Genetic engineering approaches to improve bioethanol production from maize
François Torney, Lorena Moeller, Andréa Scarpa and Kan Wang

Biofuels such as bioethanol are becoming a viable alternative to fossil fuels. Utilizing agricultural biomass for the production of biofuel has drawn much interest in many science and engineering disciplines. As one of the major crops, maize offers promise in this regard. Compared to other crops with biofuel potential, maize can provide both starch (seed) and cellulosic (stover) material for bioethanol production. However, the combination of food, feed and fuel in one crop, although appealing, raises concerns related to the land delineation and distribution of maize grown for energy versus food and feed. To avoid this dilemma, the conversion of maize biomass into bioethanol must be improved. Conventional breeding, molecular marker assisted breeding and genetic engineering have already had, and will continue to have, important roles in maize improvement. The rapidly expanding information from genomics and genetics combined with improved genetic engineering technologies offer a wide range of possibilities for enhanced bioethanol production from maize.

Addresses
Center for Plant Transformation, Plant Science Institute and Department of Agronomy, Iowa State University, Ames, Iowa 50011, USA
Corresponding author: Wang, Kan (kanwang@iastate.edu)

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Introduction
The world energy demand is increasing steadily as the human population grows and economic development progresses. However, the current predominant energy source — the fossil fuel supply — is limited. This emphasizes the need to complement fossil-fuel-based energy sources with renewable energy sources, such as agricultural biomass (see Glossary) [1]. Maize, currently one of two major biofuel (see Glossary) crops in the United States, represents 31% of the world production of cereals and occupies a little over one fifth of the worldwide cereal-dedicated land [2]. In addition, maize is the second largest biotech crop (see Glossary) grown world wide, after soybean, and a little over 10% of its cultivated surface is dedicated to biotech varieties [3].

To date, most maize genetic engineering (see Glossary) has been performed using a few genotypes that are amenable to transformation and regenration, but which do not always have the desired agronomic attributes [4,5] (see Figure 1). Improving our ability to introduce transgenes directly into inbred or elite genetic backgrounds is crucial for bioethanol production, because it reduces the time required for transgene introgression into elite maize lines. Other enabling technologies under development aim to improve the quality of transgene expression. These include tissue or developmental stage specific transgene expression, stringently regulated and induced gene expression [6**, site-specific integration of the transgenes [7], expression of multiple transgenes, and gene stacking (i.e. adding transgenes sequentially in a genome) [8,9].

The net energetic benefit of using maize, mainly its starch component [1], for bioethanol production has been extensively reviewed [10**,11] and is still debated among experts [11–13]. Our focus will be on the various possibilities that genetic engineering can offer to increase bioethanol production from maize (see Figure 2). This can be addressed from at least two angles: modifying biomass properties to reduce processing costs or increasing biomass yield (see Glossary) and reducing agricultural inputs. We will review the latest studies on maize biology related to these aspects. Promising work in other species that could lead to improved bioethanol production in maize will also be discussed.

Genetic engineering to modify biomass properties
Two key parts of maize plants can be converted into bioethanol: the kernel, which is mainly made of starch, and the stover, which is predominantly made of lignin and cellulosic (cell wall) components. To convert them effectively into fermentable sugars for ethanol production, a range of approaches using genetic engineering have been explored. One strategy is to modify the characteristics and properties of starch or lignocellulose so that they can be converted more readily to the desired products. The other strategy is to introduce biomass conversion enzymes into plants so that they can aid the conversion process more effectively.

Starch composition
Today, ethanol from maize is produced almost exclusively from starch. The technologies and processes for
Starch is composed of two glucose polymers, amylose and amylopectin. In amylose, glucose units are linked in a linear fashion by α1-4 linkages. Amylopectin, by contrast, is more branched and about 5% of its glucose units are linked by α1-6 linkages. Normal maize starches contain about 20–30% of amylose and 70–80% amylopectin. The amylose/amylopectin ratio in starch affects its physical and physicochemical properties, such as gelatinization and recrystallization [17]. Alteration in starch structure can be achieved by modifying genes encoding the enzymes responsible for starch synthesis, many of which have more than one isoform [15,18]. Transgenic lines with modified expression of specific starch synthases, starch branching enzymes or starch debranching enzymes are being generated in attempts to produce starch granules with increased or decreased crystallinity, and thus altered susceptibility to enzymatic digestion (M James, personal communication). Another strategy is to reduce the energy requirements for the starch to ethanol conversion process. For example, gelatinization is the first step in bioethanol production from starch. It is conceivable that a modified starch with decreased gelatinization temperature might require less energy for the conversion process. Recent research showed that expression of a
recombinant amylpullulanase in rice resulted in starch that when heated to 85 °C was completely converted into soluble sugars [19].

