A Comparative Review of Genetic Modification Tools for Crop Improvement: Efficiency, Scalability, And Usability

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There have been significant advancements in the field of crop genome editing over the past two decades. However, while many of these methods have been tested individually, there is limited information comparing these methods against one another concerning widespread applicability, scalability, and efficiency. In this review, we discuss the various applications, benefits, and drawbacks of various genome editing tools with regard to efficiency, efficacy, and ease-of-use in the context of crop modification. Zinc finger nucleases, one of the earliest technologies for precise genome editing, demonstrated the feasibility of double-stranded break-inducing genome editing tools in crops. Yet, widespread applicability of ZFNs are limited by the target sequence size and relative complexity in comparison to the newer technologies. Transcription activator-like effector nucleases have demonstrated ease-of-use; however, they remain complex to design. The CRISPR-Cas9 system has the potential for widespread agricultural applications due to its less complex design. These potential applications could have significant effects on crop yield and the ability to sustain crops in areas otherwise not suitable for agricultural use, thus increasing food availability for a growing population. This review emphasizes the potential of CRISPR-Cas9 as the most scalable and user-friendly tool for agricultural applications, highlighting future research directions for genome editing.

Introduction

The global population is projected to reach nine billion by the year 2050 while crop yields in the U.S. only grow at 1.49% annually, potentially producing an inconsistency between the food availability and the growing population[1,2]. In addition to a growing population, diseases and pests also pose a threat to crops, leading to decreased crop yields[3]. Genetically modified organisms (GMOs) present a solution to diseases and pests. GMOs are organisms that have had alterations to their genetic material through gene editing and gene transfer techniques to achieve desirable traits, such as, in the context of agriculture, increased resistance to disease and pests, nutritional content, and crop yield. GMOs provide new avenues of pursuing sustainability in farming and crop security in otherwise unfarmable areas of the globe[3]. GMOs or more widely, genome editing, has advanced significantly with various techniques that enable precise modifications to an organism's DNA. Many protein-based tools for genome editing such as the CRISPR-Cas9 system, transcription activator-like effector nucleases (TALENs), and zinc finger nucleases (ZFNs) work by creating double-strand breaks at specified sites in an organism's genome[4,5]. These technologies have remained at the forefront of research on crop genome editing, and thus, present the most viable avenues for future advancements. Homologous recombination (HR) and non-homologous end joining (NHEJ) have been utilized for both gene knock-in, where HR works in combination with a lab-designed repair template, and gene knock-out, respectively. HR and NHEJ work in combination with tools such as TALENs, CRISPR-Cas9, and ZFNs which create intentional double-strand breaks to be repaired through HR or NHEJ. While the outcomes of these methods have been explored individually, few studies compare the benefits and limitations of these methods to one another. Different methods of genome modification have varying benefits and drawbacks, and evaluating such differences can prove useful in determining the most effective overall method of genome modification in crops.

Methodology

In this review, we explore the different applications, advantages, and limitations of various genome editing tools, focusing on their efficiency, effectiveness, and ease of use in crop modification. A comprehensive literature search was conducted using multiple databases, including PubMed, Scopus, Web of Science, JSTOR, and Google Scholar. The search was initiated in July 2024 and covered studies published from 2018 to 2024. The review included original research articles, reviews, and conference proceedings that provided substantial insights into the development, applications, and challenges of gene editing technologies in crop species. Specific criteria for inclusion were studies on CRISPR-Cas9, ZFNs, and TALENs as tools for crop genome editing, research done on model crops, and studies reporting experimental outcomes. Studies on gene editing technology

applied to non-crop species, theoretical articles, and papers published prior to 2018 were excluded. Extracted data were synthesized and categorized into several areas, including mechanisms of function, applications in crop modifications, efficacy and specificity, as well as limitations. A comparative analysis was conducted to highlight the strengths and limitations of each technology. Quality assessment of the studies was performed based on the clarity of reporting, study design, sample sizes, and reproducibility.

