Training on Application of Genome Editing Tools for Sustainable Development in Agriculture

Training Manual

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Foreword

Genome editing, or genome engineering, or gene editing, is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. Unlike early genetic engineering techniques that randomly inserts genetic material into a host genome, genome editing targets the insertions to site-specific locations. The basic mechanism involved in genetic manipulations through programmable nucleases is the recognition of target genomic loci and binding of effector DNA-binding domain (DBD), double-strand breaks (DSBs) in target DNA by the restriction endonucleases (FokI and Cas), and the repair of DSBs through homology-directed recombination (HDR) or non-homologous end joining (NHEJ).

The country is now self-sufficient in foods (crops, fish, meat and egg). Research Institutes and extension departments are working hard and implementing various activities to increase the production and quality of the food. One of the goals of the present government is to produce and supply safe and quality foods to build a talented nation, which can be achievable though the use of genome editing. To meet this objective Livestock Division of BARC is going to organize a training program on 'Application of Genome Editing Tools for Sustainable Development in Agriculture' for the Scientists and Teachers of different institutes and universities.

The purpose of this manual is providing support to increase professional knowledge for the agricultural scientists, whom are the target users of this manual. It is an attempt to use genome editing in the agricultural sector in Bangladesh. This manual describes the main tools of the genome editing and discusses how they are being used to apply in the agricultural sector and to further understanding of production. Scientists/Officers/Teachers from BARI, BRRI, BLRI, BFRI, BJRI, BINA, BWMRI NIB, DLS, university of SAU and Lal Teer Livestock Development BD. Ltd. will attend in this training program.

I also acknowledge hard works and the sincere efforts of Dr. Mohammad Rafiqul Islam, Chief Scientific Officer, Livestock Division, BARC who was involved in this exercise. Finally, I would like to extend my sincere thanks to Livestock division, BARC for hard work in preparing this training manual and organizing training course.

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CONTENT

Advancement and Transformation of Agriculture in Bangladesh

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Introduction

Bangladesh has risen to become the 35th largest economy in the world. Agriculture is the center of our economy, decreasing poverty and ensuring food security. Agriculture stands as one of the primary driving forces behind Bangladesh's economy and livelihood, making a significant contribution to the GDP while maintaining an impressive growth rate. Around 40% of the country's workforce is engaged in the agricultural sector. Since gaining independence in 1971, the nation confronted significant food shortages. However, shortly after achieving independence, Bangabandhu Sheikh Mujibur Rahman, recognized as the Father of the Nation, acknowledged the pivotal role of agriculture and implemented bold measures to drive agricultural development, embracing the concept of the Green Revolution. Currently, Bangladesh has attained self-sufficiency in staple grains, fruits, fish, meat, milk, and egg production. Moreover, the country stands on the brink of achieving self-sufficiency in vegetable production. Despite the notable progress, the country's agricultural sector is facing several challenges notably population growth, declining arable land and soil fertility, climate vulnerability, fragile ecosystems (coastal zone, Barind and drought prone areas, haor and flash flood areas, Chattogram hill tracts, river systems and estuaries, urban areas), socio-economic factors and production risk factors (market price fluctuation) (Figure 1). Overcoming these obstacles is vital to ensure food and nutrition security for the nation.

To address the above challenges, the government has taken up some productive national policies and plans like National Agriculture Policy 2018, National Agricultural Extension Policy 2020, National Agricultural Mechanization Policy 2020, National Agricultural Marketing Policy 2023, 8th Five Year Plan 2021-25, Bangladesh Perspective Plan 2041 and Bangladesh Delta Plan 2100. Moreover, agricultural technology innovation by the scientists is in line with the effort to address the great challenge of nourishing the increasing population by growing more food on the decreasing arable land. BARC is entrusted with the responsibility of overseeing and coordinating agricultural research and development (R & D) activities in collaboration with a diverse array of stakeholders and partner organizations. These partners encompass public entities such as NARS (National Agricultural Research System) and universities, as well as private and non-governmental organizations.

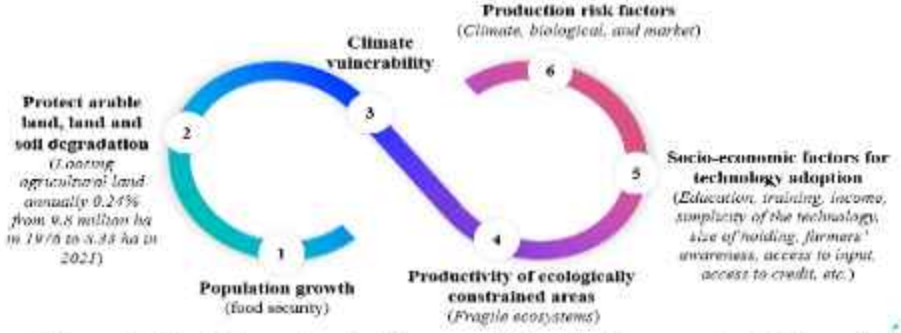


Figure 1: Six (6) major challenges of Agriculture sector in Bangladesh

Major Advancement/Achievements of Agriculture in Bangladesh

Bangladesh has now secured a position among the top 10 global producers of rice (3rd position, after China and India), jute, jackfruit, mango, onion, tea, vegetables, potatoes, fishes and livestock products (Figure 2). The main drivers of this advancement/achievements include increased allocation of revenues to agricultural ADP (Annual Development Plan), agricultural policy reforms, technological innovations, and increased access to agricultural inputs, through price subsidy and production supports. The country has achieved self-sufficiency in rice since 2000 and become surplus from 2005 by 0.37 million ton (M t) to 2021 by 7.55 M t (Figure 4-5; Table-1).

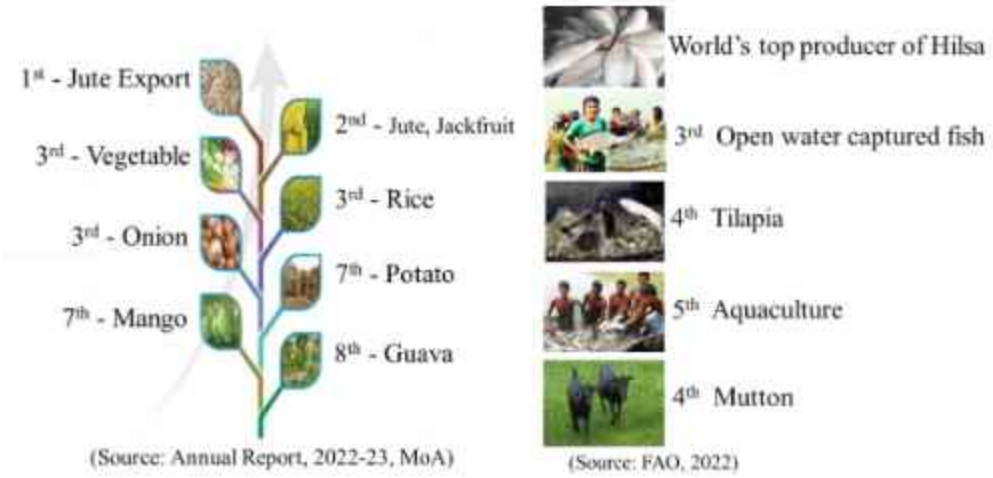


Figure 2: Major achievements of Agriculture under global ranking.

Production Statistics of Crops, Fisheries and Livestock Sectors

a) Crop sectors

Table-1: Production trends of major crops

Crops	Production (Lakh metric ton/Jute-Lakh bale)				
	2006	2009	2018	2022	2023
Rice*	265.30	313.17	362.79	389.36	390.95
Wheat	7.35	8.49	11.53	11.67	11.70
Maize	5.22	7.30	38.93	56.30	64.22
Potato	41.61	52.68	103.17	110.58	104.32
Pulses	2.75	1.96	10.39	8.38	8.79
Oil seeds	3.29	8.40	9.70	12.32	16.04
Vegetables	20.33	29.08	159.54	216.70	225.41
Jute	46.19	46.78	88.95	84.32	84.58

(Source: MoA Annual Report, 2022-23; MoA achievement in last 10 years, 2018. *Milled rice)

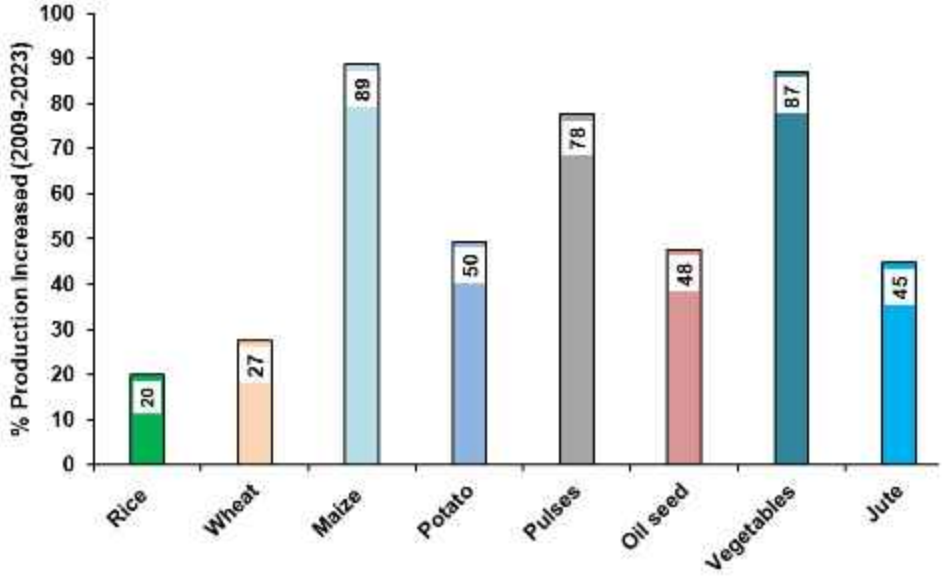


Figure 3: Production increased (%) of major crops from 2009 to 2023 (Source: Table 1).

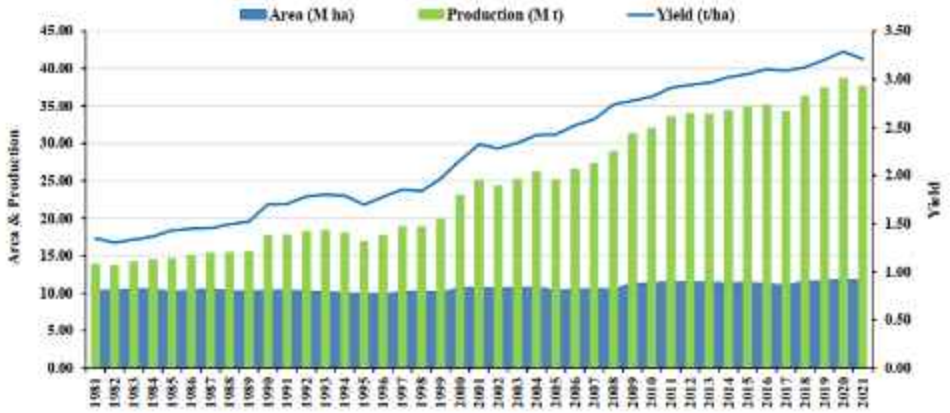


Figure 4: Area, production, and yield of rice in Bangladesh (Source: BBS, 2022).

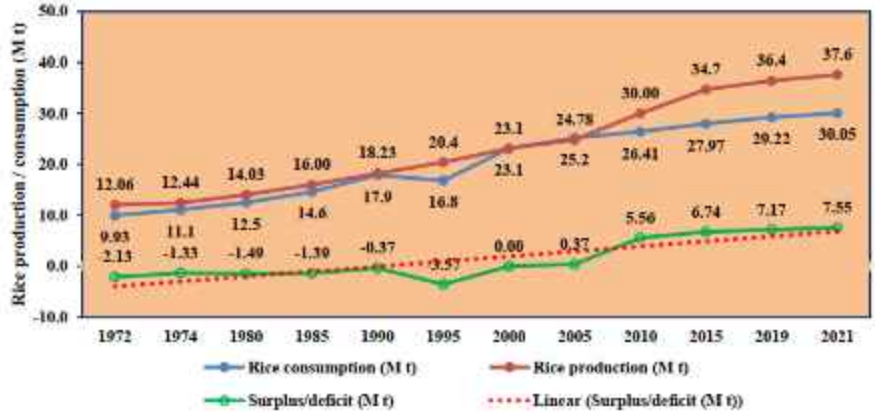
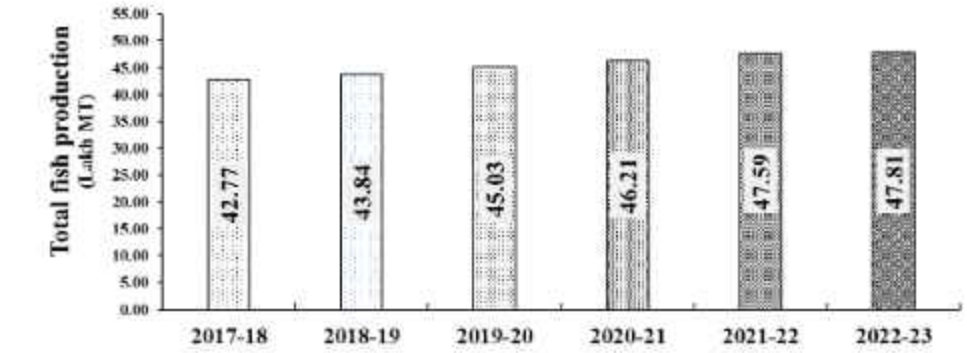
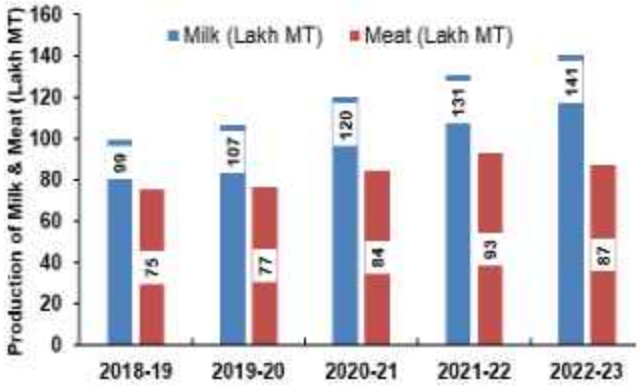


Figure 5: Trend of rice production, deficit to surplus (Source: Bokhtiar & Samsuzzaman, 2023)

b) Fisheries and Livestock sectors





Egg (Billion in Number)

2022-23	: 23.38
2021-22	: 23.35
2020-21	: 20.58
2019-20	: 17.36
2018-19	: 17.11

Figure 6: Production scenarios of fish (upper panel), milk, meat and egg (lower panel).

Transformation of Agriculture in Bangladesh

The agricultural landscape in Bangladesh has undergone a notable transformation, occurring primarily through two key ways: the shift from the *green revolution* to the *gene revolution*, and the restructuring of *subsistence agriculture* into a more *commercially* oriented model. Presently, the nation has embarked on the journey of the Fourth Industrial Revolution (4IR). This agricultural transformation entails a shift in the agri-food system from subsistence and farm-centric approaches to a more commercialized, productive, and off-farm-centered standard. Bangladesh has made significant strides in achieving food security and is currently placing emphasis on ensuring the availability of nutritious and safe food.

a) Green revolution

Dr. Norman E Borlaug, recognized as the pioneer of the Green Revolution, spearheaded global initiatives that substantially elevated crop production. Commencing his efforts in a wheat field in Mexico in 1940, he successfully developed the semi-dwarf high-yielding variety (HYV) known as *miracle wheat* within two decades through hybridization. The remarkable surge in wheat and rice production during the late 1960s marked a transformative moment in global food production, achieved through the adoption of HYVs coupled with the application of fertilizers, irrigation, and pesticides. The Green Revolution, however, had yet to make an impact on the soils of Bangladesh until the initiation by the Father of the Nation, Bangabandhu Sheikh Mujibur Rahman, in the early 1970s under

the banner of "*Sabuj Biplob*" (Green Revolution). While this undertaking successfully led to increased food production, it also incurred a notable loss in biodiversity.

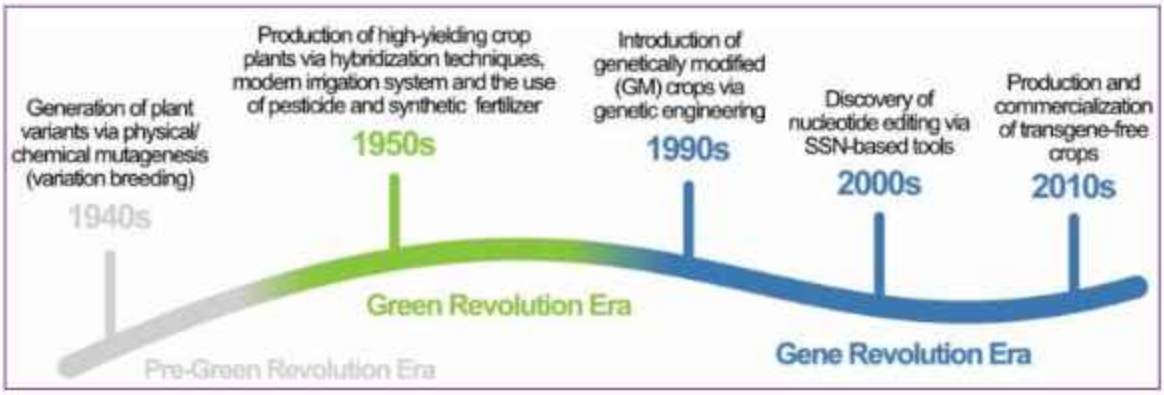


Figure 7: A roadmap showing Shift from Green Revolution to Gene Revolution Era (Source: Hamdan *et al.* 2022; *Plants.* https://doi.org/10.3390/plants11101297). This era - rapid innovations in biotechnology field provide alternative strategies to further improve crop yield, quality, and resilience towards biotic and abiotic stresses.

During the 1990s, it became evident that relying solely on HYVs developed through traditional breeding methods was insufficient to achieve significant advancements in crop production. The extensive use of inputs such as synthetic pesticides and fertilizers had deteriorated soil and environmental health, while the gradual process of conventional breeding appeared inadequate to meet the anticipated yield gains necessary for ensuring food security amid a growing population. Consequently, the concept of biotechnology emerged, initiating a paradigm shift from the *Green Revolution* to the *Gene Revolution*. This shift was marked by the introduction of molecular breeding and genetic engineering techniques, signifying a transformative approach to agricultural innovation (Figure 7).

In the late 20th century, strides in biotechnology, hybridization, high throughput selection techniques, mutation, and transgenic technologies paved the way for the development of genetically improved crop varieties. Emphasis on transgenic breeding to enhance resistance against insects and diseases intensified during this period. Despite progress, challenges surrounding the cost, efficiency, bioethics, regulatory frameworks, and public acceptance of genetically modified crops, exemplified by the case of Golden Rice, have constrained the widespread adoption of these techniques in crop cultivation. In response to these challenges, recent breakthroughs in genome editing technologies, particularly the CRISPR-Cas9 system, have emerged as a transformative tool. This technology facilitates targeted and precise genetic modifications in crop plants at an accelerated pace, fostering the potential for a rapid transition towards precision speed breeding for enhanced crop improvement.

b) Commercial agriculture

In its early stages, higher agricultural production in Bangladesh was primarily centered on subsistence farming. Over time, as the country attained self-sufficiency in food production, particularly in rice, the agricultural landscape has progressively shifted from *subsistence* to *commercial* farming. Evidently,

many farmers are now placing emphasis on cultivating vegetables, fruits, and flowers with significant commercial value, following the cultivation of rice.

The commercialization of agriculture is being pursued through the promotion of value addition to agricultural commodities, with a particular focus on horticultural products like jams, jellies, pickles, and more. This approach not only supports agri-businesses but also establishes crucial links between farmers and both local and international markets. Central to commercial farming are high-value crops, which have become the focal point for educated youths seeking to build their fortunes. Examples of such high-value crops include exotic fruits like dragon fruit and strawberries, as well as vegetables like capsicum, broccoli, and carrots.

The following initiatives need to be taken to the transformation of *subsistence agriculture* to *commercial* one:

- Highly productive technology
- High value and nutrient dense crop variety
- Safe and quality food
- Climate resilient technology/variety
- Digital agriculture (attracting the youth and woman)
- Mechanization (cost minimum, labour shortage)
- Precision agriculture (adapting with 4IR)
- New entrepreneur (e.g. ago-processing)
- Capacity development (Human resources and facilities in R & D)

Introduction to Genome Editing-Importance, Applications, and General Aspects

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Introduction

Genome editing is a revolutionary biotechnology that allows precise modifications to DNA sequences in living organisms. It involves targeted alterations in the genetic material to correct mutations, improve traits, and study gene functions. Recent advancements, particularly in CRISPR-Cas9 technology, have significantly enhanced the efficiency, accuracy, and accessibility of genome editing, leading to groundbreaking applications in agriculture, medicine, and biotechnology.

1. Importance of Genome Editing Genome editing is crucial for various reasons:

- **Precision in Genetic Modifications:** Unlike traditional breeding or genetic engineering, genome editing allows targeted changes without introducing foreign DNA.
- **Advancement in Medicine:** It provides tools for treating genetic disorders such as sickle cell anemia, cystic fibrosis, and cancer.
- **Agricultural Improvement:** Enhances crop resilience, increases yield, and develops pest-resistant varieties.
- **Environmental Benefits:** Supports bioengineering strategies to develop stress-tolerant plants and eco-friendly pest control methods.
- **Scientific Research:** Aids in understanding gene functions and disease mechanisms through gene knockout and modification studies.

History and Evolution of Genome-Editing Tools

The development of genome-editing technologies has gone through several milestones:

a. Early Genome-Editing Techniques

- **1970s–1980s: Recombinant DNA Technology**
  - Tools like restriction enzymes and DNA ligases allowed scientists to cut and paste DNA fragments.
- **1990s: Homologous Recombination**
  - Gene targeting in mice and other model organisms became possible, but it was time-consuming and inefficient.

b. The Rise of Engineered Nucleases

- **Zinc-Finger Nucleases (ZFNs) (1990s)**
  - Artificial proteins that bind specific DNA sequences and cut them using a nuclease domain.
  - Challenges: Complex protein engineering and high costs.
- **TALENs (Transcription Activator-Like Effector Nucleases) (2009)**
  - Improved specificity compared to ZFNs.
  - Challenges: Labor-intensive design and assembly.

2. General Aspects of Genome Editing Several key aspects define genome editing:

- **Genome Editing Technologies:**
  - **CRISPR-Cas9:** The most widely used tool due to its simplicity, efficiency, and cost-effectiveness.
  - **Zinc Finger Nucleases (ZFNs):** Older technology, less commonly used due to complexity.
  - **TALENs (Transcription Activator-Like Effector Nucleases):** More precise than ZFNs but still more complex than CRISPR.
  - **Base Editing & Prime Editing:** Newer technologies that allow even more refined changes to DNA without introducing breaks.
- **Mechanism of Action:**
  - Recognition of target DNA sequences via guide RNA (in CRISPR)
  - Creation of a double-strand break (DSB) by a nuclease enzyme
  - DNA repair mechanisms (non-homologous end joining or homology-directed repair) introduce changes
- **Ethical Considerations:**
  - Safety concerns in human applications
  - Regulatory challenges in agriculture and medicine
  - Societal and moral implications of gene editing, including germline modifications

3. Applications of Genome Editing

- **Biomedical Applications:**
  - Treatment of inherited genetic diseases (e.g., muscular dystrophy, hemophilia)
  - Development of gene therapies for cancer and neurodegenerative disorders
  - Creation of disease models for research
- **Agricultural Applications:**
  - Development of high-yield and climate-resilient crops
  - Engineering pest-resistant and disease-resistant plants
  - Reduction of allergenic compounds in food crops
- **Industrial and Environmental Applications:**
  - Biofuel production through genetically modified microorganisms
  - Engineering bacteria for waste decomposition and pollution control
  - Production of synthetic biology products such as bioplastics

This lecture describes three foundational technologies—clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9), transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs). Three technologies—CRISPR-Cas9, TALE nucleases, and zinc-finger nucleases—have facilitated a genome-editing revolution. But several challenges (e.g., effectively treating human diseases) remain.

In recent years, the emergence of highly versatile genome-editing technologies has provided investigators with the ability to rapidly and economically introduce sequence-specific modifications into the genomes of a broad spectrum of cell types and organisms. The core technologies now most commonly used to facilitate genome editing, shown in Figure 1, are (1) clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9), (2) transcription activator-

like effector nucleases (TALENs), (3) zinc-finger nucleases (ZFNs), and (4) homing endonucleases or meganucleases.

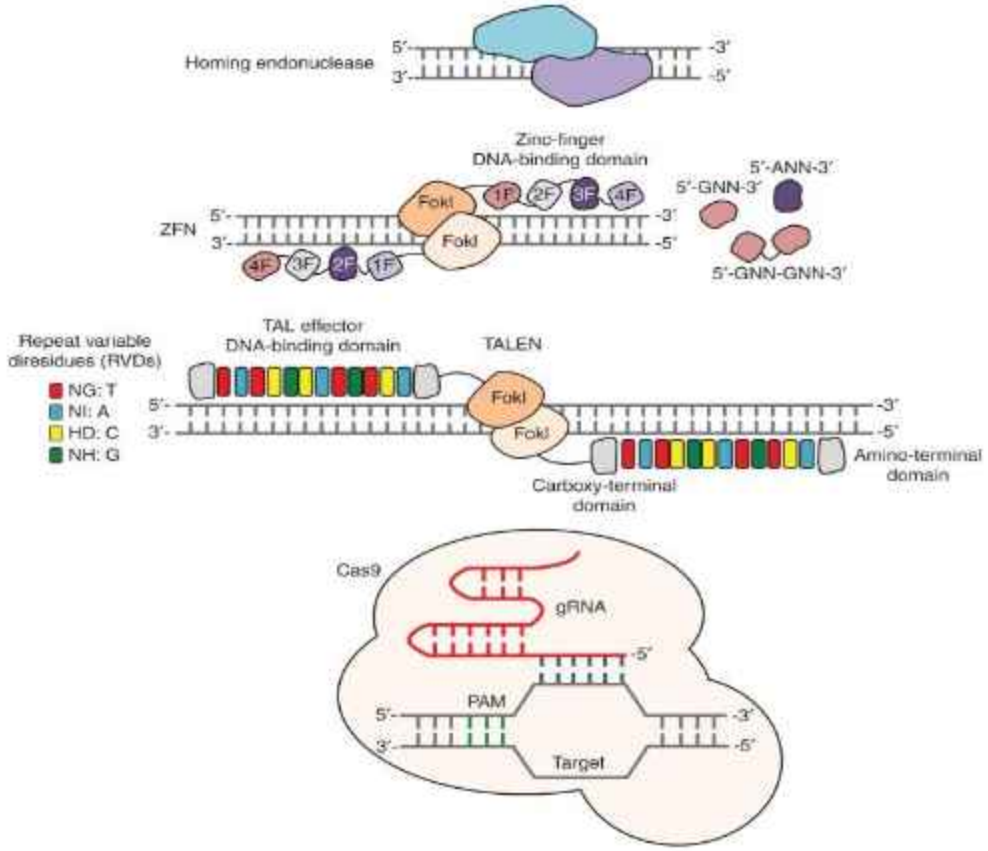

Figure 1. Genome-editing technologies. Cartoons illustrating the mechanisms of targeted nucleases. From *top* to *bottom*: homing endonucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector (TALE) nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9). Homing endonucleases generally cleave their DNA substrates as dimers, and do not have distinct binding and cleavage domains. ZFNs recognize target sites that consist of two zinc-finger binding sites that flank a 5- to 7-base pair (bp) spacer sequence recognized by the FokI cleavage domain. TALENs recognize target sites that consist of two TALE DNA-binding sites that flank a 12- to 20-bp spacer sequence recognized by the FokI cleavage domain. The Cas9 nuclease is targeted to DNA sequences complementary to the targeting sequence within the single guide RNA (gRNA) located immediately upstream of a compatible protospacer adjacent motif (PAM). DNA and protein are not drawn to scale.

The diverse array of genetic outcomes made possible by these technologies is the result, in large part, of their ability to efficiently induce targeted DNA double-strand breaks (DSBs). These DNA breaks then drive activation of cellular DNA repair pathways and facilitate the introduction of site-specific genomic modifications. This process is most often used to achieve gene knockout via random base insertions and/or deletions that can be introduced by nonhomologous end joining (NHEJ) (Fig. 2A). Alternatively, in the presence of a donor template with homology to the targeted chromosomal site,

gene integration, or base correction via homology-directed repair (HDR) can occur (HDR) (Fig. 2B) (see Fig. 2 for an overview of other possible genome-editing outcomes) Indeed, the broad versatility of these genome-modifying enzymes is evidenced by the fact that they also serve as the foundation for artificial transcription factors, a class of tools capable of modulating the expression of nearly any gene within a genome.

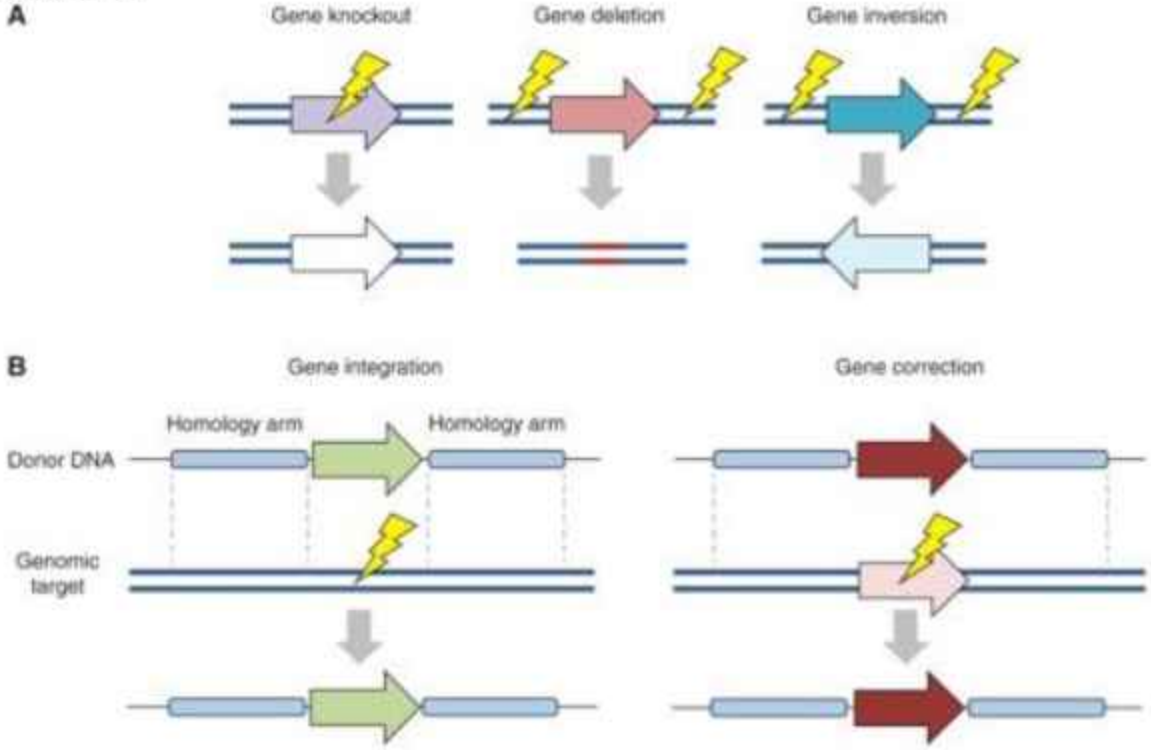

Figure 2. Genome-editing outcomes. Targeted nucleases induce DNA double-strand breaks (DSBs) that are repaired by nonhomologous end joining (NHEJ) or, in the presence of donor template, homology-directed repair (HDR). (*A*) In the absence of a donor template, NHEJ introduces small base insertions or deletions that can result in gene disruption. When two DSBs are induced simultaneously, the intervening genomic sequence can be deleted or inverted. (*B*) In the presence of donor DNA (plasmid or single-stranded oligonucleotide), recombination between homologous DNA sequences present on the donor template and a specific chromosomal site can facilitate targeted integration. Lightning bolts indicate DSBs.

Key principles of genome editing, emphasizing many of the engineering advances that have laid the groundwork for the creation, refinement, and implementation of the current suite of genome-modifying tools.

Zinc-Finger Nucleases

ZFNs, which are fusions between a custom-designed Cys_2-His_2 zinc-finger protein and the cleavage domain of the FokI restriction endonuclease, were the first targeted nuclease to achieve widespread use. ZFNs function as dimers, with each monomer recognizing a specific "half site" sequence—typically nine to 18 base pairs (bps) of DNA—via the zinc-finger DNA-binding domain (Fig. 1). Dimerization of the ZFN proteins is mediated by the FokI cleavage domain, which cuts DNA within a

five- to seven-bp spacer sequence that separates two flanking zinc-finger binding sites. Each ZFN is typically composed of three or four zinc-finger domains, with each individual domain composed of ~30 amino acid residues that are organized in a ββα motif. The residues that facilitate DNA recognition are located within the α-helical domain and typically interact with three bps of DNA, with occasional overlap from an adjacent domain. Using methods such as phage display, a large number of zinc-finger domains recognizing distinct DNA triplets have been identified. These domains can be fused together in tandem using a canonical linker peptide to generate polydactyl zinc-finger proteins that can target a wide range of possible DNA sequences. In addition to this "modular assembly" approach to zinc-finger construction, selection-based methods for constructing zinc-finger proteins have also been reported, including those that consider context-dependent interactions between adjacent zinc-finger domains, such as oligomerized pool engineering (OPEN). In addition, specialized sets of validated two-finger, zinc-finger modules have been used to assemble zinc-finger arrays, including those that take context-dependent effects into account.

One major concern associated with the use of ZFNs for genome editing (in addition to all targeted nucleases) is off-target mutations. As a result, several approaches have been undertaken to enhance their specificity. Among the most successful of these has been the creation of obligate heterodimeric ZFN architectures that rely on charge–charge repulsion to prevent unwanted homodimerization of the FokI cleavage domain, thereby minimizing the potential for ZFNs to dimerize at off-target sites.

Unlike TALENs and CRISPR-Cas9, the difficulty associated with constructing zinc-finger arrays has hindered their widespread adoption in unspecialized laboratories. In particular, it remains challenging to create zinc-finger domains that can effectively recognize all DNA triplets, especially those of the 5′-CNN-3′ and 5′-TNN-3′ variety. As a result, ZFNs lack the target flexibility inherent to more recent genome-editing platforms. Nevertheless, the potential for ZFNs to mediate specific and efficient genome editing is evidenced by ongoing clinical trials based on ZFN-mediated knockout of the human immunodeficiency virus (HIV)-1 coreceptor CCR5 for treatment of HIV/acquired immune deficiency syndrome (AIDS) and a planned clinical trial based on site-specific integration of the factor IX gene into the albumin locus to treat hemophilia B (Clinical Trial ID: NCT02695160).

TALE Nucleases

TALE proteins are bacterial effectors. In 2009, the code used by TALE proteins to recognize DNA was uncovered. In a matter of months, this discovery enabled the creation of custom TALENs capable of modifying nearly any gene. Like ZFNs, TALENs are modular in form and function, comprised of an amino-terminal TALE DNA-binding domain fused to a carboxy-terminal FokI cleavage domain. Also like ZFNs, dimerization of TALEN proteins is mediated by the FokI cleavage domain, which cuts within a 12- to 19-bp spacer sequence that separates each TALE binding site (Fig. 1). TALEs are typically assembled to recognize between 12- to 20-bps of DNA, with more bases typically leading to higher genome-editing specificity. The TALE-binding domain consists of a series of repeat domains, each ~34 residues in length. Each repeat contacts DNA via the amino acid residues at positions 12 and 13, known as the repeat variable diresidues (RVDs). Unlike zinc fingers, which recognize DNA triplets, each TALE repeat recognizes only a single bp, with little to no target site overlap from adjacent domains. The most commonly used RVDs for assembling synthetic TALE arrays are: NI for

adenine, HD for cytosine, NG for thymine, and NN or HN for guanine or adenine. TALE DNA-binding domains can be constructed using a variety of methods, with the most straightforward approach being Golden Gate assembly. However, high-throughput TALE assembly methods have also been developed, including FLASH assembly, iterative capped assembly, and ligation independent cloning, among others. More recent advances in TALEN assembly, though, have focused on the development of methods that can enhance their performance, including specificity profiling to uncover nonconventional RVDs that improve TALEN activity, directed evolution as means to refine TALE specificity, and even fusing TALE domains to homing endonuclease variants to generate chimeric nucleases with extended targeting specificity (discussed in more detail below).

Compared to ZFNs, TALENs offer two distinct advantages for genome editing. First, no selection or directed evolution is necessary to engineer TALE arrays, dramatically reducing the amount of time and experience needed to assemble a functional nuclease. Second, TALENs have been reported to show improved specificity and reduced toxicity compared to some ZFNs, potentially because of their increased affinity for target DNA (Meckler et al. 2013) or perhaps a greater energetic penalty for associating with base mismatches. However, TALENs are substantially larger than ZFNs, and have a highly repetitive structure, making their efficient delivery into cells through the use of lentivirus (Holkers et al. 2013) or a single adeno-associated virus (AAV) particle

challenging. Methods for overcoming these limitations have emerged as TALENs can be readily delivered into cells as mRNA (Mahiny et al. 2015; Mock et al. 2015) and even protein (Cai et al. 2014; Liu et al. 2014a), although alternative codon usage and amino acid degeneracy can also be leveraged to express RVD arrays that might be less susceptible to recombination (Kim et al. 2013a). In addition, adenoviral vectors have also proven particularly useful for mediating TALEN delivery to hard-to-transfect cell types (Holkers et al. 2014; Maggio et al. 2016).

