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# Site-directed Mutagenesis's Prospective Uses in Enhancing Cereal Crops

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

By 2050, there will be 9.8 billion people on the planet, which means that food production must grow by 50% globally. Using conventional plant breeding techniques alone may not be able to meet these increasing demands. A crop's yield, nutritional value, and stress tolerance may all be precisely and quickly improved with the use of genetic engineering tools like site-directed mutagenesis. The most recent site-directed mutagenesis methods—such as genome editing with CRISPR/Cas9—and their uses for crop improvement are covered in this study. A comparison is made between the

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advantages and disadvantages of various methods. The main goals for improving crops are discussed, such as the genes involved in yield, nutrient biosynthesis, disease and insect resistance, and abiotic stress tolerance. We highlight notable applications of site-directed mutagenesis in crops. We evaluate the field's current problems, including unclear regulations and off-target consequences. Though further study and the creation of policies are required, site-directed mutagenesis holds up a lot of hope for providing future food demands in a sustainable manner. This paper summarizes the potential applications of site-directed mutagenesis in targeted crop development, both in the present and the future.

*Keywords: Site-directed mutagenesis; gene modification; off target effect; CRISPR/Cas9; crop improvement.*

## 1. INTRODUCTION

Achieving sustainable food supply for the world's expanding population, which is predicted to reach 10 billion people by the year 2050 – requires improving crop production and nutritional quality (FAO, 2017). To create better crop varieties, traditional breeding methods as well as technological approaches have been applied. But more improvements are required to boost nutrition, increase yields, and provide resilience to biotic and abiotic challenges [1]. Site-directed mutagenesis refers to a group of molecular biology approaches that allow for precise, targeted alteration to be made to the DNA sequence of an organism, a possible method for quickly introducing advantageous mutations into crops without introducing foreign DNA [2]. In contrast to the random mutagenesis used in conventional mutation breeding, site-directed mutagenesis enables precise, targeted modifications to native plant genes [3].

There are presently three different approaches of site-directed mutagenesis that are known: vector-based, PCR-based, and nuclease-based [4]. Through methods like CRISPR-Cas9, genome editing and oligonucleotide-directed mutagenesis, scientists may modify certain genes to activate desired features or deactivate unwanted genes [5]. Compared to previous mutant breeding methods, site-directed mutagenesis provides a more accurate and efficient method for enhancing crops genotypes. The potential to precisely modify genes and regulatory components, which allows researchers to modify certain protein functions or expression patterns, is the main benefit of site-directed mutagenesis. Instead of depending on the random mutagenesis and screening methods utilized in conventional plant breeding, this allows for practical advancements [6].

Site-directed mutagenesis, a precise genetic engineering technique, holds immense promise for advancing agricultural biotechnology, particularly in the realm of cereal crop enhancement [7]. This method involves intentional alterations to specific DNA sequences, enabling scientists to modify genes with a high degree of accuracy. By targeting key genetic loci, researchers can develop cereal crops with desirable traits such as increased yield, improved nutritional content, and enhanced resistance to diseases and environmental stressors. As the global demand for food continues to rise, the application of site-directed mutagenesis in cereal crop improvement offers a sustainable solution to meet these challenges. The prospective uses of site-directed mutagenesis extend beyond basic crop improvement, encompassing a broad spectrum of agricultural innovations. This technique facilitates the study of gene function and the creation of novel plant varieties tailored to specific environmental conditions. By leveraging these genetic modifications, it is possible to develop cereals that thrive in diverse climates and soils, thereby contributing to food security and agricultural sustainability [8]. The ability to precisely edit genes also opens up possibilities for reducing the use of chemical inputs, such as fertilizers and pesticides, further promoting environmentally friendly farming practices. As we delve into the potential applications of site-directed mutagenesis, it becomes evident that this technology is poised to play a pivotal role in shaping the future of cereal crop production [9].

## 2. SITE-DIRECTED MUTAGENESIS TECHNIQUES FOR AGRICULTURAL ENHANCEMENT

Single or multiple sites within the genome can be mutated through the use of site-directed mutagenesis. Till date, three different techniques of site directed mutagenesis are adopted for enhancing crops. They are vector based, PCR based and nuclease based site directed

mutagenesis (Fig. 1) and (Table 1) [10]. One of the first strategies for site-directed mutagenesis was the use of vectors. These include inserting the target gene into a plasmid vector by sub cloning it, mutating the target gene using methods such as cassette mutagenesis or single-primer mutagenesis, and looking for mutants once the bacteria have been transformed [11]. The affordability and ease of use of vector-based techniques are their benefits. But their effectiveness is usually low, and the length of sequence that may be altered is restricted [12]. The other approach is the PCR based methods.

