Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol

Mariam B. Sticklen

Abstract | Biofuels provide a potential route to avoiding the global political instability and environmental issues that arise from reliance on petroleum. Currently, most biofuel is in the form of ethanol generated from starch or sugar, but this can meet only a limited fraction of global fuel requirements. Conversion of cellulosic biomass, which is both abundant and renewable, is a promising alternative. However, the cellulases and pretreatment processes involved are very expensive. Genetically engineering plants to produce cellulases and hemicellulases, and to reduce the need for pretreatment processes through lignin modification, are promising paths to solving this problem, together with other strategies, such as increasing plant polysaccharide content and overall biomass.

Finite petroleum reserves and the increasing demands for energy in industrial countries have created international unease. For example, the dependence of the United States on foreign petroleum both undermines its economic strength and threatens its national security[1]. As highly populated countries such as China and India become more industrialized, they too might face similar problems. It is also clear that no country in the world is untouched by the negative environmental effects of petroleum extraction, refining, transportation and use. For these reasons, governments around the world are increasingly turning their attention to biofuels as an alternative source of energy.

The biofuel that is expected to be most widely used around the globe is ethanol, which can be produced from abundant supplies of biomass from all land plants and plant-derived materials, including animal manure, starch, sugar and oil crops that are already used for food and energy. In addition, ethanol has a low toxicity, is readily biodegradable and its use produces fewer air-borne pollutants than petroleum fuel. The growth of feedstock crops for bioethanol production also reduces greenhouse gas levels, mainly because of the use of atmospheric carbon dioxide in photosynthesis. Although the conversion of biomass to ethanol and the burning of ethanol produce emissions, the net effect can be a large reduction in greenhouse gas emissions compared with petroleum fuel, meaning that the use of bioethanol does not contribute to an increase in net atmospheric carbon dioxide[2].

Starch- and sugar-derived ethanol already make a relatively small but significant contribution to global energy supplies. In particular, Brazil produces relatively cheap ethanol from the fermentation of sugarcane sugar to supply one quarter of its ground transportation fuel. In addition, the United States produces ethanol from corn grain. However, even if all the corn grain produced in the United States were converted into ethanol, this could only supply about 15% of that country's transportation fuels. Meeting US fuel requirements using starch would mean that corn grain production must be increased or corn grain be diverted from other uses. For example, 50.8% of total US corn grain production is currently used for livestock feed[3], and the conversion of corn grain into ethanol has already increased the prices of meat and dairy products.

The future production and use of ethanol that is obtained from cellulosic matter, supplemented by grain ethanol, has been predicted to decrease the need for petroleum fuel[1]. The main advantages of using cellulosic matter over starch and sugar for ethanol include the abundant supply of cellulosic biomass as compared with the limited supplies of grain and sugar. In addition, starch and sugar that are used for the production of ethanol compete with food supplies. Therefore, it is advantageous to use non-food crops and the waste from food crops for bioethanol production. Furthermore, the use of cellulosic biomass allows bioethanol production in countries with climates that are unsuitable for crops such as sugarcane or corn. For example, the use of rice

Department of Crop and Soil Sciences, Michigan State University, East Lansing, Michigan 48824, USA.
e-mail: stickle1@msu.edu
doi:10.1038/nrg2336

straw for the production of ethanol is an attractive goal given that it comprises 50% of the word's agronomic biomass.

Serious efforts to produce cellulosic ethanol on an industrial scale are already underway. Notably, in 2006, US president George W. Bush announced the goal of reducing 30% of foreign oil requirements by 2030 by using crop biomass for biofuel production. As a result, the Department of Energy announced the funding of three major biofuel centres and the establishment of six cellulosic ethanol refineries, which, when fully operational, are expected to produce more than 130 million gallons of cellulosic ethanol per year[3,4], Other than the Canadian Iorgen plant, no commercial cellulosic ethanol plant is yet in operation or under construction. However, research in this area is underway and funding is becoming available around the world for this purpose, from both governmental and commercial sources. For example, British Petroleum have donated half a billion dollars to US institutions to develop new sources of energy — primarily biofuel crops.

Presently, several problems face the potential commercial production of cellulosic ethanol. First, the high costs of production of cellulases in microbial bioreactors. Second, and most important, are the costs of pretreating lignocellulosic matter to break it down into intermediates and remove the lignin to allow the access of cellulases to biomass cellulose. These two costs together make the price of cellulosic ethanol about two to three-fold higher than the price of corn grain ethanol. Plant genetic engineering technology offers great potential to reduce the costs of producing cellulosic ethanol. First, all necessary cell-wall-degrading enzymes such as cellulases and hemicellulases could be produced within the crop biomass so there would be no need, or only minimal need, for producing these enzymes in bioreactors. Second, plant genetic engineering technology could be used to modify lignin amount and/or configuration in order to reduce the needs for expensive pretreatment processes. Finally, future research on the upregulation of cellulose and hemicellulose biosynthesis pathway enzymes for increased polysaccharides will also have the potential to increase cellulosic biofuel production.

In this Review, I first provide an overview of the process of cellulosic ethanol production, including a brief description of the nature of the plant cell wall as a source of biomass, and the enzymes that are used in the cellulosic conversion process. I then focus on the potential for plant genetic engineering to overcome the challenges described above.

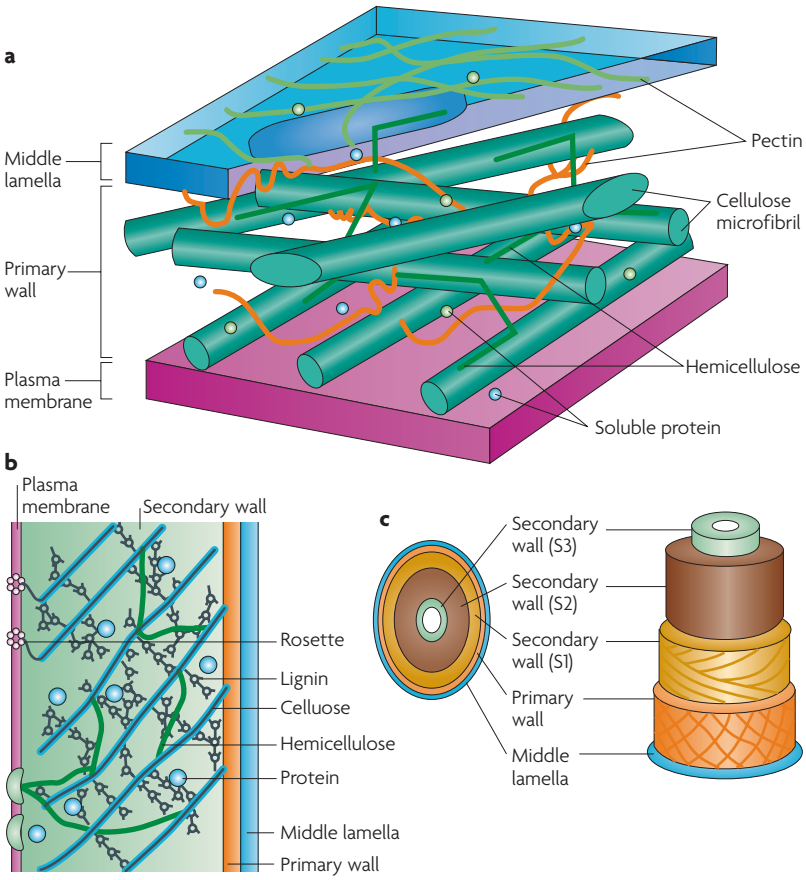


Figure 1 | **Plant plasma membrane and cell-wall structure. a** | Cell wall containing cellulose microfibrils, hemicellulose, pectin, lignin and soluble proteins. **b** | Cellulose synthase enzymes are in the form of rosette complexes, which float in the plasma membrane. **c** | Lignification occurs in the S1, S2 and S3 layers of the cell wall.

The basics of cellulosic ethanol production

Feedstock crops and lignocellulosic biomass. The factors that affect the suitability of potential new feedstock crops around the globe for bioethanol production are complex, and relate to country- and region-specific agricultural practices, market forces, and political as well as biological issues. These factors include land availability, locally accepted cropping systems, and types and forms of transportation fuel. In addition, the current status of a particular species in terms of its development as a crop (for example, the development of breeding strategies) is another important issue; in terms of biology, the feedstock crops that have so far been recommended for conversion to cellulosic ethanol have a high amount of cellulosic biomass. These include corn, rice, sugarcane, fast-growing perennial grasses such as switchgrass and giant miscanthus, and woody crops such as fast-growing poplar and shrub willow[5,6]. Depending on where they are planted, the ideal characteristics of non-food cellulosic crops are: use of the C4 photosynthetic pathway; long canopy duration; perennial growth; rapid growth in spring (to out-compete weeds); high water-usage efficiency; and possibly partitioning of nutrients to subterranean storage organs in the autumn.

