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CRISPR-Mediated Engineering of Lignin Biosynthesis to Reduce Plant Biomass Recalcitrance: Advances, Trade-offs, and Future Directions

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ABSTRACT

Growing demands on fossil fuels and population growth have increased the need for sustainable and renewable energy sources on a worldwide scale. Lignocellulosic biomass can be used as a feedstock to make biofuels. However, a variety of challenges, such as low yields and expensive treatment costs, prevent biomass commercialization due to its recalcitrant nature. One of the primary sources of this resistance is lignin, a substantial component of the cell wall. The ability to precisely alter the genes involved in lignin formation has been made possible by recent advancements in CRISPR/Cas-based genome editing, opening up new possibilities to improve biomass quality without sacrificing plant growth. This paper discusses current developments in CRISPR-based lignin engineering, targetable lignin biosynthesis genes, and associated agronomic and phenotypic results. Furthermore, it highlights critical challenges, including the need for precise regulation, integration of multi-omics techniques, long-term field evaluation, and balancing biomass processability with plant health for sustainable bioenergy production.

INTRODUCTION

Energy is one of the driving forces behind economic expansion. However, the extensive use of fossil fuels led to several negative environmental issues, including global warming, air pollution, and depletion of non-renewable resources [1]. These challenges have accelerated the demand for renewable and sustainable energy sources, with biofuels gaining attention. Biofuels are produced from organic materials such as lignocellulosic crops, agricultural leftovers, and other biomass sources. Microbial fermentation is typically used to convert biomass into fermentable sugars [2]. Second-generation biofuels, derived from lignocellulosic biomass, corn, wheat straw, and sugarcane residues, offer a promising alternative to fossil fuels [3]. Lignocellulosic biomass is composed of three major components, cellulose (40-60 wt%), hemicellulose (20-40 wt%), and lignin (10-25 wt%), and represents an abundant and renewable resource for biofuel production [4]. However, biofuel production from lignocellulosic biomass is hindered by the recalcitrant nature of lignin [5]. Lignin restricts biomass digestibility by reducing cell wall accessibility and inhibiting enzymatic hydrolysis [6]. Genetic manipulation of lignin production has emerged as a promising tool for enhancing biomass conversion efficiency. Enzymatic digestibility can be improved by downregulating lignin-related genes, which can lead to unfavourable impacts on plant growth and

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development [7]. Recent breakthroughs in genome editing technologies, particularly CRISPR/Cas9, have made it possible to target lignin production genes precisely to change lignin content and composition in a variety of crops, including barley, switchgrass [8, 9].

Here, several research gaps are filled to assist in striking a balance between biomass quality and plant health, enabling the more economical and efficient generation of sustainable biofuel from lignocellulosic biomass. This study aims to investigate the agronomic and phenotypic effects of lignin alteration and outlines important lignin biosynthesis genes that CRISPR/Cas systems target. It also discusses biosafety laws about altered plants, research gaps, and present difficulties.

Lignin Biosynthesis and Its Role in Biomass Recalcitrance

Cellulose, hemicellulose, and lignin are the three main constituents of lignocellulosic biomass [10]. In the lignin and hemicellulose matrix, cellulose creates beta 1-4 glycosidic bonds between glucose molecules to produce microfibrils [11]. Plants are given a strong structural framework by lignin, a heteropolymer comprising p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monolignol that encircles cellulose microfibrils in a complicated branching network [5]. As a plant ages, its lignin content rises. Only a few S units are seen in the early phases of lignin deposition, whereas the integration of H and G units begins at lignification. Coniferyl alcohol and sinapyl alcohol are then added to create a mixture of G and S units during secondary wall development [12]. The lignin mass percentage ranges from 9 to 30% for the majority of woody feedstock species, while it is believed to be lower for Agave, ranging from 5 to 16% depending on the species [13]. Lignin is resistant to hydrolysis; therefore, lowering the bulk fraction of lignin or changing its structure are crucial objectives for overcoming the recalcitrance and enhancing saccharification.

Target Genes in the Lignin Biosynthetic Pathway

The phenylpropanoid pathway is a complicated process that involves many enzymes for monolignol production and lignin biosynthesis. These enzymes can be targeted to reduce the lignin content in plants. The CRISPR-Cas tool can be used to mutate the genes that are involved in their synthesis.

