

## Invited Expert Review

# Genetic Engineering of Energy Crops: A Strategy for Biofuel Production in China [Free Access]

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### Abstract



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Biomass utilization is increasingly considered as a practical way for sustainable energy supply and long-term environment care around the world. In concerns with food security in China, starch or sugar-based bioethanol and edible-oil-derived biodiesel are harshly restricted for large scale production. However, conversion of lignocellulosic residues from food crops is a potential alternative. Because of its recalcitrance, current biomass process is unacceptably expensive, but genetic breeding of energy crops is a promising solution. To meet the need, energy crops are defined with a high yield for both food and biofuel purposes. In this review, main grasses (rice, wheat, maize, sorghum and miscanthus) are evaluated for high biomass production, the principles are discussed on modification of plant cell walls that lead to efficient biomass degradation and conversion, and the related biotechnologies are proposed in terms of energy crop selection.

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### Introduction

Over the past three decades, a fossil-energy-based economy has been booming in China. As a result, its energy expenditure has doubled, leading to become the second biggest energy consumer in the world (Li et al. 2010). According to a report in 2009, China annually used 2.74 billion Mg coal, 0.39 billion Mg petroleum (including 51% import), and 88.70 billion cubic meters of natural gas. By estimates, there are only 114.50–189.20 billion Mg of coal reserved for 60–100 years and 15 billion Mg petroleum for 30 years in China. The Energy and Resource Institute of the National Development and Reform Commission predicted that, in the year 2020, annual petroleum demand will reach 0.45–0.61 billion Mg including 60%–70%

import. To reduce carbon dioxide emissions by 1.2 billion Mg and oil import, Chinese authorities recently drafted a new 10-year plan of developing non-fossil energy that covers 15% of total energy consumption with 2% renewable energy. It includes bioenergy production that annually reaches the standard of 0.24 billion Mg coal (<http://finance.qq.com>).

To reach the bioenergy goal, biomass quality and quantity become crucial factors. Recently the related topics have been extensively discussed on the biomass resources, ecological distribution, developmental history, and biofuel development policies of energy plants in China and beyond (Chen et al. 2009; Tian et al. 2009; Zhang et al. 2009; Wang et al. 2009; Zhou and Thomson 2009; Walker 2010), but the underlying strategies have not well been described. Despite the potential

for biomass quality of energy plants to be evaluated in terms of the cost of biomass pretreatment and the efficiency of lignocellulose degradation (Carroll and Somerville 2009), the solution to biomass quality improvement is still in dispute, due to limited available data. In this review, we precisely defined the concept of energy crops, and propose the practicable strategies, based on our partially unpublished data, for selection of energy crops through three major approaches (germplasm collection, mutant selection and genetic manipulation) towards high biofuel production in China.

### Potential Energy Crops for Biofuel Production

With 22% population and 7% arable land over the world, China has to hold a long-term policy for food security by avoiding any competition from biomass-based applications. It determines all arable lands reserved for growing food crops rather than used for starch/sugar-based bioethanol and edible-oil-derived biodiesel products (Chen et al. 2009; Tian et al. 2009; Wang et al. 2009; Zhang et al. 2009; Zhou and Thomson 2009). Alternatively, conversion of lignocellulosic residues of food crops is a promising solution. In fact, approximately 0.7–0.9 billion Mg residues are harvested each year, and half can be used for biofuel purposes rather than being burnt into ash in the farming field (Chen et al. 2009). Apart from these agro-residue resources in China, more biomass resource is demanded. To meet the need, approximately 0.1 billion hectares of marginal lands that are not applicable for plantation of high-yield and high-quality food crops, can be a great alternative for growing biomass-rich energy plants (Yan et al. 2008).

