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REVIEW ARTICLE

Revolutionizing Agriculture: Harnessing CRISPR/Cas9 for Crop Enhancement

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Abstract Plant crops serve as essential sources of nutritional sustenance, supplying vital nutrients to human diets. However, their productivity and quality are severely jeopardized by factors such as pests, diseases, and adverse abiotic conditions. Addressing these challenges using innovative biotechnological approaches is imperative for advancing sustainable agriculture. In recent years, genome editing technologies have emerged as pivotal genetic tools, revolutionizing plant molecular biology. Among these, the CRISPR–Cas9 system has gained prominence due to its unparalleled precision, streamlined design, and heightened success rates. This review article highlights the profound impact of CRISPR/Cas9 technology on crop improvement. The article critically examines the breakthroughs, ongoing enhancements, and future prospects associated with this cutting-edge technology. In conclusion, the utilization of CRISPR/Cas9 presents a transformative shift in agricultural biotechnology, holding the potential to mitigate longstanding agricultural challenges.

Keywords CRISPR/Cas9 · Crop improvement · Genome editing · ZFNs · TALENs · MegaN

Introduction

The worldwide populace persists in its rapid expansion [1], yet there remains an unsatisfactory increase in the accessibility of food resources [2]. Projections suggest that the world's population could reach 10 billion by the year 2050. This rapid population growth, combined with the adverse effects of climatic conditions, poses potential challenges to food security. This situation could be exacerbated by diminishing arable land and declining crop yields. Recent findings from the International Rice Research Institute (IRRI) reveal that approximately every 7.7 s, one hectare of fertile land is lost. The impact of this loss could be even more significant if the current acceleration in global temperatures persists [3, 4]. To ensure food security, there is a pressing need to nearly double crop yield capacities and develop cultivars that are highly resilient to various stresses, as emphasized by Jinek et al. [5]. Horticultural practices, including the cultivation of vegetables, fruits, spices, tubers, and medicinal plants, play a pivotal role in the economy. These crops contribute significantly to both food and nutritional security. With the expanding global population, it becomes imperative to enhance agricultural productivity to sustain a consistent food supply. The advancement of next-generation crops is of paramount importance, given that conventional breeding methods have been extensively employed but are time-consuming [6]. An alternative approach is transgenesis, though its adoption depends on public acceptance for commercialization. Recent progress in recombinant DNA technology involving nucleases like ZFNs, TALENs, and CRISPR/Cas9 has demonstrated its effectiveness in precisely modifying targeted genomic locations [7, 8]. This technique is currently extensively utilized in diverse agricultural crops, and its widespread adoption is eagerly anticipated in the coming years.

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Genome Editing

Genome editing comprises a collection of sophisticated molecular techniques that enable the precise, efficient, and targeted modification of specific nucleotide sequences. Researchers utilize this technology to delve into the genome's capabilities more profoundly and to develop crops that exhibit resistance to pests, offer improved nutritional content, and can be grown in arid environments [9]. The application of genome editing methods based on site-specific nucleases (SSNs) has demonstrated widespread genetic modification across a diverse range of plant species over the past generation. SSNs function by utilizing endonucleases capable of cleaving DNA within a specific region of the genome. The active region of the SSN is tethered to it through either a DNA-binding domain or an RNA sequence. These SSNs are responsible for inducing double-stranded breaks (DSBs) in the targeted DNA. Repair mechanisms like nonhomologous end joining (NHEJ) and homology-directed recombination (HDR) are employed to repair these DSBs, resulting in insertions/deletions (INDELS) and substitutions in the host locus [5]. Upon targeting the specific nucleotide sequences, cellular DNA repair processes lead to changes in gene expression at the designated sites.

Engineered nucleases used in genome editing encompass several types, including designed Meganuclease (MegaN), Zinc finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs) and the Clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) nuclease system. These techniques have facilitated efficient and direct modifications of the genome in a rapid and cost-effective manner.

Engineered Meganuclease (MegaN)

Meganucleases (MegaN) are naturally occurring endonucleases that were first discovered in the late 1980s. These endonucleases possess the ability to identify and cleave extensive nucleotide sequences, ranging from 12 to 40 base pairs, which exhibit substantial variations across diverse genomes. Among exemplary meganucleases are I-SceI, sourced from yeast mitochondria, and I-CreI, derived from algal photosynthetic enzymes. Despite their scarcity in important genomes, meganucleases have been engineered to recognize sequences beyond their original targets. Due to the somewhat longer recognition sites, there is a greater risk of cleavage and subsequently more minor off-target effects. Conversely, the adoption of engineered meganucleases has been comparatively limited when compared to other contemporary nucleases, even though the challenge of adapting meganucleases to recognize new specificities remains [10].

