1 Genetic Engineering of Bioenergy Crops toward High Biofuel Production

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CONTENTS

1.1 INTRODUCTION

Fossil fuels have been widely used as a major energy source, which represents more than 85% of the total energy consumption for economic and social development around the world (Carroll and Somerville 2009). However, global consumption of fossil fuels and their impact on climate change due to greenhouse gas emissions have inspired the development of renewable and sustainable bioenergy for transportation fuels and industrial chemicals (Himmel et al. 2007; Solomon 2010). Bioenergy is considered as a renewable energy derived from biological sources that can be used for heat, electricity, fuel, and chemical products (Yuan et al. 2008; Himmel and Bayer 2009). Globally, land plants can produce approximately 200 billion tons of biomass per year, 70% of which is hypothesized to derive from plant cell walls, an unprecedented resource for biofuel production (Pauly and Keegstra 2008). Over the past years, the first generation of bioenergy systems, including starch- or sugar-based ethanol or plant oil–derived biodiesel, has already made a relatively small but significant contribution to global energy supplies. Technologies for the production of bioethanol from starch and biodiesel from lipid have become matured worldwide (Demirbas 2008; Soccol et al. 2010). However, the limited availability of resources and nonsustainability of food-based bioethanol, as well as their competitions with food supply, have triggered a paradigm shift toward the production of lignocellulosic bioenergy (Campbell et al. 2008). The second generation of biofuels such as the ethanol produced from lignocellulosic biomass has received increasing research and development interest (Goh et al. 2010; Mabee and Saddler 2010).

The production of lignocellulosic ethanol involves three major processes: biomass pretreatment, lignocellulose enzymatic digestibility, and sugar fermentation. Biomass recalcitrance, which is

resistant to the pretreatment and enzymatic deconstruction, makes the current biomass process unacceptably expensive (Akin 2007; Himmel et al. 2007). As such, considerable efforts have been made recently to increase the lignocellulosic conversion rate (Li et al. 2013a; Zhang et al. 2013). In spite of these efforts, difficulty remains with biomass pretreatment and enzymatic degradation. Although the extreme pretreatment conditions, such as strong acid/base or extreme temperature/pressure, have been widely used for biomass pretreatment, they are costly and may cause environmental pollution. As biomass recalcitrance is essentially determined by the composition of plant cell wall and the interlinking of wall polymers, the selection of energy crops by genetic modification of plant cell wall is hence considered to be a promising solution to this problem. Although plant cell walls are mainly composed of cellulose, hemicelluloses, and lignin, their structural diversity and functional complexity make genetic engineering of energy crops difficult. This chapter describes major grass plants as desirable energy crop candidates and discusses various potential approaches for the genetic breeding thereof.

1.2 ENERGY CROPS AND BIOMASS RESOURCES

Lignocellulosic biomass from agricultural biological resources has been regarded as the primary source of biofuels and other value-added products (Carroll and Somerville 2009; Pauly and Keegstra 2010). Sustainable biofuel production depends on the selection of energy crops with high biomass production under various environmental conditions. Hence, energy crops maximize biomass yield per acre with minimum inputs (i.e., irrigation, fertilizer, and pesticides) and exhibit value-added traits that enhance their use as biofuel feedstock. In addition, the quality of energy crops is determined by their structural characteristics that are favorable for the harvest and processing for bioethanol and other chemical products.

Desirable energy crops can be grown on second-tier agricultural lands because the production of biofuels should not compete with the food supply on arable soils in the long run. Accordingly, energy crops can be divided into three groups based on their biomass composition: sugar- and starch-rich plants (e.g., cassava and sugarcane), lipid-rich plants (e.g., rapeseed, sunflower, and oil palm), and cellulose-rich plants (e.g., poplar, eucalyptus, and grasses). For instance, a number of C_4 grass species have been found to generate high yields of biomass with minimal inputs, such as giant *Miscanthus* (*Miscanthus giganteus* L.), switchgrass (*Panicum virgatum* L.), and sweet sorghum (*Sorghum bicolor* L.).

