 55.1	29.8 - 58.1	6.46	16.4
ARA5	2013	6c	61.1	51.1 - 83.8	46.1 - 88.8	6.37	19.9
ARA5	2014	6c	63.1	55.1 - 71.2	51.1 - 87.8	9.10	26.4
SacR1	2013	3b	23.6	16.0 - 30.6	12.0 - 55.0	4.01	11.0
SacR2	2013	3c	7.0	2.0 - 11.6	0.0 - 14.6	6.43	16.1
SacR3	2013	5c	58.8	54.2 - 61.8	44.0 - 74.7	3.92	10.2
SacR4	2013	6b	33.8	25.8 - 42.8	18.5 - 51.9	4.31	14.7
SacR5	2013	6c	55.1	44.0 - 65.1	39.0 - 71.2	4.44	15.3
SacR6	2013	6c	90.8	83.8 - 99.8	79.8 - 107.8	5.96	21.9
SacR7	2013	10b	52.2	44.8 - 56.7	40.8 - 59.7	4.19	10.7
CC1	2014	4b7b	32.9	24.8 - 42.5	15.5 - 48.7	4.26	11.4
CC2	2013	4d7d	30.7	26.7 - 36.0	17.1 - 39.0	4.12	12.8
CC3	2013	8c	7.5	3.0 - 12.5	0.0 - 34.8	3.75	9.6
CC4	2014	8c	28.8	26.6 - 32.8	22.6 - 34.8	5.65	14.7
HC1	2014	6b	44.7	35.8 - 49.9	29.8 - 75.2	3.79	9.2
HC2	2014	6c	71.8	64.1 - 79.8	54.1 - 85.8	4.78	11.4
GC1	2013	3c	5.0	0.0 - 15.6	0.0 - 19.6	4.61	12.3
GC2	2013	5a	33.9	24.8 - 34.9	20.8 - 36.9	3.66	8.9
XC1	2013	6b	9.2	4.2 - 15.8	2.2 - 22.5	4.97	14.1
XC2	2013	6b	40.8	32.8 - 44.7	28.8 - 49.9	5.82	16.8
XC3	2013	6c	129.0	122.0 - 137.8	108.8 - 145.8	4.08	14.2
HD1	2014	1c	37.3	35.3 - 46.3	31.0 - 50.3	3.93	9.8
HD2	2014	3c	0.0	0.0 - 4.0	0.0 - 7.0	3.89	9.4
HD3	2014	6d	0.0	0.0 - 5.0	0.0 - 9.0	3.60	8.9

LG = linkage group, PVE = percent variance explained

3.3. QTL mapping of miscanthus biomass quality traits

QTL analysis was performed to investigate associations between genomic regions and stem composition and conversion traits. In a combined QTL analysis carried out on the male and female map simultaneously a total of 73 QTLs were found to be associated with cell wall composition and conversion efficiency characters with LOD scores ranging from 3.58 – 9.10 (Table 3). Heterozygosity was uncovered in 46 loci of the male parent and 27 loci of the female parent, but these loci might be partly the same. Twelve out of the 73 QTLs were found in both growth seasons. In the combined analysis significant QTLs were located across 19 out of the total of 33 male and female linkage groups (Figure 3). For several traits, QTLs were observed to be present in roughly the same genomic position in presumably homeologous linkage groups in both parental maps.

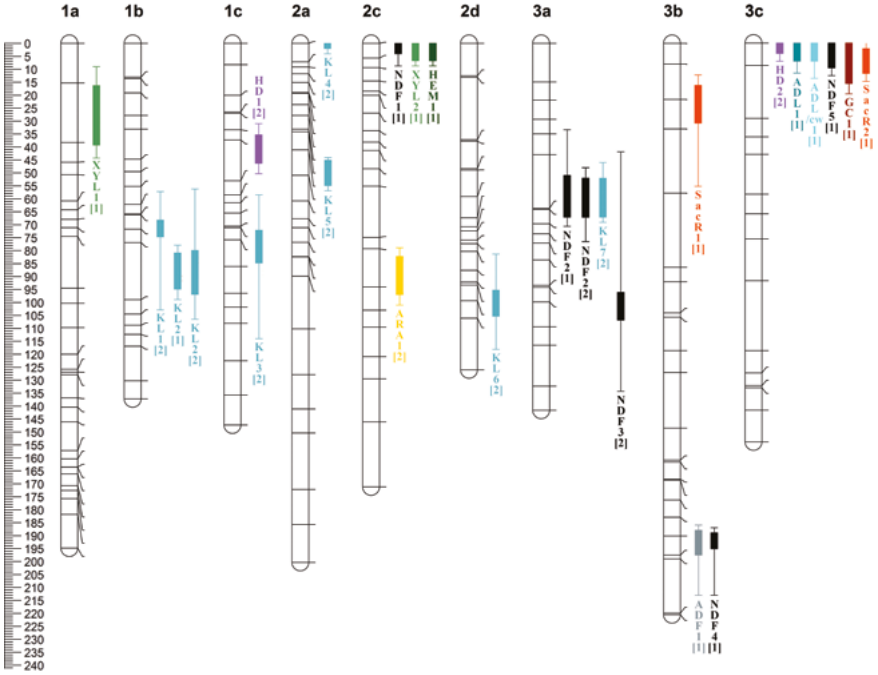

Figure 3. Distribution of QTLs identified for biomass composition and conversion efficiency across 19 linkage groups of two genetic maps of M. sinensis. Linkage groups designated with 'a' or 'b' originate from the female map, while those designated with 'c' or 'd' originate from the male map. QTLs designated with '[1]' were observed in the second growth season of the population, while those designated with '[2]' were observed in the third growth season.

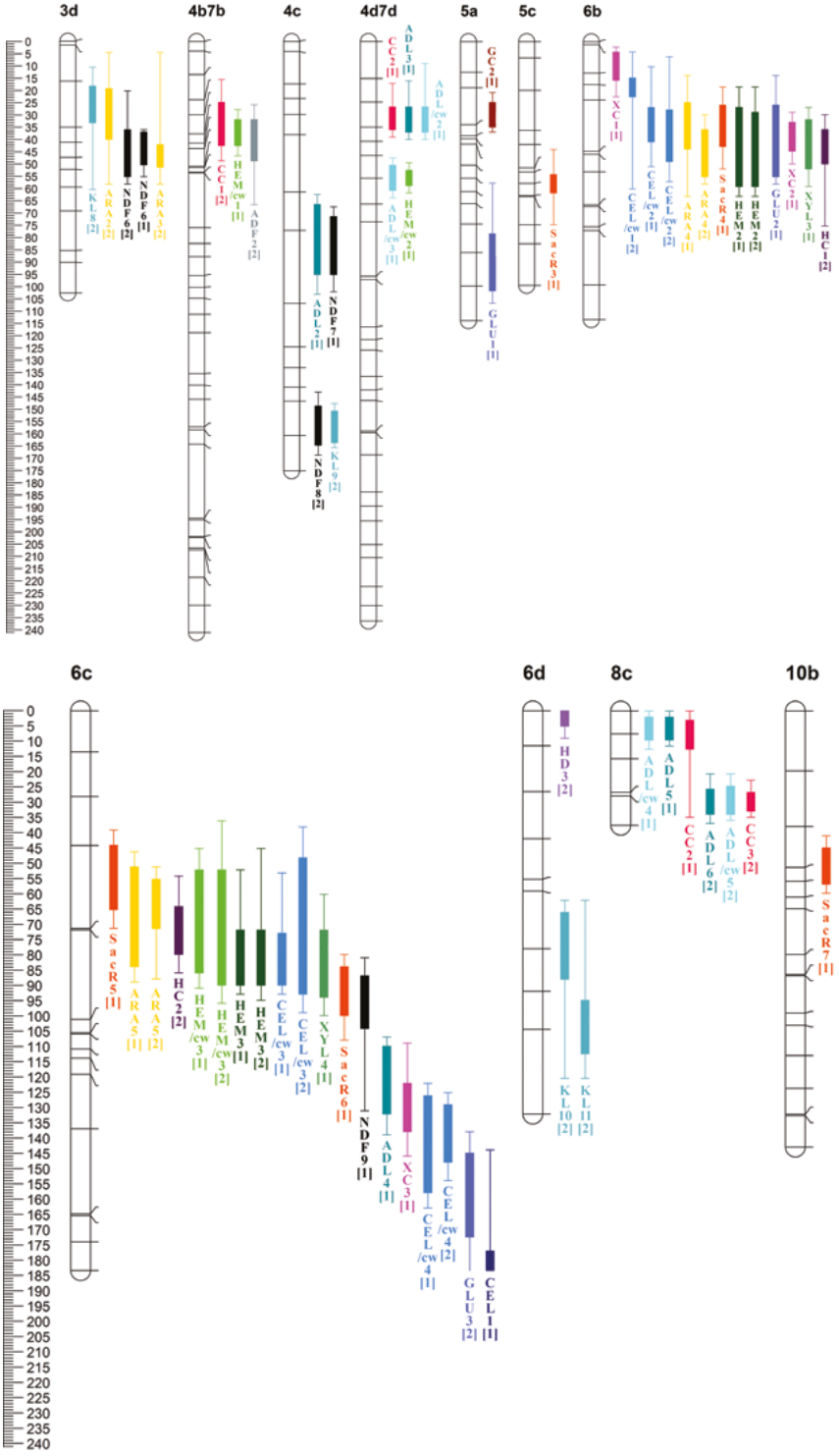

Figure 3. Continued

Out of the 73 QTLs that were observed, 9 were associated with stem cell wall, 5 with cellulose, 6 with hemicellulosic polysaccharides, 22 with lignin and 12 with neutral sugar contents (Table 3). These QTLs cumulatively explained 9 - 78 % of the observed genotypic variation in different compositional characters across both growth seasons, taking into account their appropriate classification in male or female map (Figure 4). The large amount of QTLs found to be associated with lignin content can partly be explained by the fact that three different lignin characters (ADL, ADL/cw and KL) were evaluated. However, QTLs associated with KL did not co-localize with QTLs for ADL or ADL/cw (Figure 3). Two major-effect QTLs were identified for CEL/cw in linkage groups of the male parent that together explained 46% of the observed genotypic variation (Figure 4). These may be interesting targets for further study.

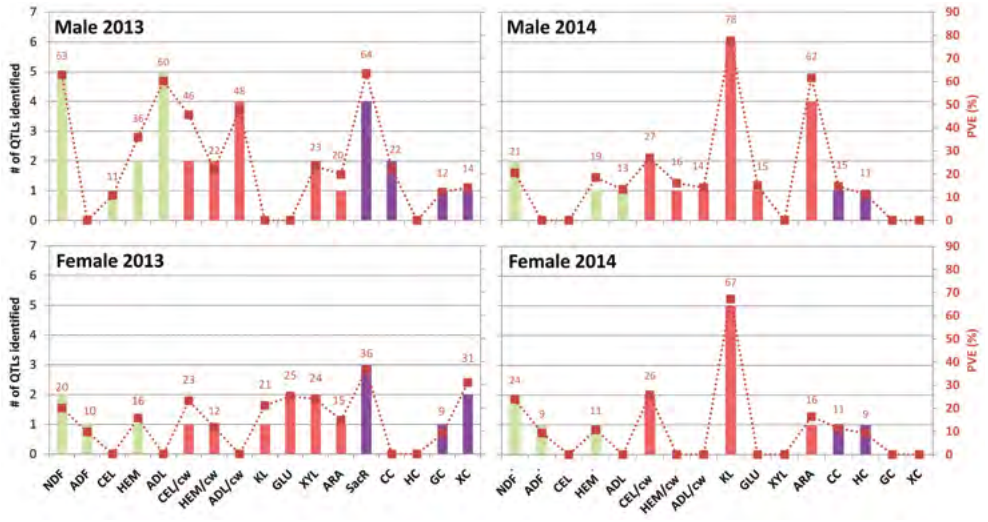

Figure 4. Summary of identified QTLs for stem composition and conversion efficiency characters in the male and the female map across two growth seasons. Colored bars indicate the total number of QTLs identified for each trait. Points on the line graph indicate the accumulated percentage of variance (PVE) explained by the QTLs.

A total of 18 QTLs were found for conversion efficiency characters with LOD-scores ranging from 3.65 – 6.43, among which 7 for SacR, 4 for cellulose conversion, 2 for hemicellulose conversion, 2 for glucose conversion and 3 for xylose conversion (Table 3). These QTLs cumulatively explained 9 - 64 % of the observed genotypic variation in different conversion characters across both growth seasons, taking into account their appropriate classification in male or female map (Figure 4). QTLs for SacR and GC co-localized on linkage group 3c and QTLs for SacR, HC and XC co-localized on linkage groups 6b and 6c (potentially homologous groups). However, most QTLs for the different conversion characters did not co-localize and seem to be independently controlled characters (Figure 3). On linkage groups 3c, 4d7d, 6c and 8c QTLs for conversion efficiency characters co-localized with QTLs for lignin characters. Particularly strong evidence for co-localization of QTLs for these traits was found on linkage group 8c, where QTLs for ADL/cw, ADL and CC were identified in two separate genomic regions in both

growth seasons. On linkage groups 4b7b, 6b and 6c QTLs for conversion efficiency characters co-localized with QTLs for accumulation of hemicellulosic polysaccharides. A big clustering of co-localized QTLs were observed on linkage groups 6b and 6c, possibly indicating the presence of a master-regulator affecting cell wall biosynthesis. QTLs for the same traits co-localized in both clusters, suggesting that 6b and 6c are homologous linkage groups. Some QTLs for conversion efficiency characters did not co-localize with any of QTLs for compositional characters evaluated in this study, suggesting that other unidentified compositional characters are affecting conversion efficiency. One such character, for example, could be the content of hydroxycinnamic acids, such as *para*-coumaric or ferulic acids, which were recently identified as key factors conversion efficiency in miscanthus (van der Weijde et al., 2016a).

3.4. Comparative analysis of QTLs in miscanthus and sorghum

In addition to identifying QTLs for miscanthus biomass composition and conversion characters, an objective of this study was to demonstrate that by aligning the genetic map of miscanthus to the physical map of *Sorghum bicolor*, the exchange of information from genetic studies across species is facilitated and a wealth of information becomes available for the genetic improvement of miscanthus. For this particular objective the heading date of the genotypes used in this study was scored in both growth seasons, as this is a trait that normally has a high heritability in miscanthus and was previously mapped in miscanthus (Gifford et al., 2015). Due to the high level of synteny between miscanthus and sorghum QTLs found in one species might have corresponding QTLs in homologous regions in the other. In this study, 3 QTLs were identified for heading date, located on linkage groups 1c, 3c and 6d (Table 3). A QTL for heading date on the linkage group that aligns with Sb03 was also identified by Gifford *et al.,* (2015) on the same position at the end of the chromosome arm (position 6 – 9 cM) as HD2 in this study. In addition, a QTL for heading date was consistently reported in sorghum on the end of the chromosome arm of Sb06 (Murray et al., 2008; Felderhoff et al., 2012; Zou et al., 2012), which is in accordance with HD3 found in this study.

Similarly, QTLs for NDF are reported in sorghum on chromosomes Sb02, Sb03, Sb04 and Sb06 (Murray et al., 2008; Shiringani and Friedt, 2011), which may correspond to the QTLs for NDF found in this study on the corresponding linkage groups 2c, 3a, 3c, 4c and 6c (Table 3, Figure 3). The QTL on chromosome Sb03 was reported to have a strong effect and explained a large fraction of the observed variation in a sorghum mapping population (Murray et al., 2008). The strong effect of this QTL in sorghum may explain why the presumably corresponding QTL was detected on both the female and the male map in both growth seasons (NDF2 on linkage group 3a and NDF6 on linkage group 3d). QTLs for ADL were identified on Sb03, Sb04, Sb06, Sb07 and Sb08 in sorghum (Murray et al., 2008; Shiringani and Friedt, 2011), which may correspond to QTLs for ADL in this study, which were observed on all of the corresponding linkage groups (Table 3, Figure 3). Similar to the clusters of QTLs for different traits that co-localized on miscanthus linkage groups 6b and 6c, a cluster of co-localizing QTLs, including

QTLs for cellulose and hemicellulosic polysaccharide accumulation, was observed in sorghum chromosome Sb06 (Shiringani and Friedt, 2011). In a number of genetic studies in sorghum that mapped conversion efficiency characters QTLs for conversion efficiency repeatedly mapped to chromosome Sb03, Sb04 and Sb07 (Wang et al., 2011; Vandenbrink et al., 2013; Wang et al., 2013). In this study QTLs for SacR and GC were located on corresponding linkage groups 3b, 3c, 4b7b and 4d7d. However, several QTLs also mapped to linkage groups that correspond to sorghum chromosome Sb06, for which no QTLs associated with conversion efficiency were detected in sorghum so far. These represent potentially previously unidentified loci affecting conversion efficiency.

The facts that (1) several QTLs were identified in both growth seasons and (2) that for several QTLs potentially corresponding QTLs were mapped to the same chromosome or sometimes even chromosomal segment in sorghum or a corresponding homeologous linkage group in miscanthus provide some indications that these QTL truly contain genetic controllers for the traits of interests. Characterization of these QTLs however needs further evaluation studies. The alignment of this miscanthus genetic map to the *Sorghum bicolor* physical map facilitates the exchange of information between the two species, as well as to other grass species with a syntenic relationship to sorghum. Novel tools, such as the Orphan Crop Genome Browser (http://www.bioinformatics.nl/denovobrowser/db/species/index) provide excellent opportunities to exploit such phylogenetic relationships to annotate the genome of miscanthus. Using this tool the regions in the sorghum genome that are homeologous to the QTLs mapped in miscanthus in this study can be easily examined for putative orthologous genes that are reported to affect cell wall compositional characters in crops such as sorghum, maize or rice. To our knowledge this is the first report of QTLs for biomass composition and conversion efficiency characters in miscanthus. These results are a first step towards the development of marker-assisted selection programs in miscanthus to improve biomass quality for biofuel production.

