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Review article

Modifying plants for biofuel and biomaterial production

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Summary

The productivity of plants as biofuel or biomaterial crops is established by both the yield of plant biomass per unit area of land and the efficiency of conversion of the biomass to biofuel. Higher yielding biofuel crops with increased conversion efficiencies allow production on a smaller land footprint minimizing competition with agriculture for food production and biodiversity conservation. Plants have traditionally been domesticated for food, fibre and feed applications. However, utilization for biofuels may require the breeding of novel phenotypes, or new species entirely. Genomics approaches support genetic selection strategies to deliver significant genetic improvement of plants as sources of biomass for biofuel manufacture. Genetic modification of plants provides a further range of options for improving the composition of biomass and for plant modifications to assist the fabrication of biofuels. The relative carbohydrate and lignin content influences the deconstruction of plant cell walls to biofuels. Key options for facilitating the deconstruction leading to higher monomeric sugar release from plants include increasing cellulose content, reducing cellulose crystallinity, and/or altering the amount or composition of noncellulosic polysaccharides or lignin. Modification of chemical linkages within and between these biomass components may improve the ease of deconstruction. Expression of enzymes in the plant may provide a cost-effective option for biochemical conversion to biofuel.

Introduction

Fossil oil is used as an energy source, predominantly as fuel for transportation and in the production of diverse biomaterials. Replacement of fossil oil with biofuel derived from plant biomass (Henry, 2010a) has the potential to greatly reduce greenhouse gas emissions (Kerr and Service, 2005; Schubert, 2006). Two stages are key for the utilization of plants for biofuel/biomaterial production; first, the deconstruction of plant biomass to release fermentable sugars, and second, the biochemical conversion of the sugars by microbial action (yeast bacteria or algae) to produce the required end products. The 'biochemical' route (Figure 1) provides the flexibility to deliver diverse fuels and biomaterials. Efficiencies in these two stages will drive the industrial use of plants as feedstocks to generate price competitive biofuel. The suitability of plants as a feedstock for industrial conversion to biofuels and biomaterials varies depending upon the type of plant biomass and the processes used (Somerville, 2006). Plants with the biomass composition required to match the deconstruction and fermentation process will lead to higher yields of biofuel per unit of plant biomass.

The first-generation conversion technologies using plants for biofuels relied on conversion of nonstructural carbohydrates (sugars and starches). Second-generation conversion technologies aim to access the much greater quantities of sugars in the less accessible structural carbohydrate fraction of plant biomass (Demirbas, 2005; Lynd *et al.*, 1991; Somerville, 2006, 2007) and are more likely to benefit greatly from modification of biomass chemistry. Plant biotechnology may provide an opportunity to greatly improve the composition of plant biomass for the industrial production of biofuels (Davies *et al.*, 2010). Here, we review the options for improving plant biomass, primarily for ease of conversion and increased end-product yield. The focus of this review is on enhancing the value of biomass to support biochemical conversion to sugars for the production of biofuels or biomaterials (Figure 1). Much of plant biotechnology is devoted to improving the productivity of plants as crops for food or industrial use. A very large number of genes are involved in the control of plant growth and productivity in agriculture, and these will not be reviewed here. This review will focus specifically on ways to support improvements in biomass composition.

The challenge of sustainable biofuel production and the key role of biomass composition

The sustainable manufacture of biofuels and biomaterials on a large scale remains a technical challenge. The use of large land areas and water may compete with food production and environmental protection including biodiversity conservation (Henry, 2010b). Biofuel manufacture may not deliver the desired greenhouse gas reductions if substantial emissions are associated with biomass haulage and production, and conversion to or transport of biofuels. The minimization of the environmental footprint of biofuel production will require the careful selection of biomass production systems and biofuel conversion technologies that are efficient in the use of land and water and require low levels of greenhouse gas emission. Two factors determine the sustainable yield of biofuel, the total biomass production and the efficiency of conversion to fuel. Plant biotechnology has potential to contribute to both the amount of biomass produced and the

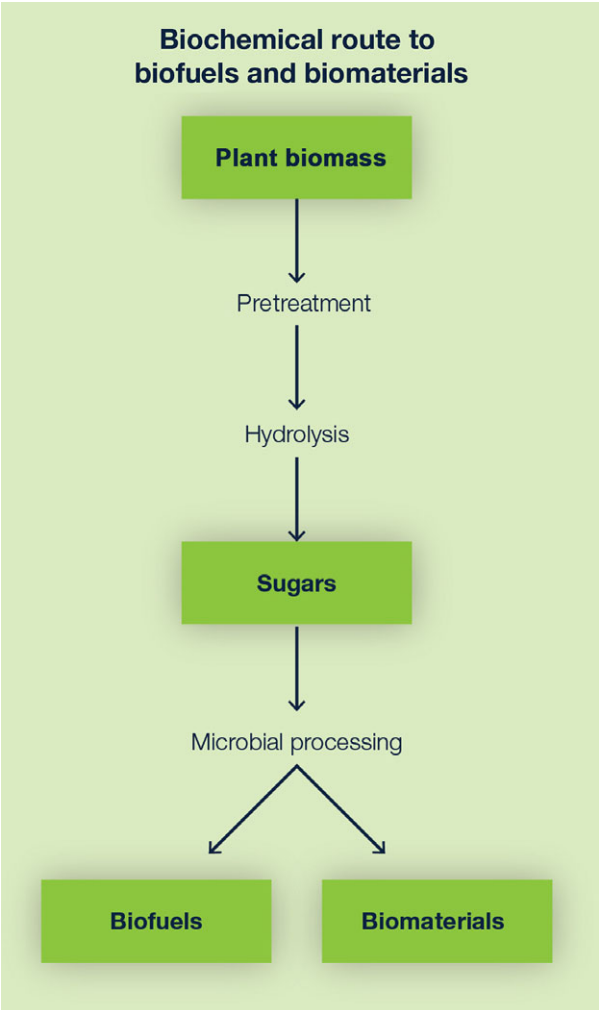

Figure 1 Biochemical route to biofuels and biomaterials. The carbohydrates in plant biomass can be converted to sugars in processes that may involve a pretreatment followed by hydrolysis with enzymes or acids. Microbes (yeast or bacteria) are then used to convert the sugars to fuel molecules or other biomaterial molecules or precursors (e.g. for organic polymers, plastics or other high value biochemicals).

composition of the biomass (determining potential conversion efficiency). Improvements in biomass composition have the potential to dramatically improve the sustainability of biofuel production by greatly increasing ease of both biomass conversion and fuel yield. Land areas required to replace fossil fuels at any level of biomass yield per hectare and efficiency of conversion to biofuel can be easily calculated (Henry, 2010a). This analysis defines the biofuel yields that are required from current or improved biomass to satisfy fuel requirements with production from available land resources.