Zinc Finger Nuclease-Based Engineering

Zinc finger nucleases (ZFNs), a class of synthetic nucleases, have brought a revolutionary impact to the realm of programmable nucleases. ZFNs were created by fusing multiple zinc finger DNA-binding domains to the non-specific cleavage property of the restriction endonuclease FokI [7, 9]. This amalgamation allows protein molecules to discern DNA sequences that are only a few nucleotides apart. The paired endonucleases form a dimer, enabling them to cleave double-stranded DNA [11]. Additionally, each motif within the zinc finger array recognizes a distinct 3-nucleotide complementary strand. This offers the flexibility of selecting a variable sequence to suit the desired target. ZFNs were initially applied for sequence-specific mutagenesis in tobacco during the early 2000s, marking one of the earliest instances where engineered endonucleases identified and modified chromosomal DNA [12]. Despite these remarkable achievements, the utilization of ZFNs in agriculture has been constrained, primarily due to factors like the technical intricacies of the production process and the limited availability of target sites compared to more recently developed techniques in functional genomics.

Transcription Activator-Like Effector Nucleases (TALENs)

To enhance the effectiveness, reliability and accessibility of genome editing, the TALEN (transcription activator-like effector nucleases) technology was introduced in 2011. This innovation stemmed from the discovery of transcription activator-like effectors (TALEs) [13]. Much like ZFNs, TALEN constructs synthetic proteins utilizing a flexible arrangement of DNA-binding domains linked to the non-specific cleavage site of FokI. Each unit consists of 33–35 amino acids and recognizes a single nucleotide. The terminal segment, often comprising 20 amino acids, is termed a "half-repeat." The specific amino acids at positions 12 and 13 determine the nucleotide recognition pattern in DNA (e.g., NI identifies adenine, HD identifies cytosine, NG recognizes thymine, while NN recognizes both guanine and adenine) [14]. These naturally occurring TAL effectors offer segmentation advantages that facilitate genome editing in TALENs. Such repeats are harnessed in TALENs to target distinct genetic regions for expression. In conjunction with TAL effector assemblies, additional TALENs, gene-specific activators and regulatory proteins are employed as gene-targeting agents [15]. Compared to Meganucleases and ZFNs, TALENs provide greater flexibility, hence are more commonly used in plant genome editing. However, the complexity of multiple assays poses challenges in the efficient production and distribution of TALENs within plant tissue.

Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR/Cas9 Nuclease System

Much like TALENs, the CRISPR–Cas system draws inspiration from biological processes. Originally discovered within the adaptive immune systems of bacteria and archaea, CRISPR–Cas nucleases play a role in targeting and cleaving foreign nucleic acids based on instructions encoded by CRISPR-associated (Cas) nucleases. Importantly, this mechanism ensures that prokaryotes employing the CRISPR/Cas system do not target their own genomes, as these representations are not found among externally linked prokaryotes. For a target DNA sequence to be recognized and cleaved, a specific short sequence element within a lateral or segmental context is required. The pioneer of genetic modification tools within this system was the *Streptococcus pyogenes* CRISPR–Cas9 system (CRISPR–SpCas9), commonly referred to as "CRISPR–Cas9" [16, 17]. However, it's essential to note that CRISPR–Cas9 pertains only to the traits shared by CRISPR–SpCas9 and its orthologs, to avoid confusion. In the proposed CRISPR–Cas9 system, both spacer-containing CRISPR RNA (crRNA) and single guide RNA (sgRNA) contribute to the enhancement of crRNA (tracrRNA). The later is necessary for the development of the functional unit. The sgRNA guides the nuclease complex to a specific DNA region, resulting in the cleavage of the targeted nucleotide sequence. Cas9's structure is intricate, featuring two nuclease domains, RuvC and HNH [9, 18]. The use of the CRISPR–Cas9 system has brought about various advancements in plant genome engineering. Techniques such as cloning, transferring into plant cells and simplified design have contributed to a high success rate in genome editing approaches [19]. Work flow of Genome Editing utilizing the Cas9 System is depicted in Fig. 1. A Comparative Analysis of Genome Editing Tools is given in Table 1.

Challenging and Unique CRISPR/Cas9 Variations

CRISPR/Cas9 genome editing, a groundbreaking technique, is re-shaping the landscape of genetic manipulation by inducing small cuts in the double-stranded genome of the target organism. This method harnesses the SpCas9 enzyme derived from *Streptococcus pyogenes*. Since its inception in 2013, this technology has been widely embraced by researchers who continue to explore its versatile applications in genome editing. Although Cas9 remains remarkably efficient, its utilization in gene editing is not without limitations [26]. For the successful binding and fragmentation of the target genome, Class 1 enzymes rely on multisubunit proteins, which are abundantly present. "Class 2" predominantly encompasses Type II and Type VI effectors. Both classes of effectors facilitate the recognition and cleavage of target nucleic acids, as well as crRNA. Class 2 Type V and Class 2 Type VI utilize distinct domains, while Class 2 Type II employs a single Ruv domain. Similarly, Class 2 Type V employs a combination of Cas9, RuvC, and HNH nuclease domains [27].