Perennial energy plants and annual food crops (e.g., rice, wheat, maize, and sweet sorghum) are the most common sources for bioenergy production worldwide (Ragauskas et al. 2006; Carpita and McCann 2008; Taylor 2008; Hodgson et al. 2011). *Miscanthus* (Dohleman and Long 2009) and switchgrass (Schmer et al. 2008) have been the focus for many research and development activities in Europe and the United States. *Miscanthus* and sweet sorghum species are currently considered as leading energy feedstock candidates in China (Xie and Peng 2011). On the other hand, rice, wheat, and maize are major food crops that also provide approximately 75% biomass resources of total agricultural residues in China and elsewhere. Potential energy food crops should maintain a high grain yield and good quality for the food supply with easy destruction of cell walls in their straws/stalks for biofuel production. Compared with food crops, energy plants should have their advantages for growth in different geographical regions and exhibit high use efficiency of resources such as light and water.

In order for large amounts of biomass supply, genetic breeding and biotechnology can be applied to increase the biomass yield in food crops and energy plants as shown in Table 1.1. The selection of energy crops from natural germplasm resources and genetic mutagenesis pools is considered as an initial approach. On the whole, genetic breeding strategies include traditional breeding and related molecular approaches such as the use of genetic markers and genome mapping. Hence, the genetic engineering of energy crops is essential to the discovery of key genes associated with plant growth traits for increasing biomass quantity and enhancing plant growth and resistance to various environmental stresses.

TABLE 1.1
List of the Potential Representative Energy Plants and Energy Crops

Species	Photosynthetic Type	Genome Size (Mbp)	Biomass Yield (Tons/ha Year)	Bioethanol Yield (Tons/ha Year)
Switchgrass	C_4	1,372–1,666	14–20	4–5
Miscanthus	C_4	4,300–6,800	60–80	6–8
Rice	C_3	430	15–30	1.5
Wheat	C_3	16,000	15–30	1.5
Maize	C_4	2,500	15–45	1.5
Sweet sorghum	C_4	740	60–80	4–6

Source: Data modified from Xie, G. and Peng, L., *J. Integr. Plant Biol.,* 53(2), 143, 2011.

1.3 PLANT CELL WALL AND BIOMASS RECALCITRANCE

Plants consist of many types of cells that form typical primary and secondary cell walls. Plant cell walls significantly differ in the chemical compositions and structural features of monocots and dicots as shown in Table 1.2 (Grabber et al. 2000; Vogel 2008; Lionetti et al. 2010; Scheller and Ulvskov 2010). There are two typical primary cell walls in plants: Type I comprises cellulose microfibrils cross-linked with xyloglucans and embedded in a complex matrix of pectins and proteins (Whitney et al. 1999), and type II is rich in glucuronoarabinoxylans (GAXs) and mixed-linkage glucans (MLGs) (Carpita 1984; Burton and Fincher 2012). In secondary cell walls, less branched or unbranched hemicelluloses are interlinked with the surface of cellulose fibrils via hydrogen bonds, whereas the side chains of hemicelluloses are covalently bonded with lignin to create enzyme-impenetrable cross-linking networks. Lignin–carbohydrate complexes are also thought to exclude water and prevent chemical or enzyme-catalyzed deconstruction of cell walls, predominantly determining the biomass recalcitrance (Chundawat et al. 2011). To date, the knowledge about the fine structures of plant cell walls is very limited, which hinders

TABLE 1.2
Components (% DW) of Cell Wall in Grass and Dicot Plants

	Grass Cell Wall		Dicot Cell Wall	
Components	Primary	Secondary	Primary	Secondary
Cellulose	20–30	35–45	15–30	45–50
Xylans	20–40	40–50	5	20–30
MLG	2–15	Minor	Absent	Absent
XyG	2–5	Minor	20–25	Minor
(Gluco)mannan	2	0–5	3–15	2–5
GAX	20–40	40–50	5	Absent
Pectins	5	0.1	20–35	0.1
Structural proteins	1	Minor	10	Minor
Phenolics	1–5	0.5–1.5	Minor	Minor
Lignin	Minor	20	Minor	7–10

Sources: Referred from Vogel, J., *Curr. Opin. Plant Biol*., 11(3), 301, 2008; Scheller, H.V. and Ulvskov, P., *Annu. Rev. Plant Biol*., 61, 263, 2010.

the identification of the crucial factors of biomass recalcitrance (Xie and Peng 2011; Huang et al. 2012; Li et al. 2013b; Zhang et al. 2013).