Lignocellulosic biomass is recalcitrant to the process of deconstruction, to release sugars, due to the cell wall structure (Blanch *et al.*, 2011; Himmel *et al.*, 2007; Somerville *et al.*, 2004). Large amounts of enzymes are often required because lignin restricts the access of hydrolytic enzymes to the polysaccharides and may also inhibit these enzymes (Chen and Dixon, 2007; Keating *et al.*, 2006; Simmons *et al.*, 2010). Lignocellulosic biomass derived from various tissues or plant species may be structurally dissimilar and require different deconstruction approaches to yield fermentable sugars for biofuel production (Carere *et al.*, 2008; Chandra *et al.*, 2007; Feldman and Wegener, 1985). The cost of lignocellulosic biomass and its deconstruction, which usually involves some form of pretreatment followed by hydrolysis of the polysaccharides (enzymatic and or chemical), are the two major costs which dictate the final cost of fuel production for a given conversion process (Lynd *et al.*, 2008). Development of plant varieties with a more desirable biomass composition should contribute to a more reliable supply of useable biomass and support investments in biofuel production. Biomass with a more desirable composition will have a higher value as a substrate for these processes. Composition may be a critical attribute, for biomass producers, justifying production because of a higher value and for processors ensuring a more efficient process and potentially delivering higher biofuel yields. A thorough understanding of the chemistry and structural components of lignocellulosic biomass will be necessary to aid in the selection/generation of the best combination of biomass for any conversion process.

Biomass composition

Lignocellulosic biomass contains a complex assortment of constituents including cellulose, noncellulosic polysaccharides, lignin, ash, protein and compounds termed extractives, for example chlorophyll, waxes, oils, terpenes and phenolics (Browning, 1963; Carroll and Somerville, 2009). Knowledge of biomass composition provides specific targets for the modification of plants to produce biomass with desired biofuel traits. The carbon content of biomass ultimately limits the theoretical yield of advanced high carbon fuels. Genetic modification of plants by targeting genes involved in carbohydrate or lignin biosynthesis may be an option to generate modified biomass with increased deconstruction efficiency.

Cellulose

Cellulose, a linear chain of β-1,4-linked D-glucopyranose units, is the most abundant biopolymer on earth, yielding approximately 400–600 million tons of dry mass per year in the USA alone (Perlack and Stokes, 2011). The cellulose fibres provide plant cell walls with strength and rigidity, and control cell morphology. Extensive hydrogen bonding between cellulose chains coupled with beta-linkages connecting glucan units (as opposed to alpha-linkages found in starches) results in considerable recalcitrance to depolymerizing enzymes. Additionally, the ratio of crystalline to amorphous cellulose has been implicated as being a significant factor in glucose production, as cellulase enzymes are reported to degrade amorphous cellulose more efficiently (Laureano-Perez *et al.*, 2005). The deconstruction of plants with high cellulose content should translate to larger amounts of available glucose for subsequent conversion to biofuels or biomaterials.

Noncellulosic cell wall polysaccharides

Noncellulosic polysaccharides are key components of the matrix of plant cell walls in which the cellulose fibres are embedded. They provide further strengthening of the cell walls by cross-linking with both cellulose and lignin. Deconstruction of noncellulosic polysaccharides results in a mixture of monosaccharides including arabinose, galactose, glucose, mannose, rhamnose and xylose, as well as acetic, glucuronic and ferulic acids, depending on the plant species (Esteghlalian Ali *et al.*, 2000; Wyman *et al.*, 2005).

Diverse noncellulosic polysaccharides are found in plant biomass, the variation of which can be best understood by

examining the phylogenetic relationships between plants (Figure 2) and their evolutionary divergence (Harris, 2005). For example, the cell wall of switchgrass is very different to the cell wall of woody biomass such as poplar, as these species are long diverged. The range of polysaccharides encountered is illustrated in Table 1. Knowledge of the noncellulosic polysaccharides in many plants is very limited. Early perspectives that defined differences between monocotyledon and dicotyledon cell walls have been updated as our understanding of angiosperm phylogeny (Figure 2) has advanced (Henry and Harris, 1997). Deconstruction of each of these polymers may require individual optimization. Improved knowledge of this fraction of biomass may require the development of improved analytical methods for rapid characterization of noncellulosic polysaccharide composition and structure.

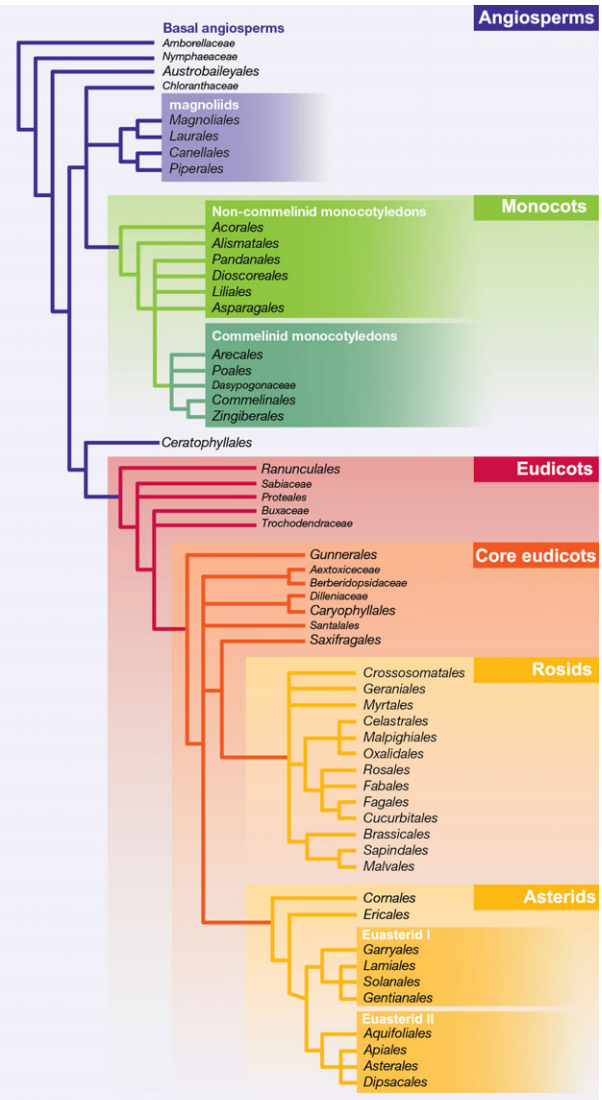

Figure 2 Plant phylogeny: the evolution of different plant groups has involved divergence in biomass composition. Major plant groups (based upon Chase, 2005) may be associated with differences in cell wall composition (Table 1). For example, the noncommelinid monocotyledons (and specifically the grasses) have specialized cell walls, and plants in this group may require different conversion technologies. The chemistry of some group such as the basal angiosperms has not been extensively characterized.