The discovery of RNA-dependent RNase enzyme systems from Class 2 Type II (FnCas9) and Class 2 Type VI (C2c2) has paved the way for innovative genome editing approaches. The *Leptotrichiashahii* bacterium houses the Class 2 Type II C2c2 effector, which is controlled by a single crRNA. This bacterium can be trained to cleave specific ssRNA molecules containing appropriate protospacers. Notably, these effectors exhibit selective cleavage of ssRNAs at varying distances from the crRNA binding site, rather than targeting adenine sequences. Structurally, they consist of two HEPN domains housing catalytic residues. Binding of C2c2 is regulated by a crRNA secondary structure comprising at least one 24-nt stem-loop motif, along with a 22–28-nt complementary sequence to the RNA protospacers. Additionally, a mononucleotide protospacer-flanking site (PFS) consisting of adenine, uracil, or cysteine must be present at the 3' end of the protospacer [27, 28]. In 2013, another RNase-based system was uncovered in the *Francisell* bacterium [29]. This system, known as FnCas9, targets bacterial mRNA without requiring a protospacer adjacent motif (PAM) and can impact gene expression. Notably, this enzyme demonstrated efficacy in combating hepatitis C virus (HCV) in Huh-7.5 cells through RNA inhibition. By attacking both positive and negative strands of the virus's RNA, FnCas9 disrupts viral RNA translation and replication. FnCas9 exhibits a degree of tolerance for mismatches of up to three to six base pairs at the 3′ or 5′ end; however, mismatches exceeding six base pairs lead to a complete loss of activity. Furthermore, this enzyme can also target DNA [30]. These enzymes can be employed in conjunction with viral vectors to modify plants, imparting desired traits. Viral vectors enable high and transient expression of foreign genes for editing. This was effectively demonstrated in instances such as targeted mutagenesis in *Nicotiana benthamiana* and *Petunia hybrida* using the tobacco rattle virus (TRV).

The larger size of SpCas9 (4.2 kb) prevents its expression in plants using the tobacco rattle virus. To address this size constraint, smaller genome editing enzymes from various bacteria—such as SaCas9 (3.2 kb) from *Staphylococcus aureus*, St1Cas9 (3.4 kb) from *Streptococcus thermophilus*, and NmCas9 (3.2 kb) from *Neisseria meningitidis* have been identified as alternatives. These Class 2 Type II immune system enzymes employ the RuvC and HNH domains to cleave double-stranded DNA. Moreover, this group of enzymes specifically targets DNA at a designated site, typically spanning 21–24 nucleotides (nt) in length, located around PAM motifs like 5′-NNGRRT-3′, 5′-NNNRRT-3′, 5′-NNAGAAW-3′, and 5′-NNNNGMTT-3′. In these sequences, N represents

