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Genetic and biotechnological approaches for biofuel crop improvement

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Research and development efforts for biofuel production are targeted at converting plant biomass into renewable liquid fuels. Major obstacles for biofuel production include lack of biofuel crop domestication, low oil yields from crop plants as well as recalcitrance of lignocellulose to chemical and enzymatic breakdown. Researchers are expanding the genetic and genomic resources available for crop improvement, elucidating lipid metabolism to facilitate manipulation of fatty acid biosynthetic pathways and studying how plant cell walls are synthesized and assembled. This knowledge will be used to produce the next generation of biofuel crops by increasing fatty acid content and by optimizing the hydrolysis of plant cell walls to release fermentable sugars.

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Introduction

Biofuels are commonly defined as fuels derived from renewable biological products and are often regarded as an attractive, 'green' alternative to fossil sources of energy due to their potential contribution to lowering carbon dioxide emissions [1]. Globally, plants produce an estimated 200 billion tones of biomass per year [2] in the form of sugars, polysaccharides, oils and other biopolymers, representing an unprecedented resource for biofuel production. However, despite its abundance and potential environmental benefits, the efficient and sustainable use of plant biomass for energy purposes remains a challenging endeavor, requiring major investments in science and technology [3,4].

With the exception of sugar cane ethanol, biofuels are a nascent industry in many parts of the world. A few of the commercialized products include bioethanol derived from corn starch and biodiesel obtained from plants with a high content in fatty acids such as soybean, canola and sunflower (Figure 1). However, the status of corn and soybean as major food crops, coupled to the fact that yields of starch and plant oil are too modest to cover the huge demand of transportation fuels has prompted the development of alternative biofuel production based on lignocellulosic biomass [4–6]. Lignocellulose, composed of the polysaccharides cellulose and hemicellulose, and lignin, a phenolic polymer, is the most abundant biomaterial on earth [2,7]. Most lignocellulosic feedstocks in consideration are perennial, non-food grasses such as switchgrass and *Miscanthus*, as well as woody plants such as poplar (Table 1).

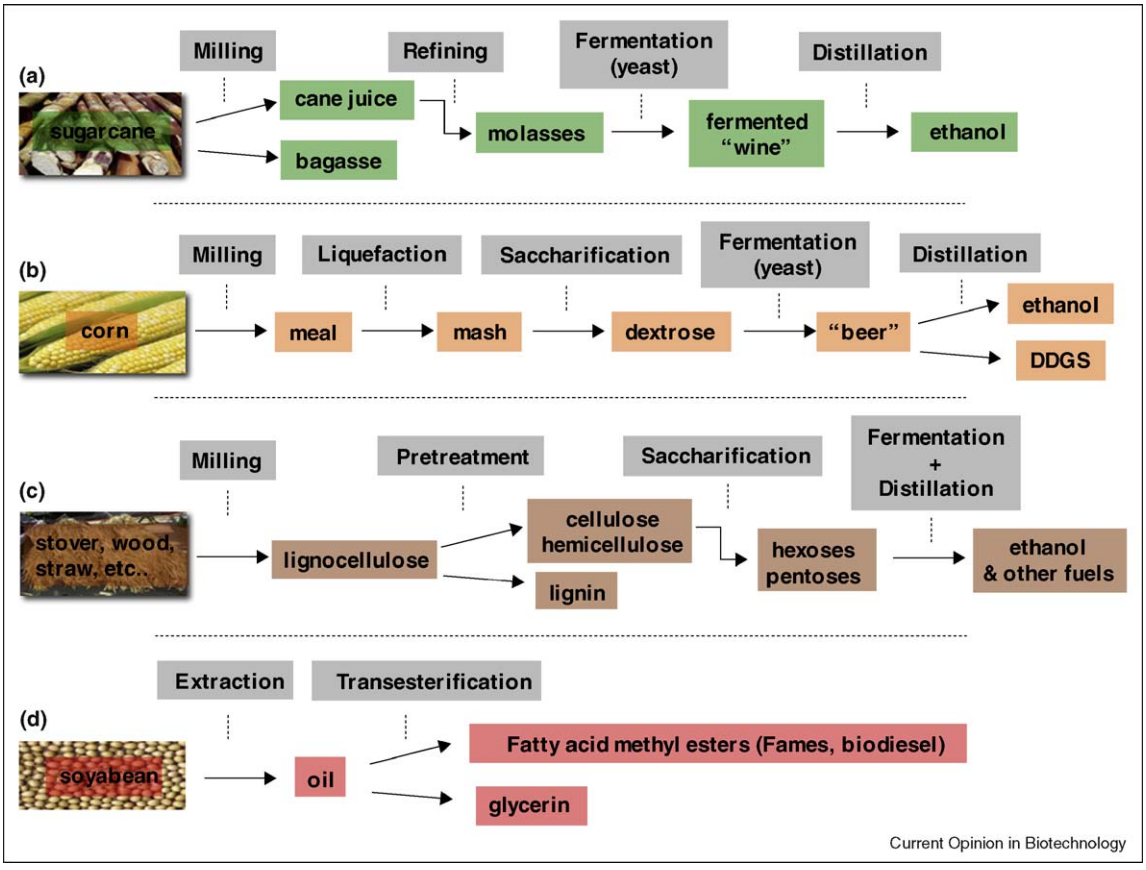
None of the current and potential crops has been domesticated or bred for improved polysaccharide or oil extraction for biofuel production. For this reason, biofuel research is focused towards understanding the plant biomass characteristics and traits that need to be modified to optimize crops for biofuel production. The wealth of genetic and genomic resources in model plants such as rice, *Arabidopsis* and *Brachypodium* are being used to answer fundamental scientific questions that cannot be addressed directly using potential biofuel crops. This review will discuss the recent advances in understanding lignocellulosic biomass recalcitrance and lipid metabolism for increasing oil production, and the current strategies for improving crops for biofuel production.

