Biotechnology for Biofuel Production

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Contents

Abstract The commercial production of lignocellulosic biofuels relies heavily on a reduction in production costs. These costs are high in part due to the challenge of biomass deconstruction and the complex nature of the secondary cell wall. The removal of lignin from the carbohydrates, and the subsequent or concurrent hydrolysis of the polysaccharides into monomers for fermentation continue to hamper large commercialization efforts. One solution for this is to tailor biomass for the production of fuels. Here we review work in the field of plant biotechnology, with a focus on dicots, to alter the secondary cell wall, produce plant made enzymes, increase biomass production, and create sterile lines. We conclude by laying out future directions for research to support the production of cost-effective lignocellulosic fuels.

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1 Introduction

Global dependence on fossil fuels continues despite improved understanding of the climate implications of these emissions (Quéré et al. 2018). The ecological cost of these fuels as well as the high demand and concerns over energy security have led to the need for development of green energy sources such as biomass derived fuels (Rodionova et al. 2017). These biofuels are derived from the chemical conversion of organic material from organisms such as plants or algae as opposed to the nonrenewable sources from which fossil fuels arise. The two primary types of biomass derived fuels are first generation and second generation, or lignocellulosic, biofuels (Rodionova et al. 2017). First generation fuels utilize starch from potential feed and food sources such as corn kernels or wheat, whereas second generation fuels are produced from the structural carbohydrates in plant secondary cell walls (SCW; Baig et al. 2019). One of the primary benefits of lignocellulosic fuels is their independence from lands used for food production (Correa et al. 2019). The biomass used for the production of these second generation fuels can be sourced from agricultural waste, or from dedicated energy crops that may be grown on land that is less optimal for agriculture (Rocha-Meneses et al. 2017).

Lignocellulosic fuels are derived from the SCW, a thick structural cell wall layer located between the plasma membrane and the thinner primary cell wall (McCahill and Hazen 2019). The SCW is comprised of the polyphenol lignin, as well as cellulose and hemicellulose, both of which can be hydrolyzed into carbohydrate monomers, and fermented into the biomass derived fuel (Wang et al. 2016; Mahon and Mansfield 2019). Cellulose is the largest carbohydrate component of the SCW, making up approximately 40–50% of softwood and hardwood trees (Rocha-Meneses et al. 2017; Zhong et al. 2019). Cellulose is formed of D-glucose units with β-1,4 glycosidic bonds that can be broken into cellobiose and then cleaved into glucose molecules (Rodrigues Mota et al. 2018). This is typically done through enzymatic hydrolysis, using multiple cellulases to allow for a high glucose yield to be fermented into ethanol (Rodrigues Mota et al. 2018; Kumar et al. 2019; Liu et al. 2019; Chu et al. 2019). Glucose from cellulose is the primary desired product from hydrolysis of the SCW for use in biofuel synthesis; however, hemicellulose can also be utilized. Hemicellulose, contributing approximately 30% of the cell wall, is less abundant and more complex than cellulose, and varies compositionally across species. Hemicellulose is composed of carbohydrates such as xylose, arabinose, and mannose rather than singularly glucose (Wang et al. 2016; Rocha-Meneses et al. 2017). Hemicellulose within the SCW serves primarily to form a complex cross-linked structure with cellulose and lignin, providing strength and rigidity to the plants (Petridis and Smith 2018); however, these sugars can also be used for biofuel production (Dodd and Cann 2009). They are more readily broken down into monomer sugars than cellulose, though acetic acid is a major byproduct of hydrolysis due to acetylation (Liu et al. 2019). The acetyl groups, from xylan in particular as it is the most abundant polysaccharide in hemicellulose, become free and form

acetic acid in the pretreatment process, leading to hemicellulose solubilization and inhibition of ethanol fermentation (Liu et al. 2019; Tian et al. 2019; Chu et al. 2019). While pretreatment increases the accessibility of cellulose, this process prevents hemicelluloses from additionally being easily used for biofuel production. The third major component of the SCW is lignin, which contributes about 20–30% of wood in hardwoods and softwoods but is not currently utilized for biofuels (Rocha-Meneses et al. 2017; Kim et al. 2019). Lignin provides rigidity and mechanical strength to the plant and is a complex phenolic polymer which crosslinks with xylan and other hemicelluloses (Xie et al. 2016; Madadi et al. 2017; Terrett and Dupree 2019). Due to this, lignin contributes to extreme recalcitrance within the plant, making the SCW difficult to break down into lignocellulosic fuel. While not utilized in second generation fermentable fuels, there is potential for lignin to be a valuable source for other types of fuels such as jet fuel (Shen et al. 2019).

