GENETIC ENGINEERING OF LIGNIN BIOSYNTHESIS TO ENHANCE PLANT TRAITS: APPLICATIONS IN BIOENERGY, AGRICULTURE, AND INDUSTRY- A REVIEW

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Abstract

Lignin is the second most prevalent metabolite in plants after cellulose. It is a complex polymer that functions as a "backbone" for plants, providing mechanical support for stability and acting as a defense mechanism against pathogens and pests. The production of lignin involves the phenylpropanoid pathway, which generates several natural compounds that can be found in plants, including those in the lignin biosynthetic pathway. Throughout the decade, the production of lignin has become a major topic in enhancing plant traits due to its unique properties. Genetic engineering has paved the way for researchers to study lignin more effectively, offering potential benefits for the production of crops with improved traits. Tools such as CRISPR/Cas9 and RNA interference (RNAi) technology have made lignin studies more efficient, helping industries like agriculture and bioenergy become more sustainable. This review highlights genetic engineering approaches to lignin biosynthesis and their applications in the bioenergy and agricultural industries.

Keywords: CRISPR/Cas9, genetic engineering, lignin, MYB, phenylpropanoid pathway

Introduction

Lignin is a biopolymer composed of phenylpropanoid units derived mainly from cinnamyl alcohols known as monolignols. It is also one of the most common organic polymers in the world, second only to cellulose (Riseh et al., 2024). Lignin contains three types of monolignols: sinapyl alcohol (S unit), coniferyl alcohol (G unit), and *p*-coumaryl alcohol (H unit), each with a different type of aromatic ring structure as shown in **Figure 1**. These monolignols are connected through oxidative polymerization to form lignin structures with their composition and strength varying based on the type of plants (Vanholme et al., 2010). After polymerization, lignin, along with hemicellulose, is embedded within cellulose in the cell wall to form a structure widely known as the cell wall matrix, which provides mechanical

support for plants (Rao et al., 2023). This lignin-cellulose-hemicellulose complex plays a significant role in the transportation of water and nutrients, while also providing mechanical support and resistance against diseases in the plants (Li et al., 2022).

