Bioscience Methods 2024, Vol.15, No.6, 315-326
http://bioscipublisher.com/index.php/bm

Research Insight Open Access

Application of CRISPR/Cas9 in Wheat Genetic Improvement

Yanxin Ma, Shuo Yang, Shuping Lang
Jiaxing Academy of Agricultural Sciences, Jiaxing, 314016, Zhejiang, China
Corresponding author: littlelsp@163.com

Bioscience Methods, 2024, Vol.15, No.6 doi: 10.5376/bm.2024.15.0031
Received: 24 Sep., 2024
Accepted: 05 Nov., 2024
Published: 24 Nov., 2024

Preferred citation for this article:
Ma Y.X., Yang S., and Lang S.P., 2024, Application of CRISPR/Cas9 in wheat genetic improvement, Bioscience Methods, 15(6): 315-326 (doi: 10.5376/bm.2024.15.0031)

Abstract This study discusses the application of CRISPR/Cas9 technology in wheat genetic improvement, highlighting its potential in overcoming traditional breeding limitations. The study covers the working principles of CRISPR/Cas9, its advantages in precise genome editing, and its utility in enhancing traits such as disease resistance, stress tolerance, yield, and nutritional content in wheat. Case studies illustrate successful implementations where susceptibility genes were edited to bolster disease resistance, and specific genes were targeted to improve stress tolerance and grain quality. Despite its potential, challenges such as low editing efficiency, off-target effects, and regulatory hurdles remain. Nonetheless, the integration of CRISPR/Cas9 with other methods shows promise for future wheat breeding, aiming to ensure food security amidst growing global demands.

Keywords CRISPR/Cas9; Wheat genetic improvement; Disease resistance; Stress tolerance; Genome editing

1 Introduction

Wheat (*Triticum aestivum* L.) is one of the most crucial staple crops globally, providing a significant portion of the daily caloric intake for millions of people. It is cultivated on more land area than any other commercial crop and continues to be the most important food grain source for humans. The global demand for wheat is expected to rise due to population growth and changing dietary preferences, making its improvement vital for food security (Haque et al., 2018; Liu et al., 2022).

Despite its importance, wheat production faces numerous challenges. These include biotic stresses such as diseases caused by pathogens, and abiotic stresses like drought, salinity, and extreme temperatures, which significantly impact yield and quality (Ahmad et al., 2020; Nazir et al., 2022). Traditional breeding methods have been employed to address these issues, but they are often time-consuming and less precise. Enhancing disease resistance, stress tolerance, yield, and quality through conventional breeding has proven to be insufficient to meet the growing demands and environmental challenges (Jaganathan et al., 2018; Ansari et al., 2020).

The advent of CRISPR/Cas9 technology has revolutionized the field of genetic engineering, offering a precise, efficient, and versatile tool for genome editing. CRISPR/Cas9 allows for targeted modifications at specific genomic loci, enabling the introduction of desirable traits and the elimination of detrimental ones with unprecedented accuracy (Arora and Narula, 2017; Haque et al., 2021). This technology has shown great promise in improving various crop traits, including disease resistance, stress tolerance, and nutritional quality, making it a powerful tool for wheat genetic improvement (Liu et al., 2022; Erdoğan et al., 2023). Compared to other genome editing tools like TALENs and ZFNs, CRISPR/Cas9 is faster, cheaper, and more efficient, making it an attractive option for crop improvement (Haque et al., 2018; Eş et al., 2019).

This study aims to provide a comprehensive overview of the application of CRISPR/Cas9 technology in wheat genetic improvement. It will cover the current state of research, highlight successful case studies, and discuss the potential challenges and future prospects of using CRISPR/Cas9 in wheat breeding. By examining the advancements and limitations of this technology, this study hopes to shed light on its potential to address the pressing challenges in wheat production and contribute to global food security.

2 Overview of CRISPR/Cas9 Technology

2.1 Basic working principles of CRISPR/Cas9

The CRISPR/Cas9 system, derived from the adaptive immune system of bacteria, has revolutionized genome editing due to its simplicity and efficiency. The system consists of two main components: the CRISPR sequences and the Cas9 protein. CRISPR sequences are short, repetitive DNA sequences found in the genomes of bacteria and archaea, which, when transcribed, form CRISPR RNA (crRNA). These crRNAs guide the Cas9 protein to specific DNA sequences in the genome, where Cas9 introduces double-strand breaks (DSBs) (Arora and Narula, 2017; Bao et al., 2019). The DSBs are then repaired by the cell's natural repair mechanisms, leading to targeted mutations.

The Cas9 protein is an endonuclease that can be programmed to cut DNA at specific sites by using a single-guide RNA (sgRNA). The sgRNA is a synthetic fusion of crRNA and a trans-activating crRNA (tracrRNA), which simplifies the system by combining the targeting and binding functions into a single molecule (Bao et al., 2019; Montecillo et al., 2020). This RNA-guided mechanism allows for precise targeting of genomic sequences, making CRISPR/Cas9 a versatile tool for genetic engineering.

2.2 The role of sgRNA design in targeted editing

The design of the sgRNA is crucial for the specificity and efficiency of the CRISPR/Cas9 system. The sgRNA contains a 20-nucleotide sequence that is complementary to the target DNA sequence, guiding the Cas9 protein to the correct location in the genome (Shan et al., 2014; Doench et al., 2015). The precision of this targeting is essential to minimize off-target effects, which can lead to unintended mutations in the genome. Various computational tools and design rules have been developed to optimize sgRNA sequences, enhancing their on-target activity while reducing off-target effects (Doench et al., 2015; Manghwar et al., 2020).

Effective sgRNA design involves selecting target sites that are unique within the genome and ensuring that the sgRNA has a high binding affinity for the target sequence. Additionally, the presence of a protospacer adjacent motif (PAM) sequence, typically NGG for the commonly used Streptococcus pyogenes Cas9, is necessary for Cas9 binding and cleavage (Montecillo et al., 2020; Zhang et al., 2023). Advances in deep learning and other computational methods have further improved the accuracy of sgRNA design, enabling more efficient and precise genome editing (Zhang et al., 2023).

2.3 Advantages and limitations of the CRISPR/Cas9 system in plant gene editing

The CRISPR/Cas9 system offers several advantages for plant gene editing. Its simplicity and versatility allow for the rapid generation of targeted mutations, facilitating the study of gene function and the development of new plant traits (Kim et al., 2017; Bao et al., 2019). The system can be used to create knockouts, insertions, and precise modifications, making it a powerful tool for crop improvement. Additionally, the ability to multiplex, or target multiple genes simultaneously, further enhances its utility in complex plant genomes (Arora and Narula, 2017; Montecillo et al., 2020).

