



INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Editing The F5H Gene In Sorghum Bicolor Using CRISPR/Cas Technology For Improved Abiotic Stress Tolerance (Drought)

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Abstract:

Sorghum bicolor, a vital drought- and heat-tolerant cereal crop, faces increasing challenges from abiotic stressors. Lignin, a complex phenolic polymer in plant cell walls, plays a crucial role in structural integrity, water transport, and defense mechanisms. However, excessive lignin accumulation can limit plant flexibility, growth, and industrial applications, while its composition affects responses to abiotic stresses such as drought. Using CRISPR-Cas9 genome editing have enabled precise modifications of lignin biosynthetic pathways, offering novel strategies to optimize lignin content and composition for improved stress resilience. By targeting gene involved in monolignol biosynthesis, such as F5H, can change or fine-tune lignin monomer content of the G and S units, balancing mechanical strength with stress adaptability. This approach enhances drought tolerance particularly in the face of climate change. Enhancing its rigidity to environmental stress through genetic modification holds significant promise for improving crop productivity in marginal lands.

Keywords: Ferulate 5-hydroxylase; sorghum bicolor, Lignin, CRISPR/Cas9

Introduction

Sorghum (*Sorghum bicolor*) is a highly resilient cereal crop that plays a crucial role in global food security, particularly in arid and semi-arid regions. It exhibits remarkable tolerance to drought and heat stress, making it a staple crop in many developing countries where water scarcity is a significant challenge. However, despite its natural adaptability, sorghum remains vulnerable to abiotic stressors, including extreme temperatures, soil salinity, and water deficiency. These environmental pressures can significantly reduce yield and biomass accumulation, threatening agricultural sustainability. In response to these challenges, advanced biotechnological approaches, particularly CRISPR-Cas9 genome editing, offer innovative solutions to enhance sorghum's stress resilience. One promising target for genetic modification is lignin, a fundamental component of plant cell walls.

Lignin is a complex phenolic polymer that provides structural support, facilitates water transport, and contributes to plant defense mechanisms. It is primarily composed of three monolignols: p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, which determine the mechanical properties and physiological responses of the plant. While lignin is essential for plant survival, excessive lignin deposition can lead to reduced flexibility, impaired growth, and lower digestibility, limiting its utility for biofuel production and forage quality. Furthermore, its composition significantly influences a plant's ability to cope with abiotic

stresses such as drought and high temperatures. Modifying lignin biosynthesis through targeted genome editing could optimize its accumulation and composition, balancing structural integrity with stress tolerance.

CRISPR-Cas9 genome editing has emerged as a revolutionary tool in plant biotechnology, allowing precise modifications in key genes involved in lignin biosynthesis. One of the most promising targets in sorghum is ferulate 5-hydroxylase (F5H), a key enzyme in the monolignol pathway that influences the ratio of G and S lignin units. By fine-tuning the expression of F5H, researchers can alter lignin composition, potentially enhancing sorghum's adaptability to environmental stressors. A higher proportion of S units generally results in more flexible lignin, which can improve drought tolerance by maintaining efficient water transport and reducing cell wall rigidity under stress conditions. Such modifications not only improve plant resilience but also enhance biomass processing efficiency, benefiting bioenergy applications.

The integration of CRISPR-Cas9 technology for lignin modification in sorghum presents a sustainable approach to improving crop performance under climate change scenarios. By optimizing lignin content, researchers can develop sorghum varieties that maintain structural integrity while adapting to harsh environmental conditions. This genetic enhancement could be particularly beneficial for marginal lands, where sorghum serves as a critical food and fodder source. As global temperatures rise and drought events become more frequent, such advancements will play a pivotal role in securing agricultural productivity and food security.

Overall, leveraging CRISPR-Cas9 for targeted modifications in the lignin biosynthetic pathway provides a novel strategy to enhance sorghum's abiotic stress tolerance. Future research should focus on integrating multi-gene editing approaches, assessing field performance, and understanding the trade-offs between lignin modification and overall plant fitness. By doing so, scientists can unlock the full potential of sorghum as a climate-resilient crop, ensuring its viability in the face of environmental uncertainties.

Genes of Lignin Biosynthesis Involved in Abiotic Stress Responses in Sorghum bicolor

Lignin biosynthesis in *Sorghum bicolor* is regulated by a network of structural genes encoding enzymes involved in the phenylpropanoid pathway (Wei, R et. al 2020). These genes play a critical role in determining lignin content and composition, which in turn affects the plant's mechanical strength, water transport efficiency, and adaptability to abiotic stresses such as drought, heat, and salinity. Modulation of these genes can enhance stress resilience while maintaining optimal plant growth (Li, H. et. al 2020).