CRISPR-Cas9

The CRISPR-Cas9 system, which has a role in adaptive immunity in bacteria, is the most recent addition to the genome-editing toolbox. In bacteria, the type-II CRISPR system provides protection against DNA from invading viruses and plasmids via RNA-guided DNA cleavage by Cas proteins. Short segments of foreign DNA are integrated within the CRISPR locus and transcribed into CRISPR RNA (crRNA), which then anneal to *trans*-activating crRNA (tracrRNA) to direct sequence-specific degradation of pathogenic DNA by the Cas9 protein. In 2012, Charpentier, Doudna, and co-workers reported that target recognition by the Cas9 protein only requires a seed sequence within the crRNA and a conserved protospacer-adjacent motif (PAM) upstream of the crRNA binding site. This system has since been simplified for genome engineering and now consists of only the Cas9 nuclease and a single guide RNA (gRNA) containing the essential crRNA and tracrRNA elements (Fig. 1). Because target site recognition is mediated entirely by the gRNA, CRISPR-Cas9 has emerged as the most flexible and user-friendly platform for genome editing, eliminating the need for engineering new proteins to recognize each new target site. The only major restriction for Cas9 target site recognition is that the PAM motif—which is recognized by the Cas9 nuclease and is essential for DNA cleavage—be located immediately downstream of the gRNA target site. The PAM sequence for the *Streptococcus pyogenes* Cas9, for example, is 5′-NGG-3′ (although in some cases 5′-NAG-3′ can be tolerated). Several studies have now shed light on the structural basis of DNA recognition by Cas9, revealing that

the heteroduplex formed by the gRNA and its complementary strand of DNA is housed in a positively charged groove between the two nuclease domains (RuvC and HNH) within the Cas9 protein, and that PAM recognition is mediated by an arginine-rich motif present in Cas9. Doudna and colleagues have since proposed that DNA strand displacement induces a structural rearrangement within the Cas9 protein that directs the nontarget DNA strand into the RuvC active site, which then positions the HNH domain near target DNA, enabling Cas9-mediated cleavage of both DNA strands.

The Cas9 nuclease and its gRNA can be delivered into cells for genome editing on the same or separate plasmids, and numerous resources have been developed to facilitate target site selection and gRNA construction, including E-CRISP, among others. Although Cas9 boasts the highest ease of use among the targeted nuclease platforms, several reports have indicated that it could be prone to inducing off-target mutations. To this end, considerable effort has been devoted to improving the specificity of this system, including using paired Cas9 nickases, which increase gene-editing specificity by requiring the induction of two sequential and adjacent nicking events for DSB formation, or truncated gRNA that are more sensitive to mismatches at the genomic target site than a full-length gRNA.

Standard Operating Procedures for Research and Release of Genome Edited Plants in Bangladesh

Dr. Md. Aziz Zilani Chowdhury
Ex Member Director (Crops Division) and
Consultant, Crop Zoning Project, BARC

কৃষিই সমৃদ্ধি

Translated in English from Bangla

Government of the People's Republic of Bangladesh
Ministry of Agriculture
Research-1 Section
www.moa.gov.bd

Memo no. 12.00.0000.062.99.005.23.646 Dated: 26 Agrahan 1430 / 11 December 2023

Subject: Approval of 'Standard Operating Procedures for Research and Release of Genome Edited Plants of Categories SDN-1 and SDN-2 in Bangladesh'.

Reference: Bangladesh Agricultural Research Council's Memo no.12.20.0000.004.99. 30.2023-2776, Date: 18-11-2023

With the above reference the approved SOP entitled 'Standard Operating Procedures for Research and Release of Genome Edited Plants of Categories SDN-1 and SDN-2 in Bangladesh' related to develop desired crop varieties after deletion/addition of specific genetic traits through advance 'Genome Editing' technology is hereby forwarded as per direction of competent authority.

11/12/2023
Mohammad Zahirul Islam
Deputy Secretary
Phone: 223351198
Email: dsresearch@moa.gov.bd

Executive Chairman
Bangladesh Agricultural Research Council
Farmgate, Dhaka

Memo no. 12.00.0000.062.99.005.23.646 Dated: 26 Agrahan 1430 / 11 December 2023

Copy forwarded for kind Information (Not According to the Seniority)

1. PS to Honorable Minister, Ministry of Agriculture, Bangladesh Secretariat, Dhaka
2. PS to Secretary, Ministry of Agriculture, Bangladesh Secretariat, Dhaka
3. PO to Joint Secretary, Research Wing, Ministry of Agriculture, Bangladesh Secretariat, Dhaka
4. PO to Joint Secretary, Research Branch, Ministry of Agriculture, Bangladesh Secretariat, Dhaka

11/12/2023
Mohammad Zahirul Islam
Deputy Secretary

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Standard Operating Procedures for Research and Release of Genome Edited Plants of Categories SDN-1 and SDN-2 in Bangladesh

Bangladesh Agricultural Research Council
Ministry of Agriculture
Government of the People's Republic of Bangladesh

December, 2023

Contents

1. Preamble

The 'National Agricultural Policy 2018' of Bangladesh has given importance to achieve poverty alleviation, food and nutritional security and agricultural growth in Bangladesh. The policy lays special importance to the use of modern techniques including biotechnology for development of stress tolerant, disease resistant and nutritious crop varieties. Genome editing is a revolutionary technology that enables both precise and efficient targeted modification in the genome of plants thus accelerating the pace of plant breeding. It is being used extensively by scientists all over the world to make desirable improvements in different crop plants such as cereals, pulses, oilseeds, fruits, and vegetables.

Genome editing uses site directed nucleases (SDNs) to make a desired change at the specific location (s) in the genome that may either be a small deletion, a substitution, or the insertion of a number of nucleotides or bases of a particular gene of a targeted plant species/crop variety. Such targeted edits result in improved traits in the plants. Based on the mechanism used in editing and the magnitude of the resulting changes, genome editing is primarily categorized as SDN-1, SDN-2, and SDN-3. The SDN-1 and SDN-2 categories of genome edited plants do not contain any transgene(s) and are indistinguishable from conventionally bred varieties. Therefore, many countries, including Argentina, Brazil, Canada, England, India, Japan, Philippines, and USA consider genome edited plants like conventionally bred varieties. Plant scientists are using genome editing to deal with serious challenges being faced viz. climate change, pests, diseases, nutritional quality etc. A good number of genome edited plants such as tomato, soybean, corn etc. are now cultivated in the farmers' fields in many countries.

Considering the precision and speed of crop improvement using genome editing technology, several research organizations, institutes under the National Agricultural Research System (NARS), National Institute of Biotechnology (NIB) Universities, private and public research laboratories in Bangladesh have initiated efforts to apply genome editing for improvement of crop plants for specific traits including climate-resilient, high quality and high yielding traits.

The Ministry of Agriculture, Government of the People's Republic of Bangladesh is responsible for variety registration for both notified and non-notified crops in Bangladesh. Variety registration procedures are followed for crops developed using conventional breeding methods. MOA has prepared the "Standard Operating Procedures for Research and Release of Genome Edited Plants of Categories of SDN-1 and SDN-2 in Bangladesh" to facilitate the research and release of genome edited crops that meet the needs of the farmers of Bangladesh and consumers.

2. Objective

The purpose of this SOP is to facilitate the research and release of genome edited plants falling under the categories of SDN-1 and/or SDN-2 in Bangladesh.

3. Definitions

Exogenous introduced DNA: DNA introduced into cell/tissue for genome editing including entire plasmid vector construct sequences. For the purposes of this document, exogenous introduced DNA includes the entire coding sequences for genome editing reagents, selectable markers, intact gene regulatory elements (e.g., promoters, transcriptional activators or modifiers, termination signals), and vector backbone. In SDN-2 genome edited plants, the sequences integrated at the ectopic locus to serve as homologous DNA template are considered as exogenous DNA, whereas any changes introduced at the target locus through genome editing would not come under the definition of exogenous introduced DNA.

Genome editing: A group of techniques used to make precise and targeted alterations including modification, insertion, replacement, or deletion of DNA sequences from an organism's genome. Genome editing uses site directed nucleases (SDNs) to introduce a DNA break.

Site-directed Nuclease: Abbreviated as SDN is an enzyme programmed to recognize a specific sequence within the genome of an organism and cleaves the DNA usually creating a double strand break.

SDN-1: Involves the unguided repair of a targeted DNA break by the natural endogenous DNA repair mechanism of the host organism such as non-homologous end joining. The spontaneous repair of this break can lead to a mutation causing gene silencing, gene knock-out, or a change in the activity of a gene. The SDN-1 genome edited plants produced will be free from exogenous/foreign DNA. These mutations can be base substitution/indels/deletions including large deletions or structural changes. These resultant mutations are comparable to those occurring in nature, obtained through conventional mutagenic treatments or natural variation found in the primary/secondary gene pool.

SDN-2: Involves a template-guided repair of a targeted DNA break using an externally supplied template sequence. The donor carries one or several small mutations flanked by two sequences matching both ends of the DNA break, and is thus recognized as a repair template, allowing the introduction of the mutation(s) at the target site. The resultant mutant carries a modified sequence, leading to an altered expression profile of the gene and/or altered activity of the encoded protein/RNA. Thus, the edited version could be regarded as an allelic form comparable to those available in the primary/secondary gene pool.

Transgene: A DNA fragment or gene from a non-cross compatible species.

Transgenesis: The process of introducing an exogenous DNA fragment from a non-cross compatible species into the genome of a given cell and the propagation of such a fragment thereafter.

4. SOPs for research and registration/release of genome edited plants of SDN-1 and SDN-2 categories

4.1 Initiating Research and Development

4.1.1 Research and development on genome edited plants in Bangladesh must be conducted with authorization from **Institutional Oversight Body (IOB)** (***Annexure I***). The laboratory facilities, materials and the procedures must be approved by the **IOB**.

4.1.2 Laboratories involved in the research and development of genome edited plants must be monitored regularly by the **IOB**.

4.1.3 Standard laboratory practices for ensuring safety must be maintained.

4.2 Suggested procedure for handling genome edited plants

4.2.1 Progenies of individual T0 events should be maintained separately. Seeds obtained through selfing of individual T1 plants should be raised from a single plant to progeny row.

4.2.2 Investigators are recommended to provide the following information to the Institutional Oversight Body.

4.2.2.1 Numbers of T0 transformants generated.

4.2.2.2 Inform the generation in which mutation in the target site is detected, and the homozygous or heterozygous status of mutation.

4.2.2.3 Maintenance of mutation either at homozygous or heterozygous state.

4.2.2.4 The generation at which the homozygosity is attained.

4.2.2.5 Generation at which genome edited lines were found to be free of exogenous introduced DNA.

4.2.2.6 Provide DNA sequence information of the target sites and their proximity of the edited plants together with wild (mother plant) type.

4.2.2.7 Provide information that shows non-specific edits have not happened other than target site(s).

4.3 Variety registration procedure for SDN-1 and SDN-2 Genome Edited plants

4.3.1 Once investigators have developed genome edited plant(s) which is free from any exogenously introduced DNA and intend to take SDN-1 and/or SDN-2 plants out of containment conditions, they shall submit data in the prescribed format to an Institutional Oversight Body (IOB) established for this purpose and information for confirmation per *Annexure II*, that establishes that the plant no longer contains any transgenes or foreign DNA.

4.3.2 **IOB** to review data for the following:

4.3.2.1 Regularly monitor the progress of genome editing research activities.

4.3.2.2 Review data that establishes category(ies) of genome editing (SDN-1 and/or SDN-2).

4.3.2.3 Confirmation of genome editing at target locus/loci using DNA sequencing.

4.3.2.4 Whether selfing and/or backcrossing has been carried out to segregate any exogenously introduced DNA?

4.3.2.5 Whether any DNA-free method such as RNA-protein complex was used for genome editing?

4.3.2.6 Evidence to confirm that the genome edited plant is free from exogenous introduced DNA.

4.3.2.7 Were any unintended phenotypic changes observed on the genome edited plant whether it was selected/ segregated out?

4.3.2.8 Whether information on identical allele(s) already documented? If yes, information to be provided.

4.3.3 Applicant shall use protocols/methods prescribed in section 5 of this document to show that the genome edited plants are free from exogenous introduced DNA.

4.3.4 After the information provided according to ***Annexure II*** is examined and found to be satisfactory, the Institutional Oversight Body shall submit the recommendations along with ***Annexure II*** and their confirmation on the absence of any exogenously introduced DNA to the "Evaluation Committee for Genome Edited Plants" established by the Bangladesh Agricultural Research Council (BARC) as given in ***Annexure III***.

4.3.5 In the case of methods other than specified in the SOPs have been used to demonstrate the absence of any exogenously introduced DNA, the same may be verified and highlighted by the **IOB**.

4.3.6 The recommendations of the **IOB** must include:

4.3.6.1 Scientific evidence based on which the proposal by the applicant for the absence of any exogenously introduced DNA has been approved.

4.3.6.2 In the case of methods other than specified in the SOPs have been used to demonstrate the absence of any exogenously introduced DNA, the same may be verified and highlighted in the minutes.

4.3.7 Once the recommendations of **IOB** (along with ***Annexure II***) are confirmed by the Evaluation Committee for Genome Edited Plants of BARC, **IOB** shall communicate the decision to the applicant to proceed with registering or release of the plant following the same procedure as used for conventional breeding for Notified/Non notified crops of that plant species in Bangladesh. The applicant multiplies certain amount of seed of genome edited plants for the necessary trial of notified or non-notified crops.

4.3.8 After getting the certification of SDN-1/SDN-2, the proposal of the institution/organization for the variety release of genome edited plants should be submitted to the respective authorities following the Seed Rules-2020 (Bangladesh Gazette published on Tuesday, June 9, 2020) for conventionally bred plants.

4.3.9 If the plant is a notified crop, the proposal will be submitted to Director of Seed Certification Agency (SCA) through fulfilling the **FORM-10 (*Annexure IV*)** of the Seed Rules 2020 in case of Notified Crops for the purpose of Variety Release and Notification by the Ministry of Agriculture, and thereby the application will have to be executed through the procedures as indicated below.

4.3.9.1 The Director SCA will perform necessary field evaluation and submit the field evaluation report to the meeting of the Technical Committee (TC) of the National Seed Board (NSB).

4.3.9.2 The TC will verify the technical merit of the report through in-depth evaluation in the meeting of the TC, and thereby recommend the crop variety to the NSB through specifically mentioning the average yield, growth duration (life cycle), source of variety, and other necessary other relevant varietal traits including mentioning the category of genome editing (SDN-1 or SDN-2).

4.3.9.3 If the proposed variety is being qualified, and it is developed either by the process of SDN-1 or SDN-2, the meeting of the NSB will approve it and send it to the Seed Wing of the Ministry of Agriculture for issuing certificate to the concerned applicant for registration of the variety.

4.3.10 If the plant is a non-notified crop, the proposal will be submitted to the Director General of Seed Wing or Secretary of NSB by fulfilling the prescribed **FORM-11 (*Annexure V*)** of the Seed Rules 2020 in case of Non-Notified Crops for the purpose of Variety Registration by the Ministry of Agriculture, and for its final approval as the seeds developed through conventional breeding.

4.4 Record Keeping

4.4.1 All records associated with research and development involving the use of genome edited plants will be maintained by the applicant/s (based on ***Annexure II***) and must be submitted to the **IOB**.

4.4.2 The **IOB** shall archive copies of the records for all applications submitted for exemptions for a minimum of five (5) years, whether or not the regulated material is granted the exemption.

5. Process Diagram for Approval procedures

When a genome edited plant is developed using either SDN-1 or SDN-2 method and the final product is free from any exogenous introduced DNA transgene or foreign gene as confirmed by the **IOB** and the Evaluation Committee for Genome Edited Plants at BARC, the respective institute/ university should proceed with registering or releasing of the plant following the same procedure as used for conventional breeding of that plant species in Bangladesh.

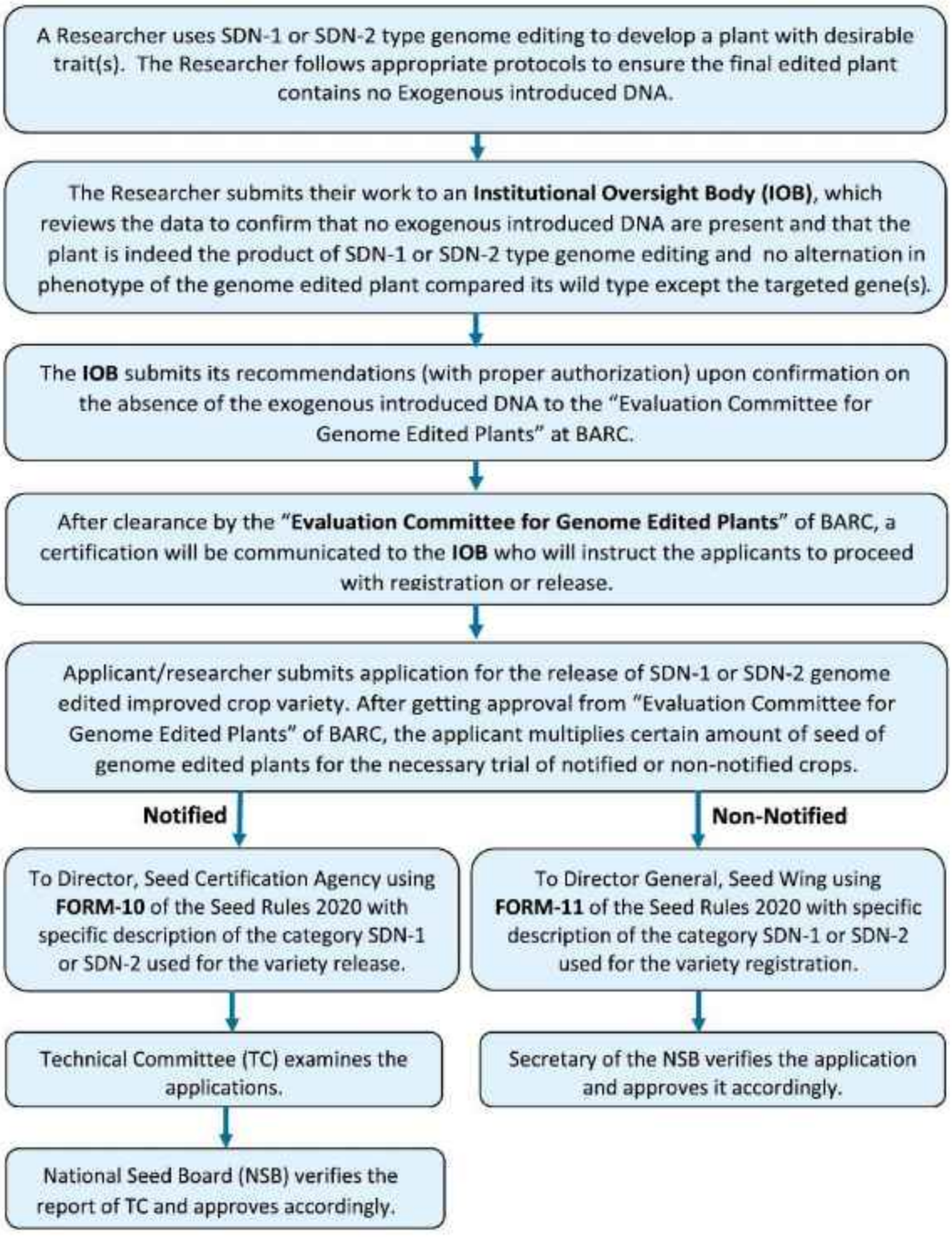

Fig. 1. Process diagram for research and release of genome edited plants of categories of SDN1 and SDN2 in Bangladesh

6. Protocols to demonstrate that a genome edited plant is free from exogenous introduced DNA

The two steps described below are the recommended protocol to show that the genome edited plants of SDN-1 and/or SDN-2 categories are free from any exogenously introduced DNA.

6.1 Absence of selectable/scorable marker

The final edited plant lines must be sensitive to the selection reagents (antibiotics/herbicide/ any other) at the concentration used for selecting the plants having exogenous introduced DNA. In the case of a scorable marker, the final edited plant line should be phenotypically negative for the same. Seeds of a segregant line harboring the any exogenously introduced DNA should be used as the positive control, and the parental genotype used for genome editing as the negative control.

6.2 Overlapping PCR/nested PCR

6.2.1 Total genomic DNA is to be used as the template for PCR amplification.

6.2.2 The primers must be chosen such that the amplicons cover the full length of the any exogenously introduced DNA (full length of the vector DNA).

6.2.3 The intended amplicons should be at the maximum of 500 bp in size and the overlap between consecutive amplicons should be at least 50 bp.

6.2.4 For each amplification reaction, three PCR positive controls must be used:

6.2.4.1 A well-known endogenous low copy gene (actin/tubulin, etc.) of the same species using the same amount of the same genomic DNA sample of the final edited lines as mentioned above.

6.2.4.2 The genomic DNA of a segregant line harboring full or part of the any exogenously introduced DNA (using the same primer pairs and the same amount of genomic DNA used for overlapping PCR).

6.2.4.3 Genomic DNA of the parental genotype spiked with at the most $1/1000^{th}$ (*w/w*) of the full-length purified vector DNA used to develop the genome edited line (using the same primer set used for overlapping PCR).

6.2.4.4 The amount of template DNA and the PCR conditions should be such that a clear band of the expected size is visible in all the positive controls.

6.2.4.5 No amplification should be detected in any of the reactions with primers directed against any exogenously introduced DNA for the final genome edited line, while clear amplification should be detected in all the positive controls by ethidium bromide- stained agarose gel electrophoresis.

6.3 Use of other methods

Evidence of the absence of exogenous introduced DNA with alternative methodologies/ technologies may be considered by the Institutional Oversight Body if the methods have the same level of stringency as the methods described above.

7. Review of the SOPs

These SOPs will be reviewed periodically by Bangladesh Agricultural Research Council (BARC) in line with technological advancements, particularly the methods that are to be used to show that genome edited plants are free from exogenous DNA.

Annexure I

Composition of Institutional Oversight Body (IOB)

An Institutional Oversight Body may be formed in each university and public institute/organization working towards development of SDN-1 and/or SDN-2 categories of genome edited plants. IOB will be formed at each institute/organization following the consent/approval of the head of the institute/organization/authorized person. The Institutional Oversight Body will have five members including experts in relevant fields such as-

1. Biotechnology/ Molecular Biology
2. Breeding/ Genetics
3. Agronomy
4. Horticulture etc.

Institute may co-opt members of relevant expertise from other institutions.

Annexure II

Format for Information and Review of SDN-1 and/or SDN-2 genome edited plants

Researcher Information	
Name of the Principal Investigator, Co-PI, Research Team	
Institution	
Chair of the Institutional Oversight Body	
Additional Information (collaborators involved in performance of the SOP)	

Information on the Genome Edited Plant		
Sl No.	**Item**	**Information to be provided**
1	Name of the plant species and genotype	
2	Targeted trait(s)	
3	Reference number or identifying name	
4	Category of genome editing: SDN-1 and/or SDN-2	
5	Targeted genomic region • Name of the gene(s) or locus/loci • Specific region of the gene or locus (promoter, terminator, other regulatory elements, coding region, intron, etc.) • Nucleotide sequence of the parental allele • Specific site(s) chosen for editing (in case of SDN-2)	
6	Genome editing method used including CRISPR- Cas, TALEN, ZFN, RNPs, Base editing, prime editing, etc.	
7	Details of vector, gene construct, editing reagents (molecular tools) including maps and nucleotide sequences	
8	The method used for delivery of gene editing reagents (Agrobacterium-mediated, Biolistic, etc.)	

Sl No.	Item	Information to be provided
9	Current Generation of the genome edited plant(s) (T0, T1, T2, etc.)	
10	Data on Phenotypic expression of the target trait(s) as assessed under containment conditions, if applicable	
11	Molecular data for confirmation of the targeted editing a. By sequencing of the parental and the modified allele(s) using Sanger or other Sequencing technologies with a minimum 10X coverage of the edited region(s) and base quality of a minimum Phred score 30 b. Sequence difference between parental and modified/edited allele through sequence alignment	
12	Whether the mutation is homozygous or heterozygous. Provide evidence for inheritance of the mutation through two generations using sequencing.	
13	Whether selfing and/or backcrossing has been carried out to segregate the any exogenously introduced DNA? Provide information.	
14	Whether any DNA-free method such as RNA-protein complex was used for genome editing? If yes, provide information.	
15	Provide evidence to confirm the absence of exogenous introduced DNA in genome edited plant by phenotypic selection (sensitivity to herbicide/ antibiotic, or absence of scorable marker) and using overlapping PCR/ Nested PCR/other appropriate methodology (as per Section 6).	
16	In case, any unintended phenotypic changes were observed on the genome edited plant whether it was selected/ segregated out. If selected, provide information.	
17	In the case of nutrition-related traits, provide data on the targeted nutritional trait in comparison to the parental line.	

Annexure III

Composition of the "Evaluation Committee for Genome Edited Plants"

Bangladesh Agricultural Research Council (BARC) will establish an "Evaluation Committee for Genome Edited Plants" to independently evaluate the proposals submitted by the Institutional Oversight body and certify that the genome edited plant is free from any exogenously introduced DNA. This committee will be convened by the Executive Chairman of BARC and Member Director (Crops), BARC will be the Member Secretary. Members of this committee will consist of five distinguished scientists from research institutes and universities working in the area of genome editing. In addition, an international relevant reputed expert may be co-opt in the committee. In case of any IPR issues arise for releasing genome edited crops, concerned authority will take necessary steps.

The Terms of Reference for the Independent Evaluation Committee for Genome Edited Plants will be as follows:

1. Receiving the proposals from the Institutional Oversight Body of any universities and public institutes.
2. To certify that the genome edited plant is free from any exogenously introduced DNA.
3. Within 30 days the committee will recommend the certified material to the institute for further processing of registration/release as notified/non-notified crop.
4. The "Evaluation Committee for Genome Edited Plants" will develop a format of certification of generated product through SDN 1 and SDN 2.

Note: There will be a provision of honorarium as per government rules for the meeting of "Evaluation Committee for Genome Edited Plants"

Annexure IV

FORM-10 of the Seed Rules 2020 in case of Notified Crops (English version)

FORM- 10
[See Rule 11, Sub- Rule (2)]
Application for Releasing of Notified Crop Variety

To
Director
Seed Certification Agency

Date:

1. Name and Address of Applicant:
2. Registration Number of Seed Dealer (Valid/ Updated):
3. Name and Address of person in- charge of the development of the proposed variety:
4. The variety from which the proposed variety is developed
   (A) Variety Name in Bangla:
   (B) Variety Name in English:
   (C) Botanical/ Scientific Name of Variety:
   (D) Station Number:
   (E) Name of the proposed Variety (in Bangla and English):
5. Source of proposed variety
   (A) Introduction:
   (B) Country of Origin/ Place and Name of Organization:
   (C) Original Station Number:
   (D) Pedigree Number:
   (E) Parentage:
6. Ecological requirement of the proposed variety:
   (A) Season:
   (B) Soil:
   (C) Water:
   (D) Other Information:
7. Agronomical requirement of the proposed variety
   (A) Method of Cultivation:
   (B) Seed rate per hectare (kg):
   (C) Spacing/Planting Distance (cm/meter):
   (D) Population per hectare:
   (E) Fertilizer requirement per hectare(kg):
   (F) Growth duration of crop in the field in days (seed to seed):
8. Describe, if special processing is needed for the product to be used.
9. Name of usable part of the crop:

10. Description of any tests performed on disease and insect reactions:
    (A) Natural (number of tested seasons/ number of years):
    (B) Artificial tests:

11. Description of the distinctive features of the proposed Variety:

12. Description of tests listed below (Result of yield in metric ton per hectare) conducted:
    (A) Advanced Yield Trial (AYT):
    (B) Regional Yield Trial (RYT):
    (C) Advanced Line Adaptive Research Trial (ALART):
    (D) Agronomical Trial:
    (E) On Farm Yield Trial:
    (F) Participatory Varietal Trial (PVT):

13. (A) Source of Plant Breeding Material:
    (B) Method of Plant Variety Development for proposed Variety:

14. (A) Overall Morphology of proposed Variety:
    (B) Identification features of Variety:

15. (A) Appropriateness of the Variety in the
    Agro- Ecological Zone:
    (B) Description of appropriate Crop Layout, if applicable:

16. Description of optimal Farm Management Practices including fertilizer and water management:
    (A) Planting:
    (B) Fertilizer application:
    (C) Water management:

17. (A) Yield Trial Result and Description of proposed Variety:
    (B) Comparative difference between Varietal Characteristics of the best variety and the proposed Variety:
    (C) If there is a suggestion to withdraw any species, its Name:

18. Crop Harvest and Collection Method :

19. (A) Good Post-Harvest Processing and Storing Method (If new method is needed, its description):
    (B) Storing Test Result:
        (1) In natural condition:
        (2) Air- conditioned (Special type/ method):

20. (A) Physical Components (Size, Shape, Weight, etc.) (if applicable):
    Size/ Shape:
    Texture:
    Color:
    Weight of One Thousand Grains (Gram) (Except Potato/ Sugarcane):
    Seed Dormancy:

(B) Chemical Ingredients, Nutritional Status and Cooking Utility Techniques (For edible substances):
(C) Recovery Ratio (if applicable):
(D) Breaking Ratio of usable crop parts (if applicable):

21. Diseases and Insect-Pests reaction:

22. Part used as Seed:

23. (A) Seed producing method (Special precautions, separation standards, seed vitality extension for at least 12 months and special warehousing requirements taken for inbred or hybrid):
(B) List of Morphologically Most Similar Varieties and Species:
(C) List of differences according to DUS Test from list of Most Similar Variety or Species (Proof is needed to be Submitted):

24. (A) Who will produce Breeder Seed and Where:
(B) Quantity of Breeder Seed that can be supplied in every season / per year:
(C) Who will produce Foundation Seed and Certified Seed and consent of the producers has been obtained
(D) When DAE will be able to undertake the demonstration of this variety in the farmers' fields in collaboration with the variety development organization and how many demonstrations.
(E) A Bangla Leaflet is enclosed herewith indicating all information as mentioned above and incorporating Post-Harvest and Seed production Technology.

25. Additional Information:

Signature, Date and Seal of Applicant

The following documents will have be attached with the Application Form

1. Copy of Seed Dealer Registration Certificate.
2. Declaration by the applicant to be responsible for preserving the quality and characteristics of the variety.
3. Non-government organizations are required to submit a copy of the Certificate of Membership of Bangladesh Seed Association.

FORM-10 of the Seed Rules 2020 in case of Notified Crops (Bangla version)

ফরম-১০

[বিধি ১১ এর উপ-বিধি (২) দ্রষ্টব্য]

নিয়ন্ত্রিত ফসলের জাত ছাড়করণ বা নিবন্ধন আবেদন

বরাবর
পরিচালক
বীজ প্রত্যয়ন এজেন্সি

তারিখ :

১। প্রতিষ্ঠানের নাম ও ঠিকানা:

২। বীজ ডিলার নিবন্ধন নম্বর (বৈধ/হালনাগাদ):

৩। প্রস্তাবিত জাতের উন্নয়নের জন্য দায়িত্বপ্রাপ্ত ব্যক্তির নাম ও ঠিকানা:

৪। যে জাত হইতে প্রস্তাবিত জাতের উদ্ভব হইয়াছে তাহার
- (ক) বাংলা নাম:
- (খ) ইংরেজি নাম:
- (গ) উদ্ভিদতাত্ত্বিক/বৈজ্ঞানিক নাম:
- (ঘ) স্টেশন নম্বর:
- (ঙ) জাতের প্রস্তাবিত নাম (বাংলা ও ইংরেজি):

৫। প্রস্তাবিত জাতের উৎস:
- (ক) সূচনা:
- (খ) উৎস দেশ/স্থান ও প্রতিষ্ঠানের নাম:
- (গ) মূল স্টেশন নম্বর (Number):
- (ঘ) বংশ পরিচয় নম্বর (Pedigree Number):
- (ঙ) প্যারেনটেজ (Parentage):

৬। প্রস্তাবিত জাতের পরিবেশগত (Ecological) চাহিদা:
- (ক) মৌসুম:
- (খ) মৃত্তিকা:
- (গ) পানি:
- (ঘ) অন্য কোনো তথ্য:

৭। প্রস্তাবিত জাতের কৃষিতাত্ত্বিক (Agronomical) চাহিদা:
- (ক) চাষ পদ্ধতি:
- (খ) প্রতি হেক্টরে বীজের হার (কেজি):
- (গ) রোপণ দূরত্ব:
- (ঘ) প্রতি হেক্টরে গাছের সংখ্যা:
- (ঙ) প্রতি হেক্টরে সারের প্রয়োজনীয়তা:
- (চ) মাঠে ফসলের জীবনকাল (বীজ হইতে বীজ):

৮। পণ্য ব্যবহারের জন্য যদি বিশেষ প্রক্রিয়াজাতকরণের প্রয়োজন হয়, তাহা হইলে উহার বিবরণ:

৯। ফসলের ব্যবহারযোগ্য অংশের নাম:

১০। রোগ এবং পোকার প্রতিক্রিয়ার উপর কোনো পরীক্ষা করা হইয়া থাকিলে উহার বিবরণ:

(ক) প্রাকৃতিক (পরীক্ষিত মৌসুম/বৎসরের সংখ্যা):

(খ) কৃত্রিম:

১১। প্রস্তাবিত জাতের স্বাতন্ত্র্য বৈশিষ্ট্যের বর্ণনা :

১২। নিম্নলিখিত পরীক্ষাগুলোর বর্ণনা (টন/হে:):

(ক) অগ্রগামী সারির ফলন পরীক্ষা (AYT):

(খ) আঞ্চলিক ফলন পরীক্ষা (RYT):

(গ) অগ্রগামী সারির অভিযোজন পরীক্ষা (ALART):

(ঘ) কৃষিতাত্ত্বিক পরীক্ষা:

(ঙ) খামারে ফলন পরীক্ষা:

(চ) কৃষকের মাঠে পরীক্ষা (PVT):

১৩। (ক) প্রজনন দ্রব্যের উৎস:

(খ) প্রস্তাবিত জাত উন্নয়নের পদ্ধতি:

১৪। (ক) প্রস্তাবিত জাতের সামগ্রিক অঙ্গসংস্থান (Morphology):

(খ) জাত শনাক্তকারী বৈশিষ্ট্য:

১৫। (ক) কৃষি-পরিবেশ অঞ্চলে (Agro-Ecological Zone) জাতটির উপযুক্ততা:

(খ) উপযুক্ত শস্য বিন্যাসের বর্ণনা, যদি থাকে:

১৬। সার ও পানি ব্যবস্থাপনাসহ অনুকূল চাষ পরিচর্যার বর্ণনা:

(ক) রোপণ:

(খ) সার প্রয়োগ:

(গ) পানি ব্যবস্থাপনা:

১৭। (ক) ফলন পরীক্ষার ফলাফল এবং প্রস্তাবিত জাতের বর্ণনা:

(খ) সর্বোত্তম জাতের বৈশিষ্ট্যের সহিত তুলনামূলক পার্থক্য:

(গ) কোনো প্রজাতির প্রত্যাহারের পরামর্শ থাকিলে তাহার নাম:

১৮। শস্য সংগ্রহ পদ্ধতি:

১৯। (ক) প্রক্রিয়াজাতকরণ এবং গুদামজাতকরণের পদ্ধতি (কোনো নূতন পদ্ধতির প্রয়োজন হইলে তাহার বর্ণনা):

(খ) গুদামজাতকরণ পরীক্ষার ফলাফল:

(১) প্রাকৃতিক অবস্থায়:

(২) শীতাতপ নিয়ন্ত্রিত (বিশেষ প্রকার/পদ্ধতি):

২০। (ক) ভৌত উপাদান (আকার, আকৃতি, ওজন ইত্যাদি) (প্রযোজ্য ক্ষেত্রে):

আকার/আকৃতি:

বুনট (Texture):

বর্ণ (Colour):

এক হাজার দানার ওজন (গ্রাম) (আলু/ইক্ষু ব্যতীত):

বীজের সুপ্ততা:

(খ) রাসায়নিক উপাদান, পুষ্টিগত অবস্থা এবং রান্নার উপযোগিতা কৌশল (ভোজ্য দ্রব্যের ক্ষেত্রে):

(গ) পুনরুদ্ধারের অনুপাত (Recovery ratio) (প্রযোজ্য ক্ষেত্রে):

(ঘ) ফসলের ভোগ্য অংশ ভাঙ্গার অনুপাত (প্রযোজ্য ক্ষেত্রে):

২১। রোগ বালাইয়ের প্রতিক্রিয়া:

২২। বীজ হিসাবে ব্যবহৃত অংশ:

২৩। (ক) বীজ উৎপাদনের পদ্ধতি (ইনব্রিড বা হাইব্রিডের জন্য গৃহীত বিশেষ সতর্কতা, পৃথকীকরণ মান, বীজের জীবনীশক্তি কমপক্ষে ১২ (বার) মাস পর্যন্ত বর্ধিতকরণ এবং বিশেষ গুদামজাতকরণের প্রয়োজনীয়তা):

(খ) অঙ্গসংস্থানগতভাবে (Morphologically) কাছাকাছি (Most Similar) জাত বা প্রজাতিসমূহের তালিকা:

(গ) ডিইউএস (DUS) টেস্ট এর আলোকে কাছাকাছি (Most Similar) জাত বা প্রজাতির তালিকা হইতে পার্থক্যের তালিকা (প্রমাণপত্র দাখিল করিতে হইবে);

২৪। (ক) কে বা কোথায় প্রজনন বীজ উৎপাদন করিবে:

(খ) মৌসুমওয়ারী/বাৎসরিক কী পরিমাণ প্রজনন বীজ সরবরাহ করা যাইতে পারে:

(গ) কে ভিত্তি বীজ ও প্রত্যায়িত বীজ উৎপাদন করিবে এবং উৎপাদনকারীর মতামত নেওয়া হইয়াছে কি না:

(ঘ) ডিএই যখন কৃষকের মাঠে জাত উন্নয়ন সংস্থার সহযোগিতায় প্রদর্শনী গ্রহণে সমর্থ হইবে তখন কতগুলি প্রদর্শনী করিতে হইবে:

(ঙ) উপরে উল্লিখিত সকল তথ্য ও ফসল সংগ্রহোত্তর এবং বীজ উৎপাদন সংবলিত একটি বাংলা প্রযুক্তি অনুলিপি এতদ্‌সঙ্গে সংযোজিত।

২৫। অতিরিক্ত তথ্যাবলি :

আবেদনকারীর স্বাক্ষর, তারিখ ও সিল

আবেদনপত্রের সহিত নিম্নবর্ণিত কাগজপত্র সংযুক্ত করিতে হইবে:

১। বীজ ডিলার নিবন্ধ সনদের কপি।

২। আবেদনকারী কর্তৃক জাতটির গুণগতমান ও বৈশিষ্ট্যসমূহ সংরক্ষণের দায়-দায়িত্ব বহন করিবার ঘোষণাপত্র।

৩। বেসরকারি প্রতিষ্ঠানসমূহকে বাংলাদেশ সীড এসোসিয়েশনের সদস্য মর্মে সনদপত্রের কপি দাখিল করিতে হইবে।

Annexure V

FORM-11 of the Seed Rules 2020 in case of Non-Notified Crops (English version)

FORM- 11
[See Rule 11, Sub- Rule (3)]
Application for Registration of Non-Notified Crop Variety

To
The Director General, Seed Wing
Secretary, National Seed Board
Ministry of Agriculture
Bangladesh Secretariat, Dhaka- 1000

1. Name and Address of Applicant
2. Seed Dealer Registration Number (Valid/ Updated): Date:
3. Name and Address of Applicant (Person in- charge for the development of proposed Variety):
4. The Variety from which the proposed Variety has been developed-
   (A) Variety Name in Bangla:
   (B) Variety Name in English:
   (C) Botanical/ Scientific Name of the Variety:
5. Name of proved Variety for Registration (In Bangla and in English):
6. Ecological requirement of the proposed Variety:
   (A) Introduction:
   (B) Country of Origin / Place and Name of Organization
7. Name and Address of Breeder:
8. Agronomical requirement of the proposed Variety:
   (A) Cultivation Method:
   (B) Seed rate per hectare (in kg):
   (C) Planting Distance/Spacing (in cm/meter):
   (D) Number of seedlings per hectare:
   (E) Requirement of fertilizer per hectare (in kg):
   (F) Growth duration of crop in the field in days (seed to seed):
9. Describe, if special processing is needed for the product to be used.
10. Name of usable part of the crop
11. Description of any tests performed on disease and insect reactions: Natural (Number of Trial Seasons/ Number of Trial Years):
12. Description of features of proposed Variety:
    (A) Identification features:
    (B) Other special features:

13. Description of tests listed below (Result of yield in metric ton per hectare) conducted:
    (A) Regional Yield Trial (RYT):
    (B) On Farm Yield Trial:
    (C) Yield Trial on Farmers' Field:
    (D) Agronomical Trial:

Signature, Date and Seal of Applicant

The following documents will have be attached with the Application Form:

1. Copy of valid/ updates Seed Dealer Registration Certificate;
2. Declaration by the applicant to be responsible for preserving the quality and characteristics of the Variety; and
3. Private sector Organization is required to Submit Copy of the Certificate of Membership of Bangladesh Seed Association.