Table 1 Cell wall polysaccharides in plants (based upon Harris, 2005)

Polysaccharide type	Plant group
Cellulose	
Callose	
Mixed link glucan	Poales
Xyloglucans	
Fucogalactoxyloglucans	Eudicotyledons, noncommelinid monocotyledons
Arabinoxyloglucans	Asterids, Laminales
(Galacto-)glucomannan	
(Galacto-)manan	Fabales
Heteroxylans	
4-O-methylglucronoxylans	
Glucuronoarabinoxylans	Commelinid monocotyledons
4-O-methylglucuronoarabinoxylans	
Homogalacturonan	Eudicotyledons, noncommelinid monocotyledons
Xylogalacturonan	
Apoigalacturonan	
Rhamnogalacturonan I	Eudicotyledons, noncommelinid monocotyledons
Rhamnogalacturonan II	Eudicotyledons, noncommelinid monocotyledons

Lignin

Lignin is the second most abundant biopolymer on the planet after cellulose. Lignin is comprised of three monomeric phenylpropene molecules, namely syringyl (S), guaiacyl (G) and ρ-hydroxyphenol (H), linked together forming a complex, three-dimensional polymer (Sarkanen and Ludwig, 1971). The function of lignin includes the strengthening of plant cell walls, providing resistance against microbial attack, and playing a vital role in water transport by reducing cell wall permeability (Boerjan *et al.*, 2003; Ralph *et al.*, 2004; Sarkanen and Ludwig, 1971). The biosynthesis of lignin is outlined in Figure 2. The evolution of S lignin has been a more recent adaptation resulting from evolution of the specificity of enzymes such as ferulate 5-hydroxylase and caffeic acid/5-hydroxyferulic acid O-methyltransferase. The ratio of S/G moieties in the cell wall can aid in determining the degradability of lignin. Some studies have shown S units to be more reactive (Li *et al.*, 2010; Tsutsumi *et al.*, 1995), while others have demonstrated increased sugar yields when S/G ratios have been decreased (Jung *et al.*, 2012a). Plants with low lignin content have been assessed to be more easily degraded enzymatically by allowing the enzymes greater accessibility to cellulose (Chen *et al.*, 2009). Lignin can also irreversibly bind cellulase enzymes, thereby reducing their catalytic potential for sugar release, making its reduction highly desirable (Esteghlalian Ali *et al.*, 2000).

Cross-linking

Cross-linking in plant cell walls occurs between cellulose, noncellulosic polysaccharides and lignin. The cell wall is composed of a network of cross-linked noncellulosic polysaccharide and lignin, sheathing the cellulose fibres from degradation. Currently, pretreating of biomass aims to diminish this network,

allowing solvents and enzymes greater penetration into plant cell walls for cellulose deconstruction (Blanch *et al.*, 2011). Hydroxycinnamates, such as ferulate in grassy cell walls, have been implicated as having a leading role in the development of lignin–carbohydrate complexes, as well as polysaccharide–polysaccharide linkages (Ralph, 2010). Physical and chemical treatments are unable to sever the recalcitrant linkages between ferulate and lignin.

Ash

Ash, in biomass, is a combination of inorganic constituents, such as silicon, potassium, calcium, sulphur and chlorine, that cannot be transformed into energy. These components defend the plant against attack by pests (Si), provide nutrients for the plant (K, Ca, S) and control mechanisms such as the opening and closing of stomata (Bakker and Elbersen, 2005). The ash content of biomass negatively affects the thermochemical conversion to sustainable products (Jenkins *et al.*, 1998). Additionally, the burning or pyrolysis of a mixture of switchgrass and coal has been measured to release 50% more silica into the atmosphere than the levels quantified by the burning of coal alone (Blevins and Cauley, 2005). Effects of ash content on biochemical conversion of plants have not been extensively studied; however, low ash can be considered desirable in biomass for biofuel production.

Advances in biomass analysis

Knowledge of the specific feedstock cell wall composition, such as cellulose, noncellulosic polysaccharide, lignin and hydroxycinnamic acid content and the chemical composition of these fractions, can greatly facilitate the selection of plants possessing key chemical traits for downstream applications. This vital information remains unmeasured for a wide variety of plants. Traditional chemical analysis of plant biomass remains challenging. Analysis of plant cell wall polysaccharides and their structures is complicated by the wide diversity of structures found. Lignin is difficult to measure because of the diversity of chemical structures found within this fraction and the variation in the contributions of these moieties in lignin from different sources. With the development of high-throughput analytical techniques, such as vibrational spectroscopy, rapid screening of diverse potential biofuel feedstocks is now possible (Lupoi *et al.*, 2013). However, more detailed analysis of the chemical structures present in specific biomass types may contribute to new insights into options for biomass improvement as a substrate for biofuel production. Further advances in chemical analysis tools will be important for biofuel crop development.

Targets for biomass manipulation

Plants have been subjected to selection for food traits but remain effectively undomesticated in relation to biofuel production. This lack of selection suggests the potential for very rapid and significant improvement in the composition of plants as biofuel crops. However, some components of biomass may interfere with conversion to biofuel. Modification of the plant to reduce the levels of these inhibiting moieties may make a significant contribution to increased biofuel production efficiency.

The chemistry of plant cell walls plays an unequivocal role in the ability to convert biomass to sustainable bio-products. The ideal, natural feedstock would contain chemical traits such as high cellulose and low lignin content, an optimal lignin S/G ratio, low ash content. While high-throughput methodologies allow the rapid screening of biomass to identify which plants possess these phenotypes, many plants do not meet these ideals. Manipulation of the structure of the plant cell walls (Harris and DeBolt, 2010) in species grown for biofuel production is a key strategy to improve biofuel production efficiency. The amount and composition of cell wall polysaccharides (including cellulose and noncellulosic polysaccharides), lignin (amount and composition), the total amount of cell wall and cross-linking of cell wall components need to considered as targets for genetic improvements of biomass.

The role of biotechnology

Cell wall polysaccharide modification

The highly water-insoluble nature of cellulose and some other cell wall polysaccharides makes efficient conversion to sugars difficult. The polysaccharide composition of the cell wall is a major determinant of facile chemical or biochemical conversion to simple sugars for use in biofuel production. Cellulose, the main component of plant biomass and the greatest source of carbon for energy, exists primarily as a highly ordered structure lacking surface area as a result of hydrogen bonding (Hall *et al.*, 2010; Mosier *et al.*, 2005; Zheng *et al.*, 2009). Noncellulosic polysaccharides including neutral polysaccharides and acidic polysaccharides (pectins) surround cellulose microfibrils within plant cell walls, contributing to biomass recalcitrance (Cosgrove, 2005; Mellerowicz and Sundberg, 2008). However, sugars released from these polymers may also contribute to fuel production. Noncellulosic polymers with high glucose content may be especially valuable as they may add to the glucose recovered from cellulose hydrolysis. Polymers of other sugars may be of less value unless conversion technologies are available.