1. Phenylpropanoid Pathway Genes

The lignin biosynthetic pathway begins with the conversion of phenylalanine into hydroxycinnamate precursors, followed by a series of enzymatic reactions leading to the formation of monolignols (Yadav, V. et al 2020). Key structural genes involved in this pathway include:

- **Phenylalanine Ammonia-Lyase (PAL)**
 - Function: Catalyzes the first step in the phenylpropanoid pathway, converting phenylalanine into cinnamic acid.
 - Abiotic Stress Response: Upregulated under drought and heat stress to enhance lignin biosynthesis and reinforce vascular tissues.
- **Cinnamate 4-Hydroxylase (C4H)**
 - Function: Converts cinnamic acid into p-coumaric acid, a precursor for lignin monomers.
 - Abiotic Stress Response: Regulates lignin precursor availability under environmental stress conditions.

➤ 4-Coumarate-CoA Ligase (4CL)

- Function: Catalyzes the formation of p-coumaroyl-CoA, a crucial intermediate in lignin biosynthesis.
- Abiotic Stress Response: Increased expression during drought and oxidative stress to support cell wall fortification.

2. Monolignol Biosynthesis Genes

- These genes control the synthesis of the three main lignin monomers: p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units.

➤ Caffeoyl-CoA O-Methyltransferase (CCoAOMT)

- Function: Converts caffeoyl-CoA into feruloyl-CoA, a precursor for both G- and S-lignin.
- Abiotic Stress Response: Enhances lignin deposition in roots and stems to improve drought resistance.

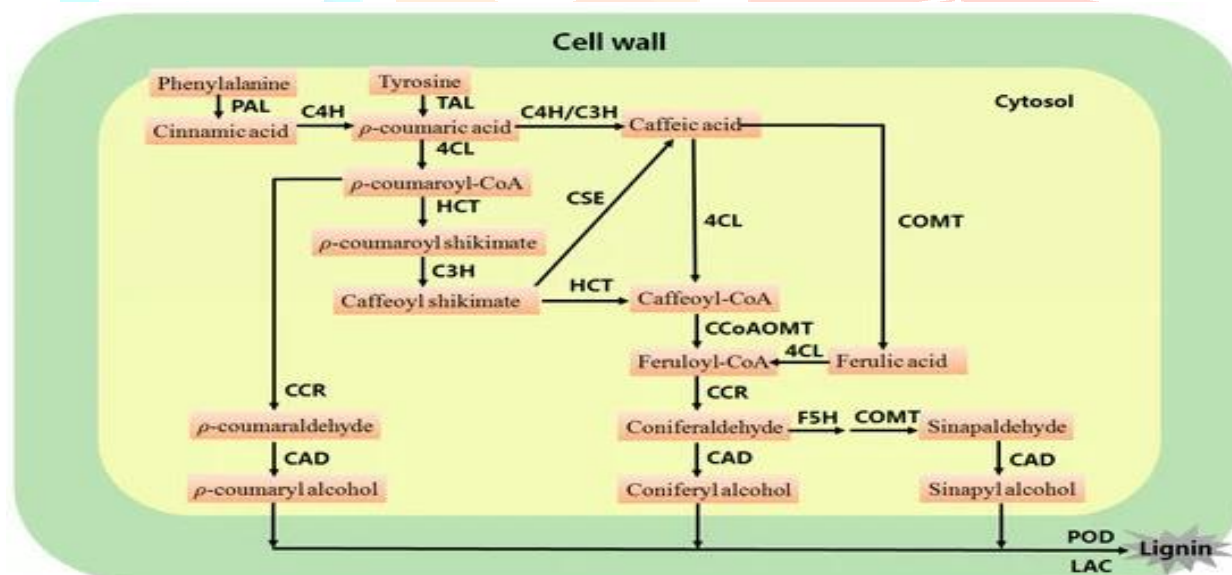
➤ Caffeic Acid O-Methyltransferase (COMT)

- Function: Involved in the methylation of caffeic acid, contributing to S-lignin formation.
- Abiotic Stress Response: Helps in balancing lignin composition for flexible cell walls under variable environmental conditions.

➤ Ferulate 5-Hydroxylase (F5H)

- Function: Converts coniferyl aldehyde into sinapyl aldehyde, leading to increased S-lignin content.
- Abiotic Stress Response: Fine-tuning F5H expression using CRISPR-Cas9 can optimize lignin composition for drought tolerance and improved water-use efficiency (Kaleh, A. M et al. 2024).

Fig. Lignin biosynthesis pathway in Monocots



Enzymes: Lignin biosynthetic pathway. Phenylalanine ammonia-lyase (PAL), Tyrosine ammonia-lyase (TAL), Cinnamate 4-hydroxylase (C4H), 4-Coumarate CoA ligase (4CL), Hydroxycinnamoyl-CoA shikimate / Quinatehydroxycinnamoyltransferase (HCT), p-coumarate 3-hydroxylase (C3H), Caffeoyl shikimate esterase (CSE), Caffeoyl-CoA O-methyltransferase (CCoAOMT), Caffeic acid O-methyltransferase (COMT), Ferulate 5-hydroxylase (F5H), Cinnamoyl CoA reductase (CCR), Cinnamyl alcohol dehydrogenase (CAD), Peroxidase (POD) and Laccase (LAC). Hydroxyphenyl Unit (H Unit), Catechyl Unit (C Unit), Guaiacyl Unit (G Unit) and Syringyl Unit (S Unit) of lignin.