Despite the potential value of biofuels from an economic and environmental perspective, there are numerous biological challenges related to lignocellulosic biofuel production, ranging from expensive resource requirements to natural barriers against SCW breakdown. Recalcitrance, broadly defined as the resistance of the SCW to the release of sugar for conversion into biofuel, is one of the primary challenges existing in the cost-effective production of lignocellulosic biofuels (Gilna et al. 2017). Crystalline cellulose microfibrils interact with hemicelluloses and lignin within the SCW, forming lignin–carbohydrate complexes which make access and extraction of celluloses and hemicelluloses expensive in terms of both cost and time (Liu et al. 2019). Biomass must undergo pretreatment to increase cellulose accessibility through decrystallization and chain disentanglement, as well as to separate lignin from the desired polysaccharides before fermentation (Seidl and Goulart 2016; Ghasemi et al. 2017). Various approaches to reduce the challenge of recalcitrance through genetic manipulation of the cell wall chemistry and structure have been examined, and recent work will be explored in this paper. In particular, research has focused on decreasing the lignin content of the plant cell wall, but often reduced lignin compromises plant growth through impairment of vascular system development (Coleman et al. 2008; Voelker et al. 2011; Pereira et al. 2018). Alternative approaches have involved modifying the crystallinity of cellulose or altering cellulose or hemicellulose levels, allowing for more susceptibility to enzymes in fermentation (Bali et al. 2016). One such study has had some success in manipulating putative "recalcitrance" genes in poplar to reduce xylan levels, finding no reduced growth traits but improved saccharification (Biswal et al. 2015).

In addition to the challenge of accessing the carbohydrates within the plant biomass, there is the challenge of producing large amounts of biomass rapidly. This can require a large input of fertilization. Numerous studies have assessed the impact of various nutrient fertilizers and have found a significant increase in biomass with their application in a variety of plant species including poplar and eucalyptus (Cooke et al. 2005; da Silva et al. 2016). The most commonly studied nutrient is nitrogen (N). Only 30–50% of nitrogen applied to crops is utilized by the plants, leaving a large percentage of N in the environment (Hodge et al. 2000; Masclaux-

Daubresse et al. 2010). Although N is abundant, N leaching acts as an environmental pollutant. The N runoff is transported from crops to rivers and other bodies of water which leads to eutrophication, or the overabundance of a nutrient. As a result, the mass addition of N to crops contributes to harmful algae blooms, anoxic waters, and other serious concerns (Le Moal et al. 2019). Improving plant uptake of N in order to reduce the cost of fertilization and the loss of N to the environment is an important challenge that must be addressed for the success of lignocellulosic fuels. In addition to these fertilization requirements, there may be more intense irrigation requirements, particularly as water becomes more and more depleted with changing climates (Kattel 2019). Agriculture accounts for approximately 70% of water use from freshwater systems, an estimate subject to change as biofuel production grows to replace fossil fuels (Pastor et al. 2019). With greater understanding of lignocellulosic biomass and improvements in nutrient and water use, biomass feedstock production will be less ecologically expensive.

Finally, these challenges of improved biomass quantity and quality are most likely to be addressed by biotechnological solutions. This requires that plants produced by these methods can be used in the field for the production of biomass. Current research is also addressing both methods to induce sterility and also assessing the environmental impact of these plants (Strauss et al. 2017). This work is integral to the success of lignocellulosic biofuel production.

Research into second generation biofuels utilizes multiple different species. *Arabidopsis thaliana* acts as a useful dicot model organism, while members of the *Populus* family, Eucalypts, and willow are also commonly studied, as trees which can produce large amounts of biomass on non-agricultural lands, making them potential biofuel feedstocks. Many other species, both monocots and dicots, also hold potential for use in the production of biomass for lignocellulose fuels; for the purpose of this review we focus on dicot species.

2 Modification of Secondary Cell Wall Biosynthesis

There are four major steps involved in the biochemical conversion of lignocellulosic biomass to ethanol: biomass production, pretreatment to disrupt the cell wall, enzymatic hydrolysis to release glucose monomers from structural carbohydrates, and the fermentation of monomeric carbohydrates into ethanol (Fig. 1; Huang et al. 2019). Higher biofuel yield or easier pretreatment can be achieved by using biomass that possesses lower cell wall recalcitrance and the use of suitable chemical pretreatment for the specific biomass being used (de Souza et al. 2019). Strategies aimed at changing the composition of the SCW have provided some success in reducing recalcitrance. Additional strategies for improving yield include increasing the carbohydrate content of the cell wall, reducing the crystallinity of the cellulose, or altering the complexity of the cell wall structure.