Microbial conversion of sugars to fuel molecules is often complicated by the presence of a mixture of sugars. Because of the challenge of processing mixed sugars, a primary pathway that is currently being pursued to design ideal biofuel feedstocks is to increase the cellulose content. The biosynthesis of cellulose is a process that involves members of a large family of cellulose synthases (Endler and Persson, 2011). Increased cellulose content may be achieved by manipulation of these enzymes. A mutant *Arabidopsis* cellulose synthase gene, *CES3A* (Sahoo *et al.*, 2013), expressed in tobacco resulted in more efficient enzymatic saccharification of cellulose. However, more research in the modification of cellulose biosynthesis will be required to provide options for enhanced cellulose biosynthesis without the loss of biomass production.

Modifications of noncellulosic cell wall polysaccharides may aim to reduce this component of the cell wall in favour of more cellulose or may seek to improve the potential of this fraction to contribute to sugar and fuel yield. Advances in understanding of the biosynthesis of xyloglucans (York and O'Neill, 2008) and arabinoxylans (Mitchell *et al.*, 2007) are providing new options to explore manipulation of these cell wall components. The complexity and diversity of these polymers may require the modification of many different and species-specific genes.

Increases in cellulose following diversion of carbon from other key cell wall components such as lignin and xylan have been routinely reported (Chabannes *et al.*, 2001; Leplé *et al.*, 2007). Another technique explored to render a feedstock more advantageous for biofuel production is the specific reduction of xylans. The xylose content of *irx7, irx8* and *irx9* mutant *Arabidopsis* plants was elegantly reduced by up to 23% following the alteration of

the spatial and temporal distribution of xylan from the secondary cell wall to the xylem vessels (Petersen *et al.*, 2012). These mutants are known to contain less xylose when compared to wild-type levels, but suffer from stunted growth and resultant reductions in cellulose content. Following a hot water pretreatment, saccharification sugar yields were significantly improved by up to 42% in the modified *Arabidopsis*. The desirable phenotypes of the altered plants, lower xylose and lignin, did not come at the expense of mechanical stability. The authors concluded that this engineering strategy should be readily transferable to other biofuel crops.

In poplar, hemicellulose disruption through RNA interference (RNAi) of *PoGT47C*, a glycosyltransferase, resulted in a significant reduction in secondary cell wall thickness that accompanied an increase in enzymatic saccharification and glucose yield after pretreatment (Lee *et al.*, 2009). Xylose content in the supernatant was decreased to 43%–77% that of the wild type following a 1 N KOH extraction, whereas xylose left in the plant cell wall was reduced to 54%–79% compared to the wild type. The amounts of extractable S and G lignin slightly increased in the transgenic trees. Glucose remaining in the plant cell wall was 56%–87% lower compared to the wild type, concurrent with previous studies that revealed deficiencies in cellulose biosynthesis with glucuronoxylan down-regulation. An increase of up to 48% in glucose concentration was measured in the transgenic trees relative to the wild-type poplar. These experiments illustrate the challenge of modifying cell wall structure without inducing downstream pleiotropic growth effects.

The exogenous addition of *RabG3bCA* from *Arabidopsis* into poplar resulted in a 9.8% increase in glucan content without an increase in other biomass constituents (Jung *et al.*, 2013). Higher yields from enzymatic hydrolysis were quantified due to the increased glucan content. The overexpression of an endoglucanase *cell*, from *Arabidopsis thaliana*, led to increased polysaccharide content in poplar (Shani *et al.*, 2004). A 10.8% increase in cellulose and a 5.7% increase in noncellulosic polysaccharide content was observed, while lignin remained unaffected.

Lignin modification

One of the most prevalent modification schemes used to improve biomass degradability is the reduction of lignin in plant cell walls, often through the suppression of key enzymes in the biosynthetic pathway (Simmons *et al.*, 2010). Pretreatment of lignocellulosic biomass to remove lignin and its inhibitory effects is the most expensive aspect of biofuel production, preventing its economical use in the energy market (Li *et al.*, 2008; Mansfield, 2009; Sticklen, 2006). The lignin in the secondary cell walls is more branched and more resistant to degradability than that in the primary cell wall (Grabber, 2005), making reduction in lignin in these walls even more important.

Lignin biosynthesis (Figure 3) involves several enzymes including phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), coumarate 3-hydroxylase (C3H), caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT), ferulate 5-hydroxylase (F5H), 4-hydroxycinnamate CoA ligase (4HCL), cinnamoyl-CoA reductase (CCR), caffeoyl-CoA O-methyltransferase (CCoAOMT) and cinnamyl alcohol dehydrogenase (CAD) (Grima-Pettenati and Goffner, 1999). Depending on the dedication of the enzymes to the lignin biosynthesis pathway, intervention to manipulate these enzymes may lead to changes in the production of lignin and other pathways that could affect the growth and development of the plant (Grima-Pettenati and Goffner, 1999). Among those, COMT and CAD are terminal enzymes in the pathway, and altering these enzymes normally has little or no impact on plant growth and development (reviewed in Jung *et al.,* 2012a) while manipulating CCR and CCoAOMT and others may have pleiotropic effects (Grima-Pettenati and Goffner, 1999). CAD has been found to influence both the quality and composition of the lignin in the plant cell walls (Saathoff *et al.*, 2011). Lignin modification may involve down-regulation of lignin synthesis enzymes such as CAD (to modify lignin content) and COMT (to modify lignin composition and content), reduce level of *p*-coumarate esters and ferulate ethers, reduce deposition of S and G lignin and increase deposition of phenylpropane units or aldehydes in lignin (Grabber, 2005).

Multiple studies have shown that reduction in lignin results in higher saccharification or ethanol yields. For example, lignin biosynthesis was down-regulated by six diverse pathways in alfalfa (Chen and Dixon, 2007). These authors demonstrated a strong negative correlation between lignin content and sugar released by enzymatic hydrolysis. S/G ratios were not found to correlate with glucose or xylose yields due to a high proportion of

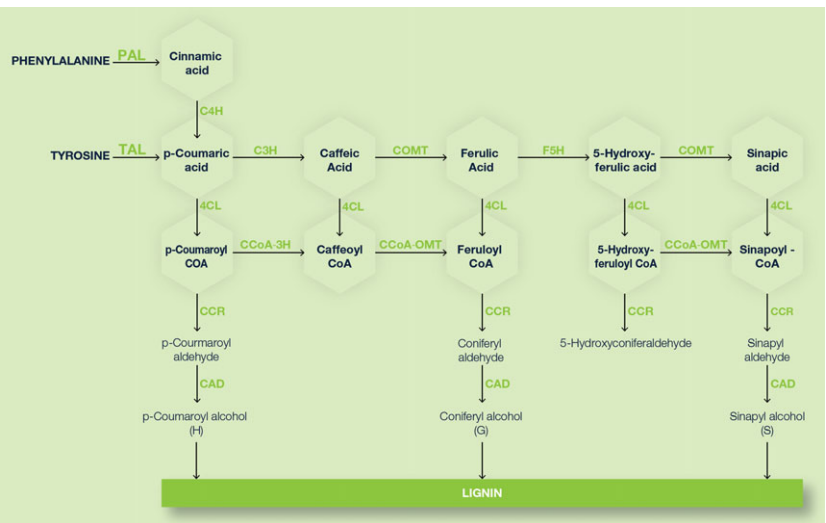

Figure 3 Lignin Biosynthesis pathways: PAL, phenylalanine ammonia-lyase; TAL, tyrosine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, 4-hydroxycinnamate 3-hydroxylase; COMT, caffeic acid 3-O-methyltransferase; F5H, ferulate 5-hydroxylase; 4CL, 4-coumarate: CoA ligase; CCoA-3H, coumaroyl-CoA 3-hydroxylase; CCoA-OMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; and CAD, cinnamyl alcohol dehydrogenase (adapted from Spangenberg *et al.*, 2001).