3. Lignin Polymerization Genes

- **Cinnamyl Alcohol Dehydrogenase (CAD)**

- Function: Converts cinnamyl aldehydes into their respective alcohols, essential for lignin polymerization.
- Abiotic Stress Response: Upregulated in response to salt and drought stress to enhance cell wall rigidity.

- **Peroxidases (PRX) and Laccases (LAC)**

- Function: Catalyze the final polymerization of monolignols into lignin.
- Abiotic Stress Response: Increased expression under oxidative and drought stress to reinforce structural integrity.

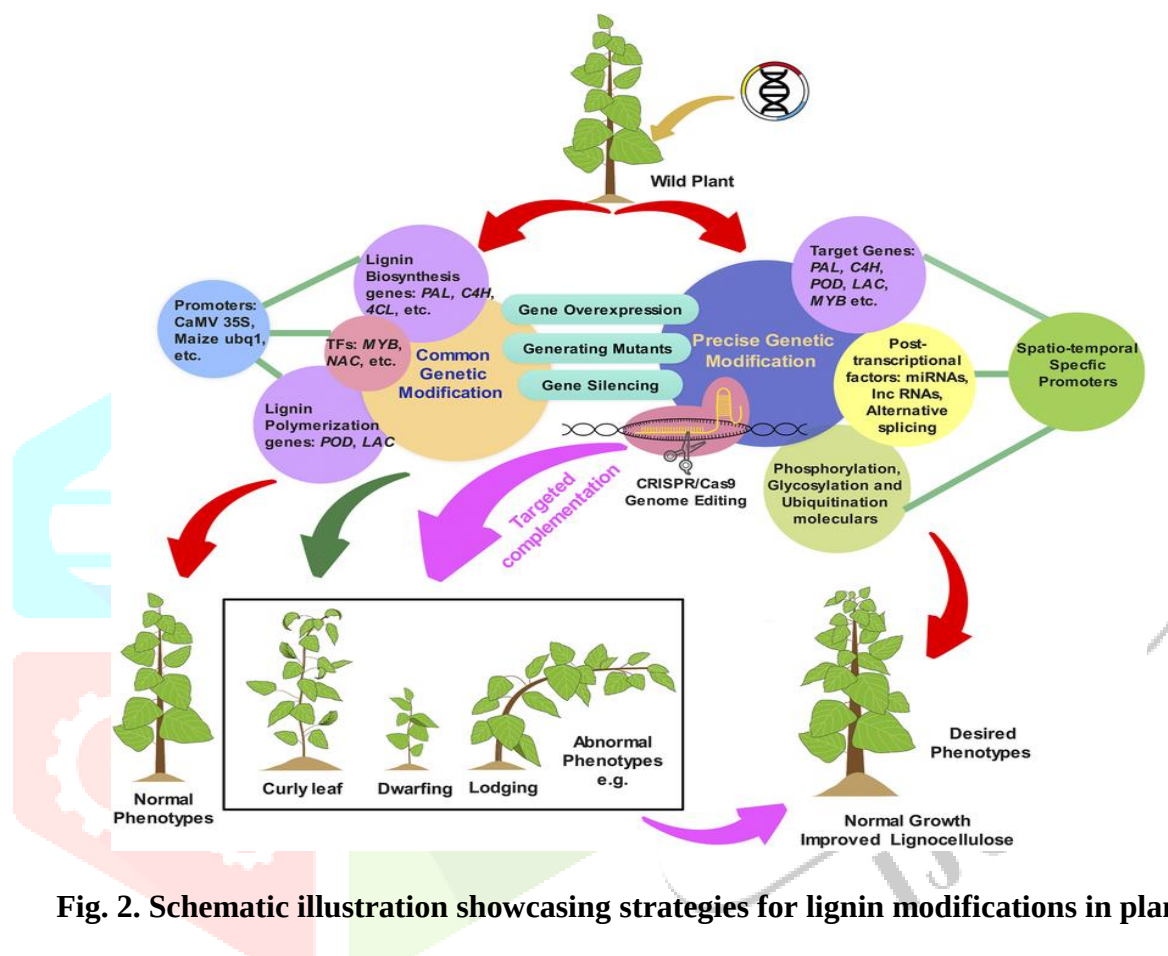


Fig. 2. Schematic illustration showcasing strategies for lignin modifications in plants

Water Stress and Drought: Role of Lignification and the F5H Gene

Extensive research has demonstrated that plant tolerance to drought stress is closely linked to the degree of lignification (Liu, D., et al. 2023). Lignin plays a crucial role in maintaining cell wall integrity, regulating water transport, and protecting plant tissues from dehydration-induced damage. Under drought conditions, the expression levels of several lignin biosynthesis-related genes, including **Cinnamyl Alcohol Dehydrogenase (CAD)**, cytochrome P450 enzymes (**CYP450**), and **S-Adenosyl-L-Methionine Synthetase (SAMS)**, have been found to fluctuate significantly. For instance, in maize leaves, changes in the expression of these genes directly influenced lignin deposition, suggesting that lignin content can serve as a reliable indicator for evaluating drought tolerance in maize.