The source of lignocellulosic biomass is the plant cell wall (FIG. 1), which has important roles in determining the structural integrity of the plant, and in defence against pathogens and insects[7]. The structure, configuration and composition of cell walls vary depending on plant taxa, tissue, age and cell type, and also within each cell wall layer[8,9]. The basic structure of the primary cell wall is a scaffold of cellulose with crosslinking glycans, and there are two types of primary cell wall, which are classified according to the type of crosslinks. Type I walls are present in dicotyledonous plants and consist of equal

Box 1 | **Key components of the plant cell wall**

Cellulose

Plants produce about 180 billion tons of cellulose per year globally, making this polysaccharide the largest organic carbon reservoir on earth[76]. Cellulose makes up 15–30% of the dry mass of primary and up to 40% of secondary cell walls, where it is found in the form of 30 nm diameter microfibrils. Each microfibril is an unbranched polymer with about 15,000 anhydrous glucose molecules that are organized in β-1,4 linkages (that is, each unit is attached to another glucose molecule at 180° orientation). The microfibrils are lined up parallel to each other and consist of crystalline regions, within which cellulose molecules are tightly packed. Cellulose also has amorphous or soluble regions, in which the molecules are less compact, but these regions are staggered, making the overall cellulose structure strong[7]. So far, cellulose is the only polysaccharide that has been used for commercial cellulosic ethanol production, probably because it is the only one for which there are commercially available deconstructing enzyme mixtures.

Hemicellulose

Cellulose microfibrils are coated with other polysaccharides such as hemicellulose or xyloglucans. All dicotyledonous cell walls and about half of monocotyledonous ones consist mainly of xyloglucans. However, in the commelinoid monocotyledons, such as cereals and other grasses, cell walls mostly consist of glucuronoarabinoxylans. Depending on the plant species, 20–40% of the plant cell-wall polysaccharides are hemicellulose. Like cellulose, hemicellulose could be converted into fermentable sugars by enzymatic hydrolysis for the production of cellulosic ethanol.

Pectin

About 35% of dicotyledonous plant dry matter is made up of pectin, a mixed group of various branched, hydrated polysaccharides that are abundant in galacturonic acid. Pectin is mostly made up of homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II, arabinans, galactans and arabinogalactans[7]. Pectin polysaccharide has roles in forming connections between plant cells, adjusting pH and ion balance, recognizing foreign molecules to alert the cell to the presence of microorganisms or insects, and establishing cell-wall porosity[77,78]. Pectin is not considered important for the production of cellulosic ethanol.

Lignin

Lignin is a major constituent of secondary cell walls, and accounts for about 10–25% of total plant dry matter. Lignin is composed of a complex of phenylpropanoids (aromatic compounds) linked in a network to cellulose and xylose with ester, phenyl and covalent bonds[7]. Neither the mechanism of association of lignin with cell-wall polysaccharides nor the lignin biosynthetic pathway is well understood[35]. Lignin has an important role in protecting the plants against invasion by pathogens and insects, and lignin deposition is thought to be increased in response to attack by these invaders[10].

amounts of glucan and xyloglucan embedded in a matrix of pectin. Type II walls are found in cereals and other grasses, have glucuronoarabinoxylans as their crosslinking glucans, and lack pectin and structural proteins[7]. Polysaccharides, such as cellulose, hemicellulose and pectin (BOX 1), are abundant in plant primary cell walls and can be hydrolysed to provide fermentable sugars for bioethanol production. They contain various combinations of constituent sugars, all of which are initially produced from glucose[7].

The plant secondary cell wall contains cellulose, hemicellulose and lignin (BOX 1). The cellulose microfibrils of the secondary cell wall are embedded in lignin, and in this context function like steel rods embedded in concrete, but with less rigidity. In tree trunks, there are three layers of secondary cell wall, which are called the S1, S2 and S3 lamellae, resulting from different arrangements of cellulose microfibrils. The S1 layer is the outermost layer, is produced first and contains helices of microfibrils. The S2 and S3 are the inner layers and have no helices.

The cellulosic ethanol production process. To produce cellulosic ethanol, lignocellulosic biomass is harvested from the feedstock crop, compacted (fresh or dry) and transported to a cellulosic ethanol refinery where it is stored, ready for conversion. The biomass is then pretreated with extreme heat and/or chemically in order to break it down into intermediates and remove the lignin; this is followed by detoxification, neutralization and separation into its liquid and solid components. The latter are then hydrolysed using enzymes that are produced in microbial bioreactors from bacteria or fungi. Finally, sugars are separated and fermented to produce ethanol (FIG. 2).

Cell-wall-deconstructing enzymes. Cell-wall polysaccharides can be converted into fermentable sugars through enzymatic hydrolysis using enzymes such as cellulases and hemicellulases (BOX 2). Lignin is the main barrier to such conversion as it prevents cell-wall hydrolysis enzymes from accessing polysaccharides[10,11]. Therefore, heat and/or chemical pretreatment processes are being developed and used to break down cell walls into intermediates and remove lignin to allow the exposure of cellulose to cellulases. These enzymes are produced naturally by a range of microbial species. As biofuels research increases in the twenty-first century, an increasing number of bacteria and fungi will be studied for their ability to convert cell-wall polysaccharides into fermentable sugars for biofuels. Many cell-wall-deconstructing enzymes have been isolated and characterized, and more are under investigation, particularly with the hope of finding more enzymes that can resist higher conversion temperatures and a range of pHs during pretreatment — presently the two most important limiting factors in the production of cellulosic ethanol. At present, commercial cellulases are produced as a combination of microbial enzymes. A future goal is the commercial production and use of hemicellulases to increase the output of five- and six-carbon fermentable sugars. Certain commercial hemicellulases are available, but are not suitable for biofuel production.

Genetic manipulation of feedstock crops

Genetic engineering of most food crop species is well established, using either *Agrobacterium tumefaciens* or gene-gun-mediated gene transfer. Among biomass feedstock crops, rice, maize, sorghum, poplar and switchgrass[12] are efficiently transformable at commercially acceptable levels. In terms of other relevant species, *Agrobacterium* has been known to genetically transform dicotyledonous crops (including fast-growing woody plants such as willow and poplar), which are natural hosts for *Agrobacterium*. Although *Agrobacterium* does not infect monocotyledonous plants such as cereals and perennial grasses in nature, certain strains have been shown to transform rice, corn, wheat, barley, sorghum and switchgrass.

Whether *Agrobacterium* or gene-gun transformation is used, the main challenge is genotype-nonspecific genetic transformation of these crops: among many species and cultivars, generally only one or two are ideal

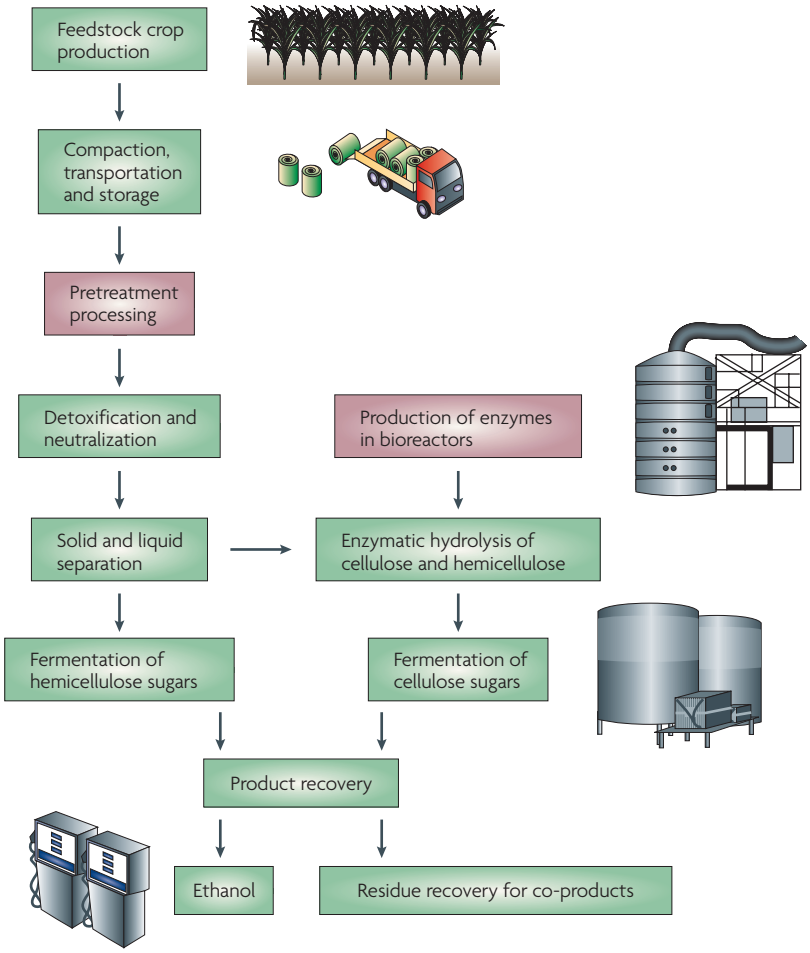


Figure 2 | **Overview of cellulosic ethanol production.** Flow chart showing the steps in the production of cellulosic ethanol from feedstock crops.