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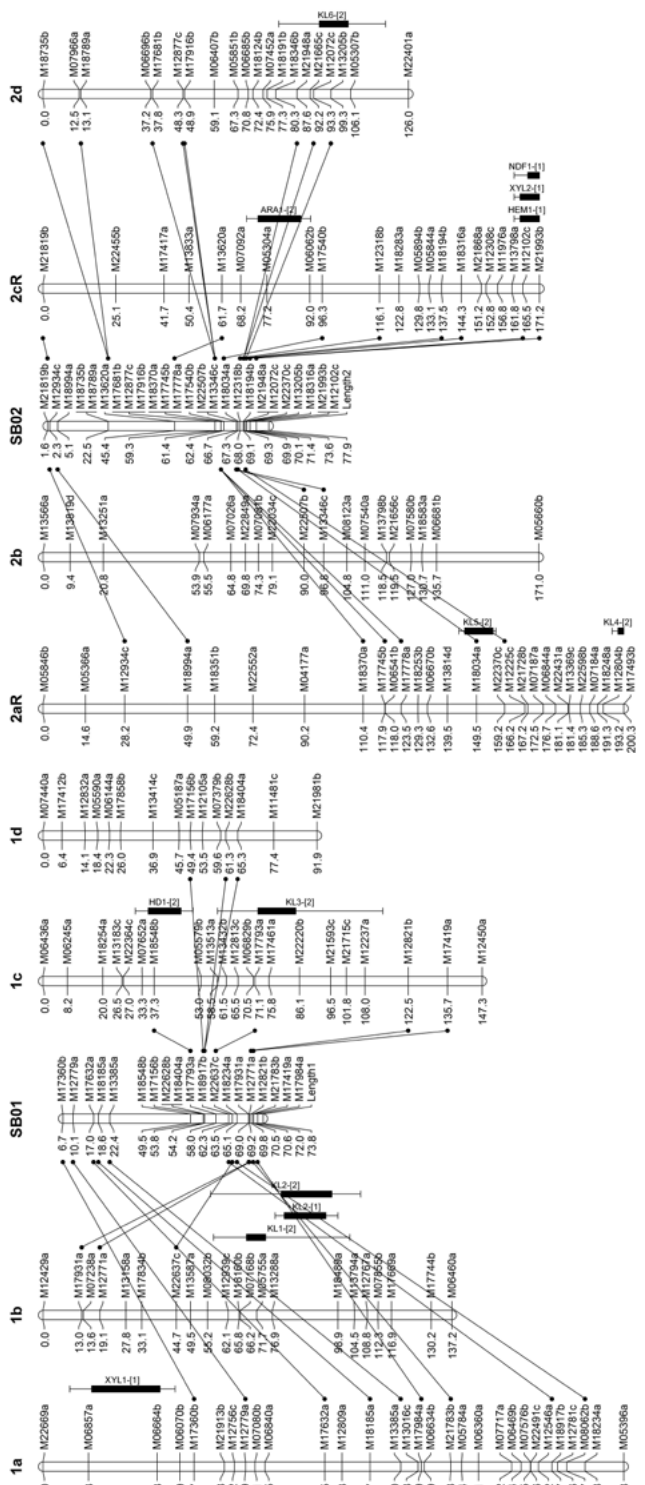
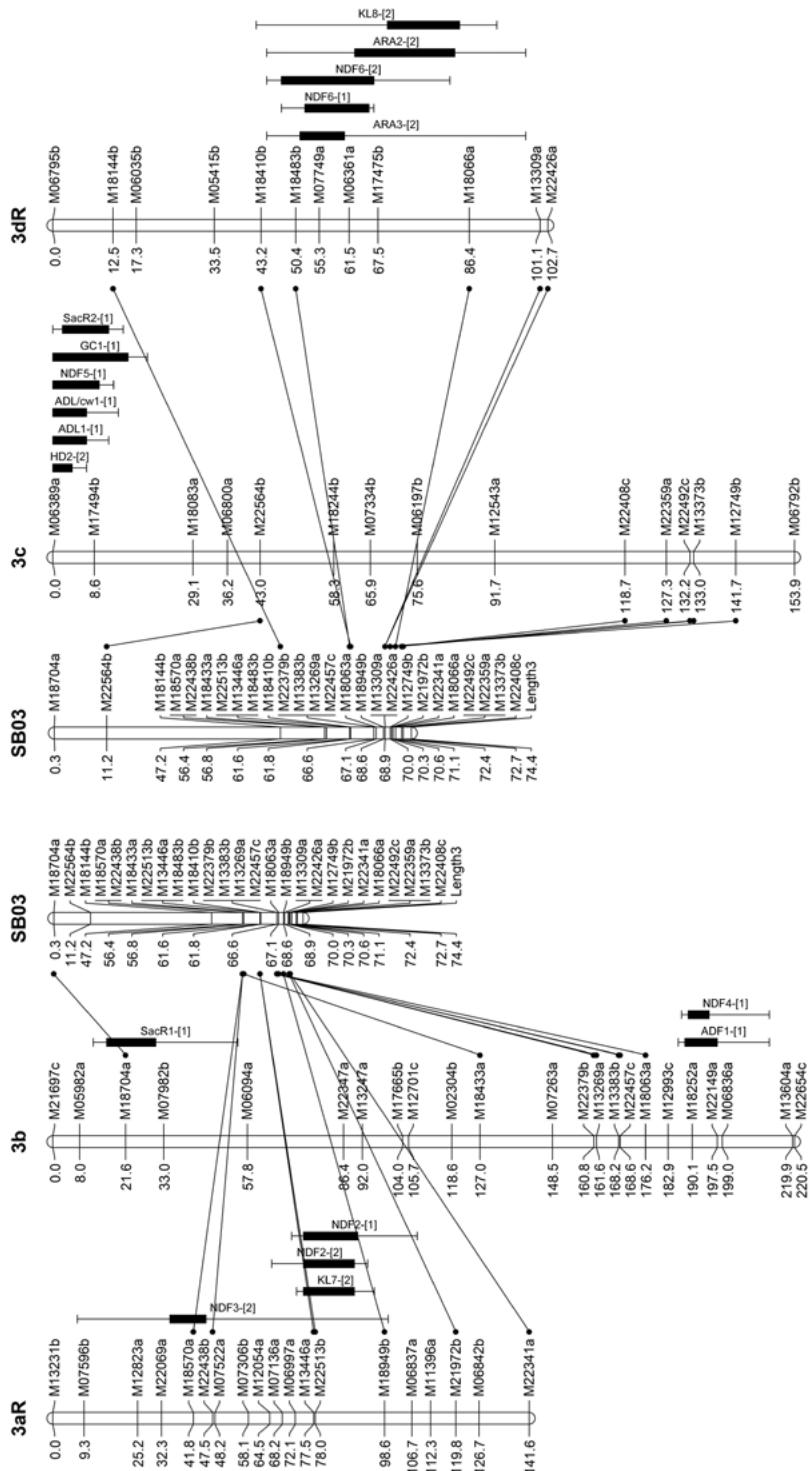
Supplementary Data



Supplementary Figure S1. Combined representation of the male and female linkage groups of the M. sinensis genetic map in cM and the physical map of Sorghum bicolor in Mbp. Homologous markers were defined using NCBI blast. Linkage groups designated with 'a' or 'b' originate from the female map, while those designated with 'c' or 'd' originate from the male map. Linkage groups with an added 'R' are reversed to facilitate comparison to sorghum. QTLs designated with '[1]' were observed in the second growth season of the population, while those designated with '[2]' were observed in the third growth season.



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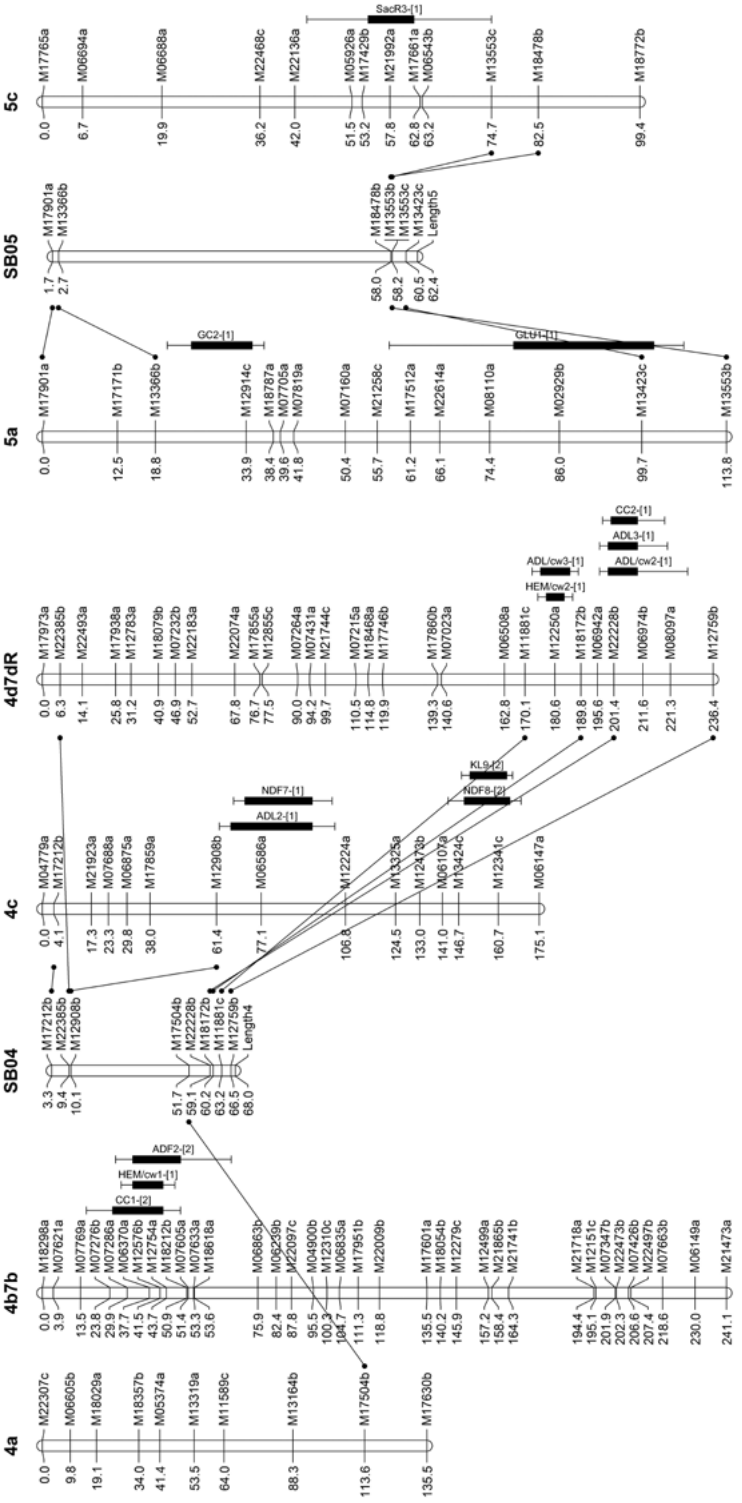

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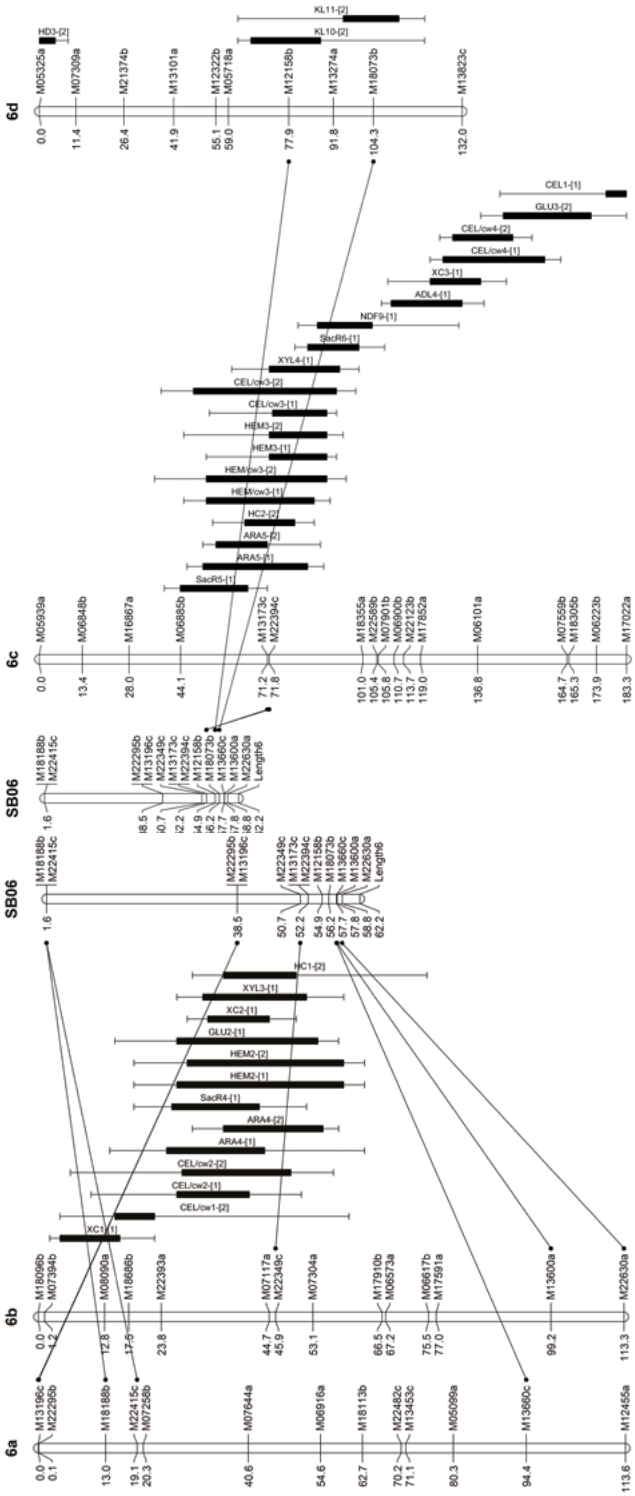

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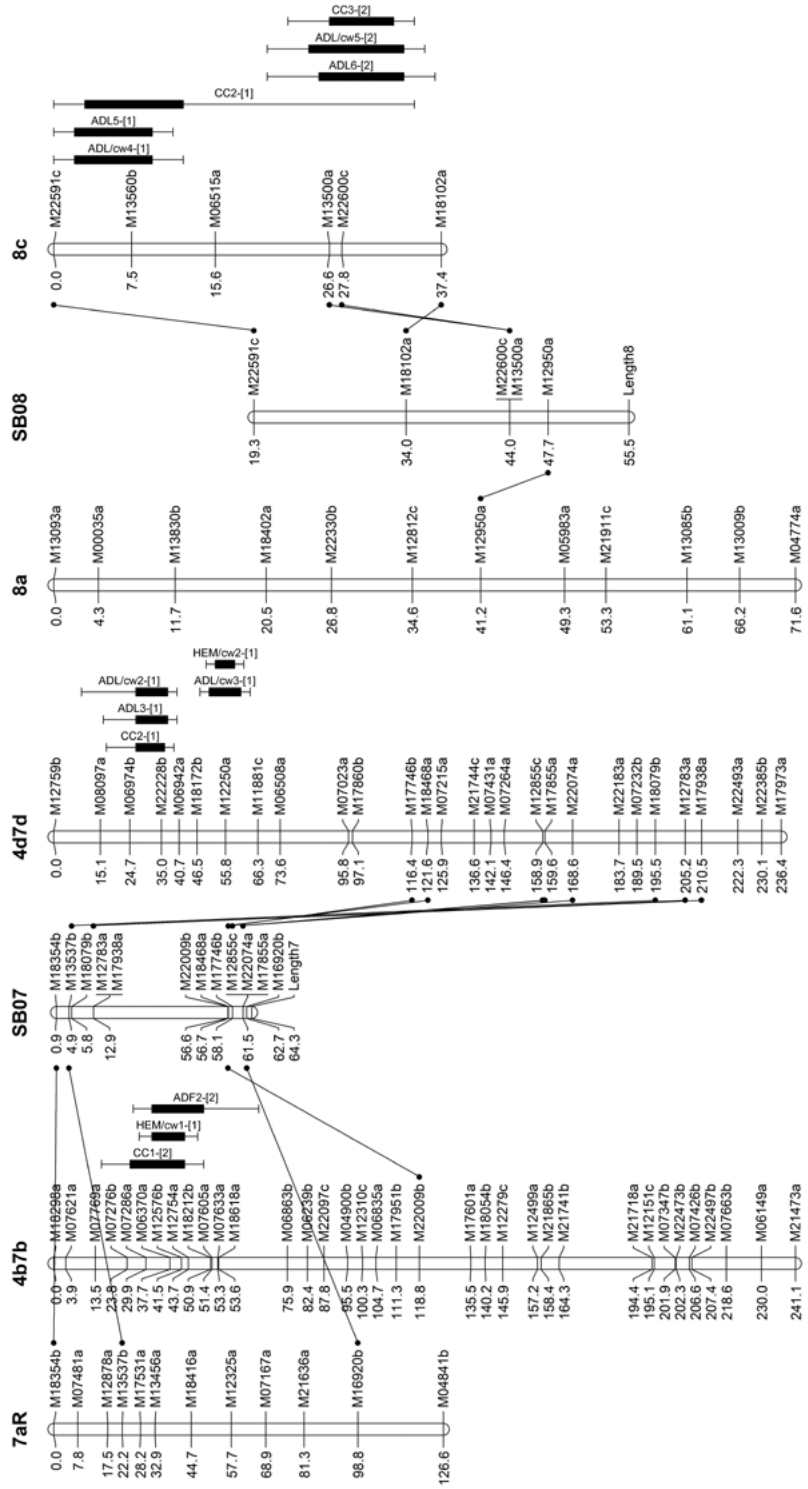