Genetic modification of plant cell walls in energy crops is considered a promising solution to recalcitrance. The discovery of appropriate genes is an initial step, and potential genetic engineering approaches should be considered in energy crops (Chen and Dixon 2007; Ambavaram et al. 2011). Such approaches involve altering wall polymer cross-linking, reducing lignocellulose crystallinity and lignin levels, increasing hemicellulose contents, and adding foreign cellulase enzymes and/or other components into cell walls (Weng et al. 2008; Xie and Peng 2011).

1.4 SELECTION OF NATURAL GERMPLASM RESOURCES

Perennial grasses, such as switchgrass and *Miscanthus*, are rich in natural germplasm resources. They have high efficiencies of using water and nutrient and are characterized by their wide geographic distribution and high biomass productivity. The selection of natural germplasm resources is considered as an initial and essential step in energy crop breeding. It not only can determine valuable genetic materials for energy crop breeding but also may directly select energy plants for biofuel production (Xie and Peng 2011).

Switchgrass (*Panicum virgatum* L.), which is a C_4 perennial forage grass, is currently being evaluated as a biofuel crop in North America and Europe. It has two distinct ecotypes: lowland tetraploid plants ($2n = 4x = 36$) with a tall coarse stature and exceptional biomass and upland octoploid plants ($2n = 8x = 72$) with a short stature and low biomass yield (Bouton 2007). Owing to the large variability within available switchgrass populations with respect to important agronomic traits, such as tillering, biomass digestibility, and biomass production, this important forage and biofuel crop holds great potential for germplasm improvement. As biomass yields of switchgrass vary greatly depending on cultivar, year, and locations, four switchgrass genotypes with similar cellulose and hemicellulose composition but significantly different lignin contents, especially in the ratio of lignin monomers and considerable levels of *p*-coumaric acid and ferulic acid, have been selected. In addition, the full genome sequence of switchgrass will allow us to use marker-assisted breeding to improve biomass yield and quality.

Miscanthus, which is another important C_4 perennial plant, has the highest biomass yield among grass plants. It was primarily originated from East Asia and the nearby Pacific islands, and 14 species of which have been identified to date. More than 1000 natural *Miscanthus* sp. accessions, including four major species (*Miscanthus sacchariflorus*, *Miscanthus lutarioriparius*, *Miscanthus sinensis*, and *Miscanthus floridulus*), have been collected in China (Xie and Peng 2011). Each species represents different ecological and geographical types, making it a diverse germplasm resource. Two hundred representative accessions with diverse cell wall composition and wide biomass digestibility have been previously determined to select possible *Miscanthus* species as energy plants (Xie and Peng 2011; Huang et al. 2012). Due to their distinct biomass degradation, several *Miscanthus* materials were selected as energy plants for biofuel production in this study as shown in Table 1.3. In addition, a genetic model based on the integrative analysis between cell wall composition and biomass digestibility is herein proposed for the potential genetic breeding of *Miscanthus* as biofuel feedstock.

1.5 SCREENING OF PLANT CELL WALL MUTANTS

During evolution, plants have to construct their typical cell walls to complete their life cycles rather than meet the requirements for biofuel production. Due to the diversity of plant cell wall structures and the complexity of their functions, the direct genetic modification of plant cell walls can unexpectedly lead to the alterations in growth and development of plant cell (Torney et al. 2007; Vega-Sánchez and Ronald 2010). Alternatively, large-scale screening of plant cell wall mutants is considered as practicable work. Mutagenesis of energy crops can be conducted by means of T-DNA

TABLE 1.3
Cell Wall Composition and Degradability of *Miscanthus* Ecotypes under Different Pretreatments[a]

	Cell Components (%)			Heat Degradation Efficiency (%)		Alkaline Degradation Efficiency (%)		Acid Degradation Efficiency (%)	
Ecotype	Cellulose	Hemicellulose	Lignin	C6-Sugar	C5-Sugar	C6-Sugar	C5-Sugar	C6-Sugar	C5-Sugar
MI10	28.25	38.74	33.01	19.07	3.87	47.07	24.54	31.98	31.31
MI108	33.33	38.11	28.56	8.59	2.60	45.86	26.06	30.61	33.96
MI1	44.50	27.95	27.56	3.29	1.24	21.78	14.08	14.56	24.63