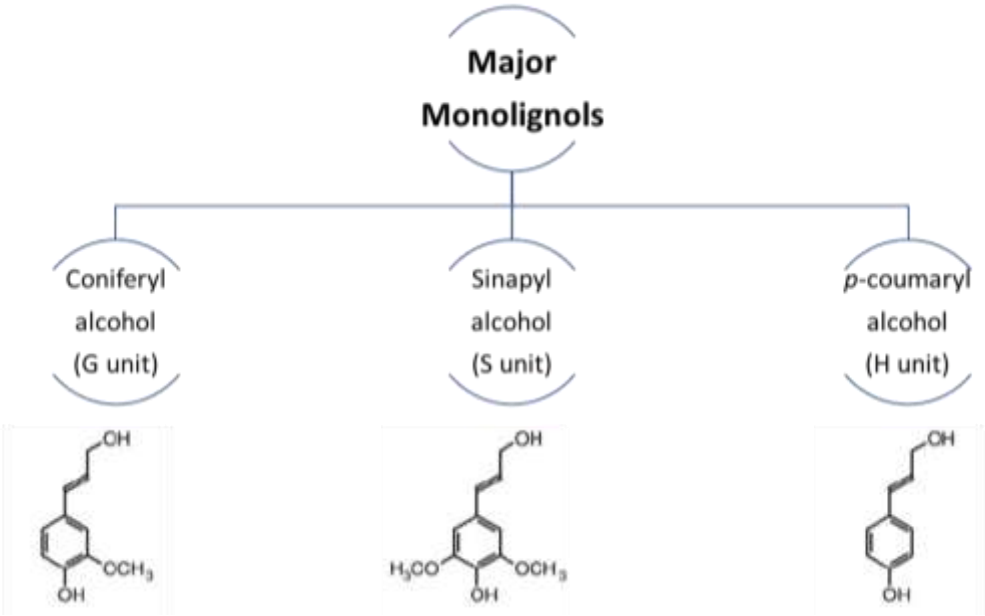

Figure 1 Structure of monolignols

Literature Review

Lignin Biosynthetic Pathway

In plants, the biosynthesis of metabolites is controlled by phenylpropanoid pathway, in which phenylalanine is converted into several intermediates that eventually become secondary metabolites. The lignin biosynthetic pathway that is illustrated in **Figure 2** involves several enzymes such as phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), cinnamyl alcohol dehydrogenase (CAD), and 4-coumarate: CoA ligase (4CL). These enzymes are responsible for the production of monolignols, involving processes like deamination, hydroxylation, methylation, and reduction. Understanding lignin formation within this pathway and the ability to regulate it has become an intriguing topic of study. Previous research has identified lignin biosynthetic genes in important crops, such as *PAL*, *4CL,* and *CAD* genes in oil palm (Yusuf et al., 2018a; 2022). The identification of these genes will facilitate investigations into their role in regulating lignin biosynthesis, potentially aiding in crop improvement (Yusuf et al., 2018b). Once monolignols are formed, the compounds are glycosylated to form lignin, which is then transported to the cell walls (Liu et al., 2018). Then, the glycosylated monolignols are transported through the cell membrane into the apoplast, where the glucose moiety is removed from the monolignols (Liu et al., 2018). Peroxidases and laccases catalyze the polymerization of the monolignol radicals, forming the lignin polymer before being integrated in the secondary cell walls of specialized plant cells such as xylem tracheids and sclerenchyma fibers. The polymerization process occurs within the plant cell walls, with lignin filling the spaces between cellulose, hemicellulose, and pectin components. By understanding the mechanism of lignin biosynthesis, genetic manipulation of lignin formation can lead to the development of plants with modified lignin content, benefiting several processing industries. For example, in the biofuel industry, reducing lignin content in plant biomass allows for greater biofuel production (Wang et al., 2022).