FORM-11 of the Seed Rules 2020 in case of Non-Notified Crops (Bangla version)

ফরম-১১

[বিধি ১১ এর উপ-বিধি (৩) দ্রষ্টব্য]

অনিয়ন্ত্রিত ফসলের জাত ছাড়করণ বা নিবন্ধন আবেদন

বরাবর
মহাপরিচালক, বীজ অনুবিভাগ/সচিব, জাতীয় বীজ বোর্ড
কৃষি মন্ত্রণালয়
বাংলাদেশ সচিবালয়, ঢাকা-১০০০

১। প্রতিষ্ঠানের নাম ও ঠিকানা:

২। বীজ ডিলার নিবন্ধন নম্বর (বৈধ/হালনাগাদ): তারিখ :

৩। আবেদনকারীর নাম ও ঠিকানা (প্রস্তাবিত জাতের উন্নয়নের জন্য দায়িত্বপ্রাপ্ত ব্যক্তি):

৪। যে জাত হইতে প্রস্তাবিত জাতের উদ্ভব হইয়াছে তাহার—

(ক) বাংলা নাম:

(খ) ইংরেজি নাম:

(গ) উদ্ভিদতাত্ত্বিক/বৈজ্ঞানিক নাম:

৫। নিবন্ধনের জন্য প্রস্তাবিত নাম (বাংলা ও ইংরেজি):

৬। প্রস্তাবিত জাতের পরিবেশগত (Ecological) চাহিদা :

(ক) সূচনা:

(খ) উৎস দেশ/স্থান ও প্রতিষ্ঠানের নাম:

৭। প্রজননবিদের নাম ও ঠিকানা:

৮। প্রস্তাবিত জাতের কৃষিতাত্ত্বিক (Agronomical) চাহিদা:

(ক) চাষ পদ্ধতি:

(খ) প্রতি হেক্টরে বীজের হার:

(গ) রোপণ দূরত্ব:

(ঘ) প্রতি হেক্টরে গাছের সংখ্যা:

(ঙ) প্রতি হেক্টরে সারের প্রয়োজনীয়তা:

(চ) মাঠে ফসলের জীবনকাল (বীজ হইতে বীজ):

৯। ফসলের উৎপাদিত পণ্য ব্যবহারের জন্য যদি বিশেষ প্রক্রিয়াজাতকরণের প্রয়োজন হয়, তাহা হইলে উহার বিবরণ:

১০। ফসলের ব্যবহারযোগ্য অংশের নাম:

১১। রোগ এবং পোকার প্রতিক্রিয়ার উপর কোনো পরীক্ষা করা হইয়া থাকিলে উহার বিবরণ: প্রাকৃতিক (পরীক্ষিত মৌসুম/বৎসরের সংখ্যা):

১২। প্রস্তাবিত জাতের বৈশিষ্ট্যের বিবরণ :

(ক) শনাক্তকারী বৈশিষ্ট্য:

(খ) অন্যান্য বিশেষ বৈশিষ্ট্য:

১৩। নিম্নলিখিত পরীক্ষার বর্ণনা (ফলন: টন/হেক্টর):

(ক) আঞ্চলিক ফলন পরীক্ষা (RYT):

(খ) খামারে ফলন পরীক্ষা:

(গ) কৃষকের জমিতে ফলন পরীক্ষা:

(ঘ) কৃষিতাত্ত্বিক পরীক্ষা:

আবেদনকারীর স্বাক্ষর, তারিখ ও সিল

আবেদনপত্রের সহিত নিম্নবর্ণিত কাগজপত্র সংযুক্ত করিতে হইবে:

১। বীজ ডিলার নিবন্ধ বৈধ/হালনাগারদ প্রত্যয়নপত্রের সনদের কপি;

২। আবেদনকারী কর্তৃক জাতটির গুণগতমান ও বৈশিষ্ট্যসমূহ সংরক্ষণের দায়-দায়িত্ব বহন করার ঘোষণাপত্র; এবং

৩। বেসরকারি প্রতিষ্ঠানসমূহকে বাংলাদেশ সীড এসোসিয়েশনের সদস্য মর্মে সনদপত্রের কপি দাখিল করিতে হইবে।

8. ACKNOWLEDGEMENTS

Expert Committee on Policies for Gene Edited Plants in Bangladesh, Bangladesh Academy of Sciences (BAS) and contributors of different National Agricultural Research System (NARS) institutes & universities for finalization of the SOPs.

Expert Committee on 'Policies for Gene Edited Plants in Bangladesh', BAS

Sl.	Name and Affiliation	Designation
1.	**Professor Dr. Zahurul Karim** Vice President, Bangladesh Academy of Sciences	Chairperson
2.	**Professor Dr. Haseena Khan** Secretary, Bangladesh Academy of Sciences	Member Secretary
3.	**Maj. Gen. (Retd.) Professor Dr. ASM Matiur Rahman** Fellow, Bangladesh Academy of Sciences	Member
4.	**Professor Dr. Zeba Islam Seraj** University of Dhaka and Fellow, Bangladesh Academy of Sciences	Member
5.	**Professor Dr. Mirza Hasanuzzaman** Sher-e-Bangla Agricultural University and Fellow, Bangladesh Academy of Sciences	Member
6.	**Professor Dr. Md. Tofazzal Islam** Bangabandhu Sheikh Mujibur Rahman Agricultural University and Fellow, Bangladesh Academy of Sciences	Member
7.	**Dr. Md. Aziz Zilani Chowdhury** Former Member Director (Crops) Bangladesh Agricultural Research Council	Member
8.	**Dr. Md. Salimullah** Director General, National Institute of Biotechnology	Member
9.	**Dr. Vibha Ahuja** Chief General Manager, Biotech Consortium India Limited	Member
10.	**Professor Dr. Rakha Hari Sarker** University of Dhaka & Country Coordinator, SABP	Member
11.	**Professor Dr. Aparna Islam** Biotechnology Program, BRAC University	Member
12.	**Dr. Mohammad Khalequzzaman** Director (Research), Bangladesh Rice Research Institute	Member
13.	**Dr. Md. Abdullah Yousuf Akhond** Director (Research), Bangladesh Agricultural Research Institute	Member
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Homologous Recombination Repair (HRR): Concept, Mechanisms, Methods, and Applications

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Introduction

Homologous recombination (HR) is a fundamental biological process that ensures the precise exchange of genetic material between homologous DNA sequences. This mechanism plays a critical role in maintaining genomic stability, repairing DNA damage, and facilitating genetic diversity. HR is widely utilized in genetic engineering, molecular biology, and therapeutic applications.

This document explores the **concept, mechanisms, methods, and applications** of homologous recombination in detail.

Concept of Homologous Recombination

Homologous recombination is a process in which genetic exchange occurs between two DNA molecules or segments with identical or highly similar nucleotide sequences. It is a crucial mechanism for DNA repair and genetic variation.

Key Characteristics of HR:

1. **Sequence Homology:** Requires a homologous sequence as a template for repair or recombination.
2. **High Fidelity:** Ensures error-free repair, reducing the risk of mutations.
3. **Essential for Genetic Stability:** Prevents chromosomal abnormalities and maintains genome integrity.
4. **Conserved Across Species:** Found in bacteria, yeast, plants, and mammals, indicating its evolutionary importance.

Biological Functions of HR:

- **DNA Repair:** Repairs double-strand breaks (DSBs) caused by radiation, chemicals, or replication stress.
- **Meiotic Recombination:** Promotes genetic diversity during gamete formation in sexually reproducing organisms.
- **Restarting Stalled Replication Forks:** Prevents replication failure and maintains genome integrity.
- **Chromosome Segregation:** Ensures accurate chromosome distribution during cell division.
- **Genetic Engineering:** Used for gene targeting and transgenic organism creation.

Mechanisms of Homologous Recombination

The HR process involves several steps mediated by key enzymes and protein complexes. It primarily follows two pathways:

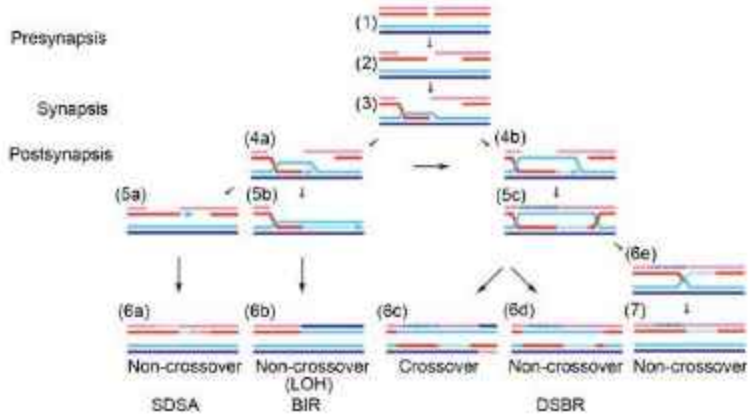

Figure 01: Pathways of recombination in DSB repair. Homologous recombination can be conceptually divided into three stages: presynapsis, synapsis, and postsynapsis. During presynapsis, DSB ends are recognized and processed to a 3'-OH ending single-stranded tail (steps 1-2). In synapsis, DNA strand invasion by the Rad51-ssDNA filament generates a D-loop (step 3). At least three different pathways are proposed after the D-loop intermediate. In synthesis-dependent strand annealing (SDSA, steps 4a - 5a - 6a), the invading strand is disengaged after DNA synthesis and annealed with the second end, leading to localized conversion without crossover. This process may involve multiple rounds of invasion, synthesis, and disengagement. In break-induced replication (BIR, steps 4a - 5b - 6b), the D-loop is assembled into a full replication fork, copying the entire distal part of the chromosome to result in loss-of heterozygosity (LOH). In double-strand break repair (DSBR, steps 4b - 5c - 6c-e - 7), both ends of the DSB are engaged, either by independent strand invasion or by second end capture, leading to double Holliday junction formation. The junction can be processed by either a resolvase into non-crossover or crossover products (steps 6c and d) or dissolved by a mechanism involving BLM-mediated branch migration and TOPOIIIα-catalyzed dissolution of a hemi-catenane (step 6e), leading exclusively to non-crossover products (step 7).

1. Double-Strand Break (DSB) Repair Pathway

This is the most well-characterized HR mechanism. It occurs in several sequential steps:

Step 1: Recognition and Processing of DSB

- DSBs trigger the recruitment of the **MRN complex (MRE11-RAD50-NBS1)** in eukaryotes.
- The **MRN complex** processes the broken DNA ends, generating **3′ single-stranded overhangs**.
- **Exonucleases (e.g., Exo1, DNA2)** further resect the DNA strands.

Step 2: Strand Invasion and D-loop Formation

- The **RAD51 recombinase**, assisted by **BRCA1 and BRCA2**, binds to the single-stranded DNA, forming a nucleoprotein filament.
- This complex searches for a homologous sequence on the sister chromatid.
- Strand invasion occurs, leading to the formation of a **displacement loop (D-loop)**.

Step 3: DNA Synthesis and Holliday Junction Formation

- DNA polymerases extend the invaded strand using the homologous template.
- The second broken end is captured, leading to the formation of **Holliday junctions (HJ)**.

Step 4: Branch Migration and Resolution

- The HJ undergoes **branch migration**, facilitated by helicases like **BLM and WRN**.
- The structure is resolved by endonucleases (**GEN1, MUS81-EME1 complex**), leading to crossover or non-crossover outcomes.

2. Synthesis-Dependent Strand Annealing (SDSA) Pathway

- Instead of forming a Holliday junction, the invading strand is displaced after DNA synthesis.
- The newly synthesized DNA anneals back to the original strand.
- No crossover occurs, ensuring non-mutagenic repair.

Methods to Study Homologous Recombination

1. Molecular and Genetic Techniques

- **Yeast Two-Hybrid Assay:** Detects protein-protein interactions in HR pathways.
- **Chromatin Immunoprecipitation (ChIP):** Identifies HR protein binding sites.
- **Recombination Reporter Assays:** Measures HR frequency in cells using GFP or antibiotic resistance markers.
- **CRISPR/Cas9-based Targeting:** Introduces DSBs to assess HR-mediated repair.

2. Microscopy-Based Approaches

- **Fluorescence In Situ Hybridization (FISH):** Visualizes homologous recombination events.
- **Live-cell Imaging:** Tracks real-time HR dynamics using fluorescent tags.

3. Biochemical Methods

- **Electrophoretic Mobility Shift Assay (EMSA):** Studies HR protein-DNA interactions.
- **DNA Fiber Assays:** Monitors DNA synthesis during HR repair.

Applications of Homologous Recombination

HR has numerous applications in genetics, medicine, and biotechnology.

1. Gene Editing and Genetic Engineering

- **Gene Targeting:** Used to introduce specific genetic modifications in model organisms.
- **Transgenic Organisms:** HR-based knock-in and knock-out models in mice and plants.
- **Precision Medicine:** Customizing gene therapies for inherited disorders.

2. Cancer Research and Therapy

- **Targeting BRCA1/2 Mutations:** HR-deficient cancer cells are susceptible to **PARP inhibitors (e.g., Olaparib)**.
- **Synthetic Lethality Approaches:** Exploiting HR defects in tumors for selective cancer treatment.

3. Drug Discovery and Screening

- **Identifying DNA Repair Modulators:** Screening for compounds affecting HR proteins.
- **Developing Chemotherapy Strategies:** Combining HR inhibitors with DNA-damaging agents.

4. Crop Improvement and Agricultural Biotechnology

- **Genome Editing in Plants:** HR-based modifications in crops for better yield and disease resistance.
- **Gene Replacement Strategies:** Producing stress-resistant plants through HR-mediated transgene integration.

5. Microbial and Evolutionary Studies

- **Horizontal Gene Transfer Analysis:** Investigating HR in bacterial genetic exchanges.
- **Studying Evolutionary Relationships:** Tracing species divergence using recombination rates.

Conclusion

Homologous recombination is a vital process for maintaining genetic stability and diversity. Its role in DNA repair, meiosis, and genetic engineering makes it a powerful tool in both fundamental biology and applied sciences. Advances in HR-based genome editing have revolutionized medicine, agriculture, and biotechnology, paving the way for novel therapeutic and research applications. Understanding and harnessing HR will continue to drive innovations in genetics, molecular biology, and personalized medicine.

RNA Editing and Site-Directed RNA Editing Using ADAR: Concept, Mechanisms, Methods, and Applications

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Introduction to RNA Editing

RNA editing is a post-transcriptional modification process that alters the nucleotide sequence of RNA transcripts, thereby diversifying the proteome beyond what is encoded by the genome. This process enables cells to regulate gene expression dynamically, influencing protein function, stability, and localization. RNA editing plays a crucial role in various biological processes, including development, neurological functions, and immune response.

Types of RNA Editing

There are two primary types of RNA editing in eukaryotes:

1. **Adenosine-to-Inosine (A-to-I) Editing**: This is catalyzed by the adenosine deaminase acting on RNA (ADAR) enzyme. Inosine is recognized as guanosine during translation, leading to codon changes and altered protein synthesis.
2. **Cytosine-to-Uracil (C-to-U) Editing**: This is catalyzed by apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) enzymes, which convert cytosine (C) into uracil (U), impacting protein coding sequences and regulatory elements.

Among these, **A-to-I editing by ADAR is the most common and well-characterized type of RNA editing in mammals.**

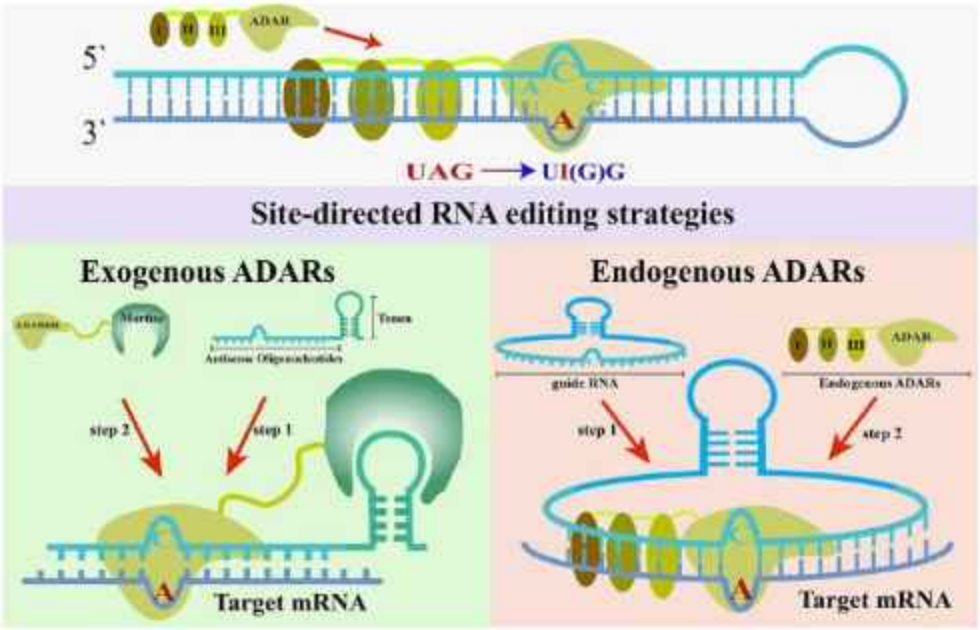

Figure 01: Site-Directed RNA Editing Using ADAR

Adenosine Deaminases Acting on RNA (ADAR): Concept and Mechanism

ADAR enzymes mediate **A-to-I RNA editing** by deaminating adenosine residues within double-stranded RNA (dsRNA) structures. This process plays an essential role in neuronal function, immune response, and transcriptome diversity.

Types of ADAR Enzymes

There are three main members of the ADAR family in mammals:

1. **ADAR1**: Ubiquitously expressed, with two isoforms (p110 and p150). ADAR1 p150 is induced by interferons and regulates immune responses.

2. **ADAR2**: Highly expressed in the brain, responsible for editing neural transcripts like the glutamate receptor subunit GRIA2.
3. **ADAR3**: Primarily found in the brain but is catalytically inactive, potentially acting as a regulatory molecule.

Mechanism of ADAR-Mediated A-to-I Editing

1. **Recognition of Double-Stranded RNA (dsRNA):** ADAR binds to dsRNA structures within pre-mRNAs, typically formed by inverted repeat sequences or intronic complementary regions.
2. **Deamination Reaction:** ADAR catalyzes the hydrolytic deamination of adenosine to inosine.
3. **Functional Consequences:** Since inosine is read as guanosine by the translation and splicing machinery, A-to-I editing can lead to amino acid substitutions, alternative splicing, altered RNA stability, or differential protein interactions.

Biological Roles of A-to-I RNA Editing

- **Neurotransmission Regulation:** ADAR2-mediated editing of the glutamate receptor (GRIA2) affects ion channel permeability, impacting neuronal excitability.
- **Innate Immunity:** ADAR1 prevents aberrant activation of the innate immune system by editing endogenous dsRNA, reducing its recognition by pattern recognition receptors (PRRs).
- **mRNA Stability & Processing:** Editing can influence alternative splicing and RNA degradation pathways.

Site-Directed RNA Editing Using ADAR

Site-directed RNA editing (SDRE) is an engineered approach to harness ADAR-mediated A-to-I editing for targeted RNA modifications. This technique enables researchers to edit specific adenosines in RNA transcripts, providing a powerful tool for gene regulation and therapeutic applications.

Strategies for Site-Directed RNA Editing

1. **Recruitment of Endogenous ADAR:**
    - Guide RNAs (gRNAs) with complementary sequences are designed to bind near the target adenosine.
    - The dsRNA structure formed recruits endogenous ADAR enzymes for site-specific editing.
2. **Engineering ADAR Variants:**
    - Catalytically active ADARs can be fused with RNA-binding domains to target specific sequences.
    - Mutant ADARs with improved specificity have been developed to enhance efficiency.
3. **Chemical & Small-Molecule Approaches:**
    - Small-molecule modulators can enhance ADAR activity for therapeutic applications.

Methods for Site-Directed RNA Editing

1. **Synthetic Guide RNA Approaches**:
    - Guide RNAs complementary to the target mRNA sequence are designed to form dsRNA structures.
    - Endogenous ADAR enzymes recognize and edit the target site.
2. **ADAR-Protein Engineering**:
    - Fusion of catalytically active ADAR domains with RNA-targeting proteins (e.g., Cas proteins, Pumilio).
    - Enables precise control over editing specificity and efficiency.
3. **CRISPR-Based ADAR Editing (REPAIR System)**:
    - The REPAIR (RNA Editing for Programmable A-to-I Replacement) system uses catalytically active ADAR2 fused to a catalytically dead Cas13 to direct site-specific editing.
    - Provides a programmable and efficient approach to RNA editing.

Applications of Site-Directed RNA Editing

1. Therapeutic Applications

- **Genetic Disease Correction:** RNA editing can transiently correct mutations without altering the genome, making it a safer alternative to CRISPR gene editing.
- **Neurological Disorders:** ADAR-mediated editing has been explored for treating conditions like amyotrophic lateral sclerosis (ALS) and epilepsy.
- **Cancer Therapy:** Targeted RNA editing can modify oncogene expression or alter drug resistance mechanisms.

2. Functional Genomics and Research Tools

- **Protein Function Modulation:** RNA editing allows for amino acid substitutions without permanent genome modifications.
- **Regulation of Alternative Splicing:** Site-directed editing can influence exon inclusion or exclusion.

3. Biotechnological and Synthetic Biology Applications

- **Synthetic RNA Circuits:** Engineered ADAR systems enable RNA-based gene regulation.
- **Molecular Recording:** RNA editing events can be used to track cellular history and environmental responses.

Advantages of RNA Editing over DNA Editing

- **Reversible and Dynamic:** RNA editing is transient and allows temporal control.
- **Minimized Off-Target Effects:** Unlike CRISPR, RNA editing does not induce double-strand DNA breaks.
- **Tissue-Specific Regulation:** ADAR expression can be controlled in specific tissues for localized editing.

Challenges and Future Directions

- **Editing Efficiency:** Enhancing the specificity and efficiency of ADAR-mediated editing remains a key challenge.
- **Delivery Methods:** Developing efficient delivery systems (e.g., lipid nanoparticles, viral vectors) for ADAR editors is critical.
- **Immune Response:** Minimizing immune recognition of modified RNAs is necessary for therapeutic applications.
- **Expanding Targeting Range:** Improving the targeting precision of ADAR enzymes to edit more diverse sequences.

Conclusion

RNA editing, particularly **A-to-I editing by ADAR**, plays a crucial role in transcriptome regulation, neuronal function, and immune modulation. Site-directed RNA editing offers a powerful approach for precision medicine, allowing for transient and reversible gene modifications. Advances in ADAR engineering and guide RNA design continue to enhance the potential of RNA editing for treating genetic disorders, modulating protein function, and developing novel biotechnological applications. As research progresses, site-directed RNA editing may become a mainstream tool in molecular medicine and synthetic biology, offering a safer and more controllable alternative to permanent genome editing techniques.

Base Editing: Concept, Mechanisms, Methods and Applications

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Concept

Base editing is a type of genome editing – a way to make targeted changes to the sequence of a piece of DNA.

CRISPR, refers to genome editing approaches that use a CRISPR guide RNA molecule to find their target. More accurately, CRISPR is an acronym that stands for clustered regularly interspaced short palindromic repeats. It describes patterns observed in bacterial DNA that code for RNA guide molecules that target specific DNA sequences. These guide molecules pair with a CRISPR-associated protein (Cas), which cuts the DNA when the guide RNA. These bacterial molecules have been harnessed by scientists to create CRISPR-based genome editing technology.

The most common type of CRISPR, CRISPR/CAS9, consists of a guide RNA modified to target a particular DNA sequence plus Cas9, which cuts through both strands of the DNA once it finds its target. Once created, this break in the DNA allows for DNA to be added or removed at the target site; conveniently, the cell's own repair mechanisms will fix the breaks.

Base editing uses a CRISPR guide RNA to bind its target sequence, but instead of cutting the DNA strand, it chemically changes one DNA base letter into another. So, base editing is part of the wider family of CRISPR genome editing approaches, but it does not work in the 'cut and paste' way most associated with CRISPR/Cas9.

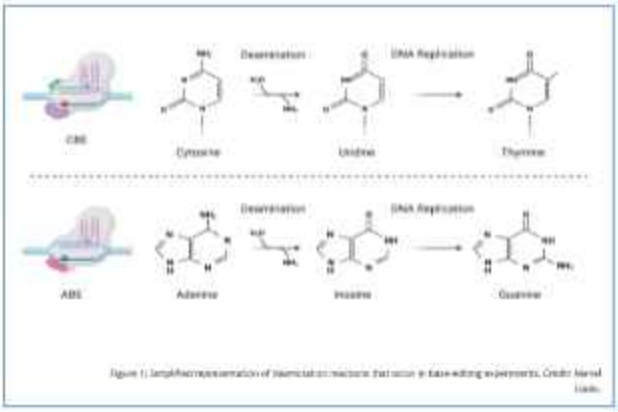
What is base editing used for?

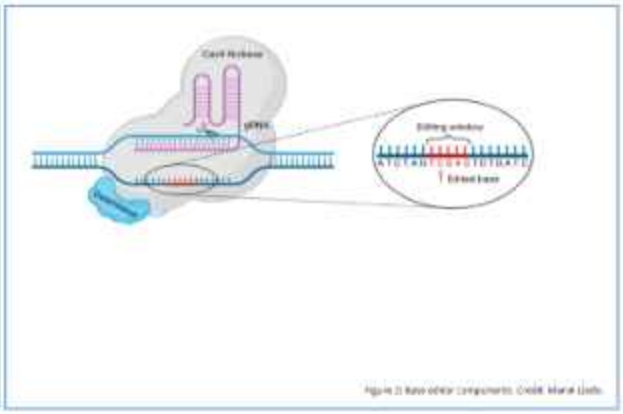
- Base editing is best suited to making single-nucleotide changes to a DNA sequence. Because it chemically changes the target letter, it can only make one change at a time. This has advantages and disadvantages.
- CRISPR/Cas9 can make much larger changes: it can remove or insert pieces of DNA into the target strand. However, to do this it makes double-stranded breaks in the DNA, which carries the risk of errors being introduced. These errors can include cuts in the wrong part of the genome (off-target effects) and unintended indels (where fragments of DNA are lost or gained when the break is repaired).
- CRISPR-derived base editors (BEs) have been developed as a versatile technology to generate targeted point mutations without the need for generating DSBs and providing homology repair templates, thereby enabling editing in HDR-deficient cells.
- BEs are modular fusions of a RuvC-inactivated nickase version of Cas9 with a nucleotide deaminase enzyme.
- Initially, two classes of BEs were developed. Cytosine BEs (CBEs), which contain catalytic domains derived from cytidine deaminases such as APOBEC1, as well as an uracil glycosylase inhibitor (UGI) domain, mediate C-to-T conversion.
- In turn, adenine BEs (ABEs) generate A-to-G conversions using an adenosine deaminase domain from the tRNA-specific deaminase TadA that has been engineered by directed evolution to act on ssDNA.
- Upon binding of the Cas9 module, BEs deaminate a cytosine or adenine within an "editing window" in the PAM-distal segment of the displaced non-target DNA strand to uracil or inosine, respectively.
- These are read out during DNA replication as thymine and guanine, respectively, inducing transition point mutations. Since their invention, the original CBE and ABE editors have gone through several design iterations to improve activity and reduce the amount of deaminase-induced off-target edits, and Cas12a BEs have also been developed.
- The base editing repertoire has also been expanded to also cover A-to-C, A-to-Y, and C-to-G transversions.
- Due to their largely predictable editing outcomes, BEs have been applied for genome-wide knockout and mutational screens.
- The precision of BEs makes them suitable for therapeutic corrections of diseases caused by single-point mutations.

What are the components of a base editor?

Base editors are generated by coupling two separate proteins capable of very specific functions, as illustrated in Figure 2. The components of a typical base editor include:

- **Cas9 nickase (nCas9):** This is a version of Cas9 that has been modified through mutations in one of the two main amino acid residues responsible for the DNA cleavage activity of Cas9. Thus, nCas9 is still able to pair with a gRNA and find the DNA sequence complementary to the gRNA spacer, but is only able to nick one strand of the DNA.
- **Nucleoside deaminase:** This is an enzyme capable of removing an amino group from a specific type of nucleoside. The deaminase fused to the nickase – be it a cytosine deaminase (e.g. APOBEC) or an adenosine deaminase (e.g. engineered TadA) – will dictate whether the base editor is a CBE or an ABE. CBEs contain cytosine deaminases, while ABEs typically contain adenine deaminases (though several groups have recently engineered CBEs from the conventional TadA adenosine deaminase domain (6-8)).



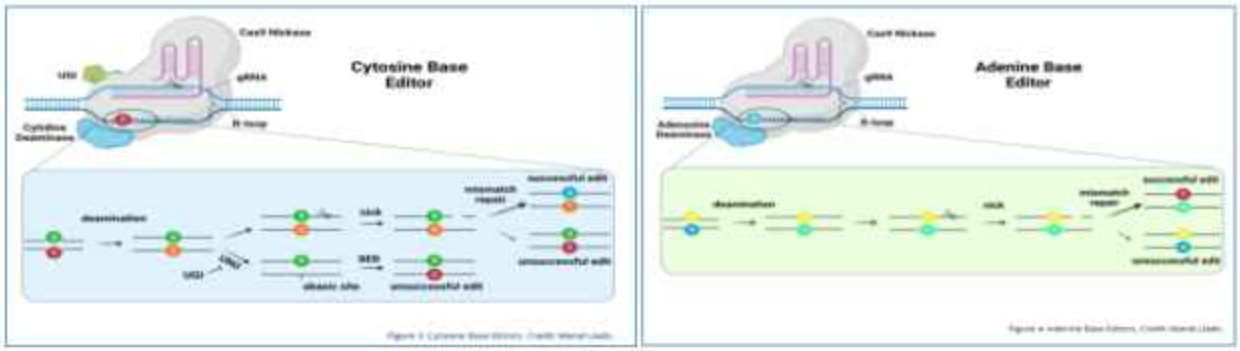


The Cas9-deaminase fusion protein complex is targeted to a specific DNA locus by a **guide RNA (gRNA)**. Once the base editor has bound to its target sequence, the deaminase can modify bases within the exposed non-target strand (NTS) of the target site R-loop. The area of the target locus in which bases can be modified is called the **base editing window** (see Figure 2). Depending on the specific intended modification, other factors may be included in the base editor complex to increase efficiency or alter the outcome of the base substitution.

Mechanisms

How does base editing work?

- Once the base-editing complex recognises the target DNA sequence complementary to the gRNA spacer, Cas9 mediates the denaturing of the double-stranded DNA (dsDNA) to form a structure known as an R-loop, which exposes a short stretch of single-stranded DNA (ssDNA) in the non-complementary strand to the activity of the deaminase (see Figure 3; also called the non-target strand).
- This allows the deaminase to chemically modify bases inside the base editing window, which is normally 5-10bp long depending on the deaminase, and somewhat distal from the protospacer adjacent motif (PAM) within the target site.
- The first generation of base editors encountered severe efficiency issues that had to be solved. For example, **CBEs** mediate the deamination of cytosines, generating a uracil (U) base, which can then be read by the cell's DNA polymerases as thymine.
- However, human cells are very efficient at DNA repair, and especially at dealing with deaminated bases via the base-excision repair (BER) pathway. Thus, uracil can be recognised and eliminated by the enzyme uracil DNA N-glycosylase (UNG) during the initiation of BER. This drastically limits the efficiency of C-to-T edits and can lead to reduced C-to-T purity (and increased C-to-A or C-to-G edits).
- To overcome this issue, second-generation CBEs included a third component, namely a uracil glycosylase inhibitor (UGI) fused together with Cas9 and the cytosine deaminase. UGI inhibits the action of UNG on the edited DNA, thus conserving the uracil base and achieving 3-fold higher editing efficiency and purity (Ref. 1, and see Figure 3).



- In addition, the DNA mismatch repair mechanisms of the cell are tasked with detecting and resolving any incorrect nucleotide pairings into correct pairings (A-T and C-G).
- Since the deamination of a cytosine, which will be paired with a guanine, will lead to an incorrect T-G pairing, this will be repaired by the cell, either by substituting the U for a C (C-G pairing) or by substituting the G for an A (T-A pairing).
- This cut in the complementary (=unedited) strand, directs the mismatch repair machinery to utilise the base-edited strand as a template for repair, thus biasing the repair towards the introduced edit, and further improving efficiency (Figure 3).

- **ABE**s work in a very similar way. In this case, the deamination of adenine generates an inosine residue, which is interpreted by the DNA polymerases as a guanosine. Thus, an A-to-G edit is achieved (Figure 4). However, no UGI or equivalent inhibitor is necessary since inosine residues are not excised like uracil residues.
- One step that was very important for the generation of ABEs was making an adenine deaminase that could edit the DNA, since naturally occurring adenine deaminases only edit RNA.
- Directed mutagenesis efforts were successful in generating a version of the *E.coli* tRNA adenosine deaminase (TadA) that can edit the DNA.
- Interestingly, editing efficiency was higher when the ABEs included one mutated TadA (TadA*) and one wildtype enzyme.
- ABEs are very important because G-C to A-T mutations represent the most frequently reported pathogenic point mutations, thus this type of base editing has the greatest potential for clinical applications.
- More recent ABEs have been developed that have improved editing efficiencies.
-

Applications in healthcare

Base editing has the potential to positively impact healthcare because it carries a lower risk of creating indels, and because single-nucleotide changes are associated with many genetic diseases in humans, including sickle cell disease and Tay-Sachs disease.

In a recent article, we described how base editing was used to make four single-nucleotide changes to the genome of blood stem cells in order to create a CAR-T cell therapy for a young girl with otherwise untreatable leukaemia.

When making CAR-T cells, the genome editing takes place outside the body, so there is an opportunity to check for errors before the cells are given to the patient. However, base editing is being looked at as a possible tool for in-body gene editing: as part of a major study into cardiomyopathy, researchers are hoping to develop gene therapies using base editing. This is because many inherited cardiomyopathies result from single-nucleotide changes.

In July 2022, a familial hypercholesterolaemia patient in New Zealand became the first to receive an in-body base-editing gene therapy as part of a clinical trial aiming to permanently deactivate the *PCSK9* gene in liver cells. In some people, *PCSK9* causes high levels of low-density lipoprotein cholesterol, which, over time, causes arteries to clog and can result in heart attacks and strokes. The therapy, known as VERVE-101, was created by biotechnology company Verve Therapeutics

What are the limitations of base editing?

Despite its advantages over other gene-editing technologies, base editing presents some limitations that are currently being addressed by scientists all over the world.

Some of the limitations are similar to those of conventional Cas9-based editing approaches regarding targeting, off-target editing and delivery. Thus, already existing Cas9 variants that were developed to solve those issues can be used to construct base editors able to overcome the same issues. When it comes to *in vivo* delivery, the increased size of the base editor complex compared to regular Cas9 complicates its delivery. To address this hurdle, the intein system, which is used to package other Cas9 products into adeno-associated viral vectors, has also been used for *in vivo* delivery of base editors. However, other, base editing-specific limitations have arisen:

- **Requirement for precise positioning of the base editor edit window:** To achieve efficient editing, base editors must be capable of recognising PAMs at an appropriate distance upstream of the intended edit (to position the edit window accordingly). Fortunately, various Cas9 PAM variants have been developed that utilise alternate PAMs, enabling more flexible base editing across the genome (15-17).
- **Unwanted edits in the editing window:** In some cases, when trying to edit a specific base, for example a cytosine, another cytosine inside the editing window can be deaminated as well, leading to unwanted effects. To avoid this, base editor variants with narrower editing windows have been developed by introducing mutations in the deaminase enzymes.
- **Indel generation:** Since new-generation base editors introduce single-strand nicks in the non-edited strand, in some cases the excision of an edited base by the BER can lead to a DSB. The repair of DSBs leads to the generation of INDELs. Strongly inhibiting the BER pathway is one approach to avoid DSB formation.
- **Off-target RNA editing:** An interesting and unexpected observation made by some scientists was that base editors could also produce unwanted edits to RNA, independently of Cas9 activity, which could lead to unpredictable effects. Efforts to solve this issue have focused on engineering the deaminase protein to reduce its activity on the RNA.

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PEG (Protein-Enabled Gene Editing): Concept, Mechanisms, Methods and Applications

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Concept

- Protein-based genome editing technologies utilize engineered proteins to make precise modifications to an organism's DNA.
- These tools have revolutionized genetic research and biotechnology by enabling targeted alterations to the genome, including gene knockout, correction of mutations, and insertion of new genetic material.
- Key technologies in protein-based genome editing include Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), Meganucleases, and CRISPR-Cas systems.
- Protein-based genome editing technologies have transformed genetic research and biotechnology by providing powerful tools for targeted manipulation of genomes across various organisms.
- Each technology—ZFNs, TALENs, meganucleases, CRISPR-Cas systems—offers unique advantages and mechanisms suited for different applications in research and therapeutic contexts.
- With advancements such as base editing and prime editing further expanding capabilities while enhancing precision and reducing off-target effects, these tools continue to shape our understanding of genetics and pave the way for innovative treatments in medicine and agriculture.