However, the CRISPR/Cas9 system also has limitations. One of the main challenges is the potential for off-target effects, which can lead to unintended genetic changes. This is particularly problematic in plants with complex and polyploid genomes, such as wheat, where distinguishing between homologous sequences can be difficult (Kim et al., 2017; Cui et al., 2019). Moreover, the efficiency of CRISPR/Cas9-mediated editing can vary depending on the target site and the delivery method used. Despite these challenges, ongoing research and technological advancements continue to improve the precision and efficiency of the CRISPR/Cas9 system in plant gene editing (Jiang et al., 2013; Cui et al., 2019).

3 Wheat Disease Resistance Improvement

3.1 The threat of major wheat diseases to yield

Wheat is a staple crop globally, but its production is significantly threatened by various diseases, notably stripe rust (*Puccinia striiformis f.* sp. *tritici*) and leaf rust (*Puccinia triticina*). These diseases can cause substantial yield losses, with stripe rust alone capable of reducing wheat yields by up to 70% in severe epidemic years (Yuan et al.,

2020). Leaf rust, on the other hand, is known for its widespread occurrence and ability to cause yield losses of up to 50% under favorable conditions for the pathogen (Kumar et al., 2020; Wang and Li, 2024). The economic impact of these diseases is profound, necessitating the development of resistant wheat varieties to ensure food security.

The evolution of virulent pathotypes of these rusts poses a continuous threat to wheat production. For instance, the emergence of new virulent strains of stripe rust has led to frequent epidemics, challenging the durability of existing resistant varieties (Figure 1) (Kumar et al., 2020). Similarly, leaf rust has shown a high degree of adaptability, with new races overcoming previously effective resistance genes. This dynamic nature of pathogen evolution underscores the need for innovative approaches, such as CRISPR/Cas9, to develop durable disease resistance in wheat.

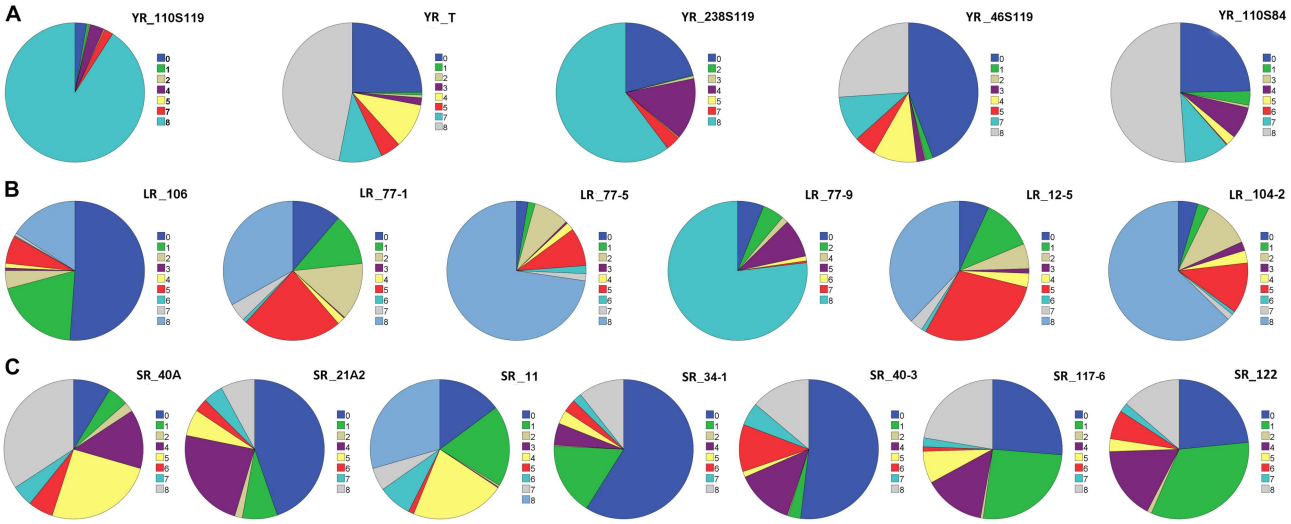

Figure 1 Pie chart representation of seedling response against (A) five pathotypes of stripe rust (YR), (B) six pathotypes of leaf rust (LR), and (C) seven pathotypes of stem rust (SR) of rust association mapping panel (RAMP) (Adopted from Kumar et al., 2020)
Image caption: The color legend on the right side of each pie chart represents the infection type (IT) score. The magnitude of arc length is directly proportional to the frequency of genotypes showing corresponding IT scores (Adopted from Kumar et al., 2020)

3.2 Case Studies on using CRISPR/Cas9 to knock out susceptibility genes for enhanced disease resistance

CRISPR/Cas9 technology has been successfully employed to enhance disease resistance in wheat by targeting and knocking out susceptibility genes. One notable example is the targeting of the *TaHRC* and *Tsn1* genes, which confer susceptibility to *Fusarium* head blight (FHB) and tan spot, respectively. By using CRISPR/Cas9-mediated genome editing, researchers were able to generate wheat plants with mutations in these genes, resulting in enhanced resistance to these diseases (Karmacharya et al., 2023). This approach not only improves disease resistance but also provides insights into the functional roles of these genes in wheat-pathogen interactions.

Another significant case study involves the identification and disruption of 33 susceptibility genes (*S* genes) related to various wheat diseases, including stripe rust, leaf rust, and powdery mildew. The down-regulation or deletion of these *S* genes using CRISPR/Cas9 has been shown to improve disease tolerance in wheat. This strategy highlights the potential of CRISPR/Cas9 to target multiple genes simultaneously, thereby providing a robust and comprehensive approach to enhancing disease resistance in wheat (Taj et al., 2022).

3.3 Identification and targeted editing strategies of disease resistance genes

The identification of disease resistance genes is a critical step in developing resistant wheat varieties. Genome-wide association studies (GWAS) have been instrumental in mapping resistance genes for major wheat diseases. For instance, a study identified several quantitative trait loci (QTLs) associated with resistance to stripe rust, leaf rust, and stem rust in a diverse panel of spring wheat genotypes. These QTLs, located on various chromosomes, provide valuable targets for CRISPR/Cas9-mediated editing to enhance disease resistance (Kumar et al., 2020).