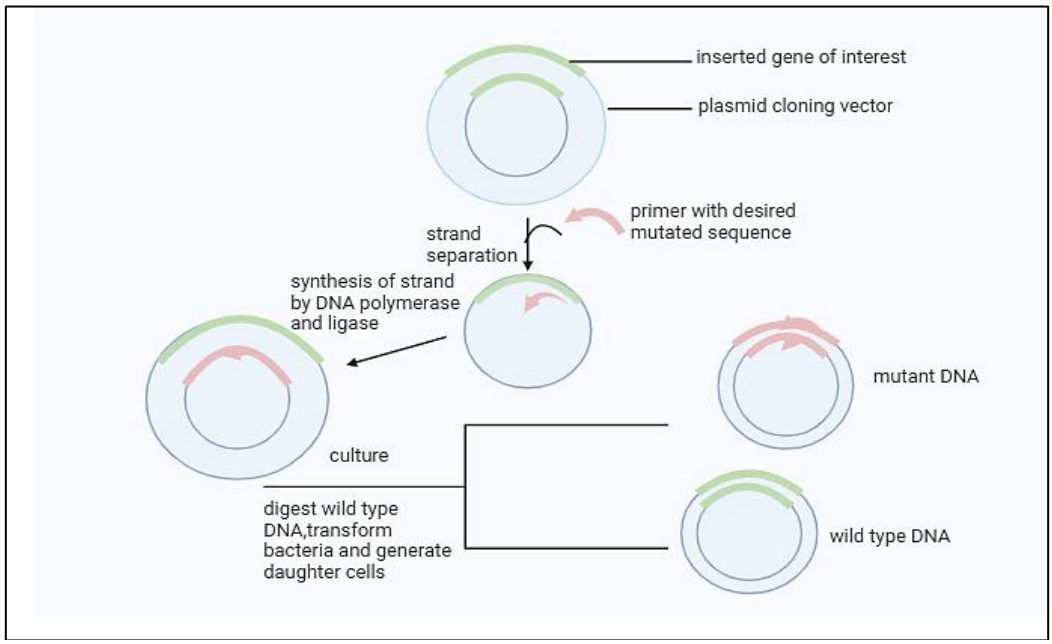


**Fig. 1. General illustration of vector based technique**

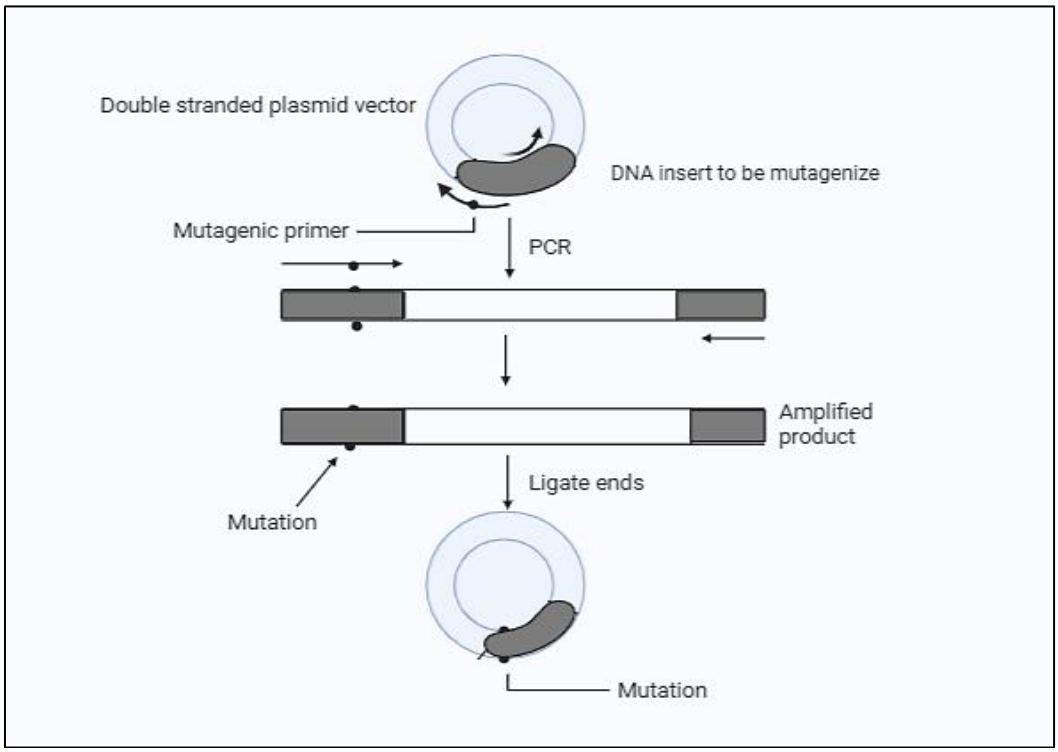


**Fig. 2. General illustration of PCR based technique**

**Table 1. Comparison of different nuclease- based techniques for site directed mutagenesis**

| Features | CRISPR/Cas9 | TALENs | ZFNs |
|---|---|---|---|
| Enzyme source | Found in Bacteria and Eukaryotes | Eukaryotes | Bacteria (Streptococcus sp.) |
| Targeting mechanism | Guide RNA: DNA base pairing | TALE protein: DNA binding | Zinc finger protein: DNA binding |
| DNA cleavage and repair mechanisms | Single- or double-strand break induced by Cas9 | Double-strand break induced by FokI | Double-strand break induced by FokI |
| Targeting length | Flexible, targets 20+ bp sequences + PAM sequence | 30-40 bp sequences | 18-24 bp sequences |
| Rate of mutation (%) | Twenty (20) | Twenty (20) | Ten (10) |
| Design complexity | Easy, design guide RNA | Difficult, complex protein engineering | Difficult, complex protein engineering |
| Multiplexity | Easy | Moderate | Limited |
| Accuracy | High but somehow may have off targets | Less | Less |
| Transmission mode | RNA/DNA | Protein/RNA | Protein/RNA |
| Most suitable for | Gene knockout, transcriptional regulation, base editing | Gene knockout, transcriptional regulation, | Gene knockout, transcriptional regulation, |
| Off- target effect | Moderate | Less | Less |
| **References** | [13-15] | [16,17] | [18,19] |

PCR-based techniques made use of polymerase chain reaction to enable more effective site-directed mutagenesis without the need for subcloning (Fig. 2). The megaprimer approach, inverse PCR, and overlap extension PCR are examples of common PCR procedures [20]. PCR-based methods are simple to use and may alter big constructs with a reasonably high efficiency. However, they call for a number of stages and a unique primer design for every mutation site. Site-directed mutagenesis has been transformed more recently by nuclease-based techniques such as CRISPR/Cas9, TALENs, and