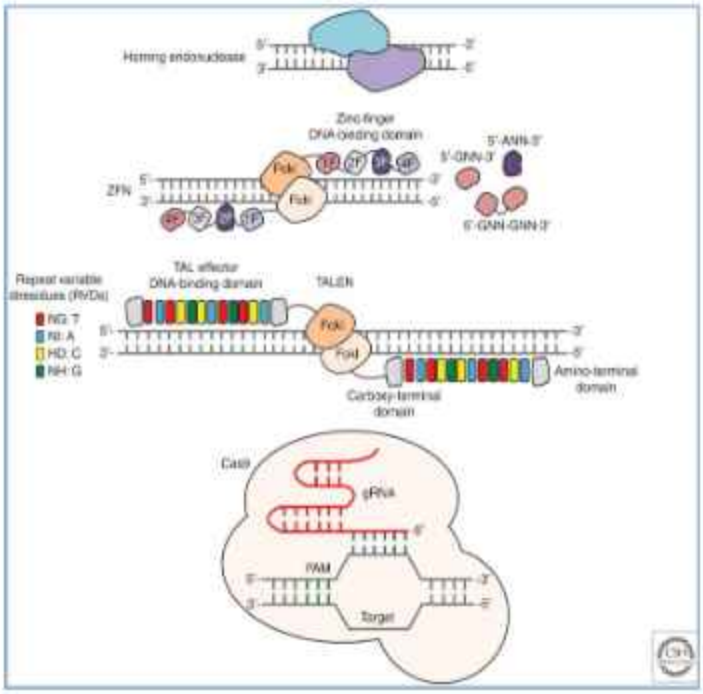
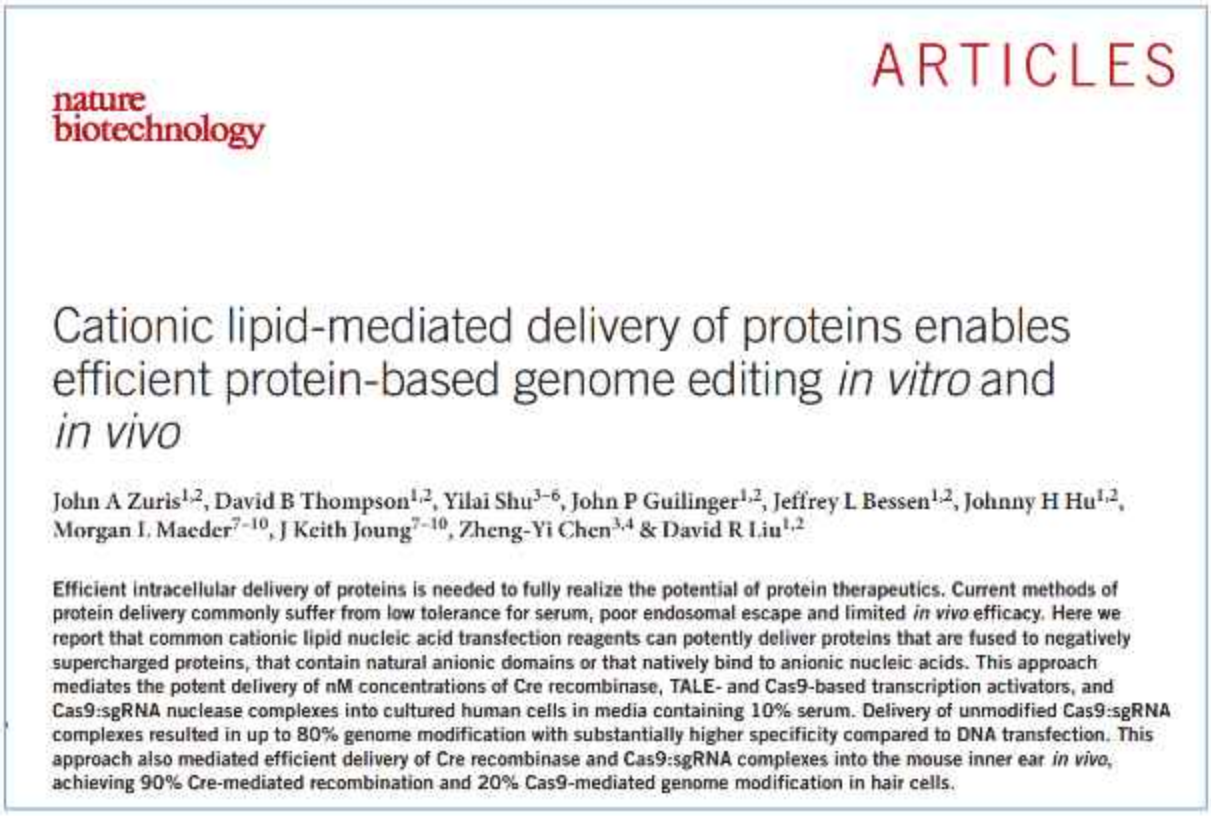


Figure: Genome-editing technologies. Cartoons illustrating the mechanisms of targeted nucleases. From *top* to *bottom*: homing endonucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector (TALE) nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9). Homing endonucleases generally cleave their DNA substrates as dimers, and do not have distinct binding and cleavage domains. ZFNs recognize target sites that consist of two zinc-finger binding sites that flank a 5- to 7-base pair (bp) spacer sequence recognized by the FokI cleavage domain. TALENs recognize target sites that consist of two TALE DNA-binding sites that flank a 12- to 20-bp spacer sequence recognized by the FokI cleavage domain. The Cas9 nuclease is targeted to DNA sequences complementary to the targeting sequence within the single guide RNA (gRNA) located immediately upstream of a compatible protospacer adjacent motif (PAM).



ARTICLES

nature biotechnology

Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing *in vitro* and *in vivo*

John A Zuris[1,2], David B Thompson[1,2], Yilai Shu[3–6], John P Guilinger[1,2], Jeffrey L Bessen[1,2], Johnny H Hu[1,2], Morgan I. Maeder[7–10], J Keith Joung[7–10], Zheng-Yi Chen[3,4] & David R Liu[1,2]

Efficient intracellular delivery of proteins is needed to fully realize the potential of protein therapeutics. Current methods of protein delivery commonly suffer from low tolerance for serum, poor endosomal escape and limited *in vivo* efficacy. Here we report that common cationic lipid nucleic acid transfection reagents can potently deliver proteins that are fused to negatively supercharged proteins, that contain natural anionic domains or that natively bind to anionic nucleic acids. This approach mediates the potent delivery of nM concentrations of Cre recombinase, TALE- and Cas9-based transcription activators, and Cas9:sgRNA nuclease complexes into cultured human cells in media containing 10% serum. Delivery of unmodified Cas9:sgRNA complexes resulted in up to 80% genome modification with substantially higher specificity compared to DNA transfection. This approach also mediated efficient delivery of Cre recombinase and Cas9:sgRNA complexes into the mouse inner ear *in vivo*, achieving 90% Cre-mediated recombination and 20% Cas9-mediated genome modification in hair cells.

Fig. An example of protein-enabled genome editing (Nature, Oct, Zuris et al., 2024)

Zinc Finger Nucleases (ZFNs)

Concept: ZFNs are synthetic proteins that combine a DNA-binding domain made of zinc finger motifs with a DNA-cleaving domain derived from the FokI restriction enzyme. Each zinc finger can recognize a specific triplet of nucleotides, allowing for the design of ZFNs that can target unique sequences in the genome.

Detailed Mechanism:

1. Design and Construction: ZFNs are engineered by assembling multiple zinc finger motifs, each recognizing a specific base pair. Typically, three to six zinc fingers are linked to form a functional DNA-binding domain that can recognize a target sequence of 9-18 base pairs.
2. Binding to Target DNA: The ZFN binds to its specific target sequence in the genome through the interaction of its zinc finger domains with the DNA bases.
3. Dimerization and Cleavage: The FokI nuclease domain is inactive as a monomer but dimerizes upon binding to adjacent ZFNs on opposite strands of the DNA. This dimerization activates the nuclease activity, resulting in a double-strand break (DSB) at the target site.
4. Cellular Repair Mechanisms: The cell repairs the DSB primarily through two pathways:
   - Non-Homologous End Joining (NHEJ): This pathway often results in insertions or deletions (indels) at the break site, leading to gene disruption.
   - Homology-Directed Repair (HDR): If a donor template is provided, HDR can be utilized for precise gene editing by incorporating desired sequences into the genome.

Transcription Activator-Like Effector Nucleases (TALENs)

Concept: TALENs are another class of engineered nucleases that utilize transcription activator-like effectors (TALEs) from Xanthomonas bacteria. TALEs can be engineered to bind specific DNA sequences, providing a customizable approach for genome editing.

Detailed Mechanism:

1. Design and Construction: TALENs consist of TALE repeat domains that can be engineered to recognize specific nucleotide sequences. Each TALE repeat typically recognizes one base pair, allowing for highly specific targeting.
2. Binding to Target DNA: The designed TALEN binds to its target sequence through the interaction of its TALE domains with the DNA bases.
3. Dimerization and Cleavage: Similar to ZFNs, TALENs are fused with the FokI nuclease domain, which requires dimerization for activation. Upon binding to adjacent target sites on opposite strands of DNA, the FokI domains dimerize and cleave both strands, creating a DSB.
4. Cellular Repair Mechanisms: The repair processes following TALEN-induced DSBs are similar to those described for ZFNs:
   - NHEJ: Frequently leads to indels that disrupt gene function.
   - HDR: Can be harnessed for precise edits if a homologous repair template is provided.

Meganucleases

Concept: Meganucleases are naturally occurring endonucleases that recognize long DNA sequences (typically 12-40 base pairs). Their inherent specificity makes them valuable tools for targeted genome editing.

Detailed Mechanism:

1. Design and Engineering: Meganucleases can be engineered by modifying their recognition sites through protein engineering techniques such as directed evolution or rational design.
2. Binding to Target DNA: The engineered meganuclease binds specifically to its target sequence based on its unique recognition properties.
3. Cleavage Mechanism: Once bound, meganucleases cleave both strands of DNA at their recognition site, creating a DSB.
4. Cellular Repair Mechanisms: Similar repair pathways apply:
   - NHEJ: Often results in gene disruption through indels.
   - HDR: Allows for precise modifications if a suitable donor template is available.

CRISPR-Cas Systems

Concept: CRISPR-Cas systems represent a groundbreaking advancement in genome editing technology derived from bacterial adaptive immune systems. The most widely used system is CRISPR-Cas9, which utilizes a guide RNA (gRNA) to direct the Cas9 nuclease to specific genomic locations.

Detailed Mechanism:

1. gRNA Design: A gRNA is designed to complementarily bind to a 20-nucleotide target sequence within the genome adjacent to a protospacer adjacent motif (PAM), typically "NGG" for Cas9.
2. Formation of Ribonucleoprotein Complex: The gRNA forms a complex with the Cas9 protein, creating an active ribonucleoprotein complex capable of recognizing and binding to the target DNA sequence.
3. Target Recognition and Binding: The gRNA guides Cas9 to the target site through base pairing between the gRNA and complementary bases in the target DNA.
4. DNA Cleavage: Upon successful binding, Cas9 induces a conformational change that activates its nuclease activity, resulting in cleavage of both strands of DNA and generating a DSB.
5. Cellular Repair Mechanisms: Similar repair pathways as previously described:
   - NHEJ: Often leads to indel formation disrupting gene function.
   - HDR: Can be employed for precise edits when provided with an appropriate donor template

Types of Genome Editors

1. Nuclease-Based Editors

Nuclease-based editors primarily include ZFNs, TALENs, and CRISPR-Cas systems that create DSBs at targeted sites in the genome.

2. Base Editing

Base editing is a novel genome editing approach that enables the direct, irreversible conversion of one DNA base into another without introducing DSBs or requiring donor templates. This technique utilizes engineered deaminase enzymes fused to a catalytically impaired Cas protein to achieve precise nucleotide modifications.

Cytosine Base Editors (CBEs):

CBEs employ a fusion of a cytidine deaminase enzyme with a catalytically inactive or nickase Cas9 (dCas9 or nCas9). Guided by an sgRNA, the complex binds to a target DNA sequence, and the deaminase converts cytosine to uracil within a specific editing window. During DNA replication or repair, uracil pairs with adenine, resulting in a permanent C•G to T•A transition. This method allows for precise editing of point mutations associated with various genetic diseases.

Adenine Base Editors (ABEs):

ABEs are designed to convert adenine to guanine. They consist of an engineered adenine deaminase fused to dCas9 or nCas9. The complex targets a specific DNA sequence, where the deaminase converts adenine to inosine, which is recognized as guanine by the cell during replication, leading to an A•T to G•C transition. This approach expands the scope of base editing to correct mutations that CBEs cannot address.

Base editing offers several advantages, including high efficiency and reduced risk of unintended insertions or deletions since it does not rely on DSBs. However, limitations include the potential for off-target deaminase activity and the restriction to specific types of base transitions. Ongoing research aims to refine these systems to enhance their specificity and broaden their applicability.

3. Prime Editing

Prime editing is an advanced genome editing technique that enables precise insertions, deletions, and all possible base-to-base conversions without requiring DSBs or donor DNA templates. This method utilizes a fusion protein comprising a Cas9 nickase and a reverse transcriptase enzyme, guided by a prime editing guide RNA (pegRNA) that specifies the target site and encodes the desired edit.

Mechanism of Prime Editing:

- The pegRNA directs the Cas9 nickase to introduce a single-strand nick at the target site.
- The reverse transcriptase then uses the pegRNA as a template to synthesize the desired genetic alteration directly onto the nicked DNA strand.
- Cellular repair mechanisms subsequently incorporate the edited strand into the genome, achieving the intended modification without creating DSBs.
- Prime editing offers remarkable versatility, capable of performing a wide range of genetic edits, including precise point mutations, small insertions, and deletions.
- Its ability to make exact changes without relying on DSBs reduces the risk of unintended mutations and genomic instability.

TALENs (Transcription Activator-Like Effector Nucleases): Concept, Mechanisms, Methods and Applications

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Concept

New molecular breeding techniques use gene-editing tools such as clustered regularly interspaced short palindromic repeats (CRISPR), transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and homing endonucleases or meganucleases.

CRISPRs have become the most popular of the genome editing techniques. Yet, TALENS are extremely precise and have additional capabilities, such as targeting any DNA sequence, discriminating between methylated and unmethylated DNA targets, and modifying DNA within organelles such as mitochondria.

What are TALENs?

- TALENs, or transcription activator-like effector nucleases, are engineered nucleases that allow for precise and effective genome editing in living cells.
- Transcription activator-like effectors (TALEs) are proteins made and used by plant pathogenic bacteria to control plant genes during infection.
- In nature, TALEs bind to plant DNA sequences and activate genes. Binding is targeted to specific DNA sequences through amino acid repeats of the TALE protein that recognize specific DNA bases by a set of biochemical rules or "code".
- Researchers can use this code to customize TALEs and TALE protein fusions to bind to any desired DNA sequence.
- TALENs are protein combinations composed of two parts: one part is the TALE that targets the protein to a specific DNA sequence and the second part is a nuclease (N) that cuts DNA. Fok1 is a nuclease that is commonly used in TALENs (Figure 1).

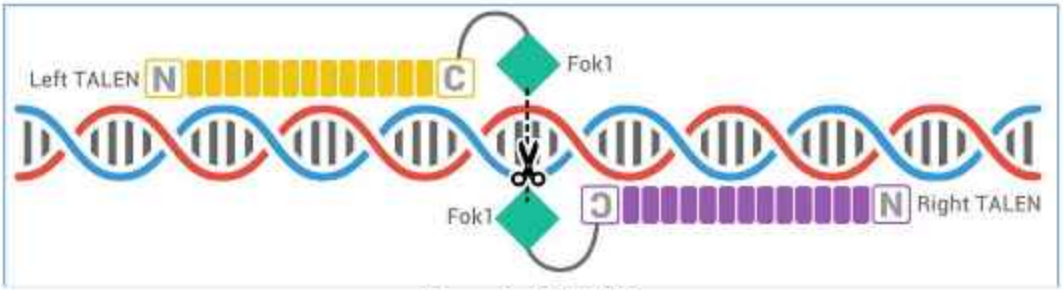


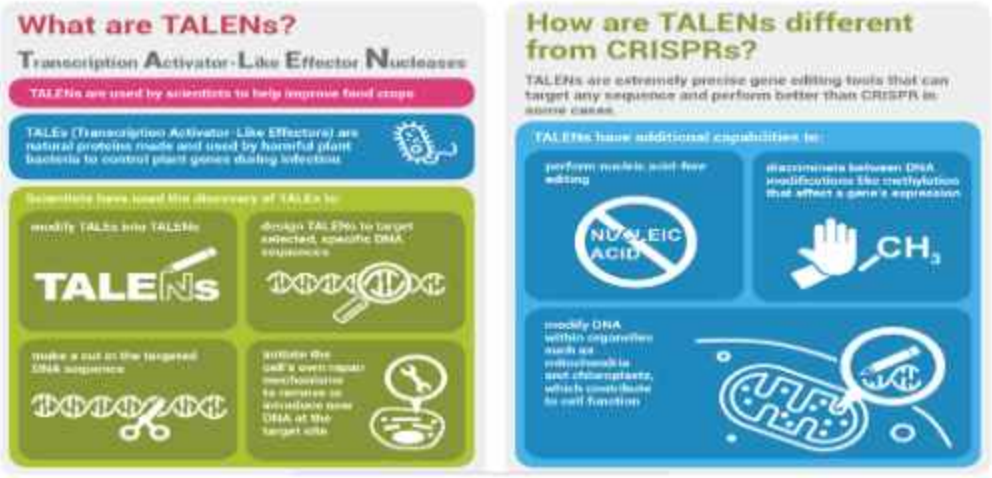
Figure 1. TALENs

- TALE-nuclease fusions cut the DNA, making a precise double-strand break. This break initiates the plant cell's existing DNA repair mechanisms which may remove DNA bases around the cut or incorporate new DNA at the position of the break.
- The outcome is either small insertions or deletions that create sequence diversity, as often found in nature, leading to new functions or inactivating existing ones.
- Additionally, new genes can be inserted.[2,3] These molecular scissors provide incredibly precise means to fine-tune traits in a targeted, predictable fashion anywhere in the genome.
- Similar to ZFNs, TALENs are modular proteins, but the structure of TALEN DNA binding domains makes them simpler to design.[4]

Mechanisms

- In gene-editing technology, TALENs have shed light on new opportunities.
- TAL effector proteins are produced by *Xanthomonas* bacteria, which are plant pathogens.

- A catalytic domain from *Flavobacterium okeanokoites* (Fok I) has been identified and termed TALEs and can cut any gene sequence in higher frequency.

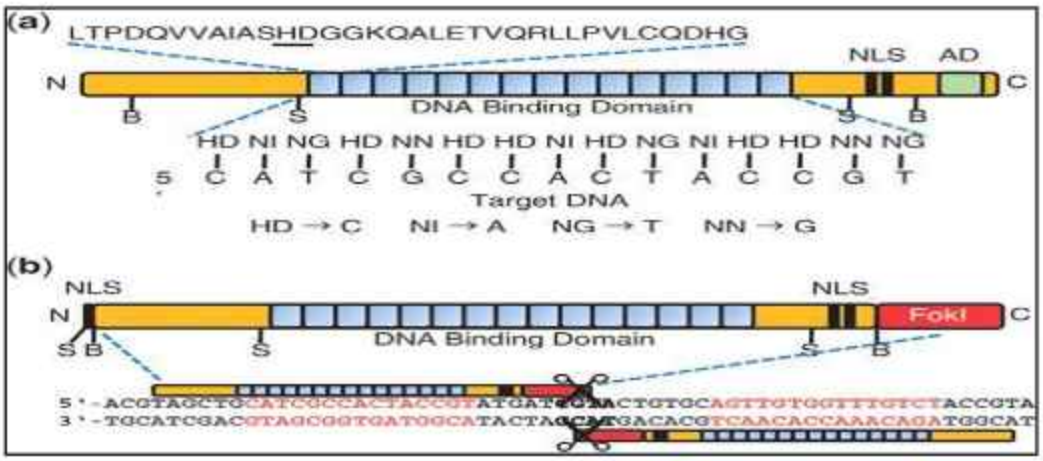


Methods

Conduction of TALEN genome editing experiment:

1. Design and Construction of TALENs: TALENs are composed of two main components: a customizable DNA-binding domain and a nuclease domain. The DNA-binding domain consists of repeating amino acids that recognize specific DNA base pairs. By assembling these repeats, we can create a TAL protein that binds to a desired DNA sequence
2. Delivery of TALENs into Cells: Once the TALENs are constructed, they need to be delivered into the target cells. This can be achieved through various methods.
3. Targeted DNA Cleavage: The TALENs bind to the targeted DNA sequence, and the FokI nuclease domain creates a double-stranded break (DSB) at the specified location.
4. DNA Repair Mechanisms: The DSB created by TALENs is repaired through one of two endogenous cellular mechanisms: nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is an error-prone process that can introduce frameshift mutations, effectively silencing the gene. HDR allows for the insertion of a new, defined DNA sequence using a homologous DNA "donor sequence"
5. Gene Silencing or Insertion: By utilizing NHEJ, TALENs can induce gene silencing. With HDR, a specific DNA fragment can be precisely inserted into the genome.

New uses and improvements to TALENs continue to emerge. For example, in 2020, a rapid TALEN preparation protocol was developed based on the widely used Golden Gate TALEN and TAL Effector Kit 2.0. The protocol involves the use of a set of linear monomers, a Fok1 backbone plasmid, and a straightforward pipeline to assemble the ready-to-use TALEN expression. This TALEN construction pipeline has high reproducibility, reliability, and an efficiency of over 80%. Furthermore, it was shown that these customized TALENs performed with better editing efficiency than CRISPR.[6]



TAL effector and TALEN structure. (a) Structure of a naturally occurring TAL effector. A consensus repeat sequence is shown with the repeat-variable di-residue (RVD) underlined. The sequence of RVDs determines the target nucleotide sequence. The four most common RVDs, on which our designs and plasmids are based, are shown with their most frequently associated nucleotide. Some evidence suggests that the less common RVD NK (not displayed) has greater specificity for G than NN does and for that reason our plasmid set also includes NK modules. (b) Structure of a TALEN. Two monomeric TALENs are required to bind the target site to enable FokI to dimerize and cleave DNA. NLS, nuclear localization signal(s); AD, transcriptional activation domain; B, BamHI; S, SphI.

Applications for Crop Improvement

TALENs can be applied to help protect plants from the effects of climate change. Genes that play a role in resistance to pests, diseases, or protection from harsh environmental conditions, such as drought and salinity, can be edited to enhance resilience. TALENS have also been used to improve the quality of products derived from crops.

Soybeans

Oils with low polyunsaturated fats are considered to be a healthier alternative to those high in polyunsaturated fats which can be hydrogenated and produce unhealthy trans-fatty acids. Soybean lines with low levels of polyunsaturated fats were developed using TALENs by introducing stacked mutations in two fatty acid desaturase 2 genes (*FAD2-1A* and *FAD2-1B*) which confer changes in fatty acid desaturase 3A (*FAD3A*). This led to oleic acid levels of over 80% and linoleic acid levels under 4%.[8]

Now gene-edited soybean plants produce premium quality high-oleic soybean oil sold as Calyno by Calyxt. It became available in the U.S. market in 2019, making it the first commercialized product from a gene-edited plant.[9,10]

Rice

TALENs have been used in rice, one of the world's most important food crops, to engineer resistance to the destructive disease, bacterial blight, caused by *Xanthomonas oryzae*. The rice gene *OsSWEET14* encodes an essential function for *Xanthomonas* to infect and cause bacterial blight. By editing the regulatory region of the gene, researchers generated heritable disease resistance.[11]

Additionally, non-aromatic rice varieties were transformed into aromatic rice varieties using TALENs. TALENs were engineered to target and disrupt the *OsBADH2* gene, resulting in the production of the major fragrance compound 2-acetyl-1-pyrroline.[12, 13]

Potato

The commercial potato variety, Ranger Russet, suffers from browning, bitter taste, and high levels of potentially carcinogenic acrylamides. TALENs were used to knockout the vacuolar invertase gene (*Vlnv*) producing improved properties. The amounts of reducing sugars were undetectable, and chips produced from these potatoes had a reduced level of acrylamide and a more desirable light color.[14]

To reduce cholesterols and related toxic steroidal glycoalkaloids, TALENs targeting the *SSR2* gene were delivered into potato using a transient *Agrobacterium* transformation method. In the resulting potato lines the *SSR2* gene was inactivated and no TALEN transgene remained in the plant. This new method can help to accelerate the use of genome editing technology to benefit crops like potato that do not have parent lines with homozygous genomes, such as inbred lines.[15]

Maize

To develop TALEN tools for corn (maize), scientists made stable, heritable mutations at a visible marker, the *glossy2* (*gl2*) locus. Transgenic lines containing mutations were obtained and three of the novel alleles were able to confer a glossy phenotype. The use of TALENs in maize is a powerful tool for genome mutagenesis, discovery of gene function, and trait improvement.[16]

Wheat

Whereas many organisms have one set of chromosome pairs, bread wheat varieties carry three pairs, increasing the complexity for gene editing. Scientists attempted to engineer resistance to powdery mildew, a widespread disease, by testing both TALENS and CRISPR-Cas9 on the MILDEW-RESISTANCE LOCUS (*mlo*) gene. They succeeded in making TALEN-induced mutations in all six sets of *mlo* genes, resulting in plants with essentially complete resistance to the wheat powdery mildew pathogen.[17]

With the need to double food production to feed the projected population of 10 billion by 2050, TALENs and other new breeding innovations are essential tools to ensure food security.

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ZFNs (Zinc Finger Nucleases): Concepts, Mechanisms, Methods and Applications

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Introduction

Zinc finger nuclease (ZFN) is an artificially engineered hybrid protein consists of a series of zinc finger protein domains, fused to a cleavage domain of *Fok I* endonuclease. Zinc finger domains can be engineered to target specific desired DNA sequences and this enables zinc-finger nucleases to target unique sequences within complex genomes. By taking advantage of endogenous DNA repair machinery, these reagents can be used to precisely alter the genomes of higher organisms. Alongside CRISPR/Cas9 and TALEN, ZFN is a prominent tool in the field of genome editing. Genome editing with the use of zinc finger nucleases (ZFNs) has been successfully applied to a variety of eukaryotic cells. Engineered ZFNs are able to target and manipulate genes through site-specific double-strand DNA breaks followed by non-homologous end joining or homologous recombination mechanisms. Consequently, this technology has considerable flexibility, which can result in either a gain or loss of function of the targeted gene. In addition to this flexibility, gene editing with ZFNs may enable persistent long-term gene modification without continuous transfection. As a powerful molecular tool for gene editing, ZFN has been widely used for gene knock-in and knock-out in a variety of cell types and organisms. This emerging field has significant promise as a therapeutic strategy for genetic diseases, infectious diseases and oncology.

Discovery of ZFNs

Homologous recombination (or homology-directed repair, HDR) is a powerful gene targeting technique in which DNA sequences are transferred from one sister chromosome to another. A particular gene may be corrected or inactivated in cells or living organisms by this type of genetic recombination. However, the frequency of HDR, particularly in higher eukaryotic cells, is as low as 1 per million treated cells. Nevertheless, double-strand breaks (DSBs) can stimulate recombination efficiency several thousand-fold, approaching gene targeting frequencies as high as 29% without selection. The method that induces a DSB at a specific site promote the frequency of gene targeting and potentially have significant therapeutic utility. The need for such an approach spurred the development of zinc finger nucleases (ZFNs).

The zinc finger motif was first discovered in transcription factor IIIA and exhibited specific DNA binding in eukaryotic cells. The binding domain of the zinc finger can insert its α-helix into the major groove of DNA in a sequence-specific manner. The C2H2 zinc finger is the most common DNA binding domain in humans, with nearly 1,000 different zinc finger motifs identified in transcription factors. This predominance in nature to bind specifically to DNA sequences provides the framework for their therapeutic use. Moreover, linking different zinc fingers together in a subunit has enabled investigators to design targeted ZFNs specifically to almost

Components of ZFNs and the mechanism

The subunit of the ZFN is composed of three domains: 1) a nonsequence-specific cleavage domain at the C-terminal mediated by the restriction enzyme *FokI*; 2) a DNA-binding zinc finger domain, Cys2-His2 (C2H2), at the N-terminal, essential for its specificity; and 3) a peptide linker that connects the zinc finger domain with the nuclease. The restriction enzyme *FokI* induces a DNA double strand break as a catalytic dimer. Thus, for DNA cleavage to occur, two zinc finger subunits must bind to the gene target sequence in the opposite orientation, leading to *FokI* dimerization (Figure 1). In addition to its nuclease activity, effects of engineered zinc fingers on DNA extend to artificial transcription factors and methylases.

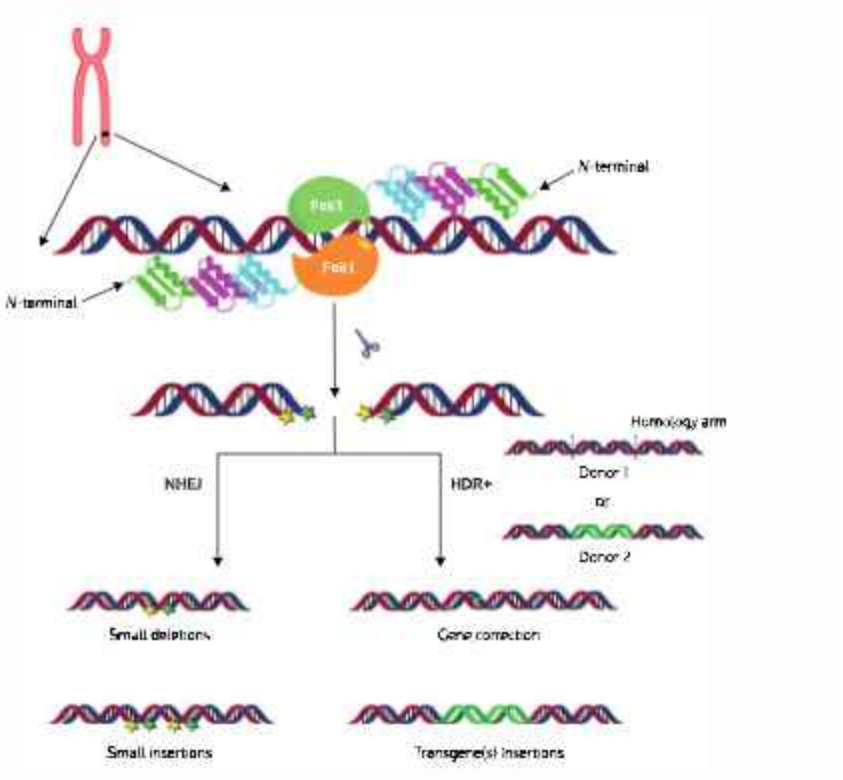

Figure 1. The components and mechanisms of zinc finger nuclease (ZFN). ZFN is composed of a three-finger binding domain and a *Fok*I nuclease cleavage domain that are connected by a linker. Each finger binds to three base pairs. Site-specific double-strand break (DSB) can be induced by ZFN at endogenous loci. The repair process leads to small gene deletions or insertions in the non-homologous end joining (NHEJ) pathway. Corrected gene or transgene (one or multiple) can be integrated into the mutant gene by an alternative mechanism, homology-directed repair (HDR), while co-expressing ZFN and donor DNA.

With each finger recognizing about three base pairs, a three-finger subunit of ZFN binds to nine base pairs on the DNA. Typically, 2 ZFN subunits containing 6-12 zinc fingers (or 3-6 zinc finger pairs), respectively, bind to between 18 and 36 nucleotides. Several studies have indicated that ZFNs with a higher number of zinc fingers (four, five and six finger pairs) have increased specificity. Comparison of a pair of 3-finger ZFNs and a pair of 4-finger ZFNs detected off-target cleavage in human cells at 31 loci for the 3-finger ZFNs and at 9 loci for the 4-finger ZFNs. In some cases, however, the activity of ZFNs may be reduced with five- and six-paired ZFNs compared to three- or four-paired ZFNs (16). Moreover, it was found that subunit affinity for the DNA sequence was critical in determining ZFN activity and may be more important than the number of fingers, that is, the subunits with lowest and highest binding affinity had reduced activity compared to the subunit with intermediate activity.

The DNA DSB induced by ZFN at endogenous loci can be repaired primarily by two pathways in eukaryotic cells: error-prone non- homologous end joining (NHEJ) or HDR in the presence of a donor DNA (Figure 1). Because small base pair

insertions or deletions can be directly induced by the NHEJ-driven DNA repair process, knockout of specific genes within eukaryotic cells can be readily achieved by this ZFN-mediated approach. More complicated modi- fications of DNA may also be accomplished by ZFN with the NHEJ pathways. For example, with two pairs of ZFNs, large deletions of 15 megabases occurred efficiently. For HDR modifications, single or multiple transgenes can be introduced into the DSB site by co- expression of ZFN and donor DNA. With donor DNA homologous to the sequences flanking the DSB, HDR mediated by ZFN can be quite versatile, including insertion of marker genes, replacement of mutant with wild-type genes, or insertion of different transgenes at the same or different loci on chromosomes. Although the NHEJ pathway can be used in all eukaryotic cells without extensive knowledge of the sequence of the targeted gene, HDR can only be used in eukaryotic cells/organisms in which the gene sequence of the targeted locus is known. The option of utilizing a selection marker as part of donor DNA can be advantageous for HDR, particularly if tar- get modification with zinc fingers is quite low. Moreover, NHEJ occurs primarily in G_1 of the cell cycle, whereas HDR occurs primarily in G_2. Consequently, HDR activity of ZFN was increased by folds in cells treated with vinblastine, which increased the number of cells in G_1.

Applications

The use of site-specific nucleases for therapeutic purposes represents a paradigm shift in gene therapy. ZFN-induced HDR has been used to directly correct the disease-causing mutations associated with X-linked severe combined immune deficiency (SCID), haemophilia B, sickle-cell disease, and α_1-antitrypsin deficiency. Moreover, ZFNs have been used to genetically repair Parkinson's disease-associated mutations within the SNCA gene in patient-derived human iPS cells. Targeted gene knockout via ZFN-induced NHEJ-mediated repair has also proven a potentially powerful strategy for combating HIV/AIDs. ZFNs have been used to confer HIV-1 resistance by disabling the HIV co-receptor C-C chemokine receptor type 5 (CCR5) in primary T cells and hematopoietic stem/progenitor cells. This approach is currently in clinical trials). More recently, ZFN-mediated targeted integration of anti- HIV restriction factors into the *CCR5* locus has led to the establishment of T cells that show near-complete protection from both R5 and X4-tropic strains of HIV. Additionally, ZFNs has been used to improve the performance of T cell-based immunotherapies by inactivating the expression of endogenous T cell receptor genes. While the overall utility of site-specific nucleases is currently limited in somatic cells, continued progress in stem cell research, including the production and manipulation of iPS cells, will ultimately open countless new directions for gene therapy, including treatments based on autologous stem cell transplantation.

Prime Editing: Concept, Mechanisms, Methods and Applications

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1. Introduction:

The blueprint of life, DNA, holds the secrets to the development, function, and health of every living organism. Traditionally, understanding gene function involved laborious genetic screens or studying organisms with spontaneous mutations. However, the advent of targeted and multiplexed genome editing techniques has revolutionized our ability to precisely manipulate DNA, ushering in a new era of biological research in crop plant improvement. Introducing desired genetic modifications into plant genomes plays an important role in crop improvement. Genome editing technologies have transformed the field of molecular biology, allowing scientists to precisely modify DNA sequences in various organisms. The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based genome editing allows researchers to modify targeted genomic DNA in a flexible and precise manner. To date, gene editing systems have reached the fourth stage; i.e., the first, second, and third stages depend on unconstrained homologous recombination (HR)-mediated strategy, genome editing-assisted HR, and genome editing with various DNA double-strand break (DSB) repair pathways such as non-homologous end-joining and microhomology-mediated end-joining, respectively. Finally, in the fourth stage, DSB-free precision gene editors such as base editor and prime editor became available.

2. Targeted genome editing by prime editors (PEs)

Targeted genome editing by prime editors (PEs) is a new and advanced method pricewise genome editing which first reported by Anzalone et al., in 2019. Prime editors (PEs) open up a new era of intentional editing of the genome, widely contributing to the functional genomics study. The recently established prime editor (PE) system is regarded as next-generation gene-editing technology. This methodology can install any base-to-base change as well as insertions and deletions without the requirement for double-stranded break formation or donor DNA templates; thus, it offers more targeting flexibility and greater editing precision than conventional CRISPR-Cas systems or base editors.

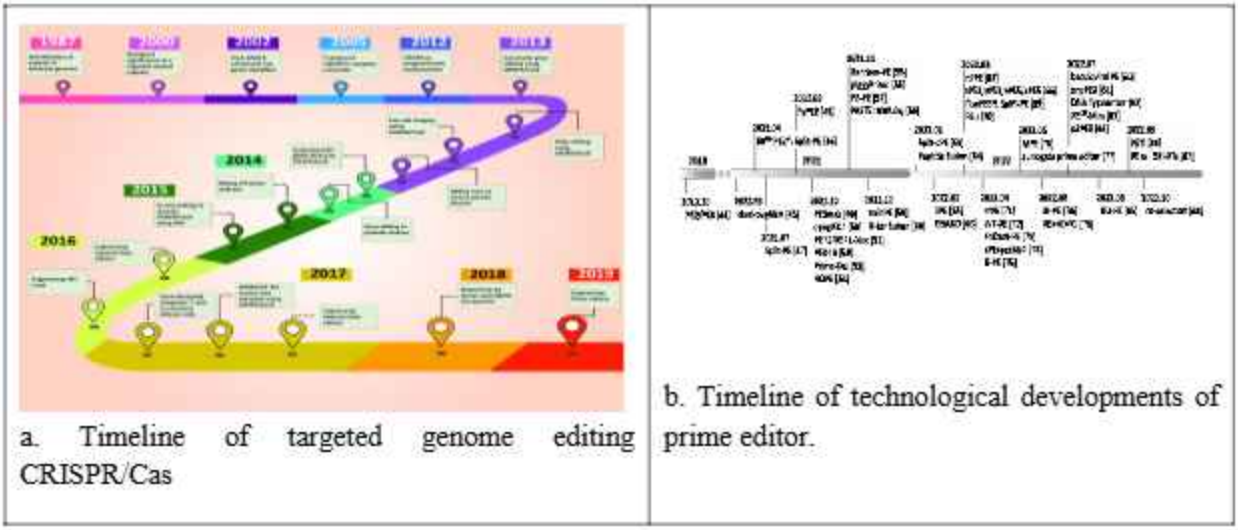
2.1 Evolution of targeted genome editing

The story of targeted genome editing begins with the discovery of enzymes known as nucleases. These molecular scissors have the remarkable ability to cleave DNA strands at specific sequences. By harnessing these nucleases, researchers can introduce double-strand breaks (DSBs) at a desired location within the genome. The cell's natural repair mechanisms then mend these breaks, providing an opportunity to introduce desired modifications. Two prominent tools for targeted editing are Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs). Both utilize custom-designed proteins that recognize specific DNA sequences and guide the nuclease activity. However, the design process for these proteins is complex and labor-intensive, often requiring significant expertise and resources. This limitation hindered the widespread adoption of these early editing techniques.