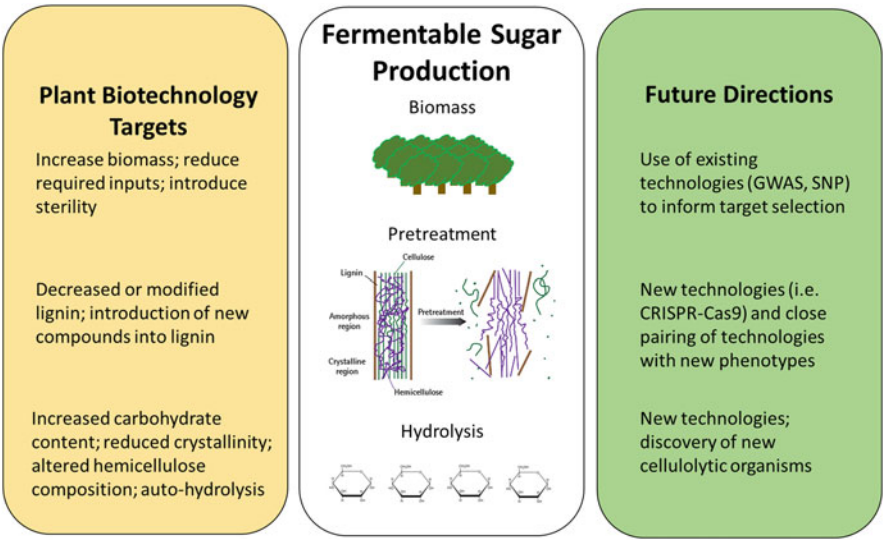

Fig. 1 Schematic of current targets of biotechnology and the expected future directions for continued advancement in the production of lignocellulosic biofuels

2.1 Lignin

Lignin is one of the major components of the SCW and is comprised of dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G), and non-methoxylated (p-hydroxyphenyl, H) phenylpropanoid units which are derived from *p*-hydroxycinnamyl alcohols (Martínez et al. 2005). It provides a rigid structure to the plant cell wall and, in processing, restricts the hydrolysis of cellulose and hemicelluloses (Isikgor and Becer 2015). Removal of lignin is therefore a necessary step in the production of lignocellulosic biofuel (Bušić et al. 2018).

The pretreatments required for the removal of lignin are very costly and potentially toxic to the environment (Hamelinck et al. 2005). The capability to produce biofuel feedstocks with reduced lignin would consequently have positive impacts in many capacities, and the modification of lignin gene expression has been well explored (reviewed in Chanoca et al. 2019). However, when there is a reduction in lignin content, there are other potentially negative impacts on the plant, including increased susceptibility to pests, decreased resistance to drought, and reduced growth (Moura et al. 2010). A number of strategies exist for reducing or altering lignin in plants; however, some recent reviews have emphasized that strictly reducing the amount of lignin throughout the plant is unlikely to be the solution to improving biomass for biofuel production (Mahon and Mansfield 2019; Muro-Villanueva et al. 2019). There are still other options addressing lignin recalcitrance, including manipulating expression of transcription factor genes controlling the

lignin biosynthesis pathway. Transcription factors in the NAC and MYB families are well-known regulators of SCW biosynthesis (Nakano et al. 2015). Controlling the expression of these transcription factors can result in trees with a low-lignin phenotype in a more scripted way than the direct manipulation of individual lignin biosynthesis genes (Zhang et al. 2018). In addition, CRISPR could be used to edit specific genes, rather than reducing their expression, to improve lignin phenotypes (Chanoca et al. 2019).

While noting the growth penalties regularly associated with reductions in lignin, Mahon and Mansfield (2019) proposed research directions focused on minimizing association of adjacent polymers within the cell wall. Muro-Villanueva et al. (2019) advocated for a more directed modification of lignin that protects the vessels responsible for water transport and reduces the accumulation of phenolics, intermediates in the formation of lignin, using vessel specific complementation.