Cell wall composition
Maize stover (leaves and stalks) constitutes a large part of agricultural biomass. Ethanol production from non-grain portions of plants is referred to as cellulosic or lignocellulosic ethanol. Lignocellulose is composed of 30% hemicellulose, 44% cellulose and 26% lignin [20]. The structural crosslinking of these polymers presents a physical barrier to hydrolytic enzymes used in the ethanol conversion process, limiting its efficient usage for bioethanol production. Altering cell wall composition, mainly lignin, has long been contemplated as an option to enhance the efficiency of biomass conversion to ethanol [1].

Lignin is a vital component of the plant cell walls. It is responsible for the rigidity required for plant architecture, provides physical protection against pathogens and aids water transport in the xylem [21,22]. However, during the process of converting biomass into bioethanol, lignin limits the availability of polysaccharides to enzymes, therefore limiting the enzymatic degradability and digestibility of biomass. Maize brown midrib mutants (bm) with an altered lignin biosynthetic pathway have a naturally reduced lignin content and higher digestibility. Two transgenic approaches have successfully mimicked one of these mutant phenotypes (bm3) [23,24]. Piquemal et al. [24] used a maize caffeic acid o-methyltransferase (COMT) antisense gene construct and showed decreased COMT activity and lignin content in the transgenic maize. He et al. [23] obtained similar results using a sorghum O-methyltransferase antisense construct in maize, where transgenic plants showed increased digestibility. These studies show the feasibility of using plant transformation to modify the lignin biosynthetic pathway and to alter the lignin profile of maize.

As anticipated, altering plant lignin composition or content can lead to undesired agronomic consequences. Early studies showed that the bm3 mutants were impaired in several agronomical traits; for example, grain and stover yields were reduced by 20% and 17%, respectively (reviewed in [25]). Arabidopsis and alfalfa genetically engineered for an impaired lignin biosynthetic pathway showed dwarfism and/or flower color change [26,27]. Currently, more basic research is required to understand the lignin biosynthetic pathway and related areas. The future genetic engineering strategy should be a holistic approach to obtain maize with

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maximum digestibility in lignocellulose and minimum reduction in agronomic performance.

**Biomass conversion enzymes**

Although lignocellulosic feedstocks derived from corn stover could be used for conversion to bioethanol, two major limitations to the process are the costs of transport and processing of biomass. One solution is to produce microbial cellulase enzymes in the plant cells to facilitate the conversion of fermentable sugars in planta during the biomass to bioethanol conversion process [28**]. Expression of the catalytic domain of the thermostable 1,4-β-endoglucanase (E1) of *Acidothermus cellulolyticus* in maize [29*] proves the concept that maize can be used as a biofactory for cellulose-degrading enzymes. Even though expression of E1 has not achieved desirable levels, targeting the enzymes to specific subcellular compartments or tissues has shown to be effective in allowing the plants to accumulate higher levels of recombinant enzymes [30,31].

In addition to subcellular targeting of these enzymes, it is also important to express these cell wall degrading enzymes during appropriate developmental stages, rather than over the entire lifetime of the plants. Controlled expression would help to avoid undesired effects on agronomic performance such as lodging or susceptibility to diseases. A senescence-induced promoter might be used to drive cellulase expression in senescing maize. Ideally, the gene should be expressed at the end of the growing season or during post-harvest operations. Other approaches include the use of plant endogenous genes to promote cell wall deconstruction; for example, expansins, a group of hydrogen bond-breaking proteins thought to loosen the cell wall during normal plant growth and development, might be such candidates [32].

**Genetic engineering to improve biomass yield**

Biomass yield is a complex trait. Although several biotech crop lines engineered for yield enhancement are currently being tested [33], the majority of genes involved in the trait remain elusive. Biomass yield increase and stabilization can be achieved through understanding and enhancing mechanisms such as stress tolerance [34,35**,36,37] and carbohydrate metabolism [38].