Biofuel crops

A list of plants and plant models for biofuel research and development is presented in Table 1. Unlike the crops currently used for commercial biofuel production, biofuel crops have been chosen on the basis of high yield in low input agricultural settings [8]. This approach is crucial because it will reduce the amount of land needed to produce the biofuel crops and at the same time avoid the need to add fertilizers and pesticides to maximize production as is common for food crops [6].

The molecular genetic, genomic and biotechnological resources for candidate biofuel crops are limited at this time, but are growing in number. Methods for reliable genetic transformation using *Agrobacterium* have been developed for switchgrass, *Jatropha*, poplar and *Brachypodium* [9–12], paving the way for genetic engineering approaches to crop improvement. For example, the successful engineering of a functional metabolic pathway for

Figure 1



Biofuel production from different crops. **(A)** Ethanol production from sugarcane. Stems are milled to extract cane juice, with bagasse as side product that is used for burning to produce electricity to power the whole process. Sugars (molasses) are refined and used for yeast fermentation into ethanol. **(B)** Ethanol production from corn starch. Corn grains are milled to produce flour, also known as meal, which is hydrated and pretreated at high temperatures in cookers with thermostable alpha amylase to extract fermentable sugars. Saccharification into dextrose is further achieved by enzymatic hydrolysis with glucoamylase for yeast fermentation into ethanol. A side product from the process, distilled dried grain with solubles (DDGS) is used for animal feed. **(C)**. Biofuel production from straw, stover, wood and other cellulosic materials. Biomass is milled to reduce particle size; a whole array of chemical or physical pretreatments at high temperatures can be used to separate polysaccharides from lignin, including dilute acid or base hydrolysis, ammonia fiber expansion (AFEX), and ionic liquids. Polysaccharides are further broken down into C5 and C6 sugars by enzymatic saccharification with cellulases and hemicellulases, and used for fermentation into ethanol by yeast or engineered bacteria, such as *E. coli*. C5 sugar conversion is less efficient than C6 fermentation, however, and the majority of fuel is produced from glucose (C6) conversion. Lignin byproducts can be used to generate energy to power the processing plant. **(D)** Biodiesel production from plant oils. Oil is extracted from seeds and used for transesterification using sodium hydroxide and methanol to produce fatty acid methyl esters (FAMEs). Glycerin, a side product of transesterification of fatty acids is used to make soap or other value added products.