As highlighted by Mahon and Mansfield (2019), the strategy to introduce labile structures into lignin has proved valuable as a method to create lignin that is more easily removed (Eudes et al. 2012; Wilkerson et al. 2014). Eudes et al. (2012) used a bacterial hydroxycinnamoyl-CoA hydratase-lyase gene to produce rare lignin monomers they termed DO reducers (C_6C_1 monomer) to reduce the polymerization of lignin. Although this proved effective in altering the lignin structure, there was still a small growth penalty. Wilkerson et al. (2014) expressed a feruloyl–coenzyme A (CoA) monolignol transferase (FMT) gene from *Angelica sinensis* in poplar to drive the production of monolignol ferulate conjugates in lignin. These conjugates, which introduce readily digestible ester bonds into the lignin, allow for near doubling of glucose release following a mild alkaline pretreatment, with no growth deficit. Further analysis on these lines showed improved response to ionic liquid pretreatments (Kim et al. 2017).

As explored in Muro-Villanueva et al. (2019), another option is the reduction of lignin biosynthesis in fibers only, which would serve to reduce the negative effects of cell wall modification on plant growth and development. This could potentially be achieved through knockout of a gene, with complementation of the same gene using a vessel specific primer, or by targeting gene knockout to fibers. Both of these options have been explored. De Meester et al. (2018) used a vessel specific artificial promoter Secondary Wall NAC Binding Element of the xylem cysteine protease 1 to reintroduce cinnamoyl coenzyme A reductase 1(ccr1) into the *ccr1 Arabidopsis* mutant line. This resulted in recovery of biomass levels beyond that of wild type and a resultant glucose release per plant of nearly four times that of the wild type. Smith et al. (2017) demonstrated reduced lignification and increased saccharification in *Arabidopsis* by using a fiber specific promoter driving the expression of the same gene. Liang et al. (2019) used a fiber specific promoter (NST3/SND1) to drive Cas9 expression to knock out the expression of hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT). The CRISPR edited *HCT* lines were not different in size from wild type, but did display the expected increase in H lignin. Taken together, this work suggests that improvements in biomass quality can be

made without detrimental impacts on plant growth if the modification is well targeted.

An additional interesting approach takes advantage of enzyme promiscuity to overload an enzyme in the lignin biosynthetic pathway with a non-traditional substrate in order to alter lignin production. Eudes et al. (2016) engineered *Arabidopsis* to produce protocatechuate, which they define as a HCT competitive inhibitor. By providing a nontraditional substrate to this lignin biosynthesis enzyme, the lignin content was reduced and saccharification increased.

2.2 Cellulose

Cellulose is the most abundant component of the SCW and is seen as an almost inexhaustible resource for greener fuels and products (Klemm et al. 2005). This, in addition to its pure composition of glucose monomers, makes cellulose the most desired portion of the SCW for the production of lignocellulosic biofuels. The synthesis of cellulose is facilitated by cellulose synthase (CesA) proteins which interact with one another to form a rosette-shaped plasma membrane-spanning CesA complex (CSC) (Holland et al. 2000; Li et al. 2016). Three isoforms of *CesA*, *CesA4*, *CesA7*, and *CesA8*, are essential to SCW biosynthesis of cellulose, and come together at the cell plasma membrane to link glucose chains into cellulose fibers (Li et al. 2016; Xi et al. 2017; Hu et al. 2018; Speicher et al. 2018). These CSCs synthesize cellulose microfibrils from β-1,4-linked glucose arranged in linear chains with cellobiose as the repeating unit (Rodrigues Mota et al. 2018; Turner and Kumar 2018). The linear glucan chains are arranged by the CSCs into a multilaminar structure where the chains interact with their neighbors through hydrogen bonds to form cellulose microfibrils (Zhang et al. 2015; Lindh et al. 2016; Xi et al. 2017). These cellulose microfibrils provide the framework for the cell wall and are the primary load-bearing component of the SCW (Mueller and Malcolm Brown 1980; Ruel et al. 2012). Studies have examined the potential in overexpressing SCW CesAs to improve cellulose content but have been unsuccessful. However, a study looking at the impact of increasing the expression of primary cell wall *CesAs*, including *CesA2*, *CesA5*, and *CesA6*, found improved cellulose synthesis and plant growth in *Arabidopsis* (Hu et al. 2018). Other research promoted the biosynthesis of cellulose and SCW thickening in *Arabidopsis* through hydrogen peroxide (H_2O_2) production under salt stress signaling CesA activity (Shafi et al. 2019).

