Review Article

Marker-Assisted Breeding Techniques for the Development of Gluten-Free Wheat Varieties: A Comprehensive Review

Abstract

Ingestion of gluten proteins from wheat, barley and rye poses a significant health risk. The sole treatment for various disease is a lifelong adherence to a strict gluten-free diet. Conventional breeding methods have limitations in producing wheat varieties that maintain baking quality due to the complexity of the wheat genome and the multitude of gluten genes.This review explores the use of RNA interference (RNAi) silencing and CRISPR/Cas9 gene editing techniques. Efficient screening and selection processes for identifying lines with reduced celiac disease epitopes at both the DNA and protein levels, as well as maintaining baking quality, are discussed. The integration of gene editing for the production of wheat products holds significant potential in meeting the growing demand for safer dietary options while ensuring compliance with regulatory standards and addressing the complex challenges associated with disease management. Marker-assisted selection (MAS) offers several advantages over conventional selection methods in plant breeding, including timesaving, cost-effectiveness and goal-oriented outcomes. This review aims to delineate various molecular markers, encompassing sequence-tagged sites (STS), simple sequence repeats (SSR), genotyping by sequencing (GBS), single nucleotide polymorphism (SNP) arrays, exome capture, Competitive Allele Specific PCR (KASP), cleaved amplified polymorphic sequences (CAPS), semi-thermal asymmetric reverse PCR (STARP), and genotyping by target sequencing (GBTS). Additionally, we compile quantitative trait loci (QTL)/genes and their associated markers, which hold potential utility in MAS applications.The rapid advancement of wheat genomics is poised to expedite marker development, QTL mapping, gene cloning, and wheat breeding processes.

Keywords: Gene Editing, Molecular Markers, Polymorphism, Allele Specific PCR, QTL

Introduction

Gluten intolerance and related disorders, including celiac disease (CD) and non-celiac gluten sensitivity (NCGS), have spurred an urgent need for the development of gluten-free wheat varieties[1-2]. Celiac disease, in particular, affects millions worldwide, with symptoms ranging from mild discomfort to severe gastrointestinal distress and malabsorption issues[3]. Moreover, non-celiac gluten sensitivity presents its own set of challenges, often with symptoms similar to CD but lacking the characteristic autoimmune response[4]. In response to these health concerns and the growing demand for gluten-free options, researchers and breeders have turned to marker-assisted breeding techniques as a promising avenue for creating wheat varieties that are safe for consumption by gluten-sensitive individuals.

Marker-assisted breeding (MAB) techniques have revolutionized plant-breeding practices by enabling the selection of desirable traits with precision and efficiency[5-6]. By leveraging molecular markers linked to specific genes or traits of interest, breeders can bypass the lengthy and labour-intensive process of traditional phenotypic selection[7]. This targeted approach not only accelerates the breeding cycle but also enhances the probability of success in developing wheat varieties with improved traits, such as reduced gliadin content and enhanced nutritional profiles[8].

In recent years, the booming field of wheat genomics has provided invaluable insights into the genetic makeup and regulatory mechanisms governing gluten composition and related traits[9-10]. Advances in high-throughput sequencing technologies, coupled with bioinformatics tools for data analysis, have facilitated the identification of key genes and regulatory elements underlying gluten synthesis, storage, and immunogenicity[11]. As a result, researchers are now equipped with a wealth of genetic resources and markers that can be exploited to develop gluten-free wheat varieties through marker-assisted breeding strategies[12].

This comprehensive review aims to provide an in-depth exploration of marker-assisted breeding techniques for the development of gluten-free wheat varieties. By synthesizing the latest research findings and technological advancements in the field, we seek to elucidate the potential of MAB approaches in addressing the complex challenges associated with gluten intolerance and wheat-related disorders[13]. Specifically, we will delve into the following key aspects:

1. ***Molecular Markers and Genomic Resources:*** We will discuss the diverse array of molecular markers utilized in marker-assisted breeding, including sequence-tagged sites (STS)[14], single nucleotide polymorphisms (SNPs)[15], simple sequence repeats (SSRs), and genotyping-by-sequencing (GBS) technologies [16]. Additionally, we will highlight the availability of genomic resources, such as

reference genome sequences and genetic maps, which serve as foundational tools for marker development and QTL mapping in wheat.