H lignin when hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (*HCT*) or *C3H* was down-regulated. These transgenics also led to >50% reduction in lignin content compared to the wild-type alfalfa stems. Gressel (2008) has indicated that two to four times as much cellulose becomes available for enzymatic degradation for every one per cent diminution in total lignin. Down-regulating of CCR in poplar resulted in up to 50% reduction in lignin content, resulting in a concomitant increase in cellulose content (Leplé *et al.*, 2007). Similar conclusions were obtained through suppression of CAD or COMT in poplar (Pilate *et al.*, 2002). Although the authors measured reductions in lignin S/G ratios, the transgenic trees exhibited improved pulping characteristics. A recent study examined reducing lignin content while preventing subsequent vessel collapse (Yang *et al.*, 2013). The promoter of a crucial lignin gene, *C4H,* was swapped for a vessel-specific promoter of transcription factor *VND6*, leading to enhanced vessel formation. A further modification strategy allowed the augmentation of polysaccharide content, generating a novel two-pronged approach to increasing monomeric sugar yields following enzymatic saccharification. These discoveries formulate the general conclusion that reductions in total lignin through modification of the biosynthesis pathway can reduce or eliminate costly pretreatment requirements to render biomass accessible to enzymes. However, improved release of sugars from noncellulosic polysaccharides as a function of reduced lignin was not observed in alfalfa, canary grass and switchgrass when tested at different stages of development correlating with different levels of lignin (Dien *et al.*, 2006).

Some progress has been made towards modification of the lignin components of grass biomass in species used for biofuel production. For example, in sugarcane, 10 different enzymes have been found in the lignin pathway including those involved in hydroxylation, methylation and side chain reduction of monolignol precursors (Jung *et al.*, 2012a). *COMT* alone involves at least 31 different expressed sequence tags (ESTs) in the complex sugarcane genome which could be homologous genes/alleles (Jung *et al.*, 2012a). In sorghum, 14 *CAD*-like genes at seven genomic locations have been identified (Oraby *et al.*, 2007). Reduced expression of lignin biosynthesis enzymes was first achieved by using antisense oligonucleotides, but more recently, RNAi technology has been more commonly used (Sticklen, 2008). Using RNAi suppression to down-regulate *COMT* in sugarcane by 67%–97% resulted in the lignin content being reduced by 3.9%–13.7% (Jung *et al.*, 2012a). Down-regulation of *COMT* in maize enhanced the digestibility of the biomass (Boudet *et al.*, 2003), and Down-regulation of *CAD* in switchgrass resulted in either an overall decrease in lignin or an altered structure. (Fu *et al.*, 2011; Saathoff *et al.*, 2011).

Lignin monomeric composition (S lignin, G lignin and H lignin) and their ratios affect the conversion efficiency (Jung *et al.*, 2012a). Using RNAi suppression of *COMT* in sugarcane, the lignin S/G ratio was reduced from 1.47 in the wild type to values ranging between 1.27 and 0.79 and resulted in increase of the sugar yield of up to 29% without pretreatment (Jung *et al.*, 2012a). In switchgrass, down-regulation of *COMT* also resulted in a reduction of the S/G ratio and increased ethanol yield by 38% (Fu *et al.*, 2012).

Reduced expression of lignin biosynthesis genes can be achieved by manipulating the expression of *PvMYB4* a key transcript factor from *Panicum virgatum*. Overexpression of *PvMYB4* in switchgrass reduced lignin content by 40%–70%, altered the wall phenolic content and reduced recalcitrance in transgenic biomass (Shen *et al.*, 2012). Some grass mutants of COMT and CAD have been reported including maize *bm1* mutants with altered CAD enzyme, brown midrib *Sorghum*, a *COMT* gene in maize with altered lignin subunit composition and sugarcane mutants with modified lignin content (Li *et al.*, 2008).

Alterations in lignin S/G ratio have also been explored to make lignin more amenable to degradation. The overexpression of *F5H* led to significant increases in syringyl monomer content in poplar lignin (Stewart *et al.*, 2009). S moieties were quantified as high as 97.5% (S/G ratio ~38) when measured with standard wet chemistry techniques as well as nuclear magnetic resonance, nearly 30% higher than detected in wild-type poplar. This transgenic poplar also exhibited nearly a twofold increase in acid-soluble lignin content.

The addition of coniferyl ferulate into the lignin biosynthetic pathway of maize led to a greater degradability when alkaline pretreatments were employed and therefore greater enzymatic saccharification sugar yields (Grabber *et al.*, 2008). The coniferyl ferulate was added to partially replace some of the coniferyl alcohol precursor. As the coniferyl ferulate concentration increased from 20% to 60%, the amount of alkali-soluble lignin escalated from 12% to 18%. The authors reasoned that pectin and hemicellulose removal should be improved due to the more degradable lignin. Carbohydrate yields following enzymatic saccharification were significantly improved after initial decreases in lignin content, but eventually plateaued regardless of continued lignin reduction, indicating that the presence of lignin was not the sole parameter controlling cell wall degradation.

The expression of a bacterial 4-hydroxycinnamoyl-CoA hydratase/lyase (4HCHL) in *Arabidopsis* led to a reduction in the degree of lignin polymerization (Eudes *et al.*, 2012). The propanoid side chain of hydroxycinnamic lignin precursors was cleaved, resulting in the accumulation of hydroxybenzaldehyde and hydroxybenzoate moieties. This triggered a decrease in the molecular weight of lignin, as well as improved saccharification yields.

In other studies, down-regulation of 4-coumarate: CoA ligase (*Pt4CL1*) led to decrease in lignin content in aspen (*Populus tremuloides*) (Dean, 2005). Targeting this enzyme along with HCT and CH3 may be targets in reducing lignin content and increasing sugar release in grass or woody species. A recent study (Wilkerson *et al.*, 2014) demonstrated that monolignol ferulate transferase can be used to introduce chemically labile linkages into the lignin backbone.