The structure of cellulose provides a number of challenges for biofuel production. One of these is crystallinity index, associated with larger degrees of polymerization and microfibril orientation (Xi et al. 2017). Cellulose exists in two primary forms within the SCW depending on the organization and compactness of microfibrils (Sun et al. 2016). Crystalline cellulose represents the majority and is relatively resistant to hydrolysis processes aiming to break down the cellulose into glucose molecules. The second form of cellulose is amorphous and includes areas with low molecular order

or weaker inter- and intramolecular hydrogen bonds and less organization (Karimi and Taherzadeh 2016; Hattori and Arai 2016). Amorphous regions are much more easily hydrolyzed due to their greater ability to adsorb water as opposed to crystalline cellulose. Due to the difficulties in hydrolyzing cellulose I, or native cellulose, even after the removal of lignin, studies have begun to examine the potential benefits of a synthetic allomorph of cellulose called cellulose II (Karimi and Taherzadeh 2016). Cellulose II has an antiparallel arrangement of its chains and a monoclinic lattice arrangement, contributing to decreased crystallinity, increased surface area, and greater hydrophilicity, all of which are ideal for biofuel production (Nagarajan et al. 2017).

2.3 *Hemicellulose*

Hemicellulose is the third major component of the plant SCW. In dicots, the majority of hemicellulose is glucuronoxylan (xylan), but there are also smaller amounts of glucomannan and galactoglucomannan (Kumar et al. 2016). Hemicellulose interacts closely with lignin and cellulose, which contributes to the complexity of the SCW. Pectin, an additional but minor component of the SCW, contributes to this complexity through forming a gel-like matrix in which hemicellulose and cellulose are embedded (Tomassetti et al. 2015).

Using targeted RNAi construct-driven downregulation of a gene encoding galacturonosyl transferase 12, *GAUT12* (*IRX8*), a gene involved in glucuronoxylan and pectin biosynthesis, in poplar led to increased biomass production and decreased recalcitrance (Biswal et al. 2015). In a later study, when GAUT12 was overexpressed, the resultant plants had decreased biomass and increased recalcitrance (Biswal et al. 2018b). This gene also impacts the formation of homogalacturonan (HG), a pectin, which is a minor part of the SCW. When overexpressed, the HG content in the plants increased and when silenced, the HG content decreased, following the same pattern as xylan levels. In other work by the same group, downregulation of *GAUT4*, a gene encoding enzyme responsible for pectin polysaccharides and glycan biosynthesis, resulted in increased biomass and improved saccharification (Biswal et al. 2018a), along with decreased pectins including HG and reduced crosslinking between carbohydrates and lignin (Li et al. 2019). Additionally, endopolygalacturonase (*EPG*), a well-known pectinase, has been examined as a potential additive to enzymatic hydrolysis in order to decrease the recalcitrance of pectin in the SCW. Experiments utilizing EPG found increased efficiency of hydrolysis and decreased recalcitrance (Latarullo et al. 2016).

The modification of hemicellulose results in some of the same growth challenges as the modification of lignin and cellulose. Recommendations include the use of wood-specific promoters or heat inducible enzymes or promoters (Donev et al. 2018).

3 Enzyme Production in Plants

Hydrolytic enzymes are essential for the breakdown of cellulose and hemicellulose into fermentable carbohydrate monomers. These enzymes include cellulases and hemicellulases (Niu et al. 2019). Both these enzyme classes work together to break down the SCW by hydrolyzing cellulose and hemicellulose respectively. They are of high importance in various industries beyond biofuels for the production of glucose and have been extensively studied (Srivastava et al. 2018). They are produced by many types of organisms, including fungi, plants, and animals. Cellulases from bacteria and fungi, including *Trichoderma reesei,* are often used in lignocellulosic biofuel production and are generally produced in microbes (Mojsov 2016; Singh et al. 2019). This microbial production of cellulases can be very expensive (Srivastava et al. 2018); an alternate production method may be producing them in the biofuels crop itself (Xiao et al. 2016).

There are three major families of cellulase enzymes: endoglucanase, exoglucanase, and β-glucosidase. These enzymes work together to hydrolyze the β-1,4 links in the cellulose chains (Mojsov 2016). Endoglucanase works within the cellulose chain where it breaks bonds in no particular pattern. Exoglucanase is much more specific as it hydrolyzes bonds near the end of the glucose chains. Exoglucanase releases β-cellobiose and other short fermentable oligosaccharides (Srivastava et al. 2018). β-glucosidase further breaks down the β-cellobiose into a simple sugar, β-glucose (Mojsov 2016).