Upstream Genes (PAL, C4H, 4CL)

Phenylalanine ammonia-lyase (PAL) catalyses the first step of the phenylpropanoid pathway by changing phenylalanine into trans-cinnamic acid. It is coded by multiple genes having different isoforms, abundant in wood-forming tissues, and localized in the endoplasmic reticulum [14]. Cinnamate 4-hydroxylase (C4H) is a cytochrome P450 monooxygenase of the CYP73A family. It catalyses the hydroxylation of cinnamic acid to form p-coumaric acid in the lignin synthesis pathway. It has two primary isoforms: Class I is involved in lignin production and expressed in the endoplasmic reticulum; Class II is involved in stress responses and has tissue-specific expression [15]. 4-Hydroxycinnamoyl-CoA (4CL) initiates the synthesis of hydroxycinnamoyl-CoA esters. In Eucalyptus, many 4CL genes form clusters and are involved in lignin synthesis with class I isoforms and localized in the endoplasmic reticulum. Several phenylpropanoid-derived pathways involve class II and 4CL-like proteins [16].

Midstream Genes (HCT, C3H)

Shikimate hydroxycinnamoyl transferase (HCT) converts hydroxycinnamoyl groups into shikimate or quinate, creating the intermediates needed for additional hydroxylation processes. Several HCT gene clusters, with distinct isoforms, are expressed in specific lignifying tissues [17]. *p-Coumarate* 3-hydroxylase (C3H) is a CYP98A cytochrome P450 enzyme that hydroxylates p-coumaroyl shikimate or quinate esters to form caffeoyl derivatives. Many C3H isoforms are known to exist and are expressed in the ER. Pathway regulation and lignin composition may be influenced by functional heterogeneity among isoforms [18].

Downstream Genes (CCoAOMT, CCR, F5H, COMT, CAD)

Caffeoyl-CoA O-methyltransferase (CCoAOMT) is a crucial precursor of lignin monomers. Feruloyl-CoA is created when CCoAOMT catalyses the methylation of caffeoyl-CoA. Two main CCoAOMT isoforms are found in the cytoplasm and are significantly expressed in lignifying tissues [19]. Cinnamoyl-CoA reductase (CCR) catalyses the reduction of hydroxycinnamoyl-CoA esters to aldehydes. Only some isoforms are functionally linked to lignin production [20]. Ferulate 5-hydroxylase (F5H) is a cytochrome P450 enzyme that belongs to the CYP84 family; it aids in the production of syringyl lignin by facilitating the hydroxylation of ferulate derivatives. The most highly expressed F5H isoforms are found in the ER and play a key role in lignin production [21]. Caffeic acid O-Methyltransferase (COMT) catalyses methylation processes, converting hydroxylated intermediates into precursors of sinapyl alcohol. COMT is one of the most prominent lignin biosynthetic enzymes in Eucalyptus, indicating that it plays an important role in lignin production. There are several COMT-like genes, but only a few are important for lignification [22]. Cinnamyl Alcohol Dehydrogenase (CAD) catalyses the last stage of monolignol biosynthesis, which transforms cinnamyl aldehydes into their corresponding alcohols. Only some part of the many CAD isoforms is closely linked to the formation of lignin [23]. In addition to biosynthesis, chitinases, laccases, and dirigent proteins influence lignin polymerization and structure [24]. Laccases are essential enzymes that catalyse the oxidative polymerisation of monolignols into lignin without the need for H_2O_2 [25]. Dirigent-like proteins are involved in regulating the

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structure of lignin and aid in the stereoselective binding of monolignols [26]. Although their functional roles are still unclear, these targets provide an alternate approach by altering the architecture of lignin rather than its quantity. The Phenylpropanoid pathway of lignin biosynthesis was analyzed [10] (Figure 1).