The emergence of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system (CRISPR/Cas9) revolutionized targeted editing. This ingenious system, derived from a natural bacterial defense mechanism, offers a much simpler and more affordable approach. CRISPR/Cas9 utilizes a short RNA molecule called guide RNA (gRNA), which can be easily programmed to recognize a specific DNA sequence. The gRNA acts as a guide for the Cas9 protein, directing it to cleave the target DNA with remarkable efficiency and precision. While targeted editing allows for precise modifications at a single location, multiplex genome editing takes the field a step further. This technique enables the simultaneous editing of multiple genes within a single cell. This capability opens doors to a range of exciting possibilities. However, due to some limitation of CRISPR/Cas9 method viz., unreliable repair system and

possibility of undesired insertion and deletion at DNA nicked point, scientists have developed a more precise method which is called prime editing (PE).

Prime editing (PE) is a recent and promising technique in the field of genome editing. Unlike CRISPR-Cas9, which relies on creating double-strand breaks in the DNA, prime editing (PE) allows for more precise modifications without introducing breaks. Because of its usefulness and versatility, prime editor (PE) has rapidly become one of the mainstream technologies of precision gene engineering. The timeline of worldwide technological developments is presented in Figure 1.



a. Timeline of targeted genome editing CRISPR/Cas

b. Timeline of technological developments of prime editor.

Figure 1. Timeline of targeted genome editing CRISPR/Cas (a), and prime editor (b)

2.2 Mechanism of targeted gene editing with prime editing

In 2019, David Liu and coworkers described a new method of genome-editing technology, called the prime editing (PE) system, which enables all types of nucleotide conversions, targeted insertions, and deletions without an exogenous DNA template or double-strand breaks. Anzalone et al., 2019 (Nature: 576) first showed the prime editor (PE) performance the improvement and applications of PE tools in diverse organisms have brought much excitement into the field. Prime editing combines the powerful DNA-scanning and sequence-identification capabilities of the CRISPR-Cas9 system with a reverse transcriptase enzyme, which uses an RNA template to synthesize a new single-strand DNA sequence and insert it into the DNA. The prime editing method consists of Cas9 nickase with a reverse transcriptase (RT) derived from Moloney murine leukemia virus (M-MLV) and prime editing guide RNA (pegRNA). The pegRNA contains sgRNAs for recognizing the target sequence in the genome, primer binding site (PBS) for initiation of reverse transcription, and RNA donor sequence for the reverse transcriptase encoding the desired edit. RNA-guided Cas9 nickase introduces a single-strand nick at the non-complementary strand of the DNA (PAM-containing strand) to expose a 3$^{\prime}$OH group that hybridizes with the PBS in the pegRNA, allowing the initiation of reverse transcription by the RT. The associated RT extends the 3$^{\prime}$ flap by copying the edit sequence of the pegRNA, resulting in the formation of two intermediate, redundant ssDNA structures: the 3$^{\prime}$ flap that contains the edited sequence and the original, unedited 5$^{\prime}$ flap sequence. These 3' and 5' DNA flaps compete with each other. Although the non-edited 5$^{\prime}$ flap is thermodynamically favored to hybridize with the complementary strand, it is degraded by cellular endonucleases,

leading to the incorporation of the edited 3′ flap. The formed heteroduplex DNA, containing edited and non-edited strands, is resolved, and the desired modification is introduced into both DNA strands by ligation and DNA mismatch repair.

2.2.1 Components of Prime Editing

The fundamental components of a prime editing system include:

I. Prime Editor Fusion Protein: This fusion protein consists of two critical domains:

- Nickase: A modified Cas9 protein that introduces a single-strand break (nick) in the target DNA.
- Reverse Transcriptase (RT): An enzyme responsible for copying RNA into DNA. In prime editing, RT synthesizes a new DNA strand based on the information provided by the prime editing guide RNA (pegRNA).

II. Prime Editing guide RNA (pegRNA):

- Protospacer: The region complementary to the target DNA sequence.
- Scaffold: A structural RNA that guides the pegRNA to the target site.
- Primer Binding Site: A sequence where RT initiates DNA synthesis.
- Reverse Transcription Template: The RNA template that RT uses to synthesize the complementary DNA strand.

II. Additional RNA molecules (in some prime editing systems):

- These components enhance efficiency and specificity for different target organisms or compact the system for viral delivery.

2.2.2 Mechanism of action

I. Nickase binding and cleavage:

- The prime editor (nickase) binds to the target DNA at the protospacer adjacent motif (PAM) site.
- It introduces a single-strand break (nick) in the non-target DNA strand.

II. Primer Annealing and Reverse Transcription:

- The pegRNA hybridizes with the target DNA, guided by the scaffold and primer binding site.
- RT synthesizes a complementary DNA strand using the reverse transcription template.

III. DNA Repair and Edit Incorporation:

- The newly synthesized DNA strand replaces the original non-target strand.
- Equilibration between the edited 3' end and the unedited 5' end, endogenous 5' end cleavage, and DNA repair result in the stable incorporation of the desired edit in the genome.

In brief, the vector PE:pegRNA binds to the target DNA, and Cas9 nicks only one strand, generating a flap. The PBS (primer binding site), located on the pegRNA, binds to the DNA flap and the edited RNA sequence is reverse transcribed using the reverse transcriptase. The edited strand is incorporated into the DNA at the end of the nicked flap, and the target DNA is repaired with the new reverse transcribed DNA. The original DNA segment is removed by a cellular endonuclease. This leaves one strand edited, and one strand unedited. In the newest PE system, PE3 and PE3b, the unedited strand can be corrected to match the newly edited strand by using an additional standard guide RNA. In this case, the unedited strand is nicked by a Cas9 nickase and the newly edited strand is used as a template to repair the nick, thus completing the edit.

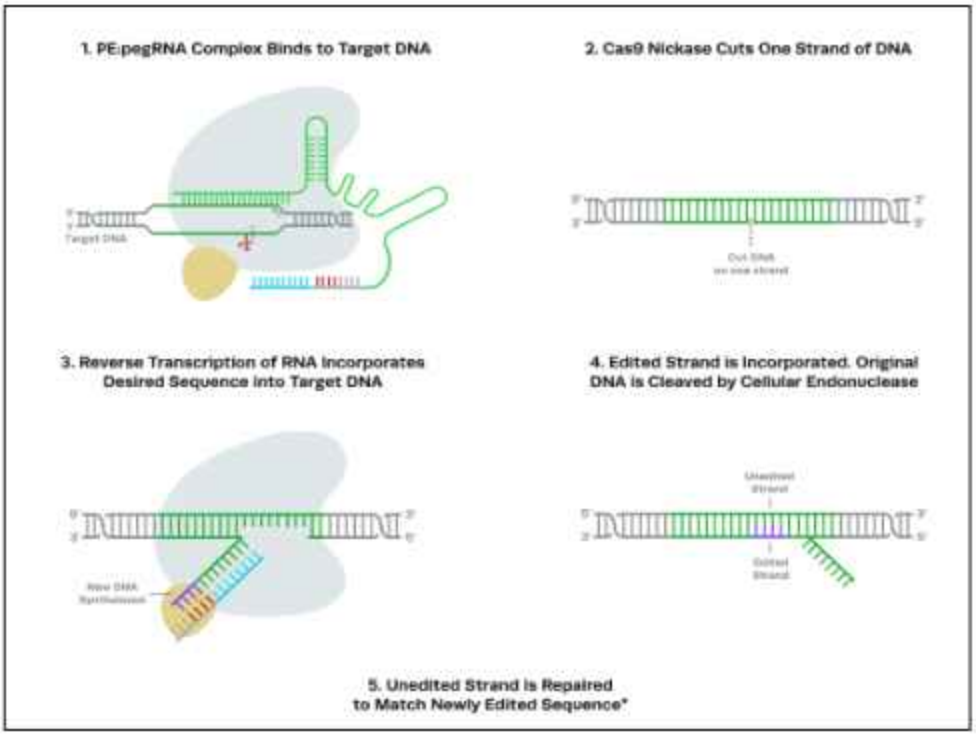


Figure 2. Mechanism of prime editing (PE)

Basically, PE employs a nickase SpCas9 (nCas9) that can generate an R-loop at the target site and single-stranded breaks (SSBs) on the non- target strand (nCas9 (H840A) for binding to a prime editing guide RNA (pegRNA). The SSB of the non-target strand releases a 3' single-stranded end that can anneal to the primer binding site (PBS) of a 3' extension of the pegRNA that is pre- designed to contain the PBS and reverse transcriptase (RT) template having the desired bases to be installed into the genome site (Figure 3). Then, an RT peptide fused to the nCas9 (H840A) acts on the primed heteroduplex by adding deoxynucleotides to the 3'-OH of the nicked end corresponding to the code of the RT template. The de novo synthesized product appears as a 3'flap that could be fixed into the genome via competition with the original sequence of the 5' nicked end (Anzalone et al., 2019; Chen and Liu, 2023). The step may involve DNA damage repair pathways such as mismatch repair (MMR) (Figure 3). Theoretically, PE can be used to install various types of precise gene editing, such as all types of base substitutions, DNA sequence insertion and deletion. Moreover, the technique is efficient and precise in animals (Anzalone et al., 2019) and plants (Lin et al., 2020), though different loci often bring various efficiencies of editing.

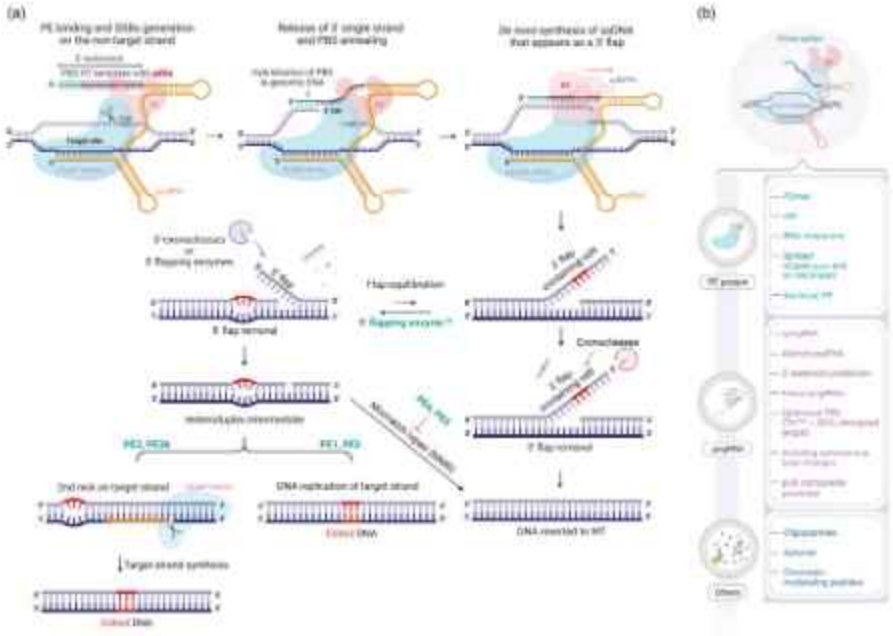
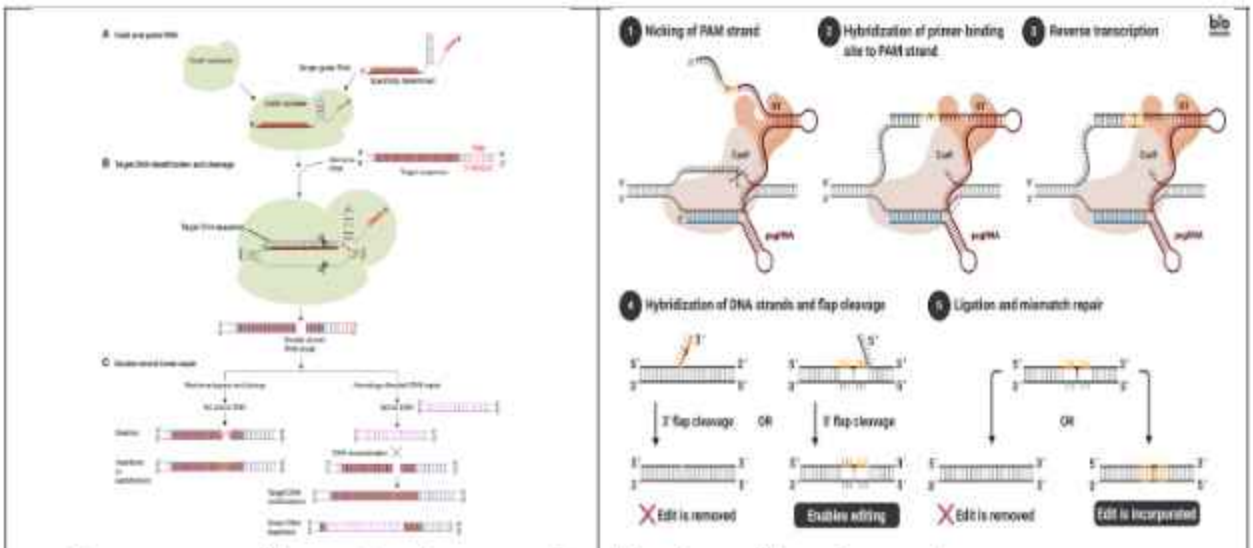


Figure 3. The Overview of the PE mechanism. (a) Schematic diagram illustrating the components involved in the PE mechanism. (b). Components of the PE system and various approaches that have enhanced prime editing efficiency. [Source: TienV.Vue et al, 2024, Plant Biotechnology Journal]

2.3 Prime editing vs. CRISPR-Cas9 editing

The prime editing system was demonstrated to be more effective than CRISPR/Cas9- mediated HDR and revealed significantly fewer off-target effects than Cas9. Compared to base editing, PE offers all possible base conversions. However, prime editors cannot be used for large DNA insertions or deletions, while CRISPR/Cas9 systems can do this.



(a) Genome editing with Crispr-Cas9 for single targeted gene

(b) Prime editing of targeted gene

Figure 4. Mechanism of CRISPR-Cas9 editing (a) VS. Prime editing (b)

2.4 Advantages of prime editing over CRISPR-Cas9:

- ❖ Precision: Minimizes off-target mutations.
- ❖ Simplified Protein Engineering: No extensive modifications needed.
- ❖ Expanded Editing Range: Can achieve edits not possible with CRISPR-Cas9 alone

2.5 Applications of prime editing

The prime editing system has enormous potential to improve the precision of genome editing in plants. Prime editing has increased the scope and capabilities of CRISPR/Cas9 for precision modification of plant genomes to obtain the targeted gene/allele replacement and base transition/transversion. However, challenges remain in the applications of prime editing techniques that require further research, such as low editing efficiency and short editing window. By improving the size of the introduced indels and reducing the editing of byproducts, the prime editing technique can be made more versatile. The plant prime editing system can be further optimized to improve crops in a user-defined, cost-effective manner, without compromising other elite agronomic tra

CRISPR Cas9 in Genome Editing: Biology, Mechanism, and Relevance

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CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats–CRISPR-associated protein 9) is a revolutionary genome-editing tool that allows precise, targeted modifications to DNA in living organisms. Its simplicity, efficiency, and versatility have transformed research in biology, agriculture, and medicine.

1. The CRISPR-Cas Revolution

- **Discovery of CRISPR in Bacteria (1987)**
  - Scientists first observed CRISPR sequences in *Escherichia coli* without understanding their function.
- **2005–2007: Role of CRISPR in Bacterial Immunity**
  - Researchers found that CRISPR sequences help bacteria recognize and destroy viral DNA by storing snippets of past invaders.
- **2012: Repurposing CRISPR for Genome Editing**
  - Jennifer Doudna and Emmanuelle Charpentier developed the CRISPR-Cas9 system as a programmable tool for precise DNA editing.
- **2013: Application in Plants and Animals**
  - Feng Zhang demonstrated CRISPR's potential for editing the genomes of mammalian cells.

2. Mechanism of CRISPR-Cas9: From Bacterial Immunity to Crop Improvement

a. CRISPR-Cas9 in Bacterial Immunity

- CRISPR is a natural defense mechanism in bacteria and archaea against invading viruses (bacteriophages).
- Mechanism:
  1. **Acquisition**: Bacteria capture snippets of viral DNA and integrate them into the CRISPR locus as "spacers."
  2. **Expression**: The CRISPR locus is transcribed into a long RNA, which is processed into small guide RNAs (gRNAs).
  3. **Interference**: Cas proteins, guided by the gRNAs, recognize and cut matching viral DNA, neutralizing the infection.

b. Repurposing CRISPR-Cas9 for Genome Editing

- In genome editing, scientists mimic bacterial immunity by designing synthetic guide RNAs (sgRNAs) to target specific DNA sequences.
- Mechanism:
  1. **Recognition**: The sgRNA binds to the target DNA sequence via complementary base pairing.
  2. **Cleavage**: Cas9 introduces a double-strand break (DSB) at the target site.
  3. **Repair**: The cell repairs the DSB through:
     - **Non-Homologous End Joining (NHEJ)**: May introduce insertions or deletions (indels) that disrupt gene function.
     - **Homology-Directed Repair (HDR)**: Enables precise insertion of desired sequences using a repair template.

c. Applications in Crop Improvement

- CRISPR-Cas9 has been widely adopted for:
  - Creating disease-resistant crops by knocking out susceptibility genes.
  - Developing stress-tolerant crops to combat drought, salinity, and heat.
  - Enhancing crop yield and quality traits like grain size, fruit ripening, and nutrient content.
  - Targeting multiple genes simultaneously for complex trait improvement.

3. Overview of CRISPR Systems: Cas9, Cas12, and Cas13

a. Cas9: The Classic Workhorse

- Most widely used CRISPR system for genome editing.
- Cuts DNA to create double-strand breaks.
- **Key Features**:
  - Recognizes a protospacer adjacent motif (PAM) sequence (e.g., NGG for *Streptococcus pyogenes* Cas9).
  - High efficiency in a wide range of organisms.
- **Applications**:
  - Gene knockout, gene insertion, transcriptional regulation, and epigenome editing.

b. Cas12: A Versatile Alternative

- Recognizes and cleaves both single-stranded and double-stranded DNA.
- **Key Features**:
  - Broader PAM requirements (e.g., TTTV for *Cas12a*).
  - Creates staggered (sticky) cuts, which are useful for specific genome-editing applications.
- **Applications**:
  - Multiplex editing, diagnostic tools (e.g., SHERLOCK), and epigenetic studies.

c. Cas13: Targeting RNA

- Unique among CRISPR systems as it targets RNA instead of DNA.
- **Key Features**:
  - No permanent changes to the genome.
  - Can degrade or edit RNA transcripts in real-time.
- **Applications**:
  - Gene silencing, studying RNA function, and combating RNA viruses in crops.

Comparison of CRISPR Systems:

Feature	Cas9	Cas12	Cas13
Target Molecule	DNA	DNA	RNA
Cut Type	Double-strand break	Single-/double-strand	Single-strand cleavage
PAM Sequence	NGG (narrow)	TTTV (broad)	None required
Applications	Genome editing	Precision editing	RNA editing

There are many protein tools in the CRISPR toolbox. While CRISPR-Cas10 is a functional CRISPR system, it's not as widely used as CRISPR-Cas9 in many applications because Cas10 primarily degrades single-stranded DNA instead of creating precise double-stranded breaks, making it less ideal for targeted gene editing; it's better suited for applications where broad-spectrum nucleic acid degradation is desired, like defense against viruses in bacteria.

Key points about CRISPR-Cas10:

- **Mechanism:**
  Unlike Cas9, which cuts double-stranded DNA at specific sites, Cas10 primarily degrades single-stranded DNA, which limits its precision for gene editing.
- **Type III CRISPR system:**
  Cas10 belongs to the Type III CRISPR system, which is more focused on degrading invading nucleic acids rather than precise genome editing.
- **cOA production:**
  A unique feature of Cas10 is its ability to synthesize cyclic oligoadenylates (cOAs), which can trigger additional cellular responses against invading pathogens.

When might CRISPR-Cas10 be useful?

- **Antiviral defense:**
  Due to its single-stranded DNA degradation activity, Cas10 can be particularly useful in studying and potentially manipulating viral genomes.
- **RNA targeting:**

Some research explores using Cas10 to target RNA molecules by engineering the guide RNA to bind to RNA instead of DNA

CRISPR-Cas11 is a functional CRISPR system, it's not as widely used as CRISPR-Cas9 because in many bacterial systems, the "cas11" gene is often "hidden" within another gene, requiring special conditions to express it properly, making it difficult to utilize in research and therapeutic applications compared to the more readily accessible Cas9 protein; essentially, researchers need to work harder to access and utilize the Cas11 component effectively.

Key points about Cas11:

- **Hidden Translation:**

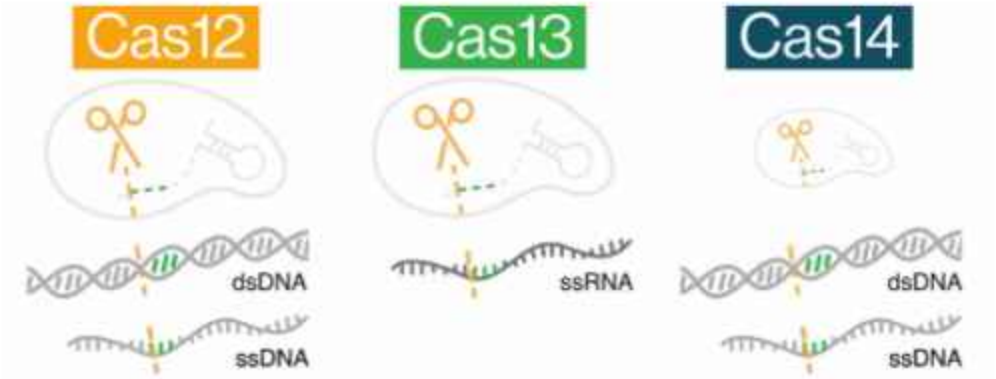
  In certain types of CRISPR systems (like Type I-B, I-C, and I-D), the Cas11 protein is produced through a process called "internal translation," meaning it is encoded within another gene, like Cas8, which can be challenging to express in other organisms like human cells.

- **Importance for Cascade Complex:**

  Despite being "hidden," Cas11 is crucial for the proper function of the Cascade complex, which is necessary for targeting DNA in these CRISPR systems.

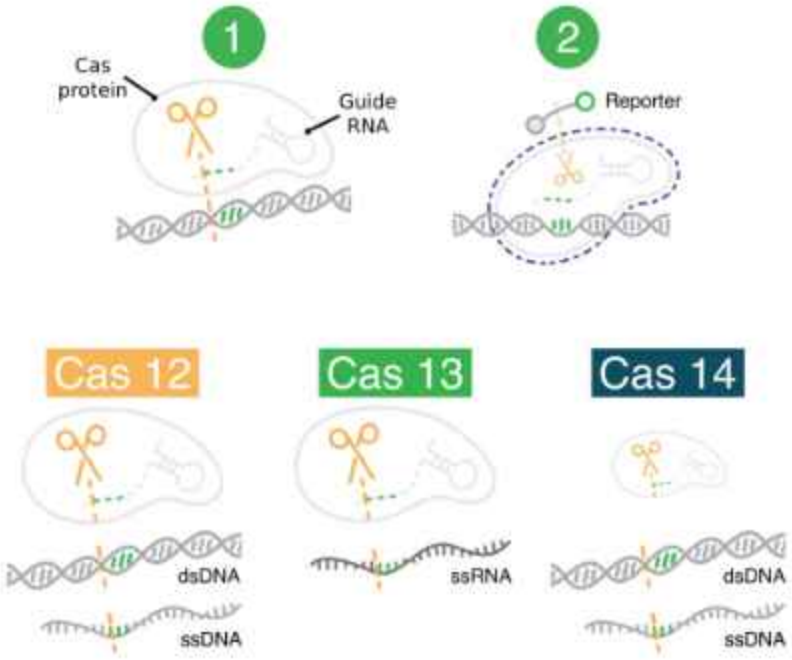
- **Potential for Applications:**
  Recent research has shown that by identifying and expressing the "hidden" Cas11 protein, scientists can utilize CRISPR-Cas3 systems (which rely on Cas11 for full functionality) more effectively in human cells.

Cas system is suited to a particular suite of uses. For example, the common CRISPR protein Cas9 is often used for genome editing. It is not suited for CRISPR diagnostics. In this blog post, we'll introduce you to the proteins behind CRISPR diagnostics: Cas12, Cas13, and Cas14.



CRISPR diagnostics make use of non-specific cutting
CRISPR diagnostics have two key components:

1. Protein-guide molecule complexes. These first cut specific nucleic acid sequences that the user wants to detect. After cutting a user-specified sequence, these complexes non-specifically cut other nucleic acids.
2. Modified nucleic acids (reporters). These produce a visual signal when cut. They are only cut if the user-specified nucleic acids are cut first. These modified nucleic acids make it easy to observe when the user-specified nucleic acids have been detected (cut).



Top: The two components of CRISPR diagnostics. 1: Protein-guide molecule complexes that cut user specified nucleic acid sequences. 2: Modified nucleic acids that are cut non-specifically after the user-specified nucleic acid sequences. These produce a visual cue signaling that the user-specified nucleic acids have been detected. Bottom: The Cas12, Cas13, and Cas14 protein-guide molecule complexes. These are capable of cutting the indicated types of user-specified nucleic acid sequences. In a CRISPR diagnostic, these would go on to cut modified nucleic acids nonspecifically.

In diagnostics, it's critical that non-specific cutting comes after specific cutting. Nonspecific cutting (sometimes called "collateral," "trans," or "indiscriminate") results in cleavage of the modified nucleic acids. The visual signal produced by the modified nucleic acids then shows that the user-specified sequence has been detected. If non-specific cutting came first, CRISPR diagnostics would always produce a visual signal. They would be useless.

Cas12, Cas13, and Cas14 are families of proteins used in CRISPR diagnostics. They form the protein portion of the "protein-guide molecule complexes" described above. Individual family members come from specific species of bacteria and archaea. Yet, all members of a given family share certain characteristics. Characteristics important for CRISPR diagnostics are displayed in table 1 and discussed below.

Cas12

The Cas12 proteins directly bind to and cut user-specified DNA sequences. They can cut either single or double stranded DNA. Once a Cas12 protein cuts its DNA target, it begins to shred single stranded DNA non-specifically. Thus Cas12-based diagnostics can only directly detect DNA. They must be combined with proteins that convert RNA into DNA to detect RNA. Cas12 proteins are on the larger side of the CRISPR diagnostic proteins.

They come in at ~1,300 amino acids long.

Users specify Cas12 DNA targets using 42-44 nt RNA molecules.

Cas12 dsDNA targets are restricted in that they must be found near short stretches of DNA known as protospacer adjacent motifs (PAMs). For some Cas12 proteins, the PAM sequence is TTTN. Importantly, Cas12-based diagnostics cannot detect DNA sequences without PAMs.

Cas12 proteins can readily distinguish very similar dsDNA sequences. This feature is lost when the target sequence is ssDNA.

Cas13

The Cas13 proteins directly bind and cut user-specified RNA sequences. After cutting a target, Cas13 proteins non-specifically cut other RNA molecules. Thus, they can directly detect RNA, but not DNA. To detect DNA, Cas13-based diagnostics must be combined with proteins that convert DNA into RNA.

Like Cas12, Cas13 proteins are on the larger end of the CRISPR diagnostic proteins at ~1,400 amino acids long.

Like Cas12, the Cas13 RNA guide molecule is relatively short at ~64 nt.

Cas13 proteins do not have strong targeting restrictions. Yet, their RNA targets can adopt structures that are difficult to cut. These structural constraints limit the targets detectable by Cas13-based diagnostics.

Cas13 will cut RNA sequences that are 1 nt off from the user-specified sequence. Thus, researchers must carefully test Cas13-based diagnostics when using them to distinguish between very similar sequences.

Cas14

The Cas14 proteins bind to and cut user-specified, single-stranded or double stranded DNA. To detect RNA, Cas14-based diagnostics must be combined with proteins that convert RNA into DNA.

The Cas14 proteins are on the smaller end at 400 – 700 amino acids. However, their guide RNAs are on the longer side at ~140 nt.

Cas14 proteins have no targeting restrictions when cutting ssDNA. They are highly versatile. For dsDNA targeting, Cas14 proteins require T-rich PAM sequences like TTTA.

Cas14 proteins can readily distinguish between very similar ssDNA sequences.

Table 1: Cas12, Cas13, and Cas14 properties important for CRISPR diagnostics

Protein Family	Cas12a	Cas13	Cas14
Rough protein length (amino acids)	~1,300	~1,400	~400 - 700
Single guide molecule size (nucleotides, nt)	42-44 nt	~64 nt	~140 nt
Targeted nucleic Acids (DNA or RNA)	DNA (ss or ds)	RNA (ss)	DNA (ss or ds)
Non-specifically cut nucleic acids (DNA or RNA)	DNA (ss)	RNA (ss)	DNA (ss)
Targeting restrictions	dsDNA targets must be near TTTN	Weak requirements dependent upon family member. Activity is also constrained by RNA secondary structure	None for ssDNA. dsDNA targets must be near T-rich sequences like TTTA
Accuracy	Effectively discriminates between targets off by one bp (dsDNA)	Difficulty discriminating between targets off by one bp	Effectively discriminates between targets off by one bp (ssDNA)

Conclusion

The CRISPR-Cas9 technology has revolutionized genome editing by making it simpler, faster, and more accessible. Its applications in crop improvement are transformative, offering solutions to address food security, climate change, and nutritional challenges. Emerging systems like Cas12 and Cas13 expand its versatility, paving the way for new advancements in agriculture and beyond.

CRISPR-Cas9 experimental design for a selected crop trait (success in Bangladesh)

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Dhaka University

Objectives: To develop insect resistant rice variety using clustered regularly interspaced short palindromic repeats (CRISPR) Cas9 genome editing tool.

Target gene: Serotonin biosynthesis gene, CYP71A1 (LOC_Os12g16720)

Target gene sequence:

TTGATGCAATTAATTCCACATTAATTCGCTGTTTCAACATCCATCTTACTAGTTAGAGTACTACACAAGTATACACTATCTGTCGCAT
TCCACACCTTGATCTCAGATAACGTTGATCTCTTCAACTCATGTGATAAATCTAATGAAAATCTTCAGTGCCCAACGTGTGCATCTAC
CTCTCCTCCAACCAACCACCCACTATATAAACCCTTCCCCACTTAACCACGGTTAGTTCATACCAACACATCTCCATTGCTGCTTAAGT
TTCTTGGACCAAACGTGCACCCCAAGTGTTCGACGATATGGAGCTCACCATGGCGTCGACGATGTCGCTCGCGCTGCTCGTGCTCTC
CGCGGCGTACGTGTTGGTCGCGTTGAGGAGGAGCCGGTCGTCGTCGTCAAAGCCACGGCGGCTGCCGCCGTCGCCGCCGGGGTG
GCCGGTGATCGGGCACCTCCACCTCATGTCCGGCATGCCGCACCACGCGCTGGCCGAGCTGGCGCGCACCATGCGCGCGCCGCTGT
TCCGGATGCGGCTGGGGAGCGTGCCGGCGGTGGTGATCTCCAAGCCGGACCTCGCCCGCGCCGCGCTCACCACCAACGACGCCGC
GCTGGCGTCGCGGCCGCACCTGCTCTCCGGCCAGTTCCTGTCGTTCGGCTGCTCCGACGTGACGTTCGCGCCGGCGGGGCCGTACC
ACCGGATGGCGCGCCGCGTGGTGGTGTCGGAGCTCCTGTCGGCGCGTCGCGTCGCCACGTACGGCGCCGTCAGGGTCAAGGAGCT
CCGCCGCCTGCTCGCGCACCTCACCAAGAACACCTCGCCGGCGAAGCCCGTCGACCTCAGCGAGTGCTTCCTCAACCTCGCCAACGA
CGTGCTCTGCCGCGTCGCGTTCGGCCGCCGGTTCCCGCACGGCGAGGGCGACAAGCTCGGCGCGGTGCTCGCCGAGGCGCAGGAC
CTCTTCGCCGGGTTCACCATCGGCGACTTCTTCCCCGAGCTCGAGCCCGTCGCCAGCACCGTCACCGGACTCCGCCGCCGCCTCAAG
AAGTGCCTCGCCGACCTCCGCGAGGCCTGCGACGTGATCGTGGACGAACACATCAGCGGCAACCGCCAGCGCATCCCCGGCGACC
GCGACGAGGACTTCGTCGACGTCCTCCTCCGCGTCCAGAAATCCCCCGACCTCGAGGTCCCCCTAACCGACGACAATCTCAAGGCCC
TCGTCCTGGACATGTTCGTCGCCGGCACGGACACCACGTTCGCGACGCTGGAGTGGGTGATGACGGAGCTAGTCCGCCACCCACG
GATCCTCAAGAAGGCGCAGGAGGAGGTCCGGCGAGTCGTCGGCGACAGCGGCCGCGTCGAGGAGTCCCACCTCGGCGAGCTCCA
CTACATGCGCGCCATCATCAAGGAGACGTTCCGGCTGCACCCGGCGGTGCCGTTGCTAGTGCCGCGCGAGTCCGTCGCGCCGTGC

Design of guide RNA and synthesis of target primers

The target site (5′-TGGTCGCGTTGAGGAGGAGC-3′) of serotonin synthesis gene (CYP71A1) was designed in the following format and purchased from the company consist of a protospacer adjacent motif (PAM) lying within the CYP71A1 coding sequence.

Target-Sense: 5-CAG-gRNA sense; 5′- CAGTGGTCGCGTTGAGGAGGAGC - 3′ (forward)

Target-Anti: 5-AAC-gRNA Anti; 5′- AACGCTCCTCCTCAACGCGACCA - 3′ (reverse)

The above designed oligonucleotide sequence of target insertion part of CYP71A1 gene was purchased from Macrogen company (Humanizing Genomics, Seoul, Korea) via Biotech Concern (Dhaka, Bangladesh) and stored in -20°C freezer before use.

Construction of Cas9/gRNA recombinant vector

The Cas9/gRNA vector, VK005–01 was purchased from Chinese company (Viewsolid Biotech, China, http://www.v-solid.com) which harbors the rice U6 promoter. The gRNA target sequence was inserted into the VK005–01 following the protocol described by company. Briefly, the target forward and reverse primers were diluted to 10μM and mixed according to the following ratio (Table 36). After mixing the solution was treated with the following condition; 95°C for 3 min then slowly cooled to room temperature (25°C). The cool mixture

was used to cloned into the Cas9/gRNA vector following the company protocol. The cloning component and their amount were listed in Table 37. The mixing amount (10 µl) was reacted at 16°C for 2 hours. After 2 hours, the 5-10 µl was transformed into DH5α competent cells following standard protocol. The transformation product was kept for culture in LB plate at 37°C for overnight. Then recombinant Cas9/gRNA-CYP71A1 vector was selected, and cultured in 5 ml LB solution overnight at 37°C. DNA was extracted from cultured recombinant vector and target part of the genome was amplified using PCR. Electrophoresis was applied to observe the amplified part of the DNA. The final recombinant Cas9/gRNA vector was checked by sequencing. The purified recombinant Cas9/gRNA vector DNA was sent to National Institute of Biotechnology (NIB), Savar, Dhaka for sequencing. The sequencing product was analyzed by comparing with original target sequence of CYP71A1.

Table 1. List of primers used in this study

Gene		**Primer sequence (5′→3′)**	**Purpose**
CYPV	F:	CAGTGGTCGCGTTGAGGAGGAGC	Vector construction
	R:	AACGCTCCTCCTCAACGCGACCA	
CYPSEQ	F:	AAATCTTCAGTGCCCAACG	Target confirmation
	R:	GGAGCAGCCGAACGACA	
HPT	F:	TGCTCCATACAAGCCAACC	Genotyping
	R:	TGTCCTGCGGGTAAATAGC	
gRNA	F:	TGGTAGAAGTCGGAGATGT	
	R:	CTTTCCCTTTGTATTGCTG	
Cas9	F:	TACTGAACTCCGAAATCTG	
	R:	CAACGGTGGCTTACTCT	
CYP71A1	F:	CACCATCGGCGACTTCTTCCC	qRT-PCR
	R:	AGCTCCGTCATCACCCACTCC	
OsEDS1	F:	CATTCCAAGAACGAGGACACTG	
	R:	CAAGACTCAAGGCTAGAACCGA	
OsPAD4	F:	CCAACATGTACCGCATCAAG	
	R:	GGTTGTTTCGGTGGTAGTGG	
OsPAL	F:	GCACATCTTGGAGGGAAGCT	
	R:	GCGCGGATAACCTCAATTTG	
OsICS1	F:	TATGGTGCTATCCGCTTCGAT	
	R:	CGAGAACCGAGCTCTCTTCAA	
OsNPR1	F:	TTTCCGATGGAGGCAAGAG	
	R:	GCTGTCATCCGAGCTAAGTGTT	
OsPR1	F:	GGCAACTTCGTCGGACAGA	
	R:	CCGTGGACCTGTTTACATTTTCA	

Gene		Primer sequence (5′→3′)	Purpose
OsASα1	F:	AATTTGGGTCAGCACTACAG	
	R:	AACTTTGTCTTCTGCTTTCGA	
OsASα2	F:	CAGTTTGGTACACCTTTGAAG	
	R:	ACAAACATCTTCCTTCTCTGT	
OsASβ1	F:	ATGAACTTACCATAGAGGATG	
	R:	ATGATCCTCTTGCCTTCTGG	
OsASβ2	F:	GATATCACCGTGGAAGAAATT	
	R:	CATGAGCCTCCCTTCGTGG	
TDC	F:	ATGACCTGCCTCGACTGCACC	
	R:	CTTGTTCAGCCGCTCCATCAG	
AANAT	F:	GGGCTGCGGCAACTTGGTCC	
	R:	GCTGGCACTAAAATCTGGGGTACC	
ASMT	F:	TACCGTCCATGACGGCG	
	R:	CGGCCGCCTTCTCGACA	
OsActin	F:	CAGCACATTCCAGCAGAT	
	R:	GGCTTAGCATTCTTGGGT	

Table 2. Component and their amount for preparation of target gene of interest.