the production of polyhydroxybutyrate (PHB) in transgenic switchgrass has been recently reported, suggesting that complex traits can be engineered in this dedicated biofuel crop [13••]. Additionally, a protocol for generating switchgrass protoplasts for use in transient gene expression experiments has been recently published [14], potentially allowing for rapid testing of candidate genes for functional analyses. Tissue culture techniques for the propagation of *Miscanthus* and *Jatropha* explants have also been developed [15–17]. The genomes of *Brachypodium*, poplar, sorghum and maize have been recently sequenced [18–21], while genome projects for switchgrass and oil palm are in progress [22]. These resources will be instrumental in developing the tools for functional genomic and proteomic assays, and will allow comparative genomic approaches between model species and biofuel crops to become a reality. These tools will greatly complement basic research currently aimed at understanding plant biomass characteristics that can be targeted for the design of better yielding and more efficiently processed biofuel crops via metabolic engineering.

Targeting plant oil metabolism

Plant seed storage oils, in the form of triacylglycerols (TAGs), are excellent sources for the generation of biodiesel due to their high chemical similarity to fossil oils

Table 1

Biofuel crops and model species

Species	Family	Biomass type	Genome status[a]	Reference
Strictly Models				
Arabidopsis thaliana	Brassicaceae	Lignocellulose/fatty acids	Complete	[55]
Brachypodium distachyon	Poaceae	Lignocellulose	Draft	[18]
Models and potential crops				
Oryza sativa (rice)	Poaceae	Lignocellulose	Complete	[56]
Zea mays (corn)	Poaceae	Lignocellulose/starch	Draft	[21]
Sorghum bicolor	Poaceae	Lignocellulose/sucrose	Draft	[20]
Glycine max (soybean)	Fabaceae	Fatty acids	Draft	[57]
Brassica napus (canola/oilseed rape)	Brassicaceae	Fatty acids	Draft[b]	Not applicable
Strictly crops				
Panicum virgatum (switchgrass)	Poaceae	Lignocellulose	In progress	[22]
Miscanthus x giganteus	Poaceae	Lignocellulose	Not available	Not applicable
Populus trichocarpa (poplar)	Salicaceae	Lignocellulose	Draft	[19]
Eucalyptus globulus	Myrtaceae	Lignocellulose	In progress	[22]
Jatropha curcas	Euphorbiaceae	Fatty acids	Not available	Not applicable
Elaeis guineensis (oil palm)	Arecaceae	Fatty acids	In progress	[22]
Saccharum officinarum (sugarcane)	Poaceae	Sucrose	Not available	Not applicable

[a] Draft genome indicates that the genome sequence is available and published but is still in the early versions of the annotation process.
[b] The genome was sequenced in 2009 by Bayer Crop Science but is not publicly available.

[23,24••]. Biodiesel is produced by the transesterification of plant TAGs with methanol in the presence of acid or alkali to produce fatty acid methyl esters (FAMEs) (Figure 1). Current biofuel crops, such as soybean and *Jatropha*, have either low or unpredictable oil yields [24••,25]. In addition, the quality of biodiesel produced is highly dependent on both the type and abundance of the fatty acids in seed storage organs. Thus, increasing oil content in plants and redirecting the biosynthesis of fatty acids for accumulation of specific types are needed to achieve optimal biodiesel production.

Fatty acid metabolism involves the conversion of sucrose, a product of photosynthesis, into TAGs. The pathway starts in the plastid where fatty acid chains are elongated while conjugated to acyl carrier proteins (ACP) and ends in the endoplasmic reticulum (ER) where acyl CoAs are converted to diacylgrycerol and subsequently to TAG by the diacylglycerol acyltransferase (DGAT) enzyme [23]. Increase in oil content in seeds has been achieved by manipulation of the expression levels of enzymes involved in synthesis of TAG. Overexpression of a fungal DAGT2 enzyme in soybean seeds led to a 1.5% increase in oil content [26•]. A similar phenotype was observed by overepxression of a *DGAT* cDNA in *Arabidopsis* [27]. An alternative means to increase seed oil content in plants has been recently achieved by activation of the fatty acid biosynthetic pathway: overexpression of two soybean transcription factors in Arabidopsis, *Dof4* and *Dof11*, increased total fatty acid and lipid seed content [28]. Dof4 and Dof11 seemed to activate lipid biosynthesis in *Arabidopsis* by activating acetyl CoA carboxylase and long-acyl-CoA synthase, respectively [28], both enzymes involved in fatty acid biosynthesis.