2. ***Trait Mapping and Gene Discovery:*** Through comprehensive QTL mapping studies and genome-wide association analyses, researchers have identified genomic regions associated with gluten content, composition, and immunogenicity in wheat. We will explore the latest findings on QTLs and candidate genes implicated in gluten synthesis, storage proteins, and related biochemical pathways[17]. Furthermore, we will discuss the implications of gene cloning and functional characterization in elucidating the genetic basis of gluten-related traits and facilitating targeted breeding efforts.
3. ***Breeding Strategies for Gluten-Free Wheat Varieties:*** Building upon the insights gained from trait mapping and gene discovery, we will examine various breeding strategies employed to develop gluten-free wheat varieties[18]. From marker-assisted backcrossing and genomic selection to gene editing and transgenic approaches[19], we will evaluate the efficacy, feasibility, and regulatory considerations associated with each method. Moreover, we will highlight case studies and success stories highlighting the application of marker-assisted breeding techniques in wheat improvement programs worldwide.

In summary, this review aims to provide a comprehensive overview of marker-assisted breeding techniques for the development of gluten-free wheat varieties. By elucidating the underlying principles, challenges, and opportunities in wheat breeding, we hope to inspire further research and collaboration towards the realization of safer, healthier wheat products for individuals with gluten intolerance and related dietary restrictions. Through interdisciplinary efforts and technological innovations, we envision a future where gluten-free wheat varieties are not only accessible but also synonymous with nutritional excellence and culinary delight.

Wheat proteins, encompassing non-gluten proteins such as amylase/trypsin inhibitors (ATI), have been implicated as triggering factors in the development of celiac disease[20]. Recent comparative studies on the nutritional attributes of ancient and modern wheat varieties have affirmed that breeding improves the gluten quality of wheat. This improvement is evident in both its technical performance, leading to the creation of high-quality baking products, and its allergenic potential (refer to Figure 1). Specifically, contemporary cultivars exhibit a higher gluten index associated with increased gluten content. The breeding process has also substantially decreased wheat-dependent exercise-induced anaphylaxis (WDEIA) by reducing the presence of a significant allergen[21]. However, both ancient and modern cultivars display similar levels of α and β-type gliadin content, with the former being linked to celiac disease toxicity[22]. Despite the presence of allergens in wheat grains, there exists extensive genetic diversity within wheat germplasm. Further research is necessary to develop cultivars with diminished reactivity and/or increased secondary health-promoting components suitable for consumption. This presents an opportunity for fertilization practices, particularly nitrogen fertilization, to influence the yield and quality of wheat production.