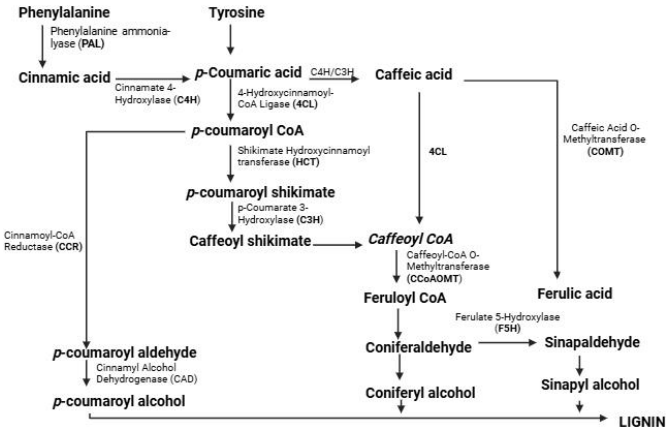

Figure 1: The Phenylpropanoid Pathway of Lignin Biosynthesis

Comparative Analysis of Target Genes

PAL, C4H, 4CL control carbon flux into the pathway. Although editing these genes typically results in significant lignin reduction, the disruption of vital metabolic processes frequently causes severe growth abnormalities [15, 16]. The production and distribution of lignin precursors is regulated by midstream genes (HCT, C3H). Studies have shown that mutations in these enzymes can significantly reduce the concentration of lignin, but the outcomes have been inconsistent, ranging from enhanced saccharification to severe dwarfism. This variation is a reflection of route flexibility and metabolic compensation [17, 18]. More intriguing targets are downstream genes (CCoAOMT, CCR, F5H, COMT, and CAD). Editing these genes frequently modifies the lignin's composition rather than its total concentration, particularly the S/G ratio, which has an impact on biomass digestion. For example, F5H and COMT alteration increases syringyl lignin, enhancing processing efficiency without a large growth penalty [21, 22]. CAD downregulation, which increases digestibility without appreciably reducing lignin concentration, further highlights the importance of compositional engineering over bulk reduction [23]. No single gene target provides an optimal solution. Upstream targets maximize lignin reduction, whereas downstream targets offer a better balance between biomass quality and plant performance. Therefore, combinatorial and fine-tuned editing strategies are more effective than single-gene knockouts.

CRISPR Technologies Applied to the Lignin Pathway

CRISPR/Cas9 is a ground-breaking genome editing tool to modify the genome by introducing double breaks in the DNA, directed by sgRNA. The Cas9 enzyme is responsible for locating and attaching itself to target DNA sequences. After binding, Cas9 marks the site of DNA cleavage by generating a protospacer adjacent motif (PAM). The combination of two RNA molecules, trans-activating crRNA (tracrRNA) and crRNA, known as single-guide RNA (sgRNA), facilitates the assembly of Cas9 in this process [27]. A single DNA construct knocks out the expression of an endogenous gene involved in lignin monomer biosynthesis while simultaneously expressing an altered version of the gene's open reading frame that is not cut by the Cas9 system, complementing the introduced mutation in the lignin biosynthesis pathway. CRISPR/Cas9 has several advantages over traditional genetic engineering. Its precision allows specific targeting and reduces unintentional off-target effects and genetic manipulations; efficiency produces first-generation homozygous mutants [28]. It targets multiple genes at a time and modifies complex traits with minimal growth penalty in lignin-reduced mutants [29]. Research has been done on different plants like barley, switchgrass, poplar, etc., to reduce their lignin content using CRISPR/Cas9.

Quantitative Comparison of Lignin Engineered Plants

Multiplex CRISPR editing in poplar achieved significant lignin reduction (up to 51.7%), surpassing single-gene edits like CSE (~29%). While barley COMT1 editing resulted in 14% lignin decrease but a 34% increase in glucose recovery, 4CL1 mutants in switchgrass demonstrated up to 30% lignin reduction with the biggest improvements in sugar release. Multi-gene targeting (e.g., CCR and CAD) in poplar reduced lignin by 20–40% and increased ethanol yield by 30–50%, outperforming single-gene approaches, although it required extensive haploid screening [30, 31]. Similarly, compared to woody species, maize PAL knockout resulted in a 15–25% decrease in lignin and a ~40% increase in glucose release, along with faster regeneration. Studies showed defense-related lignification is improved by regulatory gene editing (e.g., WRKY16), but growth is severely limited. CRISPR-based strategies show improved efficiency with lower penalties (~0–5%) and 1.2–4-fold increases in saccharification when compared to traditional RNAi approaches, which reduce lignin by ~20–30% but suffer 10–20% growth penalties [32-34]. These results demonstrate that while increased lignin reduction is associated with increased biofuel yield, the best results require striking a balance between biomass digestibility, plant growth, and structural integrity.

Research Trends in Global Lignin Engineering

Global trends highlight CRISPR for bioenergy crops, with market growth from 415 M (2025) to 600 M (2034), with Cas9 (65% share) shifting to base/prime editors (8.2% CAGR). The US focuses on therapeutic crossover, Europe on poplar for forestry, and Asia-Pacific on plant applications (such as barley and switchgrass). AI-optimized sgRNAs, RNP delivery for perennials, and multiplex edits aimed at

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phenylpropanoid redundancy, projected 22% CAGR to $6.92 B by 2030, are among the trends. Low-lignin grasses are given priority in CRISPR trials that target biofuels [35] (Table 1).