Similarly, in **Leucaena leucocephala** seedlings subjected to mannitol-induced drought stress, an increased accumulation of **Cinnamoyl-CoA Reductase (CCR)** protein was observed, leading to enhanced lignification of the cell wall. This response is thought to reinforce vascular tissues, reduce water loss, and improve overall drought resilience. However, drought-induced changes in lignin content can vary among plant species. In **Populus trichocarpa**, for example, total lignin levels in young shoots and mature stems remained largely unchanged under drought stress. Interestingly, a significant reduction in the **syringyl/guaiacyl (S/G) ratio** was observed in young shoots, which was associated with the downregulation of **S-lignin biosynthetic genes**,

particularly **Caffeic Acid O-Methyltransferase (COMT)** and **Ferulate 5-Hydroxylase (F5H)** (Han, X. et al 2022).

The Specific Role of the F5H Gene in Drought Adaptation

Among lignin biosynthesis genes, **F5H (Ferulate 5-Hydroxylase)** plays a crucial role in modulating the ratio of **S-lignin (syringyl)** to **G-lignin (guaiacyl)** in plant cell walls. The **F5H** enzyme catalyzes the hydroxylation of ferulic acid derivatives, which is a key step in the formation of syringyl monolignols (Shigeto, J. et al 2017). An optimal S/G ratio is essential for maintaining a balance between structural rigidity and flexibility, which influences plant mechanical strength and water transport efficiency.

Under drought stress, the downregulation of **F5H** in some species, such as *P. trichocarpa*, suggests that plants may reduce S-lignin biosynthesis to alter cell wall composition, possibly to improve water conservation or reinforce cell walls against dehydration. However, this response varies depending on species, tissue type, and growth stage. In herbaceous plants like sorghum (*Sorghum bicolor*), **F5H** expression levels may be critical for determining how lignin composition adapts to prolonged water stress. A reduction in **F5H** activity may lead to a lower S/G ratio, increasing the proportion of guaiacyl lignin, which is associated with more condensed and rigid cell walls (Pazhany, A. S., & Henry, R. J. (2019)). This adaptation may enhance drought tolerance by reinforcing vascular tissues and reducing water loss through transpiration.

Future Prospects for Enhancing Drought Tolerance Through F5H Modulation

Despite significant progress in understanding lignin biosynthesis and its structural modifications under drought conditions, further research is needed to explore the full potential of **F5H** as a target for improving drought tolerance in crops like sorghum. CRISPR-Cas9 genome editing provides a powerful tool for precisely modulating **F5H** expression, allowing researchers to fine-tune the lignin composition to optimize drought resistance without compromising plant growth. Future studies should focus on:

- Investigating the tissue-specific regulation of **F5H** under varying drought intensities.
- Assessing the impact of modified **F5H** expression on plant water-use efficiency and biomass accumulation.
- Exploring potential trade-offs between lignin composition and other physiological traits essential for drought resilience.

By integrating **F5H**-targeted genetic modifications with advanced breeding strategies, it may be possible to develop sorghum varieties that maintain structural integrity, improve water transport efficiency, and enhance overall drought adaptation, ensuring sustainable crop production in water-limited environments.

Final lignin polymerization in the cell wall is controlled by peroxidases and laccases, which influence stress responses (Tobimatsu, Y., et al 2019).

Role of F5H in Flooding Stress Response

Flooding imposes severe stress on plants by creating a low-oxygen environment due to soil waterlogging, disrupting normal growth and development. Among the key genes involved in lignin biosynthesis, **Ferulate 5-Hydroxylase (F5H)** plays a crucial role in modifying lignin composition, particularly by influencing the **syringyl (S) to guaiacyl (G) lignin ratio**. Under flooding conditions, significant downregulation of **F5H** has been observed in plant roots (Cheng, T. et al 2024), as reported in studies on **gray poplar (*Populus × canescens*)**, where multiple lignin biosynthetic genes, including **F5H**, exhibited reduced expression.

The suppression of **F5H** under flooding stress suggests a shift in lignin composition, potentially leading to a lower proportion of S-lignin (Cheng, T et al 2024). This reduction may result in weaker, less flexible cell walls, making root tissues more susceptible to structural damage and impairing their ability to withstand

prolonged waterlogging. Since S-lignin contributes to the reinforcement of secondary cell walls, a decline in **F5H** activity could negatively impact plant resilience to hypoxic conditions by altering xylem integrity and reducing efficient water transport (Riaz, M. W. et al 2023).