[a] The degradability was subjective to hexoses/pentoses (percentage of total cell walls) released from both pretreatment and 0.4% cellulase digestion (Angel Comp. Limited, China). Heat degradation, the powders (40 mesh) of mature stem were heated at 121°C for 20 min, and the remaining residues incubated with cellulase for 48 h; alkaline/acid degradation, the powders treated either with 1% (w/w) NaOH for 2 h at 50°C or with 1% H_2SO_4 for 20 min at 121°C, and the remaining residues incubated with cellulase as just described.

insertion knockout, transposon insertion, as well as chemical (e.g., ethyl methanesulfonate-induced) and physical (e.g., cobalt-60 irradiation) treatments. The ideal cell wall–altered mutants should be able to maintain normal plant growth and similar grain and biomass yields but show enhanced biomass digestibility as compared with the wild types. Hence, these mutants can be directly used as energy crops for the production of biofuels. On the other hand, their identification would provide novel genes for genetic manipulation of such energy crops.

The selection of mutants is specifically helpful for the genetic breeding of energy food crops due to their inadequate natural germplasm resources. Rice, wheat, and maize provide major food sources worldwide. However, the enormous amounts of biomass residues from food crops have not been optimized for biofuel production. Their biomass is principally composed of cellulose (30%–45%), hemicelluloses (20%–30%), and lignin (25%–35%) (Pauly and Keegstra 2008, 2010). Through the genome sequencing of two rice cultivars, functional genomics and several microarray platforms have been established (Matsumura et al. 1999; Gowda et al. 2004; Nobuta et al. 2007). Rice transformation has been advanced using methods such as gene silencing and overexpression techniques (Miki and Shimamoto 2004; Nishimura et al. 2006). The variation-induced collections of tagged lines (Tos17, Ac/Ds, and T-DNA) and chemically or physically induced mutant lines (Hirochika et al. 2004; Zhang et al. 2006) have facilitated the discovery of new genes that determine cell wall properties. Recently, large-scale rice mutants have been investigated as an energy and food crop candidate (Xie and Peng 2011). Distinct from previously identified cell wall mutants that show abnormal phenotypes, such as dwarfism, irregular xylem (IRX), and even lethality (Goubet et al. 2003; Desprez et al. 2007), most of the rice mutants selected have exhibited agronomic traits and grain yields similar to those in the wild type but with a remarkable alteration in cell wall composition and structure. Under various mild physical and chemical pretreatments, several elite mutants demonstrated enhanced biomass enzymatic digestibility as shown in Table 1.4.

In maize, the uniform mutator (Mu) population has been developed by introgressing Robertson's Mu, an insertional mutation element, into established maize inbreds W22 and B73 (McCarty et al. 2005; Settles et al. 2007), and a database has been established to facilitate high-throughput molecular analysis of Mu-tagged mutants and gene knockouts (http://www. maizegdb.org/documentation/uniformmu/). The project of cell wall genomics has successfully identified a set of mutants in which cell wall biosynthesis–related genes are disrupted by a Mu element. On the other hand, four spontaneous brown midrib (*bm*) mutants in maize were recognized by the reddish-brown coloration of the vascular tissue in the leaf blade and sheath. The *bm1* mutation affects the lignin biosynthetic enzyme cinnamyl alcohol dehydrogenase (CAD) (Halpin et al. 1998), the *bm2* mutant contains

TABLE 1.4
Cell Wall Composition and Digestibility of Rice and Maize Mutants under Different Pretreatments[a]

	Cell Components (%)			Heat Degradation Efficiency (%)		Alkaline Degradation Efficiency (%)		Acid Degradation Efficiency (%)	
Mutants	**Cellulose**	**Hemicellulose**	**Lignin**	**C_6-Sugar**	**C_5-Sugar**	**C_6-Sugar**	**C_5-Sugar**	**C_6-Sugar**	**C_5-Sugar**
Rice									
RG53	34.13	33.73	32.14	77.0	7.20	75.40	23.50	68.30	30.00
RC2	38.81	37.44	23.76	21.80	4.80	55.00	29.60	42.80	32.50
RG37	46.67	29.28	24.05	22.00	4.70	37.10	21.90	35.10	25.90
WT	46.47	28.27	25.26	24.90	4.40	56.10	19.40	43.40	20.40
Maize									
CM10	31.12	34.64	34.24	37.40	7.40	57.10	29.10	47.80	29.80
CM9	41.30	29.01	29.69	23.10	6.40	47.50	25.60	39.60	26.70
WT	43.08	28.68	28.24	30.40	6.00	52.70	28.80	39.30	26.40

