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Genetic Engineering for Enhanced Lignin Digestibility in Forage Maize (*Zea maize* L.): From Brown Midrib Mutations to CRISPR-Based Approaches

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Forage maize (*Zea mays* L.) is a critical livestock feed resource, yet lignin-mediated biomass recalcitrance significantly limits its nutritional value. This review examines biotechnological strategies for improving lignin digestibility through genetic engineering approaches. Brown midrib (bm) mutations affecting six characterized loci (*bm1-bm6*) have provided fundamental insights into lignin metabolism and demonstrate digestibility improvements of 15-58%. Modern CRISPR-Cas9 technology enables precise modifications without regulatory constraints of transgenic approaches. Transcriptional engineering through MYB and NAC family regulators offers sophisticated pathway control strategies. Current research confirms that strategic lignin modifications can substantially improve neutral detergent fibre digestibility while maintaining acceptable agronomic performance through careful genetic background selection and precision breeding approaches.

Keywords: Forage maize, lignin biosynthesis, brown midrib mutations, CRISPR-Cas9, digestibility

Introduction

Forage maize represents a cornerstone of modern livestock nutrition, with over 3.5 million hectares cultivated annually for silage production. The nutritional quality of maize silage depends on carbohydrate accessibility, particularly cellulose and hemicellulose from cell walls. However, lignin creates substantial barriers to enzymatic digestion, forming cross-links with polysaccharides that limit microbial access during ruminal fermentation. Lignin, comprising 15-20% of maize biomass, provides structural integrity and pathogen resistance but reduces neutral detergent fibre digestibility (NDFD) to 40-50% in conventional varieties. This limitation directly impacts livestock energy intake and production efficiency, creating economic challenges for producers. Traditional approaches have exploited naturally occurring brown midrib mutations, which provide visible markers for altered lignin metabolism. While these mutations demonstrate significant digestibility potential, they often exhibit agronomic penalties including reduced yields and increased disease susceptibility. Recent biotechnological advances offer unprecedented opportunities for precision lignin engineering. CRISPR-Cas9 technology enables targeted modifications without introducing foreign DNA, addressing regulatory concerns while providing sophisticated control over lignin biosynthesis. These tools, combined with comprehensive understanding of regulatory networks, promise development of superior forage varieties that optimize both digestibility and agronomic performance.

Lignin Biosynthesis in Maize

Pathway Architecture

Lignin biosynthesis in maize involves a complex phenylpropanoid pathway converting aromatic amino acids into monolignol precursors. Genomic analyses have identified 117 genes across 10 enzyme families participating in lignin synthesis, representing substantial expansion compared to model plants. This gene multiplication reflects maize's polyploid evolutionary history and provides functional redundancy influencing engineering strategies.

The pathway initiates with phenylalanine ammonia-lyase (PAL) enzymes, which in maize possess bifunctional activity enabling utilization of both phenylalanine and tyrosine substrates. Sequential reactions involve cinnamate 4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase (HCT), cinnamoyl-CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid O-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD).

Maize-Specific Features

Maize lignin exhibits unique characteristics including tricin incorporation as an additional monomer, p-coumaroylation of syringyl units, and extensive ferulate cross-linking between lignin and arabinoxylan. These modifications create specific engineering opportunities while influencing digestibility enhancement strategies. Ferulate esters forming covalent bridges particularly impact enzyme accessibility during digestion.

Brown Midrib Mutations: Genetic Models for Engineering

Characterized Brown Midrib Loci

Six brown midrib loci (*bm1-bm6*) have been identified in maize, each affecting different lignin metabolism aspects (Table 1). The *bm1* mutation affects CAD2, resulting in aldehyde incorporation into lignin and increased extractability. The *bm3* mutation, affecting COMT, demonstrates the most consistent digestibility improvements with 20-45% increases in NDFD alongside 20-40% lignin reductions, though often with 5-20% yield penalties.

Table 1. Brown Midrib Mutations and Their Effects on Forage Quality

Gene	Enzyme	Lignin Reduction	Digestibility Improvement	Yield Impact
bm1	CAD2	10-21%	10-58%	Minimal
bm2	MTHFR	7-17%	21-58%	Minimal
bm3	COMT	20-40%	9-45%	5-20% reduction
bm4	FPGS	10-14%	43%	Minimal
bm5	4CL1	10-20%	18-22%	Minimal
bm6	GCH1	6-9%	3%	6% height reduction

One-Carbon Metabolism Connections

The *bm2*, *bm4*, and *bm6* mutations affect methylenetetrahydrofolate reductase (MTHFR), folylpolyglutamate synthase (FPGS), and GTP cyclohydrolase 1 (GCH1), respectively, revealing crucial connections between lignin biosynthesis and one-carbon metabolism. These genes demonstrate that lignin synthesis depends on adequate S-adenosylmethionine availability for methylation reactions. The *bm6* mutation provides particularly valuable insights into metabolic flux regulation. GCH1 disruption redirects p-coumaroyl-CoA from lignin toward anthocyanin biosynthesis through enhanced chalcone synthase activity. This redirection occurs through altered protein interactions between GCH1 and phosphatidylethanolamine-binding protein 15, illustrating sophisticated regulatory mechanisms controlling pathway allocation.