Hemicellulases make up another family of enzymes that hydrolyze the various polysaccharides that compose hemicellulose. As hemicellulose is comprised of a complex subset of heteropolymers made up of multiple carbohydrates, the family of hemicellulase enzymes is also diverse and includes xylanases and mannanases. These enzymes work together to break down the polymer into monomeric sugars (Gupta et al. 2016). Though cellulase research is more prevalent, *in planta* expression of hemicellulases has also been shown to be a valuable tool for decreasing plant recalcitrance (Tsai et al. 2017). Hemicellulose is primarily made up of xylan, which is broken down into the monomer sugar xylose with the help of the hemicellulases endo-β-xylanase and β-xylosidase (Niu et al. 2019). Co-expression of a β-glucosidase with a xylose isomerase resulted in better xylose production efficiency in vitro since β-glucosidase is often inefficient and the rate-limiting step in xylose production (Niu et al. 2019).

Lignin modifying enzymes (LMEs) are another group of enzymes involved in plant cell wall break down. Laccases and peroxidases are the most common and best studied types of LMEs in plants (Bilal et al. 2018). These enzymes break down the complex matrix of lignin that envelops the SCW and limits the accessibility of the desired carbohydrates. Lignin degradation is a complicated process as there is no single method by which the enzymes work, but research in this area has shown that these enzymes are capable of reducing lignin in plants when their amino acid codons are altered by site-directed mutagenesis to create higher efficiency (Madzak et al.

2006). A bacterial-produced peroxidase, DypB, was expressed in tobacco and targeted specifically to either the endoplasmic reticulum (ER) or the cytosol (Ligaba-Osena et al. 2017). In both cases, the growth of the plants was not negatively impacted despite a 200% increase in saccharification when compared to the wild type tobacco plants likely due to a decrease in lignin in the transgenic plants. The reduction of lignin, which increased access to the cellulose, was attributed to its depolymerization by DypB (Ligaba-Osena et al. 2017). Ectopic expression of peroxidase genes can also alter cellulose accumulation and metabolic pooling which impacts growth and development.

Current research is primarily working with *in planta* expression of enzymes as an alternative or supplement to applying microbial derived enzymes. One of the potential concerns associated with overexpressing desired enzymes in the plants is stunted growth and poor development in the transgenic plants. Many recent studies have shown that it is possible to overcome this challenge by targeting the enzymes to various cellular components such as the apoplast and the ER as reviewed by Park et al. (2016). For example, when overexpressing an endoglucanase TrCel5A in tobacco, it was shown that targeting the enzymes to the ER did not result in detrimental effects on plant growth, contrary to a previous study where apoplast targeting of the same enzyme had a growth penalty (Klose et al. 2013, 2015).

Apoplast-targeted expression of a hyperthermophilic endoglucanase from the bacteria *Thermotoga neapolitana* in hybrid poplar resulted in plants with altered cell wall compositions and biomass that was easier to breakdown. One of the transgenic lines did not require pretreatment in order to reach the same level of glucose release as the pretreated wild type plants (Xiao et al. 2018). Similar results were seen with apoplast-targeted expression of hyperthermophilic endoglucanase and xylanase genes in *Arabidopsis* with no detrimental impact on height or fresh weight but an increase in glucose release by enzymatic hydrolysis (Mir et al. 2017).

Overexpression of cellulases is clearly a viable option for improving lignocellulosic biomass for biofuel production. In addition to avoiding harmful effects on plant growth, there is an increased rate of digestion of the SCW. Pretreatment and enzymatic hydrolysis, which together break down the SCW into fermentable sugars, are expensive and time-consuming steps in the biofuel production process, but overexpressing cellulases has shown to be as effective and more convenient, as reviewed in Akram et al. (2018).

4 Improving Biomass Production

4.1 Nutrients

Another major goal with regards to plant biotechnology for biofuels production is increasing biomass yield. In order to maximize growth, plants often require supplemental nutrients in the form of fertilizers. In addition to altering nutrient availability,

it is possible to increase growth by manipulating the genes that regulate or directly participate in nutrient uptake and allocation.

Phosphorus and N are the most well-studied nutrients, as they are most often growth limiting. Phosphorus tends to be growth limiting in old soils due to a limited amount of mobile phosphorus (Wiersum 1962), whereas N is limiting in young soils in part due to rapid mineralization of available N (Wiersum 1962; Stanford and Smith 1972).