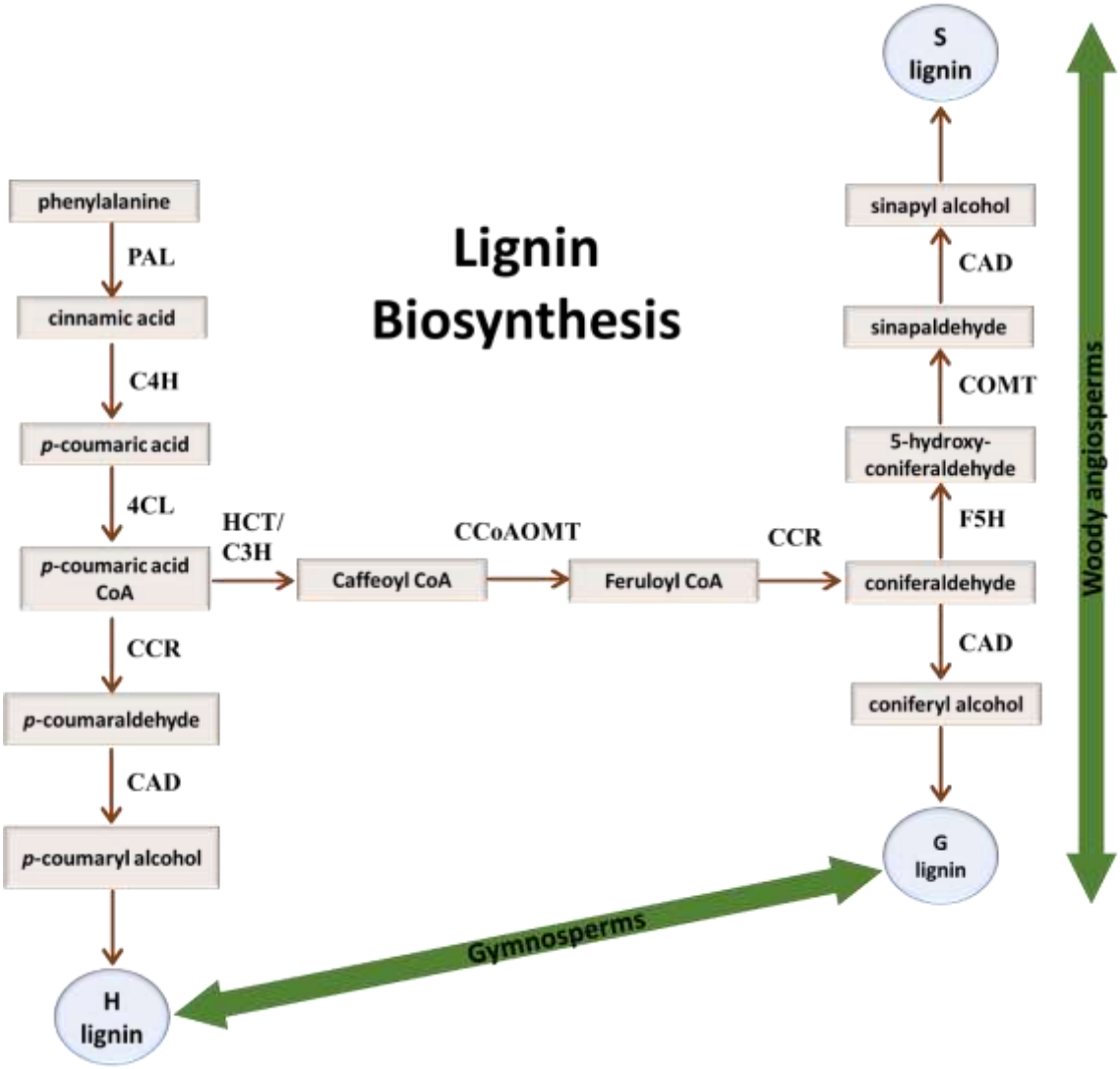

Figure 2 The General Lignin Biosynthetic Pathway. Phenylalanine Ammonia-Lyase (PAL), Cinnamate 4-Hydroxylase (C4H), 4-Coumarate: CoA Ligase (4CL), Cinnamoyl-CoA Reductase (CCR), Cinnamyl Alcohol Dehydrogenase (CAD), Hydroxycinnamoyl-CoA Shikimate (HCT), *p*-Coumarate 3-Hydroxylase (C3H), Caffeoyl-CoA *O*-Methyltransferase (CCoAOMT), Ferulate 5-Hydroxylase (F5H), Caffeic Acid *O*-Methyltransferase (COMT)

MYB Transcription Factor

Lignin biosynthesis in plants is regulated by proteins known as transcription factors. Researchers have pinpointed several essential transcription factors involved in this process, including OsTCP19 in rice, PbrMYB24 in pear, ZmMYB69 in maize, and CmHLB in chrysanthemum (Lv et al., 2024; Qiang et al., 2022; Xue et al., 2023; Zhao et al., 2022). These transcription factors are known to regulate genes associated with lignin biosynthesis, cellulose formation and mechanical strength in plant stems and fruits. Furthermore, the regulatory network governing lignin biosynthesis includes MYB and NAC transcription factors, which play a significant role in determining the timing and location of lignin deposition in response to developmental and environmental signals, particularly during stressful conditions (Choi et al., 2023).

MYB proteins are the most extensively studied group of transcription factors due to their diverse roles in the production of secondary metabolites such as flavonoid and anthocyanin (Xue et al., 2023). The first *MYB* gene identified in plants was the maize *C1* gene, which is involved in anthocyanin biosynthesis (Wu et al., 2022). Since then, numerous *MYB* genes have been identified and characterized across various plant species (Kranz et al., 2000). MYB proteins have two distinct regions: one is an N-terminal conserved MYB DNA-binding domain consisting of amino acid sequence containing 52 amino acids. The amino acids will create three α-helices, which form helix-turn-helix (HTH) structure for DNA recognition. The other region is a diverse C-terminal modulator region, which is responsible for regulatory activity. The MYB family is categorized based on the number of MYB domains at the N-terminal, such as R1-MYB (1R-MYB), R2R3-MYB (2R-MYB), R1R2R3-MYB (3R-MYB) and R0R1R2R3-MYB (4R-MYB).

The 1R-MYB group is involved in various metabolic activities, such as cellular morphogenesis, secondary metabolism, organ morphogenesis, phosphate starvation, chloroplast development, and circadian regulation in plants (Ambawat et al., 2013). The most abundant MYB type is the R2R3-MYB, where most lignin transcription factors in plants are categorized. Besides lignin, R2R3-MYB transcription factors are also involved in flavonoid and anthocyanin biosynthesis. The 3R-MYB type often produces proteins involved in various plant developmental processes, such as organ morphogenesis, chloroplast development, and responses to phosphate starvation (Feng et al., 2017). The 4R-MYB class is the smallest, and its functions remain relatively unknown. **Table 1** shows the MYB transcription factors that are regulating lignin biosynthesis in plants.