Given the importance of **F5H** in lignin modification, targeted regulation of this gene through **genome editing tools like CRISPR-Cas9** could offer promising strategies for improving flooding tolerance in crops like sorghum (*Sorghum bicolor*). Fine-tuning **F5H** expression may help maintain an optimal lignin balance, ensuring structural stability while minimizing growth constraints under excess water conditions. Further research is needed to understand how modulating **F5H** influences root adaptability and overall plant survival in flood-prone environments.

Role of F5H in Salt Stress Response

Salt stress significantly impacts plant growth by disrupting ion homeostasis, causing oxidative damage, and affecting cell wall composition (Dabravolski, S. A., & Isayenkov, S. V. (2023)). Lignin biosynthesis plays a crucial role in plant adaptation to salinity, with key genes regulating its content and composition. Among these, **Ferulate 5-Hydroxylase (F5H)** is essential for modifying lignin structure by converting guaiacyl (G) lignin precursors into syringyl (S) lignin, which influences cell wall flexibility and stress tolerance.

Comparative transcriptomic studies in salt-stressed plants have revealed that lignin biosynthesis genes, including **F5H**, exhibit species- and tissue-specific responses to salinity. In salt-sensitive species like *Populus × canescens*, **F5H** expression is often downregulated, leading to a reduced S-lignin content and potentially compromising cell wall integrity (Han, X. et al 2022). In contrast, salt-tolerant species may regulate **F5H** expression to maintain an optimal lignin composition, enhancing cell wall reinforcement and preventing excessive ion leakage.

The involvement of **F5H** in salt stress responses suggests that modulating its expression could improve plant resilience to salinity. By **fine-tuning F5H activity using CRISPR-Cas9 genome editing**, researchers can optimize the S/G lignin ratio to balance structural rigidity with stress adaptability. Future studies should explore how targeted modifications of **F5H** influence water retention, ion transport, and overall salt tolerance in crops like *Sorghum bicolor*, offering new strategies for improving productivity in saline-affected soils.

Role of F5H in High-Temperature Stress Response

Rising global temperatures impose significant stress on plants, necessitating adaptive mechanisms to maintain cellular integrity and physiological functions. Among these mechanisms, lignin biosynthesis plays a crucial role in reinforcing cell walls and mitigating heat-induced damage (Wang, S. et al 2023). **Ferulate 5-Hydroxylase (F5H)**, a key enzyme in the lignin biosynthetic pathway, is responsible for converting guaiacyl (G) lignin precursors into syringyl (S) lignin, which influences cell wall flexibility and resistance to environmental stresses.

Under high-temperature stress, changes in **F5H** expression can directly affect lignin composition (Liu, D. et al 2023). Studies in various plant species indicate that heat stress alters the balance of lignin monomers, with an observed increase in both G- and S-lignin content while reducing hydroxyphenyl (H) lignin levels. This shift in lignin composition suggests that **F5H** plays a role in adjusting cell wall properties to enhance thermal tolerance (Shafiei, R. et al 2023). In heat-stressed plants such as *Coffea arabica*, the increased S-lignin content, likely regulated by **F5H**, contributes to improved structural stability and protection against oxidative stress.

By modulating **F5H** expression, plants may optimize their lignin composition to withstand prolonged heat exposure. Advanced genome-editing technologies like **CRISPR-Cas9** offer an opportunity to fine-tune **F5H** activity, potentially enhancing thermotolerance in crops like *Sorghum bicolor*. Future research should explore how targeted modifications of **F5H** influence lignin biosynthesis under heat stress and how these changes contribute to maintaining growth and productivity in warming climates.

Role of F5H in Low-Temperature Stress Response

Cold stress significantly alters plant gene expression and metabolism, impacting various biological functions, including cell wall reinforcement through lignin biosynthesis. **Ferulate 5-Hydroxylase (F5H)**, a key enzyme in the lignin biosynthetic pathway, plays a critical role in modulating lignin composition by converting guaiacyl (G) lignin precursors into syringyl (S) lignin. This modification influences the mechanical properties of the cell wall, enhancing plant resilience to freezing temperatures (Wang, S. et al 2023).

Studies on different plant species have shown that low temperatures can induce **F5H** expression, leading to an increase in S-lignin content. For instance, in *Saccharum* spp. (sugarcane), prolonged exposure to cold (14.5°C for 75 days) resulted in **upregulation of F5H in the pith**, correlating with increased lignin accumulation. This suggests that **F5H-mediated lignin biosynthesis contributes to cold adaptation by reinforcing cell walls, preventing cell collapse, and mitigating dehydration stress caused by ice crystal formation**. In contrast, other lignin biosynthetic genes, such as **ShCAD2, ShCOMT1, and ShCCoAOMT1**, were downregulated in the rind, leading to reduced lignin content (Dos Santos, A. B. et al 2015), indicating a tissue-specific lignin remodeling strategy under cold stress.