candidates for genetic transformation. For example, among all switchgrass genotypes, only two cultivars can be efficiently genetically engineered. The biological basis of this issue is not well understood, but could partly be due to the transformability of the targeted *in vitro* explants. For example, a system has been developed for the genetic transformation of a range of different cultivars of cereal crops (such as maize, oat and barley) by gene-gun bombardment of multiple meristem primordial explants[13] A similar system might enable the genetic transformation of a wider range of cultivars of switchgrass and other perennial grasses.

Breeding strategies are also likely to have an important part in the improvement of relevant feedstock species for cellulosic bioethanol production. Food and feed crops have been improved from their wild ancestors for many decades through breeding for better seed yields and resistance to biotic and abiotic factors. The newly emerging biomass crops such as switchgrass and miscanthas are essentially wild populations, and like food and feed crops they require years of traditional breeding and related molecular approaches such as genetic markers and genome mapping. Along these lines, it is encouraging to note that sequencing of the switchgrass genome by the Joint Genome Institute is pending.

Production of hydrolysis enzymes in plants

At present, plant cell-wall hydrolysis enzymes are expensively produced in microbial bioreactors for commercial use. Plants are already used industrially for the production of enzymes and other proteins, carbohydrates, lipids, industrial polymers and pharmaceuticals[14–16]. Expertise is available for plant genetic transformation, farming of transgenic crops and harvesting, transporting and processing the plant matter[16]. Attention is now turning to the heterologous expression of plant cell-wall-deconstructing enzymes in plants so that they can be produced more cheaply for use in cellulosic hydrolysis.

Cell-wall hydrolysis enzymes can potentially be produced in all feedstock crops that are to be used for cellulosic ethanol production. The plant-based production of these enzymes has a crucial advantage, in that growing transgenic plants in the field requires a much lower energy input than microbial production of these enzymes. As many of the cell-wall hydrolysis enzymes

Box 2 | **Cell-wall-deconstructing enzymes**

Cellulases

Three types of cellulase are needed to deconstruct cellulose into glucose. These include endoglucanase (E1; E.C. 3.2.1.4), exoglucanase or cellobiohydrolase (E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21)[25,79]. In the hydrolysis process, endoglucanase first randomly cleaves different regions of crystalline cellulose, producing chain ends. Exoglucanase then attaches to the chain end and threads it through its active site, cleaving off cellobiose units. The exoglucanase also acts on regions of amorphous cellulose with exposed chain ends without the need for prior endoglucanase activity. Finally, β-glucosidase breaks the bonds between the two glucose sugars of cellobiose to produce monomers of glucose[80].

Hemicellulases

For cellulases to access cellulose, the hemicellulose surrounding it must be removed. While cellulose consists of a single monosaccharide and type of bond, hemicelluloses are amorphous and diverse. Since the main constituent of hemicellulose is β-1,4-xylan, the most abundant class of hemicellulase is xylanase, which can have both endo- and exo-activity[80].

Ligninases

Lignin degradation by microorganisms is poorly understood. The most effective lignin-degrading microbes in nature are thought to be white rot fungi[81], especially *Phanerochaete chrysosporium* and *Trametes versicolour*. The three main families of lignin-modifying enzymes that are produced by fungi are laccases, manganese-dependent peroxidases and lignin peroxidases[82–84].

Table 1 | **Heterologous expression of cell-wall-deconstructing enzymes in plants**

Plant	Transgenic enzyme	Subcellular storage compartment	References
Arabidopsis thaliana	*Acitothermus celluluolyticus* $E1_{CAT}$	Apoplast	25
Tobacco	*A. celluluolyticus* E1 and $E1_{CAT}$	Apoplast	79,85
Tobacco	*A. celluluolyticus* E1	Endoplasmic reticulum	85
Tobacco	*Clostridium thermocellum* XynZ	Apoplast	86
Tobacco	*A. celluluolyticus* E1	Chloroplast	85,87
Tobacco	Maize β-glucosidase	Chloroplast	42
Tobacco	*A. celluluolyticus* $E1_{CAT}$	Chloroplast	79
Tobacco	*Thermomonospora fusca* E2 and E3	Cytosol	88
Tobacco	*Trichoderma reesei* CBH1	Cytosol	89
Tobacco	Human β-glucosidase	Cytosol	90
Tobacco	*C. thermocellum* $XynA_{CAT}$	Cytosol	91
Tobacco	*A. cellulolyticus* E1	Cytosol	18,27
Potato	*A. celluluolyticus* E1	Apoplast	27
Potato	*Streptomyces olivaceoviridis* XynB	Apoplast	92
Potato	*A. celluluolyticus* E1	Chloroplast	87
Potato	*A. celluluolyticus* E1	Vacuole	87
Potato	*T. fusca* E2	Cytosol	38
Potato	*T. fusca* E3	Cytosol	88
Potato	*S. olivaceoviridis* XynB	Cytosol	92
Alfalfa	*T. fusca* E2 and E3	Cytosol	88
Rice	*A. celluluolyticus* $E1_{CAT}$	Apoplast	20
Rice	*C. thermocellum* $XynA_{CAT}$	Cytosol	93
Barley	*Rumen Neocallimastix patriciarum* XynA	Cytosol	94
Maize	*A. celluluolyticus* $E1_{CAT}$	Apoplast	95
Maize	*A. celluluolyticus* $E1_{CAT}$	Apoplast	19

E1, E2 and E3, endoglucanases (endocellulases); CAT, catalytic domain; XynA, XynB and XynZ, xylanases (hemicellulases); CBH1, celluobiohydrolase 1.

identified so far are of bacterial origin, codon alteration of the coding region is usually needed to ensure efficient expression in plants; this is a straightforward procedure that is widely used for the heterologous expression of microbial proteins in eukaryotes. Another potential issue is misfolding of the enzymes in their new environment, a point that is explored below.

When expressed in plants, accumulation of the cell-wall hydrolysis enzymes in subcellular compartments is preferred over their accumulation in cytosol. When targeted for accumulation in subcellular compartments, these enzymes are more likely to display correct folding and activity, glycosylation, reduced degradation and increased stability, as compared to production and accumulation in the cytosol[14,17]. The heterologous enzymes can be extracted from fresh or dry transgenic crop biomass as part of the plant total soluble protein (TSP), which can then be added to pretreated crop biomass for conversion into fermentable sugars[18–20]. Extraction of TSP from fresh or dry matter is quick and easy, and could be included in ethanol production facilities. However, research is needed to determine the stability of the biological activity of extracted plant-produced hydrolysis enzymes in TSP when stored under freeze conditions for different periods of time before their use in hydrolysis.

Two other important and related areas for further research are increasing the levels of production and the biological activity of the heterologous enzymes. For example, the E1 enzyme from *Acidothermus cellulolyticus* has been produced in rice at almost 5% plant TSP and in maize at about 2% TSP, but these levels need to be increased to about 10% TSP for complete hydrolysis without the need for addition of microbially produced endoglucanase. It has been shown that expressing just the catalytic domain of these enzymes results in a higher level of expression (TABLE 1). Another method of increasing the level of enzyme production is to genetically engineer the chloroplast genome instead of the nuclear genome. Because the chloroplast genome of most flowering plants is maternally inherited, chloroplast transgenesis also provides the benefit of transgene containment, which is important for crops with out-crossing wild relatives. Genetic transformation of chloroplast genomes is now possible in most dicotyledonous crops, including poplar.

However, chloroplast transgenesis of most cereal and perennial grass feedstock crops is not yet an easy task[21], in part because cereal crops cannot be regenerated from leaf or cotyledon explants. Efforts made in this regard have resulted in the ability to achieve heteroplastomic chloroplast transformation (in which a fraction of the chloroplasts in a single plant are transformed). Other than for poplar[22], homoplasmic chloroplast transformation (in which every chloroplast carries the transgene) is not yet possible for any feedstock biomass crop.