Altering the carbon flux to TAG biosynthesis by affecting the supply of glycerol 3-phosphate, which is another TAG substrate in addition to fatty acids in the ER, is an alternative way of increasing oil accumulation in plant seeds. Indeed, overexpression of the yeast glycerol 3-phosphate dehydrogenase (*ghpd1*) gene in canola seeds increased the lipid content by 40% [29].

Lowering the levels of both saturated and polyunsaturated fatty acids while increasing the amount of monounsaturated fatty acids, such as oleate (C18:1) or palmitoleate (C16:1) are important targets for optimal FAME production [24••]. This can also be achieved by manipulation of TAG biosynthesis. In soybean, reduced levels of the saturated fatty acid palmitate were obtained by downreglation of *FATB*, an acyl-ACP thioesterase, causing the accumulation of oleic acid up to 85% from 18% in the wild type [30]. A *Jatropha* cDNA encoding a putative *FATB* homolog, *JcFATB1*, has been recently cloned [31]. Downregulation of *JcFATB1* in transgenic *Jatropha* can be attempted to increase levels of oleic acid in seeds, especially in highland accessions that have been shown to accumulate higher amounts of polyunsaturated linoleic acid [32].

An attractive approach to increase overall yield of oils for biodiesel production is to engineer oil accumulation in vegetative tissues, such as leaves. Slocombe *et al.* have identified a mechanism for significant oil accumulation in senescing leaves of *Arabidopsis* mutants compromised in fatty acid breakdown (such as *cts2*) and by overexpression of the seed development transcription factor *LEC2* [33••]. Senescent leaves of *cts2* accumulated significant amounts of TAGs (up to 2% leaf dry weight) compared to wild

type, while senescent leaves overexpressing LEC2 accumulated TAGs around 1% leaf dry weight [33••]. This study raises the possibility of engineering biofuel crops with senescence inducible promoters that either suppress fatty acid breakdown or induce the seed development program in leaves.

Finally, lipid metabolism research can be expanded to better understand the plant wax biosynthetic machinery. Plant waxes accumulate alkanes derived from fatty acids, among other compounds, and a better understanding of alkane biosynthesis can lead to the production of hydrocarbons as a next generation of biofuels [34].