Supplying adequate levels of N typically results in increased plant growth and biomass production. Poplar trees subjected to deficient, control, and fertilized N levels were assessed for changes in biomass accumulation and concentrations of amino acids, hormones, and N across tissues. N deficient trees allocated their growth in opposing patterns to that of N normal and N fertilized, with N deficient trees expending resources to increase root biomass and N normal and N fertilized trees having increased above ground biomass (Luo et al. 2019). While a deficient N status resulted in decreased growth, there is a limit to the increase in growth that can be afforded by increased N supply. Providing 2 mM of N resulted in significantly increased leaf biomass compared to no additional N, and 50 mM of N also resulted in increased biomass, the plants developed syllepsis which persisted for the entire experiment (Cooke et al. 2005). Syllepsis, the growth of lateral branches in the same year as bud formation, has previously been shown to have a positive impact on total biomass production (Moreno-Cortés et al. 2012). The plasticity of growth in relation to available nutrients allows plants to respond to their immediate environment, but can also be useful to identify opportunities to modify the plant to improve uptake and use of nutrients.

Altering the expression of genes involved in N metabolism can also have significant impacts on plant growth. Overexpression of cystolic glutamine synthetase (GS1) has been shown to improve yield in a number of species, and in poplar has been shown to also alter the cell wall chemistry (Jing et al. 2004; Coleman et al. 2012). In addition, there is evidence that poplar overexpressing GS1 has increased nitrate uptake, making it ideal for phytoremediation in addition to having improved biomass production (Castro-Rodríguez et al. 2016). Overexpression of GS1 in *Betula pubescens* caused increased root growth and root complexity, as well as increases in amino acid concentrations. Glutamic acid, aspartic acid, and glutamine along with auxin were significantly increased in transgenic plant shoots compared to wild type controls (Lebedev et al. 2018). Improving the growth rate of plants has important implications for the production of fuels from biomass, and success using genetically modified plants with altered N metabolism (reviewed in Cánovas et al. 2018) may also reduce the need to increase fertilization.

Many studies also assessed phosphorus fertilization in tandem with N. In *Arabidopsis*, increased N availability resulted in increased concentrations of N, potassium, and magnesium in the leaves of *Arabidopsis*, while increased phosphorus availability resulted in increased concentrations of phosphorus and calcium in leaves with a reduction in carbon concentration (Yan et al. 2019a). This result implies that increasing phosphorus fertilization will increase the concentration of other required

elements but may reduce carbon acquisition. Another study in *Arabidopsis* found that supplemental N increases growth of leaves and stems while supplemental phosphorus increases stems and fruiting bodies (Yan et al. 2019b). N and phosphorus are highly linked and should be researched in tandem to create the best possible increase in growth. This is due in part to genes encoding for phosphorus transporters such as PHOSPHATE2 (PHO2) being regulated by available nitrate and expressed under phosphorus starvation in correlation with nitrate transporters such as NITRATE TRANSPORTER 1.1 (NRT1.1). Crosstalk between NRT1.1 and PHO2 was observed, as each impacts the expression of the other in periods of phosphorus deficiency (Medici et al. 2019).

4.2 Hormones

In addition to the improvements in growth that can be induced by altering the plant metabolism of nutrients, altering hormones can also impact plant growth and biomass production. One study found that gibberellin, auxin, and brassinosteroids all contribute to growth of apple trees, and manipulation of brassinosteroid concentration caused an increase in internode length, stem biomass, and leaf biomass. In addition, increases in expression of cell growth related genes such as *MYB2*, *CESA*, and *CYCD1* were observed (Zheng et al. 2019). Consistent with this, overexpression of *CYP85A3*, a brassinosteroid biosynthesis gene, resulted in increased diameter, internode length, and height in transgenic poplar relative to wild type (Jin et al. 2017).

Abscisic acid is a classic plant hormone known not only for repressing leaf abscission and preventing bud break, but also for inducing drought tolerance. When overexpressed in poplar, abscisic acid was found to increase biomass production under drought conditions (Yu et al. 2019). Jeon et al. (2016) found that overexpressing *PdGA20ox1*, gibberellin 20 oxidase 1, increased biomass production, SCW thickening, glucose levels, and xylem differentiation in both plant species relative to wild type. Unlike previous studies, they also found decreased negative effects on root formation when expressing *PdGA20ox1* under the control of a xylem specific promoter as opposed to a constitutive promoter (Eriksson et al. 2000; Mauriat et al. 2014; Jeon et al. 2016). Manipulating genes involved in hormone biosynthesis may lead to new ways to increase biomass without requiring additional agricultural inputs.