The subcellular targeting of heterologously expressed hydrolysis enzymes is important for several reasons. Such targeting keeps the foreign enzymes away from cytoplasmic metabolic activities, avoiding potential damage. It also allows higher levels of enzyme accumulation and can increase enzyme stability through reduced exposure to proteases. Targeting can also enable better folding of proteins in subcellular compartments where there are molecular chaperones, and keeps the cell-wall degrading enzymes away from host cell walls. Retention signal peptides have been characterized for targeting plant proteins to the endoplasmic reticulum (ER), the apoplast, chloroplasts, vacuoles and mitochondria. These subcellular compartments have various features that make them more or less desirable for the accumulation of different proteins. For example, increased protein folding and assembly, and high levels of protein accumulation have been achieved by targeting heterologous antibodies to the secretory pathway instead of allowing them to remain in the cytosol[23]. The ER is an excellent potential compartment for the targeting of cell-wall-deconstructing proteins: it has an oxidizing environment and is abundant in molecular chaperones, with few proteases[15]. In addition, studies have shown that proteins are more stable when they are retained in the lumen of the ER, causing 2–10-fold greater activity compared with when they are secreted in the cytosol[24].

Other factors that might affect the levels of biological activity of heterologous enzymes need further investigation. For example, the importance of matching the optimal pH of the targeted enzymes with the pH of their targeted compartment is currently unclear. It is possible to produce biologically active enzymes in certain subcellular compartments, and directly use these enzymes for hydrolysis upon their extraction from transgenic plants. It is also possible to produce the hydrolysis enzymes in compartments in which they might lose biological activity, but then to activate these enzymes by chemical treatments during extraction. As pH is one of the factors required for the biological activity of enzymes, it might be difficult to exactly match the pH needed for ideal biological activity of certain enzymes in certain subcellular compartments. For example, the pH of chloroplasts is around 7.5 at night and 8.0 during the day[14], and therefore the enzymes accumulated in chloroplasts might not always have the same biological activity.

Several microbial hydrolysis enzymes have already been produced in plants through subcellular targeting (TABLE 1). However, most research has been performed on tobacco and alfalfa, which are not feedstock biofuel crops. As indicated above, the cytosol might not be an ideal location for the accumulation of heterologous molecules because of potential interference with metabolic activities. The apoplast has been selected in many cases, assuming that this compartment is the most spacious and therefore capable of accumulating large quantities of heterologous proteins[25]. However, this compartment is of most use for the accumulation of hydrolysis enzymes that are thermophilic (biologically active at higher temperatures). Should the heterologous enzymes become active at *in situ* temperatures, the enzymes would degrade plant cell walls before lignification. Targeting the same enzyme to several compartments in the same plant might increase the level of enzyme production, as shown for xylanase targeting to either chloroplasts or peroxisomes individually compared with its production in both compartments in the same plant[26].

Another important factor to be considered in this respect is the bioconfinement of genetically engineered biomass crops. For example, plants could be genetically engineered in a tissue-specific manner under regulation of the Rubisco promoter so that hydrolysis enzymes are not produced in seeds, flowers and roots[27]. Several other methods of bioconfinement could be used to produce genetically engineered biomass crops while avoiding concerns about the transfer of transgenes from genetically modified crops to their cross-breedable relatives through pollen flow[21].

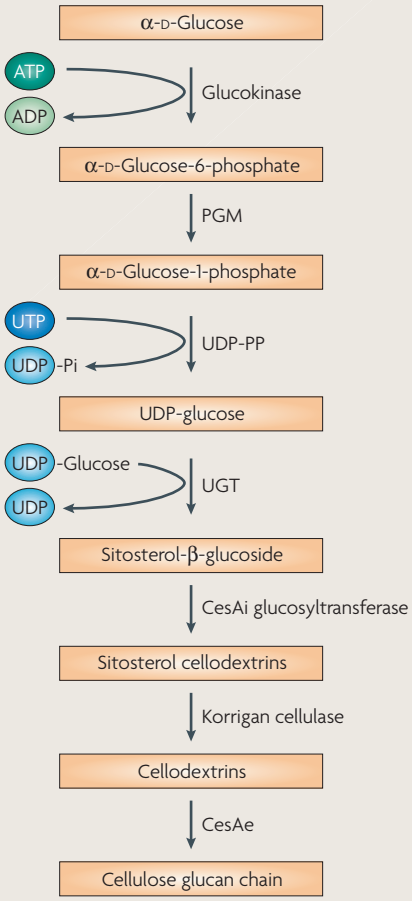
Increasing plant cellulosic biomass

Increasing cell-wall polysaccharide content. Functional genomics and mutant studies have played important parts in the identification of genes that are involved in both cellulose (BOX 3) and hemicellulose biosynthesis. Although cellulose biosynthesis has been studied for decades, most steps in this pathway are not yet well understood[28–33]. For example, even in studies of model plants such as *Arabidopsis thaliana*, most of the cellulose biosynthesis pathway enzymes have been identified based on hypothetical modelling, without confirmation that they actually have the roles that are shown in BOX 3. Current understanding of the hemicellulose biosynthesis pathway is even less complete. Future studies will be geared towards an improved understanding of the biosynthesis of these plant cell-wall polysaccharides, and towards their genetic manipulation to increase polysaccharides for improved cellulosic biofuel production. Recent large grants for biofuel research such as those from British Petroleum are aimed at these issues.

Increasing the overall biomass. Increased overall feedstock biomass could also be achieved by genetically modifying feedstock plants. This could include modification of plant growth regulators. For example, transgenic hybrid poplar with increased gibberelin biosynthesis displayed improved growth and an increase in biomass[34], probably owing to the effects of gibberelin on plant height. There are also several other potential routes to increasing overall plant biomass[35]. Assuming there are no limitations to the supplies of water, fertilizer or sunlight, feedstock biomass is the product of the solar radiation over the cropping duration, corrected

Box 3 | **Cellulose biosynthesis**

Cellulose synthesis involves several steps. First, glucokinase utilizes water soluble α-D-glucose and one phosphate molecule of an ATP molecule to produce α-D-glucose-6-phosphate, which is converted by phosphoglucomutase (PGM) to α -D-glucose-1-phosphate. Following this step, UDP-glucose pyrophosphorylase (UDP-PP) removes one organic phosphate from α-D-glucose-1-phosphate to produce UDP-glucose, which is soluble in the cytoplasm and is the precursor for the generation of microcrystalline cellulose. There are three more steps associated with polymerization of UDP glucose and formation of glucan chains: chain initiation, chain elongation and chain termination. In chain initiation, UDP-glucosyltransferase (UGT) transfers a glucose residue from UDP-glucose into a sitosterol molecule on the cytoplasmic face of the plasma membrane, forming sitosterol β-glucoside and releasing an UDP. Sitosterol β-glucoside functions as a primer for cellulose biosynthesis in plants. It uses the cellulose synthase initiation factor (CesAi) glucosyltransferase enzyme and the glucose of an UDP-glucose molecule to initiate glucan polymerization by synthesizing lipid-linked oligosaccharides called sitosterol cellodextrins (SCDs) in the cytoplasm. The SCDs then flip to the outer face of the plasma membrane and bind to the elongation cellulase synthase (CesAe). Then, the SCDs use korrigan cellulase enzyme to cleave the sitosterol molecule from cellodextrin. The cellodextrin has a small water-soluble glucan chain. Following this step, the cellodextrin uses the membrane-bound sucrose synthase (SuSy) enzyme to extend the glucan chain into a 36-mer growing chain by adding UDP-glucose molecules. Finally, termination of the glucan chain occurs. Therefore, the glucan chains are derived from soluble α -D-glucose units that polymerize through β-1,4-glycosidase bonds. The glucan chains then extrude into the plant cell wall where they coalesce to form microfibrils. In microfibrils, the multiple hydroxyl group of the glucose residues of one glucan chain form hydrogen bonds with oxygen molecules of another glucan chain, resulting in firm side-by-side chains of glucans with high tensile strength cellulose microfibrils.



Antisense oligonucleotides
Short synthetic pieces of DNA that are designed to bind to their target mRNA through base pairing. As a result, they inhibit the expression of the target mRNA, causing inhibition of translation, splicing or transport of the target mRNA, or degradation of the DNA–RNA hybrid by RNase H.