There are 38 oilseed crops, three sugar-producing crops, five starch-producing crops and 18 species rich in lignocellulose in China (Table 1). Of these species, rice, wheat and maize

are major food crops that can provide about 75% biomass resources of total agricultural residues (Li et al. 2010), and C<sub>4</sub> grasses such as sweet sorghum and miscanthus can be considered as candidates of energy non-food plants (Carpita and McCann 2008; Hodgson et al. 2010; Sang 2010). The energy (food) crops should maintain high yield and good quality of grain/oil for food supply with easy destruction of cell walls in their straws/stalks for biofuel production, whereas the energy (non-food) plants should have high yield of total biomass product with an efficient degradation of cell walls. It is also defined that energy non-food plants should grow very well in marginal lands (Tang et al. 2010).

### Natural Germplasm Resources for Energy Plant Discovery

Bioenergy refers to 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). Principally, starch- and sugar-derived ethanol or plant oil-derived biodiesel is regarded as the first generation biofuel that has already made a relatively small but significant contribution to global energy supplies (Demirbas 2007; Soccol et al. 2010; Sivakumar et al. 2010). The second generation biofuel derived from lignocellulosic residues is predicted to be used in the near future (Mabee and Saddler 2010; Goh et al. 2010; Berndes et al. 2010). Accordingly, energy plants can be divided into three groups subjective to their biomass composition: sugar- and starch-rich plants (for instance, cassava, and sugarcane), lipid-rich plants (rapeseed, sunflower and oil palm), and cellulose-rich plants (poplar, eucalyptus and grasses such as miscanthus and sweet sorghum). Over the past years, technology about bioethanol and biodiesel conversion from starch- and lipid-producing plants has become mature over the world. For

**Table 1. Estimated bioethanol production of major energy crops in China in 2007<sup>a</sup>**

| Energy plants/crops  | Estimated planted area (10 <sup>6</sup> ha) | Straw dry yield per hectare (Mg/ha/year) | Bioethanol production per hectare (Mg/ha) | Total straw dry yield per year (10 <sup>6</sup> Mg/year) | Total bioethanol production per year (10 <sup>6</sup> Mg/year) |
|----------------------|---|--|---|--|--|
| Sugar-based          |   |  |   |  |  |
| Sugar cane           | 1.5   | 50–70                                    | 4–6                                       | 90   | 8  |
| Starch-based         |   |  |   |  |  |
| Cassava              | 3.0   | 20–40                                    | 2–4                                       | 90   | 6  |
| Sweet potato         | 4.0   | 10–20                                    | 3–5                                       | 60   | 12   |
| Lignocellulose-based |   |  |   |  |  |
| Miscanthus           | 7.0   | 50–70                                    | 3–5                                       | 420  | 28   |
| Sweet sorghum        | 3.2   | 10–30                                    | 3–5                                       | 64   | 12   |
| Rice                 | 12  | 10–25                                    | 1–2                                       | 186  | 18   |
| Maize                | 27  | 10–30                                    | 2–4                                       | 305  | 60   |
| Wheat                | 6.5   | 15–30                                    | 1–2                                       | 109  | 10   |

<sup>a</sup>The estimated data modified from Yan et al. (2008), Yuan et al. (2008) and Zhang et al. (2010).

example, since 1975 Brazil has launched a national effort to convert sugarcane into ethanol with a current production at 13.5 million Mg/year. The United States has used maize starch for bioethanol at 16.5 million Mg/year (Demirbas 2007; Spiertz and Ewert 2009; Berndes et al. 2010; De Witt et al. 2010). In China, starch bioethanol is mainly produced from the decayed and aged maize, rice and wheat grains at 1.33 million Mg/year (Zhou and Thomson 2009).

As a contrast, lignocellulose ethanol production, because of its recalcitrance, is still under development (Akin 2007; Himmel et al. 2007). In fact, a great effort has been made to increase the lignocellulose conversion rate, but the difficulty remains with two crucial factors: biomass pretreatment and enzymatic degradation. It is determined by cellulose crystallinity and lignin linking-styles of the plant cell walls (Nguyen et al. 2007, 2010). In spite of extreme pretreatment conditions that can be a solution, such as strong acid/base, or extreme temperature/pressure, it leads to a negative economic profit of biofuel production together with a secondary environmental pollution (Boudet et al. 2003). Therefore, discovery of energy crops would provide a solution to a bottleneck situation. Without doubt, characterization of germplasm resources is an initial and essential work. Not only can it indirectly find out valuable genetic materials for energy crop breeding, but also directly select energy plants. As described above, sweet sorghum and miscanthus are recommended to grow at the first priority in the marginal land in China, because of their diverse natural germplasm resources, rich biomass, efficient lignocellulose degradation and good adaptation to various environmental conditions. In particular, sweet sorghum is suitable for growing in the north of China, whereas miscanthus is considered in the south.