SL No.	Component	Amount (µl)
1	Forward primer (target sense)	5
2	Reverse primer (target anti)	5
3	ddH_2O	15
Total		25

Table 3. Component and their amount of cloning procedure

SL No.	Component	Amount (µl)
1	Cas9/gRNA vector (Vk005-01)	1
2	Forward primer (prepared above)	1
3	Reverse primer (prepared above)	1
4	Solution 1	1
5	Solution 2	1
6	ddH_2O	6
Total		10

Agrobacterium mediated genetic transformation of rice

The rice varieties, BRRI dhan87, BRRI dhan89 and BRRI dhan92 were used in this study. For callus induction, 100 healthy seeds of each variety were manually dehusked and then washed 2–3 times with autoclaved sterile

double distilled water. Seeds were surface sterilized with 70% ethanol (v/v) for 90 s followed by 4–5 times with sterile double distilled water. Seeds were further surface sterilized with 50% (v/v) commercial bleach with gentle shaking for 20 min followed by 5–6 times wash with sterile double distilled water. Seeds were dried on autoclaved Whatman paper (3 mm) for an hour. Twenty–25 seeds were inoculated per plate (100 mm x 90 mm) on callus induction media (CIM) and incubated at 26 ± 2°C in dark conditions. The compositions of media used are given in Table 38. Previous studies used CIM with 2,4-D (2.5 mg/l) alone or combination of 2,4-D (2.5 mg/l) and 6-BAP (0.25 mg/l) for different indica varieties (Sahoo et al. 2011). For BRRI rice varieties, combination of 2,4-D (2.5 mg/l) and 6-BAP (0.25 mg/l) were used. Further, maltose can influence the degree of differentiation and thus the efficiency of regeneration in different plants (Strickland et al. 1987; Chu et al. 1990; Jain et al. 1995; Kumar et al. 2005). Hence, maltose was used as a carbon source in the CIM in this study.

Agrobacterium mediated transformation was carried out following method of Sahoo and Tuteja (2012) with some modifications. Media compositions used in this study are given in Table 31. A single colony of *Agrobacterium tumefaciens* strain LBA4404 harbouring Cas9/gRNA-CYP71A1 constructs were inoculated in 5 ml of liquid of LB medium and incubated at 28°C for 24 h in a rotary shaker. Depending on the growth of the culture, 0.5-1 ml of the primary culture was inoculated in 100 ml of LB liquid medium with Rifampicin (Duchefa) 10 mg/l and Kanamycin (Duchefa) 50 mg/l, and incubated at 28°C over-night in a rotary shaker at 200 rpm. The next day, *Agrobacterium* culture with an absorbance 0.6–1.0 was centrifuged for 20 min at 4000 rpm at 20°C to pellet the cells. The pellet was resuspended in 10–20 ml of resuspension medium containing 150 lM acetosyringone. The microcalli were immersed in resuspension medium with gentle shaking in an incubator shaker for 20 min. After infection, the media was discarded, the calli were blotted on a filter paper and air-dried for 5–10 min in a laminar flow hood. The microcalli were transferred on to co-cultivation media and incubated in dark at 25°C for 48 h.

Excess *Agrobacterium* was removed by washing the calli for 4–5 times with sterile double distilled water until no turbidity observed in water, followed by sterile double distilled water containing 300 mg/l cefotaxime. Then, the calli were blotted to dry on sterile Whatman no. 1 paper and transferred to selection medium (SM1) containing 50 mg/l hygromycin, 300 mg/l cefotaxime and 200 mg/l Timenitin. Calli were maintained in the SM1 medium in growth incubator at 26 ± 2°C dark for 15 days. After 15 days of selection, the resistant calli were selected and transferred to selection medium (SM2) containing 300 mg/ 1 cefotaxime and 50 mg/l hygromycin, and cultured in growth incubator at 26 ± 2°C dark for 15 days. After two rounds of selection, white portions of proliferated calli were isolated and transferred to fresh selection medium (SM2) and maintained in growth incubator at 26 ± 2°C dark for 10–15 days. The resistant proliferated calli were isolated and transferred to regeneration medium containing 35 mg/l hygromycin and kept in dark for 1 week and then transferred to 16 h light/8 h dark photoperiod for 10–15 days. The regenerated healthy shoots were separated and transferred on rooting media and kept in light for 10–15 days. Twenty-four (24) rooted plants were then transplanted in earthen pot and kept in greenhouse for full grown.

Table 4. List of media used for *Agrobacterium* mediated transformation of rice cv. BRRI dhan87, BRRI dhan89 and BRRI dhan92

Name of the media	Composition
Callus induction media (CIM)	MS Salts with B5 vitamins, 300mg/L Casein hydrolysate, 560mg/L L-Proline, 36g/L Maltose monohydrate, 0.4% Gelrite dissolved in double distilled water then adjust the pH to 5.8. Autoclave the media and after cooling to room temperature then add 2.5mg/L 2-4D and 0.25mg/L 6-BAP.
Yeast extract Mannitol medium (YEM)	Yeast extract 1g/L, Mannitol 10g/L, NaCl 1g/L, MgSO4. 7H2O 0.2g/L, K2HPO4 0.5g/L dissolved in double distilled water then adjust the pH to 6.8-7.0. Autoclave and store the media at room temperature.
Yeast extract Mannitol Agar medium (YEMA)	To YEM medium, add 1.5% Agar.
Resuspention medium (RSM)	½ MS salts with 2% sucrose and adjust the pH to 5.3-5.4 autoclave and store at room temperature.
Co-cultivation medium (CCM)	½ MS Salts with B5 vitamins 300mg/L Casein hydrolysate, 560mg/L L-Proline, 36g/L Maltose monohydrate, 0.4% Gelrite dissolved in double distilled water then adjust the pH to 5.8. add 150µM Acetosyringone was added to media.
Selection medium-1 (SM-1)	To the CIM medium, add 300mg/L Cefotaxime sodium salt, 200mg/L Timentin, 50mg/L Hygromycin.
Selection medium-2 (SM-2)	To the CIM medium, add 300mg/L Cefotaxime sodium salt 50 mg/L Hygromycin.
Regeneration medium (RGM)	MS salts, 36g/L Maltose monohydrate adjust the pH to 5.8 then add 0.8-1% Agarose carein 300mg/L. Autoclave the media and add 2.5mg/L 6-BAP and 0.5mg/L NAA, 35mg/L Hygromycin, kinetin.
Rooting medium (RM)	½ MS salts, 36g/L maltose then adjust the pH to 5.8 then add 0.25% Gelrite (Sigma-Aldrich). After autoclaving add 0.05mg/L NAA.

Molecular characterization of genome edited plants

After 20 days of transplanting in pot, leaf of each plant was collected, and genomic DNA was extracted from putative T0 plants. The purified each T0 plant genomic DNA was used as template for confirmation by PCR using SpCas9 specific primers. To identify the mutation in CYP71A1 gene in Cas9 PCR confirmed T0 plants, the CYP71A1 genomic region covering the gRNA target regions was sequenced by Sanger sequencing method. The DNA sequences of the T0 plants were analyzed using DSDecodeM (http://dsdecode.scgene.com/) (Liu et al. 2015; Ma et al. 2015) and CRISPR-ID (Dehairs et al. 2016) tools. Seeds from this sequence confirmed mutants will be used to raise T1 plants.

In the production of CYP71A1 knockout (CYP71A1-KO) rice plant, a 20 bp fragment (5'-TGGTCGCGTTGAGGAGGAGC -3') of CYP71A1 gene was successfully cloned into the transfer vector, Cas9/gRNA. Electrophoresis and sequencing results confirmed the generated recombinant Cas9/gRNA contained the target sequence of interest (Fig. 1 and 2). Successful recombinant Cas9/gRNA-CYP71A1 vector was transformed into *Agrobacterium tumefaciens* LBA4404 competent cell. Electrophoresis confirmed the successful recombinant Agrobacterium with target gene of interest was confirmed by PCR (Fig. 3) and used for

co-cultivation. Calli of BRRI dhan87, BRRI dhan89 and BRRI dhan92 were developed using tissue culture technique (Figs. 4). Successful calli were co-cultivated with recombinant *Agrobacterium*. Calli were cultured with shoot and root inducing medium in MS media supplemented with different hormone and antibiotic. Shoot was developed from callus and healthy shoot was transferred to root inducing media in glass bottle. After 20 days in rooting media, rooted plants were transplanted in plastic pot and kept in greenhouse for further growth. Cas9 specific primers were used to confirm the genome edited plants. Electrophoresis results confirmed that five plants harbored Cas9/gRNA vector (Fig. 5).

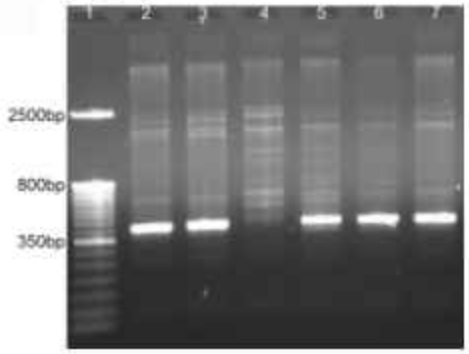

Fig. 1. Electrophoresis of PCR product amplified by target primers of CYP71A1 and Cas9 vector. Lane 1: DNA lader (50bp); lane 2 - 7: Recombinant Cas9 vector DNA.

```
CYP71A1          ----TGGTCGCGTTGAGGAGGAGC---------------------------------------- 20
Cas9/gRNA-CYP71A1 TCCTGGTCGCGTTGAGGAGGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATC 60
                  **********************

CYP71A1          ---------------------------------------------------------------- 20
Cas9/gRNA-CYP71A1 AACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTACTAGTTTTGATCTTGAAAGATCT 120

CYP71A1          ---------------------------------------------------------------- 20
Cas9/gRNA-CYP71A1 TTTATCTTTAGAGTTAAGAACTCTTTCGTATTTTGGTGAGGTTTTATCCTCTTGAGTTGG 180

CYP71A1          ---------------------------------------------------------------- 20
Cas9/gRNA-CYP71A1 TCATAGACCTATTCATGGCTCTGATACCAATTTTTAAGCGGGGGCTTATGCGGATTATTT 240

CYP71A1          ---------------------------------------------------------------- 20
Cas9/gRNA-CYP71A1 CTTAAATTCATAAGGGGTTTTACGGGCATAGGGTATAAATACAAGCATTCCCTTGTTGTT 300

CYP71A1          ---------------------------------------------------------------- 20
Cas9/gRNA-CYP71A1 TCTAAGTATAGTAGCGTACCTCTATCAATTTCCATCTTCTTACCTGCACAGGGCCGCAAC 360

CYP71A1          ---------------------------------------------------------------- 20
Cas9/gRNA-CYP71A1 CTTCT------------------------------------------------------- 385
```

Fig. 2. Alignment of the original target site of CYP71A1 and the sequence of recombinant Cas9/gRNA-CYP71A1. * indicates the similarity between original target sequence and recombinant Cas9/gRNA-CYP71A1 site.

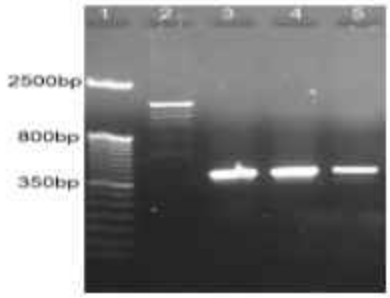

Fig. 3. Electrophoresis of PCR product amplified by target primers of CYP71A1 and Cas9 vector. Lane 1: DNA ladder (50bp); lane 2: Blank Agrobacterium LBA4404 and 3 - 5: Recombinant Agrobacterium LBA4404.

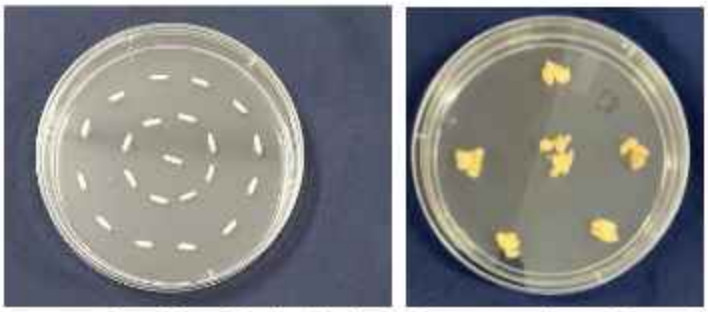

Fig. 4. Development of callus. Dehusked seeds were placed in callus induction plate (left side) and callus developed from seed culture plate (right side).

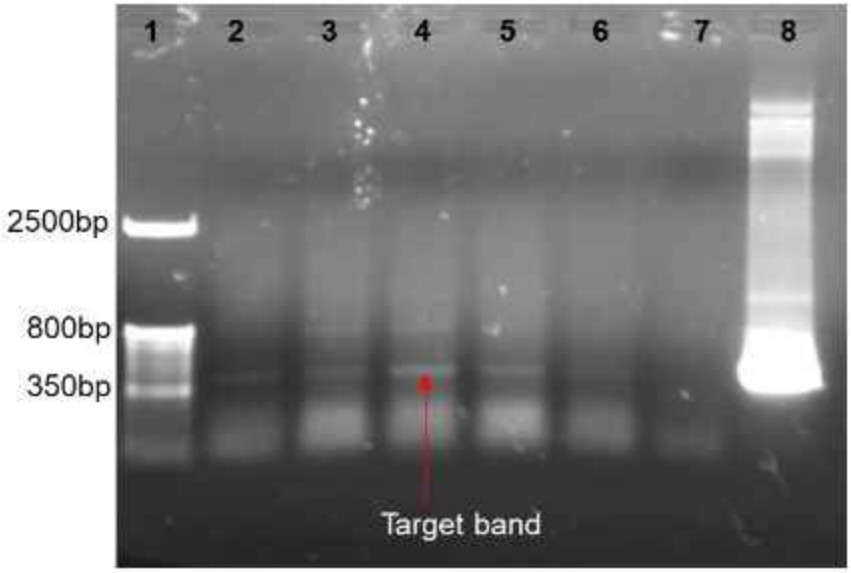

Fig. 5. Electrophoresis of PCR product amplified by SpCas9 primers. Lane 1: DNA ladder (50bp); lanes 2 - 6: CRISPR Cas9 edited plants (mutants); lane 7: BRRI dhan87 (control) and lane 8: Cas9/gRNA-CYP71A1 recombinant vector.

RNAi (RNA Interference): Concept, Mechanisms, Methods, and Applications

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Introduction to RNA Interference (RNAi)

RNA interference (RNAi) is a **highly conserved, post-transcriptional gene silencing mechanism** that regulates gene expression by degrading specific mRNA molecules. This process plays a crucial role in **gene regulation, antiviral defense, and transposon silencing** in eukaryotic cells. RNAi is mediated by **small RNA molecules**, including **small interfering RNAs (siRNAs)** and **microRNAs (miRNAs)**, which guide the RNA-induced silencing complex (RISC) to target mRNAs for degradation or translational repression.

Discovered in **1998 by Andrew Fire and Craig Mello**, RNAi has become a powerful tool in functional genomics, biotechnology, and therapeutic applications, offering a precise method to silence genes of interest.

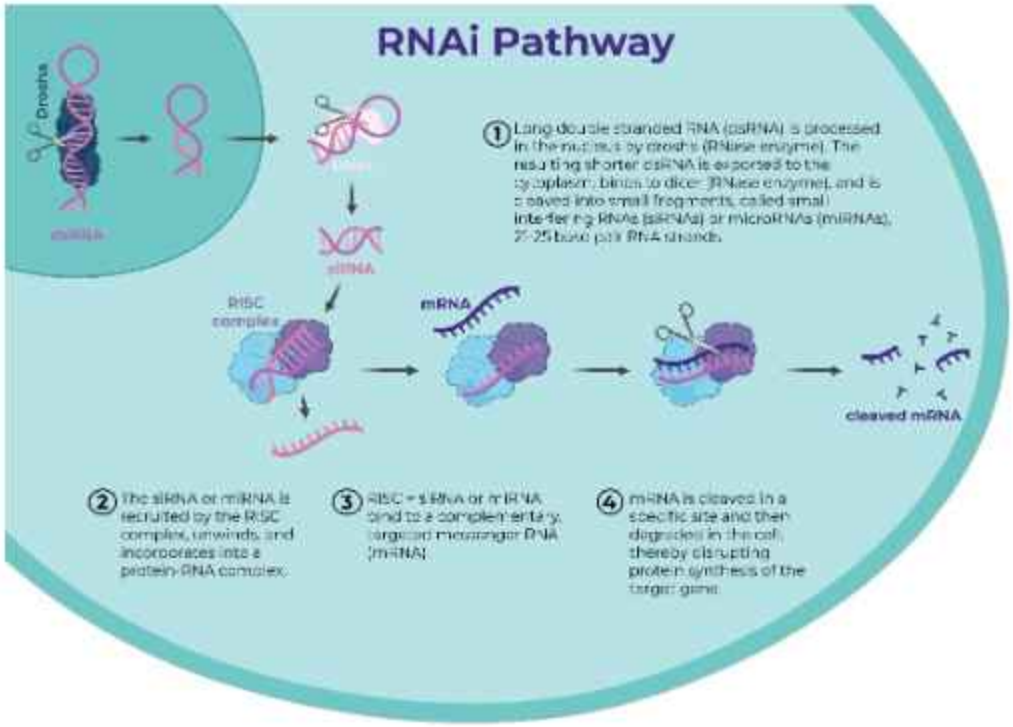

Figure 01: RNA interference pathway. The term RNA interference (RNAi) was coined to describe a cellular mechanism that uses the gene's own DNA sequence to turn it off, a process that researchers call silencing. In a wide variety of organisms, including animals, plants, and fungi, RNAi is triggered by double-stranded RNA (dsRNA). During RNAi, long dsRNA is cut or "diced" into small fragments ~21 nucleotides long by an enzyme called "Dicer". These small fragments, referred to as small interfering RNAs (siRNA), bind to proteins from a special family: the Argonaute proteins. After binding to an Argonaute protein, one strand of the dsRNA is removed, leaving the remaining strand available to bind to messenger RNA target sequences according to the rules of base pairing: A binds U, G binds C, and vice versa. Once bound, the Argonaute protein can either cleave the messenger RNA, destroying it, or recruit accessory factors to regulate the target sequence in other ways.

Concept of RNA Interference (RNAi)

RNAi operates through sequence-specific interactions between small RNA molecules and their complementary mRNA targets, leading to gene silencing. The two main categories of small RNAs involved in RNAi are:

1. **Small interfering RNAs (siRNAs)**: Derived from **exogenous** double-stranded RNA (dsRNA) or experimentally introduced RNA molecules.
2. **MicroRNAs (miRNAs)**: Derived from **endogenous** precursor RNA molecules transcribed from the genome.

Both siRNAs and miRNAs interact with **Argonaute proteins** within the RISC complex to mediate **mRNA cleavage, degradation, or translational repression**.

Mechanisms of RNA Interference

1. Small Interfering RNA (siRNA) Pathway
The siRNA pathway primarily defends against exogenous genetic elements, such as viral RNA and transposons.

Steps in the siRNA Mechanism:

1. **dsRNA Processing**:
    - Long dsRNA molecules (from viral infections or laboratory introduction) are recognized and cleaved by **Dicer**, an RNase III enzyme.
    - This generates **21-23 nucleotide siRNA duplexes** with **5' phosphate and 3' hydroxyl overhangs**.
2. **RISC Loading and Activation**:
    - One strand of the siRNA (the guide strand) is loaded onto the **RNA-induced silencing complex (RISC)**, which contains an **Argonaute protein**.
    - The passenger strand is degraded, leaving the guide strand available for target recognition.
3. **Target Recognition and Cleavage**:
    - The guide strand directs RISC to **perfectly complementary** mRNA targets.
    - The Argonaute protein **cleaves** the target mRNA, leading to rapid degradation and **gene silencing**.

2. MicroRNA (miRNA) Pathway
The miRNA pathway is a critical mechanism of **endogenous gene regulation**, influencing **development, differentiation, and homeostasis**.

Steps in the miRNA Mechanism:

1. **miRNA Biogenesis**:
    - miRNAs are transcribed as **primary miRNA (pri-miRNA)** by RNA polymerase II.
    - pri-miRNA is processed in the nucleus by **Drosha** and **DGCR8** into a precursor miRNA (pre-miRNA).
    - pre-miRNA is exported to the cytoplasm via **Exportin-5**.
2. **Processing by Dicer**:
    - In the cytoplasm, **Dicer** cleaves pre-miRNA into a **~22 nucleotide miRNA duplex**.
3. **RISC Loading and Gene Silencing**:
    - The miRNA duplex is loaded onto RISC.
    - The **guide strand** (mature miRNA) directs RISC to **partially complementary** target mRNAs.
    - This results in **translational repression or mRNA degradation**.

Methods of RNAi Application

RNAi technology has revolutionized functional genomics, drug discovery, and disease treatment. Several methods exist to **introduce siRNA or miRNA into cells**:

1. Chemical Synthesis of siRNA
- **Synthetic siRNAs** can be designed to target specific genes and transfected into cells.
- Commonly used in **cell culture and therapeutic research**.

2. Vector-Based RNAi

- **Plasmids, viral vectors, or shRNA (short hairpin RNA)** constructs are used to express siRNA or miRNA inside cells.
- Allows **stable and long-term gene silencing**.

3. Lipid Nanoparticle Delivery

- Lipid nanoparticles (LNPs) encapsulate siRNA molecules for efficient delivery.
- Used in RNAi-based **therapeutic drugs**, such as **patisiran (Onpattro)** for treating hereditary transthyretin-mediated amyloidosis.

4. CRISPR-Based RNAi (CRISPRi)

- CRISPR interference (CRISPRi) utilizes **catalytically inactive Cas9 (dCas9)** fused to repressors.
- Can be used for long-term and reversible gene silencing.

Applications of RNA Interference

1. Gene Function Studies

- RNAi is widely used in **functional genomics** to **knock down gene expression** and study gene roles.

2. Therapeutic Applications

- **Viral Infections**: RNAi-based drugs are being explored to target **HIV, Hepatitis B, and SARS-CoV-2**.
- **Cancer Treatment**: RNAi therapies are designed to silence oncogenes, improving cancer treatment.
- **Neurodegenerative Diseases**: RNAi is being tested for conditions like **Huntington's disease and ALS**.
- **Genetic Disorders**: RNAi offers **mutation-specific silencing** in diseases like **Duchenne Muscular Dystrophy**.

3. Agricultural Applications

- **Crop Improvement**: RNAi is used to enhance resistance to pests and diseases.
- **Pest Control**: RNAi-based insecticides target essential genes in crop pests.
- **Biofortification**: Modifying crops to improve nutrient content using RNAi.

4. Biotechnological and Industrial Applications

- **Synthetic Biology**: RNAi can be used for metabolic engineering in microbes for biofuel and pharmaceutical production.
- **Environmental Remediation**: RNAi approaches help in reducing the expression of harmful genes in pathogens.

Challenges and Future Perspectives of RNAi

Despite its vast potential, RNAi faces several **challenges**:

- **Off-Target Effects**: Non-specific gene silencing can affect unintended targets.
- **Delivery Issues**: Efficient delivery of siRNA to specific tissues remains a major hurdle.
- **Immune Response**: Exogenous siRNA can trigger an innate immune response.
- **Transient Effects**: siRNA-based silencing is **temporary**, requiring repeated administration.

Future Directions

- **Next-Generation RNAi Therapies**: Improved delivery methods, such as **engineered nanoparticles and cell-penetrating peptides**.
- **Artificial miRNA Engineering**: Developing synthetic miRNAs for **precise gene modulation**.
- **Combining RNAi with CRISPR**: Hybrid strategies to achieve more robust gene regulation.
- **Expanding RNAi to New Organisms**: RNAi applications in non-model organisms for **biodiversity conservation**.

Conclusion

RNA interference (RNAi) is a revolutionary gene-silencing tool with **broad applications in research, medicine, and biotechnology**. Its ability to specifically target genes **without modifying the genome** makes it an attractive alternative to genome-editing technologies. While challenges remain in delivery and specificity, advancements in RNAi-based therapeutics and genetic engineering continue to enhance its potential for **precision medicine, agriculture, and industrial applications**. As RNAi technology evolves, it holds great promise for treating **genetic disorders, viral infections, and cancer**, while also benefiting **crop improvement and biotechnology industries**. Continued research and development will further unlock the potential of this powerful gene regulation tool.

Homology-Independent Targeted Integration (HITI): Concepts, Mechanism, Mrthods and Applications

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Concept

The non-homologous end-joining (NHEJ) repair pathway operates continuously throughout the cell cycle, making it a potentially efficient mechanism for targeted gene insertion in whole organisms, as it is utilized preferentially over homology-directed repair (HDR) by most cells and organisms. Typically, NHEJ-mediated repair results in small insertions or deletions (indels) at the target site, leading to gene disruption. This has been the primary application of NHEJ, particularly in gene inactivation using CRISPR-Cas9 technology, which has gained widespread popularity due to its ability to create disruptions via indels.

Previously, integrating large DNA fragments via NHEJ has been inefficient and imprecise, limiting its widespread use. To enhance efficiency, one strategy has been to combine NHEJ with in vivo ligation (termed Bendcapture^), utilizing compatible overhangs created in the donor DNA and the genomic target site by molecular scissors such as ZFNs. This approach facilitates pairing of cohesive ends from the donor and target, resulting in donor insertion at a single orientation. However, the recreated cleavage target sites in the product can lead to re-cleavage until mutations, such as deletions by exonucleases, occur, resulting in imprecise junctions. Additionally, exonucleolytic deletions may occur in the donor DNA upon initial introduction into the cell before protection by ZFNs. Integration of the donor DNA in the opposite orientation is challenging due to the lack of complementary cohesive ends between the donor and genomic target sites. Nonetheless, exonuclease digestion may expose areas of microhomology, enabling integration via microhomology-mediated end-joining (MMEJ). Both pathways result in stable products but occur at low frequencies with imprecise junctions.

While the FokI nuclease of ZFNs and TALENs generates suitable cohesive ends for end-capture, Cas9 creates blunt ends unsuitable for this process. Recent advancements involve fusing FokI to an inactivated Cas9 to create overhanging ends for end-capture. Alternatively, CRISPR can be used with Cpf1, which generates overhanging ends. End-capture efficiency is optimized when plasmids are cleaved after cell introduction. However, the efficiency of site-specific genomic insertion by NHEJ with end-capture is not significantly higher than HDR. In instances where blunt ends are created by CRISPR-Cas9 cleavage, DNA insertion by NHEJ without end-capture has been explored. However, efficiencies remain low, with the presence of indels at insertion junctions. Despite challenges, some studies have achieved large DNA insertions using CRISPR-Cas9 and NHEJ, albeit with frequent indels at junctions. Optimization of CRISPR-Cas9 and NHEJ integration methods, such as those described in the HITI techniques, may address these limitations.

Mechanisms of Homology-Independent Targeted Integration

Homology-independent targeted integration (HITI) is a genome engineering approach that enables precise DNA integration at specific genomic loci without the requirement for homology-directed repair (HDR) or homologous recombination. Unlike traditional genome editing methods that rely on homology-based repair mechanisms, HITI exploits the cellular repair machinery to insert exogenous DNA sequences at predetermined genomic sites in a homology-independent manner.

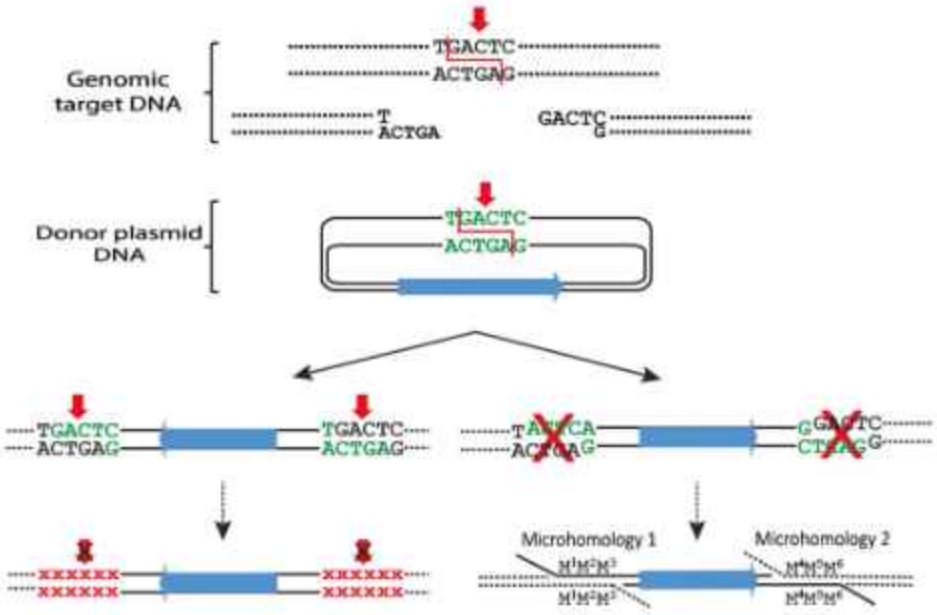


Figure 01: End-capture of cohesive ends. Diagram of Bend capture^ of a piece of donor DNA at the genomic site of a nuclease-induced (red arrows) DSB. Two pathways could result in stable products, but at low efficiency and with imprecise junctions. Left: the overhanging ends of the donor DNA are complementary to the overhanging ends of the genomic target site, since both the donor and genomic DNAs were cleaved by the same ZFN. However, end capture recreates the target sites that can be re-cleaved. Recutting and religation will be repeated until the target site sequence is altered by mutation and can no longer be cleaved by the programmable nuclease. Small red Xs indicate the mutated sequences. Right: donor DNA that inserts with an opposite orientation to that shown in the left pathway will have incompatible ends to the genomic target sites, but it can be integrated via microhomology with very low efficiency. (Source: doi: 10.1007/s00412-018-0677-6)

Here's a step-by-step description of the mechanism of HITI:

Design of HITI Cassette:

- The HITI cassette consists of the desired DNA sequence to be integrated into the genome, flanked by sequences that facilitate its insertion into the target locus.
- The cassette typically includes a promoter sequence to drive expression of the inserted DNA, along with any regulatory elements necessary for gene expression.

Delivery of HITI Cassette:

- The HITI cassette is delivered into target cells using various methods, such as viral vectors, transfection, or electroporation.
- The delivery system ensures the efficient uptake of the HITI cassette into the nucleus of target cells, where genomic integration will occur.

Endonuclease-Mediated DNA Cleavage:

- Optionally, a site-specific endonuclease (e.g., CRISPR-Cas9) may be used to induce a double-strand break (DSB) at the target genomic locus.
- The DSB serves as a trigger for the cellular DNA repair machinery to initiate the integration process.

Non-Homologous End Joining (NHEJ):

- In the absence of homologous donor DNA, the predominant repair pathway for DSBs is non-homologous end joining (NHEJ).
- NHEJ ligates the broken DNA ends together, often resulting in small insertions or deletions (indels) at the repair junctions.

Integration of HITI Cassette:

- The HITI cassette contains short regions of homology (microhomologies) flanking the inserted DNA sequence.
- Microhomologies at the ends of the HITI cassette anneal to the genomic DNA adjacent to the DSB site, facilitating the precise integration of the cassette into the genome.

Gap Filling and Ligation:

- DNA repair synthesis occurs to fill any gaps between the inserted DNA and the genomic DNA, ensuring seamless integration.
- DNA ligases catalyze the sealing of nicks in the DNA backbone, completing the integration process.

Expression of Integrated DNA:

- Once integrated into the genome, the inserted DNA sequence is under the control of the promoter within the HITI cassette, allowing for its expression in the host cell.

Validation and Characterization:

- The efficiency and accuracy of HITI-mediated integration can be assessed using molecular techniques such as PCR, sequencing, and functional assays.
- Validated HITI clones can be further analyzed to confirm the stable expression of the integrated DNA sequence and its functional impact on cellular phenotype.

HITI offers a versatile and efficient method for targeted genome editing and gene insertion in a wide range of cell types and organisms, with potential applications in basic research, biotechnology, and gene therapy.

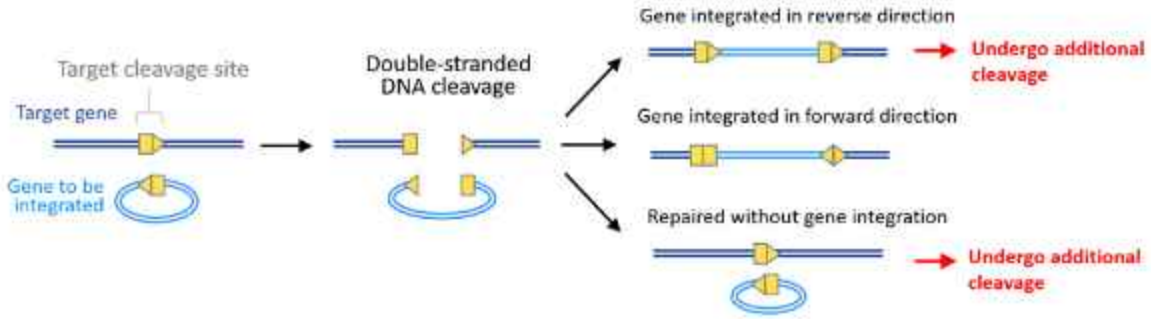


Figure 02: Scheme of HITI method, which uses NHEJ-mediated targeted integration. If cleavage is repaired without gene integration or with integration in reverse direction, DNA undergoes additional cleavage until forward gene integration or gRNA can no longer bind to target cleavage sequence due to errors from NHEJ repair.

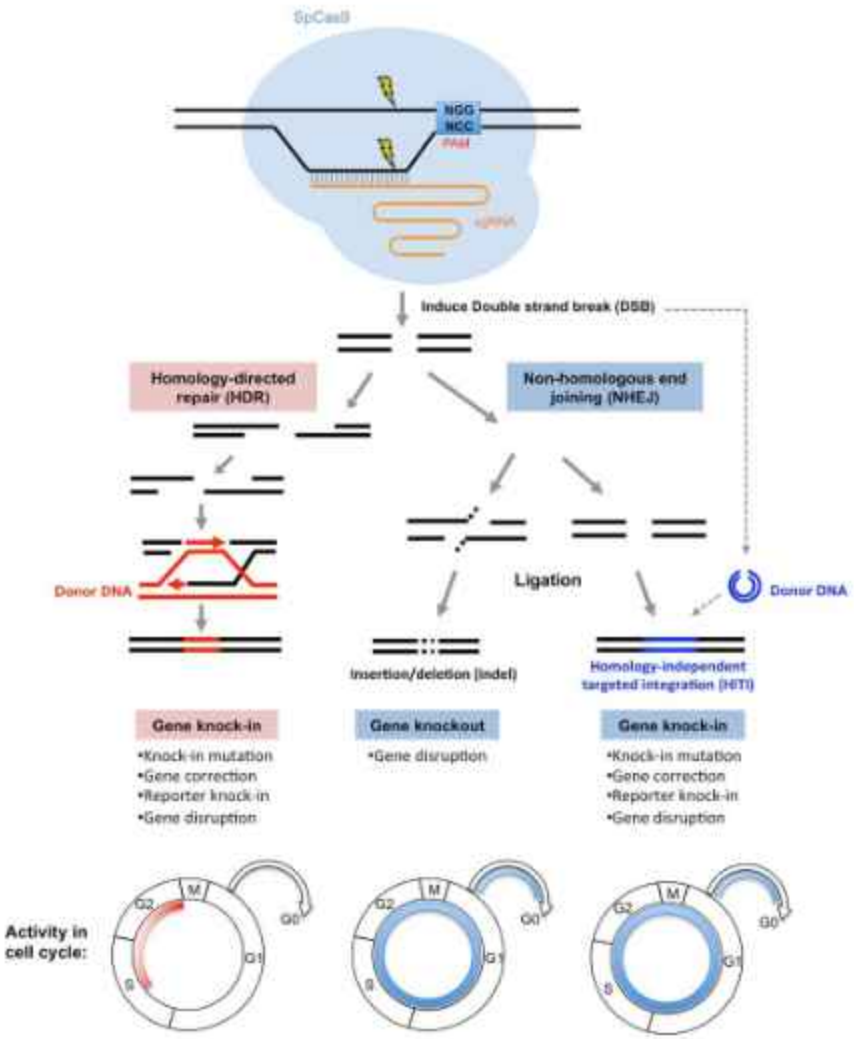
Methods of Homology-Independent Targeted Integration

Homology-independent targeted integration (HITI) is a method used to precisely insert DNA sequences into specific genomic loci without relying on homologous recombination. Here's a step-by-step overview of the HITI method:

Design of Donor DNA: The first step in HITI involves designing the donor DNA, which typically consists of the desired DNA sequence flanked by regions of homology to the target genomic locus. These homology arms facilitate the integration of the donor DNA into the target site.

Preparation of CRISPR-Cas9 System: Next, the CRISPR-Cas9 system is prepared. This involves designing and synthesizing guide RNAs (gRNAs) that target the Cas9 nuclease to the desired genomic locus where integration will occur. The gRNAs are designed to create double-strand breaks (DSBs) at the target site.
Cell Culture and Transfection: Cells are cultured under appropriate conditions and transfected with the CRISPR-Cas9 system, including the gRNAs targeting the desired genomic locus, along with the donor DNA.

DSB Induction: Once the cells are transfected, the CRISPR-Cas9 system induces DSBs at the target genomic locus. The Cas9 nuclease creates precise cuts in the DNA, generating free ends that are suitable for integration of the donor DNA.



Donor DNA Integration: The donor DNA, containing the desired DNA sequence flanked by homology arms, is introduced into the cells. The homology arms facilitate the precise insertion of the donor DNA into the target genomic locus through non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ).

Repair and Integration: The DSBs created by the CRISPR-Cas9 system are repaired by the cellular DNA repair machinery, which includes NHEJ and MMEJ pathways. In HITI, the donor DNA is integrated into the genomic locus by these repair pathways using the homology arms as templates for repair.

Selection and Screening: After integration, cells containing the desired genomic modifications are selected and screened. This may involve the use of selectable markers included in the donor DNA or other screening methods to identify cells with the desired integration events.