ZFNs

Zinc finger nucleases (ZFNs) use a combination of multiple zinc finger domains (ZFDs), which are used to recognize a specific DNA triplet[4]. Once the target sequence is recognized, a FokI nuclease domain–a site-specific restriction endonuclease–creates double-strand breaks at specific sites[5]. Once a double-strand break is created, the sequence is repaired through the cell's natural repair mechanisms, namely HR[6]. Applicability of ZFNs as a means of genome modification has been demonstrated in an agricultural setting. In one study, researchers tested ZFN efficacy in hexaploid bread wheat, and in all samples tested, found intentional double-strand breaks, indicating that their ZFNs operated with high efficiency and efficacy in hexaploid wheat[7]. This also demonstrates that ZFNs can navigate the complex genomes of polyploid plants. ZFNs are one of the earliest tools developed for genome editing, and have played a foundational role in the creation of other protein-based genome editing methods, as they demonstrated the feasibility of using double-strand breaks in combination with HR as a means of genome modification[6].

Despite their application in agriculture and their significance in the development of gene editing technology, ZFNs have several limitations. In a study in which ZFNs were used to modify pluripotent stem cells, researchers designated ten potential off-target sites based on sequence similarity and, in the 184 clones analyzed, found one off-target mutation[8]. Thus, the potential for off-target effects exists when using ZFNs. Another limitation when using ZFNs is their complexity, and as such, they are less accessible to those without extensive expertise in designing ZFDs. Since each ZFD recognizes a DNA triplet rather than a single nucleotide, designing a domain to match each nucleotide of the target sequence is a complex process that requires expertise in manufacturing domains in order to create an accurate and optimized ZFN, and the target range is limited to roughly 18-bp in length[9]. Additionally, the development, synthesis, and validation of an effective ZFN can take months[9]. Although ZFNs are an established technology with extensive research, their lack of accessibility due to the expensive, complex design and development process is a significant limitation in the face of newer, more streamlined gene editing tools such as TALENs and the CRISPR-Cas9 system[9]. ZFNs do, however, offer a distinct advantage in cases requiring high precision and specificity.

TALENs

TALENs utilize TAL effectors (TALEs), naturally occurring proteins found in plant pathogens, as a DNA binding domain called TALE repeats[10]. TALE repeats work in tandem with engineered, site-specific endonuclease domains to create double-strand breaks at a specified target site, which are then repaired through HR or NHEJ[11]. Unlike ZFNs, TALE repeats have a modular structure which provides a one-to-one correspondence with the target sequence, meaning each domain recognizes a single nucleotide and can be assembled based on this correspondence, whereas a ZFD must be designed to recognize a DNA triplet[9]. As such, TALE repeats can be designed to target a specific sequence through custom array assembly, and these assemblies can be made or extended to any base pair (bp) length, whereas ZFNs are typically limited to 18-bp in length[12]. Thus, the manufacturing of TALENs is easier in comparison to ZFNs, and can be created in days rather than months[9,13]. TALE repeats are highly efficient at binding with target DNA sequences and limiting off-target effects, with affinity rates reaching 96%, similar to that of the CRISPR-Cas9 system[13]. In one study, both TALENs and ZFNs were used to target the same site in the CCR5 gene, an HIV coreceptor, in which TALEN demonstrated significantly fewer off-target mutations in comparison to the ZFN[14]. The study also indicated that TALENs resulted in less cell toxicity in comparison to ZFNs suggesting that TALENs operate with a very low rate of off-target effects in comparison to ZFNs[14]. TALENs have also demonstrated their potential for agricultural applications. In one study, researchers used TALENs to create rice exhibiting a strong resistance to bispyribac-sodium (BS) in comparison to controls, showing the promise of TALENs as a means of precise genome modification in crops[15].

There are still limitations to TALEN technology. For instance, TALENs are significantly larger in size compared to ZFNs. The size of a cDNA that encodes for a TALEN is typically 2 kb larger compared to one that encodes for a ZFN[9]. This makes TALEN delivery particularly challenging. Despite the ability to extend TALENs to greater lengths, it has been demonstrated that longer TALENs show a decrease in accuracy[12]. Though they are easier to develop in comparison to ZFNs, the design and development process of TALENs is complex and costly, presenting issues for commercial and agricultural scalability in terms of production scale. However, due to their modular structure, TALENs present a distinct advantage in cases with complex target sites.