Table 1: Summary of CRISPR/Cas-Engineering for Lignin Reduction in Plants

Study	Plant Species	Key Outcomes	Lignin Reduction	Saccharification/Glucose Release Improvement	Growth Penalty	References
CSE Knockouts	Hybrid Poplar	Mutants in a single or both genes; no morphological changes	29.1%	+25%	None	[30]
PDS Activation	Poplar	51.7% efficiency; 30/59 homozygous mutants	N/A (control study)	N/A	Not reported	[28]
HvCOMT1 Knockout	Barley	Improved biofuel yield; no morphological changes	14%	Glucose recovery: +34%; Bioethanol:14.3 g/L (+34% vs. WT); Sugar yield:0.46 g/g (+12% vs. WT)	None	[31]
CCR1 Knockout + Complementation	Poplar/ Arabidopsis	T1 vessel-specific rescue; T2 homozygous individuals show a strong negative phenotype without construct	Variable (Strong allele control)	4x sugar/plant vs. 2x mutants	Strong growth penalty without complementation (dwarfism-like); rescued to normal	[32]
WRKY16 Knockout	Tomato/ Arabidopsis (vs. *parasitic plants*)	Constant lignin accumulation for defense	Increased (sustained)	N/A (defense focus)	Compromised vegetative growth (trade-off)	[33]
RNAi Lignin	Sugarcane	Field-tested suppression	20–30%	Improved	Dwarfism; lodging; pest vulnerability	[34]

Advancement in Engineering Tools

In addition to CRISPR/Cas9, which uses sgRNA-guided Cas9 and PAM recognition to create double-strand breaks, more recent versions address precision constraints for lignin engineering. Base editors, such as adenine base editors (ABE) and cytosine base editors (CBE), allow single-nucleotide changes without breaks, changing C-G to T-A or A-T to G-C, covering about 60% of disease variants and lowering off-target risks, which is perfect for fine-tuning lignin genes like 4CL or COMT without indels. With recent proPE variants increasing efficiency 6.2-fold and accuracy to 1/101 error rate, prime editing installs any of 12 base changes, small insertions/deletions, and is appropriate for precise syringyl unit modifications in polyploid crops. While RNP complexes and lipid nanoparticles enhance delivery in resistant plants, CRISPR/Cas12a (Cpf1) provides alternative PAM sites and staggered cuts for multiplexing. These hold promise for lignin pathways, though plant applications lag behind human therapeutics [36].

Biological and Technical Limitations of CRISPR-Mediated Lignin Engineering

CRISPR/Cas has biological and technological limits despite its accuracy and effectiveness. Optimizing genome editing techniques and converting CRISPR-mediated lignin engineering into agronomically viable crops requires an understanding of these limitations.

Gene Redundancy and Metabolic Compensation

Gene redundancy and a plant's capacity to adjust physiologically after gene disruption are two of the most important biological difficulties. Numerous enzymes are involved in the phenylpropanoid pathway, which produces lignin. Alternative gene isoforms can restore the function of a knocked-out gene in a single gene knockout. Functional redundancy was shown in a study on hybrid poplar, where some mutant lines showed considerable lignin reduction while others showed modest phenotypic effects despite gene editing. The predictability of lignin engineering is limited by the densely linked metabolic networks of plants, which have the potential to reprogram biosynthesis [37].

Off-Target Effects in Complex Plant Genomes

Off-target mutations in large polyploid genomes by CRISPR are still a significant concern. Even though the CRISPR system is very precise, imperfect base pairing guided by sgRNA can cause unintended cleavage at homologous genomic sites. CRISPR/Cas systems can introduce unintended mutations at off-target sites, affecting gene function [38].

Mosaicism and Chimerism in Regenerated Plants

Mosaic plants contain a mixture of edited and unedited cells, which results in an incomplete phenotype, because CRISPR editing continues after the initial transformation event, leading to independent editing events in different cell lineages during plant development, and results in plants with fully edited cells, partially edited cells, and Unedited cells. This leads to unequal lignin distribution affecting vascular function and mechanical strength [39].

Species-Specific Challenges in Recalcitrant Crops

CRISPR-mediated lignin engineering faces species-specific barriers, especially in woody and recalcitrant crops. Polypoid species have multiple homologous copies of lignin biosynthesis genes, required to edit multiple

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alleles to get effective results. Precise spatial and temporal editing is required to prevent growth abnormalities because lignin deposition is strictly controlled during vascular differentiation [40]. Addressing these limitations is essential for the successful translation of CRISPR-mediated lignin engineering into sustainable bioenergy crops, improved forage species, and industrial biomass feedstocks.