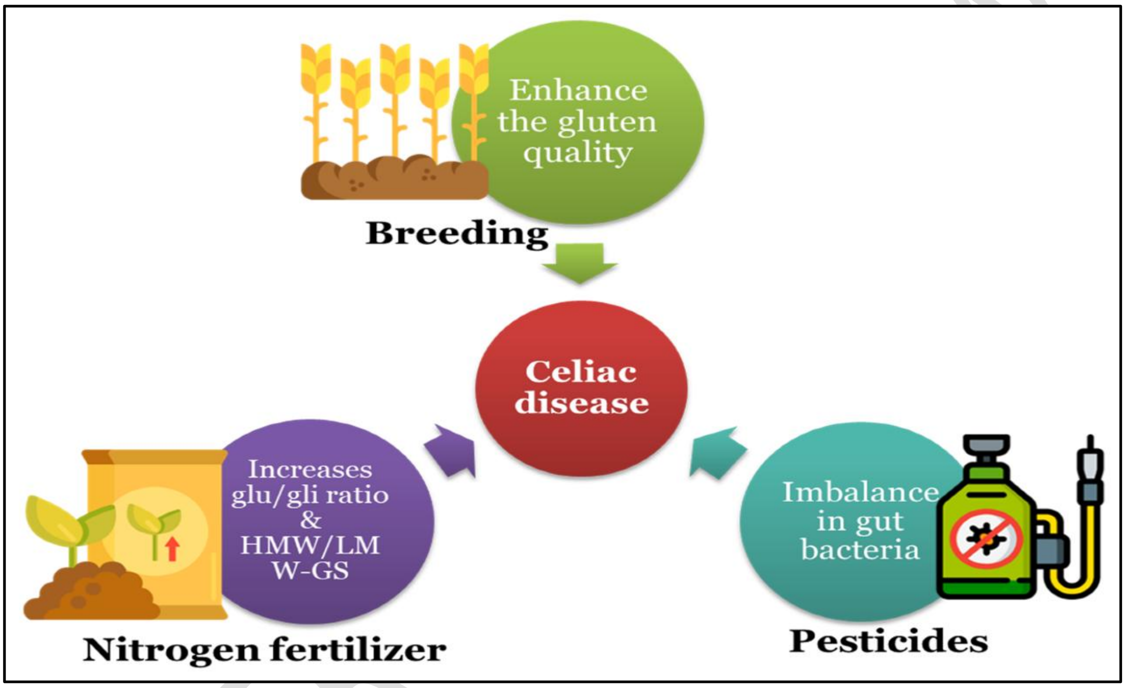

Figure 1: *The escalating prevalence of celiac disease (CD) is influenced by wheat breeding, nitrogen (N) fertilizers, pesticides (glyphosate), and assorted agronomical practices.*

Various breeding methods and strategies employed in the pursuit of gluten-free wheat varieties.

Traditional Breeding Approaches:Traditional breeding methods involve the controlled crossing of wheat varieties to introduce desired traits, followed by rigorous selection based on phenotypic characteristics[23]. Historically, these methods have relied on visual assessments of gluten content and quality, which can be subjective and time-consuming. Despite their limitations, traditional breeding approaches have played a pivotal role in the development of wheat varieties with improved agronomic performance and disease resistance[24].

Marker-Assisted Breeding (MAB):In recent years, marker-assisted breeding (MAB) has emerged as a powerful tool for accelerating the development of gluten-free wheat varieties[25]. MAB leverages molecular markers linked to genes of interest, allowing breeders to identify and select individuals carrying desired traits with precision. In the context of gluten-free wheat breeding, molecular markers associated with gluten proteins, such as gliadins and glutenin, have been utilized to screen for low or non-toxic gluten variants.

Quantitative Trait Loci (QTL) Mapping:Quantitative trait loci (QTL) mapping involves the identification and mapping of genomic regions associated with specific traits, including gluten content and composition[26]. Using molecular markers and advanced statistical techniques, researchers can pinpoint chromosomal regions harbouring genes that influence gluten-related traits[27]. QTL mapping facilitates the selection of wheat lines with favourable QTL combinations for gluten reduction or modification.

Genome-Wide Association Studies (GWAS):Genome-wide association studies (GWAS) involve the analysis of genetic variations across the entire genome to identify associations between genetic markers and phenotypic traits[28]. In the context of gluten-free wheat breeding, GWAS enables the identification of candidate genes and regulatory elements involved in gluten synthesis, storage, and immunogenicity. By elucidating the genetic basis of gluten-related traits, GWAS provides valuable insights for targeted breeding efforts.

Genomic Selection:Genomic selection integrates genomic data with phenotypic information to predict the breeding value of individuals within a population[29]. By leveraging genome-wide marker information, genomic selection enables breeders to accurately estimate the genetic merit of potential parents and select for desired traits across multiple generations. In gluten-free wheat breeding, genomic selection can expedite the identification of low-gluten or gluten-free varieties with optimal agronomic performance.

Gene Editing and Transgenic Approaches:Gene editing technologies, such as CRISPR-Cas9, offer precise and targeted methods for modifying specific genes associated with gluten content and composition in wheat[30]. By introducing targeted mutations or gene knockouts, gene-editing technologies can disrupt the synthesis of toxic gluten proteins while preserving the functionality of non-gluten components[31]. Similarly, transgenic approaches involving the introduction of foreign genes or regulatory elements offer potential avenues for enhancing gluten-free traits in wheat.

