Molecular Builders of Cell Walls of Lignocellulosic Feedstock: A Source for Biofuels

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Neeru Gandotra

Abstract

Biofuels are an alternative source of renewable and sustainable energy which can be obtained from lignocellulosic feedstock (crop or non-crop energy plants), algae, or as a by-product from the industrial processing of agricultural/food products, or from the recovery and reprocessing of products such as cooking and vegetable oil. Such biofuels are generally in the form of either bioethanol or biodiesel or biobutanol. It is necessary that improvements be made at every stage during the processing of biofuel starting from enhancing the ability of the plant to maximally utilize the solar energy to fix the CO_2 into biomass and generate greater amounts of cellulosic material. The next step in the process would be to separate the cellulose from the lignin in a cost-effective way. And finally extract ethanol from this cellulose using various methodologies such as fermentation and/or cellulose pyrolysis. Engineering the steps involved in releasing the cellulose from the other cell wall components especially lignin would reduce the cost of generating biofuels from lignocellulosic materials. Hence, an in-depth understanding of the molecular components that are involved in either the regulation or biosynthesis of lignin and consequences/limitations of altering those pathways and redirecting the flux to alternate pathways are discussed.

Keywords

Lignin • Biofuel • Lignocellulosic feedstock • Molecular builders

14.1 Introduction

Biofuels generated in a sustainable and environmentally friendly way is being increasingly considered as an alternative source of energy over the traditional fossil fuels due to their adverse effect on the environment and limited supply and reserves of fossil fuel. It is projected that there

N. Gandotra (✉)
Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, USA
e-mail: neeru.gandotra@gmail.com

K.R. Hakeem et al. (eds.), *Plant signaling: Understanding the molecular crosstalk*,
DOI 10.1007/978-81-322-1542-4_14, © Springer India 2014

would be an increase in the world energy consumption by 54 % between 2001 and 2025. Increasing demands accompanied by the low net greenhouse gas emissions have put forth the usage of lignocellulosic feedstocks to produce liquid biofuels.

Although the idea of using plants to generate energy has been around for a couple of decades, its use to replace the fossil fuels has only recently been recognized. The United States and Brazil account for almost 90 % of the global production of bioethanol, while Europe produces 53 % of biodiesel. Brazil has replaced petroleum-derived oil for their transportation needs with ethanol produced from sugarcane (Chaddad 2010). There has been an ever-increasing effort in the USA and around the world to replace the existing fuel sources with biofuels. About 30 % of fuel consumption is to be replaced by biofuels as directed by the US government.

14.2 Lignocellulosic Feedstocks as Candidates for Biofuels

So far grasses mainly of the C4 type have been a major contributor to the lignocellulosic biofuels. C4 grasses demonstrate a higher efficiency in photosynthesis by compartmentalizing the process of photosynthesis between the bundle sheath and mesophyll cells and thereby increasing the carboxylation activity of the RuBisCO enzyme to fix CO_2 and reducing the loss of carbon/energy via photorespiration. By doing so, they gain an advantage in their nutrient and water use efficiencies. All of this makes them favorable candidates with regard to biomass production. Additionally, the cell walls of grasses are special with regard to their cell wall content. Grasses contain type II cell walls, while dicots contain type I cell walls. The main polymer components of cell walls of grasses are cellulose, arabinoxylans, and lignins. Grass cell walls are characterized by a high proportion of hydroxycinnamic acids, i.e., ferulic acid (FA) and p-coumaric acid (pCA). Lignin and phenolics bound to the cell walls hinder the release of cellulose for conversion to bioethanol. The biofuel crops such as sugarcane and maize are the first-generation feedstocks. More recently the second-generation feedstocks such as switchgrass and Miscanthus are gaining importance as biofuel feedstocks since they do not compete with hampering the food security.

14.3 Challenges Associated with Incorporating Biofuel from Lignocellulosic Plants to Existing Setup

(a) One of the challenges is the inefficiency in procuring cellulose from the lignocellulosic plants due to the presence of lignin. Removal of lignin tends to be costly.
(b) The quality of the fuel obtained is different from that obtained using fossil fuels in terms of their number of carbon atoms.
(c) There is a need for specialized equipments to incorporate the bioethanol into the existing setup.
(d) Ethanol is difficult to transport and it needs to be mixed with the conventional fuel at the delivery point.