Targeted editing strategies using CRISPR/Cas9 involve precise modifications of resistance genes to improve their efficacy. For example, the *Lr34*/*Yr18* genes, which confer durable resistance to leaf rust and stripe rust, have been mapped to a specific locus on chromosome 7DS. By using CRISPR/Cas9 to introduce targeted mutations in these genes, researchers can enhance their resistance properties and potentially extend their effectiveness to other pathogens, such as powdery mildew (Spielmeyer et al., 2005). This approach not only improves resistance but also helps in understanding the genetic basis of disease resistance in wheat.

4 Wheat Stress Tolerance Improvement

4.1 Impact of abiotic stresses (e.g., drought, salinity, cold) on wheat production

Abiotic stresses such as drought, salinity, and cold significantly impact wheat production, leading to substantial yield reductions globally. These environmental factors create hostile conditions that adversely affect plant growth and development, ultimately compromising overall productivity (Zafar et al., 2020; Bhat et al., 2021; Kumar et al., 2023). Drought stress, for instance, limits water availability, which is crucial for various physiological processes in plants. Salinity stress, on the other hand, disrupts ion homeostasis and water uptake, while cold stress affects membrane fluidity and enzyme activities, all of which are vital for plant survival and growth (Bhat et al., 2021; Nazir et al., 2022; Erdoğan et al., 2023).

The economic implications of these stresses are profound, as they lead to decreased crop yields and increased production costs. Traditional breeding methods have been employed to develop stress-tolerant wheat varieties, but these approaches are often time-consuming and less effective in addressing the multifaceted nature of abiotic stresses (Jaganathan et al., 2018; Karunarathne et al., 2023). Therefore, there is a pressing need for innovative solutions to enhance wheat's resilience to these environmental challenges.

4.2 Application progress of CRISPR/Cas9 in regulating stress tolerance-related genes

The advent of CRISPR/Cas9 technology has revolutionized the field of plant genetic engineering, offering a precise and efficient method for editing stress tolerance-related genes in wheat. This genome-editing tool allows for targeted modifications at specific genomic loci, enabling the development of wheat varieties with enhanced tolerance to abiotic stresses (Zafar et al., 2020; Bhat et al., 2021; Kumar et al., 2023). Researchers have successfully utilized CRISPR/Cas9 to edit genes involved in stress response pathways, such as those regulating osmotic balance, ion transport, and antioxidant defense mechanisms (Nazir et al., 2022; Erdoğan et al., 2023; Nascimento et al., 2023).

One of the significant advancements in this area is the ability to create transgene-free plants, which are more likely to gain public acceptance and regulatory approval. By employing CRISPR/Cas9, scientists can introduce beneficial mutations without incorporating foreign DNA, thus addressing concerns related to genetically modified organisms (GMOs) (Jaganathan et al., 2018; Karunarathne et al., 2023). Additionally, the development of novel CRISPR/Cas9 variants and delivery methods has further improved the specificity and efficiency of gene editing, making it a robust tool for wheat genetic improvement (Biswas et al., 2021; Wang et al., 2022).

4.3 Successful examples of gene editing to enhance wheat stress tolerance

Several successful examples highlight the potential of CRISPR/Cas9 in enhancing wheat stress tolerance. For instance, researchers have edited the *TaERF3* gene, which plays a crucial role in the ethylene response pathway, to improve drought tolerance in wheat. The edited plants exhibited better water-use efficiency and maintained higher photosynthetic rates under drought conditions (Zafar et al., 2020; Kumar et al., 2023). Another notable example is the modification of the *TaHKT1;5* gene, which is involved in sodium transport. By knocking out this gene, scientists have developed wheat varieties with improved salinity tolerance, as the plants were able to maintain ion homeostasis more effectively (Nazir et al., 2022; Erdoğan et al., 2023).

In addition to these examples, CRISPR/Cas9 has been used to edit genes associated with cold tolerance. The *TaCBF1* gene, which is part of the C-repeat binding factor (CBF) pathway, was targeted to enhance cold tolerance in wheat. The edited plants showed increased survival rates and better growth under low-temperature conditions (Jaganathan et al., 2018; Karunarathne et al., 2023). These successful applications demonstrate the versatility and

effectiveness of CRISPR/Cas9 in addressing various abiotic stresses, paving the way for the development of resilient wheat varieties capable of thriving in challenging environments (Figure 2) (Biswas et al., 2021; Wang et al., 2022).

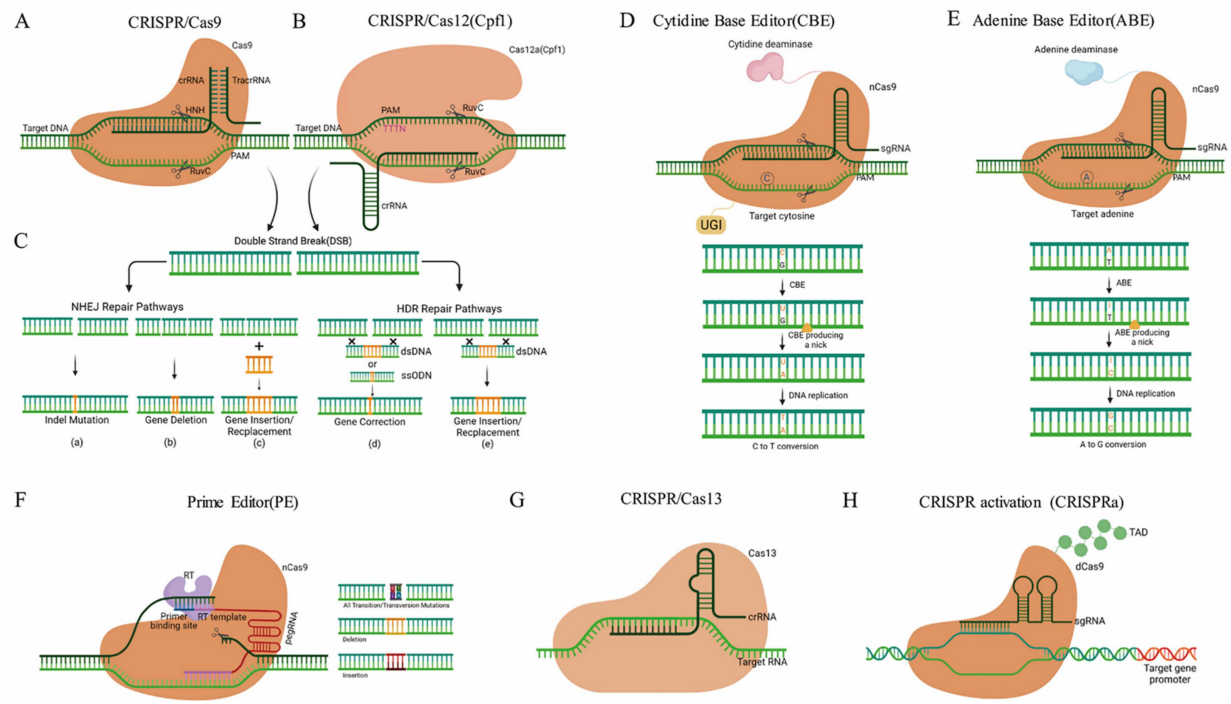

Figure 2 The methodology of major CRISPR/Cas systems (Adopted from Wang et al., 2022)