Table 1 MYB transcription factors that are involved in regulating lignin biosynthesis

Genes	Tissue level expression pattern	Bind to AC element	Overexpression phenotypes	Downregulation phenotypes	References
Snapdragon *AmMYB308* *AmMYB330*	N/D	N/D	Reduced lignin	N/D	(Tamagnone et al., 1998)
Arabidopsis *PAP1*	N/D	N/D	Increased lignin	N/D	(Borevitz et al., 2000)
Maize *ZmMYB31* *ZmMYB42*	Lignifying tissues	N/D	Reduced lignin	N/D	(Sonbol et al., 2009)
Pine *PtMYB4*	Secondary xylem	Yes	Ectopic lignin deposition	N/D	(Patzlaff et al., 2003)
Pine *PtMYB1* *PtMYB8*	Secondary xylem	N/D	Ectopic lignin deposition	N/D	(Bomal et al., 2008)

Eucalyptus *EgMYB2*	Secondary xylem	Yes	Increased wall thickening and lignin metabolism	N/D	(Goicoechea et al., 2005)
Arabidopsis *MYB58* *MYB63*	Lignifying tissues	Yes	Ectopic lignin deposition	Reduced wall thickening and lignin deposition	(Zhou et al., 2009)

N/D: not determined.

Manipulation of Lignin Content through Genetic Engineering

Genetic manipulation involves a deliberate alteration of the genetic characteristics of any organism to yield a preferred characteristic (Robert & Baylis, 2008). In relation to the amount of lignin studied within plant species, biotechnological processes are applicable to alter the number of genes that produce lignin (Riseh et al., 2024). This can be achieved through various techniques, where each technique has its working principles and applications.

One of the widely used tools for genetic manipulation is the genome-editing technology. Among the earliest examples of these technologies are Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) (Bhuyan et al., 2023). However, these tools are not as popular today due to the emergence of more advanced genome-editing techniques, such as CRISPR-Cas9. CRISPR-Cas9 is considered one of the most effective and accurate gene-editing methods in recent years. The technology works by targeting specific genes and causing double-strand breaks in the DNA (Zhang et al., 2024). These breaks are then repaired by the cell's natural DNA repair mechanisms, leading to mutations at the site of the desired insertion. Conversely, through such mutations, CRISPR-Cas9 can knock out or delete certain genes, thereby reducing their function (Li et al., 2023). This method is preferred because it is time-efficient, highly specific, and can be directed to almost any gene of interest. It can also introduce multiple gene mutations simultaneously. In the context of lignin, several studies have employed CRISPR/Cas9 to knock out lignin biosynthetic genes. For example, research conducted by Laksana et al. (2024) on sugarcane targeted SoLIM, a transcription factor that upregulated lignin formation, resulting in transgenic sugarcane with low lignin content and higher cellulose levels. Another study was performed by Jang et al. (2021) focusing on knocking out *CSE* gene in poplar, which resulted in low lignin content and an increase in biomass yield.

Another approach is the use of RNA interference (RNAi), where small RNA molecules are used to control gene expression (Nien et al., 2024). This is achieved by introducing double-stranded RNAs (dsRNAs) to silence genes from being expressed. dsRNAs are cleaved into small interfering RNAs (siRNAs), which bind to an enzyme to form an RNA-induced silencing complex, which aims at the target RNA to prevent gene expression (Muhammad et al., 2019). RNAi in plants is often associated with plant viruses, as RNAi can silence the genes of viruses that infect food crops (Koeppe et al., 2023). This method is particularly useful when a certain number of genes related to lignin biosynthesis need to be silenced. However, the use of RNAi in lignin manipulation is less popular compared with other approaches due to its high cost. Several studies on lignin have been performed using RNAi method, indicating that this approach could potentially be useful in future research (Daly et al., 2019; Jung et al., 2013).