The regulation of **F5H** under cold stress highlights its potential as a target for improving chilling and freezing tolerance in crops like *Sorghum bicolor*. Utilizing **CRISPR-Cas9 genome editing** to fine-tune **F5H** expression could enhance lignin composition in key tissues, optimizing cell wall flexibility and structural reinforcement for improved cold resilience. Further research should focus on understanding the precise mechanisms by which **F5H** contributes to lignin reorganization under low temperatures, paving the way for the development of cold-tolerant crop varieties.

Role of F5H in Heavy Metal Stress Response

Heavy metal (HM) pollution, particularly aluminum (Al) toxicity, poses a significant challenge to plant growth by disrupting cellular metabolism and damaging root systems (Ghuge, S. A. et al 2023). The plant cell wall acts as the first line of defense against heavy metal stress, with lignin playing a crucial role in immobilizing toxic ions and reinforcing structural integrity. **Ferulate 5-Hydroxylase (F5H)**, a key enzyme in the lignin biosynthetic pathway, influences lignin composition by converting guaiacyl (G) lignin precursors into syringyl (S) lignin. This balance is critical in determining the mechanical properties and adaptability of plant cell walls under HM stress.

Studies on *Camellia sinensis* (tea plants) have shown that under **Al stress (400 µM AlCl₃)**, **F5H expression is downregulated**, leading to a reduction in S-lignin content (Xiong, X. et al 2021). This decreased lignin accumulation in roots, combined with the binding of phenolic acids to Al, appears to mitigate excessive cell wall rigidification, potentially enhancing root plasticity and improving plant growth under acidic soil conditions. In contrast, other lignin biosynthetic genes, such as **ALS3 and CAD**, are induced in species like *Citrus sinensis* and *Citrus grandis* under **1.0 mM AlCl₃ stress**, leading to increased lignin deposition in roots.

The differential regulation of **F5H** under heavy metal stress suggests that modulating its expression could be a strategic approach for balancing lignin composition in crops like *Sorghum bicolor*. **CRISPR-Cas9 genome editing** offers a promising tool for fine-tuning **F5H** activity, potentially improving plant tolerance to heavy metal toxicity by optimizing lignin synthesis and reducing stress-induced growth inhibition. Further research is needed to understand how **F5H-mediated lignin modifications** contribute to heavy metal detoxification and overall plant resilience in contaminated soils.

Role of F5H in Cadmium Stress Response

Cadmium (Cd) stress significantly alters lignin biosynthesis in plants, influencing structural adaptations that enhance stress tolerance (Chen, H et al 2019). **Ferulate 5-Hydroxylase (F5H)**, a key enzyme in the lignin biosynthetic pathway, plays a pivotal role in modifying lignin composition by increasing the proportion of

syringyl (S) lignin. This structural adjustment can impact the ability of plants to cope with Cd toxicity (Vaculík, M. et al 2012).

Research on woody plants has demonstrated that lignin deposition, particularly within **Casparian strips**, serves as a protective barrier against Cd uptake (Chen, T et al 2011). In cadmium-tolerant species like *Salix caprea*, the positioning of the **Casparian strip** in roots differs between populations from contaminated and uncontaminated areas, influencing Cd accumulation patterns. While lignin-rich barriers in roots limit Cd entry, Cd accumulation in aerial tissues remains a critical factor in stress response. **The downregulation or differential expression of F5H under Cd stress** may influence lignin composition, affecting the rigidity and permeability of these barriers (Jia, S et al 2023).

Modulating **F5H** expression through **CRISPR-Cas9 genome editing** in crops like *Sorghum bicolor* could provide a novel strategy to enhance Cd tolerance. By fine-tuning lignin composition, it may be possible to **optimize cell wall flexibility and strengthen selective barriers against heavy metal absorption**, improving overall plant resilience in contaminated environments. Further research is needed to determine the precise regulatory mechanisms governing **F5H-mediated lignin modifications** under cadmium stress and their implications for phytoremediation efforts.

Role of F5H in Response to Light and Elevated CO₂ Stress

Environmental factors such as light availability and atmospheric CO₂ concentrations influence lignin biosynthesis, with **Ferulate 5-Hydroxylase (F5H)** playing a key role in modifying lignin composition.

Light Stress and F5H Regulation

Light stress, particularly shading or sudden exposure to intense sunlight, affects the structural properties of plant tissues. In response, plants adjust their **lignin biosynthetic pathways** to reinforce mechanical stability (Elias, A. A. (2009)). **F5H**, which regulates the biosynthesis of syringyl (S) lignin, may be differentially expressed under varying light conditions to optimize cell wall composition. For instance, in tree species like *Fagus sylvatica*, lignin accumulation in shaded leaves suggests an adaptive response to mechanical reinforcement (Chelli-Chaabouni, A. (2013)). While studies have primarily focused on PAL, CAD, and COMT, further investigation is needed to determine how **F5H expression changes under different light intensities** and its role in lignin modification.