Image caption: (A) CRISPR/Cas9 induces double-stranded breaks (DSBs) in DNA strands. (B) CRISPR/Cas12a cleaves the target DNA and introduces DSBs. (C) CRISPR/Cas methods can achieve different research goals: (a-c) are results of non-homologous end-joining NHEJ, and (d,e) are results of the homology-directed repair HDR repair pathways using a donor DNA template. (D – F) Base editing tools mainly include Cytidine Base Editor (CBE), Adenine Base Editor (ABE), and Prime Editor (PE). (D) CBE converts C-G base pairs to T-A base pairs at the target site. (E) ABE converts A-T base pairs to G-C base pairs at the target site. (F) PE is a new base editing system, which enables precise sequence substitution, insertion, and deletion. PE mainly consists of a Cas9 nickase (nCas9), an engineered reverse transcriptase (RT), and pegRNA. PegRNA includes PBS (Primer Binding Site) sequence and RT Template. (G) CRISPR/Cas13 consists of a Cas13, a crRNA, and a target RNA. Cas13:crRNA complexes bind target RNA and cleave the target RNA. (H) CRISPR transcriptional activation (CRISPRa) consists of a nuclease-deficient Cas9 (dCas9) and transcription activation domain (TAD). CRISPRa activates the transcription of single or multiple target genes (Adopted from Wang et al., 2022)

5 Improvement of Wheat Yield and Quality

5.1 Key traits related to yield (e.g., grain size, grain weight)

The application of CRISPR/Cas9 technology in wheat has shown significant potential in enhancing key yield-related traits such as grain size and grain weight. By targeting specific genes that regulate these traits, researchers have been able to create wheat varieties with improved yield characteristics. For instance, the manipulation of genes involved in the CLAVATA-WUSCHEL pathway, which controls meristem size, has been shown to increase grain yield in maize, suggesting similar potential in wheat (Liu et al., 2021b). Additionally, the editing of genes related to nitrogen use efficiency, such as the *ARE1* ortholog, has resulted in wheat varieties with increased grain yield under nitrogen-limiting conditions (Zhang et al., 2021a).

Moreover, CRISPR/Cas9 has enabled the precise editing of regulatory genes that control grain size and weight, leading to the development of wheat varieties with enhanced yield potential. This technology allows for the creation of new allelic variations in a much faster and more precise manner compared to traditional breeding methods (Zhang et al., 2021). The ability to target multiple genes simultaneously further enhances the potential for yield improvement, making CRISPR/Cas9 a powerful tool in wheat genetic improvement (Arora and Narula, 2017).

5.2 Research outcomes on yield improvement through the editing of regulatory genes

Recent research has demonstrated the effectiveness of CRISPR/Cas9 in improving wheat yield by editing regulatory genes. For example, the targeted mutagenesis of the *TaARE1* gene in wheat has led to increased nitrogen use efficiency and delayed senescence, resulting in higher grain yield (Zhang et al., 2021a). This study highlights the potential of CRISPR/Cas9 to enhance yield-related traits by manipulating key regulatory genes involved in nutrient utilization and plant development.

Another significant outcome is the editing of promoter regions of *CLE* genes, which are involved in the regulation of meristem size and, consequently, yield-related traits. By creating weak promoter alleles and null alleles of these genes, researchers have successfully increased grain yield in maize, providing a promising approach for similar improvements in wheat (Liu et al., 2021b). These findings underscore the potential of CRISPR/Cas9 to fine-tune gene expression and achieve desired agronomic traits.

5.3 Case studies on enhancing wheat grain quality (e.g., protein content, gluten strength) via editing

CRISPR/Cas9 technology has also been employed to enhance wheat grain quality, focusing on traits such as protein content and gluten strength. In one study, researchers targeted four genes related to grain quality: *pinb, waxy, ppo*, and *psy*. These genes are involved in determining wheat grain hardness, starch quality, and dough color. The precise editing of these genes resulted in wheat varieties with improved grain quality attributes, demonstrating the versatility of CRISPR/Cas9 in modulating complex traits (Zhang et al., 2021).

Another case study involved the use of CRISPR/Cas9 to improve the nutritional components of wheat. By targeting specific genes that influence protein content and gluten strength, researchers have been able to develop wheat varieties with enhanced nutritional profiles. This approach not only improves the quality of wheat but also addresses the growing demand for high-quality, nutritious food (Liu et al., 2021a; 2022). The ability to make precise modifications to the wheat genome opens up new possibilities for enhancing grain quality and meeting consumer preferences.

6 Improvement of Wheat Nutritional Content

6.1 The demand for improving wheat nutritional value (e.g., micronutrients, reduction of anti-nutritional factors)

The global demand for wheat with enhanced nutritional value is driven by the need to address malnutrition and improve public health. Wheat is a staple food for a significant portion of the world's population, and its nutritional enhancement can have a profound impact on human health. Traditional breeding methods have been employed to improve wheat's nutritional profile, but these methods are often time-consuming and less precise. The advent of CRISPR/Cas9 technology offers a promising alternative, enabling precise modifications to enhance the nutritional content of wheat (Arora and Narula, 2017; Eş et al., 2019; Liu et al., 2021).

Micronutrient deficiencies, such as those of iron, zinc, and vitamins, are prevalent in many parts of the world. Enhancing the micronutrient content of wheat can help alleviate these deficiencies. Additionally, reducing anti-nutritional factors, such as phytic acid, which inhibits the absorption of essential minerals, is crucial for improving the bioavailability of nutrients in wheat. CRISPR/Cas9 technology allows for targeted editing of genes involved in nutrient biosynthesis and anti-nutritional factor production, making it a powerful tool for nutritional improvement (Chen et al., 2019; Li et al., 2021; Wang et al., 2021).