zinc finger nucleases [21]. Compared to previous approaches, these nuclease-based procedures are more efficient and selective because they cause double-strand breaks at specific DNA target locations. Because of its cost, simplicity, and versatility, CRISPR/Cas9 has had the most impact [22]. It has been used on a range of crops to enhance characteristics including fruit quality, abiotic stress tolerance, and disease resistance. Even though they are used less frequently these days, TALENs and zinc finger nucleases may still be adapted for tough target areas and have a high specificity [23].

Overall, CRISPR/Cas9 has emerged as the most popular site-directed mutagenesis tool for crops because of its reasonable cost, efficiency, and multiplexed editing potential. Through the development of site-directed mutagenesis techniques, which began with vector-based methods and moved to more sophisticated PCR and now nuclease-driven procedures, genome editing has become more precise and efficient.

## 3. UTILIZATION OF SITE DIRECTED MUTAGENESIS IN CEREAL CROP IMPROVEMENT

With the ability to precisely modify genetic material to increase a variety of desired qualities, site-directed mutagenesis has become a potent tool for agricultural enhancement. Advanced genome editing technologies, especially CRISPR/Cas9, have completely changed plant breeding and speed up the creation of better crop types. Enhancing disease resistance is one of the main uses of site-directed mutagenesis in crop development. Researchers have effectively created rice crop varieties with enhanced resistance to a variety of diseases by focusing on and eliminating susceptibility genes or introducing certain mutations [24]. In the same way, wheat's MLO genes have been edited using TALEN technology to provide heritable resistance to powdery mildew [25]. By focusing on genes related to plant architecture, flowering period, and hormone pathways, site-directed mutagenesis has been shown to be beneficial in increasing agricultural yield and biomass output [26].