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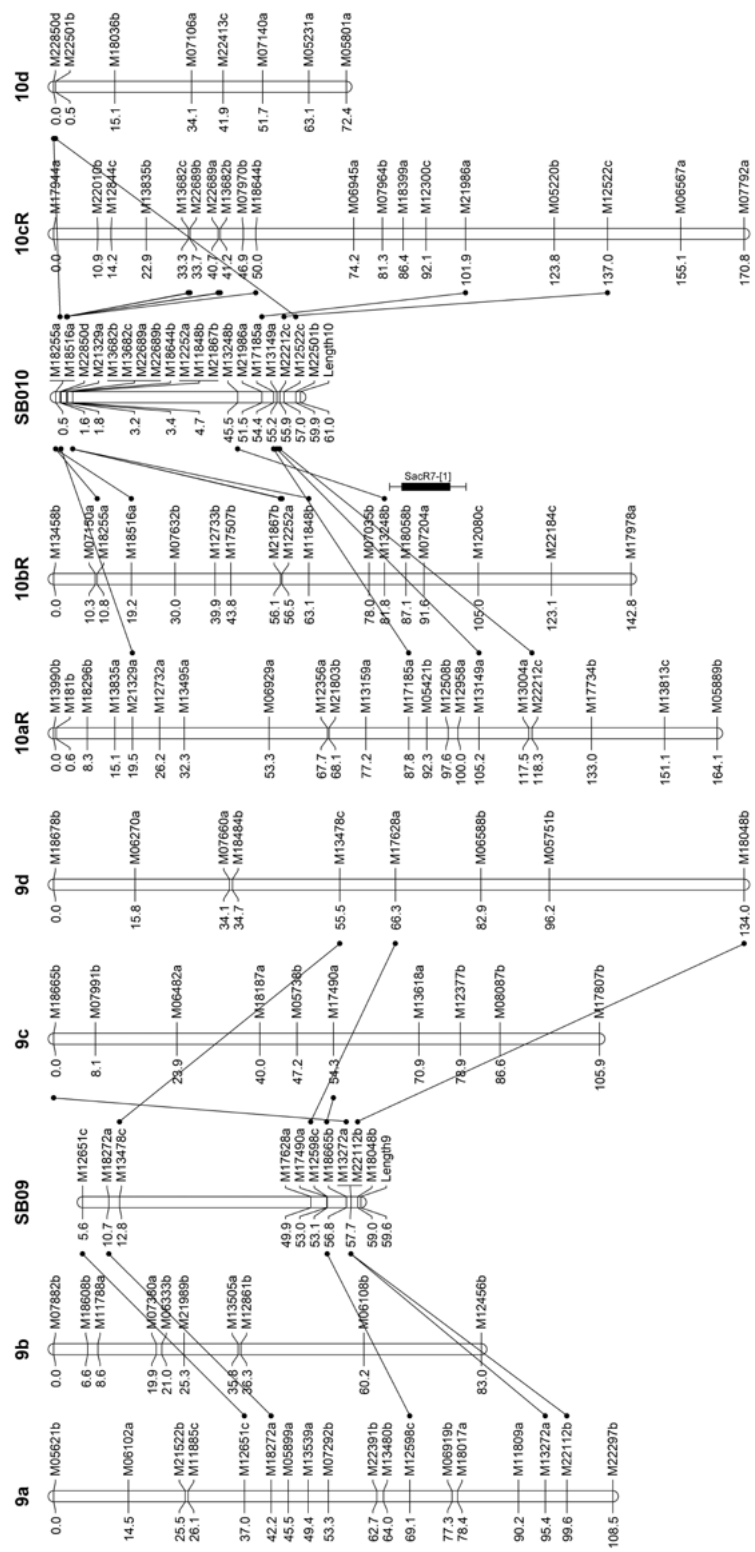

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Chapter 7

Impact of drought stress on growth and quality of miscanthus for biofuel production

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Abstract

Miscanthus has a high potential as a biomass feedstock for biofuel production. Drought tolerance is an important breeding goal in miscanthus as water deficit is a common abiotic stress and crop irrigation is in most cases uneconomical. Drought may not only severely reduce biomass yields, but also affect biomass quality for biofuel production as cell wall remodeling is a common plant response to abiotic stresses. The quality and plant weight of 50 diverse miscanthus genotypes were evaluated under control and drought conditions (28 days no water) in a greenhouse experiment. Overall, drought treatment decreased plant weight by 45%. Drought tolerance – as defined by maintenance of plant weight - varied extensively among the tested miscanthus genotypes and ranged from 30 to 110%. Biomass composition was drastically altered due to drought stress, with large reductions in cell wall and cellulose content and a substantial increase in hemicellulosic polysaccharides. Stress had only a small effect on lignin content. Cell wall structural rigidity was also affected by drought conditions; substantially higher cellulose conversion rates were observed upon enzymatic saccharification of drought-treated samples with respect to controls. Both cell wall composition and the extent of cell wall plasticity under drought varied extensively among all genotypes, but only weak correlations were found with the level of drought tolerance, suggesting their independent genetic control. High drought tolerance and biomass quality can thus potentially be advanced simultaneously. The extensive genotypic variation found for most traits in the evaluated miscanthus germplasm provides ample scope for breeding of drought-tolerant varieties that are able to produce substantial yields of high quality biomass under water deficit conditions. The higher degradability of drought-treated samples makes miscanthus an interesting crop for the production of second generation biofuels in marginal soils.

1. Introduction

Perennial biomass crops, such as miscanthus, are being developed for the production of biofuels to replace our fossil fuel based energy supply chain with a renewable and more sustainable biomass-based alternative. Miscanthus is a leading candidate crop for biomass production owing to its rapid biomass accumulation and high nutrient and water-use efficiencies (Heaton *et al.*, 2010, Jones & Walsh, 2001, van der Weijde *et al.*, 2013). In addition, miscanthus biomass typically has a high quality for biofuel production as it is characterized by low moisture and high cell wall and carbohydrate contents, which are traits that contribute favorably to the yield of fermentable sugars to be used for the production of cellulosic ethanol (Himmel & Picataggio, 2008, Wyman, 2007, Zhao *et al.*, 2012).

A consistent and predictable supply of high-quality lignocellulosic feedstocks is crucial to the success of cellulosic biorefineries (Perlack *et al.*, 2005, van der Weijde *et al.*, 2013). To achieve this, crops must be high yielding and have stable performance across diverse environments. Drought is one of the most wide-spread abiotic stresses (Chaves *et al.*, 2003, Farooq *et al.*, 2009) and the incidence of local and regional drought events is increasing worldwide due to climate change (Dai, 2013, Sheffield & Wood, 2008). In addition, miscanthus is seen as a crop with a high potential for production on marginal land, minimizing competition with food crops for arable land (Quinn *et al.*, 2015). Plants growing on marginal soils, such as eroded soils or bare lands, will regularly encounter periods with a water deficit. Unlike most food products, lignocellulosic feedstocks are considered low-value, high-volume commodities and crops like miscanthus must be produced under low-input regimes. Under these provisions, irrigation is likely to be uneconomical and/or unsustainable for the production of miscanthus biomass (Bullard, 2001). In most miscanthus crop production scenarios, particularly those involving the production of biomass on marginal soils, periods of drought stress may regularly occur (Quinn *et al.*, 2015).

Attractive characteristics of miscanthus with regard to drought tolerance include 1) that its C4 photosynthesis system is characterized by a higher water-use efficiency compared to C3 photosynthetic plants and 2) that its perennial growth habit and extensive root system enable better exploitation of soil water reserves present in deeper soil layers than annual plants (Byrt *et al.*, 2011, Heaton *et al.*, 2010, van der Weijde *et al.*, 2013). Moreover, the genus *Miscanthus* harbors extensive genetic diversity as it is adapted to a wide range of geographical conditions across East Asia (Clifton-Brown *et al.*, 2008, Clifton-Brown *et al.*, 2002). These features provide scope for the selection and breeding of stress-tolerant miscanthus varieties.

Aside from the adverse effects of drought on plant growth, drought influences virtually all plant physiological processes, including cell wall biosynthesis. These effects are important if miscanthus grown on marginal soils are to be used for biofuel production, as the composition and structural rigidity of the cell wall are key factors determining the techno-economic

efficiency of biofuel production (Himmel & Picataggio, 2008, Torres *et al.*, 2016, Wyman, 2007, Zhao *et al.*, 2012). The contents of the two main cell wall polysaccharides, cellulose and hemicellulose determine the maximum theoretically extractable content of fermentable sugars. The relative contents of the major cell wall components – particularly the content of lignin – as well as the extent of cross-linking between them are important parameters determining the efficiency of converting cell wall polysaccharides into fermentable sugars (Himmel & Picataggio, 2008, Wyman, 2007, Zhao *et al.*, 2012). One of the consequences of drought is a loss of cell turgor (Farooq *et al.*, 2009). A primary plant response to the loss of turgor is stiffening of cell walls to provide structural resistance and arrest cell extension (Moore *et al.*, 2008, Tenhaken, 2015). Longer exposure to drought stress challenges plants to modify their cell walls to sustain growth under conditions with reduced water potential (Moore *et al.*, 2008). Drought stress is thus likely to affect the biomass quality of the feedstock (Emerson *et al.*, 2014, Frei, 2013, Iraki *et al.*, 1989a, Moore *et al.*, 2008, Moura *et al.*, 2010, Pauly & Keegstra, 2010, Tenhaken, 2015).

Although the cell wall is clearly affected by drought stress, surprisingly little is known about drought-induced changes in cell wall composition (Tenhaken, 2015). Transcriptome studies often report cell wall related genes to be differentially expressed upon drought stress, but actual biochemical changes in cell wall components are sparsely investigated. Studies that have investigated biochemical changes in cell wall composition consistently report a decrease in cellulose content upon drought stress (Frei, 2013). However, both increases and decreases in contents of lignin and hemicellulosic polysaccharides upon drought stress are reported in different crops and plant tissues (Al-Hakimi, 2006, Emerson *et al.*, 2014, Guenni *et al.*, 2002, Hu *et al.*, 2009, Jiang *et al.*, 2012, Meibaum *et al.*, 2012, Moore *et al.*, 2008, Rakszegi *et al.*, 2014, Vincent *et al.*, 2005). Therefore, it is yet largely unknown how water deficits affect biomass quality of bioenergy crops.

Increasing our understanding of drought-induced cell wall modifications and their impact on biomass quality is of major importance for developing miscanthus varieties for biomass production under low-input conditions and/or on marginal soils. In this study, plant growth and the compositional quality of stem and leaf material were analyzed in 50 diverse miscanthus genotypes, comprising *M. sinensis*, *M. sacchariflorus* and interspecific hybrids, cultivated under drought and control growing conditions. To our knowledge, this is the first study to explore the magnitude of available variation in plant growth and biomass quality under drought stress in the germplasm pool of bioenergy feedstock miscanthus.

2. Material and Methods

2.1. Plant material

The experiment comprised 50 miscanthus genotypes including 35 *M. sinensis*, 8 *M. sacchariflorus* and 7 *M. sinensis × M. sacchariflorus* species. All genotypes used in this study were supplied by Wageningen University and Aberysthwyth University, in a collaboration that is part of the EU Seventh Framework Programme OPTIMISC (www.optimisc-project.eu). Like-sized tillers were split from clonal stock plants into eight separate parts, and transferred to pre-lined 1-metre pipes filled with John Innes number-3 soil (Figure 1a-b). Plants were allowed to grow with sufficient watering for 84 days prior to the start of treatment.

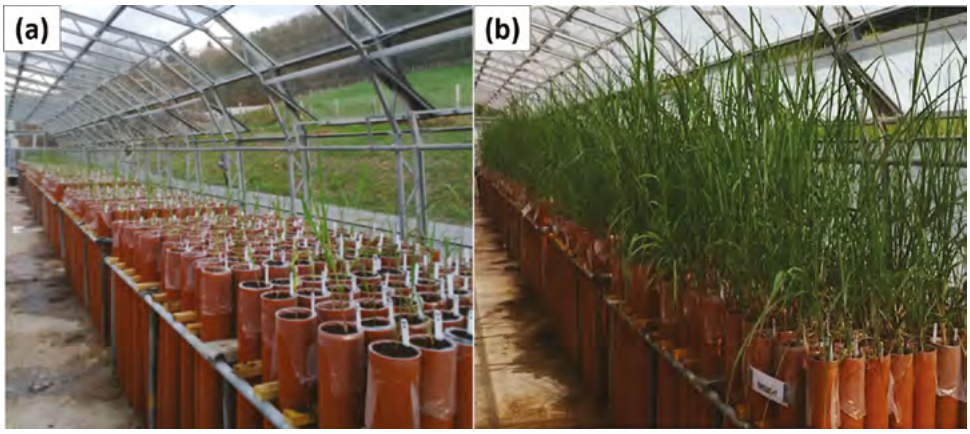

Figure 1. Establishment of 50 miscanthus genotypes (n = 500) in 1-metre pipes between March (a) and June 2014 (b) prior to screening.

2.2. Drought experiment

The experiment was designed to evaluate genotypic responses to total water withdrawal. A total of 50 miscanthus genotypes were planted in a randomised split-plot block design with four blocks. Each block was randomly split in two segments, each containing the full set of genotypes, which received one of two treatments: well-watered vs complete water withdrawal for 28 days, commencing June 2014. In total four replicates per genotype per treatment were evaluated (n = 400). The experiment was conducted in a glasshouse at IBERS (52°43'N, 04°02'W).

After 28 days of treatment all five replicate plants per genotype per treatment were harvested. Using secateurs, plant tillers were cut just above soil level. Stem (with panicle) and leaf material were separated and oven-dried to a constant dry weight (DW) at 60 °C for 72 hours to determine stem,leaf and plant weights in gram dry matter per plant, as well as

the stem:leaf ratio (g/g). Plant weight as defined here refers to the aboveground biomass (stem + leaf) of the plants. Drought tolerance was calculated as the percentage of maintained biomass under water stress (average plant weight under drought stress (n = 4) / average plant weight under control treatment (n = 4) x 100%). One genotype, OPM-17, yielded insufficient material for analysis and was excluded from the study. In order to obtain enough material for biochemical analyses the samples were pooled for stem and leaf samples independently, by randomly combining two of the four replicate samples per genotype per treatment into one of two pools. All pooled leaf and stem samples (n = 400 [50 genotypes × 2 treatments × 2 pools × 2 tissue types]) were ground using a hammer mill with a 1mm screen prior to biochemical analysis.

2.3. Biochemical analysis of the cell wall

Contents of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin contents (ADL) of stem and leaf dry matter were determined according to protocols developed by ANKOM Technology, that are essentially based on the work of Goering and Van Soest (Goering & Van Soest, 1970, Van Soest, 1967). Neutral and acid detergent extractions were performed using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY). Acid detergent lignin was determined after 3-hour hydrolysis of the ADF residue in 72% H_2SO_4 with continuous shaking. All analyses were performed in triplicate. The weight fractions of detergent fiber residues in dry matter were subsequently used to estimate the content of cell wall in dry matter (NDF%dm) and to obtain the contents of cellulose ([ADF%dm-ADL%dm]/NDF%dm × 100%), hemicellulosic polysaccharides ([NDF%dm-ADF%dm]/NDF%dm × 100%) and lignin (ADL%dm/NDF%dm × 100%) relative to the cell wall content.

2.4. Analysis of saccharification efficiency

Saccharification efficiency of the samples was assessed by the conversion of cellulose into glucose by mild alkaline pretreatment and enzymatic saccharification reactions. Reactions were carried out in triplicate using 500 mg subsamples per stem or leaf sample. All subsamples were incubated for 13 minutes with thermostable α-amylase (ANKOM Technology Corporation, Fairpoint, NY), followed by three five minute incubations with warm deionized water (60°C) in order to remove interfering soluble sugars. The remaining biomass was then subjected to a mild alkaline pretreatment, carried out in 50 ml plastic centrifuge tubes with 15 ml 2% NaOH at 50°C with constant shaking (160 RPM) for two hours in an incubator shaker (Innova 42, New Brunswick Scientific, Enfield, CT). In this study the objective of the pretreatment was not to maximize cellulose conversion but to treat samples to better allow discrimination of genotypic differences in cellulose conversion efficiency. Pretreated samples were washed two times with deionized water (5 minutes, 50°C) and once with 0.1 M sodium citrate buffer (pH 4.6, 5 minutes, 50°C).

Saccharification reactions were subsequently carried out according to the NREL Laboratory Analytical Procedure "Enzymatic saccharification of lignocellulosic biomass" (Selig *et al.*, 2008). Pretreated samples were hydrolyzed for 48 hours with 300 µl (25.80 mg of enzyme) of the commercial enzyme cocktail Accellerase 1500 (DuPont Industrial Biosciences, Leiden, NL) supplemented with 15 µl (0.12 mg of enzyme) endo-1,4-β-xylanase M1 (EC 3.2.1.8, Megazyme International Ireland, Bray, IE) in an incubator shaker (Innova 42, New Brunswick Scientific, Enfield, CT) set at 50°C and constant shaking (160 RPM). This enzyme mixture has the following reported specific activities: endoglucanase 2200-2800 CMC U/g, beta-glucosidase 450-775 pNPG U/g and endoxylanase 230 U/mg. Reactions were carried out in 44 ml 0.1 M sodium citrate buffer (pH 4.6), containing 0.4 ml 2% sodium azide to prevent microbial contamination.