In order to overcome some of the challenges mentioned above, we will discuss the biosynthesis and regulation of lignin to understand and determine the steps that can be engineered in planta to modify and/or reduce lignin content thereby improving the recalcitrance of lignin with cellulose.

14.4 Lignin Biosynthesis

Lignin is a phenolic polymer that is mainly derived from the hydroxycinnamoyl alcohols. It is found mostly in tracheophytes. Lignin provides structural rigidity for land plants to stand upright and strengthens the cell wall of tracheary elements that conduct water from the roots all the way to the tip of the plant and withstand the negative pressure from transpiration. Lignin deposition in the primary wall starts after the secondary wall formation, and the factors and mechanism that control the deposition of lignin in both types of cell wall appear to be under the

control of the same genes/enzymes (Harrington et al. 2012). There are three types of lignin, H (*p-hydroxyphenyl*), G (guaiacyl), and S (syringyl), that eventually make the plant cell wall in most grasses. The ratios and types of lignin depend on the species type, stage of development, and cell type. Grass cell wall is made up of all three types with S and G being the predominant types. A feature of grass cell wall is that it is made up of relatively high amounts of the H type of lignin compared to dicots that contain trace amounts of it (Barrierre et al. 2007; Dixon et al. 2001). Another unique feature of grasses is that they contain relatively high amounts of p-hydroxycinnamic acids, particularly pCA and FA (ferulic acid). Earlier studies have shown that these acids play an important functional role in the incorporation of lignin into the cell wall (Grabber et al. 2004). Due to the impact of the amounts of lignin on the initial steps of lignin polymerization, it has been hypothesized that FA might play a role in determining the sites of nucleation for lignification. Phylogenetic analysis done by Xu et al. (2009) suggests that expansion of the lignin biosynthetic gene families occurred after the speciation of mono- and dicotyledons which explains so many differences between monocot and dicot cell wall.

The lignin biosynthetic pathway essentially consists of ten steps (Fig. 14.1). A detailed description of the phenotypes of the mutant and transgenic plants that are affected in the lignin pathway is described in the review by Harrington et al. (2012). In general, mutation in the lignin biosynthetic genes results in reduced lignin content along with a change in the ratio of the H, G, and S content that ultimately results in a higher enzymatic digestibility of these mutants compared to wild type. If the mutation results in a visible phenotype, it resembles the brown midrib mutant phenotype. The mutations so far described have been identified in the 4CL, CCR, COMT, and CAD genes in the lignin biosynthetic pathway. Future research needs to be done to characterize the role of C4H, HCT, CCoAOMT, and F5H in terms of plant growth and lignin content.

14.5 Lignin Regulation

Natural variation for cell degradability appears to be at the regulatory mechanisms rather than the biosynthetic pathway based on the co-localization of QTLs involved with the lignin or cell wall degradability and genes present at those physical locations (Harrington et al. 2012).

Plant cell walls are highly complex and dynamic in nature. The composition of cell wall not only differs among different cell types but also varies in different microdomains of the same cell. This is achieved in part by the regulatory mechanisms controlling biosynthesis, targeted secretion, and assembly of wall components that provide such heterogeneity within and among the cell types. A number of factors such as hormones, cytoskeletal components, glycosylphosphatidylinositol-anchored proteins, phosphoinositides, sugar nucleotide supply, and coordination of wall biosynthesis are implicated in the process of cell wall biosynthesis and deposition (Zhong and Ye 2007).

There are specific transcription factors that regulate the secondary wall biosynthesis in each cell type, and the cell wall patterns appear to be initiated by the microtubule organization in those cell types. However, some NAC transcription factors might play a role in secondary wall deposition patterns although those mechanisms are not so clear yet. Transcription factors in the NAC and MYB family are the key master regulators of the secondary wall biosynthesis. These master regulators are active in different cell types, but their downstream targets appear to be the same set of genes involved in the biosynthesis of secondary wall components such as cellulose, lignin, and xylan (Yang et al. 2013). For instance, the differentiation of the vessel and fiber cell starts independently of each other, and the fiber secondary cell wall is under the control of NST1 and NST3/SND1 master regulator, whereas the vessel is under the control of VND6 and VND7 but thereafter shares the same regulatory network for lignin, cellulose, and xylan biosynthesis (Wang and Dixon 2012). These secondary wall-related NAC transcription factors (SWNs)