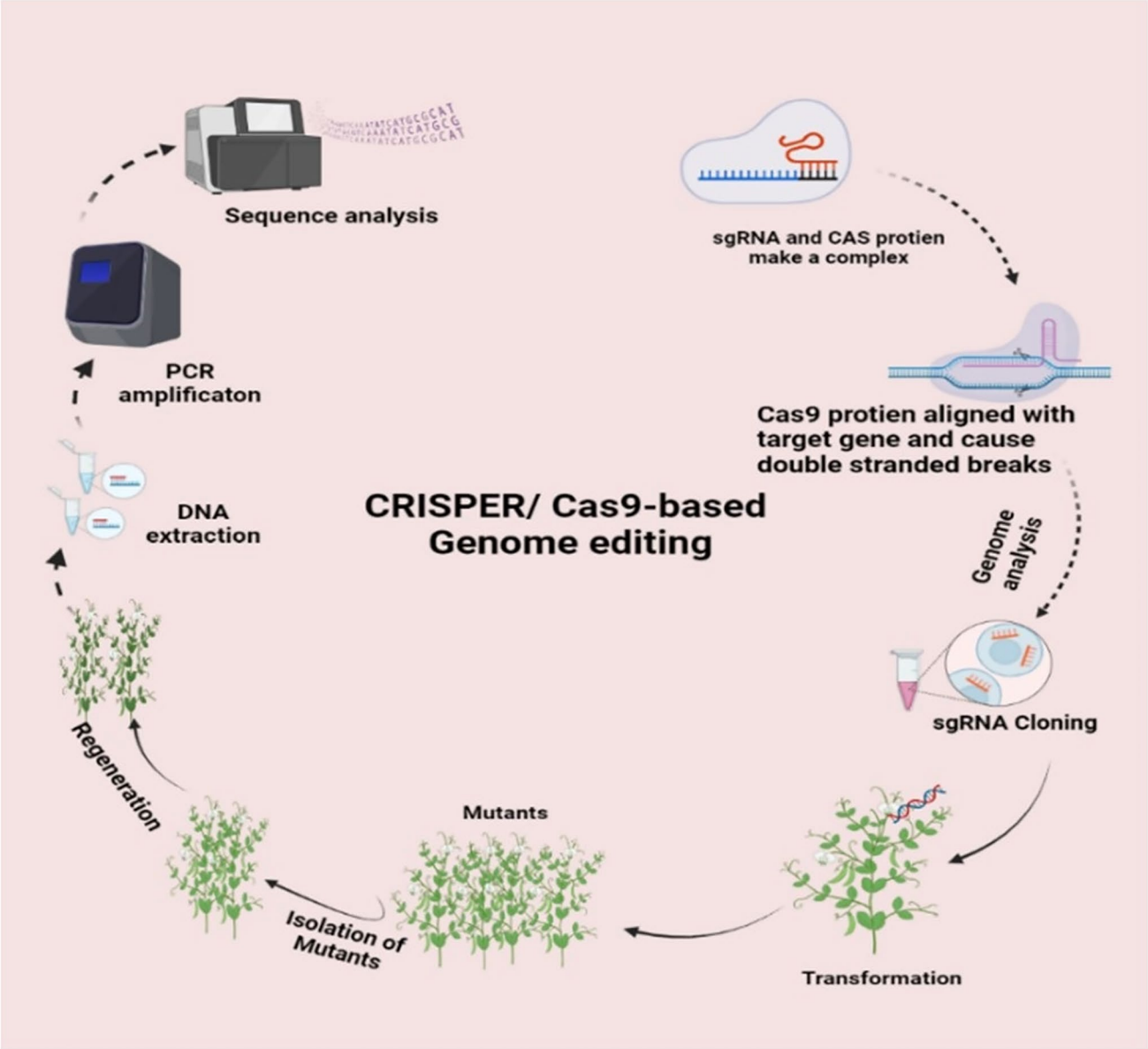


Fig. 1 Workflow of Genome Editing utilizing the Cas9 System. The Cas9 gene-editing process entails the formation of a complex between sgRNA and Cas9 protein, DNA unwinding facilitated by sgRNA, gene cleavage by Cas9, utilization of analysis tools, cloning, transformation and more. Notably, this process does not require any foreign elements for successful editing

any nucleotide, R stands for A or G, M represents A or C, and W represents A or T [31]. Research also indicates that SaCas9 effectively targeted the 5′-NNNGGT-3′ PAM sequence, achieving a notably increased mutation rate (80%) and promoting homologous recombination in the selected lines. For genome editing, these mentioned enzymes primarily focus on a considerably longer PAM sequence [32]. These unique CRISPR/Cas9 variations represent a testament to the dynamic nature of genetic research and the potential for CRISPR technology to revolutionize various fields, from medicine and agriculture to biotechnology and beyond. As scientists continue to refine and expand these variations, the possibilities for precise and targeted genome manipulation are boundless, promising groundbreaking advancements in science and medicine.

Applications of CRISPR/Cas9-Based Genome Editing System in Crop Improvement

In recent times, CRISPR/Cas9-based genome editing has made substantial advancements in enhancing various attributes of crops. However, the scientific community continues to grapple with emerging challenges while

Table 1 Comparative analysis of Genome Editing Tools and their properties

Role	ZFNs	TALENs	MNs	CRISPR/Cas9	Reference
Construction	Requires protein engineering for each individual target	Requires protein engineering for each individual target	Utilizes customizable target recognition sequences	Relies on a 20-Nucleotide sequence of sgRNA	[20]
Efficacy of target recognition	Higher	Higher	Higher	Higher	[20]
Targeting	Relies on protein-DNA interactions, which can be less predictable	Relies on protein-DNA interactions, which can be less predictable	Employs unique DNA binding domains for target specificity	Utilizes DNA-RNA interactions, which are highly predictable	[21]
Delivery	Involves the use of two ZFNs positioned around the target sequence	Requires two TALENs positioned around the target sequence	Uses customizable DNA binding proteins along with RNA guides	Employs sgRNA that is complementary to the target sequence along with the Cas9 protein	[22]
Feasibility of library construction and transformation for genome-wide screens	Technically demanding and complex	Technically demanding and complex	Enables genome-wide studies with improved accuracy	Highly feasible and practical	[23]
Kind of Action	Double-stranded break in target DNA	Double-stranded break in target DNA	Direct conversions in targeted regions	Double-stranded break in target DNA	[21]
Mutagenesis	Higher	Middle	Middle	Lower	[20]
Multiplexing	Difficult	Difficult	Difficult	Possible	[22]
Target range	Unlimited	Unlimited	Unlimited	Limited by PAM	[24]
Effects	Lower	Lower	Lower	Lower	[23]
Cost	Higher	Higher	Higher	Low	[25]
Crop Improvement	Low	Low	Low	Higher	[25]
Range	Narrow	Narrow	Narrow	Broad	[25]
Dimerization	Required	Not-Required	Not-Required	Not-Required	[23]
Types	One	One	One	Many	[23]
Future use	Medium	Medium	Medium	High	[25]

striving to enhance the quality of diverse culinary plants. CRISPR/Cas-based gene editing holds a multitude of applications, including augmenting yield, bolstering disease and pathogen resistance, fortifying herbicide resistance, and boosting stress tolerance. These applications are detailed in Table 2. Gene editing has also been pursued in developmental genes. A host of genes participate in carotenoid production, encompassing Anthocyanin 1 (ANT1), Phytoene Desaturase (SlPDS), Phytochrome Interacting Factor 4 (SlPIF4), and Phytoene Synthetase 1 (PSY1), among others. Notably, under conditions of heat stress, mutant tomato plants with the Slagamous-like 6 (SlAGL6) gene deletion exhibited parthenocarpy fruit development, which would have otherwise impeded fertilization-dependent fruit set. Targeted silencing of the eIF4E gene in melons and tomatoes resulted in resistance to RNA viruses [33, 34]. The CRISPR/Cas9 system was employed to edit the granule-bound starch synthase (GBSS) gene, yielding amylopectin but excluding amylose production [35]. Additionally, the morphology of sweet potatoes (*Ipomoea batatas*) underwent alteration using the CRISPR/Cas9 system [36]. In the case of chili peppers (*Capsicum annuum* L.), a mutation induced by CRISPR/Cas9 conferred resistance to anthracnose [37].