Elevated CO₂ and F5H Expression

Rising atmospheric CO₂ levels directly impact carbon allocation in plants, influencing lignin biosynthesis. In **hybrid poplar (*P. tremula* × *alba*)**, increased CO₂ availability enhances lignin deposition in lower and middle stems, likely due to increased carbon flux toward cell wall fortification (Pazhany, A. S., & Henry, R. J. (2019)). While studies have reported variable lignin responses in different species, **F5H may play a role in adjusting lignin composition under elevated CO₂** (Pazhany, A. S., & Henry, R. J. (2019)), particularly by modulating the S/G lignin ratio. Given that **syringyl-rich lignin contributes to greater cell wall elasticity and structural modifications**, its regulation through **F5H** could be crucial for plant adaptation to changing CO₂ environments (Pazhany, A. S., & Henry, R. J. (2019)).

Future studies should focus on **F5H expression patterns under light and CO₂ fluctuations**, providing insights into how plants optimize lignin composition for structural integrity and environmental resilience.

Regulatory Networks of F5H in Abiotic Stress Reduction

The **Ferulate 5-Hydroxylase (F5H) gene** plays a crucial role in **lignin biosynthesis**, directly influencing plant adaptation to abiotic stresses such as drought, salinity, extreme temperatures, and heavy metal toxicity. Understanding the **regulatory mechanisms** controlling F5H expression is essential for improving plant resilience (Kumar, A. V. et al 2024).

Transcription Factor Regulation of F5H Under Stress

Through our research, we utilized the **Plant Transcription Factor Database (Plant TFD)** to identify key transcription factors (TFs) that regulate **F5H expression**. Certain **TF families**, such as **MYB**, **NAC**, **WRKY**, and **bHLH**, are known to modulate lignin biosynthesis in response to environmental stress.

- **MYB TFs:** These transcription factors have been reported to directly activate or repress **F5H**, influencing the balance between **S-lignin** and **G-lignin** synthesis. Under **drought and salt stress**, MYB-mediated regulation of **F5H** can **alter lignin composition**, reinforcing cell walls to reduce **water loss and ion toxicity** (Kumar, A. V. et al 2024).
- **NAC TFs:** These are major regulators of **secondary cell wall formation**. Under **cold and heat stress**, NAC TFs can enhance **F5H expression**, leading to an optimized lignin structure that helps maintain **cell wall integrity and elasticity** during temperature fluctuations.
- **WRKY TFs:** Known for their role in **stress signaling**, WRKY TFs may influence **F5H** expression in response to **heavy metal toxicity**, reinforcing cell walls to limit metal entry and transport.
- **bHLH TFs:** These factors are involved in **oxidative stress responses**, which indirectly affect **F5H regulation** by maintaining a balance between **lignin biosynthesis and reactive oxygen species (ROS) detoxification**.

By identifying these TFs, we can better understand how **F5H expression is dynamically adjusted** in response to various abiotic stresses, enabling plants to **modulate lignin biosynthesis and improve survival**.

miRNA-Mediated Regulation of F5H Under Stress

Using the **Plant RNA Prediction Tool**, we analyzed **miRNA binding sites** associated with **F5H genes**. miRNAs serve as **post-transcriptional regulators**, fine-tuning gene expression based on environmental conditions (Kumar, A. V. et al 2024).

- **miR397 and miR528:** These miRNAs have been linked to **lignin biosynthesis pathways** by targeting genes like **LACCASEs** and **F5H**. Under **high-salinity and drought conditions**, miR397-mediated **downregulation of F5H** may reduce excessive lignin deposition, maintaining cell wall flexibility to prevent cracking or mechanical failure.
- **miR858:** This miRNA negatively regulates MYB transcription factors, which in turn influence **F5H expression**. During **cold stress**, miR858 suppression may indirectly promote **F5H activity**, leading to increased **S-lignin accumulation**, which enhances **cell wall rigidity** and prevents structural damage from ice crystal formation.
- **miR164:** Known for its role in **secondary cell wall development**, miR164 may contribute to **F5H regulation** under **heavy metal stress**, helping plants modify **lignin structure** to trap and immobilize toxic metals in root cell walls.

By visualizing these regulatory networks using the **Cytoscape tool**, we can interpret the intricate interactions between **F5H**, **TFs**, and **miRNAs**, providing valuable insights into how **lignin biosynthesis is optimized for abiotic stress reduction**. Understanding these mechanisms is essential for developing **stress-resilient crops and trees** with enhanced lignin-based defenses (Kumar, A. V. et al 2024).