Table 1 illustrates the recommended intake levels of essential amino acids expressed as grams per gram of protein, as outlined by the Food and Agriculture Organization (FAO) for different age groups, alongside the actual amino acid content found in wheat, wholemeal, and white flour. For histidine, the FAO recommends levels of 0.016 g/g protein for children aged 11–14, 0.016 g/g for adolescents aged 15–18, and 0.015 g/g for adults over 18. Comparatively, wheat, wholemeal, and white flour contain 0.022 g/g, 0.0266 g/g, and 0.0269 g/g, respectively.

Table1.Essentialaminoacidlevelsrecommended(expressedasg/gprotein).

	FAORecommendedIntakeLevels			AminoAcidsContent		
AminoAcids	**Children (Age11–14)**	**Adolescents (Age15–18)**	**Adults (Age>18)**	**Whole Wheat**	**Wholemeal Wheat**	**White Flour**
Histidine	0.016	0.016	0.015	0.022	0.0266	0.0269
Isoleucine	0.030	0.030	0.030	0.038	0.0314	0.0309
Leucine	0.061	0.060	0.059	0.067	0.0594	0.0565
Lysine	0.048	0.047	0.045	0.027	0.0288	0.0222
Methionine + cysteine	0.023	0.023	0.022	0.039	0.0363	0.033
Phenylalanine+tyrosine	0.041	0.040	0.038	0.077	0.0544	0.0514
Threonine	0.025	0.024	0.023	0.029	0.0254	0.0224
Tryptophan	0.0066	0.0063	0.006	0.012	-	0.0085
Valine	0.040	0.040	0.039	0.047	0.0388	0.0354

Wheat grain comprises the germ (2–3%), the bran (13–17%), and the endosperm (80–85%). The germ, rich in protein, lipids, and several B-vitamins, constitutes the embryo of the wheat kernel. Conversely, whole-wheat flour, incorporating the bran, offers limited protein but abundant B-complex vitamins, trace minerals, and dietary fiber. White flour, derived from the endosperm, contains most of the kernel's protein, along with iron, carbohydrates, and various B-complex vitamins such as riboflavin, thiamine, and niacin. Consumption of wheat contributes to numerous health benefits, with bread alone constituting 27% of total carbohydrate intake in the European Prospective Investigation into Cancer and Nutrition (EPIC) study populations in Figure 2. Epidemiological evidence highlights the protective effects of cereal dietary fiber and wholegrain consumption against prevalent chronic diseases associated with sedentary lifestyles, notably type 2 diabetes and cardiovascular diseases.