Furthermore, leveraging CRISPR/Cas9 technology, improvements have been made in rice's PIN5b, GS3, GW2, GW5, and GW2 genes, leading to enhanced yield. Fruit-related genes like CLV and ENO have been successfully modified by scientists to enhance fruit production. Addressing celiac disease triggered by gluten in susceptible individuals, the conserved region has been effectively altered using CRISPR/Cas9 technology, resulting in an impressive 85% reduction in immunoreactivity at wheat loci. Similarly, the potential of CRISPR genome editing to enhance Vitamin A and β-Carotene content in plants has been demonstrated [47].

Genome Editing Safety Guidelines

The advancement of crop resilience against biotic, abiotic, and extreme climatic shifts, as well as the resolution of global policy and governance challenges, heavily rely on plant genome editing techniques. In addition to discussing the current state of agriculture, this narrative delves into reflections on the principled adoption of biotechnology, alongside ethical, social and biological considerations associated with the CRISPR/Cas system. Given the

Table 2 Some reports on applications of CRISPR/Cas9 gene editing system for quality and yield enhancement in crops

Crop	Type of modification	Target gene	Target trait/organism	Result/outcome	Reference
Grapes	Gene disruption	MLO7	Powdery mildew	Mutations in target genes; disease resistance not checked	[38]
Grapevine	Gene disruption	*VvWRKY52*	*Botrytis cinerea*	Resistance to gray mold disease	[39]
Rice	Promoter disruption	*OsSWEET11, OsSWEET14*	*X. oryzae* pv. *oryzae*	Mutations in promoter, resistance not checked	[40]
Rice	Gene disruption	*OsSWEET14*	*X. oryzae* pv. *oryzae*	Resistance to bacterial blight	[40]
Rice	Gene disruption	OsERF922	*Magnaporthe oryzae*	Resistance to rice blast	[41]
Rice	Gene disruption	*OsMPK5*	Fungal and bacterial pathogens	Mutations in target, resistance not checked	[41]
Rice	Gene disruption	*eIF4G*	*Rice tungro spherical virus*	Resistance to rice tungro spherical disease	[41]
Tobacco	Gene disruption	Three viral regions *(R, CP,* and *RCR)*	*Tomato yellow mosaic virus*	Significant reduction or attenuation of disease symptoms	[42]
Wheat	Gene disruption	TaEDR1	Powdery mildew	Resistance to *Blumeria graminis f.sp.* tritici	[43]
Apple	Gene disruption	DIPM1, DIPM2, DIPM4	Fire blight disease	Mutations in target genes; disease resistance not checked	[44]
Banana	Viral genome disruption	Viral genes	*Banana streak virus* (BSV)	Inactivation of endogenous banana streak virus integrated in host genome	[45]
Citrus	Gene disruption	CsLOB1	*X. citri* subsp. *citri*	Resistance to citrus canker	[46]
Citrus	Promoter disruption	CsLOB1 promoter	*X. citri* subsp. *citri*	Enhance resistance to citrus canker	[46]

technology's limitations, ethical quandaries surrounding CRISPR have surfaced, prompting the need for both domestic and international attention to navigate questions that serve the broader public interest. Meanwhile, ongoing public discourse revolves around the guidance and regulation of novel methodologies within industrialized nations [47]. The emergence of transgenic plants endowed with nutritional, herbicide-tolerant, and insect-resistant traits has contributed significantly to the surge in genetically modified crop production. A recent estimate indicates that in 2014, 18 million farmers cultivated GMO crops across 181.5 million hectares spanning 28 countries, marking a 3–4 percent increase from 2013 figures [48]. Notable genetically modified crops recently introduced include tomato, corn, soybean, cotton, canola, rice, potato, squash, melon, and papaya. However, soybean, corn, and cotton stand out due to their extensive cultivation and pivotal role in the agricultural economies of numerous nations.

Leading the chart as the world's primary producers and exporters of genetically modified products are the United States, Argentina, and Canada [45]. In India, the management of all GMO-related activities is governed by the Environmental Protection Act (EPA), enacted in 1986. The enforcement of this law falls under the jurisdiction of the Ministry of Environment, Forests, and Climate Change (MoEF & CC) [49, 50].