Validation of Integration: Finally, the integration of the donor DNA at the target genomic locus is validated using molecular techniques such as polymerase chain reaction (PCR), sequencing, or other genomic analysis methods.

Overall, HITI provides a precise and efficient method for targeted integration of DNA sequences into specific genomic loci, offering numerous applications in genetic engineering and gene therapy research.

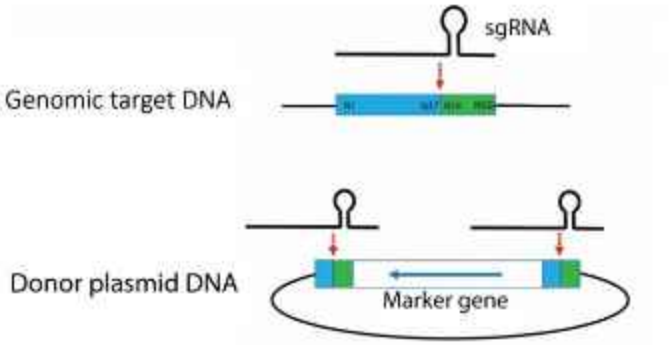


Figure 03: Homology-independent targeted integration (HITI). The sgRNA of CRISPR cleaves the same target sequence in the genome and flanking the gene of interest in the donor plasmid (the blue box indicates the beginning of the target sequence and the green box indicates the end of the target sequence). The gene of interest is subsequently inserted into the genomic target site by NHEJ in either orientation. If the sgRNA target site is re-created, the inserted DNA is presumably excised and re-inserted until finally it is in the final stable orientation where the sgRNA target site is no longer present.

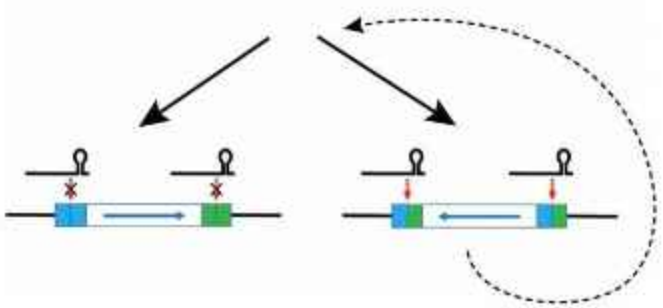


Figure 04: CRISPR/Cas9-mediated targeted genome editing. Streptococcus pyogenes Cas9 (SpCas9) forms a complex with a

single-guide RNA (sgRNA) that recognizes a specific 20-bp homologous sequence in front of the NGG PAM sequence. Once bound, these complexes induce double strand breaks (DSBs) at targeted genomic loci. These DSBs are then repaired by intracellular DSB repair machineries that can be categorized into two major types: (1) error-free homology-directed repair (HDR), which repairs DSBs using a homologous chromatid or chromosome, and (2) error-prone non-homologous end joining (NHEJ), which processes and ligates DNA ends directly. Error-prone NHEJ often introduces indels, which can disrupt the target gene. In contrast, in the presence of ectopic homologous DNA, HDR can introduce any sequence into the target site. HDR only occurs during the S-G2 phase of the cell cycle; whereas NHEJ is active throughout the cell cycle in a variety of adult cell types, making it accessible to non-dividing cells. Homology-independent targeted integration (HITI) hijacks the NHEJ pathway and inserts ectopic DNA at target sites in both dividing and non-dividing cells. (Source: doi: 0.1038/s10038-017-0352-4)

Applications in Sustainable Agriculture and Livestock:

Homology-independent targeted integration (HITI) holds significant potential for applications in sustainable agriculture and livestock breeding by enabling precise genetic modifications in crops and livestock without relying on homology-directed repair mechanisms. While HITI applications in agriculture and livestock are still in the early stages of development, several potential applications can be envisioned:

Trait Improvement in Crops:

- HITI can be used to introduce beneficial traits into crop plants, such as disease resistance, improved nutritional content, and enhanced stress tolerance.
- By precisely integrating gene cassettes containing desired traits into specific genomic loci, HITI offers a means to develop crops with improved agronomic characteristics, leading to increased yield, reduced pesticide use, and enhanced resilience to environmental stresses.

Engineering Stress Tolerance:

- Climate change is leading to more frequent and severe environmental stresses, such as drought, heat, and salinity, which adversely affect crop productivity.
- HITI-mediated integration of stress-tolerance genes into crop genomes can confer resilience to adverse environmental conditions, ensuring sustainable agricultural production in the face of climate change.

Enhancing Disease Resistance:

- Plant diseases caused by pathogens, fungi, and viruses pose significant threats to global food security.
- HITI can be employed to introduce disease resistance genes into crop plants, providing durable protection against a wide range of pathogens and reducing crop losses due to diseases.

Nutritional Enhancement:

- Malnutrition and food insecurity remain pressing challenges worldwide, necessitating the development of nutrient-rich crops.
- HITI can facilitate the integration of genes involved in the biosynthesis of essential nutrients (e.g., vitamins, minerals, amino acids) into crop genomes, leading to the production of biofortified crops with improved nutritional quality.

Livestock Improvement:

- HITI can also be applied to improve livestock breeding programs by introducing desirable traits into animal genomes.
- Integration of genes associated with traits such as disease resistance, meat quality, milk production, and feed efficiency can lead to the development of livestock breeds with enhanced productivity and resilience, contributing to sustainable livestock farming practices.

Bioremediation and Environmental Applications:

- HITI-mediated genetic modifications can be used to develop plants capable of phytoremediation, i.e., the removal of pollutants from soil and water.
- Integration of genes encoding enzymes involved in pollutant degradation pathways can enable the engineering of plants with enhanced abilities to detoxify contaminated environments, contributing to environmental sustainability.

Conservation and Biodiversity:

- HITI can aid in conservation efforts by introducing beneficial traits into endangered plant species or genetically diverse crop varieties.

- Integration of genes associated with adaptive traits or stress tolerance can enhance the resilience of endangered plant populations to environmental pressures, contributing to biodiversity conservation.

Overall, HITI offers a promising tool for precision genome engineering in agriculture and livestock, with the potential to address various challenges facing sustainable food production, environmental conservation, and human nutrition. However, continued research, regulatory oversight, and public engagement are essential to ensure responsible and ethical deployment of HITI technologies in agricultural and environmental contexts.

Microhomology-Mediated End Joining (MMEJ): Concept, Mechanisms, Methods, and Applications

Dr. Mohammad Nurul Islam
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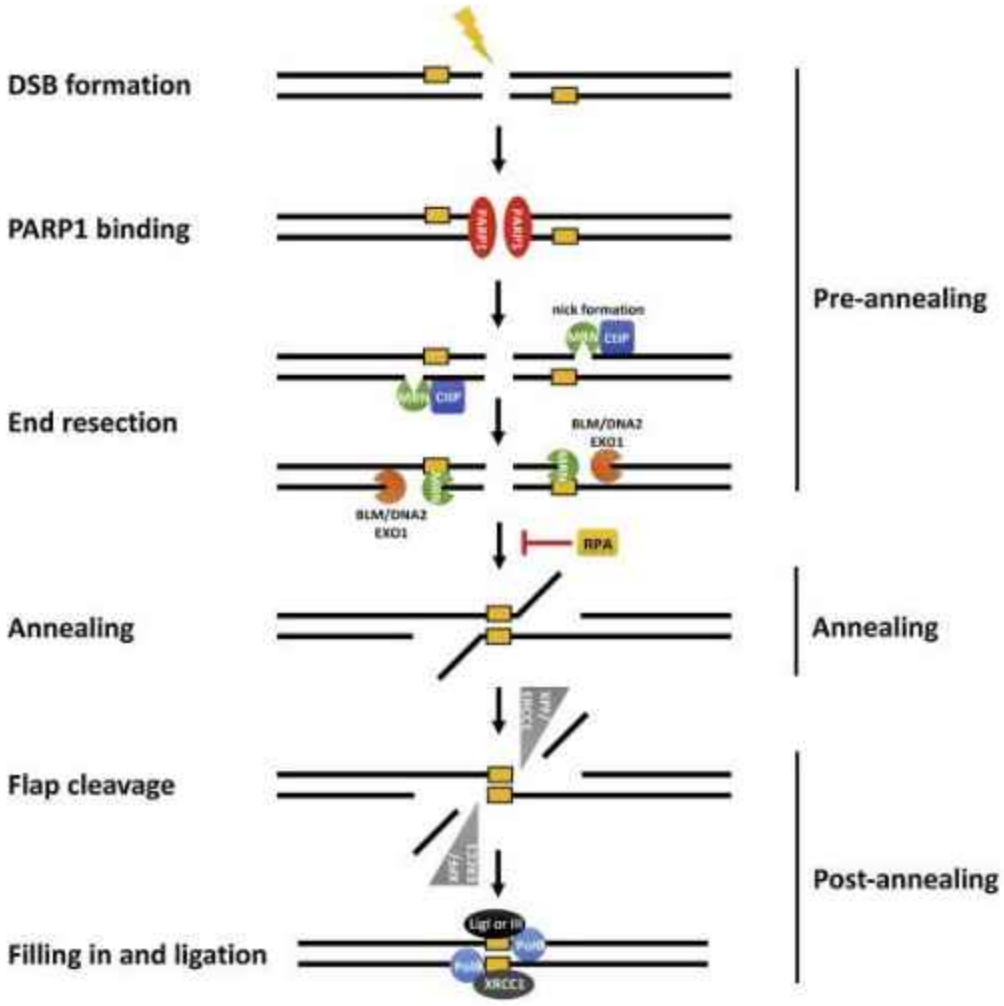
Introduction

DNA double-strand breaks (DSBs) are a severe form of genetic damage that can lead to cell death or genomic instability if left unrepaired. Cells have evolved several repair mechanisms to address DSBs, including homologous recombination (HR), non-homologous end joining (NHEJ), and microhomology-mediated end joining (MMEJ). MMEJ is an alternative repair pathway that uses short homologous sequences (microhomologies) to join broken DNA ends. It is often considered error-prone and is associated with genomic deletions and mutations.



Concept of MMEJ

Definition

MMEJ is a repair pathway that relies on short, complementary DNA sequences (5-25 base pairs) near the DSB sites to mediate repair. Unlike HR, which requires an extensive homologous sequence and a sister chromatid, or classical NHEJ,

which directly ligates DNA ends, MMEJ trims DNA to expose microhomologies, aligns them, and then joins the strands, often leading to deletions.

Characteristics

1. **Use of Microhomologies**: Short regions of sequence homology guide repair.
2. **Error-Prone Nature**: Leads to deletions and occasional insertions.
3. **Alternative to NHEJ and HR**: Operates when these pathways are unavailable or dysfunctional.
4. **Dependency on DNA Polymerases and Ligases**: Requires specific enzymes for strand resection, pairing, and ligation.
5. **Association with Disease**: Errors in MMEJ can contribute to genetic disorders and cancer progression.

Mechanisms of MMEJ

Step 1: Recognition of Double-Strand Breaks

- DSBs occur due to ionizing radiation, oxidative stress, replication errors, or exogenous agents like CRISPR-Cas9 or restriction enzymes.
- The repair pathway choice depends on factors such as the phase of the cell cycle and the availability of repair proteins.

Step 2: Resection of DNA Ends

- The broken DNA ends undergo **5' to 3' resection**, exposing single-stranded DNA (ssDNA) overhangs.
- This process is facilitated by nucleases like **CtIP, MRE11-RAD50-NBS1 (MRN) complex**, and exonucleases.

Step 3: Microhomology Searching and Annealing

- Exposed ssDNA overhangs search for complementary microhomologous sequences.
- Annealing occurs, stabilizing the broken ends through base pairing.

Step 4: DNA Polymerization and Gap Filling

- DNA polymerases, such as **Pol θ (DNA polymerase theta)**, extend the annealed strands, filling any missing nucleotides.
- This step ensures that the broken ends are bridged and prepared for final ligation.

Step 5: Ligation

- DNA ligases, such as **Ligase III or Ligase I**, complete the repair process by sealing the newly synthesized DNA strands.
- Occasionally, mismatch repair proteins like XRCC1 may assist in ligation and final structural adjustments.

Step 6: Consequences of Repair

- Due to the loss of intervening sequences between microhomologous regions, deletions are a frequent outcome.
- MMEJ can introduce genetic variations, which may be beneficial (evolutionary adaptation) or harmful (genetic disorders, cancer mutations).

Methods for Studying MMEJ

1. Reporter Assays

- Scientists use reporter constructs to analyze the frequency and accuracy of MMEJ in cells.
- Fluorescent or luminescent markers (e.g., GFP-based assays) help quantify repair efficiency.

2. Gene Knockout and Knockdown Studies

- Knockout of MMEJ-associated genes (e.g., Pol θ) using CRISPR-Cas9 helps determine their role in repair.

- RNA interference (siRNA) or shRNA techniques silence specific genes to observe changes in MMEJ activity.

3. Chromatin Immunoprecipitation (ChIP)

- ChIP assays identify proteins involved in MMEJ by isolating DNA-protein complexes and sequencing them.

4. Next-Generation Sequencing (NGS)

- NGS allows researchers to study genome-wide MMEJ activity, identifying specific deletion patterns and sequence signatures associated with this pathway.

5. Live-Cell Imaging

- Advanced microscopy techniques track DNA damage repair events in real-time, visualizing MMEJ dynamics and kinetics.

6. In Vitro Biochemical Assays

- Purified proteins involved in MMEJ are studied in test tube conditions to analyze their biochemical activities.

Applications of MMEJ

1. Genome Editing and Genetic Engineering

- **CRISPR-Cas9-Induced DNA Breaks**: When Cas9 creates DSBs, MMEJ can be harnessed to introduce specific deletions or knockouts.
- **Targeted Mutagenesis**: By designing microhomology sequences, researchers can guide specific deletions in genes of interest.

2. Cancer Research and Therapy

- **Cancer Progression**: MMEJ is upregulated in various cancers due to mutations in HR and NHEJ pathways.
- **Drug Targeting**: Inhibiting Pol θ (key in MMEJ) is a promising cancer therapy strategy.
- **Synthetic Lethality Approaches**: Tumor cells defective in HR rely on MMEJ, making them vulnerable to Pol θ inhibitors.

3. Aging and Neurodegeneration

- MMEJ contributes to genomic instability in aging cells, leading to neurodegenerative diseases like Alzheimer's and Parkinson's.
- Understanding MMEJ in neurons helps design therapeutic interventions.

4. Plant and Microbial Engineering

- MMEJ plays a role in plant genome editing, allowing efficient genetic modifications in crops.
- In microbial systems, engineered MMEJ pathways enhance genetic manipulation for biotechnology applications.

5. Genetic Disease Modeling and Therapy

- By replicating MMEJ-related mutations in model organisms, researchers study genetic diseases such as Fanconi anemia and ataxia-telangiectasia.
- Gene therapy strategies can exploit MMEJ for controlled genetic modifications.

Conclusion

Microhomology-Mediated End Joining (MMEJ) is an alternative, error-prone DNA repair pathway that plays a significant role in genomic maintenance, genome editing, cancer progression, and therapeutic targeting. While often considered a backup repair mechanism, MMEJ has unique applications in biotechnology, cancer therapy, and genome engineering. Understanding its mechanisms, associated proteins, and functional consequences is essential for both fundamental biology and medical advancements. Further research into MMEJ inhibitors, enhancers, and applications could unlock new avenues in gene therapy and precision medicine.

Primer design and multiplex CRISPR/Cas9 vector construction

Dr. Shah Alam
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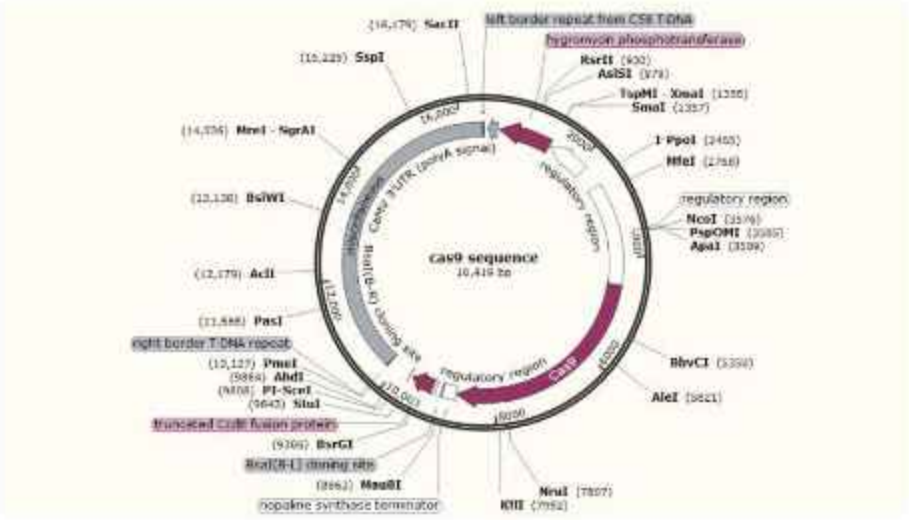
Plant Quarantine Station, Seaport, Chattogram



CRISPR/Cas9 Vector

A. Primer design

Multiplex target designs of target gene:

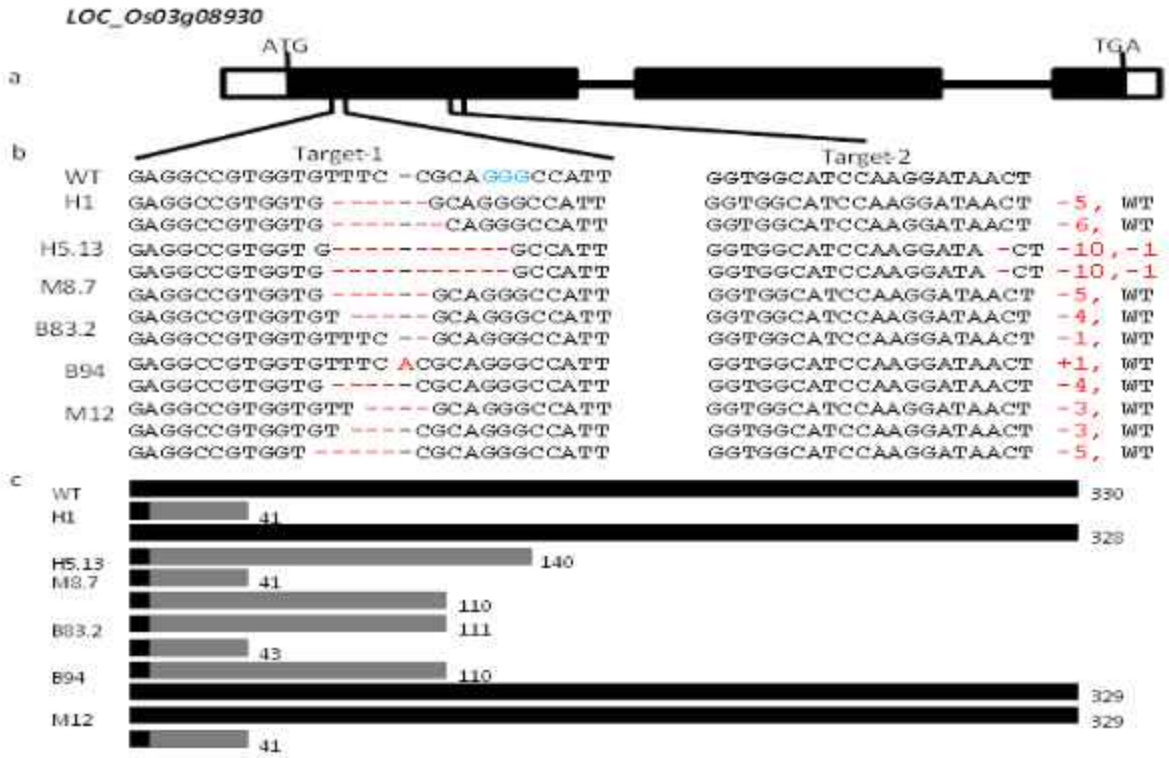
seq_id	sgRNA_id	Score	Sequence	Strand	pos	% GC
LOC_Os01g39330	Guide101	0.9312	ATTCCACTCACTCAAC CGAAGGG	+	13 71	45 %
LOC_Os01g39330	Guide 93	0.8205	GATGTCGGAGAGGAAG AGGAGGG	+	10 38	55 %

Multiplex CRISPR/Cas9 vector construction:

1. Two targets design in the exon of target gene by using CRISPR/Cas9 software
2. Preparation of Guide RNA by adding Double Distilled (DD) Water
   Note: Add DD water (Differ). Use ten times DD water
3. Preparation of Guide RNA by mixing forward (F) and Reverse (R)
   Note: From each target1 forward (F) and reverse (R), mix 2 µl and add 16 µl dd water, a total of 20 µl. Shake and Heat for 30 seconds at 90°C. And wait 20 minutes (at room temperature)
4. Ligation of two targets (1 and 2) Guide RNA with site-specific promoters

Note: Take another small 2 tubes marked B1 and B2 with a red marker, Shake and keep it 28 °C at 15 min

5. Two targets with U-F Primers and gR-R- Primers
   Note: PCR: 94°C-2m, (94°C – 10 S, 60 °C -15 S, 68 °C -20 S) Cycle-28, Cas 1 PCR about 50 min. Adjust volume up to 100 µl
6. Two targets with PpS-GGL - PGS- GG2 Primers
   Note: PCR: Cas 2: 95oC -2m, (95oC – 15 S, 60 oC -15 S, 68 oC -20 S) last 68 oC-1m, Cycle-17 about 40 minutes, after PCR, 3 µl 6x loading buffer for all and vortex & shake clearly. Check sequence, if sequence is good transfer into ecoli bacteria and keep in -80^{O} C
7. Gel Electrophoresis
   If you find clear red coloured bands with accurate sizes of U6a and U6b, cut the bands and put them in a 2 ml tube. Weight tubes and measure the accurate weight of the band (gram).
8. DNA extraction (Zymoclean GEL DNA Recovery kit).
   Place the column into a 1.5 ml tube. Add 16 DNA Elution Buffer/ dd water and centrifuge for 1 min and take Nano concentration.
9. Ligation of two targets with CRISPR/Cas9 vector
   PCR-Cas3 for 4 hours (37 ^{0} C 5 min, 10^{0} C 5min and 20^{0} C 5 min, 15 cycles)
10. Check sequence whether two targets are present in the CRISPR/Cas9 vect



Transformation of multiplex CRISPR/Cas9 vector into plant cell

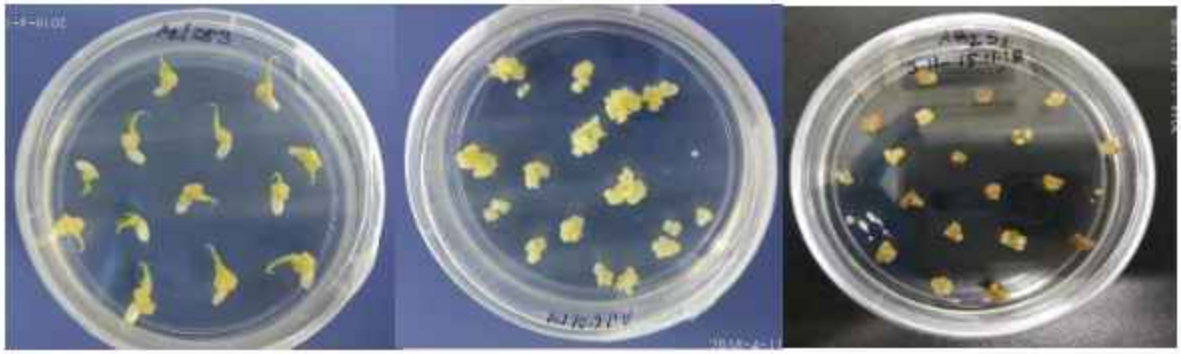
Dr. Shah Alam
Deputy Director
Plant Quarantine Station, Seaport, Chattogram

Transformation steps:

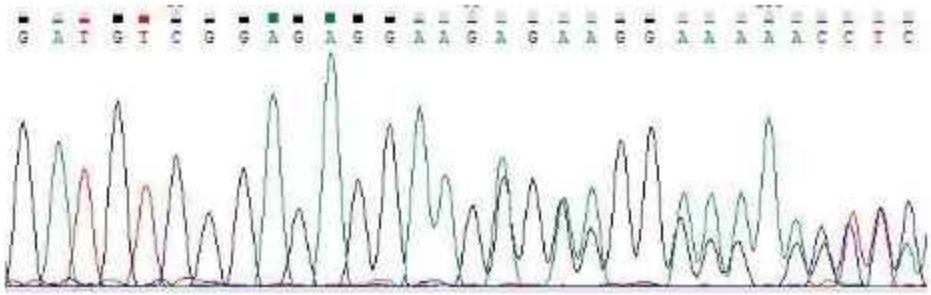
1. Confirmation of CRISPR/Cas9 Vector+ target genes sequences
2. Transformation of CRISPR/Cas9 Vector+ target genes sequences to ecoli bacteria
3. Confirmation of CRISPR/Cas9 Vector+ target genes sequences in ecoli bacteria
4. Transformation of CRISPR/Cas9 Vector+ target genes sequences to *Agrobacterium* cell
5. Confirmation of CRISPR/Cas9 Vector+ target genes sequences in *Agrobacterium* cell
6. *Agrobacterium* cell with CRISPR/Cas9 Vector+ target genes sequences Co-cultivation with one-month aged rice callus
7. Successful rice callus transfer to NBD callus induction media
8. Primary selection for callus screening
9. Secondary selection for callus screening
10. Check callus by PCR if there is any mutation
11. Transfer callus to MS-Pre-germination (PG) media for rice seedling
12. Transfer to MS-Regeneration (RG) media for rice seedlings
13. Transfer rice seedling to MS-Rooting (RT) media
14. Check mutation/edited of rice seedlings
15. Check types of mutation, homozygous or heterozygous
16. If there is heterozygous mutation, grow seedlings for one generation to get homozygous CRISPR/Cas9 edited rice seedlings
17. Collect CRISPR/Cas9 vector free CRISPR/Cas9 edited rice seedlings
18. Identify phenotypes of edited rice plants
19. Study the function of the target genes by numerous experiments
20. Publish the gene's function and use this gene for develop new rice crop

Target check in Gel-electrophoresis

Mutation Checking:



Callus to **Transgenic Plant**



Mutation detection

Allele1: ACATGATGTCGGAGAGGAAGAGGAGGGAGAAGCTC (WT) Allele2: ACATGATGTCGGAGAGGAAG-GGAGGGAGAAGCTTA

(complicated variant)

Reference: ACATGATGTCGGAGAGGAAGAGGAGGGAGAAGCTCAACGACAG CTTCCACACGCTCAGATCACTCCTCCC

The Future of Poultry: Creating Avian Influenza-Resistant Chickens Through Genome Editing

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Department of Biotechnology and Genetic Engineering
State University of Bangladesh

Avian influenza (AI), commonly known as bird flu, has been a persistent challenge in the poultry industry and a looming threat to human health. Highly pathogenic strains, such as H5N1, have caused devastating losses in poultry farms worldwide and sporadic infections in humans. Despite ongoing efforts, including vaccination and biosecurity measures, the virus continues to evolve, making control strategies less effective over time. But what if we could breed chickens that are inherently resistant to avian influenza? Scientists are now harnessing the power of genome editing to develop AI-resistant poultry, potentially revolutionizing the poultry industry and mitigating future outbreaks. This article explores groundbreaking research that modifies the ANP32 gene family using CRISPR/Cas9 technology to make chickens immune to AI.

The Devastating Impact of Avian Influenza
Avian influenza outbreaks have had catastrophic effects on global poultry industries, leading to billions of dollars in economic losses, trade restrictions, and significant threats to food security. Poultry farmers face the risk of mass culling to control the spread, while consumers experience fluctuations in supply and price.

The Science Behind Avian Influenza
AI is caused by influenza A viruses, which primarily affect birds but occasionally cross into humans and other mammals. These viruses mutate rapidly, making traditional control methods, such as vaccines, less effective over time. The spread of AI among poultry is facilitated by direct contact with infected birds, contaminated feed, and farm environments.

Why Is Avian Influenza So Dangerous?
AI is highly contagious among poultry and can spread through wild birds, making it challenging to contain. The virus's ability to mutate quickly through antigenic drift and reassortment makes it a persistent threat. If certain mutations enable the virus to spread efficiently between humans, it could lead to a pandemic similar to past influenza outbreaks.

The Role of ANP32 Proteins in Influenza Replication
Influenza A viruses rely on host proteins to replicate. One crucial factor is the ANP32 protein family, which includes ANP32A, ANP32B, and ANP32E. These proteins facilitate the viral polymerase activity required for the virus to reproduce within host cells. In chickens, ANP32A is the primary protein that supports AI virus replication, while ANP32B is inactive, and ANP32E has antiviral properties.

Researchers have discovered that avian ANP32A has a unique 33-amino-acid extension absent in mammals. This difference partly explains why AI struggles to infect humans directly. By targeting this protein in chickens, scientists aimed to disrupt the virus's replication ability, rendering the birds resistant to AI.

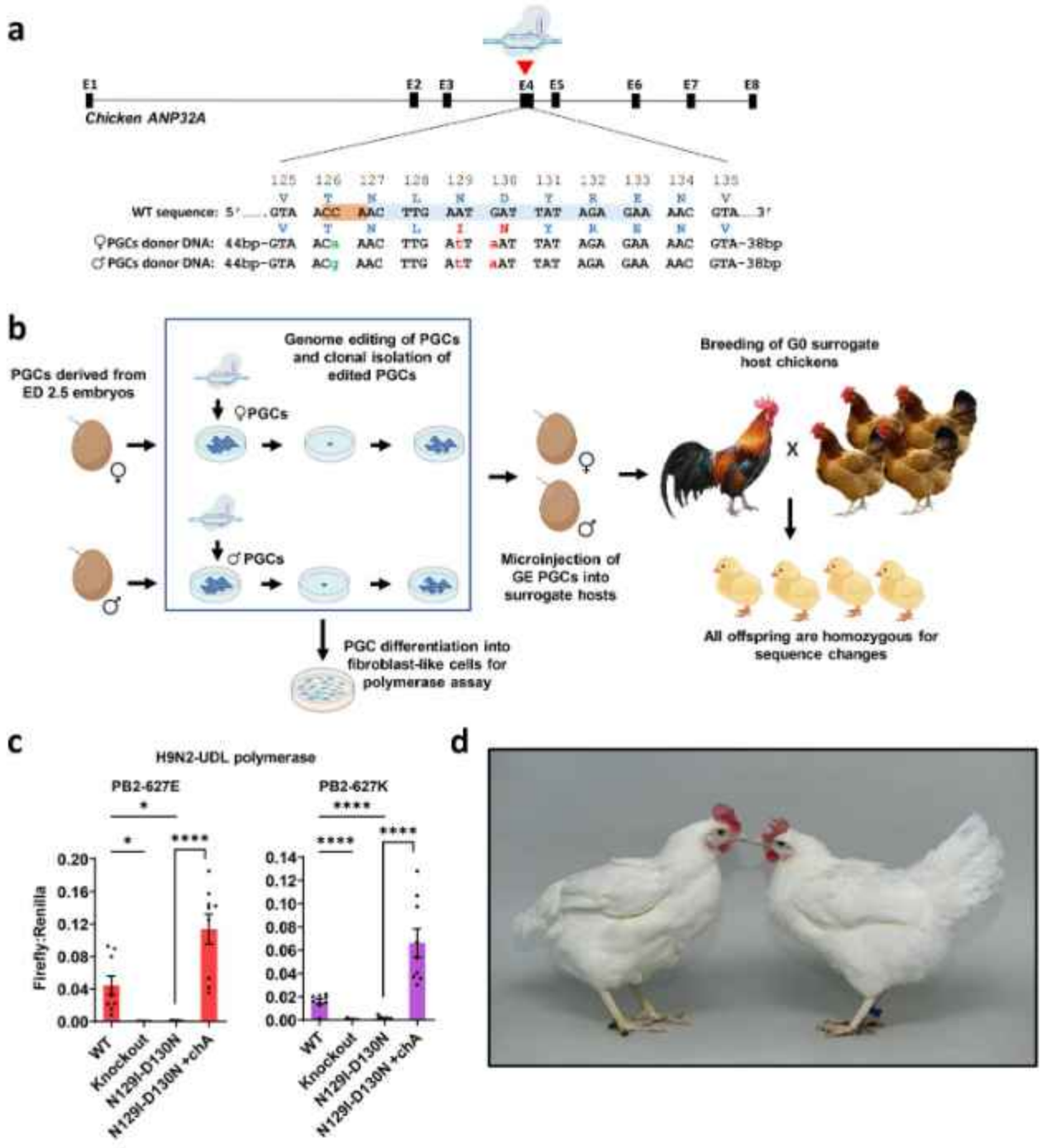

Figure 01: Breeding strategy for homozygous ANP32A$^{N129I-D130N}$ chicken. a ANP32A editing strategy: two nucleotide changes (red letters) introduce asparagine (N) position 129 (N129I) and aspartic acid (D) position 130 (D130N) missense mutations. The third nucleotide change (green letters) is a synonymous mutation in the gRNA PAM and serves as a marker control for allelic contribution from the male and female surrogate hosts. **b** Male and female PGC cultures were derived from the blood of individual chick embryos. The PGCs were edited, and clonal lines of GE PGCs were propagated and analysed. GE PGCs were differentiated into fibroblast-like cells for IAV polymerase assays. To generate GE chicks, GE PGCs were mixed with B/B dimerisation compound (to induce cell death of host embryo germ cells) and injected into iCaspase9 host embryos, which were incubated to hatch. After hatching, the surrogate hosts were raised to sexual maturity and directly mated. All offspring from eggs laid by the surrogate hosts were biallelic for the edit and contained the parent-specific PAM nucleotide change. **c** the activity of reconstituted IAV polymerase was assessed in fibroblast-like cells derived from ANP32A^{knockout} (Knockout), ANP32A$^{N129I-D130N}$ (N129I-D130N) or wild-type (WT) PGCs. Cells were transfected with avian IAV polymerase (PB2/627E - black bars) or human-adapted isoforms (PB2/627K - grey bars), Firefly minigenome reporter and Renilla reporter control plasmids and then incubated at 37 °C for 48 h. Wild-type chicken ANP32A (chA) cDNA was co-expressed with minigenome plasmids to rescue polymerase activity in ANP32A$^{N129I-D130N}$ cells. Data shown are Firefly activity normalised to Renilla plotted as mean ± SEM derived from ($n = 3$) three independent experiments each consisting of three technical replicates. Error bars represent standard error of mean (SEM). One-way ANOVA and Dunnett's multiple comparison test were used to compare polymerase activity in the GE cells with polymerase activity in WT cells. Unpaired two-tailed t-test was used to compare ANP32A$^{N129I-D130N}$ and

ANP32A$^{N129I-D130N}$ chA data. Statistical annotations are defined as *$P \leq 0.05$, ****$P \leq 0.0001$. **d** Image: wild-type (WT) hen (left) and homozygous ANP32A$^{N129I-D130N}$GE hen (right, blue ring on right shank).

How Genome Editing Was Used to Create AI-Resistant Chickens
Scientists at leading research institutions used CRISPR/Cas9 technology to introduce precise modifications to the ANP32A gene. The study involved:

- **Identifying Key Residues:** Researchers pinpointed two specific amino acids, N129 and D130, in ANP32A that are critical for viral polymerase interaction.
- **Introducing Genetic Edits:** CRISPR/Cas9 was used to replace these residues with N129I and D130N mutations, preventing viral interaction.
- **Establishing Gene-Edited (GE) Chickens:** The modified genes were introduced into primordial germ cells (PGCs), which were then used to create fertile GE chickens through sterile surrogate hosts.

Assessing the Resistance of Gene-Edited Chickens
To evaluate the effectiveness of the genetic modifications, researchers conducted controlled AI infection experiments:

1. **Low-Dose Infection (10^3 PFU of H9N2-UDL virus)**
   - 9 out of 10 GE chickens remained uninfected.
   - No onward virus transmission occurred within the GE group.
   - In contrast, all wild-type (WT) chickens became infected and transmitted the virus to sentinels.

2. **High-Dose Infection (10^6 PFU of H9N2-UDL virus)**
   - 5 out of 10 GE chickens exhibited low-level, transient virus shedding.
   - WT chickens had robust infections with significant viral shedding.
   - Only one WT sentinel housed with GE birds acquired infection, indicating minimal transmission.

The Challenge of Viral Escape Mutations
Although the genetic modification significantly reduced AI susceptibility, some GE chickens still experienced breakthrough infections at high viral loads.

Sequencing these escape viruses revealed adaptive mutations in the viral polymerase genes, such as PA-E349K and PB2-M631L. These mutations allowed the virus to:

- Utilize the edited ANP32A protein.
- Adapt to use ANP32B and ANP32E, despite their usual inefficiency in supporting viral replication.
- Enhance polymerase activity in human ANP32A and B, raising concerns about potential zoonotic adaptation.