Glucose contents in the enzymatic saccharification liquors were determined in duplicate using the enzyme-linked D-glucose assay kit (R-Biopharm, Darmstadt, DE). This assay was adapted to a 96-well microplate format and the increases in sample absorption following enzyme-mediated conversion reactions were spectrophotometrically determined at 340 nm using a Bio-Rad Microplate Reader (Bio-Rad, Richmond, CA, USA). All sample absorbance measurements were corrected using blanks, containing water instead of sample solution. Glucose release was determined by calculating the glucose content in the saccharification liquor from absorbance measurements using equation (1).

$$\text{Glucose release } (\text{mg}) = \frac{\text{V} \times \text{MW}}{\varepsilon \times \text{d} \times \text{v} \times 1000} \times \text{df} \times \Delta\text{Abs} \quad (1)$$

where *V* = final well volume (=3.02 ml); *MW* = molecular weight of glucose (=180.16 g/mol); ε = the molar extinction coefficient of NADPH (=6.3 L × mol^{-1} × cm^{-1}); *d* = light path-length (=1.016 cm); *v* = sample volume (ml); *df* = dilution factor (=10); and ΔAbs = increase in sample absorbance, corrected for the increase in blank absorbance. Cellulose conversion was calculated from the release of glucose relative to the cellulose content in the sample, as detailed in equation 2.

$$\text{Cellulose conversion } (\%) = \frac{\text{Glucose release}(\text{mg})}{\text{CC} \times 1.111} \times 100\% \quad (2)$$

where *CC* = cellulose content in the sample (in mg); and *1.111* = the mass conversion factor that converts cellulose to equivalent glucose (the molecular weight ratio of 180.16 to 162.16 g/mol for glucose and anhydro-glucose, Dien, 2010).

2.5. Analysis of miscanthus biomass using near infrared spectroscopy (NIRS)

Multivariate prediction models based on near-infrared (NIR) spectral data were developed to allow high-throughput prediction of biomass quality traits. Near-infrared absorbance spectra of stem and leaf samples were obtained using a Foss DS2500 near-infrared spectrometer (Foss, Hillerød, Denmark). Averaged spectra were obtained consisting of 8 consecutive

scans from 400 nm to 2500 nm with an interval of 2 nm using ISI-Scan software (Foss, Hillerød, Denmark). Obtained spectra were further processed by weighted multiplicative scatter correction and mathematical derivatization and smoothing treatments using WinISI 4.9 statistical software (Foss, Hillerød, Denmark). These statistical transformations of spectra help to minimize effects resulting from light scatter and differences in particle size. Parameters for derivatization and smoothing were set at 2-6-4-1, in which the first number of this mathematical procedure refers to order of derivatization, the second number to the gap in the data-points over which the derivation is applied and the third and fourth number refers to the number of data-points used in the smoothing of the first and second derivative.

For the creation of prediction models a calibration set of 110 samples was randomly selected from the complete set of samples, but with an approximate 1:1 representation of leaf and stem samples. The biochemical reference data and near-infrared spectra of the calibration samples were used for the development and validation of prediction models using WinISI version 4.9 (Foss, Hillerød, Denmark). The prediction equations were generated using modified partial least squares regression analyses (Shenk & Westerhaus, 1991) and obtained calibration statistics are reported in Table 1. Another 20 of the remaining samples were randomly selected as an external validation set to evaluate the quality of the generated prediction models. The prediction models were validated using the squared Pearson coefficient of correlation (r^2) between predicted and biochemical data of the external validation set (n = 20) and by evaluating for these samples the standard error of prediction (SEP) and its comparison to the standard error of laboratory (SEL) for each of the traits (Table 2). The prediction models were used to determine the cell wall, cellulose, hemicelluloses and lignin contents, as well as the cellulose conversion rate of all leaf and stem samples.

Table 1. Summary of calibration statistics of mPLS models used for the prediction of biomass quality traits

Trait	Samples	Mean chemical analysis	Mean NIRS prediction	r^2	SEC	SECV
Cell wall content (%dm)	104	67.38	67.14	0.99	0.56	1.25
Cellulose (%ndf)	106	45.82	45.75	0.96	0.77	1.15
Hemicellulose (%ndf)	107	47.28	47.33	0.96	0.86	1.40
Lignin (%ndf)	105	6.89	6.85	0.81	0.40	0.59
Cellulose conversion (%)	103	49.99	49.34	0.61	4.42	4.68

r^2= coefficient of determination; SEC = standard error of calibration; SECV = standard error of cross validation

Table 2. Summary of validation statistics of mPLS models used for the prediction of biomass quality traits

Trait	Samples	Slope	Intercept	r^2	SEP	SEL
Cell wall content (%dm)	19	0.78	0.13	0.86	2.36	0.51
Cellulose (%ndf)	19	0.92	0.59	0.82	1.53	0.39
Hemicellulose (%ndf)	19	0.93	-0.54	0.86	1.55	0.34
Lignin (%ndf)	19	1.01	-0.09	0.74	0.43	0.26
Cellulose conversion (%)	19	1.33	-1.63	0.73	4.62	2.99

r^2= coefficient of determination; SEP = standard error of prediction; SEL = standard error of laboratory

2.6. Statistical Analysis

General analyses of variance (ANOVA) were performed to determine the significance ($p < 0.05$) of genotype, treatment and interaction sources of variation. For growth-related traits ANOVAs were performed following the completely randomized split-plot block design of the experiment. The four original biological replicates per genotype per treatment were used as a fixed block effect with a nested split-plot on which treatment was applied. Variance analyses for biomass quality related traits were performed considering that the four biological replicates were combined into two pools. For variance analyses these two pools were considered as two independent replicates per genotype per treatment and used as a block effect. Since these pools were not actual blocks in the original experimental design they could not be used as a fixed block effect, but instead were used as a random block effect. The analyses were performed for stem and leaf samples separately following a mixed effect model (3):

$$Y_{ijk} = \mu + G_{ij} + T_k + GT_{ijk} + B_j + e_{ij} \quad (3)$$

where Y_{ijk} is the response variable, μ is the grand mean, G_{ij} is the genotype effect, T_k is the treatment effect, GT_{ijk} is the interaction term between genotype and treatment, B_j is the block effect and e_{ijk} is the residual error.

Multiple comparisons analyses were performed to distinguish significant ($p < 0.05$) genotypic differences within each treatment using Fisher's protected least significant difference (LSD) test on genotype means. The significance of differences ($p = 0.05$) in trait means between two groups of genotypes that were formed based on tolerance level were evaluated using unpaired two-sample t-tests. Correlation analyses were performed on genotype means to identify the significance, strength and direction of correlations among traits using Pearson's correlation coefficients. All statistical analyses were performed using the statistical software package Genstat, 16th edition (VSN International, Hemel Hempstead, UK).

3. Results

3.1. Drought stress affects plant weight and morphology

Growth, composition and bioconversion efficiency of 50 miscanthus genotypes were evaluated using the leaf and stem tissues of plants grown under drought stress and control conditions. The drought treatment had a significant impact on almost all evaluated traits (Tables 3 and 4). The results showed that both final plant weight and the stem:leaf ratio were significantly affected by treatment. The set of genotypes showed significant differences in genotype performance with a low residual error (Table 3).

Table 3. Tables of analyses of variance for yield and stem-to-leaf ratio of 50 miscanthus genotypes grown under drought stress compared to control conditions

Trait	Source of variation	Degrees of freedom	Mean squares	F probability.
Plant weight	*Wplot stratum*[*]	4	81.48	
(g dm per plant)	*Wplot.SplitPlot stratum*[¥]			
	Treatment	1	9687.92	<.001
	Residual	4	27.94	
	Wplot.SplitPlot.Unit stratum			
	Genotype	49	222.89	<.001
	Genotype.Treatment	49	56.67	<.001
	Residual	260	18.42	
Stem:Leaf ratio	*Wplot stratum*	4	1.15	
(g/g)	*Wplot.SplitPlot stratum*			
	Treatment	1	2.45	0.075
	Residual	4	0.43	
	Wplot.SplitPlot.Unit stratum			
	Genotype	49	0.48	<.001
	Genotype.Treatment	49	0.07	0.126
	Residual	260	0.06	

[*]Wplot = whole blocks in the experiment containing two split-plots to which treatment was applied. [¥]SplitPlot – split-plots in the experiment containing all genotypes.

The mean and the range in genotype performance for plant weight and stem:leaf ratio in control and drought conditions are displayed in Table 5. Mean plant weight under control conditions was 20.10 g per plant (n = 200), whereas under drought stress plant weight was on average 11.10 g per plant (n = 200). Drought treatment in this experiment thus reduced plant weight on average by 45%. Moreover, drought treated plants, with on average a stem:leaf ratio of 0.77, were generally more leafy than the corresponding control plants, which had on average a stem:leaf ratio of 0.91. Variation in plant weight and stem:leaf ratio among genotypes was extremely large under both stress and control conditions. Final mean plant weight of genotypes under control conditions ranged from 5.80 to 35.63 gram, while the range under drought stress was from 2.78 to 20.38 gram per plant (Figure 2). Under both drought and control conditions, leaf biomass contributed on average more to total plant weight than stem biomass (Table 5), but for some genotypes stems comprised the largest weight fraction of total plant weight.

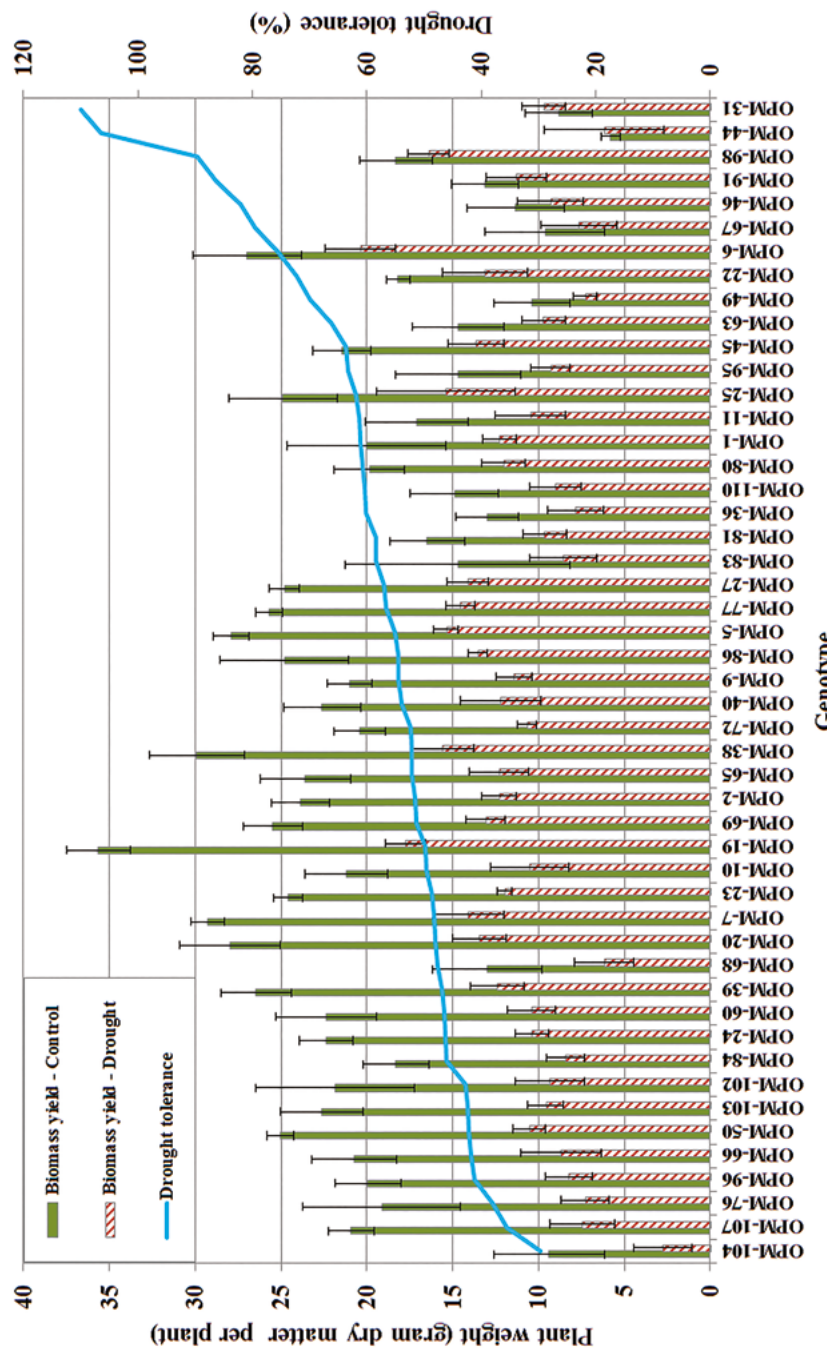

Figure 2. Plant yield of 49 miscanthus genotypes (expressed in gram dry matter per plant) with varying drought tolerance grown under drought and control conditions. Error bars indicate the standard error of a genotype mean (average of 4 replicates per genotype per treatment).

Genotypes responded very differently to the drought treatment, as shown by the variation in plant weight and drought tolerance (Figure 2) and by the significance of the genotype-by-treatment interaction term (Table 3). For example, two genotypes, OPM-6 and OPM-19, are both high yielding genotypes, but differed considerably in drought tolerance. OPM-6 was the genotype with the highest plant weight under drought stress (20.38 g per plant), which was even higher than the average plant weight (20.10 g per plant) over all genotypes under control conditions. This particular genotype had a plant weight of 26.98 g per plant under control conditions, leading to a drought tolerance of 75.53% (only a 25% reduction in plant weight due to the drought treatment). OPM-19 was the genotype with the highest plant weight under control conditions (35.63 g per plant), but was more severely affected by drought stress. Its plant weight under stress conditions was 17.75 g per plant, leading to a drought tolerance of 49.82% (a 50% reduction in plant weight due to the drought treatment). Variation in drought tolerance among all genotypes ranged from 29.60 to 109.90%. The two genotypes with a tolerance value above 100% had a higher plant weight under drought conditions than under control conditions, although the difference in mean plant weight was smaller than the variation between replicates and both genotypes were low biomass types. On the other side of the tolerance spectrum, genotypes displayed large reductions (up to 70%) in plant weight due to drought treatment. Yields in 39 of the 49 genotypes were reduced by 30 - 60% following drought treatment, with the majority of the genotypes with a high plant weight under control conditions showing yield reductions of at least 40% under drought (Figure 2).

3.2. Drought stress affects biomass quality in miscanthus

Drought treatment significantly affected most of the biomass quality traits of miscanthus, including cell wall content, cellulosic and hemicellulosic polysaccharide contents and the efficiency of cellulose conversion (Tables 4 and 5). Stem lignin content, on the other hand, was only moderately affected (p = 0.015) and drought stress had no significant effect on lignin in leaf tissues (p = 0.522). Significant differences among the set of genotypes were found for all biomass quality characteristics. Furthermore, the effects of drought on biomass quality were more apparent in some genotypes than in others, as indicated by the presence of significant genotype-by-treatment interactions for most traits (Table 4).

Overall, the biochemical composition of the stem samples of drought-treated plants compared to their respective control plants was considerably changed (Table 5). Average cell wall content decreased from 73 to 65% of stem dry matter and from 70 to 63% in leaf dry matter. Average cellulose content decreased in stem tissue, from 51 to 46%, but in leaf tissue remained 43%. In contrast, average content of hemicelluloses increased, from 42 to 47% in stem and from 50 to 51% in leaf tissue. Lignin content was not substantially different between drought-treated and control plants (Table 4), remaining at 6% in leaf and 7% in stem tissue (Table 5).

Genotypic variation for cell wall composition and cellulose conversion was extensive. Generally, genotypic variation in cell wall composition was larger in drought-treated plants compared to control plants, and compositional variation between genotypes larger for stem than for leaf tissue. In drought-treated plants, mean cell wall content ranged from 51 to 74% of stem dry matter and 57 to 70% of leaf dry matter among genotypes (Table 5). Similarly, cellulose content ranged from 39 to 49% in stem and 40 to 45% in leaf, the content of hemicellulosic polysaccharides ranged from 43 to 53% in stem and 49 to 54% in leaf and lignin content ranged from 5.7 to 8.7% in stem and 5.6 to 7.4% in leaf materials.

Table 4. Tables of analyses of variance for stem and leaf biomass quality traits of 50 miscanthus genotypes grown under drought stress compared to control conditions

		Stem			Leaf		
Trait	Source of variation	d.f.	m.s.	F prob.	d.f.	m.s.	F prob.
Cell wall content	*Pool stratum**						
(% dm)	Treatment	1	3603.55	0.004	1	2608.88	<.001
	Residual	2	14.94		2	2.58	
	Pool.Unit stratum						
	Genotype	49	82.06	<.001	49	30.57	<.001
	Treatment.Genotype	49	10.43	<.001	49	4.42	0.036
	Residual	95	2.90		96	2.87	
Cellulose	*Pool stratum*						
(% ndf)	Treatment	1	1154.58	0.009	1	39.82	0.033
	Residual	2	10.10		2	1.40	
	Pool.Unit stratum						
	Genotype	49	9.83	<.001	49	4.24	<.001
	Treatment.Genotype	49	3.24	0.020	49	1.81	0.009
	Residual	95	1.98		96	1.03	
Hemicellulose	*Pool stratum*						
(% ndf)	Treatment	1	1239.44	0.009	1	81.03	0.008
	Residual	2	11.19		2	0.64	
	Pool.Unit stratum						
	Genotype	49	12.85	<.001	49	6.35	<.001
	Treatment.Genotype	49	4.17	0.018	49	2.03	0.068
	Residual	95	2.52		96	1.42	
Lignin	*Pool stratum*						
(% ndf)	Treatment	1	8.96	0.015	1	0.01	0.522
	Residual	2	0.14		2	0.02	
	Pool.Unit stratum						
	Genotype	49	1.67	<.001	49	0.61	<.001
	Treatment.Genotype	49	0.35	0.027	49	0.22	0.002
	Residual	95	0.22		96	0.11	
Cellulose	*Pool stratum*						
conversion	Treatment	1	3486.63	0.003	1	689.99	0.001
(%)	Residual	2	9.53		2	1.04	
	Pool.Unit stratum						
	Genotype	49	54.22	<.001	49	7.03	<.001
	Treatment.Genotype	49	7.85	<.001	49	1.60	0.020
	Residual	95	2.66		96	0.98	

* Pools were created by combining stem/leaf samples of two replicated plants per genotype per treatment. Pools were analyzed statistically as two independent replications per genotype per treatment.