5 Introducing Sterility

The use of genetically modified plants as biomass for fuel production is attractive, as the characteristics produced can be challenging to obtain through breeding programs, and for some species, the breeding cycle may be very long (Klocko et al. 2018). One of the most common concerns with genetically modified trees is their potential for wide dispersal of seed and pollen. Removing flowering capability thereby inducing plant sterility is a potential way to reduce concerns about the use of genetically modified species. The goal of sterility in genetically modified trees began in 1987 and has been progressing to prevent biosafety implications of releasing modified trees into the wild (Committee on Environmental Impacts Associated with Commercialization of Transgenic Plants, Board on Agriculture and Natural Resources NRC 2002). Since then, a condition for further commercialization of genetically modified trees is the development of genetic containment strategies, but this work remains challenging due to market and regulatory restrictions (Strauss et al. 2017).

There are many possible ways to reduce the potential of gene flow. Some methods are horticultural, such as harvesting before maturity, growing varieties that cannot interbreed, or creating sterile hybrids (Lu et al. 2019). Others require the identification of specific target genes with roles in flowering and take advantage of tissue specific promoters to target reproductive tissues and induce sterility or alter timing of flowering.

Engineered induction of male sterility has been engaged to prevent pollen mediated transgene flow. The anther is required for pollen development, including the tapetum cells which aid in pollen development. In some plant species, success in male sterility has been achieved using genetic ablation techniques and tapetum specific promoters. One example is the pea Endothecium 1 (PsEND1) promoter driving the expression of barnase-barstar in tobacco (Roque et al. 2007). This approach has been effective in all plant species that have been tested to produce full anther ablation with no mature pollen grains produced (Roque et al. 2019). Other similar approaches include the overexpression of a restriction enzyme under the control of a tomato pollen specific promoter in tobacco, which was also effective in producing male sterility (Millwood et al. 2016). A male cone specific promoter derived from *Pinus radiata* (PrMC2) was used to express *BARNASE* in pine and eucalyptus tree to successfully produce male sterility (Zhang et al. 2012).

Poplar is dioecious, with potential for long distance wind transport of pollen and seed. Recently, a large field trial of poplar engineered for sterility was assessed (Klocko et al. 2018). This trial included a diverse set of approaches (23 constructs) and two female and one male poplar clones. The goal of the research was to identify modes of bisexual sterility of poplar that did not produce off target effects. This trial built on previous work by the group that showed targeting of *LEAFY* and *AGAMOUS* successfully produced sterility in a female poplar clone and reduced pollen amount and size in apple trees respectively (Klocko et al. 2016a, b). In the

field trial, these two genes in combination resulted in male sterility and female floral alterations, while *LEAFY* on its own resulted in female and male sterility (Klocko et al. 2018). Additionally from this work, *AGAMOUS* and *SEEDSTICK* were identified as strong modification targets to achieve gene containment (Lu et al. 2019).

Huang et al. (2016) found that creating a gene fusion of *SOLODANCERS* with *BARNASE* under the control of the SDS promoter resulted in ablation of both microspore and megaspore mother cells creating both male and female sterility. When the fusion was used, with the entire SDS coding region, there was no negative impact on growth and development in tobacco or *Arabidopsis* (Huang et al. 2016).

Another recent paper reported *Arabidopsis* sterility due to double mutations in the TFIIB-related factor (BRF) family, which plays important roles in RNA polymerase transcription. *BRF1* and *BRF2* are highly involved with the reproductive system, and the double mutation results in a high degree of aborted macrogametes and microgametes, and complete failure in zygote generation, inducing sterility (Zhang et al. 2019).

6 Conclusions

The challenges associated with the cost-effective production of biofuels are largely linked to the production of large amounts of high-quality biomass. Current fertilizer requirements, pretreatment methods, and enzymes for hydrolysis are costly to the environment and production process. As presented above, the use of plant biotechnology tools to produce improved plants, both in terms of increased biomass or improved biomass quality, continues to advance and is assisted by novel application of new technologies.

While significant advances have been made with existing technologies, the advent and rapid improvement of genomic and biotechnology tools such as genome editing (e.g., CRISPR) and the ability to introduce SNPs will allow for significant improvements over the status quo. Using the results of GWAS populations to identify the most relevant SNPs, the genome can be specifically tailored to produce the optimal combination for biomass production and quality (reviewed in Myburg et al. 2019). A final challenge remains in the regulation of transgenic trees and crops (Chang et al. 2018), and the high costs associated with bringing these crops to market. Improvement through plant biotechnology continues to advance the potential of lignocellulosic fuels; however, more work is needed to achieve cost competitive fuel production.

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