[a] The degradation efficiency was subjective to hexoses/pentoses (percentage of total cell walls) released from both pretreatment and 0.4% cellulase digestion (Angel Comp. Limited, China). Heat degradation, the powders (40 mesh) of mature stem were heated at 121°C for 20 min, and the remaining residues incubated with cellulase for 48 h; alkaline/acid degradation, the powders treated either with 1% (w/w) NaOH for 2 h at 50°C or with 1% H_2SO_4 for 20 min at 121°C, and the remaining residues incubated with cellulase as just described.

fewer guaiacyl and syringyl residues (Vermerris and Boon 2001), the *bm3* gene encodes the enzyme caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT) (Vignols et al. 1995), and the *bm4* mutant shares similar chemical composition with the *bm2* mutant (Barriere et al. 2004). Hydrolysis of stovers from *bm1* to *bm3* in an inbred A619 background resulted in enhanced saccharification, compared with the wild type. Several good maize mutant lines have been selected with increased biomass degradability in our laboratory as shown in Table 1.4.

Sweet sorghum (*Sorghum bicolor* L.) is a breeding line from the ordinary grain sorghum species. The stalk contains 17%–21% sugar content, and the biomass yield is 60–80 Mg ha^{-1} (Carpita and McCann 2008). The *bm* mutants were first isolated by chemical mutagenesis more than 30 years ago, and spontaneous mutants were identified since then. Most *bm* mutants were found to have an altered cell wall composition, particularly in lignin composition, and enhanced biomass saccharification (Bout and Vermerris 2003). Recently, dozens of ethyl methanesulfonate-induced mutants have been selected to show a pattern of plant growth and grain yield similar to that in the wild type. However, several mutants with high levels of soluble sugar and cell wall components in the stove and efficient biomass digestibility have been detected (Xie and Peng 2011).

1.6 GENETIC MODIFICATION OF PLANT CELL WALLS

1.6.1 Genes Involved in Cellulose Biosynthesis and Assembly

Cellulose biosynthesis has been extensively studied in higher plants. Generally, cellulose is synthesized at the plasma membrane by an enzymatic complex and deposited directly into the cell wall in a directional manner (Somerville 2006; Mutwil et al. 2008; Taylor 2008), undergoing a dynamic reorientation followed by the deposition that enables its anisotropic expansion (Anderson et al. 2010). Cellulose synthase (CESA) isoforms form rosette hexameric complexes, which presumably consist of 36 individual CESA proteins and some other accessory proteins. The CESA complexes are assembled in the Golgi apparatus and then exported to the plasma membrane via exocytosis, whereas the glucose residues come from UDP-glucose molecules that are present in the cytosol. Three major steps are associated with the polymerization of UDP-glucose and formation of glucan chains: chain initiation, chain elongation, and chain termination (Peng et al. 2002). The deposition of cellulose is oriented by an interaction between CESA protein and microtubules (Baskin 2001; Lane et al. 2001; Pagant et al. 2002). It is believed that the rosette CESA protein complexes move in the plasma membrane in a direction that is defined by cortical microtubules, thereby producing cellulose microfibrils that are deposited into the extracellular wall matrix at a location adjacent to the plasma membrane (Paredez et al. 2006; Li et al. 2013a). Many proteins may have a role in linking cortical microtubules to a membrane-associated scaffold in cellulose deposition. However, the precise roles of these proteins remain unclear (Somerville 2006; Bringmann et al. 2012; Li et al. 2012).