Cis-Acting Elements in F5H Promoter Regions and Their Role in Abiotic Stress Reduction

Cis-acting elements in the promoter regions of **F5H (Ferulate 5-Hydroxylase) genes** play a crucial role in regulating gene expression under **abiotic stress conditions**. By analyzing the **1500 bp upstream region** of **F5H genes** using the **Plant Care database**, we identified multiple regulatory elements that control F5H expression in response to **environmental stimuli**, enabling plants to fine-tune lignin biosynthesis as a defense mechanism (Kumar, A. V. et al 2024).

Key Cis-Regulatory Elements and Their Role in Abiotic Stress Tolerance

1. Stress-Responsive Elements

- **Low-Temperature Responsive Elements (LTR):** These elements facilitate **cold-induced F5H expression**, promoting **S-lignin accumulation** to strengthen **cell walls**, prevent dehydration, and protect against **freezing damage**.
- **Wound-Responsive Elements (WRE):** These are activated under **mechanical or environmental stress**, suggesting that F5H helps **reinforce structural integrity** when plants face **physical damage from wind, herbivory, or extreme weather conditions**.
- **Sialic Acid Responsive Elements (SARE):** These elements indicate potential involvement in **oxidative stress responses**, helping mitigate **ROS (reactive oxygen species) damage** by regulating **F5H-mediated lignin biosynthesis**.

2. Hormone-Responsive Elements in Abiotic Stress

- **Abscisic Acid Responsive Elements (ABRE):** ABREs regulate **F5H expression during drought stress**, enhancing lignin deposition to reduce **water loss** and maintain **cell wall elasticity**.
- **Gibberellic Acid Responsive Elements (GARE) & Auxin Responsive Elements (ARE):** While primarily linked to **growth**, these elements also contribute to **stress adaptation**, ensuring lignin biosynthesis supports both **structural reinforcement** and **developmental plasticity**.
- **Methyl Jasmonate Responsive Elements (MeJRE):** These elements suggest a role in **biotic and abiotic defense**, indicating that F5H-mediated lignin modification **strengthens barriers** against **pathogens and environmental stressors**.

3. Light and Environmental Signal Elements

- **Light Responsive Elements (LREs):** F5H expression is influenced by **light intensity and photoperiod**, which affects lignin biosynthesis in response to **shading stress** and sudden **high-light exposure**.
- **MYB Binding Sites:** MYB transcription factors play a significant role in **lignin regulation**, particularly under **drought, temperature, and heavy metal stress**, ensuring appropriate **cell wall reinforcement**.

Implications for Abiotic Stress Reduction

The identification of these **cis-acting elements** underscores the **multi-layered regulatory control** of **F5H expression**, allowing plants to **adjust lignin composition** in response to **abiotic stress factors**. This regulation enables:

- **Enhanced drought tolerance** through increased **cell wall hydrophobicity** (via ABRE elements).
- **Improved cold resistance** by reinforcing **mechanical strength** (LTR elements).
- **Heavy metal detoxification** by modulating lignin-based **metal sequestration** (MYB elements).
- **Oxidative stress reduction** via improved lignin polymerization and **ROS scavenging** (SARE and WRE elements).

These findings highlight how **F5H gene regulation** via **cis-acting elements** plays a pivotal role in **plant adaptation to harsh environmental conditions**, making it a valuable target for **genetic engineering** and **crop improvement** strategies.

Conclusion

The **genome editing of the F5H gene in Sorghum bicolor using CRISPR/Cas technology** offers a novel and targeted approach to enhancing **abiotic stress tolerance**, with a specific focus on **drought resistance**. Given the critical role of **F5H in lignin biosynthesis**, precise modifications to this gene can influence **cell wall composition and structure**, thereby improving the plant's ability to withstand **water-deficient environments**.

Our analysis of **cis-acting elements in the F5H promoter region** has revealed the presence of key regulatory elements, including **abscisic acid-responsive elements (ABA RE)**, **low-temperature-responsive elements (LTR)**, and **wound-responsive elements (WRE)**, all of which are known to be involved in **stress adaptation mechanisms**. The presence of these elements suggests that **F5H expression** is tightly regulated in response to **environmental cues**, including **drought-induced oxidative stress**. Furthermore, the identification of

MYB transcription factors and miRNA binding sites interacting with **F5H** highlights the complex regulatory networks that fine-tune its expression under **adverse conditions**.

By leveraging **CRISPR/Cas-mediated gene editing**, targeted modifications in **F5H** can be designed to optimize **lignin content and composition**, balancing **structural reinforcement and efficient water transport**. This strategy can lead to **reduced water loss, enhanced root strength, and improved overall drought tolerance**, making sorghum better suited for cultivation in **arid and semi-arid regions**. Given the increasing challenges posed by **climate change**, such genetic improvements in **drought-resilient crops** hold great potential for **sustainable agriculture, food security, and environmental resilience**.

Overall, **F5H gene editing** represents a promising genetic engineering approach to **developing stress-resilient sorghum varieties**, ensuring **higher productivity and stability in challenging climatic conditions** while minimizing the need for excessive irrigation and external inputs.

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