**Stress tolerance**

Enhanced stress tolerance in plants has been achieved mainly through the manipulation of effector genes [39] (e.g. ion transporters, biosynthetic enzymes; see Glossary) and regulatory genes (e.g. transcription factors [40] or signal transduction components [41,42]; see Glossary) from maize itself, other plants or bacterial sources.

Transgenic maize expressing δ-endotoxins from *Bacillus thuringiensis* (Bt) is the classic example of genetic engineering for (biotic) stress resistance. This biotech maize is widely used in North America and constitutes 22 million hectares worldwide [3]. Among the strategies for next-generation insect-resistant crops are the expression of broad-spectrum insecticidal proteins from plants, from bacteria other than *B. thuringiensis* and novel proteins and peptide hormones from insects [43].

Although insect damage can account for as much as 10–20% of crop loss [42] environmental (abiotic) stress has been held responsible for 69% of crop loss [44]. Common denominators are found in response to several stresses, such as the accumulation of reactive oxygen species (ROS) with deleterious effects (e.g. DNA damage and/or impairment of mitochondrial and chloroplast functions). Several excellent reviews addressing genetic engineering for abiotic stress tolerance have been recently published [34,35**] and here we will examine promising approaches centered on plant responses to oxidative stress.

Mitogen-activated protein kinases (MAPKs) are widely associated with the response to biotic and abiotic stress [45], and might be directly linked to the regulation of abscisic acid (ABA)-responsive antioxidant enzymes in maize [46]. Expression of a *Capsicum annum* MAPK in rice and expression of upstream signaling components MAP kinase kinases (MAPKKs) from tobacco in *Arabidopsis* yielded increased tolerance to a range of biotic and abiotic stresses [47,48]. Our laboratory has demonstrated the benefits of this strategy in maize, where constitutive expression of *Nicotiana* protein kinase 1, a MAPKK, enhanced freezing and drought tolerance in transgenic maize plants [41,42]. Other kinases as well as phosphatases also hold much potential in regulating signal transduction in response to stress [45].

De Block et al. [49] have successfully prevented the formation of ROS and consequently increased various stress tolerances in *Brassica napus* and *Arabidopsis*. Constitutive expression of the gene coding the antioxidant enzyme super oxide dismutase (SOD) in maize, led to increased tolerance to oxidative damage [39]. More recently, *Arabidopsis* plants with enhanced resistance to several abiotic stresses were obtained by overexpressing not a SOD gene itself, but rather a microRNA involved in the fine regulation of two SOD genes, CSD1 and CSD2 [50].

Much of the study and engineering of plant stress resistance has been in model systems [34]. For instance, a particular class of transcription factors — the dehydration-responsive element-binding protein (DREB)/C-repeat binding factor (CBF) — interact with the DRE/CRT cis-element of many stress-related genes and has been widely studied in *Arabidopsis* [35**]. Constitutive over-expression of OsDREB1A and OsDREB1B in rice resulted in improved tolerance to drought, high-salt and cold stresses [51]. A recently cloned maize homologue, *ZmDREB1A,*
enhanced cold tolerance when expressed in Arabidopsis [52]. Additionally, the overexpression of the ZmCRT Binding Factor increased cold tolerance in maize (reviewed in [40]). Results such as these indicate that many of the mechanisms used to enhance stress response pathways in model systems are applicable to maize and offer a key to reducing biomass and grain yield fluctuations, thereby ensuring steady production for biofuel.

Photosynthesis
As a C4 plant, maize has a compartmentalized photosynthetic system that uses the phosphoenolpyruvate carboxylase (PEPC) as a primary carboxylase [53]. It has been reported that transgenic maize overexpressing PEPC has improved CO₂ fixation rate and compensation point, increased fresh and dry weight, enhanced leaf surface and stomatal density, as well as water stress resistance (reviewed in [54]). Additionally, recent work in transgenic tobacco showed that increased levels of fructose-1, 6-bisphosphatase [55] and sedoheptulose-1,7-bisphosphatase [55,56], two Calvin cycle enzymes, significantly increased dry weight. Interestingly, expression of sedoheptulose-1,7-bisphosphatase also increased leaf area [56].