Genes from CESA family have been annotated in plants (Holland et al. 2000; Wang et al. 2010). In rice, OsCESA1, OsCESA3, and OsCESA8 form the CESA complex typically for primary cell wall biosynthesis, whereas OsCESA4, OsCESA7, and OsCESA9 are characterized for secondary cell wall formation (Tanaka et al. 2003; Wang et al. 2010). In maize, *ZmCESA1_9* is associated with cellulose synthesis in primary cell walls, whereas *ZmCESA10_12* is involved in secondary cell wall synthesis. In addition, several non-CESA proteins, such as KORRIGAN (KOR), COBRA, and KOBITO, have been reported to be involved in cellulose synthesis and modification (Pagant et al. 2002; Bhandari et al. 2006; Brady et al. 2007; Xie et al. 2013). The KOR proteins encode a membrane-bound β-1,4-glucanse (Nicol et al. 1998), but its precise role remains to be elucidated (Peng et al. 2002). The rice mutant brittle culm1 is a putative ortholog of AtCOBL4, which shows reduced level and an improper orientation of crystalline cellulose microfibrils (Li et al. 2003). COBRA families have 11 members in rice and 9 genes in maize (Brady et al. 2007). The spontaneous *Zea mays* brittle *stalk2* mutant is a putative glycosylphosphatidylinositol-anchored gene that plays a role in cellulose deposition into the cell wall (Ching et al. 2006; Sindhu et al. 2007). In addition, *KOBITO*

gene mutation has been demonstrated to cause a randomized cellulose microfibril orientation in newly deposited cell wall layer (Pagant et al. 2002).

CESA-interactive protein 1 (CSI1) has been identified to interact with CESA isoforms for primary plant cell wall synthesis in *Arabidopsis*. As the CSI1 mutant affects the distribution and movement of CESA complexes in the plasma membrane, CSI1 may function as a scaffold protein for the assembly of the cellulose synthase complex (CSC) or in its interaction with microtubules (Gu et al. 2010). As UDP-glucose is the major substrate for cellulose biosynthesis in the cell wall, sucrose synthase (SuSy), sucrose phosphate synthase (SPS), invertase, and UDP-glucose pyrophosphorylase (UGPase) are important candidates involved in the regulation of carbon partitioning into cellulose and starch (Albrecht and Mustroph 2003).

Furthermore, the cell wall–related NAC, MYB, WRKY, and leucine zipper transcription factors have been partially identified to regulate cellulose biosynthesis in plants (Taylor 2008; Wang and Dixon 2012). In rice and maize, *OsSWNs* (1, 3, and 7) and *ZmSWNs* (1, 3, 6, and 7) were found to serve as the secondary wall–associated NACs (Zhong et al. 2011). Overexpression of an ATAP2 family transcription factor (*SHINE/WAX INDUCER1*) caused a 34% increase in cellulose and a 45% reduction in lignin content with an altered lignin composition, improved biomass digestibility, and no compromise in plant strength and performance (Ambavaram et al. 2011).

Enzymatic hydrolysis of biomass is synergistically catalyzed by cellulases, including endoglucanases, exoglucanases, and β-glucosidases (Mosier et al. 2005; Nguyen et al. 2010). Transgenic plants expressing exogenous microbial cellulase genes have been determined to exhibit enhanced hydrolysis activity and no side effects on plant growth and biomass yield (Himmel and Bayer 2009). Furthermore, the heterologous expression of the catalytic domain of endo-1,4-beta-D-glucanase E1 in *Acidothermus cellulolyticus* targeted to the apoplast in maize can enhance the efficiency of the conversion of the polysaccharides in corn stover into glucose (Ransom et al. 2007). In rice, the overexpression of a nonglycosylated mutant of a cellulose-bound module (CBM) from bacteria has led to a 30% increase in rumen digestibility of the rice straw compared with the wild type. In addition, expansins and expansin-like proteins are cell wall proteins involved in the extension and loosening of plant cell walls, and they have also been found to serve as endogenous CBMs in plants.

Lignocellulose crystallinity is a reportedly important parameter affecting biomass enzymatic digestibility in biofuel production (Somerville 2006; Xu et al. 2012). Cellulose crystallinity refers to the ratio of crystalline regions to noncrystalline regions in cellulose microfibrils, and it can thus be determined by the degree of polymerization (DP) of β-1,4-glucans and cross-linking style of cellulose microfibrils with hemicelluloses and lignin. Hence, it is rational to hypothesize that genetic manipulation of genes associated with cellulose biosynthesis and assembly can result in an alteration of lignocellulose crystallinity. On the other hand, the reduction in lignocellulose crystallinity may somewhat affect plant growth and fitness. To avoid such effect, inducible promoters and protein signals may be applied specifically for gene expression and protein tissue localization in energy crops.