6.2 Research examples of enhancing nutritional content through CRISPR/Cas9

Several studies have demonstrated the successful application of CRISPR/Cas9 in enhancing the nutritional content of wheat. For instance, researchers have used CRISPR/Cas9 to target and edit genes involved in the biosynthesis of essential nutrients. One notable example is the editing of the *TaABCC6* gene, which has been shown to increase the bioavailability of zinc and iron in wheat grains by reducing the levels of phytic acid, an anti-nutritional factor (Cui et al., 2019; Wang et al., 2021a).

Another example is the modification of genes involved in the biosynthesis of vitamins. By targeting specific genes, researchers have been able to increase the levels of vitamins such as vitamin E and provitamin A in wheat. These modifications not only enhance the nutritional value of wheat but also contribute to better health outcomes for consumers. The use of CRISPR/Cas9 in these studies highlights its potential to revolutionize the nutritional quality of wheat (Upadhyay et al., 2013; Eş et al., 2019; Wang et al., 2021a).

6.3 Target genes for nutritional content improvement and their editing strategies

Several target genes have been identified for improving the nutritional content of wheat using CRISPR/Cas9. One key target is the *TaABCC6* gene, which is involved in the transport of phytic acid. By knocking out this gene, researchers have been able to reduce phytic acid levels, thereby increasing the bioavailability of essential minerals such as iron and zinc (Cui et al., 2019; Wang et al., 2021a). Another important target is the *TaNFXL1* gene, which has been edited to enhance the accumulation of beneficial nutrients in wheat grains (Cui et al., 2019).

The editing strategies for these genes typically involve the use of single guide RNAs (sgRNAs) to direct the Cas9 endonuclease to specific genomic loci. In some cases, multiple sgRNAs are used to create larger deletions or to target multiple genes simultaneously. This multiplexing approach allows for more comprehensive modifications and can lead to more significant improvements in nutritional content. Additionally, the use of optimized Cas9 variants, such as pcoCas9, has been shown to increase the efficiency and precision of gene editing in wheat (Cui et al., 2019; Wang et al., 2021a).

7 Optimization and Innovation in CRISPR/Cas9 Technology

7.1 Methods to improve editing efficiency

One of the primary methods to enhance the efficiency of CRISPR/Cas9 editing in wheat involves the use of modified Cas9 variants. For instance, a study demonstrated that a plant codon-optimized Cas9 (pcoCas9) yielded more consistent results compared to a codon-optimized Cas9 for expression in algae (crCas9) when targeting specific genes in wheat (Cui et al., 2019). This highlights the importance of using Cas9 variants that are specifically optimized for the target organism to achieve higher editing efficiency. Additionally, the use of ribonucleoprotein (RNP) complexes has been shown to significantly reduce the chances of off-target mutations, thereby increasing the overall efficiency of the genome editing process (Liang et al., 2017).

Another approach to improve editing efficiency is the co-expression of multiple sgRNAs targeting the same gene. This method has been successfully employed to create large deletions in wheat by using pairs of co-expressed sgRNAs, which target different sites within the same gene. This strategy not only increases the frequency of desired editing events but also facilitates the identification and characterization of these events in complex genomes like that of wheat. Furthermore, the development of efficient genotyping protocols to identify edited events in hexaploid genomes has also contributed to the optimization of CRISPR/Cas9 technology in wheat (Cui et al., 2019).

7.2 Techniques for enhancing specificity and accuracy

Enhancing the specificity and accuracy of CRISPR/Cas9-mediated genome editing is crucial to minimize off-target effects. One effective technique involves the optimization of single-guide RNA (sgRNA) parameters. Research has shown that the GC content of the six protospacer-adjacent motif-proximal nucleotides (PAMPNs) in the sgRNA is positively correlated with mutagenesis efficiency, suggesting that careful design of sgRNAs can significantly improve the specificity and efficiency of the CRISPR/Cas9 system. Additionally, the use of well-designed sgRNA plasmids at optimal concentrations has been demonstrated to efficiently generate mutations in multiple genes in a single step (Ren et al., 2014; Zhang et al., 2024).

Another promising approach to enhance specificity is the development of novel Cas proteins and engineered variants. For example, the use of CRISPR/Cas9 ribonucleoprotein (RNP) complexes has been shown to produce transgene-free mutants with a much lower chance of off-target mutations compared to traditional DNA-based CRISPR/Cas9 methods (Liang et al., 2017). This method not only improves the specificity of genome editing but also addresses concerns related to transgene integration, making it a valuable tool for precision crop breeding

(Liang et al., 2017). Furthermore, advancements in base-editing tools that enable targeted nucleotide substitutions have also contributed to the increased specificity and accuracy of CRISPR/Cas9-mediated genome editing in plants (Chen et al., 2019).

7.3 Strategies and Tool Development for Multi-Gene Editing

The development of robust CRISPR/Cas9 systems for multiplex genome editing has opened new avenues for studying gene functions and improving crop traits. A notable example is the creation of a CRISPR/Cas9 vector system that allows for convenient and high-efficiency multiplex genome editing in both monocot and dicot plants. This system utilizes a plant codon-optimized *Cas9* gene and PCR-based procedures to rapidly generate multiple sgRNA expression cassettes, which can be assembled into binary CRISPR/Cas9 vectors in a single cloning step. This approach has been successfully used to edit multiple target sites in rice and Arabidopsis, demonstrating its potential for multi-gene editing in wheat as well (Ma et al., 2015).

Another strategy for multi-gene editing involves the use of co-expressed pairs of sgRNAs targeting different sites within the same gene. This method has been shown to produce large deletions and facilitate the identification of homoeolog-specific editing events in wheat (Cui et al., 2019). Additionally, the application of CRISPR/Cas9 ribonucleoprotein (RNP) complexes has been explored for multi-gene editing, offering a DNA-free approach that reduces the risk of off-target effects and transgene integration (Liang et al., 2017). These advancements in multi-gene editing strategies and tool development are crucial for the efficient manipulation of complex genomes like that of wheat, ultimately contributing to the improvement of crop traits and agricultural productivity.

8 Challenges in Wheat Breeding Applications

8.1 Low editing efficiency and technical barriers (e.g., off-target effects)

One of the primary challenges in applying CRISPR/Cas9 technology to wheat breeding is the relatively low editing efficiency. Wheat, being a polyploid with a complex genome, presents significant difficulties in achieving precise and efficient genome edits. The efficiency of CRISPR/Cas9 in wheat is often lower compared to diploid plants, which complicates the breeding process and reduces the likelihood of obtaining the desired traits in a timely manner (Kim et al., 2017). Additionally, the identification and characterization of edited events in wheat are challenging due to its complex genome structure, which requires robust and efficient genotyping protocols to confirm successful edits (Cui et al., 2019).