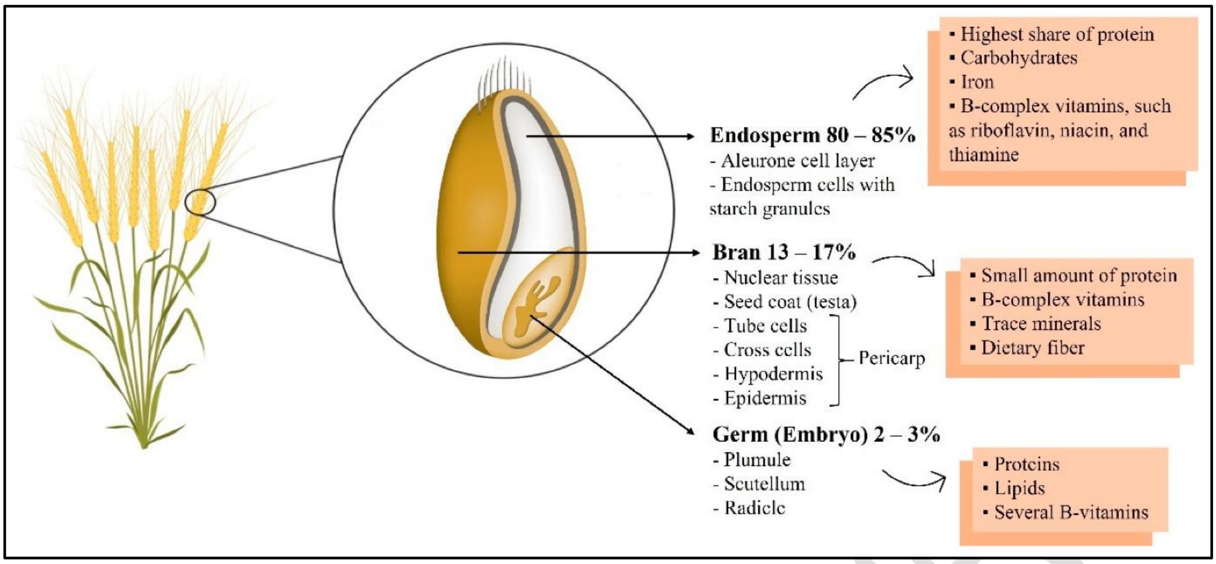

Figure 2:*Nutritional Composition and Health Benefits of Wheat Consumption*

Genomic Advancements in Wheat Breeding: Revolutionizing Crop Improvement

In the pursuit of enhancing crop productivity and resilience to environmental stresses, modern agriculture has increasingly relied on advanced genomic tools and techniques. The advent of genome sequencing, coupled with genome-wide association studies (GWAS) and CRISPR/Cas-mediated gene editing, has revolutionized wheat breeding strategies[32]. In this comprehensive exploration, we delve into the significance of these advancements and their implications for the future of wheat agriculture in Figure 3.

Genomic advancements in wheat breeding have revolutionized the field, offering unprecedented insights into the wheat genome's complexity and diversity. High-throughput sequencing technologies have facilitated comprehensive genome sequencing and annotation, providing a robust foundation for breeding programs[33]. Integration of molecular markers and quantitative trait loci (QTL) mapping has enabled precise trait selection, accelerating the development of improved wheat varieties. CRISPR/Cas9 gene editing techniques offer targeted genome modifications, allowing for rapid trait introgression and trait stacking. Overall, these genomic advancements are poised to drive significant progress in wheat breeding, enhancing crop productivity, resilience, and nutritional quality to meet the demands of a growing global population.