A recent study has shown that manipulation of a transcriptional regulatory complex, Mediator, can alter lignin in *Arabidopsis* (Bonawitz *et al.*, 2014). Plants with normal growth patterns were generated but without guaiacyl or syringyl lignin subunits and with enhanced saccharification potential. This suggests that radical modifications of lignin may be possible and that these will not necessarily limit plant performance.

Increasing the total cell wall content

Another modification strategy is to increase the proportion of the biomass that is cell wall. Sugarcane and related species are targets for this type of genetic manipulation. Increased fibre (cell wall) content in sugarcane may be desirable in varieties dedicated for biofuel production. High-fibre sugarcanes (energycanes) have been developed in several ways. *Saccharum spontaneum* and *S. robustum* have been used as parents and crosses between sugarcane, and *Miscanthus* have been used to create *Miscane* (de Siqueira *et al.*, 2013). This results in sugarcane with higher

cellulose production, disease resistance and cold and drought tolerance (Burner *et al.*, 2009). Some *Erianthus* species have also been used for this purpose (Jackson and Henry, 2011).

Overexpression of miR156 precursor in switchgrass lines increased biomass yield by 58%–101% compared to the controls with normal flowering time and improved the solubilized sugar yield and biomass digestibility but stunted growth at high level of expression (Fu *et al.*, 2012). The implication of this would be crossing the miR156 overexpressed lines with the *COMT* down-regulated lines or other lower recalcitrance transgenics to create superior switchgrass lines (Fu *et al.*, 2012).

Other modifications

The cross-linking of macromolecules in the cell wall is a major barrier to their separation in biofuel production. The cell walls of grasses have high levels of ferulic acid esterified to arabinose residues (Hatfield *et al.*, 1999). These ferulic acid substituents are able to link to form diferulates that in turn link polysaccharide molecules to one another and to lignin molecules. These dimers have been shown to significantly decrease sugar release following enzymatic hydrolysis (Grabber *et al.*, 1998). Disrupting this type of cross-linking is an important strategy for improving biomass to biofuel conversion efficiency. Benzyl ester and ether cross-linking is also known to occur in plant cell walls; however, they remain less understood due to a lack of analytical methodology facilitating their routine measurement. Reducing the level of hydroxycinnamic acid concentration in the cell wall is one strategy.

Grabber (2005) showed that the enzymatic hydrolysis of grass cell walls increased by 28% after manipulation of the linkages between and within lignin components and the cell wall carbohydrates include altering ferulate and diferulate cross-linkages, benzyl ether and ester cross-linkages. Selection of grasses through breeding or engineering for lower ferulate cross-linking or developing microbial xylanase is a preferred option relative to pretreatment using a feruloyl esterase (Grabber, 2005).

The insertion of exogenous enzymes and other proteins into plants has been explored as a way to modify biomass properties such as cellulose crystallinity. Expansin and swollenin are two such proteins that have been shown to reduce the rigidity of cellulose fibril connections (Cosgrove, 2000; Saloheimo *et al.*, 2002). While the inclusion of higher concentrations of these proteins into lignocellulosic biomass has not been extensively explored, the sentiment indicates that perhaps their addition could be a supplementary pathway to reducing cellulose recalcitrance, thereby lessening the need for pretreatments.

Species-specific approaches

Two major distinct types of plant biomass are options for biofuel production, grass species and woody species. Many plants including *Panicium virgatum* (switchgrass), *Miscanthus* spp. (miscanthus), *Sorghum bicolor* (sorghum) and *Saccharum* spp. (sugarcane) proposed for biofuel uses are from the grass family (Poaceae). Grass species have unique characteristics in addition to the diferulate cross-linkages described above. Although woody biomass represents a large source of polysaccharides for fuel conversion in the form of cellulose, the complex nature of the cell wall composition and the high levels of lignin results in inefficient enzymatic saccharification and microbial fermentation (Carroll and Somerville, 2009; Himmel *et al.*, 2007; Hinchee *et al.*, 2010). The different chemistry of these two types of biomass requires specific approaches to biomass improvement and utilization.

Grasses are part of the commelinid group of monocotyledons (Figure 2). Grass cell walls contain characteristic noncellulosic polysaccharides such as arabinoxylans and glucans (Table 1). The capture of these molecules for biofuel production requires consideration of their chemistry. Modification of the unique noncellulosic cell wall polysaccharides in grass and related species (Poales and commelinid monocotyledons) may provide novel pathways to new biomass designed for biochemical conversion. The 1,3; 1,4-β-glucans of grasses may be water soluble even at very high molecular weight, and biomass abundant in these polymers may be an attractive alternative to biomass plentiful in insoluble cellulose. The glucan synthases responsible for the biosynthesis of these polysaccharides have been identified (Burton *et al.*, 2006) and may be targets for manipulation in the development of biomass.

The main challenge in woody biomass is the high lignin content. The majority of strategies used to manipulate lignin for biofuel phenotypes in woody species have been primarily to alter gene expression within the phenylpropanoid and monolignol synthesis pathways to reduce total lignin content or alter its composition, or systemically manipulate lignin deposition by influencing cell wall transcription factors (Sticklen, 2006, 2008; Vanholme *et al.*, 2008). Most studies have focused on poplar species and hybrids, the model plant species for woody biomass based on their growth rate and rotation speed, clonal propagation and amenability to transformation by *Agrobacterium*-based methods (Jansson and Douglas, 2007). Strategies to improve biofuel characteristics in trees closely align with those used in pulp and paper industries, where lignin content and composition is manipulated to increase paper quality and pulping efficiency (Li *et al.*, 2008; Pilate *et al.*, 2002).

The first manipulations of lignin in woody biomass systems involved the transformation of poplar hybrid species with sense and antisense genes from the lignin biosynthesis pathway. Doorsselaere *et al.* (1995) transformed poplar hybrids with vectors for the up- and down-regulation of *COMT*. The down-regulation of *COMT* resulted in the sixfold reduction of the S/G lignin composition, while total lignin content remained unchanged. Baucher *et al.* (1996) altered the expression of *CAD* in transgenic poplar hybrids (*Populus tremula* x *Populus alba*) using sense and antisense vector constructs. Although transgenic trees contained lignin of similar content and composition as controls, *CAD* down-regulation resulted in biomass with red-coloured xylem tissue that was more easily digested by alkali pretreatment and increased pulping efficiency. Lapierre *et al.* (1999) further characterized these poplar transgenics with down-regulated *COMT* and *CAD* expression levels to understand the alterations to their lignin structure. The down-regulation of *COMT* resulted in lignin with an increased proportion of guaiacyl units. Upon polymerization, G moieties produce lignin with a higher proportion of biphenyl 5-5 linkages, which are resistant to cleavage during Kraft pulping. Alternatively, transgenic poplars with reduced expression of *CAD* underwent more efficient pulping as their lignin contained a high number of free phenolic groups, which increased lignin solubility. This result was later confirmed by (Pilate *et al.* (2002) where *CAD*-reduced lines were delignified with greater efficiency and with fewer chemicals. However, from a biofuels perspective, gains in pulping may not necessarily translate into greater fuel production. The altered lignin structure of *CAD*-deficient poplars may release a higher proportion of phenolics into solution during biomass pretreatment. Phenolics, such as vanillin and syringaldehyde, can inhibit

downstream biofuel processes including enzymatic saccharification and microbial fermentation (Ximenes *et al.* 2010).