Targeting lignocellulosic biomass

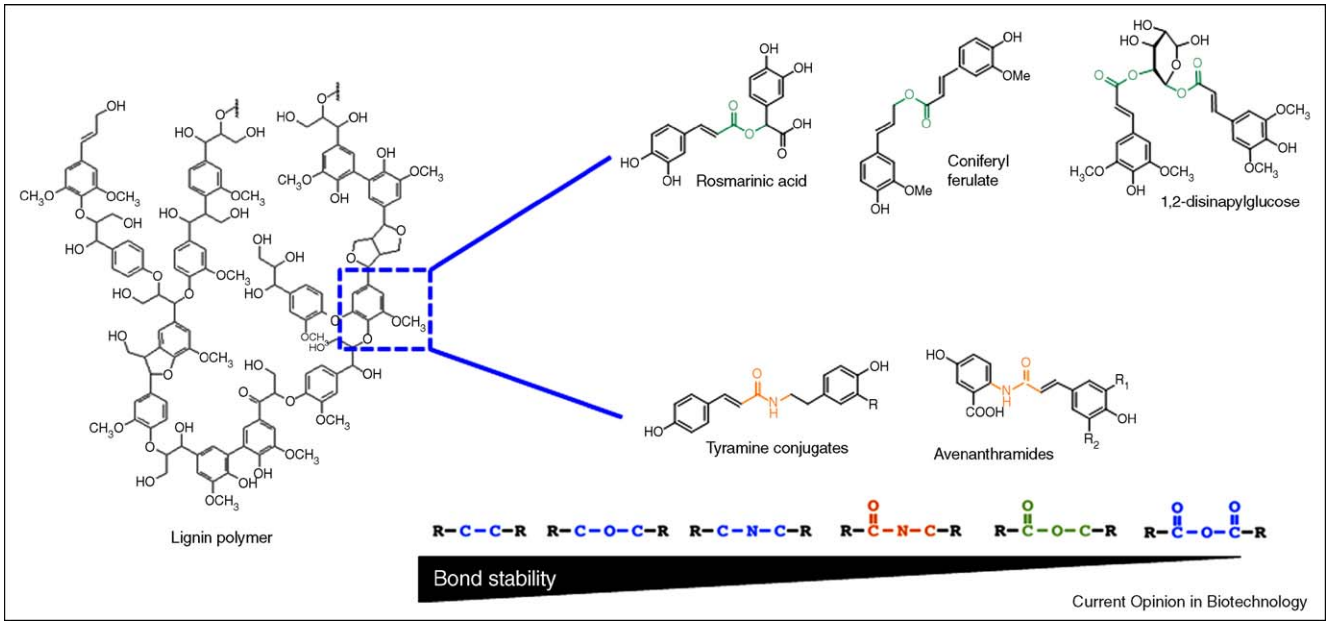
One of the major hurdles to efficient lignocellulose conversion to biofuels is the recalcitrant nature of this complex mixture of polysaccharides and lignin [3,35•]. Plants deposit cellulose, hemicellulose and lignin in their cell walls. Recalcitrance is mainly due to the heterogeneity and molecular structure of lignocellulose, where cellulose is arranged into a network of tight, inter-chain hydrogen bonds that form a crystalline core of microfibrils, embedded in a matrix of hemicellulosic polysaccharides that are covalently linked to lignin, a highly complex polymer of aromatics [3,35•]. Since ultimately the goal is to obtain cell wall sugars for conversion into liquid fuels, the highly complex nature of lignocellulose requires costly and harsh pre-treatments to gain access to monosaccharides (Figure 1). The study of how plant cell walls are synthesized, modified and degraded is one of the main areas of focus in biofuel research at the moment, with the aim of designing future bioenergy crops with improved lignocellulosic characteristics for easier and more efficient breakdown.

Extensive studies on lignocellulose quality in the forage and pulping industry have identified lignin as having a major effect in cell wall recalcitrance [35•,36,37•]. A recent study in transgenic alfalfa achieved a significant improvement in fermentable sugar release from lignocellulose by downregulating certain monolignol biosynthetic enzymes. Notably, alfalfa lines silenced for cinnamate 4-hydroxylase (C4H), hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT) and coumaroyl shikimate 3-hydroxylase (C3H) had lower lignin amounts that correlated with enhanced cell wall enzymatic hydrolysis [38]. However, increased saccharification came at the expense of low biomass yield and severe plants growth defects [38]. A related, and possibly favored, approach looked at downregulation of enzymes acting at later stages in monolignol biosynthesis, such as cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). Targeting CCR and CAD lead to generation of alfalfa lines with up to 60% and 40% improvements in enzymatic saccharification efficiency, respectively, with little to no apparent developmental defects associated [39]. However, extensive metabolomic and transcriptomic analyses have shown that silencing CCR and CAD affects many more biochemical pathways in tobacco and poplar than just lignin biosynthesis [40••,41••], suggesting that additional genes can be targeted for more efficient pathway engineering. A candidate *CCR* gene has been recently identified in switchgrass [42] as well as other lignin biosynthetic genes by comparative genome analyses in currently available plant genomes [43] that could serve as targets for pathway engineering.

Studies on the coupling reactions acting in the chemical polymerization of lignin have shown incorporation of many more compounds than the typical monolignols [44]. This plasticity can be exploited for engineering of novel lignin compositions for improved lignin extraction from biomass. For example, maize cell walls incorporating coniferyl ferulate had improved enzymatic hydrolysis and sugar release [45••]. Following this logic, lignin engineering approaches are currently being pursued at the Joint BioEnergy Institute to replace monolignols with compounds containing easily cleavable chemical linkages such as ester and amide bonds (Figure 2) (D. Loqué, personal communication and unpublished results). Monolignol replacement strategies could avoid the undesirable developmental and structural phenotypes associated with the downregulation of lignin biosynthetic enzymes in transgenic plants.