Another area where site-directed mutagenesis has shown great promise is improving the nutritional value of crops. Researchers have effectively raised the concentrations of important nutrients and elements that promote health in a variety of crops by focusing on important metabolic pathways [27]. Another important characteristic that has been addressed by site-directed mutagenesis is abiotic stress tolerance. Through the targeting of genes involved in stress response pathways, crop types with increased resistance to salt [28], drought [29], and heavy metal stressors have been created by researchers which ultimately results in the increased yield [30]. The successful use of site-directed mutagenesis in crop improvement has been made possible by ongoing developments in genome editing technology as well as the expanding understanding of molecular biology and plant genetics (Table 2). But it's crucial to address possible issues about adverse effects, legal issues, and the general acceptance of genetically modified crops. Nevertheless, there is a great deal of hope for maintaining sustainable agriculture and global food security if site-directed mutagenesis is widely used in crop modification.

The application of site-directed mutagenesis in enhancing cereal crops has shown significant promise, with various techniques such as CRISPR/Cas9, TALENs, and plasmid vectors being utilized to achieve specific genetic modifications [24-27,30]. In rice, for instance, CRISPR/Cas9 has been employed to improve disease resistance against bacterial blight by knocking out the OsSWEET13 and OsSWEET14 genes. Additionally, this technology has facilitated the enhancement of nutritional benefits, such as high tryptophan content, through vector methods introducing mutations into the OASA2 gene [31-42]. TALENs have been used to increase fragrance by knocking out the osBADH2 gene, while improved grain weight and overall yield have been targeted through CRISPR/Cas9 by mutating genes like Gn1a, DEP1, GS3, and IPA1.

**Table 2. Application of site directed mutagenesis in cereal crop improvement**

| Crops | Improved trait | Technique used | Detail | References |
|---|---|---|---|---|
| Rice | Disease resistance (bacterial blight) | CRISPR/Cas9 | Knockout of OsSWEET13 and OsSWEET14 genes | [24] |
| | Improve nutritional benefits (High tryptophan rice ) | Vector method | Introduction of S126F, Y367A, and L530D mutations into OASA2 | [27] |
| | Increase fragrance | TALENs | High fragrant rice by knocking of osBADH2 gene | [43] |
| | Improved grain weight | CRISPR/Cas9 | Gene knockout | [31,32] |
| | Yield/biomass | CRISPR/Cas9 | Mutations in Gn1a, DEP1, GS3, IPA1 genes | [32] |
| Wheat | Disease resistance (powdery mildew) | TALENs | Editing of MLO genes | [25] |
| | Increased grain number spikelet | CRISPR/Cas9 | mutation on either B or D genome | [33] |
| Maize | Increased starch contain | Plasmid vector | Lys was replaced by Asn, Glu, or Arg to improve phosphoenolpyruvate enzyme catalytic efficiency | [34] |
| | Sterile male maize developed | CRISPR/Cas9 | On zmtms5 gene (chr 2 with exon number 5) m | [35] |
| | Drought tolerance | CRISPR/Cas9 | Changing the promoter of the ARGOS8 gene | [35] |
| | Heritable genome modification | TALENs | Evaluation of mutation efficiency maize glossy2 (gl2) locus | [36] |
| Barley | Generating homozygosity in transgenic barley | CRISPR/Cas9 | producing homozygous mutants, with the Nud gene knockout resulting in naked grains | [37] |
| | Improve malting quality | CRISPR/Cas9 | Mutated the D hordein gene in the cultivar, ‘Golden Promise’. | [32] |

Wheat and maize have also benefited from these advanced genetic techniques. In wheat, TALENs have been used to edit MLO genes, conferring resistance to powdery mildew. The CRISPR/Cas9 system has increased the grain number per spikelet by inducing mutations in specific genomes. In maize, plasmid vectors have been utilized to enhance starch content by improving the catalytic efficiency of the phosphoenolpyruvate enzyme. Moreover, CRISPR/Cas9 has been instrumental in developing sterile male maize and increasing drought tolerance by modifying the zmtms5 gene and the promoter of the ARGOS8 gene, respectively [33,34]. TALENs have facilitated heritable genome modifications, with notable efficiency in the glossy2 (gl2) locus.