Another significant technical barrier is the occurrence of off-target effects, where the CRISPR/Cas9 system inadvertently edits regions of the genome other than the intended target. This can lead to unintended mutations that may affect plant growth and development negatively. Although methods such as the use of CRISPR/Cas9 ribonucleoproteins (RNPs) have been developed to reduce off-target mutations, the risk still exists and needs to be carefully managed (Liang et al., 2017). Ensuring high specificity and minimizing off-target effects are crucial for the successful application of CRISPR/Cas9 in wheat breeding (Li et al., 2021).

8.2 Ethical and regulatory challenges in gene editing

The application of CRISPR/Cas9 in wheat breeding also faces significant ethical and regulatory challenges. Public perception of genetically modified organisms (GMOs) remains a contentious issue, with concerns about the safety and long-term impacts of consuming genetically edited crops. These concerns are often amplified by a lack of understanding of the technology and its benefits, leading to resistance against the adoption of CRISPR/Cas9-edited crops (Eş et al., 2019). Ethical considerations also include the potential for unintended ecological impacts, such as the spread of edited genes to wild relatives, which could disrupt local ecosystems.

Regulatory frameworks for gene editing vary widely across different countries, creating a complex landscape for the commercialization of CRISPR/Cas9-edited wheat. In some regions, CRISPR/Cas9-edited crops are subject to the same stringent regulations as traditional GMOs, which can delay the approval process and increase the cost of bringing new varieties to market (Haque et al., 2018). Harmonizing these regulations and ensuring they are based on scientific evidence rather than public fear is ssential for the widespread adoption of CRISPR/Cas9 technology in wheat breeding (Liu et al., 2021).

8.3 Limitations and potential of CRISPR/Cas9 in commercial breeding

Despite its potential, CRISPR/Cas9 technology has limitations that need to be addressed for its successful application in commercial wheat breeding. One major limitation is the difficulty in achieving large deletions or complex genetic modifications, which are often required for significant trait improvements. While protocols have been developed to create large deletions using pairs of co-expressed sgRNAs, these methods are still not as efficient or reliable as needed for commercial applications (Cui et al., 2019). Additionally, the delivery of CRISPR/Cas9 components into wheat cells remains a technical challenge, with traditional methods often resulting in low transformation efficiencies (Arora and Narula, 2017).

However, the potential of CRISPR/Cas9 in commercial wheat breeding is immense. The technology allows for precise modifications at specific genomic locations, enabling the development of wheat varieties with improved traits such as disease resistance, drought tolerance, and enhanced nutritional content (Chen et al., 2019). The use of DNA-free methods, such as CRISPR/Cas9 RNPs, also holds promise for producing transgene-free edited plants, which could alleviate some regulatory and public acceptance issues (Liang et al., 2017). As the technology continues to advance, it is expected that these limitations will be overcome, paving the way for the widespread adoption of CRISPR/Cas9 in commercial wheat breeding (Bortesi and Fischer, 2015).

9 Concluding Remarks

The application of CRISPR/Cas9 technology in wheat genetic improvement is rapidly evolving, with significant advancements in multi-gene editing and precision breeding. The ability to target multiple genes simultaneously using multiplex sgRNA-CRISPR/Cas9 systems has been demonstrated to be highly effective in creating complex trait modifications. For instance, the successful editing of five *TaSal1* homologous genes in wheat using three gRNAs showcases the potential of this technology to address traits like drought tolerance by inducing heritable mutations across multiple loci. Additionally, the use of CRISPR/Cas9 ribonucleoproteins (RNPs) has been shown to reduce off-target effects and avoid transgene integration, making the process more precise and acceptable for commercial applications. These trends indicate a move towards more sophisticated and precise breeding techniques that can address multiple traits simultaneously, thereby accelerating the breeding process and enhancing crop resilience and productivity.

Combining CRISPR/Cas9 technology with traditional and modern breeding methods holds great promise for the future of wheat genetic improvement. Traditional breeding methods have long been used to enhance crop traits, but they are often time-consuming and less precise. The integration of CRISPR/Cas9 with these methods can significantly speed up the breeding process and increase precision. For example, the use of CRISPR/Cas9 in conjunction with marker-assisted selection can help in the rapid identification and incorporation of desirable traits. Moreover, the combination of CRISPR/Cas9 with other genome editing tools like TALENs and ZFNs can provide a more comprehensive approach to genome manipulation, allowing for the fine-tuning of gene expression and the development of crops with enhanced traits. This hybrid approach can lead to the development of wheat varieties that are not only high-yielding but also resistant to diseases and environmental stresses.

The potential of CRISPR/Cas9 technology to contribute to global food security is immense. As the global population continues to rise, the demand for food is expected to increase significantly. Traditional breeding methods alone may not be sufficient to meet this demand. CRISPR/Cas9 offers a powerful tool to enhance crop yields, improve nutritional quality, and develop resistance to pests and diseases, thereby playing a crucial role in ensuring food security. For instance, the application of CRISPR/Cas9 in editing genes related to stress responses in wheat has shown promising results in developing drought-tolerant varieties, which are essential for maintaining crop productivity under changing climatic conditions. Furthermore, the ability to produce transgene-free mutants using CRISPR/Cas9 RNPs makes the technology more acceptable to regulatory bodies and consumers, paving the way for its widespread adoption in agriculture. By enabling the rapid and precise improvement of crop traits, CRISPR/Cas9 technology can help secure a stable and nutritious food supply for the growing global population.

Acknowledgments

We would like to thank CropSci Publisher continuous support throughout the development of this study.

Funding

This research was funded by agrant from Zhejiang Science and Technology Major Program on Agricultural New Variety Breeding (2021C02064-3-4).

Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Reference

Ahmad S., Wei X., Sheng Z., Hu P., and Tang S., 2020, CRISPR/Cas9 for development of disease resistance in plants: recent progress, limitations and future prospects, Briefings in Functional Genomics, 19(1): 26-39.
https://doi.org/10.1093/bfgp/elz041

Ansari W., Chandanshive S., Bhatt V., Nadaf A., Vats S., Katara J., Sonah H., and Deshmukh R., 2020, Genome editing in cereals: approaches, applications and challenges, International Journal of Molecular Sciences, 21(11): 4040.
https://doi.org/10.3390/ijms21114040

Arora L., and Narula A., 2017, Gene editing and crop improvement using CRISPR-Cas9 system, Frontiers in Plant Science, 8: 1932.
https://doi.org/10.3389/fpls.2017.01932

Bao A., Burritt D., Chen H., Zhou X., Cao D., and Tran L., 2019, The CRISPR/Cas9 system and its applications in crop genome editing, Critical Reviews in Biotechnology, 39(3): 321-336.
https://doi.org/10.1080/07388551.2018.1554621

Bhat M., Mir R., Kumar V., Shah A., Zargar S., Rahman S., and Jan A., 2021, Mechanistic insights of CRISPR/Cas mediated genome editing towards enhancing abiotic stress tolerance in plants, Physiologia Plantarum, 172(2): 1255-1268.
https://doi.org/10.1111/ppl.13359

Biswas D., Saha S., and Dey A., 2021, CRISPR-Cas genome-editing tool in plant abiotic stress-tolerance, Plant Gene, 26: 100286.
https://doi.org/10.1016/J.PLGENE.2021.100286

Bortesi L., and Fischer R., 2015, The CRISPR/Cas9 system for plant genome editing and beyond, Biotechnology Advances, 33(1): 41-52.
https://doi.org/10.1016/j.biotechadv.2014.12.006

Chen K., Wang Y., Zhang R., Zhang H., and Gao C., 2019, CRISPR/Cas genome editing and precision plant breeding in agriculture, Annual Review of Plant Biology, 70(1): 667-697.
https://doi.org/10.1146/annurev-arplant-050718-100049

Cui X., Balcerzak M., Schernthaner J., Babic V., Datla R., Brauer E., Labbé N., Subramaniam R., and Ouellet T., 2019, An optimised CRISPR/Cas9 protocol to create targeted mutations in homoeologous genes and an efficient genotyping protocol to identify edited events in wheat, Plant Methods, 15: 119.
https://doi.org/10.1186/s13007-019-0500-2

Doench J., Fusi N., Sullender M., Hegde M., Vaimberg E., Donovan K., Smith I., Tothova Z., Wilen C., Orchard, R., Virgin H., Listgarten J., and Root D., 2015, Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9, Nature Biotechnology, 34(2): 184-191.
https://doi.org/10.1038/nbt.3437

Erdoğan İ., Cevher-Keskin B., Bilir Ö., Hong Y., and Tör M., 2023, Recent developments in CRISPR/Cas9 genome-editing technology related to plant disease resistance and abiotic stress tolerance, Biology, 12(7): 1037.
https://doi.org/10.3390/biology12071037

Eş I., Gavahian M., Martí-Quijal F., Lorenzo J., Khaneghah A., Tsatsanis C., Kampranis S., and Barba F., 2019, The application of the CRISPR-Cas9 genome editing machinery in food and agricultural science: current status, future perspectives, and associated challenges, Biotechnology Advances, 37(3): 410-421.
https://doi.org/10.1016/j.biotechadv.2019.02.006

Haque E., Taniguchi H., Hassan M., Bhowmik P., Karim M., Śmiech M., Zhao K., Rahman M., and Islam T., 2018, Application of CRISPR/Cas9 genome editing technology for the improvement of crops cultivated in tropical climates: recent progress, prospects, and challenges, Frontiers in Plant Science, 9: 617.
https://doi.org/10.3389/fpls.2018.00617

Jaganathan D., Ramasamy K., Sellamuthu G., Jayabalan S., and Venkataraman G., 2018, CRISPR for crop improvement: an update review, Frontiers in Plant Science, 9: 985.
https://doi.org/10.3389/fpls.2018.00985

Jiang W., Zhou H., Bi H., Fromm M., Yang B., and Weeks D., 2013, Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice, Nucleic Acids Research, 41(20): e188-e188.
https://doi.org/10.1093/nar/gkt780

Karmacharya A., Li D., Leng Y., Shi G., Liu Z., Yang S., Du Y., Dai W., and Zhong S., 2023, Targeting disease susceptibility genes in wheat through wide hybridization with maize expressing Cas9 and guide RNA, Molecular Plant-Microbe Interactions, 36(9): 554-557.
https://doi.org/10.1094/MPMI-01-23-0004-SC

Karunarathne S., Walker E., Sharma D., Li C., and Han Y., 2023, Genetic resources and precise gene editing for targeted improvement of barley abiotic stress tolerance, Journal of Zhejiang University-SCIENCE B, 24(12): 1069-1092.
https://doi.org/10.1631/jzus.B2200552

Kim D., Alptekin B., and Budak H., 2017, CRISPR/Cas9 genome editing in wheat, Functional & Integrative Genomics, 18: 31-41.
https://doi.org/10.1007/s10142-017-0572-x

Kumar D., Kumar A., Chhokar V., Gangwar O., Bhardwaj S., Sivasamy M., Prasad S., Prakasha T., Khan H., Singh R., Sharma P., Sheoran S., Iquebal M., Jaiswal S., Angadi U., Singh G., Rai A., Singh G., Kumar D., and Tiwari R., 2020, Genome-wide association studies in diverse spring wheat panel for stripe, stem, and leaf rust resistance, Frontiers in Plant Science, 11: 748.
https://doi.org/10.3389/fpls.2020.00748

Kumar M., Prusty M., Pandey M., Singh P., Bohra A., Guo B., and Varshney R., 2023, Application of CRISPR/Cas9-mediated gene editing for abiotic stress management in crop plants, Frontiers in Plant Science, 14: 1157678.
https://doi.org/10.3389/fpls.2023.1157678

Li C., Brant E., Budak H., and Zhang B., 2021, CRISPR/Cas: a Nobel Prize award-winning precise genome editing technology for gene therapy and crop improvement, Journal of Zhejiang University-SCIENCE B, 22(4): 253-284.
https://doi.org/10.1631/jzus.B2100009

Liang Z., Chen K., Li T., Zhang Y., Wang Y., Zhao Q., Liu J., Zhang H., Liu C., Ran Y., and Gao C., 2017, Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes, Nature Communications, 8(1): 14261.
https://doi.org/10.1038/ncomms14261

Liu H., Chen W., Li Y., Sun L., Chai Y., Chen H., Nie H., and Huang C., 2022, CRISPR/Cas9 technology and its utility for crop improvement, International Journal of Molecular Sciences, 23(18): 10442.
https://doi.org/10.3390/ijms231810442

Liu L., Gallagher J., Arevalo E., Chen R., Skopelitis T., Wu Q., Bartlett M., and Jackson D., 2021b, Enhancing grain-yield-related traits by CRISPR-Cas9 promoter editing of maize *CLE* genes, Nature Plants, 7(3): 287-294.
https://doi.org/10.1038/s41477-021-00858-5

Liu Q., Yang F., Zhang J., Liu H., Rahman S., Islam S., Ma W., and She M., 2021, Application of CRISPR/Cas9 in crop quality improvement, International Journal of Molecular Sciences, 22(8): 4206.
https://doi.org/10.3390/ijms22084206