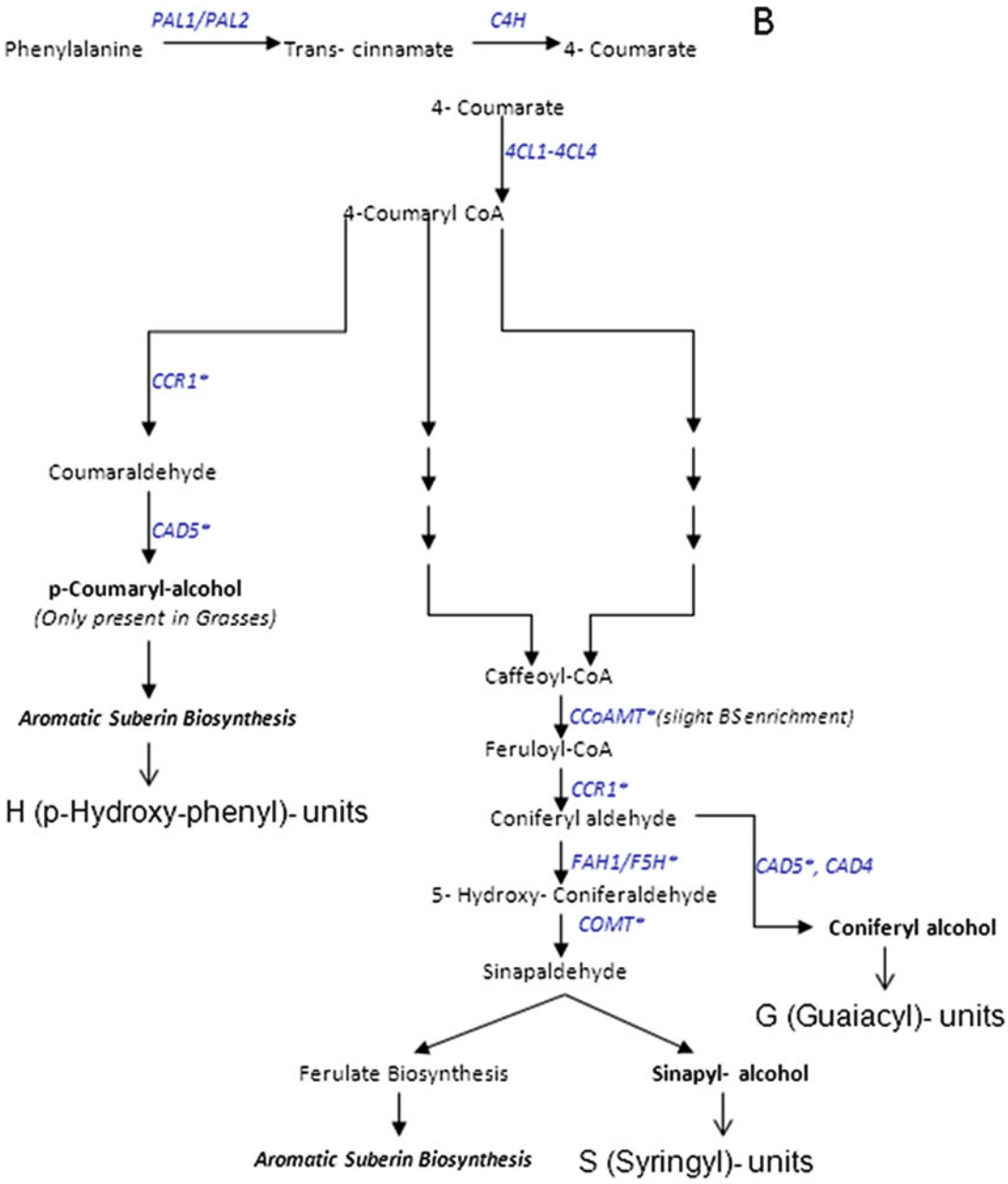

Fig. 14.1 Biosynthetic pathway of Lignin

function as the first layer of master regulators, and the MYBs act as the second layer of master switches in secondary wall formation (Fig. 14.2). The SWNs are under the control of both positive and negative feedback regulation. It was shown in pith cells that AtWRKY tx factor functions as a negative regulator of SWNs (NST2) to maintain parenchymatous identity of these cells (Wang et al. 2010).