Saccharification efficiency was significantly affected by drought treatment. In both stem and leaf materials considerably higher cellulose conversion efficiencies were achieved in drought-treated plants compared to their respective control plants. Stem cellulose conversion increased from 42% (under control conditions) to 51% (under drought treatment) (Table 5). Similarly, leaf cellulose conversion increased from 50% to 54%. Extensive variation among genotypes was found for cellulose conversion efficiency in both drought-treated and control plants. Stem cellulose conversion ranged from 43 to 62% under drought and from 38 to 51% under control conditions (Table 5, Figure 3). Less variation was observed in leaf cellulose conversion, but significant genotypic differences were detected (Tables 4 and 5).

Table 5. Variation in the performance of 50 miscanthus genotypes regarding plant weight, stem:leaf ratio and quality traits in non-stressed and drought conditions.

	Trait	Unit	Treatment	Average	Min	Max	Range	CV (%)	LSD
Plant growth	Plant weight	g dm/plant	Control	20.10	5.80	35.63	29.83	25.12	3.19
			Drought	11.10	2.78	20.38	17.60	30.51	4.24
	Stem:Leaf ratio	g/g	Control	0.91	0.55	1.49	0.94	20.35	0.12
			Drought	0.77	0.36	1.39	1.03	36.45	0.18
	Drought tolerance	%	-	57.75	29.60	109.90	80.32	-	-
Stem composition	Cell wall content	%dm	Control	73.06	62.23	78.71	16.49	1.99	2.92
			Drought	64.57	51.28	73.66	22.38	3.00	3.90
	Cellulose	%ndf	Control	51.06	47.57	53.78	6.22	2.33	2.39
			Drought	46.25	39.33	49.49	10.16	3.47	3.23
	Hemicellulose	%ndf	Control	41.57	37.97	45.33	7.36	3.18	2.66
			Drought	46.56	42.60	52.55	9.95	3.92	3.68
	Lignin	%ndf	Control	7.38	6.26	9.50	3.24	5.94	0.88
			Drought	6.93	5.67	8.67	3.00	7.15	1.00
	Glucose yield	%	Control	42.22	37.69	50.75	13.06	2.89	2.45
			Drought	50.57	43.45	62.18	18.73	3.91	3.98
Leaf composition	Cell wall content	%dm	Control	70.25	64.74	75.72	10.98	1.87	2.64
			Drought	62.92	57.10	70.46	13.36	3.18	4.03
	Cellulose	%ndf	Control	43.37	40.29	45.89	5.60	2.22	1.93
			Drought	42.50	40.48	44.58	4.11	2.51	2.14
	Hemicellulose	%ndf	Control	49.93	46.63	54.14	7.51	2.21	2.22
			Drought	51.18	48.64	54.06	5.42	2.50	2.57
	Lignin	%ndf	Control	6.35	5.40	7.12	1.72	5.65	0.72
			Drought	6.37	5.64	7.35	1.71	4.65	0.60
	Glucose yield	%	Control	50.37	46.16	52.65	6.50	1.78	1.80
			Drought	54.13	51.36	57.24	5.87	1.99	2.17

CV (%) = Coefficient of variation (root mean squared error / average × 100%), LSD = Least significant difference (0.05)

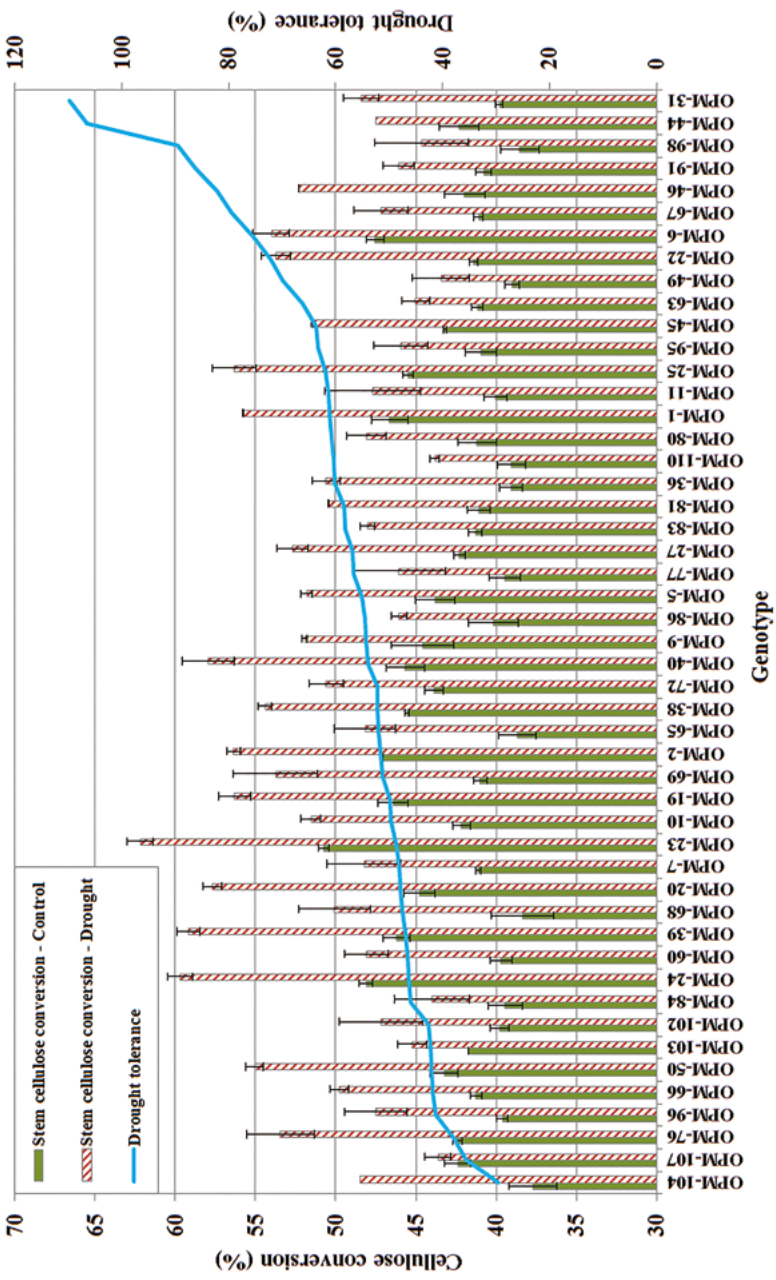

Figure 3. Cellulose conversion of stem tissues of 49 miscanthus genotypes with varying drought tolerance grown under drought and control conditions. Error bars indicate the standard error of a genotype mean (average of 2 replicates per genotype per treatment).

3.3. Cell wall composition does not play a major role in drought-tolerance

To evaluate if differences existed in response to drought between tolerant genotypes and susceptible genotypes, the top six drought-tolerant (OPM-31, 44, 46, 67, 91 and 98) and top six drought-susceptible (OPM-50, 66, 76, 96, 104 and 107) genotypes were grouped together to compare changes in plant weight and biomass quality between the two groups (Figures 4a-b). Difference in trait means between drought-treated plants and their corresponding control plants are presented and further referred to as the trait name preceded by a 'Δ' symbol. These differences are a measure of the plasticity in cell wall components, with a larger difference in cell wall composition between drought-treated and control plants indicative of greater plasticity. In the tolerant group hardly any reduction in leaf and stem biomass was observed, whereas the weight of both plant fractions was highly reduced in the susceptible group. The differences in cell wall content and biomass quality between the two groups contrasting for drought-tolerance were small. The only significant difference between the two groups was a significantly larger increase in leaf Δlignin (0.71 versus 0.02) in the tolerant group compared to the susceptible group (Figure 4a). Between these two extreme groups cell wall plasticity was found to be highly similar (Figures 4a-b).

To further investigate interrelations between drought tolerance and cell wall characters, a correlation analysis was performed on the whole set of genotype means of all traits. The primary objective was to investigate whether cell wall composition and cell wall plasticity play a role in tolerance to drought. A few significant trait associations (with low coefficients of determination) were observed between drought tolerance and biomass quality traits, including correlations with leaf cellulose and hemicelluloses content (Figure 5a, r^2 = 0.13 and -0.11, respectively) and leaf Δcellulose, leaf Δlignin and stem Δlignin (Figure 5b, r^2 = -0.08, 0.21 and 0.10, respectively). No significant correlations were found between drought tolerance and cellulose conversion. The increase in cellulose conversion in stems of drought-treated plants was highly correlated to Δhemicellulose (Figure 5c) and to cell wall content (Figure 5d).

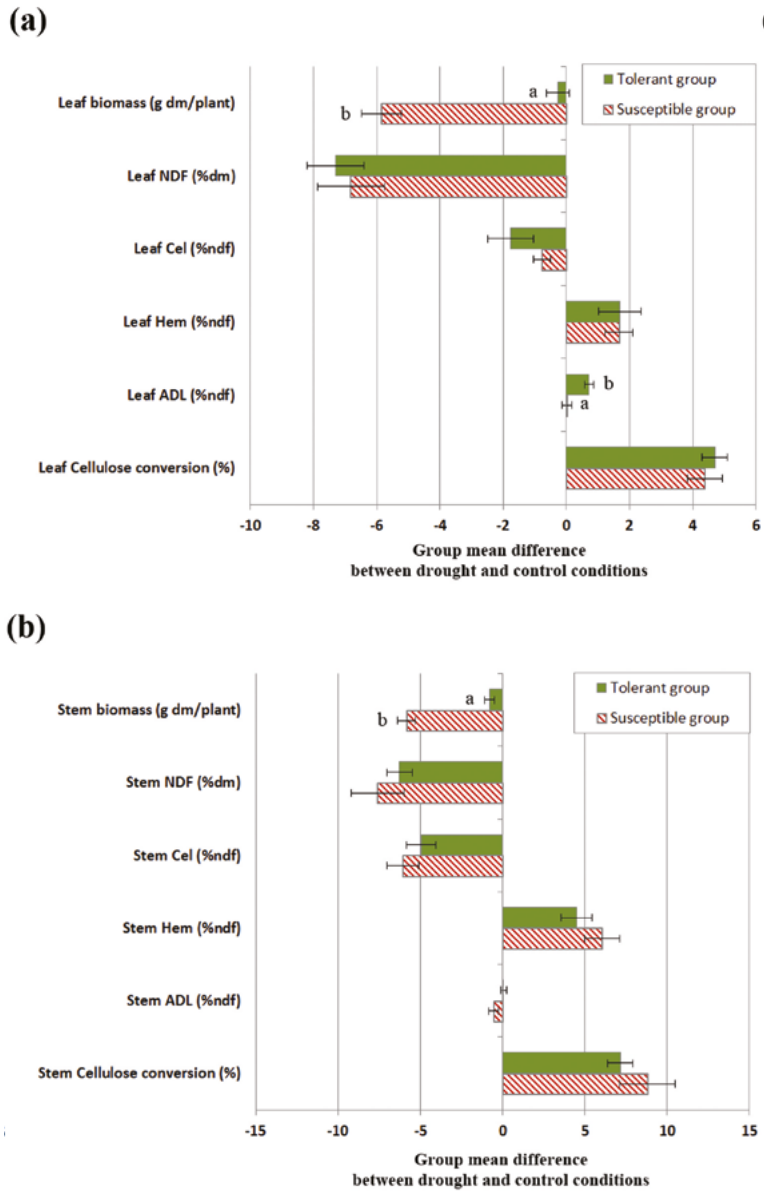

Figure 4a-b. Change in leaf (4a) and stem (4b) weight per plant and composition of drought stressed plants relative to the control plants grouped by tolerance/susceptibility to drought. Unit on x-axis is determined by the unit of the trait on the y-axis. Error bars indicate standard errors on group means (n=6 for tolerant and n=6 for susceptible group). The significance of differences in group means per trait was evaluated by unpaired two-sample t-tests. Group means per trait that have a different suffix are significantly different ($p < 0.05$).

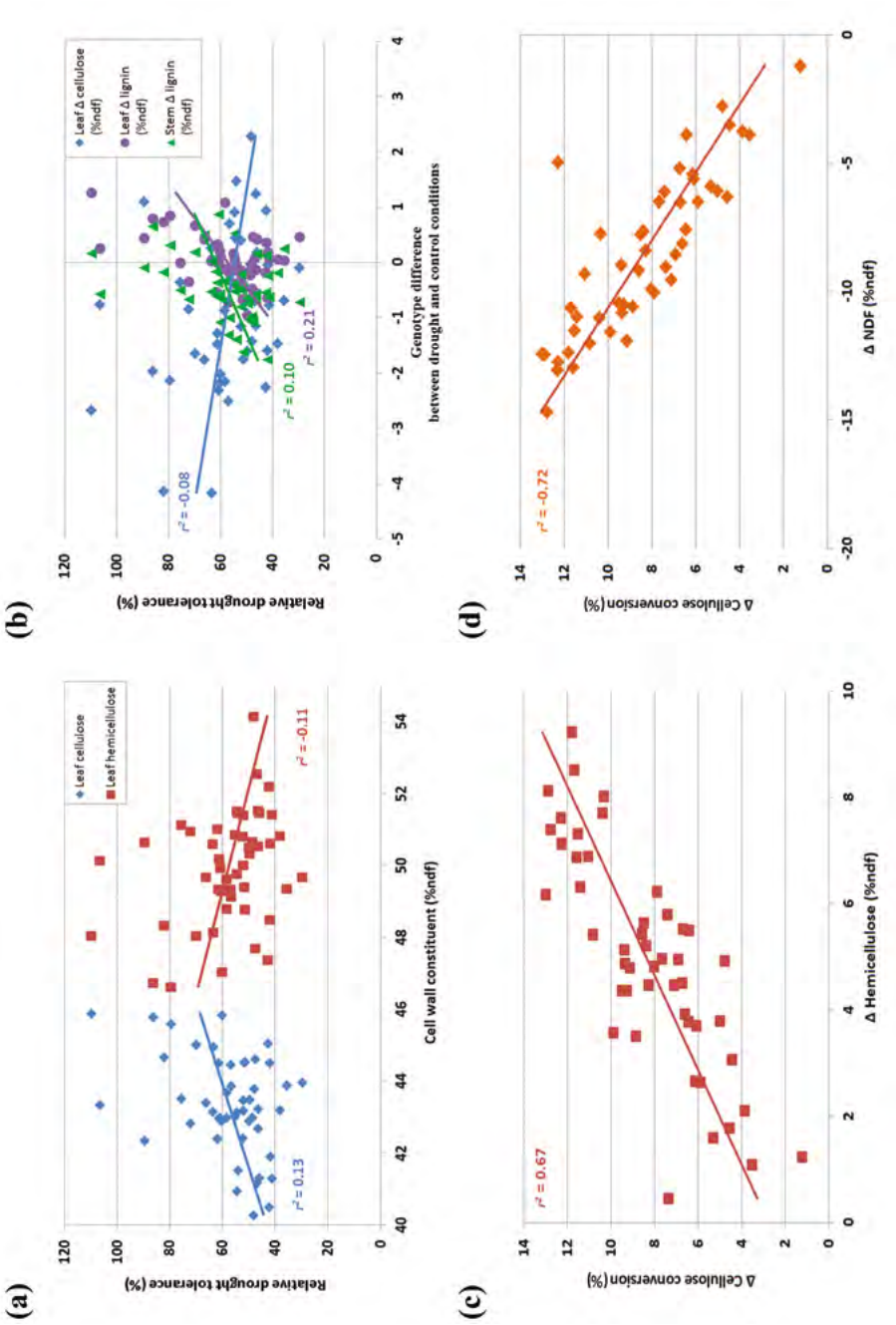

Figure 5a-d. Correlations between drought tolerance and cell wall composition (a), between drought tolerance and leaf Δcellulose, leaf Δlignin and stem Δlignin (b), between stem Δcellulose conversion and stem Δhemicellulose (c) and between stem Δcellulose conversion and stem ΔNDF (d), where Δ = genotype difference between drought and control conditions.

Discussion

3.4. Variation in drought tolerance in miscanthus

The extensive variation observed among the evaluated genotypes regarding plant weight under drought stress (2.78 – 20.38 g/plant) indicates large genotype differences in vegetative growth vigor under dry cultivation conditions. The average loss in plant weight under drought stress compared to control conditions was considerable (45%), however, the range of variation in drought tolerance among the evaluated genotypes (30 – 110 %) was comprehensive and is evidence of the suitability of this test panel for the experiments that were conducted. This indicates that the genotypes tested may interesting candidates for investigation of mechanisms underlying drought tolerance and could possibly be used in breeding programs.