RNA interference
In RNAi, long double-stranded RNAs (dsRNAs of around >200 nt) are used to silence the expression of specific target genes. Long dsRNAs are first processed into 20–25 nt small interfering RNAs (siRNAs) by the Dicer RNase III-like enzyme. SiRNAs then assemble into endoribonuclease-containing RNA-induced silencing complexes (RISCs), and subsequently guide RISCs to complementary RNA molecules, which they cleave and destroy.

by the amount of intercepting crop canopy, where the solar light energy is converted into plant dry matter. Other factors are also important contributors to overall biomass: carbon allocation[36]; uptake of carbon dioxide, nitrogen and other resources; utilization of nutrients, oxygen and water; respiration; and the synchronization of the circadian clock and external light–dark cycle[37]. An important direction for future research will be a better understanding of these factors in order to find potential targets for genetic manipulation for increased biomass.

One study involving genetic manipulation has produced promising results towards the goal of increasing biomass in a relevant crop plant. A key enzyme for starch biosynthesis in endosperm, ADP-glucose pyrophosphorylase (AGP), was expressed at higher levels by using an endosperm-specific promoter in rice. This caused an unexpected 20% increase in plant biomass[38]. How the increase in the biological activity of AGP increased the overall rice biomass is unclear. Similarly, whether manipulations of other enzymes that are associated with starch biosynthesis would shift energy from starch biosynthesis to overall biomass production, and at what level such a shift might become harmful to seed development and viability, are questions that remain to be investigated.

Decreasing the need for pretreatment

Lignin modification. Downregulation of lignin biosynthesis pathway enzymes (FIG. 3) to modify the chemical structures of lignin components and/or reduce plant lignin content is an important potential way to reduce pretreatment costs in bioethanol production from cellulosic biomass[39]. Lignin is derived from three precursors — paracoumaryl, coniferyl and sinapyl alcohols — that are synthesized in separate but interconnected pathways[35]. As the question marks in the pathway show (FIG. 3), there are important gaps in our knowledge of lignin biosynthesis. However, despite these limitations, significant progress has been made towards genetically engineering plants to modify lignin composition and content to improve cellulosic ethanol production and costs.

Lignin genetic modification was initially of interest for other industrial applications, such as to increase digestibility and decrease the necessity for bleaching in the paper industry[40–42]. Downregulation of lignin biosynthesis enzymes was initially performed using antisense oligonucleotides; however, RNA interference (RNAi) technology has also been used for this purpose.

Downregulation of 4-coumarate 3-hydroxylase (C3H) in alfalfa (*Medicago sativa*) resulted in a dramatic shift in the lignin profile and consequent altered lignin structure[43], causing improved digestibility of

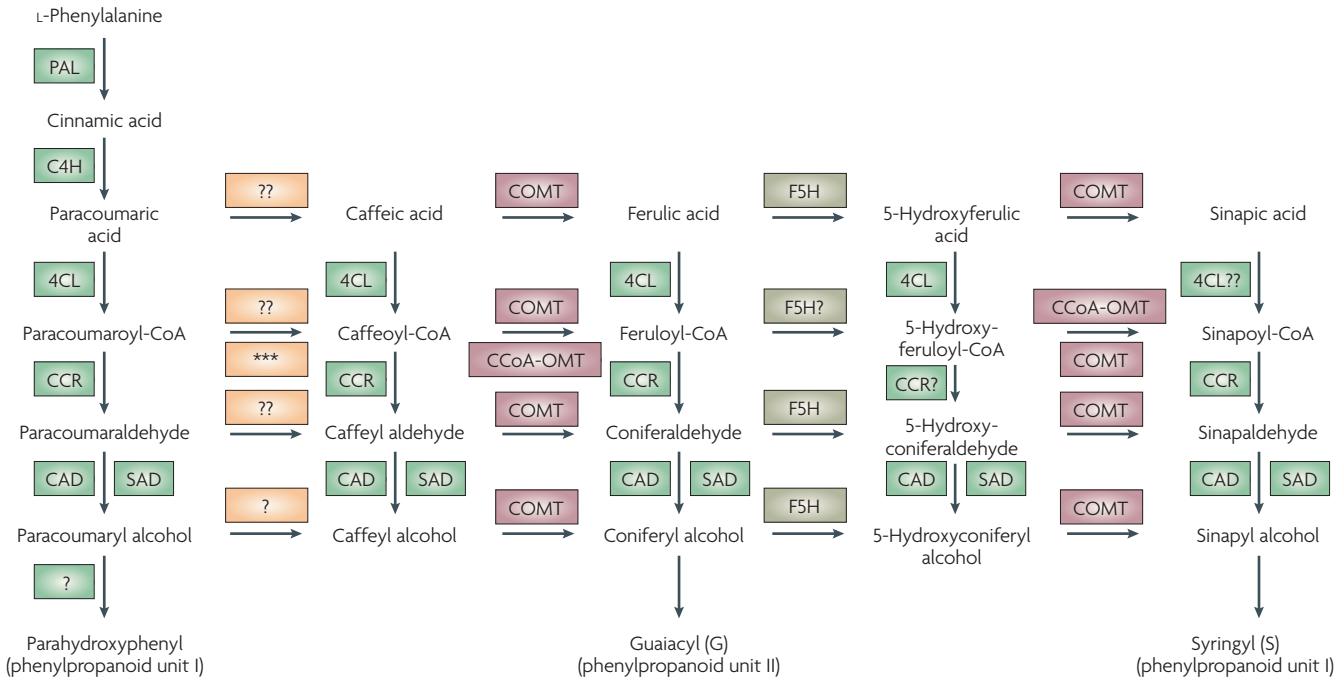


Figure 3 | **Lignin biosynthesis.** The lignin biosynthesis pathway. 4CL, hydroxycinnamate-CoA/5-hydroxyferuloyl-CoA-ligase; C3H, 4-hydroxycinnamate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, hydroxycinnamyl alcohol dehydrogenase; CCoA-OMT, *S*-adenosyl-methionine caffeoyl-CoA/5-hydroxyferuloyl-CoA-*O*-methyltransferase; CCR, hydroxycinnamoyl-CoA:NADPH oxidoreductase; COMT, caffeate *O*-methyltransferase; OMT: *S*-adenosyl-methione-caffeate/5 hydroxyferulate-*O*-methyltransferase; PAL: phenyl ammonia lyase; SAD, sinapyl alcohol dehydrogenase.

C3H-deficient alfalfa lines in ruminants[42]. In another study, downregulation of another lignin biosynthesis pathway enzyme, cinnamyl alcohol dehydrogenase (CAD) in alfalfa resulted in modification of lignin residue composition and increased in *in situ* digestibility. However, CAD downregulation in alfalfa did not result in a decrease in the amount of lignin in the plants[44]. In *Populus* spp., CAD downregulation resulted in improved lignin solubility in an alkaline medium, leading to more efficient delignification[45] and highlighting the possibility of decreasing the need for pretreatment processes in this species. Finally, suppression of another lignin biosynthesis enzyme, *O*-methyl transferase (OMT) in tobacco (*Nicotiana tabacum*) resulted in increased biomass production without decreasing the overall lignin content[46]. Future challenges include gaining a better understanding of lignin biosynthesis pathway enzymes in biomass crops and understanding the effects of downregulating each lignin biosynthesis enzyme in a wider range of relevant species.

Another strategy of interest is to divert plant carbon resources away from lignin production, which can also have additional advantages in terms of improving biomass content. For example, shifting energy from lignin biosynthesis to polysaccharide synthesis has been achieved in aspen (*P. tremuloides*). Downregulation of 4-coumarate CoA ligase (4CL) resulted in a 45% decrease in lignin content and a concomitant 15% increase in cellulose content[47]. These figures were further increased to a 52% reduction in lignin content and a 30% increase in cellulose content when coniferaldehyde 5-hydroxylase (CAld5H) was also downregulated[48]. Finally, downregulation of cinnamoyl CoA reductase (CCR) in transgenic tobacco resulted in a decrease in lignin content and a concomitant increase in xylose and glucose associated with the cell wall[49].

Importantly, the genetic manipulation of lignin biosynthesis pathway enzymes has been specifically shown to reduce the need for pretreatment processes for the production of fermentable sugars. For example, a recent proof-of-concept study showed that downregulation of six different lignin biosynthetic pathway enzymes in alfalfa — C4H (cinnamate 4-hydroxylase), HCT (hydroxycinnamoyl transferase), C3H (4-hydroxycinnamate 3-hydroxylase), CCoA-OMT (*S*-adenosyl-methionine caffeoyl-CoA/5-hydroxyferuloyl-CoA-*O*-methyltransferase), F5H (ferulate 5-hydroxylase), or COMT (caffeate *O*-methyltransferase)— could reduce or eliminate the needs for chemical pretreatment in the production of fermentable sugars[50,51]. The report indicates that some of the transgenic plants yield nearly twice as much sugar from cell walls as do wild-type plants upon conversion. The same study also indicated that downregulation of lignin biosynthesis enzymes could bypass the need for acid pretreatment. However, alfalfa is not a biomass biofuel crop and similar studies in a wide range of biomass crops are needed to confirm the usefulness of downregulating lignin biosynthesis pathway enzymes, either singly or in combination. Further investigations are also crucial to ensure that lignin manipulations do not interfere with plant structural integrity and defence against pathogens and insects[35,39].