Sweet sorghum (*Sorghum bicolor* (L.) Moench) is a breeding line from the ordinary grain sorghum species. It grows fast

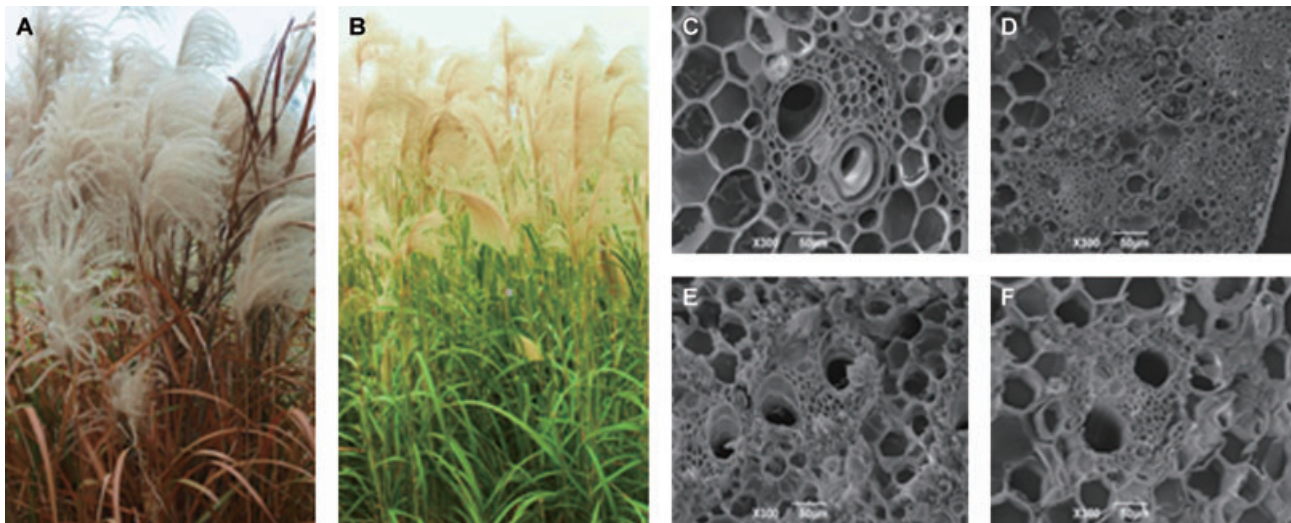
and has tolerance to drought, flooding and salt stresses. Its stalk contains 17%–21% sugar content, and weighs 60–80 Mg per ha (Carpita and McCann 2008). By estimate, it can potentially produce 20 million Mg of bioethanol in the alkaline soils in the north of China. Recently, the Chinese Academy of Sciences (CAS) has organized an international collection of potential energy plants (Li et al. 2010). Dr Haichun Jing and his colleagues in the Institute of Botany CAS have collected hundreds of diverse germplasm materials in order to find out the sweet sorghum that are of rich sugar/starch in stalk/grain and high biomass production. In addition, our laboratory has collaborated to screen out the materials that are of efficient lignocellulose degradation.