1.6.2 Genes for Hemicellulose Biosynthesis

Hemicelluloses are synthesized in the Golgi apparatus by a plethora of glycosyltransferases (GTs) and subsequently modified by different hydrolases, esterases, and lyases (Leroxel et al. 2006). Cellulose synthase-like F (CSLF) genes encode Golgi-localized glycan synthases that are involved in hemicellulose biosynthesis. The ectopic expression of two rice *CSLF* genes (*OsCSLF2* and *OsCSLF4*) in *Arabidopsis* has confirmed the functions of CSLF proteins in MLG synthesis (Burton et al. 2006). In total, 25 xylem-specific GT genes from 7 GT families (GT2, GT8, GT14, GT31, GT43, GT47, and GT61) have been recommended for hemicellulose biosynthesis (Scheller and Ulvskov 2010). Several GT families, including GT43, GT47, and GT61, may be involved in xylan biosynthesis. The identification of *Arabidopsis* IRX mutants has implicated GT8, GT43, and GT47 families as potential glucuronoxylan (GX) biosynthetic genes (Zhong et al. 2005; Pena et al. 2007; Persson et al. 2007; Brown et al. 2009). Recent research has shown that the biosynthesis of GAXs

would require at least three GTs: xylosyltransferase (XylT), arabinosyltransferase (AraT), and glucuronosyltransferase (GlcAT) (Zeng et al. 2008, 2010). Three wheat (*Triticum aestivum* L.) TaGT proteins from the GT43, GT47, and GT75 families have been identified as promising candidates involved in GAX synthesis termed as TaGT43-4, TaGT47-13, and TaGT75-4. In maize, UDP-glucose 6-dehydrogenase genes encode central enzymes of hemicellulose biosynthesis and appear to be essential for cell wall formation in young organs.

Biomass recalcitrance is collectively known as the natural resistance of plant cell walls to microbial and enzymatic deconstruction (Himmel and Bayer 2009). In nature, the types and abundance of hemicelluloses are considered to be factors of biomass recalcitrance (Gírio et al. 2010; Xu et al. 2012). As hemicelluloses are often cross-linked with lignin to form lignin–carbohydrate complexes, the degree of xylose residues substituted with arabinose residues has been considered an important parameter for the process efficiency of the cell wall (Li et al. 2013b; Zhang et al. 2013). As GAX other than MLG tightly links to lignin, MLG can be used to replace GAX by expressing CSLF and CSLH genes (Fry et al. 2008).

Feruloyl esterases play a key role in enhancing the accessibility of hydrolyzing enzymes to hemicellulose fibers by removing ferulic acid side chains and cross-linking bonds. The expression of a fungal ferulic acid esterase gene in transgenic ryegrass renders the cell walls more accessible to endoxylanases. Plant enzymes that remodel the cell wall architecture, such as expansins and xyloglucan endotransglycosylases/hydrolases (XTHs), can also be used to facilitate wall deconstruction. Expansins act to reduce hydrogen bonding by an unknown mechanism. For example, EXPB1, a β-expansin subfamily member, binds to maize cell walls, causes swelling of the maize cell wall, and facilitates the local movement and stress relaxation of arabinoxylan–cellulose networks (Yennawar et al. 2006). XTHs can generate additional modifications of the hemicellulose–cellulose networks that can potentially loosen cell wall structures. These enzymes cleave and ligate xyloglucans with new xyloglucan partners such that the wall architecture may be modified. Moreover, mannan transglycosylase/hydrolase can catalyze a transglycosylation reaction using mannan-derived oligosaccharide as a substrate. These transglycosylation activities may potentially generate novel heteropolymeric networks that impact biomass recalcitrance.