Modern Biotechnological Approaches

CRISPR-Cas9 Technology

CRISPR-Cas9 genome editing has revolutionized lignin engineering possibilities in maize by enabling precise modifications without introducing foreign DNA sequences. This technology addresses regulatory concerns while providing unprecedented control over gene function through creation of controlled allelic series that fine-tune lignin characteristics.

Delivery strategy innovations have overcome transformation limitations in elite germplasm. Transgenerational editing approaches, including haploid inducer-mediated genome editing (Hi-Edit), enable modification introduction into commercial varieties without traditional transformation bottlenecks. These methods combine haploid induction with CRISPR delivery, producing edited doubled haploids efficiently.

Multiplexed editing strategies using BREEDIT technology allow simultaneous targeting of multiple gene family members, overcoming functional redundancy that limits single-gene approaches. This capability is particularly relevant given extensive gene duplications characterizing maize lignin biosynthetic pathways.

Precision Breeding Integration

Base and prime editing technologies provide tools for creating subtle modifications that adjust enzyme activities rather than completely disrupting gene function. These approaches offer nuanced control over lignin characteristics, potentially avoiding severe phenotypic effects while achieving desired digestibility improvements.

Integration with doubled haploid technology accelerates breeding cycles from decades to years, enabling responsive adaptation to agricultural demands and rapid evaluation of engineering strategies across genetic backgrounds.

Transcriptional Regulation Engineering

MYB Family Regulators

MYB transcription factors play crucial roles in lignin biosynthesis regulation. ZmMYB31 and ZmMYB42 function as repressors, directly binding promoter elements and redirecting phenylpropanoid flux from lignin production. These proteins represent attractive targets for transcriptional enhancement of digestibility.

Hierarchical regulatory cascades control these repressor proteins. ZmMYB69 functions as an upstream activator of *ZmMYB31* and *ZmMYB42* expression, where overexpression reduces lignin content by 15-20% while improving saccharification efficiency up to 50%. This transcriptional approach offers sophisticated control mechanisms minimizing pleiotropic effects compared to direct enzyme manipulation.

NAC Family Regulation

NAC transcription factors serve as master regulators coordinating secondary cell wall formation. *ZmNST3* and *ZmNST4* control downstream MYB factors and structural genes, enabling comprehensive cell wall engineering approaches that address multiple components simultaneously rather than focusing solely on lignin content.

Metabolic Engineering Strategies

Pathway Flux Redirection

Engineering approaches redirecting metabolic flux between competing pathways offer sophisticated digestibility improvement strategies. Competition between lignin and flavonoid biosynthesis for shared precursors can be manipulated to favour more digestible cell wall components while providing additional nutritional benefits through increased antioxidant content.

Disruption of chalcone synthase regulation significantly alters phenylpropanoid precursor allocation, reducing lignin accumulation while increasing anthocyanin content. This metabolic rebalancing provides dual benefits of improved digestibility and enhanced nutritional value.

Cross-linking Modifications

Targeting fabulate cross-links between lignin and hemicellulose represents promising strategy for digestibility enhancement. Downregulation of BAHD acyltransferases involved in ferulate conjugation reduces cross-linking density without substantially affecting total lignin content, providing digestibility improvements with minimal structural impact.

Agricultural Applications

Field Performance

Comprehensive field trials demonstrate that lignin-modified varieties maintain digestibility improvements under commercial conditions. Environmental stability varies among modifications, with some genetic backgrounds showing better adaptation while retaining engineered traits.

Commercial dairy operation evaluations confirm substantial milk production efficiency improvements, validating practical benefits of lignin modification strategies. These applications demonstrate agricultural value of biotechnological interventions.

Elite Germplasm Integration

Successful implementation requires careful integration with elite breeding materials. Advanced techniques including transgenerational editing and marker-assisted introgression enable beneficial modification introduction into high-performing commercial varieties without compromising agronomic traits.

Recent studies demonstrate successful introduction of *bm6* mutations into elite varieties Zhengdan 958 and Xianyu 335, with in vitro rumen digestion assays confirming significant digestibility improvements for dry matter, organic matter, cellulose, hemicellulose, and lignin components.

Future Perspectives

Future lignin engineering will increasingly rely on integrated approaches combining multiple biotechnological tools. Systems biology understanding guides identification of optimal gene combinations for synergistic effects, while precision editing techniques enable fine-tuning of pathway activities.

Emerging technologies including base editing, prime editing, and synthetic biology approaches promise greater precision in lignin modification. These tools enable creation of novel lignin variants with enhanced digestibility while maintaining beneficial characteristics such as disease resistance and structural integrity.

Predictive models incorporating genomic, environmental, and management factors will guide engineered variety deployment across diverse production systems, optimizing lignin modification benefits for specific applications and conditions.

Conclusion

Genetic engineering for enhanced lignin digestibility in forage maize has evolved from exploiting spontaneous mutations to sophisticated biotechnological approaches targeting specific genes and regulatory networks. Modern genome editing techniques provide precise tools for implementing improvements in elite germplasm while avoiding traditional transgenic limitations. The challenge involves translating laboratory successes into commercially viable varieties delivering consistent digestibility improvements across diverse environments. Success requires continued integration of advanced biotechnological tools with conventional breeding, maintaining focus on practical applications enhancing livestock nutrition while supporting sustainable production systems.

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