Changes in the expression of *F5H*, one of the enzymes responsible for monolignol synthesis, have also generated transgenic poplars with altered lignin structure. Franke *et al.* (2000) produced transgenic poplar hybrids (*P. tremula* × *P. alba*) by *Agrobacterium*-mediated transformation with a *C4H* promoter driving the expression of F5H. The resulting transgenic poplars displayed a marked increase for lignin S:G ratios (1.90- WT; 14.17-transgenic line H) and underwent more efficient Kraft pulping, as conducted by Huntley *et al.* (2003). Transgenic trees also displayed altered lignin structure, with increases in resinol (β-β linkage) and spirodienone (β-1 linkage) content, and a virtual elimination of linkages arising from guaiacyl monolignols. Perhaps most importantly *pC4H-F5H* transgenic lines maintained their normal growth phenotype, a trait often lost during manipulation of phenylpropanoid and monolignol pathways (Hinchee *et al.*, 2009).

One of the most successful manipulations of the lignin biosynthesis pathway for improvement of biofuel phenotypes in woody species was conducted by Hu *et al.* (1999). Down-regulation by antisense constructs of *4CL* in transgenic *P. tremuloides* resulted in a 45% reduction in total lignin content and a 15% increase in cellulose. The authors postulated the increase in cellulose is an adaptive response to strengthen xylem tissue, weakened from *4CL* down-regulation. A second desirable biofuel trait that arose within the transgenic lines was increased growth, as transformants possessed thicker stems, increased weight, longer roots and more leaves. The authors postulated that the growth increase could be attributed to shifting carbon supply into primary cell metabolism pathways (Hu *et al.*, 1999), or by influencing the flavonoid synthesis, found to have role in auxin transportation (Jacobs and Rubery, 1988; Shirley, 1996).

Continued *4CL* work in hybrid poplars by Voelker *et al.* (2010) showed that down-regulation of *4CL* also correlates with decreases in lignin content, but small reductions in lignin diminished plant productivity and resulted in discoloured (reddish-brown) wood, with twice the amount of phenolic extractives as controls. This example of alterations within the lignin biosynthesis pathway illustrates the effect of pleiotropy when silencing single genes. Down-regulation of lignin pathways in poplars has also been linked to a reduction in fitness, photosynthesis, vascular collapse and hemicellulose reduction (Coleman *et al.*, 2008; Hinchee *et al.*, 2009; Leplé *et al.*, 2007). For these reasons, not all transgenic strategies are suitable for energy crops (Li *et al.*, 2008). An alternative strategy is the systemic down-regulation of the lignin biosynthesis pathway through targeting of transcription factors rather than individual genes.

Although most of the experimental work regarding the down-regulation of transcription factors has been documented in *Arabidopsis* and *Antirrhinum* (Vanholme *et al.*, 2010; Zhong and Ye, 2009), some work in woody biomass have documented the effects of MYB and LIM transcription factors, and their effect on lignification. Kawaoka *et al.* (2006) investigated the effects of the *LIM1* gene on lignification in *Eucalyptus camaldulensis*. LIM1, originally identified in tobacco, binds to AC promoter elements upstream of phenylpropanoid and monolignol genes, coordinating their expression. Silencing *LIM1* in *E. camaldulensis* resulted in the down-regulation of genes *PAL*, *C4H* and *4CL* and reduced lignin content in transgenics by 29%. Consequently, polysaccharide content increased by 5%, possibly due to shifting carbon pathways. Another transcription factor that binds AC regulatory elements is the *MYB* gene family. *EgMYB1*, isolated from *Eucalyptus grandis*, was transformed into poplar hybrids, resulting in the down-regulation of lignin, cellulose and hemicellulose transcripts and caused dwarfing of stems and leaf tissue (Legay *et al.*, 2010).

Another approach for the manipulation of lignin composition in woody biomass is the up-regulation of peroxidases and laccases that preferentially oxidize sinapyl alcohol, resulting in lignin with a higher S component. The rationale behind this strategy is to increase the S/G ratio of lignin without sacrificing overall structure (Barceló *et al.*, 2007). Silencing of lignin peroxidase, *prxA3a*, in transgenic poplars (*Populus sieboldii* × *P. gradidentata*) showed lower lignin content and a reduction of vanillin (Li *et al.*, 2003). Sasaki *et al.* (2004) investigated the action of a poplar cell wall peroxidase (CWPO-C), finding a strong preference for the oxidation of sinapyl alcohol and syringaresinol, resulting in a higher proportion of β-*O*-4 linkages in lignin. The importance of lignin composition for sugar release from pretreated woody biomass was illustrated by Studer *et al.* (2011), who demonstrated that S/G ratio has a higher impact on saccharification than total lignin content, although additional factors such as lignin acetylation must be considered when assessing woody biomass for fuels (Weng *et al.*, 2008).

An alternative to lignin alteration to improve woody biomass for biofuel production is the up-regulation of genes involved in the thickening of the secondary cell wall during plant development to increase biomass productivity and proportionally, polysaccharide content. Gibberellins, a group of plant hormones that influence growth and other developmental processes, have been shown to influence wood formation in poplar trees (Hinchee *et al.*, 2009; Sticklen, 2008). Overexpression of GA 20-oxidase, a gibberellin biosynthesis gene, resulted in faster growing trees with increased stem biomass and xylem fibres (Eriksson *et al.*, 2000). Expression of endoglucanases (EGs) to loosen the cell wall during cell expansion has been demonstrated to promote growth and cellulose deposition. Park *et al.* (2004) expressed a fungal xyloglucanase (AaXEG2) in poplar transformants, resulting in xylem tissue with increased cellulose content. Although an obvious strategy to improve cellulose content in woody biomass would be the up-regulation of cellulose synthase (*CesA*) genes, transgenic *Populus* seedlings generated by Joshi *et al.* (2011) illustrates the complex nature of woody biomass. Up-regulation constructs of *CesA8* in poplar resulted in the silencing of the introduced transgene, as well as other *CesA* genes. The resulting transgenic phenotype was slow growing with smaller leaves and roots and contained considerably less cellulose than wild-type trees (10% versus 41%, respectively). Transformants also displayed significant increases in lignin and noncellulosic polysaccharide content, and irregular xylem tissues.

Heterologous enzyme expression

The cost of conversion of biomass to biofuel can be reduced by *in planta* production of the enzymes used for conversion of polysaccharides in biomass to sugars. Low cost enzymes can be produced in dedicated enzyme crops.