Some plant defense strategies against the injurious effects of drought, such as dehydration avoidance, are rarely compatible with high biomass yields (Blum, 2005). Drought tolerance and plant yield of the genotypes included in this study were evaluated (Figure 2). Plants that achieved higher plant weights in drought conditions than in control conditions (drought tolerance > 100%) were quite small and had low plant weights in both control and drought conditions. The applied drought-treatment was potentially less harsh for small plants than for large plants; small plants need less water and are less likely to lose water due to a proportionally small leaf surface area (Blum, 2005). However, some genotypes exhibited both a relatively high plant weight and a relatively high drought-tolerance, indicating that some genotypes utilize drought tolerance strategies that to some extent could be compatible with high yield. For example, one of the more drought-tolerant (75%) genotypes, OPM-6, in this experiment achieved a plant weight in drought conditions that was similar to the plant weight of *M. × giganteus* (OPM-9) in control conditions (Figure 2). Extrapolations of the reported plant weights to estimate yield potential under field conditions should be approached with care. The current experiment is more suitable for investigating early vegetative growth than yield potential. Moreover, genotypes that had a relatively low plant weight in this experiment, might still achieve substantial yields under field conditions, perhaps by optimizing planting density. The findings reported here suggest the availability of drought-tolerant varieties in miscanthus germplasm resources that may achieve substantial biomass yields, even under dry cultivation conditions.

3.5. Drought reduces cell wall and cellulose content, while increasing hemicellulosic polysaccharides

A key objective of this study was to determine the effects of drought stress on biomass composition and conversion properties, to evaluate whether growing miscanthus under

water deficit conditions affects its biomass quality for biofuel production. Biomass composition was substantially affected by drought, with significant reductions in cell wall and cellulose content and a significant increase in hemicellulosic polysaccharides observed in plants grown under drought compared to control conditions (Table 5, Figures 4a-b). One of the most striking effects of drought was a large decrease in average cell wall content (11.62% in stem and 10.43% in leaf tissue). A drastic reduction in cell wall content was also reported in field-grown *M. × giganteus,* after evaluation of its biomass composition in a year with low precipitation as compared to a year with average precipitation (Emerson *et al.*, 2014).

It was previously shown that cultured tobacco cells subjected to osmotic stress were reduced in size and had thinner cell walls compared to untreated cells (Iraki *et al.*, 1989b). Under normal conditions, cells expand using turgor pressure and cell walls thicken when they no longer need to be elastic to accommodate cell expansion (da Costa *et al.*, 2014, Lam *et al.*, 2013). During drought plants have to act to maintain turgor, leading to a stop or slowing down of cell division and cell expansion, which reduces average cell size (Farooq *et al.*, 2009). However, if water would become available again, cell walls need to be able to accommodate cell expansion. Therefore, it is unlikely that the small-sized cells of drought stressed plants will undergo extensive premature cell wall thickening. Such physiological and developmental processes could explain the lower cell wall content found in drought stressed plants compared to the control plants.

A physiological explanation for the reduction in cellulose during drought stress may be found in the formation of osmolytes (such as soluble sugars and proline). Osmolytes are solutes formed to aid the maintenance of osmotic equilibrium in the cell under dry growing conditions and plant stress due to water deficit is associated with a disturbance of the osmotic equilibrium of cells. The production of osmolytes at the expense of cellulose biosynthesis (or financed by cellulose deconstruction) is well reported in literature (Al-Hakimi, 2006, Emerson *et al.*, 2014, Guenni *et al.*, 2002, Hu *et al.*, 2009, Jiang *et al.*, 2012, Meibaum *et al.*, 2012, Moore *et al.*, 2008, Rakszegi *et al.*, 2014, Vincent *et al.*, 2005). The fact that in stem tissue the reduction in cellulose is much more apparent than in leaf tissue may indicate that in most plants the production of osmolytes in stems is more associated with a concomitant decrease in cell wall cellulose than in leaves.

Lignin content in leaves of drought-treated plants was not significantly different from that of control plants and in stem tissue only a slight decrease in lignin content was observed. Previously a large reduction in lignin content was reported to be one of the side-effects of drought on biomass composition of field-grown *M. × giganteus* (Emerson *et al.*, 2014). However, there is no consistency amongst studies in different crops and tissues regarding the effect of drought on lignin content, with some studies reporting an increase in lignin (Guenni *et al.*, 2002, Hu *et al.*, 2009, Jiang *et al.*, 2012, Meibaum *et al.*, 2012) and some reporting a

decrease in lignin content (Al-Hakimi, 2006, Vincent *et al.*, 2005). The associations between drought stress and lignin are complex and perhaps influenced by yet uncharacterized factors that may explain discrepancies between studies. The small effect of drought on cell wall lignin content and the large effect on cell wall and cellulose content reported here were consistently observed for a diverse set of genotypes comprising three miscanthus species.

Similarly, inconsistent effects of drought on hemicellulose content were previously reported (Al-Hakimi, 2006, Emerson *et al.*, 2014, Jiang *et al.*, 2012, Moore *et al.*, 2008, Rakszegi *et al.*, 2014), whereas in this study drought-treated plants of all genotypes consistently had a significantly higher content of hemicellulosic polysaccharides compared to their respective control plants. Some of the discrepancies may also be explained by a difference in the duration of the applied drought-treatment. A long-term exposure to drought, such as the treatment applied in this study, challenges plants to alter their cell wall structure to sustain cell expansion with reduced water potential. Hemicelluloses contribute to cell wall rigidity by reinforcing the cell wall matrix through crosslinking to lignin and to cellulose fibers (Le Gall *et al.*, 2015). Lignin also provides cell wall rigidity, but is mostly deposited in mature cells that no longer require the flexibility to accommodate cell expansion (da Costa *et al.*, 2014, Lam *et al.*, 2013). Compared to lignin, hemicellulose-crosslinks are more easily broken to ensure cell wall plasticity. An increase in the relative proportion of hemicelluloses might enable cell walls of drought-treated plants to uphold their structural rigidity without compromising plasticity (Le Gall *et al.*, 2015, Tenhaken, 2015).

In this experiment the effects of drought were evaluated in a controlled greenhouse environment, in which environmental factors other than those related to the drought treatment were reduced to a minimum. Compared to the often contradictory results reported in previous studies regarding the effects of drought on biomass quality, in this study the observed effects were highly consistent for a diverse set of genotypes.

3.6. Drought improves saccharification efficiency in miscanthus

In addition to cell wall composition, drought treatment was shown to significantly affect cell wall degradability. Cellulose conversion was substantially increased in biomass samples of drought stressed plants compared to those of control plants, indicating that available cell wall polysaccharides were more easily released as fermentable sugars by pretreatment and enzymatic saccharification reactions. (Figure 3, Table 5). According to these results, the occurrence of drought during growth of bioenergy feedstocks can have highly beneficial side-effects on the processing efficiency of the biomass for the production of biofuel.

The observed increase in cellulose conversion in drought-treated plants was shown to be highly correlated to an increase in the relative proportion of hemicelluloses (Figure 5c). It has been reported that the content of hemicelluloses is positively correlated to saccharification efficiency (Torres *et al.*, 2014, van der Weijde *et al.*, 2016, Xu *et al.*, 2012). The positive effect of

hemicelluloses on cell wall digestibility was associated to a reduction in cellulose crystallinity (Xu *et al.*, 2012). Hemicelluloses, unlike cellulose, are highly branched polysaccharides that form an amorphous network through different types of crosslinks (Doblin *et al.*, 2010, Hatfield *et al.*, 1999). Hydrolytic enzymescan more efficiently penetrate the cell wall matrix during enzymatic saccharification and have a higher affinity to the cellulose substrate when the ratio of hemicellulose to cellulose in the cell wall matrix is increased (Xu *et al.*, 2012). This explains how a relative increase in cell wall hemicelluloses in response to drought treatment resulted in a reduction of cell wall recalcitrance to deconstruction.

Saccharification efficiency is often negatively correlated to cell wall content (Jung & Casler, 2006, Torres *et al.*, 2014, van der Weijde *et al.*, 2016). The reduction in cell wall content observed in drought-treated plants may be another side-effect of drought that contributes positively to saccharification efficiency. As was reported previously, drought treatment of cultured tobacco cells reduced cell wall thickness (Iraki *et al.*, 1989b). Similarly, the reduction in cell wall content observed in drought-treated miscanthus plants could be due to thinner cell walls, as discussed above. Thinner cell walls, in turn, might be more easily penetrated by hydrolytic enzymes due to increased accessible surface area compared to thicker cell walls. This could provide an explanation for the negative correlation found between ΔNDF and Δcellulose conversion (Figure 5d). However, microscopic investigations of differences in cell wall thickness were beyond the scope of this study.

3.7. Drought improves saccharification efficiency in miscanthus

Overall, growing miscanthus under drought conditions substantially affected biomass composition and saccharification efficiency, with cell walls of plants grown under drought conditions being more readily deconstructed during mild alkaline pretreatment and enzymatic saccharification. Hereby, the occurrence of drought during the growth of miscanthus biomass may contribute beneficially to its compositional quality for biofuel production, through the enhanced efficiency of releasing cell wall polysaccharides as fermentable sugars during processing. Importantly, this effect appears to occur even in genotypes that maintained high biomass yield despite drought.

3.8. Implications for breeding drought-tolerant varieties for biofuel production

These results show that genotypic variation for drought tolerance exists within miscanthus germplasm resources and that the development of drought-tolerant varieties that produce substantial biomass yields should be possible. Drought stress significantly reduced cell wall and cellulose content, which reduces the amount of structural sugars available per unit of biomass. This effect was previously reported to have a negative impact on theoretical

ethanol yields of *M. × giganteus* grown during a year with limited compared to a year with average precipitation (Emerson *et al.*, 2014). However, in the current study it was shown that drought also substantially increased cellulose conversion, which considerably enhances the techno-economic performance of bioconversion processes (Himmel & Picataggio, 2008, Torres *et al.*, 2016, Wyman, 2007, Zhao *et al.*, 2012). The occurrence of drought during the growth of miscanthus may thus beneficially affect biomass quality, through the substantial increase in cellulose conversion efficiency. The question that remains is whether in terms of the total ethanol yield per hectare, the reductions in cellulose and biomass yield associated with drought are compensated for by an increase in conversion efficiency. However, the selection of drought-tolerant high yielding genotypes should minimize any penalty.

The absence of strong correlations amongst drought tolerance and compositional characters in the set of genotypes, together with the observation that in the tolerant group similar differences in biomass composition were observed as in the susceptible group are strong indicators that biomass quality characteristics and drought tolerance are largely under independent genetic control. Hence, drought tolerance and biomass quality are not mutually exclusive breeding goals and biomass quality can be selected for independently and simultaneously, without adversely affecting drought tolerance and vice versa. The wide range of variation for the evaluated traits observed amongst this set of miscanthus genotypes provides evidence of ample scope in the miscanthus germplasm pool for breeders to improve both drought tolerance and biomass composition to supply optimized varieties for the biofuel industry.

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Chapter 8

General Discussion

1. Rationale of the research

Cellulosic ethanol is a sustainable and renewable biofuel produced from lignocellulosic biomass. As a second-generation liquid biofuel it is presently the only fossil fuel alternative in the transportation sector with the potential for displacing gasoline on a large scale and in a sustainable way (Wyman, 2008). One of the greatest barriers towards the wide-scale commercialization of cellulosic ethanol resides within our inability to efficiently deconstruct the plant cell wall to release its fermentable sugars (Himmel *et al.*, 2007, Himmel & Picataggio, 2008, Schubert, 2006, Wyman, 2007, Yang & Wyman, 2008). The plant cell wall is a rigid and highly impermeable matrix that is primarily composed of variable ratios of cellulose, hemicellulosic polysaccharides and lignin (Doblin *et al.*, 2010). There are two main objectives regarding the genetic improvement of biomass quality. The first pertains to increasing the content of cellulosic and hemicellulosic polysaccharides, which can be deconstructed and fermented into ethanol. The second objective is to increase the conversion efficiency of these cell wall polysaccharides into fermentable sugars, with lignin being considered as one of the main constituents limiting the efficient extraction of fermentable sugars from the cell wall (Chundawat *et al.*, 2011). In order to make the conversion of lignocellulosic biomass into cellulosic ethanol economically viable, the conversion efficiency of biomass feedstocks needs to be improved to allow for less severe processing conditions.

The perennial grass miscanthus is one of the most promising crops to be used as a lignocellulose feedstock for the production of cellulosic ethanol, as it is able to achieve high biomass yields under low-input cultivation conditions (Brosse *et al.*, 2012, Heaton *et al.*, 2010, Lewandowski *et al.*, 2000, Lewandowski *et al.*, 2003). The domestication of miscanthus is a case study for the utilization of natural genetic variation, as great and largely untapped genetic diversity is harbored within and among natural populations, which have adapted to a wide range of geographical conditions (Clifton-Brown *et al.*, 2008, Hodkinson *et al.*, 2015). However, for the development of high-quality miscanthus varieties that contribute to the techno-economic feasibility of cellulosic ethanol we must first acquire insights into the extent of genetic diversity in biomass quality properties in miscanthus and how we can exploit this variation to improve biomass conversion efficiency during industrial processing.

In this thesis a range of experiments were performed to increase our understanding of lignocellulose conversion efficiency of miscanthus biomass and to elucidate the molecular, genetic and environmental factors that are controlling this complex trait. The compendium of knowledge generated within the framework of this thesis will be comprehensively analyzed and contextualized in the following sections. In section two, knowledge gained in the diverse experiments presented in this thesis is combined and discussed in the light of the genetic improvement of biomass quality in miscanthus. The third section discusses the technical challenges that lie ahead with respect to the genetic improvement of biomass quality. The fourth and final section contextualizes political and societal factors that may hamper the implementation of cellulosic ethanol as a transportation fuel.

2. Knowledge gained on the genetic improvement of biomass quality in miscanthus

2.1. Genetic diversity in cell wall composition and conversion efficiency

Extensive genetic variation in cell wall composition has been reported in miscanthus, with cellulose contents reported to range from 26 - 49%, contents of hemicellulosic polysaccharides from 25 - 43% and lignin contents from 5 - 21% of stem dry matter (Allison *et al.*, 2011, Qin *et al.*, 2012, Zhao *et al.*, 2014). This range of variation is substantially larger than that reported for other promising lignocellulose feedstocks, such as switchgrass (David & Ragauskas, 2010, van der Weijde *et al.*, 2013, Vogel *et al.*, 2011), and provides ample scope for the development of miscanthus varieties with a cell wall composition tailored to the quality demands of the cellulosic ethanol industry. However, the extent of genetic diversity in conversion efficiency of miscanthus was still largely uncharacterized at the start of this thesis.

Therefore we first set out to assess the degree of genetic variation for conversion efficiency in a small subset of genotypes with distinct cell wall compositional profiles. The stem biomass composition of 66 promising *M. sinensis* clones was evaluated from the miscanthus breeding program of Wageningen UR Plant Breeding. Out of these 66 clones, eight genotypes were selected, which showed extreme phenotypes for cellulose, hemicellulosic polysaccharides and lignin contents (**Chapter 4**). These genotypes were cultivated in a field trial in Wageningen and great variation in conversion efficiency was observed among them, with variation in cellulose conversion ranging from 32 – 50% and variation in hemicellulose conversion ranging from 24 – 29% **(Table 6, Chapter 4**). Similar ranges in conversion efficiency characters were as well observed in a broader set of miscanthus germplasm, containing approximately 90 diverse genotypes of different miscanthus species including *M. sinensis*, *M. sacchariflorus* and interspecific hybrids (unpublished data). These studies indicated the availability of substantial genetic diversity in conversion efficiency of miscanthus biomass in the germplasm collection of Wageningen UR Plant Breeding, from which promising genotypes can be selected and used for breeding of varieties with a high biomass quality. Moreover, the full extent of variation is probably even broader, considering that genetic diversity in this germplasm is likely to be a fraction of the genetic diversity harbored in natural populations.

2.2. Key targets for improving conversion efficiency

This research demonstrated that variation in cellulose conversion efficiency was significantly correlated to a number of cell wall properties, some of which provided newly identified targets for genetic improvement of biomass quality. Lignin has since long been recognized

as one of the key components that limit the conversion of biomass into biofuel. However, its hydrophobic nature and chemical characteristics make the structural characterization and quantification of the complete native lignin polymer very challenging. Different protocols have been developed for quantifying lignin content, which led to highly different values, indicating that by using different protocols different lignin fractions are being quantified (**Chapter 3**). Although these fractions exhibited slightly different correlation patterns to conversion efficiency properties, it was shown that most lignin fractions (except for the acid-soluble lignin fraction) were negatively correlated to conversion efficiency and that all evaluated protocols allowed for discrimination of genetic differences in lignin content (**Chapter 3**). Consequently, all these methods are suitable for phenotyping lignin content in genetics studies.