Miscanthus is one of the C<sub>4</sub> perennial plants with the highest biomass yield among grass. It is mainly originated from East Asia and the nearby Pacific islands, and more than 11 species have been identified (Jakob et al. 2009). Because of an original center in China, Hunan Agricultural University and Wuhan Botany Garden, CAS have collected more than 1 000 natural *Miscanthus* spp. accessions including four major species (*Miscanthus sacchariflorus*, *Miscanthus lutarioriparius*, *Miscanthus sinensis*, and *Miscanthus floridulus*). Each species covers different ecological and regional types, contributing to a diverse germplasm resource. In order to select out desirable miscanthus as energy plants, our lab has attempted to assess 300 representative accessions including biomass yield, cell wall composition and lignocellulose degradation (Table 2 and Figure 1, Liangcai Peng, unpubl. data, 2010). Due to their distinct biomass degradation, the selected miscanthus materials can be used as energy plants. In addition, the genetic model can be formulated based on the relationships between cell wall composition and biomass degradation, indicating the direction towards the genetic breeding of the miscanthus that have ready biomass digestibility and positive ecological adaptations.

**Table 2. Cell wall composition and degradability of *Miscanthus* ecotypes under different pretreatments<sup>a</sup>**

| Ecotypes | Cell wall composition (%) |                |         | Heat degradation efficiency (%) |          | Alkaline degradation efficiency (%) |          | Acid degradation efficiency (%) |          |
|----------|---------------------------|----------------|---------|---------------------------------|----------|-------------------------------------|----------|---------------------------------|----------|
|          | Cellulose                 | Hemicelluloses | Lignins | C6-sugar                        | C5-sugar | C6-sugar                            | C5-sugar | C6-sugar                        | C5-sugar |
| MI 10    | 28.25                     | 38.74          | 33.01   | 19.07                           | 3.87     | 47.07                               | 24.54    | 31.98                           | 31.31    |
| MI 108   | 33.33                     | 38.11          | 28.56   | 8.59                            | 2.60     | 45.86                               | 26.06    | 30.61                           | 33.96    |
| MI 1     | 44.50                     | 27.95          | 27.56   | 3.29                            | 1.24     | 21.78                               | 14.08    | 14.56                           | 24.63    |

<sup>a</sup>The degradation efficiency is subjective to total soluble hexose or pentose sugar (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<sub>2</sub>SO<sub>4</sub> for 20 min at 121 °C, and the remaining residues incubated with cellulase as just described.



**Figure 1.** *Miscanthus* species (*Miscanthus sinensis*) as energy plants with an efficient lignocellulosic degradation upon pretreatments (Table 2) and different tissue structure in stem.

(A,B) Plant morphological traits of *Miscanthus sinensis* ecotypes (MI 1 and MI 108) growing in the field at anthesis stage.

(C,D) Scanning electron microscopic photographs of *M. sinensis* ecotype (M108, with a high biomass degradation, Table 2) showing a smooth stem transverse-section.

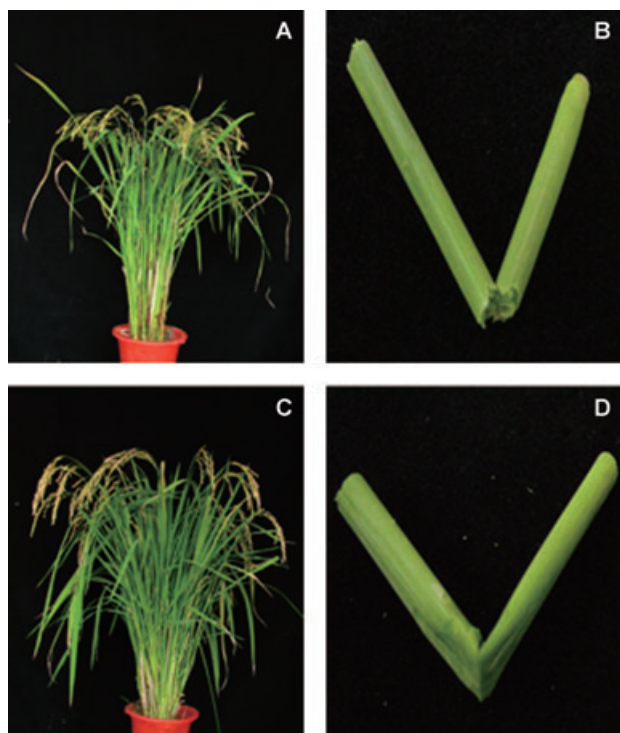
(E,F): Photographs of *M. sinensis* ecotype (M1, with a relatively low biomass degradation, Table 2) displaying a rough stem transverse-section. Scale bars indicated 50  $\mu\text{m}$ .