Enzyme production in the biomass source

Another technique being researched is the heterologous expression of genes encoding for key biomass-degrading enzymes directly in the plants (Table 2). Direct expression of enzymes in the feedstock source may act by altering the composition of the

Table 2 Examples of biomass-degrading enzymes used for *in planta* expression

Enzyme	Reference
Endoglucanase	Dai *et al.* (2000)
Cellobiohydrolase	Dai *et al.* (1999)
Xylanase	Kimura *et al.* (2003)
Lignin peroxidase	Bhat and Bhat (1997)
Manganese peroxidase	Chen *et al.* (2012) and Saha (2003)
Ferulic acid hydrolase	Buanafina *et al.* (2010)
Multifunctional hydrolases	Fan and Yuan (2010)
Cocktails (endoglucanase, exoglucanase, pectate lyase, cutinase, swollenin, xylanase, acetyl xylan esterase, beta-glucosidase and lipase)	Verma *et al.* (2010)

biomass at harvest and/or by acting during biomass processing, potentially interfering with plant growth or initiating substrate breakdown prematurely. This requires careful compartmentalization to a specific subcellular location or activation, or induction of expression late in plant development or postharvest.

Representative strategies of heterologous enzyme expression include additions of a fungal cellobiohydrolase (CBH) and a thermotolerant EG into transgenic tobacco (Dai *et al.*, 1999, 2000), thermotolerant bacterial EG and CBH in transgenic alfalfa, potato and tobacco (Ziegelhoffer *et al.*, 1999) and a thermostable xylanase from *Clostridium thermocellum* into transgenic rice Kimura *et al.* (2003). Recently, Maloney and Mansfield (2010) overexpressed an EG cellulase in poplar reducing the crystallinity of the cellulose. Endoxylanase expression in *Arabidopsis* was shown to decrease the molecular weight of xylans (Borkhardt *et al.*, 2010).

In planta enzyme expression in grasses is still in its infancy; however, high biomass yielding biofuel plants such as sugarcane, switchgrass, *Miscanthus* and *Erianthus* are potential targets for enzyme expression. Recombinant protein enzymes may be targeted to organelles such as chloroplasts, vacuoles and the endoplasmic reticulum, to separate the enzymes produced from their substrates. In sugarcane, thanks to its well-established transformation methods via *Agrobacterium*-mediated transformation, the expression of enzymes in leaves could be used to degrade the bagasse for biofuel (Manickavasagam *et al.*, 2004; Taylor *et al.*, 2008). Exoglucanases (CBH I and CBH II) and EGs have been overexpressed in genetically modified sugarcane leaves by using a maize *PepC* promoter to express at 0.05% of total soluble proteins (EG, in chloroplast) and lesser amounts of CBH I and CBH II without altering the phenotype (Harrison *et al.*, 2011). Biswas *et al.* (2006) expressed EG1 and CBH I in maize at 2.1% and 4.1% of total protein, respectively (as cited in Taylor *et al.* (2008)).

Expression of esterases (e.g. ferulic acid esterase) has been shown to improve cell wall digestibility (Buanafina *et al.*, 2010). Ferulic acid esterase was expressed in *Festuca arundinacea*, leading to the cleavage of ester bonds linking lignin to polysaccharides, and concomitant increases in digestibility and enzymatically released monomeric sugars.

Production in a specialized plant

Plants have been used to produce enzyme preparations for use in conversion of biomass to biofuels. Recent examples have provided proof of concept for the production of effective enzymes for biomass degradation in plants. Tobacco plants have been engineered to produce various enzymes. A cocktail of enzymes, endoglucanase, exoglucanase, lipase, pectate lyases, cutinase, endoglucanases, swollenin, xylanase, acetyl xylan esterase or beta-glucosidase from fungi or bacteria were expressed at high levels in tobacco chloroplasts, resulting in enzyme preparations that were highly efficient in biomass processing (Verma *et al.*, 2010). Chimeric enzymes, combining domains for different enzyme action in a single protein have been successfully expressed in tobacco (Fan and Yuan, 2010).

Enzymes involved in degrading the whole cell wall are called plant cell wall-degrading enzymes (PCDEs). PCDE targets for overexpression would include cellulose-degrading (e.g EGs, exoglucanases or CBHs and β-glucosidases) and noncellulosic cell wall polysaccharide-degrading enzymes (e.g. endoxylanases, xylosidases, arabinofuranosidases, glucuronidases, acetylxylan esterases) phenolic acid esterases (ferulic acid esterase and *p*-coumaric acid esterase) and lignin-degrading enzymes (lignin peroxidase and manganese peroxidase) (Bhat and Bhat, 1997; Chen *et al.*, 2012; Saha, 2003).

The *in planta* expression of PCDEs is mostly performed in plants for which a transformation system has been perfected such as *Arabidopsis*, tobacco, potato, corn and rice. Enzymes such as EG (E1, E2, Cel6A, Egl), exoglucanase (Cel6B, CelA/Cel6G, CBH I, E3), xylanase (XynA, XylII, XynII, xynB, xynZ) have been expressed in Arabidopsis, tobacco, potato, rice, corn, alfalfa and barley with levels of accumulation of enzymes in the plants as high as 26% of total soluble protein (reviewed in Jung *et al.,* 2012b; Taylor *et al.,* 2008). *In planta* expression allows autodegradation of the plant biomass which helps lower the cost of enzymatic pretreatment as the plant plays both roles of the host and the substrate for sugar conversion (Jung *et al.*, 2012b). The expression of the cell wall-degrading enzymes is expected to be more cost-effective using thermostable enzymes that are not functional under normal plant growth conditions, avoiding self-destruction of the plant biomass (Arruda, 2012).

Conclusions

The development of genetically improved plants for biofuel production is key to improving the efficiency and viability of sustainable biofuel and biomaterial production. Plants that deliver high yields of biomass, which can be easily converted to high-yield end products, will greatly facilitate the replacement of oil with biomass (Henry, 2010a). Strategic options include the reduction in lignin content and modification of the carbohydrate components to maximize the recovery of glucose in biochemical conversions. However, other options such as reducing cross-linking in the cell wall may also play an important role in development of improved biomass composition. Some species may offer opportunities for the development of novel biomass with unique and desirable composition (e.g. grasses with high levels of noncellulosic glucans). The use of transgenic approaches will allow direct modification of composition by altering specific genes. However, genomic analysis will also help to define loci for conventional selection in plant improvement or for targeted mutation. Advances in the analysis of the genomes of key bioenergy species (Souza *et al.*, 2011) will accelerate these developments. A detailed knowledge of the genes of cell wall biosynthesis in each species will be a platform for the development of selection strategies to target

desirable changes in cell wall composition. Advancing technologies for targeted mutagenesis of plants (Shapter *et al.*, 2013; Townsend *et al.*, 2009) offer options for major improvement in biomass composition.

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Conflict of interest

The authors declare they have no conflict of interest.

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