In **Chapter 4** we showed that traits that most favorably contributed to conversion efficiency were a high content of hemicellulosic polysaccharides, a high content of *trans*-ferulic acid, a high ratio of arabinose to xylose and a high ratio of *para*-coumaric acid to lignin content. A key concept that we postulated to underpin these results is that hemicellulosic polysaccharides and lignin fulfill similar functions to ensure the structural rigidity of the cell wall. Increasing contents of hemicellulosic polysaccharides allows for reducing lignin contents without impairing cell wall rigidity. Meanwhile, in comparison to lignin hemicellulosic polysaccharides are readily degraded during industrial processing of biomass, resulting in a positive correlation between the content of hemicellulosic polysaccharides and conversion efficiency (**Figure 3, Chapter 4**, Torres *et al.*, 2014). An additional positive effect of increased contents of hemicellulosic polysaccharides is due to an concomitant increase in their associations with the cellulose microfibrils. The study of Xu *et al.* has clearly demonstrated that through these associations, increasing hemicellulosic polysaccharides leads to a reduction in cellulose crystallinity (Xu *et al.*, 2012). The rate of enzymatic hydrolysis of cellulose is negatively affected by the level of cellulose crystallinity, as amorphous regions of the cellulose polymer are more easily digested (Hall *et al.*, 2010). Both the function of hemicellulosic polysaccharides in maintaining cell wall rigidity and its ability to interact with cellulose to reduce its crystallinity are presumably affected by the number and type of hemicellulose side-chains and their level of cross-linking within the cell wall matrix. These hypotheses were supported by the positive effects of a high ratio of arabinose to xylose and a high content of *trans*-ferulic acids on sample conversion efficiency (**Figure 3, Chapter 4**, Li *et al.*, 2013, Torres *et al.*, 2014). Slowly we are beginning to unravel the characteristics of a "quality" ideotype of lignocellulose feedstocks to improve their processing into cellulosic ethanol (and other commodities):

- A low lignin content → reduces irreversible adsorption of hydrolytic enzymes during processing and increases the accessibility of cell wall polysaccharides
- Substantial incorporation of *para*-coumaric acid into the lignin polymer → facilitates the disruption of lignin during alkaline pretreatment

- High content of hemicellulosic polysaccharides and level of crosslinking between hemicellulosic polysaccharides and between hemicellulosic polysaccharides and other cell wall components (as facilitated for example by *trans*-ferulic acids) → allows hemicellulosic polysaccharides to replace the function of lignin, resulting in increased cell wall degradability without detrimental effects on plant fitness
- High content of hemicellulosic polysaccharides and level of crosslinking to cellulose → reduces cellulose crystallinity leading to higher cellulose conversion efficiency

2.3. The influence of environmental factors on biomass quality

Cell wall biosynthesis, in particular lignin deposition, is spatially and temporally regulated during the development of the plant and like any other complex metabolic pathway, cell wall biosynthesis can be reprogrammed in response to environmental signals (Boerjan *et al.*, 2003, Pauly & Keegstra, 2010). The effect of the environment on miscanthus cell wall composition was first demonstrated by Hodgson *et al.*, who showed that trial location significantly affected contents of cellulose and hemicellulosic polysaccharides (Hodgson *et al.*, 2010). In **Chapter 5** we demonstrated in addition that total cell wall and lignin contents, as well as cellulose conversion efficiency were significantly affected by trial location. Differences between locations in mean contents of cell wall, cellulose, hemicellulosic polysaccharides and lignin in stems of mature plants ranged between 2 - 7 percentage points (**Table 5, Chapter 5**).

Moreover, we showed that differences in rate of establishment between locations also affected cell wall composition and conversion efficiency. In addition to large genotypic variation and the effect of trial location, in this study we also reported for the first time significant genotype-by-location interactions for miscanthus cell wall, cellulose, hemicellulose and lignin contents, as well as cellulose and hemicellulose conversion efficiencies. Although the phenotypic variation due to these interaction effects was relatively much smaller than the variation between genotypes and trial location, the presence of significant interaction effects indicated that some genotypes showed considerably higher sensitivity to environmental factors than other, more stable genotypes. The results suggest that selection for high conversion efficiency and low sensitivity to environmental differences could be combined and is a viable approach for breeders to minimize genotype-by-location interaction effects on biomass quality. This study demonstrated that differences between locations in cell wall composition and conversion efficiency are substantial and should be taken into account in order to match genotype, location and end-use of miscanthus as a lignocellulose feedstock.

A large environmental influence on the trait of interest is undesirable from a breeding perspective, particularly if the effect is unpredictable due to unknown and/or fluctuating environmental stimuli. Further research is therefore needed to increase our understanding

of which environmental stimuli are the cause of the observed environmental differences in mean cell wall composition and conversion efficiency values. Such a study should entail: 1) the evaluation of a number of genotypes across a large number of diverse environments, 2) extensive biochemical analysis of the biomass and 3) detailed characterization of trial locations. Through a factorial regression approach the specific environmental factors may then be identified that are responsible for the observed changes in cell wall properties. In this way the expected suitability of a certain location can be assessed on basis of a set of environmental parameters and the best production environment can be identified given certain biomass quality criteria required by the end-user.

Another approach to investigate the influence of environmental factors on biomass quality is the use of controlled experiments to study the effect of individual environmental factors. This approach is particularly useful for parameters than can relatively easily be manipulated such as temperature or water availability. Accordingly this type of experiments is highly suited to assess the effects of certain abiotic stresses, such as cold, drought and salt stress. These abiotic stresses may play a key role in environmentally derived variation in biomass quality properties, as cell wall biosynthesis genes are often reported to be differentially expressed in plants subjected to abiotic stress treatments (Frei, 2013, Le Gall *et al.*, 2015, Moura *et al.*, 2010, Tenhaken, 2015). Moreover, promising lignocellulosic crops like miscanthus may potentially be cultivated in marginal soils where certain abiotic stresses prevail. Unlike most food products, lignocellulosic feedstocks are considered low-value, high-volume commodities and crops like miscanthus must be produced under low-input regimes. In most crop production scenarios, particularly those involving the production of biomass on marginal soils, it is therefore important to evaluate the potential influence of abiotic stresses on biomass quality.

In the framework of the current thesis the influence of drought (**Chapter 7**), salinity and cold stress (unpublished data) on miscanthus biomass quality have been assessed in controlled experiments. These experiments revealed substantial changes in composition in plants subjected to these abiotic stresses. In the drought experiment, drought-treated plants were shown to have substantially lower cell wall and cellulose contents and slightly lower lignin contents, while their content of hemicellulosic polysaccharides was significantly higher compared to control plants (**Chapter 7**). It was hypothesized that the large reduction in cellulose is due to an increase in production of osmolytes, which fulfill a well-known function in plant protection to drought (Hare *et al.*, 1998).

The changes in cell wall composition observed the cold stressed and salt stressed plants followed the same trends as observed in the drought stress experiments. These results suggested that potentially a general response to cold, salt and drought stresses exists in miscanthus, resulting in plants with cell walls that proportionately contain a lower content of cellulose and lignin and a higher content of hemicellulosic polysaccharides. A physiological

explanation for this general trend may be the reinforcing of cell wall rigidity under abiotic stress through the increased biosynthesis of hemicellulosic polysaccharides. Hemicellulosic polysaccharides may be more suitable to fulfill this function under abiotic stress than lignin. Lignin is mostly deposited in mature cells that no longer require the flexibility to accommodate cell expansion (da Costa *et al.*, 2014, Lam *et al.*, 2013). Extensive premature lignification under incidental abiotic stress is probably inappropriate, as lignification is a highly energy intensive process and is not reversible after cessation of the stress. Cell wall stiffening through increasing the network of hemicellulosic polysaccharides is post-stress (or under prolonged exposure to the stress) reversible as hemicellulose crosslinks can be cleaved by OH°-radicals, xyloglucan-modifying enzymes and expansins (Le Gall *et al.*, 2015, Moore *et al.*, 2013, Rakszegi *et al.*, 2014, Tenhaken, 2015). Therefore hemicellulosic polysaccharides are more suited to maintain cell wall plasticity and therefore more suitable to bolster cell wall rigidity under abiotic stress.

A positive side-effect of abiotic stress is the result of the increased content of hemicellulosic polysaccharides, which positively affects cellulose conversion efficiency. This was demonstrated in the drought study, in which for all genotypes drought-treated plants were shown to achieve substantially higher cellulose conversion rates than their respective control plants (**Table 5, Chapter 7**). The higher degradability of biomass from plants exposed to abiotic stresses is a beneficial characteristic of miscanthus as a crop for the production of biomass on marginal soils. However, further studies are required to assess the yield potential of stress tolerant genotypes under these conditions, which could ultimately disclose whether the lower biomass yield per hectare can be compensated for in terms of economical revenue due to the higher degradability of the produced biomass.

2.4. Evaluation of biomass quality for selection purposes

Rapid and cost-efficient evaluation of biomass quality properties is necessary for screening of large numbers of biomass samples and in order to implement selection on these properties in a breeding program. Conventional biochemical methods for the determination of cell wall composition and conversion efficiency are labor-intensive, time-consuming and require expensive chemicals and equipment. This applies particularly to the determination of conversion efficiency, as this process employs a number of lengthy processing steps, including enzymatic hydrolysis during 48 hours. Even though some automated and high-throughput laboratory systems have been developed for determination of cell wall composition and conversion efficiency (Decker *et al.*, 2009, DeMartini *et al.*, 2011, Lindedam *et al.*, 2014, Santoro *et al.*, 2010, Selig *et al.*, 2010, Studer *et al.*, 2010, Zhang *et al.*, 2012), the evaluation of different biomass quality properties remain too costly and impractical for screening large numbers of samples.

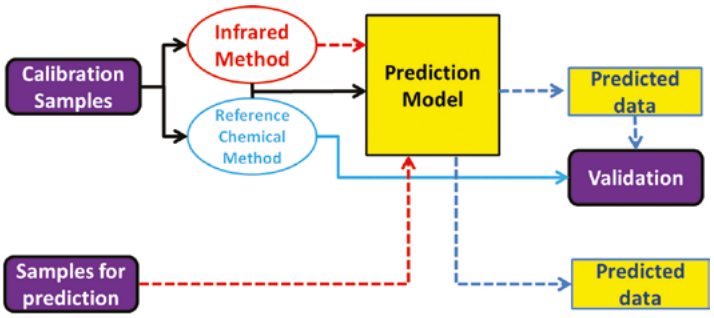

Figure 1. Flow-chart for the development, validation and use of near-infrared prediction models.

In this thesis we demonstrated that the technology of near-infrared reflectance spectroscopy (NIRS) offers a solution to these limitations. NIR technology is extensively applied in forage crops for rapid determination of forage quality properties and was shown to have a high potential for high-throughput analysis of complex traits (Marten *et al.*, 1989, Shenk & Westerhaus, 1994)1989, Shenk & Westerhaus, 1994. Obtaining NIR spectra is fast, non-destructive, requires little or no samples preparation and doesn't require the use of chemicals. However, it is a derived method that requires calibration based on biochemical methods. The principle of the method is to combine biochemical and NIR data obtained on a training population (calibration samples) to develop multi-variate prediction models that are able to predict biomass properties of novel biomass samples solely based on their near-infrared spectra (**Figure 1)**.

In this thesis accurate prediction models were developed for a wide range of biomass quality characters using either stem or leaf samples from studies with different genotypes grown under different growing conditions and at different stages of maturity (**Chapters 5, 6 and 7**). In the final stages of the thesis the biochemical data of close to 1000 diverse miscanthus samples was combined into a final 'robust' prediction model, which is far larger and contains more biochemical traits than any other prediction model for miscanthus so far reported (e.g. Allison *et al.*, 2011, Hayes, 2012, Hodgson *et al.*, 2010, Huang *et al.*, 2012, Payne & Wolfrum, 2015).

Moreover, the speed and ease of analysis of biomass quality properties using the developed prediction models make it an ideal phenotyping tool that can be implemented to aid selection for biomass quality in the breeding program. Conventional biochemical analysis is too costly and labor-intensive to incorporate on a regular basis screening of large numbers of samples, but fast and cheap progeny evaluation for biomass quality has now become possible through NIRS-based phenotyping. It should be noted, however, that NIRS-based phenotyping is slightly less accurate in discrimination of highly similar samples (for example in progeny evaluations) than in highly diverse miscanthus samples (for example grown in different locations), as shown by the slightly lower prediction accuracies in **Chapter 6** compared to **Chapters 5 and 7**. Nonetheless, the obtained results are highly promising and

the use of NIRS-based phenotyping to make informed selection decisions is currently one of the most suitable and effective strategies to improve biomass quality in miscanthus. The developed prediction models will continue to be a valuable resource for future research in miscanthus biomass quality at Wageningen University.

2.5. Developments towards marker-aided selection for biomass quality

Despite recent advances in the development of accurate and high-throughput biomass quality phenotyping tools, a major bottleneck in the evaluation of genotype performance in a perennial crop like miscanthus pertains to the need for expensive multi-year field trials to be able to evaluate the performance of fully mature plants. As described for morphological selection criteria (Clifton-Brown *et al.*, 2008) and for biomass quality criteria (**Chapter 5** and (Arnoult *et al.*, 2015a), the phenotype of immature plants is not necessarily representative of that of mature plants. Therefore, miscanthus plants should be at least two years old before the phenotype can be considered a reliable indication of the mature phenotype (**Chapter 5**). Another bottleneck is that all biomass quality properties are highly polygenic characters (**Chapter 6**) and combining favorable alleles for a number of loci is difficult using phenotypic selection.

Both limitations can be overcome by selection using genomic markers, as selections can be done on marker-phenotype at the seedling stage and marker-assisted breeding strategies can be developed for combining favorable alleles. However, our knowledge of the sequence and functional annotation of the miscanthus genome is still very limited (Głowacka, 2011). The results reported in **Chapter 6** are the first report of genetic mapping of biomass quality traits in miscanthus. We successfully developed a new genetic map for *M. sinensis*, on which we identified 73 QTL's for a wide range of quality characteristics. Most of these traits were shown to have a highly heritable and polygenic nature. These results are the starting point for evaluating the use of the markers linked to biomass quality traits in marker assisted selection to increase the frequency of favorable alleles in the miscanthus breeding pool.

In addition, through the synteny to sorghum, strategies aimed at further fine-mapping and validation of these QTLs towards potential candidate genes may be conceived. The exponential development of sequencing platforms and software tools for genetic studies in outcrossing species are bound to speed up such endeavors and will likely make marker-assisted selection for biomass quality traits available in the near-future. Moreover, advances in fundamental understanding of the genetic control of biomass quality properties in related C4 grasses, such as maize and sorghum, as well as the development of tools to facilitate the exchange of such information between diverse grass species (e.g. Orphan Crop Genome Brower - (Kamei *et al.*, 2016), GRASSIUS - (Yilmaz *et al.*, 2009), GRAMENE - (Liang *et al.*, 2008, Ware *et al.*, 2002) and CSGRqtl - (Zhang *et al.*, 2013)) and are likely to expedite progress in the genetic improvement of miscanthus (van der Weijde *et al.*, 2013).

3. Future work on the genetic improvement of biomass quality

3.1. Cross-breeding for quality and yield

One of the most important challenges facing breeders of energy crops regarding the genetic improvement of biomass quality is combining quality and yield. In **Chapter 4** the highest conversion rates and the highest biomass yields were observed in different genotypes. Although this observation was based on a small set of genotypes, it is reasonable to expect that there is a need to cross genotypes to combine good performance for both traits. Breeding of miscanthus is still in its infancy and it is not likely that natural selection within wild miscanthus species favored selection of genotypes with a high yield and cell wall properties favorable for industrial processing.

In fact, it is plausible that tall, high yielding plants require stronger and more rigid cell walls that provide more structural support. Although in none of the performed experiments we observed a strong inverse correlation between yield and conversion efficiency, we did observe a significant (p = <0.001) positive correlation between canopy height and lignin content (r = 0.75) for the plants evaluated in **Chapter 5** (unpublished data). A similar correlation coefficient between height and lignin content in miscanthus was reported by (Arnoult *et al.*, 2015b). Such a correlation could indicate that high lignin contents are required for plants to grow tall. Nonetheless, this does not necessarily exclude the possibility of combining yield and quality as there are other options for achieving high biomass yields per hectare, such as increasing stem thickness, tiller number and planting density.

3.2. Optimizing biomass composition at the tissue level.

Up to now, the majority of studies focusing on biomass quality properties for the conversion of lignocellulose into cellulosic ethanol evaluated the composition of either separated stem and leaf samples or total above ground biomass samples. However, it is known that cell wall composition differs substantially between different tissue-types and even cell-types. For example, sclerenchyma cells, the cells found directly beneath the epidermis, generally have thicker, stronger cell walls than parenchyma cells, the most numerous cell-type of plant tissues, which potentially makes the relative amount of sclerenchyma cells an important factor in biomass quality (Ding & Himmel, 2008, Himmel *et al.*, 2007). Another important feature pertains to the arrangement and density of vascular bundles, which are often highly lignified and are one of the most recalcitrant tissues (Akin, 2008).

Targeted optimization of the cell wall composition of specific cell types and increasing the relative proportion of more degradable tissues are likely to be key developments to advance biomass quality of energy crops. This type of modifications may also help to minimize adverse effects of lowering lignin content on plant fitness, for example through ‘smart

lignification' realizing low lignin contents where it is not crucial, but high lignin contents where it is helpful. However, separating plant samples into their different tissue types is not straight-forward, nor is evaluating the cell wall composition per tissue, as available routine methods for evaluation require >100 mg biomass samples and are not suitable for analysis of very fine material. For these reasons there are very few studies investigating compositional differences between tissue types and their effect on biomass quality. A promising strategy that would allow for this type of analyses is through microscopic analysis of stem-cross sections with the use of specific dyes to stain specific cell wall components, such as lignin (Lam *et al.*, 2013). In combination with automated image-analyses solutions these type of analyses could as well become relatively high-throughput.

3.3. Manipulating the transcriptional regulation of cell wall biosynthesis genes

In the past decade unprecedented progress has been achieved in elucidating the genetic control of cell wall biosynthesis in gramineous crops, leading to the identification of many genes involved in cell biosynthesis (Akin, 2008, Pauly *et al.*, 2013, Somerville, 2006). However, genetic modification techniques to know-down their expression or stimulated overexpression have so far not lived up to the expectation and did not yet result in feedstocks with improved biomass quality. Often the desired changes in cell wall composition were associated with unfavorable side-effects for plant fitness (Casler *et al.*, 2002, Pedersen *et al.*, 2005). A solution to this challenge may very well be found in increasing the fundamental understanding of the transcriptional regulation of cell wall biosynthesis genes. Cell wall composition differs between various plant tissues and cell types. Moreover, cell wall biosynthesis is differentially regulated during plant development as cell wall structure is modified to sustain cell growth and in response to environmental stimuli (**Chapter 5**), including biotic and abiotic stresses (**Chapter 7**, (Boerjan *et al.*, 2003, Frei, 2013, Kumar & Turner, 2001, Le Gall *et al.*, 2015, Moura *et al.*, 2010, Tenhaken, 2015, Vorwerk *et al.*, 2004, Wolf-Dieter, 2002, Zhong & Ye, 2007). Therefore the expression of cell wall biosynthesis genes must be controlled by complex regulatory mechanisms. In the last few years a number of studies have suggested a complex hierarchy of transcription factors involved in regulating secondary cell wall biosynthesis genes and we are beginning to unravel their regulatory pathways, particularly those related to lignin biosynthesis (Zhao & Dixon, 2011, Zhong *et al.*, 2011, Zhong *et al.*, 2008, Zhong & Ye, 2007). These studies have identified transcription factors belonging to the NAC and MYB families as key transcriptional switches turning on the secondary wall biosynthesis machinery. However, the regulation of polysaccharide biosynthesis and the signals ultimately responsible for activation of regulatory pathways in response to external factors remain elusive. Uncovering these regulatory pathways will have relevant implications to minimize the unwanted fitness-related side-effects of the manipulation of cell wall biosynthesis related genes. Increasing our understanding of these mechanisms provides opportunities for tissue or cell-type targeted genetic improvement of biomass quality.