## Cell Wall Mutants for Energy Crop Breeding

In this review, we defined energy food crops distinctive from energy non-food plants in terms of their original purpose for food supply to humans. As mentioned above, rice, wheat and maize crops provide major food sources in China, but their enormous biomass (straw/stalk) has not been well used for biofuel production. Crop biomass is principally made up of three components of cell walls: cellulose (30%–45%), a  $\beta$ -1,4-glucan polymer that is crystalline, hemicelluloses (20%–30%), branched polymers that are composed of mainly xylose and other five-carbon sugars; and lignins (25%–35%), non-carbohydrates that interlink other polymers into a robust cell wall structure and architecture (Pauly and Keegstra 2008, 2010). The properties of cellulose-crystallinity and lignin-crosslinking become a barrier that critically hinders biomass pretreatment and enzyme digestion (Chen and Dixon 2007; Abramson et al. 2010). Modification of plant cell wall structure, therefore, is the key step for improving biomass quality of energy crops. During evolution, however, plants have to construct their typical cell walls in order to complete their life cycles, rather than to meet the biofuel purpose. Because of the diversity in plant cell wall structure and the complexity of its function, the genetic modification of plant cell walls could unexpectedly lead to alteration of plant cell growth and development (Torney et al. 2007; Vega-Sánchez and Ronald 2010). Alternatively,

selection of cell wall mutants is a practicable work including three major steps: mutagenesis of the high-yield-grain crops, selection of the cell-wall-altered plants, and identification of the mutants that are of high grain yield and efficient biomass degradation.

In order for selection of cell wall mutants, we have collaborated with other labs to screen out large mutagenesis pools of rice T-DNA knockout and maize transposon insertion. Meanwhile, we have generated chemical (ethyl methane sulfonate [EMS]-induced) and physical ( $^{60}\text{Cobalt}$  irradiation) mutagenesis pools for other potentially typical mutants. Distinct from the previously identified cell wall mutants that show abnormal phenotypes as the dwarfism, irregular xylem and even lethality (Goubet et al. 2003; Desprez et al. 2007), the selected mutants displayed similar agronomic traits and grain yields to the wild type, but showed a remarkable alteration of cell wall composition. With various mild pretreatments, several mutants showed an increased rate of biomass degradation in comparison with the wild type (Figure 2, Tables 3,4, Liangcai Peng, unpubl. data, 2010). Obviously, we could take advantage of these mutants as energy crops for biofuel purposes, or use them as genetic lines for further energy crop breeding. As more mutants are selected out, an integrated analysis in combination with the above natural germplasm information, can develop potential cell wall models that are refereed as selection standards of energy crop breeding (Nothnagel and Nothnagel 2007).



**Figure 2. Elite rice (*Oryza sativa*) as a promising energy crop with high lignocellulosic degradation upon pretreatments.**

Rice mutant RC15 selected as energy crop (with a high biomass degradation, **Table 3**) showing a similar agronomic trait and grain yield (**Panel A**) as well as a brittle culm phenotype (**Panel B**), compared with wild type (**Panels C, D**).

## Cell Wall-related Genes for Genetic Manipulation

As a consequence, genetic engineering of the selected energy crops from the natural germplasm resource and mutagenesis pool is another challenge for further increasing biomass yield and biofuel production at a large scale. It relies on genetic

modification of plant cell walls by specifically altering wall polymer inter-linking and cellulose crystallinity, reducing lignin and phenolic acid ester levels, increasing specific hemicelluloses contents, and adding foreign cellulase enzymes and/or other wall proteins (Gressel 2008; Rubin 2008; Jakob et al. 2009; Abramson et al. 2010). To satisfy the above goal, selection of appropriate genes is an initial and crucial step, and the related genetic manipulation approach should be considered. In order to decrease the lignin level, we first have to select the gene that is mainly involved in lignin biosynthesis pathways. If the gene is a housekeeper, it should be partially silenced by RNAi interference rather than totally knocked out by antisense. Because of lignin reduction, the related wall proteins or other polymers should be added for complementation of cell wall strength by using gene specific-expression.