Apart from gene knockout and RNA silencing, overexpression of the genes is another strategy that can be used to alter lignin levels. This can be achieved through transgenic techniques, where a copy of a gene is placed under a strong promoter and introduced into the plant genome, thereby enhancing the output of that gene (Carvalho et al., 2016). This method can be used to increase the expression of the genes that produce enzymes capable of either reducing lignin content or altering its composition in the plant, thus enabling the development of desired characteristics for specific industrial application (Liu et al., 2014). The transgenic approach typically utilizes *Agrobacterium tumefaciens*, a soil-borne pathogen that transfers foreign DNA into the plant genome. The transformation process can be performed using either tissue culture or agroinfiltration techniques.

The application of genetic engineering allows for precise tuning of lignin biosynthesis, enabling the development of plants with specific lignin content and composition for targeted applications. Using these advanced genetic engineering technologies, scientists can develop plants with beneficial characteristics for particular purposes, such as improved biomass yield for biofuel production, enhanced raw materials for manufacturing industries, and better feedstocks for the paper and pulp industries (Sticklen, 2006). This not only increases the market value of plant-based products but also improves the sustainability factor and environmental impact of using renewable plant resources. The aforementioned studies performed by Laksana et al. (2024) and Jang et al. (2021) on their respective plant species focused on reducing lignin content. Both studies demonstrated that low lignin content leads to the production of more cellulose, and subsequently produce more biofuel.

High lignin content in plant biomass increases processing difficulty and cost, therefore, reducing lignin can significantly enhance process efficiency (Kocaturk et al., 2023). This is often achieved by downregulating lignin biosynthetic enzymes during the early steps of lignin formation. RNAi and CRISPR-Cas9 can be used to suppress the genes encoding these enzymes, thereby reducing lignin content (Mujtaba et al., 2023). Additionally, these approaches can modify lignin composition in the plant cell wall. The ratio and structure of these monolignols influence the properties of lignin (Anderson et al., 2019). By altering the biosynthetic pathways to favor certain monolignols over others, scientists can produce lignin that is more suitable for industrial processing. For instance, increasing the S content relative to G makes lignin less cross-linked and easier to break down. This can be achieved by overexpressing genes such as *ferulate 5-hydroxylase* (*F5H*), which is involved in synthesizing S units (Balk et al., 2023).

In addition to modifying the natural monolignol pathways, incorporating novel monomers into the lignin structure is another promising approach (Smith et al., 2022). This involves introducing genes from other species that produce unusual monolignols or other compounds, creating lignin with unique properties. Such modifications can improve lignin digestibility for biofuel production or enhance its suitability for specific industrial applications (Ralph et al., 2019). While reducing and modifying lignin content are common goals, there are situations where increasing lignin content is beneficial. In food crops, higher lignin content can improve the structural integrity of the plant, making it more resistant to environmental stresses (Ahmad et al., 2023). For instance, a study by Xu et al. (2020) successfully created transgenic rice by incorporating *SiMYB56* from foxtail millet, resulting in rice with higher lignin content and improved drought resistance compared to its wild-type counterpart. Another study by Wu et al. (2019) showed that overexpression of ZmMYB3R, a transcription factor in maize, enhances the drought and salt tolerance in transgenic model plants.

Conclusion

In conclusion, genetic engineering of lignin content in plants can be used to alter its structure and attributes for various applications. With the help of improvements in molecular biological methodologies, new strategies can be developed to modify the chemical properties of lignin and allow its successful application in the intended sectors of biofuel, paper, and biorefinery industries. Even as the world inclines toward the use of sustainable and renewable resources, the capacity to control the characteristics of lignin will be central to the advent of a world that is not wholly dependent on fossil resources while fighting off environmental degradation. Lastly, further research is needed to refine the genetic engineering approaches as well as to assess the effectiveness of the various prospective effects on how industrial processes aim to develop a superior green economy.

Ethics Statement

The research does not require research ethics approval.

Authors Contribution

Chong Yu Lok Yusuf conceived and designed the study. Mohd Farhan Azhari and Mohamad Shafek Hilman prepared the manuscript. Noor Azmi Shaharuddin and Mat Yunus Abdul Masani meticulously reviewed and edited the manuscript. Meilina Ong-Abdullah provided critical feedback and proofread the final version of the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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