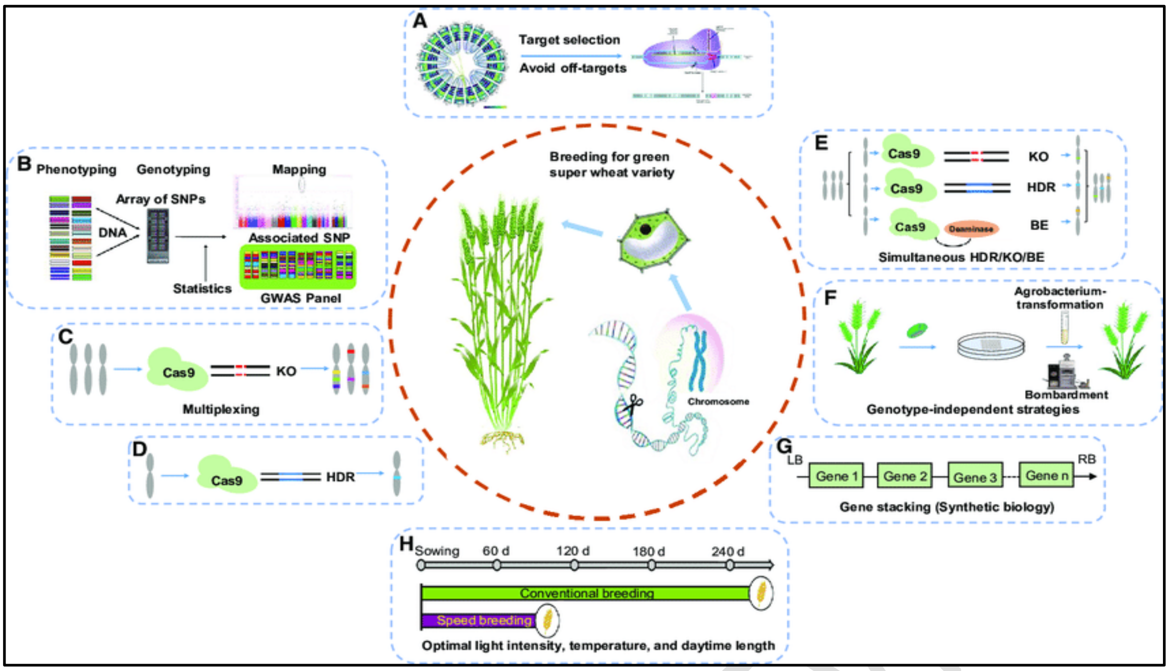

Figure 3: *Breeding of a green super wheat variety through CRISPR/Cas9*

A. Genome Sequencing: Unlocking Wheat's Genetic Blueprint

Genome sequencing serves as the cornerstone of modern crop breeding programs, offering invaluable insights into the genetic makeup of wheat. The sequencing of the wheat genome, as well as the development of pan-genome sequencing approaches, has provided researchers with a comprehensive understanding of wheat's genomic landscape. This wealth of genomic data is instrumental in various aspects of wheat breeding, particularly in the design of single-guide RNA (sgRNA) targets for CRISPR/Cas-mediated genome editing[34].

The ability to accurately identify target sequences and evaluate potential off-target effects is crucial for the precise manipulation of the wheat genome. By leveraging genome-sequencing data, researchers can select target sites with minimal off-target effects, thereby enhancing the efficiency and specificity of genome editing techniques. Moreover, pan-genome sequencing facilitates the identification of genetic variations across diverse wheat varieties, enabling breeders to exploit natural genetic diversity for crop improvement.

B. GWAS Analysis: Unraveling the Genetic Basis of Traits

Genome-wide association studies (GWAS) have emerged as a powerful tool for elucidating the genetic basis of complex traits in wheat[35]. By analyzing the association between specific genes, single nucleotide polymorphisms (SNPs), or markers and phenotypic traits, GWAS enables researchers to pinpoint genomic regions associated with desired agronomic traits[36]. This information is invaluable for marker-assisted breeding programs aimed at introgressionfavourable alleles into elite wheat varieties.

Through GWAS analysis, researchers can identify candidate genes underlying key agronomic traits such as yield, disease resistance, and abiotic stress tolerance. These insights not only accelerate the breeding process but also provide a deeper understanding of the genetic mechanisms governing trait variation in wheat. Furthermore, GWAS facilitates the identification of molecular markers linked to desirable traits, enabling breeders to develop marker-assisted selection strategies for trait improvement.

C. CRISPR/Cas-Mediated Multiplex System: Streamlining Genome Editing

The CRISPR/Cas-mediated multiplex system represents a paradigm shift in genome editing technology, allowing for the simultaneous targeting of multiple genes in the wheat genome[37]. By harnessing the programmable nature of CRISPR/Cas nucleases, researchers can efficiently generate multiple gene knockouts (KOs) in a single transformation event[38]. This multiplex editing capability not only expedites the functional characterization of gene families but also enables the engineering of complex traits through the simultaneous manipulation of multiple genes.

The CRISPR/Cas-mediated multiplex system offers unprecedented flexibility and precision in genome editing, paving the way for the development of novel wheat varieties with enhanced agronomic traits. Moreover, this technology holds promise for accelerating the breeding process by facilitating the rapid introgression of multiple desirable traits into elite wheat germplasm.

D. Investigating CRISPR/Cas-Mediated HDR: Enhancing Genome Editing Efficiency

Homology-directed repair (HDR) represents a powerful tool for precise genome editing, allowing for the precise insertion of DNA sequences at specific genomic loci[39]. While CRISPR/Cas-mediated HDR has shown great potential in model organisms, its efficiency in wheat remains to be fully elucidated. Researchers are actively investigating strategies to improve HDR efficiency in wheat, with the aim of harnessing this technology for precise gene targeting and allele replacement.

The optimization of CRISPR/Cas-mediated HDR holds significant implications for wheat breeding, particularly in the context of allele pyramiding and trait stacking[40]. By enhancing the efficiency of HDR-mediated genome editing, researchers can accelerate the development of elite wheat varieties with multiple beneficial allelesintrogressed from diverse genetic backgrounds.