Substrate-disrupting factors. Recently, a group of protein modules have been recognized that disrupt plant cell-wall substrates, potentially increasing the accessibility, and therefore efficiency, of hydrolysis enzymes. These modules mainly comprise cellulose or other carbohydrate-binding modules of the glycosyl hydrolase family that are required for polysaccharide hydrolysis. The effects of these factors on cell-wall structure, plant growth and development has also been demonstrated in transgenic plants[52] These factors are known to function synergistically with each other to disrupt plant cell-wall substrates[53,54]. A group of proteins called expansins have an important role in loosening the cell wall to allow expansion and growth[55–57]. Recently, a report[58] showed that one possible substrate for cellulosic ethanol production, corn stover, contains expansin. Another study has indicated the presence of expansins and another, unidentified molecule[59] in corn stover, both of which increase the cellulose deconstruction efficiency. Another protein called 'swollenin' has been found in the fungus *Trichoderma reesei* and has a cellulose-binding domain and an expansin-like domain, which together have a disrupting effect on crystalline carbohydrates[60]. Little is yet known about how these proteins function and whether more substrate-disrupting factors exist. Transferring the genes that encode these proteins to model plants or cellulosic biomass crops themselves, and testing the viability of the resulting, transgenic plants might provide another route towards modifying cell walls and decreasing the need for expensive pretreatment processes.

Modifying features of cellulose. Increasing cellulose solubility can increase saccharification, therefore providing another potential route to decreasing pretreatment needs. For example, in algae, exopolysaccharides such as acetan, hyalurona, alginate, levan and chitosan are water soluble. Transgenic expression of levansucrose from the bacterium *Erwinia amylovora* (which mediates the synthesis of water-soluble fructan from sucrose) increases permeability of algal cell walls[61]. Furthermore, transgenic algae expressing exogenous hyaluronan and chitin synthase in the extracellular matrix have increased cellulose production[62,63]. These studies might become important because algae can potentially be used as a source of biofuel.

The extreme complexity of the cell-wall matrix, which gives it its crystalline nature, is an important factor in the recalcitrance of cell walls to biomass release, and ways of decreasing this recalcitrance are under investigation[64]. For example, expression of cellabiose dehydrogenase (CDH) in feedstock crops might decrease cellulose crystallinity. CDH in a crude mixture of cellulases is reported to increase the degradation of crystalline cellulose[65], possibly by preventing the re-condensation of glycosidic bonds of cellulose chains that have been nicked by endocellulases[66]. In addition, CDH alters cellulose, hemicellulose and lignin *in vitro* by creating hydroxyl radicals[67]. Along similar lines, β-glucosidase has been used for some time in hydrolysis in the pulp industry[68]. In recent studies, injecting thermophilic α-glucosidase and β-glucosidase into tobacco plants converted plant tissues into fermentable sugars[69–71]; the β-glucosidase was used to decrease cellulose crystallinity[72] and therefore increase the saccharification of cell-wall polysaccharides. Finally, expression of cellulose-binding module (CBM) in tobacco decreases cellulose crystallinity[73], and its addition *in vitro* decreases pure cellulose crystallinity[74]. This effect might be due to the CBD (cellulose binding domain) hindering the transition from the cellulose polymerization phase to the crystallization phase, and therefore increasing the rate of cellulose biosynthesis[74,75]. Applying these strategies in relevant feedstock crops therefore suggests an important research direction for bioethanol production.

Conclusions

Although some important advances have been made to lay the foundations for plant genetic engineering for biofuel production, this science is still in its infancy. A general challenge will be the development of efficient, genotype-nonspecific genetic engineering systems in feedstock crops — at present only a few cultivars of each feedstock biomass crop can be efficiently transformed. There are also specific challenges relating to the various areas discussed in this Review. For example, after decades of research aimed at reducing the costs of microbial cellulases, their production is still expensive[13]. One way of decreasing such costs is to produce these enzymes within crop biomass. However, this approach has its own challenges. For example, plants have not been able to produce these enzymes at a level sufficient for complete cell-wall deconstruction (about 10% of plant total soluble proteins). As discussed above, research is particularly needed to focus on the targeting of these enzymes to multiple subcellular locations in order to increase levels of enzyme production and produce enzymes with higher biological activities. The potential also exists to produce larger amounts of these enzymes in chloroplasts, and exciting progress has been made in terms of the crops for which the chloroplast can now be genetically engineered. More efforts are needed towards the development of systems to genetically engineer chloroplasts of biomass crops such as cereals and perennial grasses[46].

There are also several challenges outside the realm of genetic engineering that need to be addressed before ethanol from cellulosic biomass can be considered as a solution to global fuel demands. These include transport and storage issues. Another important issue is whether agricultural land should be shifted from food, feed, fibre and shelter crops to biofuel crops.

Finally, there are questions over whether ethanol is the ideal biofuel. Ethanol cannot be transported through normal pipelines due to its hydrophilic nature causing pipeline corrosion, and would be expensive to transport by trains or tankers. An alternative to ethanol might be butanol, which has several advantages over ethanol as a biofuel: it is much less hydrophilic[11], produces more energy per unit, is less volatile and less corrosive than ethanol (enabling easier transportation and avoiding damage to automobile valves and gaskets), and can

be used in gasoline-powered vehicles without engine modification or adverse effects on operation. The main challenge facing the use of butanol is its production costs — it is much more difficult to produce by fermentation than ethanol because of its toxicity to the microbes that are used for fermentation, and is therefore harder to produce in relatively high concentrations. Like ethanol, butanol can be produced from starchy crops such as corn, rice, barley and sorghum. Butanol could also be produced from enzymatic hydrolysis of lignocellulosic matter, specifically using *Clostridium beijerinckii* P260, which produces enzymes that can utilize the five- and six-carbon sugars that are present in cellulosic biomass and convert them to butanol.

Whether ethanol or butanol is the main biofuel of the future, plant genetic engineering to deconstruct plant cell-wall polysaccharides, to suppress lignin biosynthesis enzymes, or to increase the level of polysaccharides or the overall plant biomass promises to have key roles in decreasing biofuel production costs.