Barley improvement through site-directed mutagenesis has focused on producing homozygous mutants and enhancing malting quality. CRISPR/Cas9 has been used to knockout the Nud gene, resulting in naked grains, and to mutate the D hordein gene in the 'Golden Promise' cultivar to improve malting characteristics [31-36]. These targeted genetic modifications not only demonstrate the versatility and precision of site-directed mutagenesis but also highlight its potential to revolutionize cereal crop production, addressing various agronomic and nutritional challenges.

## 4. CHALLENGES AND LIMITATIONS OF SITE DIRECTED MUTAGENESIS

Cereal crop enhancement using site-directed mutagenesis has great potential, but there are still many issues that need to be addressed.

### 4.1 Off Target Effect

The potential for off-target mutations, which could negatively affect agronomic parameters, is a significant disadvantage. Off-target effects are unintended mutations caused by site-directed mutagenesis that occur at genomic locations other than on-target sites. The non-specific binding activity of site-directed nucleases, such as CRISPR/Cas9, may be the cause of this.

### 4.2 Limitation for Polygenic Traits

Although site-directed mutagenesis is effective for basic, monogenic qualities, it is still difficult to improve complicated quantitative features that are regulated by multiple genes. It might be essential, but challenging, to combine numerous trait changes [38]. Since focusing on a single gene frequently results in only small increases in drought tolerance in crops [29], polygenic management of complex quantitative traits also presents challenges [39]. Optimizing methods for multiplex editing can be an active area of research.

### 4.3 Gene Characterization

Targeted mutagenesis depends on identifying and confirming the genes governing advantageous features. However, not all genes that are significant to agronomy have been found and described yet, which reduces the number of possible targets [40]. In order to increase the potential for site-directed mutagenesis, it is necessary to carry out ongoing gene identification initiatives.

### 4.4 Intricacies of Breeding

Following the introduction of targeted mutations in elite lines, additional breeding procedures may be necessary to integrate the trait alterations with the best genetic backgrounds and remove any negative effects of the change. When the breeding process needs to be controlled, variety development becomes more difficult and takes a longer period [41]. Overall, while site-directed mutagenesis has tremendous potential for crop improvement, overcoming challenges related to precision, regulation, trait complexity, gene discovery, and breeding will be important to realizing its full benefits. Continued technological and biological research is still needed.

## 5. FUTURE OUTLOOK FOR THE ROLE OF SITE-DIRECTED MUTAGENESIS IN CROP IMPROVEMENT

Site-directed mutagenesis provides tremendous opportunities to improve cereal crops through accelerated, precision breeding. The ability to precisely edit target genes underpinning complex traits offers new possibilities to develop climate resilient and nutrient-rich cereal varieties [42]. As highlighted in this review, site-directed mutagenesis can potentially enhance diverse traits in cereals related to yield, nutritional quality, biotic/abiotic stress tolerance, and post-harvest characteristics. However, several challenges need to be addressed including off-target mutations, long screening timelines, polygenic control of traits, and delivery barriers in cereals [44]. Continued efforts are required to optimize site-directed mutagenesis in cereals by

improving editing specificity, multiplexing capabilities, high-throughput screening, and innovation in delivery methods tailored for complex cereal genomes [45]. Realizing the full potential of site-directed mutagenesis in cereal improvement requires integrated efforts between scientists across disciplines, industries, policy makers and society. Responsible application built on sound science-based policies will be key to unlock the benefits of site-directed mutagenesis for global food and nutritional security while prudently addressing valid concerns. Overall, this technology holds great promise to accelerate genetic gain in cereals, provided the technical, regulatory and social challenges are appropriately addressed through collective action guided by the spirit of responsible innovation [46,47].

## 6. CONCLUSION

Site-directed mutagenesis is a powerful tool for genetic improvement in cereal crops, enabling precise and accelerated genetic improvement. It allows for the editing of specific gene supporting complex traits, creating new opportunities for developing climate-resilient and nutritionally enriched cereal varieties. However, challenges such as off-target mutations, long screening timelines, polygenic control of traits, and barriers for efficient delivery in cereals with complex genomes need to be addressed. Continued efforts are needed to improve editing specificity, optimize delivery methods, implement multiplex editing for trait stacking, and leverage speed-breeding platforms for rapid screening. Policy considerations regarding regulation and responsible application are crucial for harnessing site-directed mutagenesis for the benefit of society. Overall, this technology holds immense potential to accelerate genetic gain in cereals, provided the technical, regulatory, and social challenges are prudently addressed through collective efforts by researchers, industries, policy makers, and society guided by responsible innovation.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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