E. Facilitating Translational Breeding through Modular Genome Editing

The development of modular genome editing tools holds promise for streamlining the translational breeding process in wheat. By integrating homology-directed repair (HDR), base editing (BE), and knockout capabilities into a single module, researchers can expedite the generation of desired genetic variants in elite wheat varieties[41]. This modular approach not only accelerates the breeding process but also enables precise allele pyramiding and trait stacking, thereby facilitating the development of high-yielding and resilient wheat varieties.

F. Genotype-Independent Strategies: Overcoming Breeding Barriers

Genotype-independent strategies have emerged as a promising approach to overcome the challenges associated with genome editing in recalcitrant wheat varieties[42]. By circumventing genotype-specific barriers to transformation and regeneration, these strategies enable the efficient application of genome editing techniques across diverse wheat germplasm. This versatility empowers breeders to harness the full potential of genome editing for crop improvement, irrespective of genetic background or transformation efficiency.

G. Synthetic Biology: Revolutionizing Trait Stacking

Synthetic biology offers unprecedented opportunities for trait stacking in wheat, allowing researchers to accumulate multiple transgenes of interest within the same plant genome[43]. By leveraging synthetic biology approaches, breeders can stack beneficial traits or engineer novel phenotypes with enhanced agronomic performance. This convergence of biotechnology and breeding holds immense potential for addressing the complex challenges facing modern agriculture, from increasing yields to enhancing resilience to biotic and abiotic stresses.

H. Speed Breeding: Accelerating Crop Improvement

Speed breeding techniques enable a shortened generation time for wheat, facilitating rapid cycling and accelerated breeding cycles[44]. By optimizing growth conditions and light regimes, researchers can dramatically reduce the time required for seed harvesting and generation advancement. This rapid generation turnover not only expedites the breeding process but also allows for the timely evaluation of agronomic traits and the selection of superior genotypes. Speed breeding holds promise for revolutionizing wheat breeding programs, enabling breeders to rapidly develop and deploy improved varieties to meet the evolving needs of global agriculture[45].

The convergence of genomic advancements, including genome sequencing, GWAS analysis, and CRISPR/Cas-mediated genome editing, is revolutionizing wheat breeding strategies[46-47]. By harnessing the power of these technologies, researchers can accelerate the development of elite wheat varieties with enhanced agronomic traits and resilience to environmental stresses. As we continue to unravel the complexities of the wheat genome and refine our breeding techniques, we pave the way for a sustainable and food-secure future.

Gluten-Free Wheat Breeding Through QTL Mapping and Marker-Assisted Selection

The identification and utilization of Quantitative Trait Loci (QTL) associated with gluten-free wheat represent a pivotal step in breeding programs aimed at developing safer dietary options for individuals with gluten-related disorders. QTL mapping enables the identification of genomic regions linked to traits of interest, such as reduced celiac disease epitopes or improved baking quality, facilitating targeted breeding efforts. By pinpointing QTL associated with gluten-free traits, breeders can employ marker-assisted selection (MAS) to expedite the development of wheat varieties with improved nutritional profiles and reduced health risks[48-49]. Furthermore, QTL analysis provides insights into the genetic basis of gluten-related traits, aiding in the selection of parental lines for future crosses and accelerating the introgression of desirable traits into elite wheat germplasm. As research in wheat genomics continues to advance, the discovery and characterization of additional QTL for gluten-free traits will further enhance breeding strategies, ultimately leading to the production of safer and more accessible wheat varieties for individuals with gluten sensitivities.

Future Aspects and Challenges

Despite the promise of breeding methods for the development of gluten-free wheat varieties, several challenges and considerations persist. Maintaining agronomic performance, yield potential, and disease resistance while reducing gluten content remains a complex task. Moreover, regulatory requirements and consumer acceptance of genetically modified or gene-edited wheat varieties pose additional hurdles for breeders and researchers. Breeding methods play a pivotal role in the development of gluten-free wheat varieties, offering diverse approaches for reducing or eliminating gluten content while maintaining desirable agronomic traits. From traditional breeding methods to cutting-edge genomic technologies, breeders have a wide array of tools at their disposal for addressing the complex challenges associated with gluten intolerance. Moving forward, interdisciplinary collaborations and advancements in breeding methodologies will continue to drive progress toward the development of safe and nutritious wheat varieties suitable for individuals with gluten-related disorders.