India's comprehensive regulatory framework governing genetically modified crops falls under the purview of the Department of Biotechnology, a segment of the Ministry of Science and Technology, as well as the Ministry for Environment and Forestry. This intricate structure comprises six authoritative bodies: the State Biotechnology Coordination Committees (SBCC), the Recombinant DNA Advisory Committee (RDAC), the Genetic Engineering Appraisal Committee (GEAC), the Institutional Biosafety Committees (IBSC), and the District Level Committees (DLC). The Government Environmental Assessment Council (GEAC) in India is tasked with evaluating the environmental impact of GMO-related activities spanning research, industrial production, field applications, and environmental discharge. Crucial regulations essential for the development, environmental release, and marketing of GM crops were established by the Indian Parliament. These regulations encompass the Seeds Act of 1966, the Environment Protection Act of 1986, the Forests and Climate Change (MoEF & CC) Act, and the Seeds (Control) Order overseen by the Ministry of Agriculture. Differing perspectives within the Technical Expert Committee established by the Supreme Court of India for Safety and Guidelines for Genetically Modified Agricultural Research underline the importance of this discourse. Amidst India's infrastructure challenges and a lack of well-defined risk assessment and research protocols for genetically modified crops, the pursuit remains imperative given the nation's pressing needs. As India prepares for future deregulation, continued research, infrastructure development and robust marketing and biosafety regulations are essential and new genetically modified organism (GMO) should be assigned a registration number and date, prominently displayed on a dedicated website or portal to facilitate the commercialization of transgenic products, regardless of the country's approval process [51].

CRISPR-CHOPCHOP: A Beginner's Guide to sgRNA Design

In just a span of three years, CRISPR genome editing has brought about fundamental changes to the field of biology with its applications and acceptance continuing to expand. The need for computational tools that streamline CRISPR targeting remains constant, allowing the integration of these principles to accelerate the process of selecting appropriate targets when new CRISPR mechanisms and target selection criteria emerge. Among these tools, CHOPCHOP (https://chopchop.cbu.uib.no/), a widely recognized web application for genome editing with CRISPR and TALEN, stands as a popular choice. CHOPCHOP provides a user-friendly online platform based on the latest comprehensive research to facilitate target selection, primer generation, and restriction site identification (Fig. 2). This tool offers precise localization of various subsections, including coding regions, UTRs, splice sites, and specific exons, spanning both protein-coding and noncoding genes. It adeptly identifies potential off-target sites for all sgRNAs, automatically generates primers for target sites, and presents all relevant elements through a