1.6.3 Genes Associated with Lignin Biosynthesis

Lignin is primarily composed of guaiacyl (G), syringyl (S), and hydroxycinnamates (H) in plant cell walls (Grabber et al. 2004). Three predominant *p*-hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl, and sinapyl alcohols) form lignin monomers. Biosynthesis of lignin involves two major processes: the monolignol pathway (via the phenylpropanoid pathway) in the cytosol and polymerization of the monomers into the cell wall. In the first process, 10 enzymes are considered for monolignol biosynthesis: phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), shikimate hydroxycinnamoyltransferase (HCT), coumarate 3-hydroxylase (C3H), caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), CAD, ferulate 5-hydroxylase (F5H), and COMT (Vogt 2010). Subsequent enzymatic steps that catalyze hydroxylations and methylations produce lignin monomers (Fry et al. 2000; Lindsay and Fry 2008). Members of the pfam PF02458 gene family have been recently found to potentially act as arabinoxylan feruloyl transferases in rice (Piston et al. 2010). The global transcriptome fingerprinting in maize has identified many gene families in lignin biosynthetic pathways (e.g., three PALs, one C4H, one 4CL, two CCoAOMTs, one CCR, one COMT, one F5H1, and one CAD) (Bosch et al. 2011).

Lignin appears to have two primary effects on biomass enzymatic hydrolysis: prohibiting cellulose fiber swelling to reduce surface area access to the cellulase enzyme and preventing cellulase action on the cellulose surface (Converse and Optekar 1993). A direct approach to reduce the side effects of lignin is to alter its content and composition by regulating lignin synthesis pathways (Vanhholme et al. 2010; Sun et al. 2013). Generally, downregulation of genes early in the monolignol biosynthetic pathway, such as PAL, C4H, HCT, and C3H, is the most effective means of

reducing lignin contents. In contrast, downregulation of CCoAOMT, CCR, F5H, COMT, or CAD genes has been shown to affect their monomeric compositions (Simmons et al. 2010).

The *bm3* mutant, a well-characterized natural mutant, is defective in COMT gene and has less lignin and decreased syringyl units, resulting in improved biomass digestibility (Vignols et al. 1995). COMT maize antisense lines are also generated and exhibit a similar phenotype (Piquemal et al. 2002). The *bm1* mutant shows an altered lignin content and composition (Halpin et al. 1998). Allelic variations have also been evaluated for two CCoAOMT and COMT genes in 34 lines of maize selected for their varying digestibility (Guillet-Claude et al. 2004). In sweet sorghum, CCR catalyzes the last step of the phenylpropanoid pathway for monolignol biosynthesis. Plants contain multiple CCR-like genes, thereby complicating the rational selection of lignin-specific targets to maximize their impact. These candidate genes should be accessible to allow for genetic modifications of the content and composition of lignin in energy crops.

1.7 PERSPECTIVES

More than 1000 genes are associated with plant cell wall biosynthesis, degradation, and regulations (Yong et al. 2005; Torney et al. 2007; Vega-Sánchez and Ronald 2010). In total, 550 GT and 419 GH genes have been annotated in rice and maize (Penning et al. 2009). For the discovery of candidate genes, we can take advantage of the rice and maize mutants as described earlier and use genomic information available for the identification of the related orthologs in bioenergy crops. The close evolutionary relationship of the C_4 grasses from the Panicoid subfamily and the syntenic organization of grass genomes make it possible to determine the translation of genes that impact biomass characteristics into more genetically recalcitrant species. Notably, as major discrete transcription factors, such as NAC and MYB families, have been identified to regulate plant cell wall biosynthesis (Karpinska et al. 2004; Goicoechea et al. 2005), we may be able to increase biomass quantity and/or improve biomass quality by dynamically altering the gene expression time and level in energy grass crops. However, more genes are needed to be explored and determined in terms of the gene expression regulation during the biosynthesis of plant cell wall, which is affected by functional genotype, especially from a comparative genomics and proteomics perspective that evaluates all enzymes involved in biomass production. On the other hand, new metabolic engineering and genomics approaches hold great potential for understanding the molecular mechanism of lignocellulosic conversion into economically significant products.

1.8 CONCLUSIONS

Lignocellulose is regarded as a major biomass resource for biofuel production worldwide. Due to biomass recalcitrance, the current biofuel process is unacceptably costly. Genetic breeding of energy crops is considered a promising solution to reduce the production cost of biofuels. This chapter discussed major grass plants that can be used as potential bioenergy crops in terms of the selection of natural germplasm resources and screening cell wall mutants. Related genetic engineering strategies of cell wall were proposed to increase biomass quantity and enzymatic degradability of lignocellulosic energy crops.

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