Ma X., Zhang Q., Zhu Q., Liu W., Chen Y., Qiu R., Wang B., Yang Z., Li H., Lin Y., Xie Y., Shen R., Chen S., Wang Z., Chen Y., Guo J., Chen L., Zhao X., Dong Z., and Liu Y., 2015, A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants, Molecular Plant, 8(8): 1274-1284.
https://doi.org/10.1016/j.molp.2015.04.007

Manghwar H., Li B., Ding X., Hussain A., Lindsey K., Zhang X., and Jin S., 2020, CRISPR/Cas systems in genome editing: methodologies and tools for sgrna design, off-target evaluation, and strategies to mitigate off-target effects, Advanced Science, 7(6): 1902312.
https://doi.org/10.1002/advs.201902312

Montecillo J., Chu L., and Bae H., 2020, CRISPR-Cas9 System for plant genome editing: current approaches and emerging developments, Agronomy, 10(7): 1033.
https://doi.org/10.3390/agronomy10071033

Nascimento F., Rocha A., Soares J., Mascarenhas M., Ferreira M., Lino L., Ramos A., Diniz L., Mendes T., Ferreira C., Santos-Serejo J., and Amorim E., 2023, Gene editing for plant resistance to abiotic factors: a systematic review, Plants, 12(2): 305.
https://doi.org/10.3390/plants12020305

Nazir R., Mandal S., Mitra S., Ghorai M., Das N., Jha N., Majumder M., Pandey D., and Dey A., 2022, CRISPR/Cas genome-editing toolkit to enhance salt stress tolerance in rice and wheat, Physiologia Plantarum, 174: e13642.
https://doi.org/10.1111/ppl.13642

Ren X., Yang Z., Xu J., Sun J., Mao D., Hu Y., Yang S., Qiao H., Wang X., Hu Q., Deng P., Liu L., Ji J., Li J., and Ni J., 2014, Enhanced specificity and efficiency of the CRISPR/Cas9 system with optimized sgRNA parameters in *Drosophila*, Cell Reports, 9(3): 1151-1162.
https://doi.org/10.1016/j.celrep.2014.09.044

Shan Q., Wang Y., Li J., and Gao C., 2014, Genome editing in rice and wheat using the CRISPR/Cas system, Nature Protocols, 9(10): 2395-2410.
https://doi.org/10.1038/nprot.2014.157

Spielmeyer W., McIntosh R., Kolmer J., and Lagudah E., 2005, Powdery mildew resistance and *Lr34/Yr18* genes for durable resistance to leaf and stripe rust cosegregate at a locus on the short arm of chromosome 7D of wheat, Theoretical and Applied Genetics, 111: 731-735.
https://doi.org/10.1007/s00122-005-2058-9

Taj M., Sajjad M., Li M., Yasmeen A., Mubarik M., Kaniganti S., and He C., 2022, Potential targets for CRISPR/Cas knockdowns to enhance genetic resistance against some diseases in wheat (*Triticum aestivum* L.), Frontiers in Genetics, 13: 926955.
https://doi.org/10.3389/fgene.2022.926955

Upadhyay S., Kumar J., Alok A., and Tuli R., 2013, RNA-guided genome editing for target gene mutations in wheat, G3: Genes, Genomes, Genetics, 3(12): 2233-2238.
https://doi.org/10.1534/g3.113.008847

Wang H.P., and Li H.M., 2024, Application of molecular marker assisted selection in wheat stress resistance breeding, Triticeae Genomics and Genetics, 15(1): 1-9.
https://doi.org/10.5376/tgg.2024.15.0001

Wang T., Zhang C., Zhang H., and Zhu H., 2021a, CRISPR/Cas9-mediated gene editing revolutionizes the improvement of horticulture food crops, Journal of Agricultural and Food Chemistry, 69(45): 13260-13269.
https://doi.org/10.1021/acs.jafc.1c00104

Wang W., Tian B., Pan Q., Chen Y., He F., Bai G., Akhunova A., Trick H., and Akhunov E., 2021, Expanding the range of editable targets in the wheat genome using the variants of the Cas12a and Cas9 nucleases, Plant Biotechnology Journal, 19(12): 2428-2441.
https://doi.org/10.1111/pbi.13669

Wang Y., Zafar N., Ali Q., Manghwar H., Wang G., Yu L., Ding X., Ding F., Hong N., Wang G., and Jin S., 2022, CRISPR/Cas genome editing technologies for plant improvement against biotic and abiotic stresses: advances, limitations, and future perspectives, Cells, 11(23): 3928.
https://doi.org/10.3390/cells11233928

Yuan C., Singh R., Liu D., Randhawa M., Huerta-Espino J., and Lan C., 2020, Genome-wide mapping of adult plant resistance to leaf rust and stripe rust in CIMMYT wheat line Arableu#1, Plant Disease, 104(5): 1455-1464.
https://doi.org/10.1094/pdis-10-19-2198-re

Zafar S., Zaidi S., Gaba Y., Singla-Pareek S., Dhankher O., Li X., Mansoor S., and Pareek A., 2020, Engineering abiotic stress tolerance via CRISPR-Cas mediated genome editing, Journal of Experimental Botany, 71(2): 470-479.
https://doi.org/10.1093/jxb/erz476

Zhang B.C., Zhu J.H., Fan M., Wang W.D., and Hua W., 2024, Utilizing high-throughput phenotyping for disease resistance in wheat, Molecular Plant Breeding, 15(5): 233-246.
https://doi.org/10.5376/mpb.2024.15.0023

Zhang G., Luo Y., Dai X., and Dai Z., 2023, Benchmarking deep learning methods for predicting CRISPR/Cas9 sgRNA on- and off-target activities, Briefings in Bioinformatics, 24(6): bbad333.
https://doi.org/10.1093/bib/bbad333

Zhang J., Zhang H., Li S., Li J., Yan L., and Xia L., 2021, Increasing yield potential through manipulating of an *ARE1* ortholog related to nitrogen use efficiency in wheat by CRISPR/Cas9, Journal of Integrative Plant Biology, 63(9): 1649-1663.
https://doi.org/10.1111/jipb.13151

Zhang S., Zhang R., Gao J., Song G., Li J., Li W., Qi Y., Li Y., and Li G., 2021a, CRISPR/Cas9-mediated genome editing for wheat grain quality improvement, Plant Biotechnology Journal, 19(9): 1684-1686.
https://doi.org/10.1111/pbi.13647