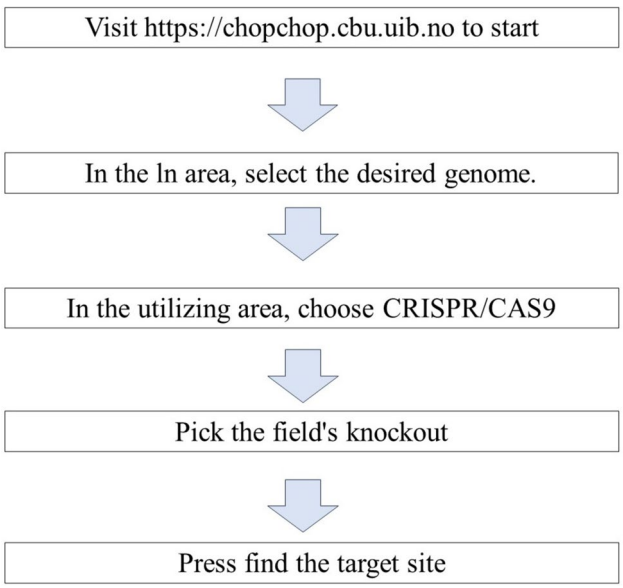
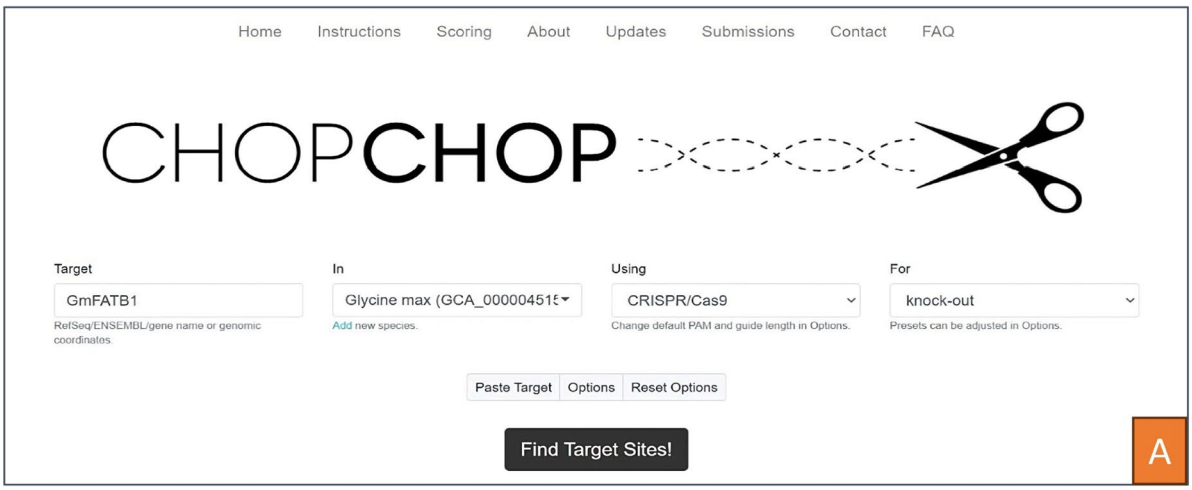
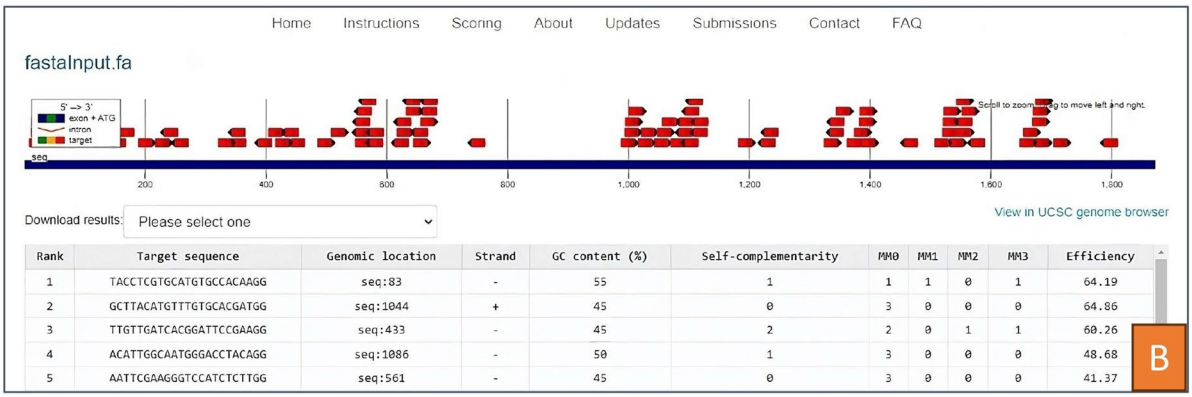
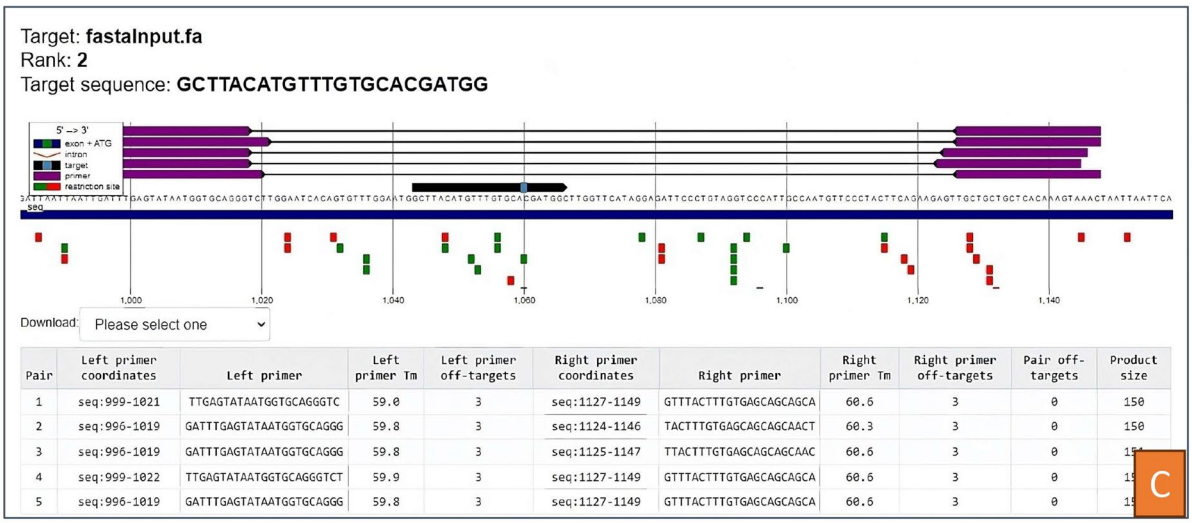


Fig. 2 Workflow of steps in designing sgRNA in CHOPCHOP tool





Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	TACCTCGTGCATGTGCCACAAGG	seq:83	-	55	1	1	1	0	1	64.19
2	GCTTACATGTTTGTGCACGATGG	seq:1044	+	45	0	3	0	0	0	64.86
3	TTGTTGATCACGGATTCCGAAGG	seq:433	-	45	2	2	0	1	1	60.26
4	ACATTGGCAATGGGACCTACAGG	seq:1086	-	50	1	3	0	0	0	48.68
5	AATTCGAAGGGTCCATCTCTTGG	seq:561	-	45	0	3	0	0	0	41.37



Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	seq:999-1021	TTGAGTATAATGGTGCAGGGTC	59.0	3	seq:1127-1149	GTTTACTTTGTGAGCAGCAGCA	60.6	3	0	150
2	seq:996-1019	GATTTGAGTATAATGGTGCAGGG	59.8	3	seq:1124-1146	TACTTTGTGAGCAGCAGCAACT	60.3	3	0	150
3	seq:996-1019	GATTTGAGTATAATGGTGCAGGG	59.8	3	seq:1125-1147	TTACTTTGTGAGCAGCAGCAAC	60.6	3	0	154
4	seq:999-1022	TTGAGTATAATGGTGCAGGGTCT	59.9	3	seq:1127-1149	GTTTACTTTGTGAGCAGCAGCA	60.6	3	0	15
5	seq:996-1019	GATTTGAGTATAATGGTGCAGGG	59.8	3	seq:1127-1149	GTTTACTTTGTGAGCAGCAGCA	60.6	3	0	15