Phenotypic and Agronomic Consequences of CRISPR-Based Lignin Modification

CRISPR/Cas editing reduces total lignin content and changes monomer ratios to increase cell wall porosity and digestibility. CRISPR/Cas tool targeting multiple genes produced mutant poplar plants with 50% reduced lignin content and up to ~28% increase in carbohydrate-to-lignin ratios, enhancing enzyme accessibility [31]. Change in lignin content, composition, or deposition can cause morphological changes in plants. Classic lignin modification leads to dwarfed stature, collapsed xylem, impaired water transport, abnormal leaf structure, or reproductive abnormalities, due to disruption in lignin's structural role [41]. CRISPR/Cas was used to mutate the SoLIM transcription factor in sugarcane. The mutant lines showed 9.7%–51.5% reduction in total lignin content, altered S/G monomer ratio, histochemical and microscopy analysis confirmed a thinner cell wall and a normal phenotype. Lower lignin sugarcane could reduce costly pretreatments in biofuel production and improve process economics, without obvious productivity loss [42].

Growth-Defense Trade-Offs

In plants, limited metabolic resources like carbon, nitrogen, and energy cause growth-defense trade-offs to maintain fitness, protective metabolism, and biomass accumulation [43]. The defence-growth barrier is compromised when lignin production is disrupted, leading to energetically induced defence responses that divert resources from growth. By returning defence gene expression and resource allocation to normal, suppressor mutations, including those affecting Mediator components, can partially restore growth. Although suppressor mutations can temporarily restore growth and defense gene expression, lignin-deficient plants frequently exhibit decreased stature due to the ongoing metabolic expenditures of induced defense responses [44]. As metabolic fluxes are complicated, quantitative knowledge of growth-defense trade-offs in lignin-modified plants is limited. Quantitative proteomics and stable isotope labelling in conjunction with LC-MS can help to clarify resource dynamics. It will be crucial to comprehend and adjust these trade-offs if lignin-engineered plants are to be successfully implemented in their natural environments [45]. It is possible to anticipate the best resource allocation for lignin-engineered crops under natural environmental settings by incorporating these observations into constraint-based metabolic models [46].

Long-Term Field Trials

The majority of research on crops modified with lignin is carried out in greenhouses or controlled environments, which do not accurately reflect environmental variability. To assess agronomic stability, stress tolerance, pathogen susceptibility, and biomass yield across seasons, long-term field studies are crucial. In the field evaluation of lignin-engineered poplar, it was observed that, despite the low lignin enhancing saccharification, various lines were more susceptible to environmental stress and exhibited altered growth performance over the years. These findings demonstrate that the enhancement in the lab digestibility might not necessarily translate into uniform field performance, and, thus, the need to conduct multi-location and multi-year assessments [47].

Limitations and Future Perspective

Despite tremendous progress, there are still a number of important research gaps, including growth penalties, vascular issues, and decreased stress tolerance. Combining multi-omics techniques is necessary to properly understand growth-defence trade-offs and metabolic compensatory mechanisms. Future research should prioritize precision editing techniques that allow for fine-tuning of lignin concentration and composition over severe reductions. The plants' resistance, which currently have lower lignin content (for example, agave has a lignin content of 5–16%), should be the focus of research. Another important gap is the insufficient field-based and long-term evaluation of lignin-engineered crops. Regulations governing genome-edited crops, including CRISPR-modified lignin variants, are always evolving on a global scale. Significant biosafety issues include off-target mutations, gene flow to wild relatives, and unforeseen ecological effects such as altered plant-microbe or plant-insect interactions. In terms of ecology, lignin loss may impair plants' natural defences and structural integrity, leaving them more susceptible to pests and illnesses that might disrupt ecosystem dynamics. Agronomic, ecological, and molecular data must be included in risk estimation frameworks before commercialization.

CONCLUSION

Lignocellulosic biomass is a significant renewable resource for biofuel production; however, lignin-induced recalcitrance is a significant limitation. CRISPR/Cas genome editing reduces lignin biosynthesis/accumulation to improve biomass processing across multiple crops, including poplar, sugarcane, Arabidopsis, and barley. Studies showed successful results with some challenges, including growth-defense trade-off and morphological

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penalties like dwarfism. Additionally, Large-scale commercialization is hindered by high production costs and a lack of field validation. Therefore, Precision editing techniques, improved knowledge of transcriptional and metabolic control, optimization of biomass quality, and plant fitness will be necessary for future advancements. Achieving this balance is necessary for the effective execution of lignin engineering for bioenergy applications.

Authors' Contribution

Conceptualization: HA
Methodology: HA
Formal analysis: HA
Writing and Drafting: HA
Review and Editing: HA

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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