The biosynthetic pathway genes for formation of the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monolignol building blocks contain AC cis-elements in their promoter regions. Both the positive and negative MYB transcription

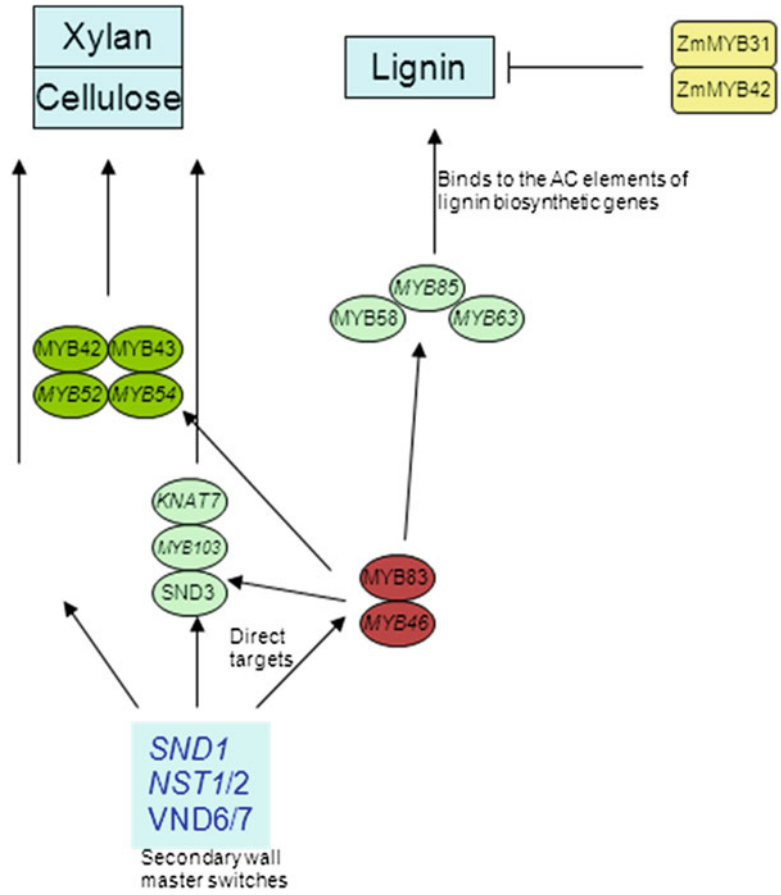

Fig. 14.2 Regulation of secondary cell wall biosynthesis

factors have been reported to bind to the AC cis-element and regulate the lignin biosynthesis. It is still unclear if they bind to the same site and how lignin synthesis is coordinated. The AC element has been proposed to coordinate G lignin biosynthesis (Zhao and Dixon 2011). However, genes specific for the synthesis of S lignin such as F5H are directly activated by NST1 (Zhao et al. 2010). Further work is required to shed some light into the details of how these regulators control lignin composition and content.

14.6 Areas for Improvement

It has been suggested that lignin deposition is a dynamic and adaptive process (Vincent et al. 2005). Lignin deposition and biosynthesis vary in terms of cell type, stage of development, tissue type, specificity, and plant species. Interfering with one of the steps in the lignin biosynthetic pathway at the whole plant level could have several detrimental effects such as loss of mechanical strength, reduced vessel integrity leading to reduced water and nutrient transport, and reduced/altered accumulation of the different subunits in the cell wall that could adversely affect the plant's response to abiotic stresses due to changes in the composition of the stress/defense lignins. Hence, a targeted approach is needed. A recent work by Yang et al. (2013) shows such an example where they document the use of cell-specific promoters fused to secondary cell wall deposition genes. Directing the synthesis of lignin just to the vessels and creating an artificial feedback loop to enhance the expression

of the NST1 (master regulator of secondary cell wall biosynthesis in fiber cells) gene in the fiber cells to generate enhanced polysaccharide deposition in its cell wall without lignin resulted in heathier plants with increased amounts of sugar that released easily from cell walls of these plants.