1. Bordetsky, A., Hwang, R., Korin, A., Lovaas, D. & Tonachel, L. *Securing America: Solving Our Oil Dependence Through Innovation* (Natural Resources Defense Council, New York, 2005)
2. Schlamadinger, B. *et al*. Towards a standard methodology for greenhouse gas balances of bioenergy systems in comparison with fossil energy systems. *Biomass Bioenergy* **13**, 359–375 (1997).
3. National Corn Growers Association. *World of corn* [online], http://www.ncga.com/WorldOfCorn/main/WorldOfCorn2007.pdf (2007).
4. Department of Energy. *DOE selects six cellulosic ethanol plants for up to $385 million in federal funding* [online], http://www.doe.gov/news/4827.htm (2007).
Shows the United States' recognition of the need to allocate funds for the design and establishment of the first six commercial cellulosic ethanol plants.
5. Knauf, M. & Moniruzzaman, M. Lignocellulosic biomass processing: a perspective. *Int. Sugar J.* **106**, 147–150 (2004).
6. Sticklen, M. B. in *Proc. 2nd Int. Ukrainian Conf. Biomass for Energy* 133–136 (Ukraine Natl Acad. Sci., Kiev, 2004).
7. Carpita, N. & McCann, M. in *Biochemistry & Molecular Biology of Plants* Ch. 2 (eds Buchanan, B., Gruissem, W. & Jones, R. L.) (John Wiley & Sons, New Jersey, 2002).
8. Ding, S. Y. & Himmel, M. E. The maize primary cell wall microfibril: a new model derived from direct visualization. *J. Agric. Food Chem.* **54**, 597–606 (2006).
9. Bothast, R. J. & Schlicher, M. A. Biotechnological processes for conversion of corn into ethanol. *Appl. Microbiol. Biotechnol.* **67**, 19–25 (2005).
10. Mosier, N. *et al*. Features of promising technologies for pretreatment of lignocellulosic biomass. *Biores. Tech.* **96**, 673–686 (2005).
11. Somerville, C. S., The billion-ton biofuels vision. *Science* **312**, 1277 (2006).
Describes the availability of lands and the needs for production of a billion ton biomass in the United States to decrease its dependency on foreign oil.
12. Somleva, M. N., Tomaszewski, Z. & Cong, B. V. *Agrobaterium*-mediated genetic transformation of switchgrass. *Crop Sci.* **42**, 2080–2087 (2002).
13. Sticklen, M. & Oraby, H. Shoot apical meristem: a sustainable explant for genetic engineering of cereal crops. *In Vitro Cell. Dev. Plant* **41**, 187–200 (2005).
14. Sticklen, M. B. Plant genetic engineering to improve biomass characteristics for biofuels. *Curr. Opin. Biotechnol.* **17**, 315–319 (2006).
15. Fischer, R., Stoger, E., Schillberg, S., Christou, P. & Twyman, R. Plant-based production of biopharmaceuticals. *Curr. Opin. Plant Biol.* **7**, 152–158 (2004).
16. Howard, J. A., & Hood, E. Bioindustrial and biopharmaceutical products produced in plants. *Adv. Agron.* **85**, 91–124 (2005).
17. Horn, M. E., Woodard, S. L. & Howard, J. A. Plant molecular farming: systems and products. *Plant Cell Rep.* **22**, 711–720 (2004).
18. Teymouri, F., Alizadeh, H., Laureano-Perez, L., Dale, B. E. & Sticklen, M. B. Effects of ammonia fiber explosion treatment on activity of endoglucanase from *Acidothermus cellulolyticus* in transgenic plant. *Appl. Biochem. Biotechnol.* **116**, 1183–1192 (2004).
19. Ransom, C. B. *et al.* Heterologous *Acidothermus cellulolyticus* 1,4-β-endoglucanase E1 produced within the corn biomass converts corn stover into glucose. *Appl. Biochem. Biotechnol.* **36**, 207–220 (2007).
20. Oraby, H. *et al.* Enhanced conversion of plant biomass into glucose using transgenic rice-produced endoglucanase for cellulosic ethanol. *Transgenic Res.* **16**, 739–749 (2007).
An excellent example of producing a cell-wall hydrolysis enzyme in rice, a globally important crop.
21. National Research Council. *Bioconfinement of Genetically Engineered Organisms* (Natl Acad. Sci., Washington D. C., 2004).
A comprehensive review of the designs and methods that could be used before production of genetically engineered organisms in order to reduce risks and public concerns.
22. Okumura, S. *et al.* Transformation of poplar (*Poplus alba*) plastids and expression of foreign proteins in tree chloroplasts. *Transgenic Res.* **15**, 637–646 (2006).
23. Schillberg, S., Zimmermann, S., Voss, A. & Fischer, R.. Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in *Nicotiana tabacum*. *Transgenic Res.* **8**, 255–263 (1999).
24. Schillberg, S. Fischer, R. & Emans, N. Molecular farming of recombinant antibodies in plants. *Cell. Mol. Life Sci.* **60**, 433–445 (2003).
25. Ziegler, M. T., Thomas, S. R. & Danna, K. J. Accumulation of a thermostable endo-1,4-D-glucanase in the apoplast of *Arabidopsis thaliana* leaves. *Mol. Breeding* **6**, 37–46 (2000).
26. Hyunjong, B., Lee, D. S. & Hwang, I. Dual targeting of xylanase to chloroplasts and peroxisomes as a means to increase protein accumulation in plant cells. *J. Exp. Bot.* **57**, 161–169 (2006).
27. Dai, Z., Hooker, B. S., Anderson, D. B. & Thomas, S. R. Improved plant-based production of E1 endoglucanase using potato: expression optimization and tissue targeting. *Mol. Breeding* **6**, 277–285 (2000).
28. Kawagoe, Y. & Delmer, D. P. Pathways and genes involved in cellulose biosynthesis. *Genet. Eng.* **19**, 63–87 (1997).
29. Arioli, T. *et al.* Molecular analysis of cellulose biosynthesis in *Arabidopsis*. *Science* **279**, 717–720 (1998).
30. Bolwell, G. P. Biosynthesis of plant cell wall polysaccharides. *Trends Glycosci. Glycotechnol.* **12**, 143–160 (2000).
31. Persson, S., Wei, H., Milne, J., Page, G. P. & Somerville, C. R. Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. *Proc. Natl Acad. Sci. USA* **102**, 8633–8638 (2005).
32. Andersson-Gunneras, S. *et al.* Biosynthesis of cellulose-enriched tension wood in *Populus*: global analysis of transcripts and metabolites identifies biochemical and developmental regulators in secondary wall biosynthesis. *Plant J.* **45**, 144–165 (2006).
33. Haigler, C. H. in *The Science and Lore of the Plant Cell Wall: Biosynthesis, Structure and Function* (ed. Hayashi, T.) (Brown Walker, Boca Raton, 2006).
34. Eriksson, M. E., Israelsson, M., Olsson, O. & Moritiz, T. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nature Biotechnol.* **18**, 784–788 (2000).
35. Sticklen, M. B. Feedstock crop genetic engineering for alcohol fuels. *Crop Sci.* **47**, 2238–2248 (2007).
36. Luo, Y., Chen, J. L., Reynolds, J. F., Field, C. B. & Mooney, H. A. Disproportional increases in photosynthesis and plant biomass in a Californian grassland exposed to elevated CO_2: a simulation analysis. *Funct. Ecol.* **11**, 696–704 (1997).
37. Dodd, A. N. *et al.* Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* **309**, 630–633 (2005).
38. Smidansky, E. D., Martin, J. M., Hannah, C. L., Fischer, A. M. & Giroux, M. J. Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase. *Planta* **216**, 656–664 (2003).
39. Ragauskas, A. J. *et al.* The path forward for biofuels and biomaterials. *Science* **311**, 484–489 (2006).
40. Boudet, A.-M. Lignins and lignification: selected issues. *Plant Physiol. Biochem.* **38**, 81–96 (2000).
41. Dean, J. F. D. in *Biotechnology of Biopolymers: From Synthesis to Patents* 4–21 (eds Steinbuchel, A. & Doi, Y.) (John Wiley & Sons, New Jersey, 2004).
42. Ralph, J. *et al.* Effects of coumarate 3-hydroxylase down-regulation on lignin structure. *J. Biol. Chem.* **281**, 8843–8853 (2006).
43. Reddy, M. S. S. *et al.* Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L). *Proc. Natl Acad. Sci. USA* **102**, 16573–16578 (2005).
44. Baucher, M. *et al.* Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (*Medicago sativa* L) and the effect on lignin composition and digestibility. *Plant Mol. Biol.* **39**, 437–447 (1999).
45. Pilate, G. *et al.* Field and pulping performances of transgenic trees with altered lignification. *Nature Biotechnol.* **20**, 607–612 (2002).
46. Blaschke, L., Legrand, M., Mai, C. & Polle, A. Lignification and structural biomass production in tobacco with suppressed caffeic/5-hydroxy ferulic acid-*O*-methyl transferase activity under ambient and elevated CO_2 concentrations. *Physiol. Plant.* **121**, 75–83 (2004).
47. Hu, W. J. *et al.* Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. *Nature Biotechnol.* **17**, 808–812 (1999).
48. Li, Y. *et al*. Processivity, substrate binding, and mechanism of cellulose hydrolysis by *Thermobifida fusca* Cel9A. *Appl. Environ. Microbiol.* **73**, 3165–3172 (2007).
49. Chabannes, M. *et al.* Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. *Plant J.* **28**, 257–270 (2001).
50. Chen, F. & Dixon, R. A. Lignin modification improves fermentable sugar yields for biofuel production. *Nature Biotechnol.* **25**, 759–761 (2007).
An excellent example of how plant lignin downregulation can reduce the needs for expensive pretreatment processes.
51. Chapple, C., Ladish, M. & Meilan, R. Loosening lignin's grip on biofuel production. *Nature Biotechnol.* **25**, 746–748 (2007).
52. Obembe, O. *et al.* Promiscuous, non-catalytic, tandem carbohydrate-binding modules modulate cell wall structure and development of transgenic tobacco plants. *J. Plant Res.* **120**, 605–617 (2007).
53. Boraston, A. B. *et al*. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochem. J.* **382**, 769–781 (2004).
54. McCartney, L. *et al.* Differential recognition of plant cell walls by microbial xylan specific carbohydrate-binding modules. *Proc. Natl Acad. Sci. USA* **103**, 4765–4770 (2006).
55. Cosgrove, D. J. Loosening of plant cell walls by expansins. *Nature* **407**, 321–326 (2000).