CRISPR-Cas9

The CRISPR-Cas9 system, derived from the immune system of various bacteria and archaea, utilizes a CRISPR array and the Cas9 endonuclease enzyme to introduce double-stranded breaks (DSBs) at a target site[4,16]. In its natural form, the CRISPR array is composed of a library of protospacers taken from foreign DNA

sequences and incorporated into repeated sequences[17]. In the context of genome modification, these protospacers are replaced with a single guide RNA (sgRNA), which designates the target sequence and DSB sites[9,18]. The sgRNA, which is created by combining CRISPR RNA (crRNA) and trans-activating CRISPR RNA into a single molecule, is engineered to be site-specific and complementary to the target sequence[18]. For effective target recognition, a protospacer adjacent motif (PAM), a short, specific sequence adjacent to the target, is required[19]. The sgRNA is tailored to ensure that the Cas9 protein recognizes the specified PAM site, and the Cas9 protein binds to the specific PAM site in order to induce DSBs at the targeted sites[20]. The PAM site acts as a differentiation marker between target and non-target sites. Different Cas9 proteins have different PAM requirements[20]. For instance, *Streptococcus pyogenes* Cas9 (SpCas9), a commonly used Cas9 protein, requires a PAM sequence of "NGG" (where "N" can be any nucleotide)[9]. Once double-stranded breaks are created, the sequence is repaired through NHEJ or HR[16,21]. Unlike ZFNs or TALENs, which require each targeting domain to be designed independently to match the target sequence, the CRISPR-Cas9 system can be adapted to any target site by subcloning the sequence into the sgRNA plasmid backbone, from which sgRNAs can be expressed[17]. The Cas9 protein does not require change whereas the endonuclease enzymes in TALENs and ZFNs are site specific and must be adapted to different targets[17]. This ease of use is a significant advantage when using CRISPR-Cas9 over ZFNs and TALENs. CRISPR-Cas9 has been widely applied in agricultural settings. For example, researchers used CRISPR-Cas9 to improve the yield and introduce different characteristics in rice based on regional demands, demonstrating the capability of CRISPR-Cas9-mediated genome editing in polyploid plants[16,21]. Researchers have also demonstrated the use of CRISPR-Cas9 to increase the grain weight and protein content of wheat by up to twelve percent compared to control models, demonstrating the ability to improve crop characteristics through CRISPR-Cas9[22].

One concern of CRISPR-Cas9 is the size of the Cas9 enzyme. The cDNA that encodes the *S. pyogenes* Cas9 is approximately 4.2 kb, meaning delivery is particularly challenging through viral vectors with limited cargo sizes[9]. However, there is widespread availability of Cas9 proteins in other organisms with varied sizes (i.e. *Neisseria meningitidis* Cas9, the cDNA for which is approximately 3.2 kb), and thus, the application of CRISPR-Cas9 for precise genome modification is not necessarily limited by size[23]. In the context of agriculture, non-viral vectors such as the tumor-inducing (Ti) plasmid or nanocapsules can be used, which typically have larger storage capacities, meaning vector size is not necessarily a limiting factor in crop genome modification[24,25]. The off-target effects of CRISPR-Cas9 when using a highly optimized sgRNA has been demonstrated to range from less than 1% to 5%, negligible compared to natural genetic variation[26,27].

Discussion

In this review, we discuss the applications, potential benefits, and drawbacks of various DSB-inducing methods of precise genome modification when applied to crop modification. We analyze the mechanisms of each method for modification and highlight their varying efficiency, ease of use, as well as scalability for agricultural and commercial uses. While the future development of ZFNs and TALENs should not be overlooked, CRISPR-Cas9 currently stands out for its advantages in regards to agriculture, primarily its ease of use, broad applicability, as well as the significant advancements of CRISPR technology over the past decade. It is important to note that much of the current research available presents the feasibility of using DSB-inducing genome editing tools in crops, and as such, there is limited information available comparing the efficacies of these methods[20]. It is also important to note that while these methods have been tested in crops, the variety of crops tested is also limited[16]. Ethical considerations, especially regarding the potential to alter crop genomes in ways that might inadvertently affect human health or animal welfare, play a significant role in the ability to integrate these techniques into agricultural practices[16]. These concerns are compounded by the fear that widespread genetic modification could reduce biodiversity, especially if modified crops outcompete wild relatives or lead to monocultures, which are more vulnerable to pests and disease[28]. Such fears have influenced policy and the ability to integrate this technology into current practices. Beyond the scope of this review, non-DSB inducing methods have been applied in crop modification successfully. For instance, two non-DSB-inducing methods, pentatricopeptide repeat proteins (PPRs) and RNA interference (RNAi), involve targeting RNA sequences either for modification or suppression of certain gene expression through mRNA degradation, and have most often been used to study specific gene function as well as gene silencing[29]. Other Cas proteins not discussed, such as CeCas12a from *Coprococcus eutactus*, have demonstrated reduced off-target effects in vivo in preliminary studies[30]. Future research should focus on addressing the limitations of each technology, particularly regarding off-target effects and delivery challenges, as well as the integration of this technology into global farming practices[18]. Creating crops that are able to withstand harsh climates would vastly increase the farmable land area available, thus increasing the food availability in unfarmable areas. Advances in delivery methods, such as nanoparticle-based systems or novel vectors, could enhance the effectiveness and accessibility of these tools, as well as facilitate the application of these technologies in other fields such as medicine[25]. Additionally, further studies are necessary to develop robust frameworks for the ethical use of these technologies, ensuring their benefits can be realized without causing long-term environmental or societal harm. Authorities should consider the adoption of risk-based frameworks focused on assessing the actual risks associated