As the demand for gluten-free wheat varieties continues to grow, the future of breeding methods holds both promise and challenges. Here are some future aspects and challenges to consider:

1. Precision Breeding Technologies:

The continued advancement of precision breeding technologies, such as gene editing and genome editing, holds immense potential for the development of gluten-free wheat varieties. These technologies offer precise control over gene expression and can facilitate the targeted modification of gluten-related genes while preserving other desirable traits. However, regulatory frameworks and consumer acceptance of genetically modified organisms (GMOs) remain key challenges in the adoption of these technologies.

2. Multi-Trait Selection:

Breeders face the challenge of balancing multiple traits, including gluten content, agronomic performance, yield potential, and disease resistance, in the development of gluten-free wheat varieties. Implementing multi-trait selection strategies, such as genomic selection and marker-assisted selection, can help breeders prioritize traits of interest while maintaining overall genetic diversity and adaptability.

3. Consumer Acceptance and Market Demand:

Despite advancements in breeding methods, consumer acceptance and market demand for gluten-free wheat varieties remain critical factors influencing adoption and commercialization. Addressing consumer preferences, nutritional quality, taste, and texture of gluten-free products is essential for driving demand and ensuring the success of gluten-free wheat varieties in the marketplace.

4. Environmental Sustainability:

Breeding methods for gluten-free wheat varieties must also consider environmental sustainability and resilience to changing climatic conditions. Developing wheat varieties that require fewer inputs, such as water and fertilizers, and exhibit tolerance to biotic and abiotic stresses can contribute to sustainable agricultural practices and long-term food security.

5. Collaborative Research and Knowledge Sharing:

Collaboration among researchers, breeders, farmers, policymakers, and industry stakeholders is crucial for addressing the complex challenges associated with gluten-free wheat breeding. Sharing knowledge, resources, and best practices can accelerate progress and foster innovation in breeding methodologies, trait discovery, and variety development.

6. Regulatory Considerations:

Regulatory frameworks governing the development and commercialization of gluten-free wheat varieties vary across regions and countries. Breeders must navigate regulatory requirements related to food safety, labeling, and biotechnology while ensuring compliance with international standards and guidelines. Clear and transparent regulatory pathways can facilitate the timely introduction of new gluten-free wheat varieties to the market.

7. Public Awareness and Education:

Increasing public awareness and education about gluten-related disorders, nutritional benefits of gluten-free diets, and the role of breeding methods in wheat improvement are essential for promoting consumer acceptance and adoption of gluten-free wheat varieties. Educating consumers about the scientific basis of gluten intolerance and the safety of gluten-free products can help dispel misconceptions and foster positive attitudes towards gluten-free alternatives.

Conclusion

The ingestion of gluten proteins from wheat, barley, and rye presents a substantial health risk, necessitating lifelong adherence to a strict gluten-free diet for affected individuals.

Traditional breeding methods face challenges in producing wheat varieties with maintained baking quality due to the complexity of the wheat genome and numerous gluten genes. However, advancements in RNA interference (RNAi) silencing and CRISPR/Cas9 gene editing techniques offer promising avenues for mitigating these challenges. Efficient screening and selection processes can identify wheat lines with reduced celiac disease epitopes while maintaining baking quality. Integrating gene-editing technologies holds significant potential in meeting the demand for safer dietary options, ensuring compliance with regulatory standards, and addressing complex disease management issues. Marker-assisted selection (MAS) offers numerous advantages over traditional methods, including time and cost-effectiveness. This review provides insights into various molecular markers and quantitative trait loci (QTL)/genes associated with MAS, which can expedite wheat-breeding processes. The rapid advancement of wheat genomics is poised to facilitate marker development, QTL mapping, gene cloning, and ultimately, enhance wheat-breeding efforts.

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