4. Implementation of cellulosic ethanol as transportation fuel

The global biobased economy is growing exponentially and the turnover of biobased industries in Europe in 2013 surpassed 600 billion Euros. Moreover, European biobased industries employed a total of 3.2 million people in 2013 (Piotrowski *et al.*, 2016). Bioenergy applications were only responsible for 13% of the 2013 turnover and only 3% of total biobased employment. This thesis has focused on the genetic improvement of biomass quality in lignocellulose feedstocks, which we believe could substantially contribute to make biofuel production more economical, in order to displace a significant amount of gasoline as a transportation fuel. However, aside from the genetic improvement of bioenergy crops, there are a number of political and societal factors that are crucially important to realize the large-scale use of cellulosic ethanol as a transportation fuel.

Of primary importance to any new industry is the need for coordinated investments along the whole product-chain. Car manufacturers are not manufacturing cars that can run on pure biofuel, as there is no consumer market demand. This demand will never exist if there is no biofuel available at gas stations. Large-scale production of biofuel requires large initial capital investments to realize biorefinery plants, which will not happen if there is no steady and reliable supply of biomass. Farmers, on the other hand, are not growing biomass crops if there is no demand for biomass from biorefineries. To overcome this impasse, simultaneous and coordinated investments along the whole product-chain are required on a regional or even national scale.

Smart policy strategies can facilitate a transition towards the large-scale use of cellulosic ethanol as a transportation fuel, such as long-term government-issued biofuel mandates, which can ensure a steady long-term market demand for biofuel that will drive investments in biorefineries. Blending of fossil fuels with (first generation) biofuel is currently widely applied, but the proportion of biofuel is only 10-15%. A consumer-determined ratio of biofuel blending at the gas station is conceivable, that would allow consumers to choose for higher ratios of biofuel blending according to their willingness to contribute to a cleaner environment. With technological improvements the economic performance of the cellulosic ethanol industry will improve. This will contribute to a smooth transition towards cars that run on pure biofuel as the costs of biofuel per mileage are becoming cheaper in comparison to gasoline. In turn, this creates a market demand for cars with engine systems that run on higher percentages of biofuel.

Currently, the market share of biobased industries is greatly accelerating due to an increasing palette of biomass-based value-chains, such as production of animal bedding, paper, mulch, particle boards, insolation materials and light-weight concrete, the extraction of plant components such as protein, chlorophyll and waxes, or the conversion of plant components into various biochemicals and biopolymers such as bioplastics, xylitol, glycerol,

levulinic acid and poly-lactic acid. Not only are many of these value-chains already profitable and have excellent potential to displace petroleum-based production processes, many of these processes can potentially be integrated in a biorefinery that is able to efficiently fractionate biomass to produce biofuels, biomaterials, food and feed products and advanced biochemicals. Making full use of all biomass components through innovative fractionation and conversion processes will greatly enhance the profitability of biobased production processes.

The value of the insights obtained in this thesis therefore extends far beyond the application of miscanthus biomass for the production of biofuel, as in many, if not all, applications, feedstock composition and degradability will remain to be an important factor dictating processing efficiency. While this is widely recognized in academic settings, commercial plant breeding of biobased crops is yet to take off. It is my strong believe that the plant breeding sector could become an important player driving the deployment of the whole biobased economy through the reduced processing costs of high quality biobased crops.

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I would also like to acknowledge some other people in the plant breeding department: **Annemarie Dechesne**, I hope you are well and healthy with a healthy new addition to the family! Thanks for all the help and advice setting up new experiments in the lab. Thanks for all the countless HPLC measurements you did and for creating a nice, safe and clean working environment in the biochemical lab. You truly deserve to be praised for all the important work you do and how well you do it! **Heleen Furrer**, you were a wonderful partner in the lab and you have helped me perform many experiments. Thanks for the pleasant company! **Claire Kamei**, although currently employed at Max Planck, I would like to thank you for the time we worked together on miscanthus in the group. Our projects aligned perfectly and I feel we complimented each other. You are a great and sociable person and I am glad to have met you! **Charlie Chen**, the last years we worked together on miscanthus and it was a good cooperation that extents into the future as you are still continuing working on salt tolerance and genetic analyses projects that partly overlapped both of our individual projects. I wish you all the best! **Gerard van der Linden**, we spend time together travelling to Dublin and Stuttgart and these trips have been fruitful as well as pleasurable, in part thanks to you. You are a great researcher, musician and very pleasant company! **Chris Maliepaard**, thanks for your expert help and advice in some of the more complex statistical and genetic analyses. You are the department's go-to guy concerning these topics and your expertise in these topics is highly valued and appreciated. You are also a very gifted, capable and patient teacher, thanks for all your time! **Peter Dinh**, we have never been office mates, but it still feels like you are. We share our belief and I feel this has connected us amidst scientists. Moreover, you are one of the happiest, most positive persons I know and it is just great to be friends with you! **Peter Bourke**, thanks for your help with the genetic analysis. And together with **Anne Giesbers** and **Jeroen van den Berg**, thanks for the great experience we shared in Nanjing. Our unforgettable trip to Huang Shan is in my memory forever! **Johan Bucher**, thanks for your optimism and countless enjoyable lunches spend together.

A special thanks is also merited to the people of **Unifarm**, particularly Gerrit Huisman, Teus van der Pol, Herman Meurs and Wim van der Slikke, which I will do in Dutch: Bedankt voor jullie fantastische zorg voor de proefvelden en de verwerking van de monsters op het ruw-lab. Jullie hebben veel plezier in jullie werk en weten er altijd een lolletje bij te maken. Maar jullie mogen ook zeer trots zijn op hoe precies en nauwgezet jullie je werk doen. Ik heb met veel plezier met jullie samen gewerkt en ook veel geleerd over de praktijk van de proefveldtechniek en monsterverwerking. Jullie worden enorm bedankt!

Of course, a special mention has to go to the **secretary of plant breeding**: Janneke van Deursen, Nicole Trefflich en Letty Dijker. You are always a great support to everyone in the department and you are all very capable in guiding us through the complexities of travel planning, expense reports, tax benefits and countless other regulatory activities. Thanks for all your help!

To all the people in **OPTIMISC**: Andreas Kiesel, Yasir Iqbal, Iris Lewandowski, Olena Kalinina (Hohenheim University, DE), Simon Fonteyne, Hilde Muyllen, Peter Lootens, Isabell Roldan-Ruiz (ILVO, BE), Laurie Huxley, Kerrie Farrar, Chris Nunn, John-Clifton Brown, Iain Donnison (Aberystwyth University, UK), Michal Mos, Graham Harding (Blankney Estates, UK), Ivan Tarakanov, Nikolay Khokhlov (Russian State Agrarian University, RU), Heike Meyer, Kai-Uwe Schwarz (Schwarz, DE), Karl Müller-Sämann (ANNA, DE), Mensure Ozguven (Konya Food & Agriculture University, TR), Heinrich Schüle (DAZ, UA), Qingguo Xi (Dongying Agricultural Institute, CN) – thank you all for the great cooperation, the scientific discussions and the fruitful project meetings over the course of the project. I feel privileged to have been part of this group of renowned miscanthus experts!

A special acknowledgement goes to my **family**: my parents, parents-in-law, sister and brothers- and sister-in-law for their support and interest in my work. Thanks for giving me renewed motivation, time and time again!

Last, but not least, I can not express in words my gratitude to my wife, **Sifra**. You have been and will always be the rock on which I stand. You have always motivated me and stimulated me and without your continuous support I would be nowhere. With you by my side I feel confident to explore what lies ahead!

My heartfelt gratitude to all of you, may your futures be bright!

About the author

Tim van der Weijde was born in Sliedrecht, Netherlands, on November 11, 1987. After being raised most of his life in Hendrik-Ido-Ambacht and attending high school in Rotterdam, Tim moved to Utrecht to study Biology at the University of Utrecht, where he obtained his Bachelor of Science degree in February 2010. In pursue of a career as a science journalist he dedicated himself during his Bachelor to writing articles on a diverse range of Biology related topics for the Biology-department issued magazine of the University of Utrecht, the BioScope, for which he acted as head-editor in the final year of his Bachelor. However, his career path soon changed as a passion for plant breeding was developed with the writing of his Bachelor thesis on "Strategies for implementing durable resistance mechanisms in plant breeding through recessive resistance genes". Fostering this passion, he then joined the TTI Green Genetics plant breeding summer school organized by Wageningen University, which made him decide to register for the Master track: Plant Sciences, with a specialization in "Plant Breeding and Genetic Resources" at Wageningen University. His master thesis focused on cell wall quality in forage maize at the Biobased Economy Group in the Plant Breeding department. The biobased-focused research in this group attracted his interest, so when he obtained his Master of Science degree in February 2012 and was offered a PhD position the decision to join this research-group was quickly made. His passion for plant breeding remained and during the course of this applied-research project he gained hands-on experience in the miscanthus breeding program at the university. In May 2016, Tim made the transition from academia to commercial plant breeding, as he started as forage grass breeder at Barenbrug Holland B.V.

List of publications

Van der Weijde, T., Huxley, L. M., Hawkins, S., Sembiring, E. H., Farrar, K., Dolstra, O., Visser, R. G. F. and Trindade, L. M. (2016). Impact of drought stress on growth and quality of miscanthus for biofuel production. *GCB Bioenergy*. doi:10.1111/gcbb.12382

Van der Weijde, T., Kiesel, A., Iqbal, Y., Muylle, H., Dolstra, O., Visser, R. G. F., Lewandowski, I. and Trindade, L. M. (2016). Evaluation of Miscanthus sinensis biomass quality as feedstock for conversion into different bioenergy products. *GCB Bioenergy*. doi:10.1111/gcbb.12355

Van Der Weijde, T., Torres, A.F., Dolstra, O., Dechesne, A., Visser, R.G.F., and Trindade, L.M. (2016). Impact of Different Lignin Fractions on Saccharification Efficiency in Diverse Species of the Bioenergy Crop Miscanthus. *BioEnergy Research* 9, 146-156.

Van Der Weijde, T., Alvim Kamei, C.L., Torres, A.F., Vermerris, W., Dolstra, O., Visser, R.G.F., and Trindade, L.M. (2013). The potential of C4 grasses for cellulosic biofuel production. *Frontiers in Plant Science* 4, 1-18.

Torres, A., Noordam-Boot, C.M., Dolstra, O., Van Der Weijde, T., Combes, E., Dufour, P., Vlaswinkel, L., Visser, R.F., and Trindade, L. (2014). Cell Wall Diversity in Forage Maize: Genetic Complexity and Bioenergy Potential. *BioEnergy Research*, 8, 187-202

Torres, A.F., Van Der Weijde, T., Dolstra, O., Visser, R.G.F., and Trindade, L.M. (2013). Effect of Maize Biomass Composition on the Optimization of Dilute-Acid Pretreatments and Enzymatic Saccharification. *BioEnergy Research* 6, 1038-1051.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Tim van der Weijde

Date: 12 September 2016

Group: Laboratory of Plant Breeding

University: Wageningen University & Research

1) Start-up phase	date
► **First presentation of your project**	
Miscanthus lignocellulose quality (BBE)'	Dec 04, 2012
► **Writing or rewriting a project proposal**	
Improving miscanthus quality for biobased products	Sep 20, 2012
► **Writing a review or book chapter**	
The potential of C4 grasses for cellulosic biofuel production, Frontiers in Plant Science 4, 2013. DOI: 10.3389/fpls.2013.00107.	2012
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	13.5 credits*

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student days 'Get2Gether', Soest, NL	Jan 29-30, 2015
EPS PhD student days 'Get2Gether', Soest, NL	Jan 28-29, 2016
► **EPS theme symposia (highly recommended)**	
EPS theme 3 symposium 'Metabolism and Adaptation', Wageningen UR	Mar 11, 2014
EPS theme 4 symposium 'Genome Biology', Wageningen UR	Dec 03, 2014
► **Lunteren days and other National Platforms**	
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 22-23, 2013
► **Seminars (series), workshops and symposia**	
Plant Breeding Research Day 2012	Feb 28, 2012
Invited seminar Bruce Dale: Why we must (and how we can) have sustainable biofuels	Mar 15, 2012
Invited seminar Rudy Rabbinge: Food security	Sep 21, 2012
Seminar Young PSG & Young ESG: Make more business with your research	Apr 23, 2013
Invited seminar 'Sustainable Agriculture'	Apr 25, 2013
Invited seminar Salvadore Ceccarelli: The efficiency of plant breeding	Sep 16, 2013
Workshop on the use of light-interception equipment	Oct 09, 2013
Bioenergy Masterclass with Jason Hill	Mar 20, 2014
All-inclusive breeding: integrating high-throughput science	Oct 16, 2014
Invited seminar 'Plant meets animal in breeding and genomics'	Jan 20, 2016
Invited seminar 'Fertilizers in food and nutrition security'	Feb 02, 2016

►	**Seminar plus**	
►	**International symposia and congresses**	
	OPTIMISC Meeting 2 - Stuttgart, Germany	Nov 26-28, 2012
	OPTIMISC Meeting 3 - Lincoln, England	Oct 07-09, 2013
	OPTIMISC Meeting 4 - Dublin, Ireland	Jun 27, 2014
	OPTIMISC Meeting 5 - Wageningen, the Netherlands	Nov 27-28, 2014
	OPTIMISC Meeting 6 - Stuttgart, Germany	Sep 11, 2015
	SunLibb/CeProBio Meeting - Wageningen	Sep 25-27, 2013
	FP7 EU projects on bioenergy crops meeting - Dublin, Ireland	Jun 25-26, 2014
	Plant Biology Europe FESPB/EPSO congress Dublin, Ireland	Jun 24, 2014
	37th Symposium on Biotechnology for Fuels and Chemicals - San Diego - USA	Apr 27-30, 2015
	Perennial Biomass Crops for a Resource Constraint World, Stuttgart, Germany	Sep 07-10, 2015
	International Agricultural Conference for Graduate Students, Nanjing, China	Oct 27-30, 2015
►	**Presentations**	
	100 years plant breeding day, Wageningen (Poster)	Aug 31, 2012
	Plant Biology Europe FESPB/EPSO congress (Poster)	Jun 24, 2014
	WUR Plant Breeding Colloquium - Miscanthus lignocellulose quality (Talk)	Dec 17, 2012
	WUR Plant Breeding Colloquium - Miscanthus: Cell Wall Quality for Biofuel Production (Talk)	Nov 04, 2013
	WUR Plant Breeding Colloquium - Screening cell wall composition and quality in the bioenergy crop miscanthus (Talk)	Nov 03, 2014
	OPTIMISC Meeting 2: Breeding Miscanthus for Bioproducts (Talk)	Nov 27, 2012
	OPTIMISC Meeting 3: Cell wall quality for biofuel production Talk)	Oct 07, 2013
	OPTIMISC Meeting 4: Miscanthus biomass quality for bioethanol production (Talk)	Jun 26, 2014
	OPTIMISC Meeting 5: Screening cell wall quality characteristics for cellulosic ethanol production (Talk)	Nov 19, 2014
	Perennial Biomass Crops for a Resource Constraint World (Talk)	Sep 09, 2015
	37th Symposium on Biotechnology for Fuels and Chemicals: Miscanthus biomass quality for biofuel (Talk)	Apr 27, 2015
	Wageningen Academy Plant Breeding: Case Miscanthus Breeding (Talk)	Jan 20, 2015
	EPS Bioenergy Course: Genetic Improvement of Miscanthus (Talk)	Nov 17, 2014
	IAAS World Congres - Transforming to a Biobased Economy while feeding the world (Talk)	Jul 08, 2015
	International Agricultural Conference for Graduate Students, Nanjing Agricultural University (Talk)	Oct 28, 2015
	Biomass and Bioenergy Research Centre, Huazhong University, Wuhan, China (Talk)	Nov 04, 2015
►	**IAB interview**	
►	**Excursions**	
	EPS - Company Visit Genetwister and In2Care	Sep 19, 2014
	Subtotal Scientific Exposure	28.5 credits*

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
PhD course 'Bioenergy production from crop plants and algae'	Nov 21-23, 2012
NIRS-training	Jan 31 & Feb 01, 2013
PhD course 'Mixed model based genetic analysis in GenStat'	Sep 02-04, 2013
Adobe InDesign	Sep 29-30, 2015
PhD course 'Genotype by environment interaction, uniformity and stability'	Oct 19-23, 2015
► **Journal club**	
► **Individual research training**	
Subtotal In-Depth Studies	4.5 credits*

4) Personal development	date
► **Skill training courses**	
Voice and presentation skill training	Oct 01 & 15, 2013
Techniques for writing and presenting a scientific paper	Dec 10,12,13, 2013
Scientific writing	Feb 05-Apr 09, 2015
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	3.4 credits*

TOTAL NUMBER OF CREDIT POINTS*	49.9

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.

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