Off-targets		
Location	Number of mismatches	Sequence (including mismatches)
NC_037622.1:113611155	0	CCATCGTGCACAAACATGTAAGC

◂**Fig. 3** Designing Guide RNAs Using CHOPCHOP Software. The process of designing guide RNAs is illustrated from **A** to **D** utilizing the CHOPCHOP bioinformatics tool. **A**—The home page of CHOPCHOP used for designing sgRNAs**. B**—Initial stage for identifying off-target regions within the desired genomic sequence. **C—**Intermediate stage for further identification of off-target regions, displaying %GC content, off-target levels, and primer sequences. **D**—Final stage depicting the completion of off-target analysis for the desired genomic sequence

flexible graphical interface, complete with information pertinent to restriction analysis for further validation [52].

Interpretations of Findings

When interpreting the results obtained from the CHOPCHOP tool, the findings are presented through a color-coded system. Within the result window, the color scheme serves as a key indicator: green signifies the absence of off-targets, orange represents a moderate number of off-targets, and red indicates a higher count of off-targets. It's important to note that when generating an appropriate sgRNA, off-targets are not necessary. Clicking on any green symbol in the results window allows users to progress to the next level of target identification. This subsequent stage provides detailed insights into primer sequences, off-target quantities, and the GC content percentage. Primer pairs are highlighted in a distinctive violet color, while corresponding restriction sites are marked by either green or red boxes, with examples like HindIII. An essential consideration is that the PAM sequence must be an integral part of the target sequence. The table presented below the results showcases the genome's location along with potential off-targets, with any mismatches depicted in red. It's worth noting that the phrase "there are no off targets" is used when no off-targets are detected in the analysis. Upon determining the final target, users are advised to copy both the target sequence and the associated primers directly from the CHOPCHOP website, ensuring accurate and reliable replication of the identified genetic information for further research or applications. Designing Guide RNAs Using CHOPCHOP Software is depicted in Fig. 3.

Envisioning the Future of CRISPR/Cas9 Technology in Crop Enhancement

In the ever-evolving world of agriculture, the quest for crop improvement and enhancement is unceasing. The demands of a growing global population, coupled with environmental challenges and changing consumer preferences, necessitate innovative solutions to ensure food security and sustainability. Among the transformative technologies on the horizon, CRISPR/Cas9 stands as a beacon of hope in revolutionizing crop enhancement. This revolutionary genome editing technology has the potential to reshape the future of agriculture, ushering in a new era of resilient, nutritious and high-yielding crops.

Moreover, the precision of CRISPR/Cas9 technology minimizes unintended genetic alterations, ensuring that the resulting crops meet safety and regulatory standards. This is in contrast to conventional genetic modification techniques, which often involve the insertion of foreign genes (transgenes) into crops, raising concerns about unintended side effects and potential ecological consequences. CRISPR/Cas9 allows for the introduction of desired traits without the use of transgenes, alleviating these concerns and making the resulting crops more acceptable to consumers and regulatory authorities. As we envision the future of CRISPR/Cas9 technology in crop enhancement, it becomes evident that this technology has the potential to unlock a multitude of possibilities. The ability to precisely edit the genetic makeup of crops offers an unprecedented level of control over their traits, opening up new avenues for crop development. However, it is important to acknowledge that the realization of this vision is not without challenges and ethical considerations. As CRISPR/Cas9 technology advances, questions surrounding intellectual property rights, equitable access, and environmental impacts must be carefully addressed. Ethical discussions about the boundaries of genetic modification and its potential consequences for biodiversity and ecosystems will play a pivotal role in shaping the future of this technology in agriculture.

Conclusion

One existing gap in the current landscape is the need for greater precision and efficiency in crop enhancement. While genome editing, including CRISPR/Cas9 technology, shows immense promise, there is room for improvement in its application. This gap can be bridged by advancing our understanding of crop genetics, optimizing delivery methods, and refining the regulatory frameworks to ensure the responsible use of these technologies. Among the cutting-edge methods for crop improvement, genome editing stands out as a promising avenue. Genome editing enables precise modification of desirable crop traits without the introduction of transgenes, ensuring safety for human health and the environment. Within this realm, CRISPR/Cas9 technology emerges as a powerful tool for enhancing crop attributes. By harnessing CRISPR/Cas9, we can revolutionize crop production, opening new avenues to combat pre- and post-harvest yield losses. This technology not only bolsters future food security but also offers a sustainable path towards reducing crop losses, thus fostering economic growth in the agricultural sector. The potential of CRISPR/Cas9 technology in crop enhancement represents a beacon of hope for

more resilient and productive agricultural practices, and with ongoing research and refinement, it holds the promise of closing the existing gap between current agricultural practices and the evolving needs of our expanding nation's economy.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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