There are more than 1 000 genes that are related to plant cell wall biosynthesis, degradation and regulations (Torney et al. 2007; Vega-Sánchez and Ronald 2010). For candidate gene discovery, we can take advantage of the rice mutants as described above, and then use any genomic information available for identification of the related orthologs. Since the *CesA* gene was first characterized for cellulose biosynthesis in cotton plant (Pear et al. 1996), the *CesA* and *CsI* superfamilies have been identified in rice, maize and other crops. Thus, *CesA/CsI* genes should be considered in use for biomass enhancement. Several non-*CesA* genes involved in cell wall synthesis can also be used for energy crop modification, such as *Korrigan*, *Cobra* and *Kobito* (Pagant et al. 2002; Lee et al. 2003; Bhandari et al. 2006). Importantly, as major transcription factors are identified for regulating secondary cell wall synthesis in *Arabidopsis*, we may directly improve quantity and quality of biomass by altering the expression time and level of these genes in energy crops. In addition, *SuSy* is another candidate gene because of its overexpression in poplar that can lead to cellulose increase by 2%–6% without any negative consequence on plant growth habits (Coleman et al. 2009). To reduce cellulose crystallinity, we can add some special microorganism-derived cellulose binding proteins into cell walls (Abramson et al. 2010).

**Table 3. Cell wall composition and degradability of rice mutants under different pretreatments<sup>a</sup>**

| Materials | Cell wall composition (%) |                |         | Heat degradation efficiency (%) |                       | Alkaline degradation efficiency (%) |                       | Sulfuric acid degradation efficiency (%) |                       |
|-----------|---------------------------|----------------|---------|---------------------------------|-----------------------|-------------------------------------|-----------------------|--|-----------------------|
|           | Cellulose                 | Hemicelluloses | Lignins | C <sub>6</sub> -sugar           | C <sub>5</sub> -sugar | C <sub>6</sub> -sugar               | C <sub>5</sub> -sugar | C <sub>6</sub> -sugar                    | C <sub>5</sub> -sugar |
| RC12      | 49.98                     | 26.70          | 23.32   | 32.52                           | 4.58                  | 48.95                               | 21.14                 | 43.00                                    | 25.17                 |
| RC15      | 38.56                     | 34.74          | 26.70   | 19.47                           | 5.39                  | 29.91                               | 27.81                 | 42.24                                    | 32.40                 |
| RC46      | 47.35                     | 29.41          | 23.23   | 25.69                           | 6.35                  | 45.66                               | 25.29                 | 38.43                                    | 26.46                 |
| RG65      | 51.27                     | 26.36          | 22.36   | 38.07                           | 5.91                  | 34.37                               | 19.49                 | 41.83                                    | 22.70                 |
| WT        | 49.90                     | 24.68          | 25.42   | 18.55                           | 4.31                  | 35.32                               | 18.79                 | 36.10                                    | 19.74                 |

<sup>a</sup>The same method as **Table 2**.

**Table 4. Cell wall composition and degradability of maize mutants under different pretreatments<sup>a</sup>**

| Materials | Cell wall composition (%) |                |         | Alkaline degradation efficiency (%) |                       | Sulfuric acid degradation efficiency (%) |                       |
|-----------|---------------------------|----------------|---------|-------------------------------------|-----------------------|--|-----------------------|
|           | Cellulose                 | Hemicelluloses | Lignins | C <sub>6</sub> -sugar               | C <sub>5</sub> -sugar | C <sub>6</sub> -sugar                    | C <sub>5</sub> -sugar |
| CM4       | 47.12                     | 25.78          | 27.10   | 25.52                               | 25.03                 | 35.66                                    | 23.29                 |
| CM31      | 45.32                     | 22.92          | 31.76   | 41.05                               | 18.72                 | 19.39                                    | 21.00                 |
| CM43      | 44.01                     | 24.80          | 31.19   | 34.59                               | 28.26                 | 29.45                                    | 25.45                 |
| WT        | 45.18                     | 28.05          | 26.77   | 31.68                               | 28.08                 | 19.71                                    | 28.41                 |

<sup>a</sup>The same method as Table 2.