The contribution of hemicellulose to plant cell wall recalcitrance is much less well understood. Reduction of xyloglucan in poplar overexpressing an *Aspergillus* xyloglucanase improved the saccharification of wood, presumably by making cellulose more accessible to enzymatic hydrolysis [46•]. Recent findings have shown that xylan content, the more abundant hemicellulosic polysaccharide present in the cell wall, affects pulping efficiency and delignification in transgenic tobacco lines downregulated for UDP-glucuronate decarboxylase, an enzyme involved in UDP-xylose production [47]. In poplar, silencing of *PoGT47C*, a glycosyltransferase homologous to *Arabidopsis FRA8* and involved in glucuronoxylan synthesis, caused an increase in glucose yield following enzymatic hydrolysis, indicating that reducing xylan content leads to improved saccharification efficiency [48]. In addition to contributing to cell wall recalcitrance, the C5 sugars in arabinoxylan are not easily converted to biofuels by current technologies [5]. Thus, decreasing or replacing C5 sugar content in hemicellulose could potentially lead to increased lignocellulosic biofuel yields. However, a better understanding of xylan biosynthesis is needed in order to identify efficient ways to modify its structure for lignocellulose improvement. Progress has been made in the recent years by the identification of several key enzymes involved in xylan synthesis in *Arabidopsis* [49•,50•,51•], but much more research is

Figure 2



Lignin engineering strategy. Replacement of monolignols with compounds harboring more easily cleavable bonds such as amide and ester linkages. Compounds with ester bonds include rosmarinic acid, coniferyl ferulate, and 1,2 disinapylglucose; amide bond-containing molecules include tyramine conjugates and avenanthramides.Figure courtesy of D. Loqué and P. Pradhan, Joint BioEnergy Institute.

needed in order to understand backbone assembly and side chain additions. Insights into xylan biosynthesis have proven it to be a very complex process, likely to differ between monocots and dicots [51•].

Targeting cellulose biosynthesis is an alternative strategy for lignocellulose improvement. Cellulose synthase complexes and other proteins assisting in the process have been identified in *Arabidopsis* and other higher plants [52]. Recently, the discovery of a transcriptional regulation mechanism via small RNAs showed that cellulose synthase and several hemicellulosic biosynthetic genes are coordinately downregulated during leaf development [53••]. Fine-tuning of this small RNA-directed pathway has great potential for metabolic engineering of the cellulose biosynthetic machinery.

Concluding remarks

The improvement of plant biomass characteristics for biofuel production is in the beginning stages. Biofuel crops have been identified and are at various levels of domestication and cultivar selection, while genetic and genomic resources for these species, including draft genome sequences and transformation protocols, are currently being developed. Major breakthroughs on the understanding of lipid metabolism and plant cell wall biosynthesis and structure are still needed to overcome low oil yields and the recalcitrance of lignocellulose, respectively, for efficient and cost-competitive conversion to biodiesel and other liquid fuels. Although a few promising targets for genetic engineering, such as overexpression of DAGT2 or glycerol 3-phosphate in oily seeds and monolignol replacement in cell walls, have been identified, the overall effects of the manipulation of these traits in dedicated biofuel crops is still lacking. It is known, for example, that modifying lignin content or structure can lead to severe developmental defects and to enhanced susceptibility to plant pathogens, while other plant cell wall modifications can actually increase resistance to biotic factors [54]. It is thus necessary to evaluate the impact of plant cell wall modifications on the overall crop fitness to reduce or avoid the negative effects that could be associated with such modifications. Important advances have been made in understanding the factors that contribute to lignocellulose recalcitrance, notably lignin and xylan content/structure, and the list of genes that can be manipulated for pathway engineering is growing. Importantly, although plant biotechnology will be key to the successful generation of energy crops, it should go hand in hand with breeding efforts targeted at maintaining or enhancing the important agronomic traits that made these plants so attractive for biofuel production to begin with, namely resistance to abiotic and biotic factors, low fertilization requirements and perennial life cycle.

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- •• of outstanding interest

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