56. **Vaaje-Kolstad, G.** *et al.* Crystal structure and binding properties of the *Serratia marcescens* chitin-binding protein CBP21. *J. Biol. Chem.* **280**, 11313–11319 (2005).
57. Yennawar, N. H. *et al.* Crystal structure and activities of EXPB1 (Zea m 1), a beta expansin and group-1 pollen allergen from maize. *Proc. Natl Acad. Sci. USA* **103**, 14664–14671 (2006).
58. Cosgrove, D. J. & Tanada, T. Use of gr2 proteins to modify cellulosic materials and to enhance enzymatic and chemical modification of cellulose. US Patent 20070166805 (2007).
59. Han, Y. & Chen, H. Synergism between corn stover protein and cellulase. *Enz. Microb. Technol.* **41**, 638–645 (2007).
60. Saloheimo, M. *et al.* Swollenin, a *Trichoderma reesei* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. *Eur. J. Biochem.* **269**, 4202–4211 (2002).
61. Han, Y. W. Microbial levan. *Adv. Appl. Microbiol.* **35**, 171–194 (1990).
62. Graves, M. V. *et al.* Hyaluronan synthesis in virus PBCV-1 infected Chlorella-like green algae, (1999). *Virology* **257**, 15–23.
63. Kawasaki, T., Tanaka, M., Fujie, M., Usami, S. & Yamada, T. Chitin synthesis in chlorovirus CVK2-infected Chlorella cells, *Virology* **302**, 123–131 (2003).
64. Himmel, M. E. *et al.* Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* **315**, 804–807 (2007).
65. Bao, W. & Renganathan, V. Cellobiose oxidase of *Phanerochaete chrysosporium* enhances crystalline cellulose degradation by cellulases, *FEBS Lett.* **302**, 77–80 (1992).
66. Ayers, A. R., Ayers, S. B. & Eriksson, K.-E. Cellobiose oxidase, purification and partial characterization of a hemoprotein from *Sporotrichum pulverulentum*. *Eur. J. Biochem.* **90**, 171–181 (1978).
67. Henriksson, G., Johansson, G. & Pettersson, G. A critical review of cellobiose dehydrogenases, *J. Biotechnol.* **78**, 93–113 (2000).
68. Breuil, C., Chan, M., Gilbert, M. & Saddler, J. N. Influence of β-glucosidase on the filter paper activity and hydrolysis of lignocellulosic substrates. *Biores. Technol.* **39**, 139–142 (1992).
69. Montalvo-Rodriguez, R. *et al.* Autohydrolysis of plant polysaccharides using transgenic hyperthermophilic enzymes. *Biotechnol. Bioeng.* **70**, 151–159 (2000).
70. Sticklen, M. B., Dale, B. E. & Maqbool, S. B. Transgenic plants containing ligninase and cellulase which degrade lignin and cellulose to fermentable sugars. US Patent 7049485 (2006).
71. Sticklen, M. B. Production of beta-glucosidase, hemicellulase and ligninase in E1 and FLC-cellulase-transgenic plants. US Patent 20070192900 (2007).
72. Jeoh, T. *et al.* Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. *Biotechnol. Bioeng.* **98**, 112–122 (2007).
73. Shoseyov, O., Shani, Z. & Levy, I. Carbohydrate binding modules: biochemical properties and novel applications, *Microbiol. Mol. Biol. Rev.* **70**, 283–295 (2006).
74. Xiao, Z. Z., Gao, P. J., Qu, Y. B. & Wang, T. H. Cellulose-binding domain of endoglucanase III from *Trichoderma reesei* disrupting the structure of cellulose, *Biotechnol. Lett.* **23**, 711–715 (2001).
75. Levy, I., Shani, Z. & Shoseyov, O. Modification of polysaccharides and plant cell wall by endo-1,4-β-glucanase (EGase) and cellulose binding domains (CBD). *Biomol. Eng.* **19**, 17–30 (2002).
76. Festucci-Buselli, R. A., Otoni, W. C. & Joshi, C. P. Structure, and functions of cellulose synthase complexes in higher plants. *Braz. J. Plant Physiol.* **19**, 1–13 (2007).
An up-to-date review of structure and function of cellulase synthase complexes in higher plants.
77. Ridley, B. L., O'Neill, M. A. & Mohnen, D. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochem.* **57**, 929–967 (2001).
78. O'Neill, M. A., Ishii, T., Albersheim, P. & Darvill, A. G.. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annu. Rev. Plant Biol.* **55**, 109–139 (2004).
79. Ziegelhoffer, T., Raasch, J. A. & Austin-Phillips, S. Dramatic effects of truncation and sub-cellular targeting on the accumulation of recombinant microbial cellulase in tobacco. *Mol. Breeding* **8**, 147–158 (2001).
80. Warren, R. A. J. Microbial hydrolysis of polysaccharides. *Annu. Rev. Microbiol.* **50**, 183–212 (1996).
81. D'Souza, T. M., Merritt, C. S. & Reddy, C. A. Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*. *Appl. Environ. Microbiol.* **65**, 5307–5313 (1999).
82. Boominathan, K. & Reddy, C. A. in *Handbook of Applied Mycology. 4. Fungal Biotechnology* (eds Arora, D. K., Elander, R. P. & Mukerji, K. G.) 763–822 (Marcel Dekker, New York,1992).
83. Hatakka, A. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiol. Rev.* **13**, 125–135 (1994).
84. Kirk, T. K. & Farrell, R. L. Enzymatic 'combustion': the microbial degradation of lignin. *Annu. Rev. Microbiol.* **41**, 465–505 (1987).
85. Dai, Z., Hooker, B. S., Quesenberry, R. D. & Thomas, S. R. Optimization of *Acidothermus cellulolyticus* endoglucanase (e1) production in transgenic tobacco plants by transcriptional, post-transcription and post-translational modification. *Transgenic Res.* **14**, 627–643 (2005).
86. Herbers, K., Wilke, I. & Sonnewald, U. A thermostable xylanase from *Clostridium thermocellum* expressed at high levels in the apoplast of transgenic tobacco has no detrimental effects and is easily purified. *Nature Biotechnol.* **13**, 63–66 (1995).
87. Dai, Z., Hooker, B. S., Anderson, D. B. & Thomas, S. R. Expression of *Acidothermus cellulolyticus* endoglucanase E1 in transgenic tobacco: biochemical characteristics and physiological effects. *Transgenic Res.* **9**, 43–54 (2000).
88. Ziegelhoffer, T., Will, J. & Austin-Phillips, S. Expression of bacterial cellulase genes in transgenic alfalfa (*Medicago sativa* L), potato (*Solanum tuberosum* L) and tobacco (*Nicotiana tabacum* L). *Mol. Breeding* **5**, 309–318 (1999).
89. Dai, Z., Hooker, B. S., Quesenberry, R. D. & Gao, J. Expression of *Trichoderma reesei* exo-cellobiohydrolase I in transgenic tobacco leaves and calli. *Appl. Biochem. Biotechnol.* **77**, 689–699 (1999).
90. Reggi, S. *et al.* Recombinant human acid β-glucosidase stored in tobacco seed is stable, active and taken up by human fibroblasts. *Plant Mol. Biol.* **57**, 101–113 (2005).
91. Kimura, T., Mizutani, T., Sakka, K. & Ohmiya, K. Stable expression of a thermostable xylanase of *Clostridium thermocellum* in cultured tobacco cells. *J. Biosci. Bioeng.* **95**, 397–400 (2003).
92. Yang, P. *et al.* Expression of xylanase with high specific activity from *Streptomyces olivaceoviridis* A1 in transgenic potato plants (*Solanum tuberosum* L). *Biotechnol. Lett.* **29**, 659–667 (2007).
93. Kimura, T. *et al.* Molecular breeding of transgenic rice expressing a xylanase domain of the *xynA* gene from *Clostridium thermocellum*. *Appl. Microbiol. Biotechnol.* **62**, 374–379 (2003).
94. Patel, M., Johnson, J. S., Brettell, R. I. S., Jacobsen, J. & Xue, G. P. Transgenic barley expressing a fungal xylanase gene in the endosperm of the developing grains. *Mol. Breeding* **6**, 113–124 (2000).
95. Biswas, G. C. G., Ransom, C. & Sticklen, M. Expression of biologically active *Acidothermus cellulolyticus* endoglucanase in transgenic maize plants. *Plant Sci.* **171**, 617–623 (2006).

FURTHER INFORMATION

Mariam Sticklen's homepage:
http://www.msu.edu/~stickle1
Joint Genome Institute:
http://www.jgi.doe.gov/sequencing/allinoneseqplans.php

ALL LINKS ARE ACTIVE IN THE ONLINE PDF