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 cell wall recalcitrance mainly depends on the types of hemicelluloses and ratio of the monomers of lignins (Girio et al. 2010). There are two major hemicelluloses in grasses: MLG ( $\beta$ -1,3- $\beta$ -1,4-glucan) and GAX ( $\beta$ -1,4-linked xylose backbone with single arabinose and glucuronic acid side chains) (Reiter 2002; Vogel 2008; Doblin et al. 2009; Carpita and McCann 2010). Because GAX other than MLG tightly links to lignins, we can use MLG to replace GAX by expressing *CsIF* and *CsIH* genes that have been characterized to catalyze MLG biosynthesis (Fry et al. 2008). Recent findings about three glycosyltransferase (TaGT) proteins participating cooperatively in the GAX polymer synthesis in wheat will extend the effort in cell wall remodeling (Zeng et al. 2010).

Lignins are primarily composed of guaiacyl (35%–49%), syringyl (40%–61%) and hydroxycinnamates (4%–15%) units. The ferulic acid and coumaric acid are also present in plant cell walls (Grabber et al. 2002). In spite of lignocellulose biodegradation being restricted by both lignins and phenolic acids esters, the ratio of coniferyl lignin to syringyl lignin is a crucial factor to determining the degree of biomass recalcitrance (Chen and Dixon 2007). In addition, esterified phenolic acids including the ferulic and *p*-coumaric acids, constitute a major chemical limitation for nonlignified cell walls biodegradation in grasses (Akin 2007; Anderson and Akin 2008). Several lignin metabolism key enzymes, such as C3H, C4H, 4-CL and CCoAOMT, have been characterized in dicot plants (Grabber et al. 2004; Xu et al. 2009; Fagerstedt et al. 2010), but only the brown midrib mutant with known lesions in lignin biosynthesis is extensively studied in corn, sorghum, and millet crops (Jakob et al. 2009). The key genes in regulating lignin biosynthesis and esterified phenolic acids formation in grasses are not clear yet. Recently, we found several rice and maize mutants that showed a distinction in lignin composition and straw/stalk biomass degradation (Liangcai Peng, unpubl. data, 2010). Gene identification of these mutants may be one of the best strategies towards the genetic modification of lignins and esterified phenolic acids in grass.

Biomass process into biofuel includes physical and chemical pretreatment, enzymatic hydrolysis and sugar fermentation. The lignocellulose hydrolysis is synergistically catalyzed by cellulases including endoglucanases, exoglucanases and  $\beta$ -glucosidases (Mosier et al. 2005; Balat et al. 2008; Abramson et al. 2010; Nguyen et al. 2010). Over the past years, several labs have attempted to express microbe cellulase genes in plants, and determined the hydrolysis activity in the transgenic plants. Accordingly, they did not observe any visible side-effect to plant growth and biomass yield (Himmel and Bayer 2009). As an effort, our lab has started to ectopically express fungi-specific cellulase genes in the selected rice and maize mutants using inducible gene promoters. In addition, we are on the way to transform lignin-hydrolysis genes of the white-rot fungi into the energy crops.

## Conclusions

Balancing food supply and biofuel production is regarded as a long-term national economy policy in China. To satisfy the above goal, selection of energy crops is a promising solution through a precise cell wall modification of food crops (rice, wheat and maize) and an extensive selection of the biomass-rich perennial plants (sweet sorghum and miscanthus) that are of high sugar level and/or high lignocellulose yield, even if grown on marginal lands. Accordingly, three practicable approaches are recommended for energy crop discovery: natural germplasm collection, cell wall mutant selection and genetic manipulation. As a result, the energy crops should remain high-yield grain with the reconstructed cell walls in their mature straw/stalk that could be efficiently converted into biofuels. Meanwhile, the genetic model is predicted in order for elucidating the dynamic relationships between plant cell wall remodeling and lignocellulose bio-converting.

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