To adjust to the high planting density currently used in agriculture, modifying plant architecture becomes another way to improve photosynthesis [37]. It has been shown recently in rice that either reducing plant hormone brassinosteroid levels or the amount of the brassinosteroid receptors results in an erect leaf phenotype [57]. These erect leaf rice plants, obtained either through mutagenesis or genetic engineering, have enhanced biomass production and grain yield under conditions of high-dense planting with no extra fertilization. It is possible that the erect leaf plants are able to enhance photosynthesis by the leaves in the lower part of the plant owing to their altered architecture [58] or are able to reduce the ‘shade avoidance syndrome’ that is considered to cause stem elongation, early flowering and decreased grain yield in dense planting conditions [59,60].

Grain yield
In 2004, 11% of the maize grain produced in the United States was used to produce ethanol from starch. It is predicted that compared with the 12.87 billion liters of starch ethanol produced in 2004, in 2007 production will reach 20.44 billion liters [1] emphasizing the importance of starch production. As the ADP-glucose pyrophosphorylase (AGP) heterotrimer catalyzes the rate-limiting step in starch biosynthesis, it is usually referred to as a key enzyme in regulating sink strength (see Glossary) in cereal seeds. Deregulation of AGP might lead to increases in plant sink strength and subsequent increases in seed and biomass yield [61–63]. Smidansky et al. [61] transformed rice and wheat [63], using the maize Shrunken2 gene Sh2r6hs coding for an AGP large subunit. Compared with control plants, both transgenic wheat and rice plants showed increased seed weight (increased by 38% and 23%, respectively) and increased biomass (increased by 31% and 22%, respectively). Recently a similar strategy in maize produced a 13% to 25% seed weight increase in AGP transgenic plants [64].

Conclusions
Genetic engineering technology presents undeniable potential for future agriculture and biofuel production, as described above. However, the acceptance of biotech-derived crops has met with skepticism and regulatory hurdles in many countries. One major public concern is the control of pollen dissemination for wind pollinated crops such as maize. Plastid genome transformation presents the advantage of limiting transmission of the transgene via pollen while preserving fertility of the plant and allowing higher transgene product production. Although transformation of plastid genomes has been achieved for a few plant species [65], it still remains to be demonstrated in maize. Male sterility offers an alternative approach to control transgene flow, an issue that will probably have a major impact on the development and routine use of biotech crops, in general, and of biofuel-destined crops in particular. Male sterility is a trait that is naturally present in certain lines but it can also be engineered. A recent demonstration of engineered male sterility used chloroplast transformation to produce completely male sterile tobacco plants [66].

It is now clear that multiple transgene strategies need to be developed to tackle complex traits, to engineer metabolic pathways and to combine the expression of different genes. Some studies have demonstrated the feasibility of such technologies [9,67], but more effort is needed to make them both applicable to bioethanol production and acceptable to the public. Indeed, the development of genetically engineered crops raises issues of legislation relating to how these technologies should be regulated and managed. Each country has its own legislation concerning plant biotechnology. Often the regulatory system lags behind the advancement of a technology. An integrated agri-biotechnology system for food, feed and fuel production is likely to be a challenge from the regulatory point of view, but will most certainly be the future for maize if it is to be bred for bioethanol production.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


A very thorough review of transgene expression control. Figure 2 of this article is especially useful. Even though published in 2004 it is still a good reference for tissue-specific promoters.


A well written review that covers the entire bioenergy field from bioenergy crops to biorefinery technologies. A must-read article for those who are interested in biofuel production.


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An interesting review on the properties of cell walls and how these properties affect the hydrolysis of biomass. It presents the concept of using dehydrogenation polymer–cell wall (DHP-CW) complexes of maize primary cell walls as a tool to study how lignin composition, structure and cross-linking affect enzymatic hydrolysis.


A thorough review on reduced-lignin plants, both mutants and genetically engineered. This article covers more than 10 species: from maize to alfalfa.


A review dedicated to strategies for altering biomass properties. The focus is on the in plants production of biomass conversion enzymes, modification of the lignin content, and increasing plant polysaccharide mass. This review is also a great source of references for a more in depth view of biofuel production related to biomass properties.


Nice example of using the maize crop biomass as a ‘green bioreactor’ for the production of cellulase enzymes. The catalytic domain of an endo-1,4-b-glucanase gene from Acidothermus cellulolyticus was expressed successfully in transgenic maize plants.