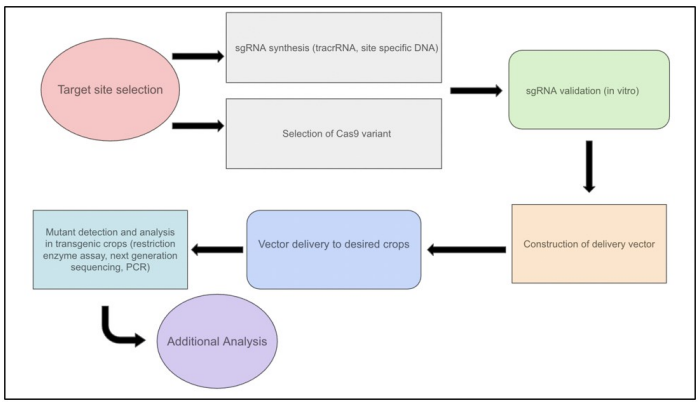

Fig. 1 Flowchart diagram of crop mutagenesis using CRISPR-Cas9. Colors serve to differentiate separate steps.

Table 1 Off-target effects, estimated cost of production, and efficiency across ZFNs, TALENs, and CRISPR-Cas9.

Editing Tool	Off-Target Effects	Estimated Cost (per unit)	Efficiency	References
ZFNs	4% – <0.1%	∼ $12,000	10% – 60%	7,8,29,36
TALENs	2% – <1%	$500 – $10,000	20% – 70%	13,15,30
CRISPR-Cas9	5% – <1%	$80 – $500	67.7% – 97.2%	27,28,31,35

Table 2 Summarized advantages and limitations of each editing tool. Optimal use cases are listed for each tool.

Editing Tool	Advantages	Limitations	Use Cases
ZFNs	• High specificity • Low rate of off-target effects • Wide range of feasible target locations	• Expensive • Low efficiency • Complex manufacturing • Long optimization and validation period	• Small batch crop genome editing, requiring high specificity
TALENs	• High specificity • Flexible target site • Modular structure • Shorter design/validation period	• Expensive • Low efficiency • Complex manufacturing process (though less complex compared to ZFNs) • Challenging delivery	• When precise editing is necessary, especially in cases where target site is complex
CRISPR-Cas9	• Highly efficient • High specificity • Relatively inexpensive • Low rate of off-target effects	• Higher rate of off-target effects compared to other tools • Delivery challenges	• Larger scale, cost-conscious production, requiring high efficiency

with the final product, allowing regulators to more effectively allocate resources to the most pressing safety concerns and facilitate approval processes. Addressing ethical issues as well as public opinion through advances in regulatory science is crucial to clarify how these technologies fit within existing legal structures and to help harmonize global standards.

1 Conclusion

With emerging concerns regarding food availability and starvation due to the growing population, applications of genome modification in agriculture have become more appealing due to their ability to create crops that can be grown in otherwise unfarmable areas. Studies have shown the applicability of ZFNs, TALENs, and CRISPR-Cas9 in agricultural settings. However, the widespread scalability of CRISPR-Cas9 due to its ease-of-use and versatility is a significant advantage over other DSB-inducing methods. Through these technologies, it is possible to create crops that not only yield more but are also more resilient to the challenges posed by climate change, improving sustainability and food availability. These crops can be tailored to thrive in diverse environments, thus expanding the areas in which they can be cultivated, and potentially reducing the pressure on existing farmlands. Investments in these agricultural applications may foster advancements in CRISPR technology and these advancements may pave the way for broader applications in other fields, with the potential to vastly improve food availability across the globe.

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