14.7 Effects of Modification

Lignin is an important part of the cell wall polymer that is required not just to provide mechanical strength but also to act as a physical barrier for protection from the invasion of pathogens. It is also important for growth and development. It is important that deep loss of lignin will render the plant unhealthy and affect its ability to grow and develop and to tolerate any kind of abiotic/biotic stress. However, moderate changes in the lignin content and changes in the composition have improved the release of cellulose from the cell wall after pretreatment. Here, we look at the effect of altered lignin mutants in terms of their ability to fight diseases.

14.8 Resistance to Diseases

Lignin synthesis has long been correlated with the plant's defense mechanism against pathogens especially since lignin is synthesized at sites of infection. Lignin acts as a physical barrier to invasion and growth of the pathogens. Could modifying the lignin structure have an effect on the plant's resistance to diseases? The phenylpropanoid pathway that synthesizes monolignols is further involved in the synthesis of other phenolic compounds such as phenolic phytoalexins, stilbenes, coumarins, and flavonoids. Some of these have roles in plant defense. An important plant defense hormone salicylic acid is derived from this phenylpropanoid pathway in some plants. Certain abiotic stresses also induce the expression and activity of the enzymes involved in this process. Altering or reducing the lignin biosynthesis could therefore have serious outcomes in the ability of the plant to withstand stress. However, some of the published reports suggest that lignin modification may not lessen the disease resistance to pathogens.

Most of the published work on plants with modified lignin via interfering with the lignin biosynthetic genes and plant-pathogen interactions is done on plants that are not bioenergy feedstock such as Arabidopsis and tobacco. This work suggests that knocking down the function of PAL, the first committed step to monolignol biosynthesis results in reduced susceptibility of tobacco plants to *Cercospora* spp. the causal agent of frogeye disease. Overexpressing PAL in tobacco resulted in an increase in the SA and other defense-related compound chlorogenic acid. Increased SA levels provided the plants resistance to the *Cercospora nicotianae,* but the resistance to TMV was unchanged. These plants however showed increased susceptibility to the insect *Heliothis virescens*. Similarly T-DNA insertion in all four PAL genes resulted in increased susceptibility to the bacterial pathogen *Pseudomonas syringae* (Huang et al. 2010). Since PAL is involved in the synthesis of a whole range of phenolic compounds, the changes in the resistance to pathogens cannot be directly attributed to changes in lignin.

Interestingly, however, reducing the expression of the HCT gene in Arabidopsis and alfalfa via antisense/RNAi results in the activation of the defense responses. In both of these genera, antisense/RNAi suppression of the HCT gene resulted in reduced growth and lignin content. It was noteworthy that even in the absence of the disease, the HCT-knockdown plants had elevated SA levels compared to the WT plants (Gallego-Giraldo et al. 2011a, b) in alfalfa. Reducing the expression of a gene SID2 (SA induction deficient 2–2) that is part of the isochorismate pathway leading to the synthesis of SA helped recover some of the growth phenotype defects. It is however unclear if the increased levels of SA can be attributed to re-funneling of some of the compounds of the phenylpropanoid pathway. There was a significant accumulation of pectin-related compounds in the cell wall of these plants which could provide additional defense to these plants.

Similar results of either increase in pathogen resistance or no change were obtained when

COMT expression was knocked down via antisense/RNAi suppression in Arabidopsis and tobacco (Sattler and Funnell-Harris 2013; Quentin et al. 2009). However, suppressing the CAD gene function resulted in increasing the susceptibility of the plants to a range of pathogens (Sattler and Funnell-Harris 2013). It is important to note that CAD suppression is an important target of the bioenergy feedstock to reduce lignin content. Similar results of increased susceptibility were reported for mutation in the gene F5H in monolignol biosynthetic pathway in Arabidopsis (Huang et al. 2009).

Although the work done on the plant-pathogen interaction in the modified bioenergy feedstocks is minimal, more light is shed from the brown midrib mutants (bmr) of maize and sorghum that have long been shown to contain reduced lignin contents. There are 5 bmr loci in maize and 7 in sorghum (Sattler and Funnell-Harris 2013).

bmr loci of maize and sorghum

	Homologous gene
Sorghum	
bmr2	4CL
bmr6	CAD
bmr12	COMT
Maize	
bmr3	COMT
bmr1	CAD

Interestingly, studies done under field conditions on the bmr mutants have revealed that mutations in the phenylpropanoid/lignin biosynthetic pathway either provide these plants with increased resistance to pathogens or cause no change in resistance (Sattler and Funnell-Harris 2013). The lesion lengths in bmr6, 12 were considerably smaller or same when inoculated with *Fusarium thapsinum* compared to their wild-type relatives and across different genetic backgrounds (Sattler and Funnell-Harris 2013; Funnell and Pedersen 2006; Funnell-Harris et al. 2010). However, the fungal growth was greater in the bmr12 plants in healthy-appearing tissues outside the necrotic discolored tissue that is defined as the lesion. Inoculations with another fungal stock pathogen *Macrophomina phaseolina* that causes charcoal rot showed that brown midrib mutants were not more susceptible to this pathogen. However, these studies relied on artificially inoculating the fungi and do not take into consideration the stalk strength that may affect the rind penetration resistance. In general mutations in the bmr genes resulted in affecting at least three different steps, i.e., 4CL, COMT, and CAD, and all of them seemed to not make these plants any more susceptible to stalk rot pathogens but may even cause an increased generalized resistance to pathogens. There could be several reasons for this: (1) There is evidence that they are hampered in their ability to synthesize structural lignins, but research needs to be done to evaluate changes in the synthesis of "defense lignin/stress lignin" in response to pathogen attack. (2) Blocking a step in the monolignol biosynthesis would result in the increase in the accumulation of the precursors that could be directed to the synthesis of other compounds that would have roles in defense response. (3) Perturbations in the synthesis of lignin a component of the cell wall might result in a generalized cell wall response that might provide additional defense to the plant.

In general reducing lignin content and altering its composition does not seem to have a tremendous change in the susceptibility of these bioenergy feedstocks to pathogens, but a case-by-case approach including field trials would need to be evaluated to determine pathogen susceptibility.

14.9 Future Directions/Emerging Technologies

Genetic modification of plants to alter lignin content can improve lignin degradation. A list of newly discovered lignin monomers has shown that lignin is able to readily copolymerize alternative units that are produced by incomplete synthesis of monolignols. An example of this has been shown in a biomimetic system by polymerizing coniferyl ferulate together with normal monolignols into the primary cell walls of maize (Grabber et al. 2008). The modified lignin incorporates easily breakable ester bonds within its backbone and

hence is easily degraded at lower temperature and under alkaline conditions. This is an area that also requires a huge study at the systems level to identify the consequences of such change on the regulation of the pathway itself and a whole range of related pathways that may alter the ability of the plant to respond to stress and pathogens. It is eminent that such studies be evaluated in the field trials where the plants are exposed to various conditions and stresses caused by environment and pests that are sometimes not possible to mimic under laboratory or greenhouse conditions. Besides the clear benefits of biotechnology in the generation of such GM plants, it is important to harness a whole host of natural mutants that may have modified cell walls that easily release the sugars from the plant cell wall.

The US Department of Energy has several recommendations to emerging alternative fuels, and those relevant to the context will be discussed further. Drop-in biofuels that are under research and development phase are substitutes for existing diesel, gasoline, and jet fuel which typically fuel vehicles that are not good candidates for electrification. These drop-in biofuels are expected to drop in directly into the existing infrastructure without any compatibility issues which are a barrier to ethanol and biodiesel.

There is more than one way to produce such fuel, and some of the potential technological pathways include upgrading alcohols to hydrocarbons by converting sugars to hydrocarbons either catalytically or via fermentation. Another would be to process biomass into bio-oil via pyrolysis or liquefaction.

There are some clear benefits of these drop-in fuels especially since they are expected to be substantially similar to their petroleum counterparts and hence do not require major modification to the existing infrastructure. They contribute to fewer greenhouse gas emissions and offer greater flexibility by allowing for replacement of diesel, jet fuel, and gasoline for products from various feedstock and production technologies.

There is a need to generate plants that are optimized for the production of biofuel via genetically modifying their cell walls without compromising their biomass or toleration to diseases and stress.

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