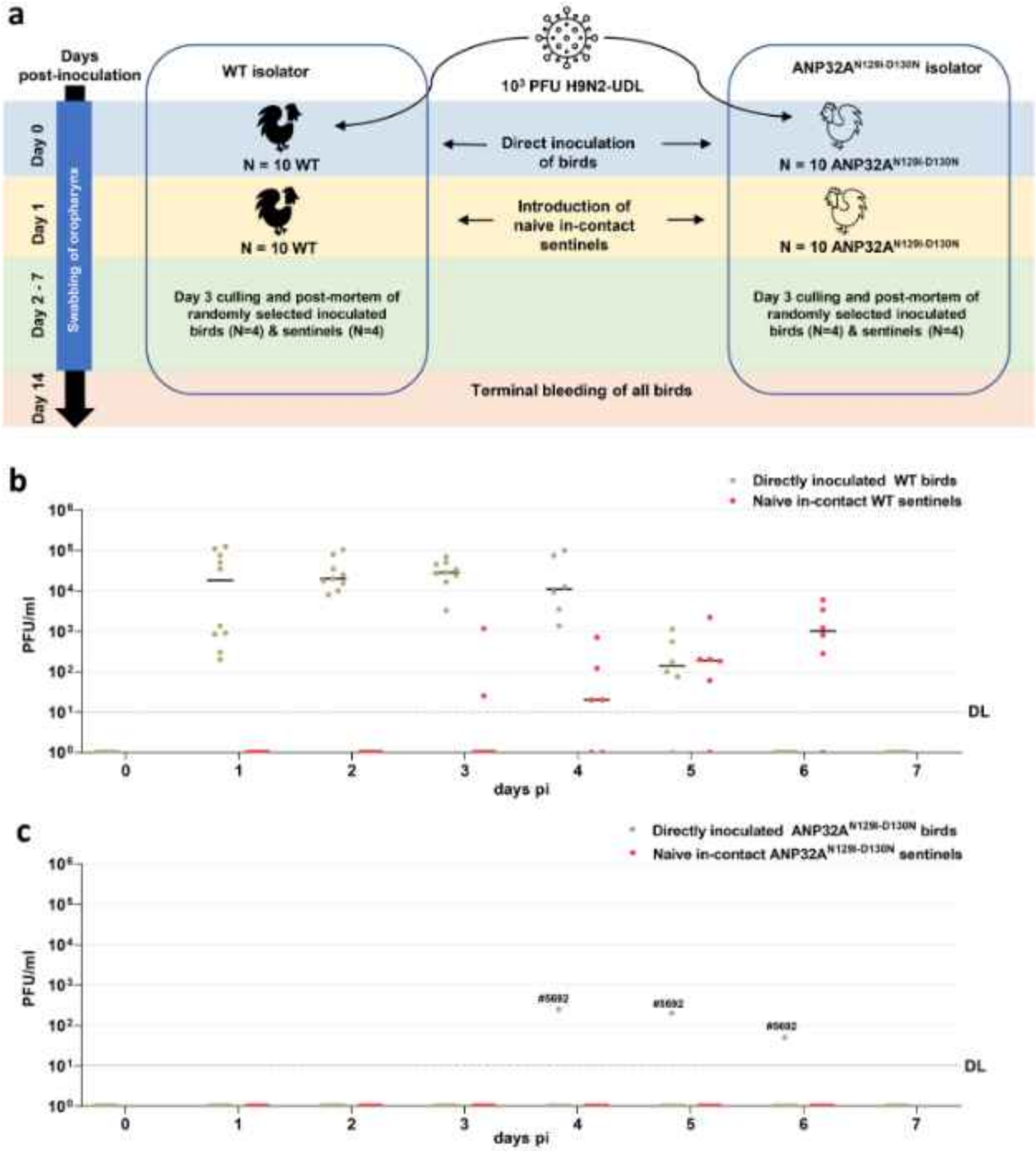

Figure 02: Assessment of low-dose IAV infection in ANP32A$^{N129I-D130N}$ chickens. a Schematic of low-dose in vivo challenge of 2-week-old chickens with H9N2-UDL influenza A virus (A/chicken/Pakistan/UDL01/08). Chickens were housed in negative pressure poultry isolators. Prior to challenge all birds were bled from the wing vein to obtain pre-infection sera. Groups of ten WT (black) chickens or ten ANP32A$^{N129I-D130N}$ (white) chickens were intranasally inoculated with 1×10^3 PFU of H9N2-UDL virus per bird. Uninoculated sentinel chickens were introduced into the isolators 24 h post infection to assess for transmission from the directly inoculated birds. Oropharyngeal cavities of each bird were swabbed daily from the day of inoculation (D0) until day 7 (D7) post-inoculation. Infectious virus titre in swabs was measured by plaque assay on MDCK cells (**b, c**). **c** Bird ID number for directly inoculated ANP32A$^{N129I-D130N}$ birds above the detection limit is indicated. DL detection limit of 10 PFU/ml for plaque assay.

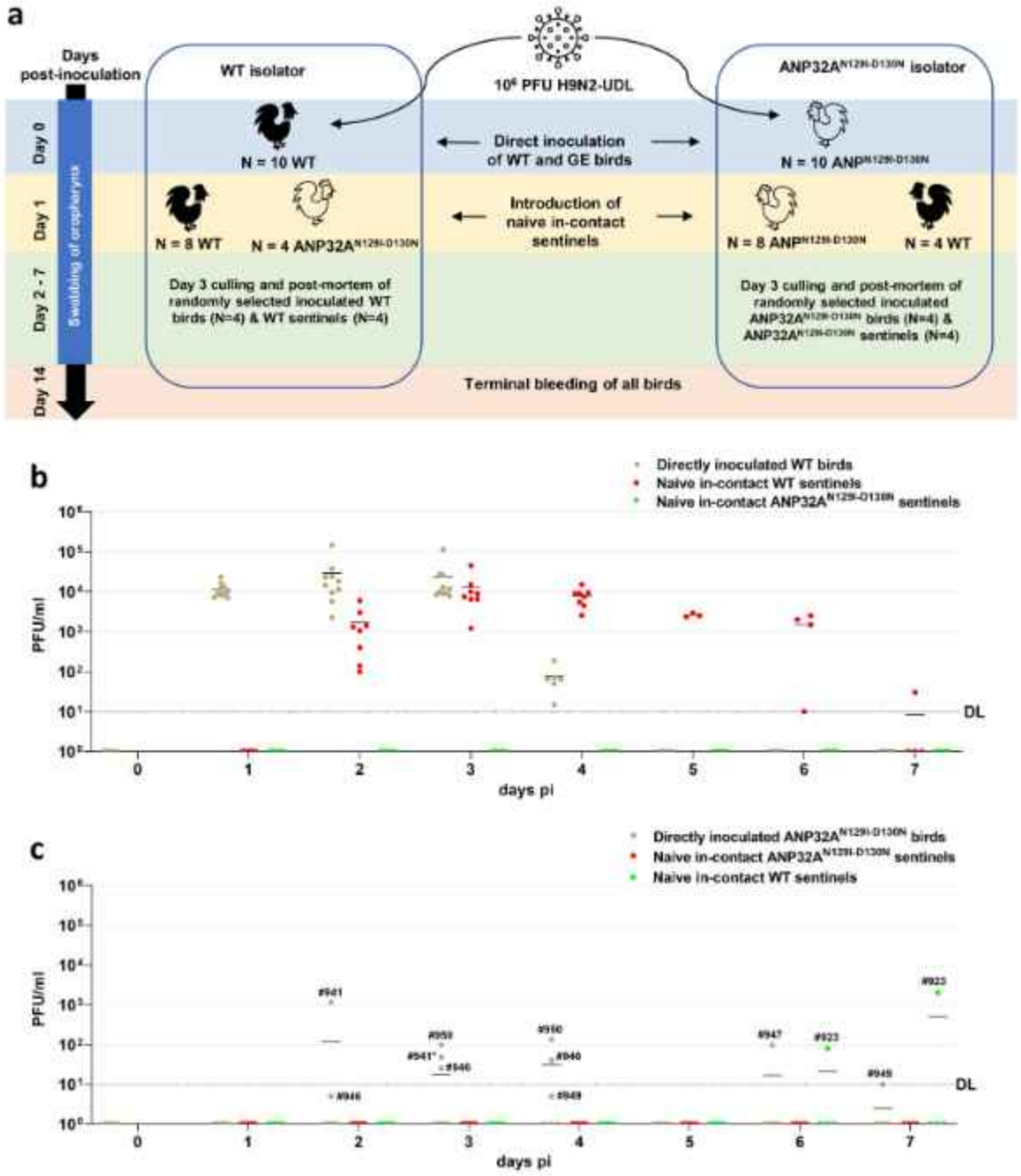

Figure 3: Assessment of high-dose IAV infection in ANP32A$^{N129I-D130N}$ chickens. a Schematic of high-dose in vivo challenge of 2-week-old chickens with H9N2-UDL influenza A virus (A/chicken/Pakistan/UDL01/08). Chickens were housed in negative pressure poultry isolators. Prior to challenge all birds were bled from the wing vein to obtain pre-infection sera. Groups of ten WT (black) chickens or ten ANP32A$^{N129I-D130N}$ (white) chickens were intranasally inoculated with 1×10^6 PFU of H9N2-UDL virus per bird. Uninoculated naive sentinel chickens were introduced into the isolators 24 h post challenge (day 1 pi) to assess for transmission from the directly inoculated birds. Oropharyngeal cavities of each bird were swabbed daily from the day of inoculation (day 0) until day 7 post inoculation. Infectious virus titre in swabs was measured by plaque assay (**b, c**). **c** Bird ID number for plaque-positive directly inoculated ANP32A$^{N129I-D130N}$ birds are indicated. Bird #941 was one of four directly inoculated ANP32A$^{N129I-D130N}$ birds culled on day 3 pi for post-mortem examination. DL detection limit of 10 PFU/ml for plaque assay.

Expanding Genetic Edits for Full Resistance

To further block viral escape, researchers developed additional genome modifications:

- **ANP32A Knockout (KO) Chickens:** Complete deletion of ANP32A reduced but did not entirely eliminate AI replication.
- **Triple Knockout (TKO) Chickens (ANP32A, ANP32B, ANP32E KO):**
    - No viral polymerase activity was detected in TKO chicken cells.
    - No viral replication occurred, even in the presence of adapted AI strains.

These findings highlight that complete resistance to AI requires eliminating all three ANP32 proteins to prevent viral adaptation.

Implications for the Poultry Industry and Public Health
The development of AI-resistant chickens has profound implications:

- **Economic Benefits:** Preventing AI outbreaks can save billions in losses from poultry culling, trade bans, and production declines.
- **Improved Food Security:** AI-resistant chickens ensure stable poultry production, benefiting global nutrition.
- **Pandemic Prevention:** Blocking AI replication in birds reduces the risk of zoonotic spillover into humans.

However, ethical, regulatory, and environmental considerations must be addressed before commercial deployment. Public perception of genetically modified animals remains mixed, and rigorous safety evaluations will be necessary to gain widespread acceptance.

Ethical Considerations and Public Perception
The idea of genetically modified livestock is often met with skepticism. Consumer concerns about food safety, animal welfare, and unforeseen ecological consequences must be addressed through transparency, education, and regulatory frameworks. Ensuring public trust in genome-edited poultry will require clear communication of benefits, risks, and long-term safety studies.

Conclusion: The Road Ahead for AI-Resistant Chickens
Genome editing presents a revolutionary strategy for tackling AI in poultry farming. By modifying the ANP32 gene family, scientists have demonstrated a viable pathway toward creating AI-resistant chickens. While additional refinements are needed to prevent viral escape mutations, this approach marks a major step forward in sustainable disease control.

As research continues, collaborative efforts between scientists, policymakers, and poultry producers will be essential to ensuring responsible adoption. If implemented successfully, AI-resistant chickens could transform the poultry industry, protect global food supplies, and mitigate future pandemic risks.

The Future of Disease-Resistant Livestock
Beyond poultry, genome editing offers opportunities to develop disease-resistant livestock across various species. Research is already underway to create pigs resistant to swine flu and cattle resistant to tuberculosis. These advancements could redefine how we approach livestock health, sustainability, and global food security.

Reference:
1. Idoko-Akoh, A., Goldhill, D.H., Sheppard, C.M. *et al.* Creating resistance to avian influenza infection through genome editing of the ANP32 gene family. *Nat Commun* **14**, 6136 (2023). https://doi.org/10.1038/s41467-023-41476-3

Transposon-Mediated Genome Editing: Concept, Mechanisms, Methods, and Applications

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Introduction

Transposable elements (TEs) are segments of DNA that have the remarkable ability to move from their original position and insert themselves into new locations within the genome. TEs are present in nearly all eukaryotic genomes, with around half of the human genome and over 80% of large plant genomes comprised of these elements. The most common types of TEs are retrotransposons, also known as class I elements, which utilize an RNA intermediate for their propagation. On the other hand, DNA transposons, or class II elements, relocate through a cut-and-paste mechanism involving DNA. When TEs insert themselves into genes, they often cause damage to those genes. As a result, both host cells and transposable elements have a shared interest in minimizing the frequency of transpositions. Transposon-mediated genome editing is an advanced molecular biology approach that utilizes mobile genetic elements, known as transposons, to modify the genetic material of an organism. Transposons, or "jumping genes," can move from one genomic location to another, facilitating DNA insertions, deletions, and modifications. This natural ability makes transposons a powerful tool for genome engineering, with applications in functional genomics, gene therapy, and biotechnology. Transposons have been extensively studied in various organisms, from bacteria to humans, revealing their role in genome evolution and genetic diversity. The ability to harness transposon activity for genome editing has revolutionized molecular biology, offering a highly efficient and cost-effective alternative to other gene-editing technologies.

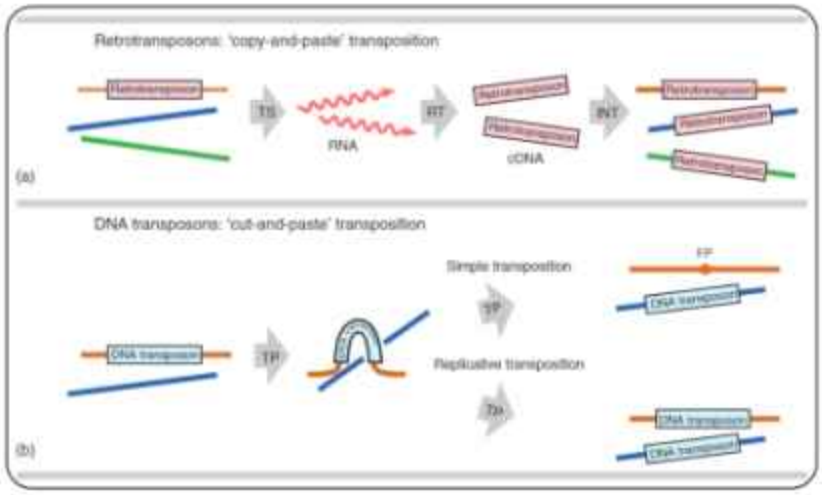

Figure 1: The two major classes of eukaryotic transposons. (a) Retrotransposons (also termed class I elements) transpose by a 'copy-and-paste' mechanism. In the first mobilization step, they are transcribed into RNA (TS), followed by reverse transcription into cDNA (RT) and integration of the cDNA (INT) into novel positions in the genome (blue and green lines). (b) DNA transposons (class II elements) move by a 'cut-and-paste' mechanism. During transposition (TP), the transposon ends associated with a target DNA (blue line), are excised from the donor site and reintegrated into the target DNA, followed by ligation of the donor-site ends. In a simple transposition reaction, the two donor site ends are sealed by nonhomologous end joining (NHEJ) which typically results in a 'footprint' (FP) with small mutations at the fusion site. More rarely, the excision site can also be repaired by homology-dependent gap repair using the homologous chromosome in diploid cells as template, resulting in replicative transposition. Helitrons, a subclass of DNA transposons, move by a different transposition mechanism that always results in replicative transposition.

Concept of Transposon-Mediated Genome Editing

Transposons are DNA sequences capable of self-mobilization within the genome through a cut-and-paste or copy-and-paste mechanism. This mobility is mediated by transposase enzymes that recognize specific DNA sequences and facilitate transposition events. The concept of transposon-mediated genome editing involves harnessing this natural ability to manipulate genomes for research and therapeutic purposes. Key aspects of this concept include:

- **Gene Insertion**: Using transposons to introduce desired genes into a target genome.
- **Gene Disruption**: Inactivating specific genes by inserting transposon sequences.
- **Targeted Mutagenesis**: Creating random or site-directed mutations using transposon-based systems.
- **Genome Labeling**: Tagging genes with transposon markers to study gene expression and function.
- **Site-Specific Integration**: Engineering transposon systems to target precise genomic loci, reducing off-target effects.

Mechanisms of Transposon-Mediated Genome Editing

Transposon-mediated genome editing involves several key mechanisms, primarily categorized into two major classes:

1. DNA Transposons (Cut-and-Paste Mechanism)

DNA transposons move by excising themselves from one location and integrating into another within the genome. This process involves:

1. **Recognition of Terminal Inverted Repeats (TIRs):** Transposase enzymes bind to specific sequences at the ends of the transposon.
2. **Excision:** The transposase cuts the transposon out of its original location.
3. **Integration:** The transposon is inserted into a new genomic site, often creating short duplications of target site sequences.

Examples of DNA transposons used in genome editing include **Sleeping Beauty (SB)** and **PiggyBac (PB)** transposon systems. These systems are widely used in genetic research due to their efficiency and relative safety compared to viral-based gene delivery systems.

2. Retrotransposons (Copy-and-Paste Mechanism)

Retrotransposons use an RNA intermediate to mobilize and integrate into new genomic sites. This process involves:

1. **Transcription:** The retrotransposon is transcribed into RNA.
2. **Reverse Transcription:** The RNA is converted back into DNA by reverse transcriptase.
3. **Integration:** The newly synthesized DNA is inserted into the genome by an integrase enzyme.

Retrotransposons include **Long Interspersed Nuclear Elements (LINEs)** and **Short Interspersed Nuclear Elements (SINEs).** These elements have played a crucial role in genome evolution and are being explored for potential genome engineering applications.

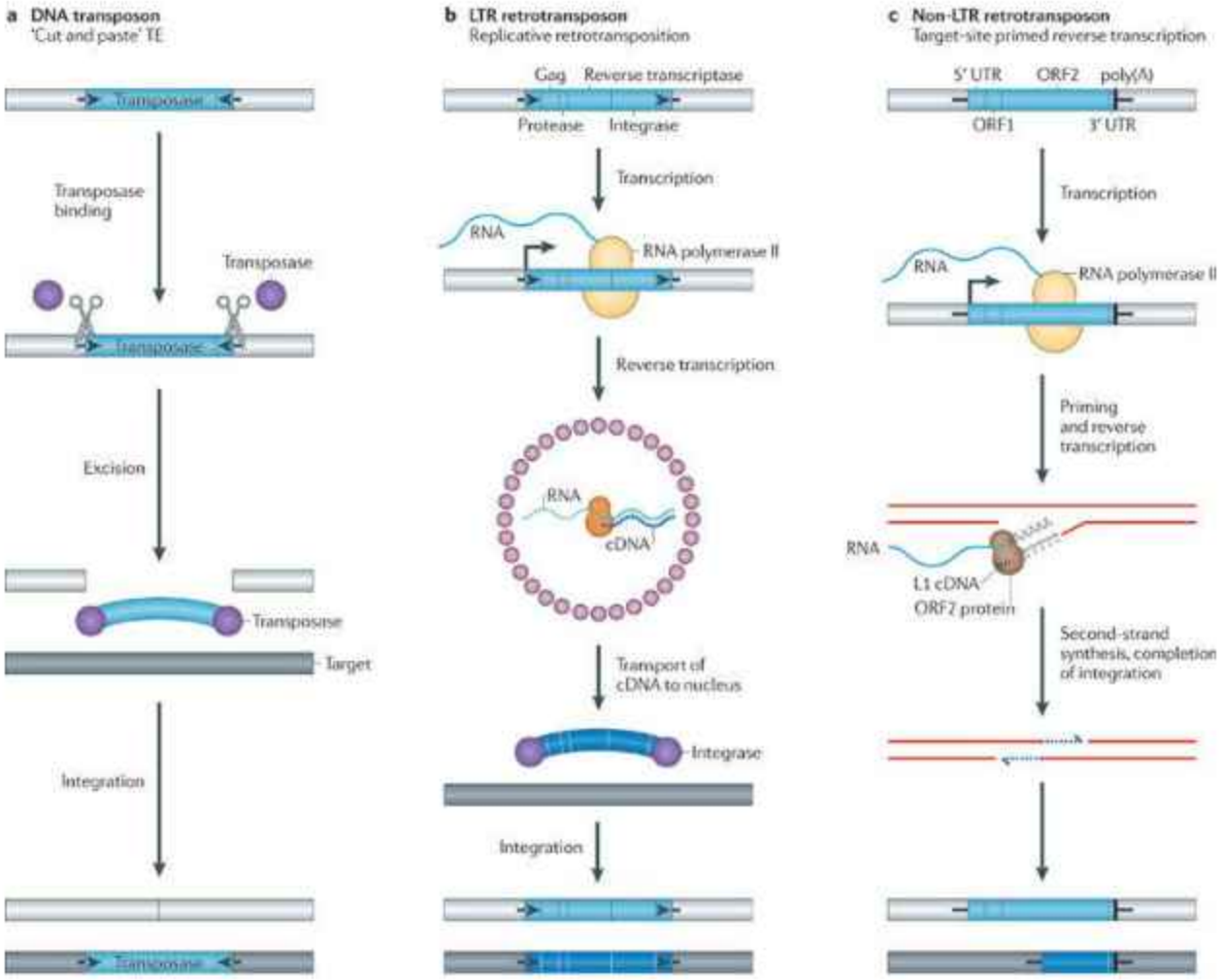

Figure 2: The diverse mechanisms of transposon mobilization.a | DNA transposons. Many DNA transposons are flanked by terminal inverted repeats (TIRs; black arrows), encode a transposase (purple circles), and mobilize by a 'cut and paste' mechanism (represented by the scissors). The transposase binds at or near the TIRs, excises the transposon from its existing genomic location (light grey bar) and pastes it into a new genomic location (dark grey bar). The cleavages of the two strands at the target site are staggered, resulting in a target-site duplication (TSD) typically of 4–8 bp (short horizontal black lines flanking the transposable element (TE)) as specified by the transposase. Retrotransposons (b and c) mobilize by replicative mechanisms that require the reverse transcription of an RNA intermediate. b | LTR retrotransposons contain two long terminal repeats (LTRs; black arrows) and encode Gag, protease, reverse transcriptase and integrase activities, all of which are crucial for retrotransposition. The 5′ LTR contains a promoter that is recognized by the host RNA polymerase II and produces the mRNA of the TE (the start-site of transcription is indicated by the right-angled arrow). In the first step of the reaction, Gag proteins (small pink circles) assemble into virus-like particles that contain TE mRNA (light blue), reverse transcriptase (orange shape) and integrase. The reverse transcriptase copies the TE mRNA into a full-length dsDNA. In the second step, integrase (purple circles) inserts the cDNA (shown by the wide, dark blue arc) into the new target site. Similarly to the transposases of DNA transposons, retrotransposon integrases create staggered cuts at the target sites, resulting in TSDs. c | Non-LTR retrotransposons lack LTRs and encode either one or two ORFs. As for LTR retrotransposons, the transcription of non-LTR retrotransposons generates a full-length mRNA (wavy, light blue line). However, these elements mobilize by target-site-primed reverse transcription (TPRT). In this mechanism, an element-encoded endonuclease generates a single-stranded 'nick' in the genomic DNA, liberating a 3′-OH that is used to prime reverse transcription of the RNA. The proteins that are encoded by autonomous non-LTR retrotransposons can also mobilize non-autonomous retrotransposon RNAs, as well as other cellular RNAs (see the main text). The TPRT mechanism of a long interspersed element 1 (L1) is depicted in the figure; the new element (dark blue rectangle) is 5′ truncated and is retrotransposition-defective. Some non-LTR retrotransposons lack poly(A) tails at their 3′ ends. The integration of non-LTR retrotransposons can lead to TSDs or small deletions at the target site in genomic DNA. For example, L1s are generally flanked by 7–20 bp TSDs.

Methods of Transposon-Mediated Genome Editing

Several methods have been developed to utilize transposons for genome engineering, including:

1. Sleeping Beauty (SB) Transposon System

- Derived from fish transposons and re-engineered for high activity in mammalian cells.

- Uses the **SB transposase** enzyme to mobilize transposons.
- Efficient for stable gene integration and insertional mutagenesis.
- Applications include gene therapy, stem cell research, and cancer studies.

2. PiggyBac (PB) Transposon System

- Originally identified in insects and adapted for use in various organisms.
- Uses a **PB transposase** enzyme to mediate precise insertions and excisions without leaving residual sequences.
- Advantageous for reversible genome modifications.
- Used in stem cell research, regenerative medicine, and drug discovery.

3. Tol2 Transposon System

- Derived from zebrafish and widely used for gene transfer in vertebrates.
- Mediates stable and efficient gene insertions.
- Often used in transgenic animal models and functional genomics.

4. CRISPR-Transposon Hybrid Systems

- Combines CRISPR/Cas9 for targeted DNA cleavage with transposase-mediated integration.
- Allows site-specific transposon insertions, improving precision in genome editing.
- Examples include **CRISPR-associated transposases (CASTs)** and **prime editing with transposon components.**
- Holds potential for therapeutic applications requiring precise gene insertions without the risks of random integration.

Applications of Transposon-Mediated Genome Editing

1. Functional Genomics

- Enables gene disruption and insertional mutagenesis to study gene function.
- Facilitates identification of essential genes and regulatory elements.

2. Gene Therapy

- Used to deliver therapeutic genes into patient cells for treating genetic disorders.
- Applied in conditions such as hemophilia, immune deficiencies, and certain cancers.
- Sleeping Beauty and PiggyBac are being explored in clinical trials for gene therapy applications.
- Potential for treating inherited diseases by correcting defective genes at the genomic level.

3. Generation of Transgenic Organisms

- Allows stable gene integration in animal models.
- Used in agricultural biotechnology for developing genetically modified crops and livestock.
- Enhances traits such as disease resistance, yield improvement, and stress tolerance.

4. Drug Discovery and Disease Modeling

- Creates disease models by inserting or disrupting disease-related genes in cells and animals.
- Used to test new drugs and therapies.
- Aids in understanding genetic contributions to disease susceptibility.

5. Regenerative Medicine

- Facilitates reprogramming of cells for regenerative applications.
- PiggyBac has been used to generate induced pluripotent stem cells (iPSCs) for tissue regeneration.
- Holds promise for developing personalized medicine strategies.

Challenges and Future Directions

Despite its potential, transposon-mediated genome editing faces several challenges:

- **Insertional Mutagenesis:** Random insertions can disrupt essential genes or regulatory elements.
- **Efficiency and Targeting:** Traditional transposon systems have low target specificity.
- **Immune Response:** Host immune reactions against transposases can limit therapeutic applications.
- **Off-Target Effects:** Risk of unintended genomic alterations.

Future developments aim to:

- Improve site-specific integration using CRISPR-transposon hybrid systems.
- Engineer more precise and safer transposase enzymes.
- Develop strategies to minimize insertional mutagenesis.
- Enhance clinical applications for gene therapy and personalized medicine.
- Expand applications in synthetic biology and genome engineering.

Conclusion

Transposon-mediated genome editing is a powerful and versatile tool in modern biotechnology. It enables gene insertions, disruptions, and modifications with applications spanning functional genomics, gene therapy, and regenerative medicine. Although challenges such as insertional mutagenesis and targeting efficiency remain, advances in hybrid genome editing systems and engineered transposases promise to make this technology more precise and clinically relevant. The future of transposon-based genome editing lies in its integration with cutting-edge molecular tools, opening new avenues for scientific and medical breakthroughs.

Epigenome Editing: Concept, Mechanisms, Methods and Applications

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Epigenome editing or epigenome engineering is a type of genetic engineering in which the epigenome is modified at specific sites using engineered molecules targeted to those sites (as opposed to whole-genome modifications). Whereas gene editing involves changing the actual DNA sequence itself, epigenetic editing involves modifying and presenting DNA sequences to proteins and other DNA binding factors that influence DNA function. By "editing" epigenomic features in this manner, researchers can determine the exact biological role of an epigenetic modification at the site in question.

The engineered proteins used for epigenome editing are composed of a DNA binding domain that target specific sequences and an effector domain that modifies epigenomic features. Currently, three major groups of DNA binding proteins have been predominantly used for epigenome editing: Zinc finger proteins, Transcription Activator-Like Effectors (TALEs) and nuclease deficient Cas9 fusions (CRISPR).

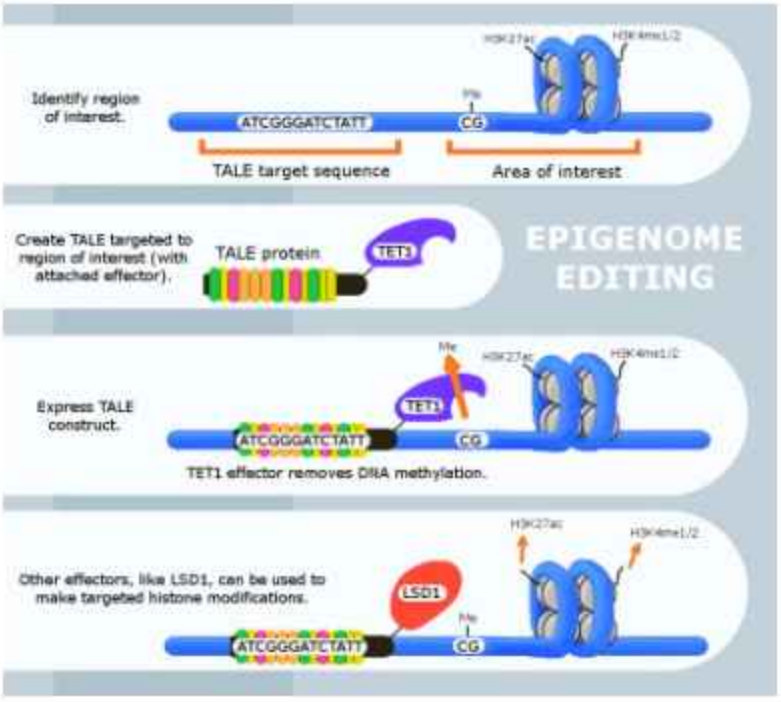

Figure: A visual overview of how TALE proteins are used for epigenome editing

General concept

Comparing genome-wide maps of epigenetic modifications with gene expression patterns has enabled researchers to attribute specific roles to these modifications, either activating or repressing gene activity. The significance of DNA sequence in governing the epigenome has been demonstrated by utilizing DNA motifs to predict the occurrence of epigenetic modifications. Further understanding of the mechanisms underlying epigenetics has been gained through biochemical and structural analyses conducted in vitro.

By employing model organisms, researchers have elucidated the functions of numerous chromatin factors through knockout experiments. However, disrupting an entire chromatin modifier can have widespread effects on the genome, which may not accurately reflect its function in a specific biological context. For instance, DNA methylation occurs at various genomic regions, including repeat sequences, promoters, enhancers, and gene bodies. While methylation at gene promoters often leads to gene silencing, methylation within gene bodies is associated with gene activation, and DNA methylation can also influence gene splicing.

The ability to precisely target and modify individual methylation sites is crucial for understanding the precise role of DNA methylation at specific loci. Epigenome editing provides a powerful tool for such analyses. Systems for editing site-specific DNA methylation, as well as histone modifications, have been developed by adapting genome editing technologies into epigenetic editing systems.

In essence, genome-targeting proteins with modified or natural nuclease functions for gene editing can be repurposed and engineered into delivery systems. These systems allow for the fusion of epigenetic modifying enzymes or domains with the targeting proteins, enabling the alteration of local epigenetic modifications upon recruitment of the modified proteins.

Targeting proteins

TALE

The Transcription Activator-Like Effector (TALE) protein possesses a unique ability to recognize specific DNA sequences determined by the composition of its DNA binding domain. This characteristic enables researchers to engineer various TALE proteins tailored to recognize specific target DNA sequences by manipulating the primary protein structure of the TALE. Typically, the binding specificity of these engineered proteins is validated using Chromatin Immunoprecipitation (ChIP) followed by Sanger sequencing of the resulting DNA fragment. Confirmation through ChIP and sequencing remains a standard requirement for all TALE sequence recognition studies. In epigenome editing applications, these DNA binding proteins are fused with effector proteins. Effector proteins utilized for this purpose include Ten-eleven translocation methylcytosine dioxygenase 1 (TET1), Lysine (K)-specific demethylase 1A (LSD1), and Calcium and integrin binding protein 1 (CIB1).

Zinc finger proteins

Researchers have explored the use of zinc finger-fusion proteins for epigenome editing purposes. Maeder et al. developed a ZF-TET1 protein capable of inducing DNA demethylation, similar to the approach with TALE proteins. These zinc finger proteins function by binding to specific DNA sequences based on their protein structure, which can be engineered to recognize desired sites. Chen et al. demonstrated successful demethylation of previously silenced genes by employing a zinc finger DNA binding domain coupled with the TET1 protein. Additionally, Kungulovski and Jeltsch achieved gene silencing through ZFP-guided deposition of DNA methylation. However, they observed that the DNA methylation and resultant gene silencing were reversible upon cessation of the trigger signal. To ensure stable epigenetic changes, the authors suggest the need for multiple depositions of DNA methylation or prolonged trigger stimuli. ZFP epigenetic editing holds promise for treating various neurodegenerative diseases, as evidenced by its potential demonstrated in recent studies.

CRISPR-Cas

The Clustered Regulatory Interspaced Short Palindromic Repeat (CRISPR)-Cas system serves as a DNA site-specific nuclease. In the extensively studied type II CRISPR system, the Cas9 nuclease forms a complex with a chimera consisting of tracrRNA and crRNA, collectively known as a guide RNA (gRNA). When the Cas9 protein binds to a DNA region-specific gRNA, it cleaves the DNA at targeted loci. However, introducing point

mutations D10A and H840A generates a catalytically-dead Cas9 (dCas9) variant, which retains the ability to bind to DNA but lacks cleavage activity. The dCas9 system has been harnessed for targeted epigenetic reprogramming, facilitating site-specific DNA methylation by fusing the DNMT3a catalytic domain with dCas9. This fusion protein, dCas9-DNMT3a, enables precise DNA methylation at targeted regions specified by the guide RNA. Similarly, dCas9 has been fused with the catalytic core of the human acetyltransferase p300, resulting in dCas9-p300, which catalyzes targeted acetylation of histone H3 lysine 27. A CRISPR variant known as FIRE-Cas9 enables the reversal of epigenetic modifications, providing a mechanism to undo changes if needed. Additionally, CRISPRoff involves a dead Cas9 fusion protein capable of heritably silencing the expression of "most genes," offering reversible modifications for gene regulation purposes.

Commonly used effector proteins

TET1 enzyme initiates the process of demethylation of cytosine residues at CpG sites. Researchers have employed TET1 to activate genes that are typically repressed by CpG methylation, shedding light on the significance of individual CpG methylation sites in gene regulation. LSD1 enzyme catalyzes the demethylation of histone H3 at lysine 4 (H3K4me1/2), consequently leading to an indirect deacetylation effect on histone H3 at lysine 27 (H3K27). This effector is particularly effective in enhancer regions of chromatin, influencing the expression levels of neighboring genes. CIB1, a light-sensitive cryptochrome, is fused with the TALE protein for targeted epigenetic editing. Another protein comprises an interaction partner, CRY2, fused with a chromatin/DNA modifier (e.g., SID4X). Upon activation by blue light illumination, CIB1 interacts with CRY2, facilitating the recruitment of the chromatin modifier to the desired genomic location. This inducible and reversible modification approach allows for precise control over epigenetic changes, minimizing potential long-term secondary effects associated with constitutive epigenetic modifications.

Applications

Studying enhancer function and activity

A group of Researchers demonstrated the editing of gene enhancer regions in the genome through targeted epigenetic modification. Their study utilized a fusion protein combining a TALE (Transcription Activator-Like Effector) domain with LSD1 (Lysine-specific demethylase 1), referred to as a TALE-LSD1 effector. This fusion protein was employed to target enhancer regions of genes, aiming to induce enhancer silencing to elucidate enhancer activity and its impact on gene control. By targeting specific enhancers and inducing their silencing, the researchers were able to deduce the activity of these enhancers and their influence on gene regulation. Locus-specific RT-qPCR (Reverse Transcription quantitative Polymerase Chain Reaction) was employed to determine the genes affected by the silenced enhancer. Alternatively, inducing silencing of enhancers located upstream of genes enabled the alteration of gene expression. Subsequent RT-qPCR analysis allowed for the examination of the effects on gene expression, thereby facilitating a detailed study of enhancer function and activity.

Determining the function of specific methylation sites

Understanding the role of specific methylation sites in regulating gene expression is crucial. To investigate this, a research group utilized a TALE-TET1 fusion protein to demethylate a single CpG methylation site. While this method necessitates numerous controls to ensure specific binding to target loci, a meticulously conducted study employing this approach can elucidate the biological function of a particular CpG methylation site.

Determining the role of epigenetic modifications directly

Epigenetic editing utilizing an inducible mechanism presents a versatile approach for studying epigenetic effects across different cellular states. In one study, a research group utilized an optogenetic two-hybrid system that combined the sequence-specific TALE DNA-binding domain with a light-sensitive cryptochrome 2 protein

(CIB1). Upon expression in cells, this system enabled the inducible editing of histone modifications, allowing researchers to determine their function within a specific cellular context.

Functional engineering

Targeted regulation of disease-related genes holds promise for advancing therapeutic interventions across a wide range of diseases, particularly in scenarios where adequate gene therapies are not yet available or are not suitable. Although the long-term and population-level effects of this approach are not yet fully understood, it has the potential to emerge as a significant tool in applied functional genomics and personalized medicine. Similar to RNA editing, epigenome editing does not entail genetic alterations and their associated risks. An exemplar of the practical application of epigenome editing emerged in 2021, where the repression of Nav1.7 gene expression via CRISPR-dCas9 demonstrated therapeutic potential in three mouse models of chronic pain. This discovery underscores the therapeutic promise of epigenome editing in addressing complex diseases. In 2022, research endeavors focused on evaluating its efficacy in various disease contexts, including reducing tau protein levels, modulating a protein implicated in Huntington's disease, targeting an inherited form of obesity, and addressing Dravet syndrome. These investigations highlight the versatility and potential of epigenome editing in therapeutic interventions, marking significant strides toward the development of innovative treatments for diverse medical conditions.

Limitations

Sequence specificity is paramount in epigenome editing and requires meticulous verification. Chromatin immunoprecipitation followed by Sanger sequencing is commonly employed to confirm the targeted sequence accuracy. However, it remains uncertain whether TALE fusion proteins might influence the catalytic activity of the epigenome modifier, particularly in cases involving complex effector proteins like the Polycomb repressive complex. Additionally, proteins utilized for epigenome editing may obstruct ligands and substrates at the target site. There's also the potential for competition with transcription factors if they are targeted to the same sequence. Furthermore, DNA repair systems could potentially reverse chromatin alterations, hindering the intended changes. To ensure reliable and reproducible epigenome editing, fusion constructs and targeting mechanisms must be carefully optimized.

Mechanisms of Epigenome editing

Epigenome editing involves modifying the chemical tags and proteins that regulate gene expression without altering the underlying DNA sequence. There are several mechanisms through which epigenome editing can be achieved:

DNA Methylation Editing: DNA methylation involves adding a methyl group to cytosine nucleotides, typically at CpG dinucleotides. This modification is associated with gene silencing. Epigenome editing techniques like CRISPR-based tools can be used to target specific DNA methyltransferases (DNMTs) to either add or remove methyl groups from specific regions of the genome, thereby altering gene expression patterns.

Histone Modification Editing: Histones are proteins around which DNA is wrapped, forming nucleosomes. Post-translational modifications of histones, such as acetylation, methylation, phosphorylation, and ubiquitination, play crucial roles in regulating chromatin structure and gene expression. Epigenome editing approaches can target histone-modifying enzymes (e.g., histone acetyltransferases, histone methyltransferases) to specific genomic loci to alter histone modifications and thereby regulate gene expression.

Chromatin Remodeling: Chromatin remodeling complexes use the energy from ATP hydrolysis to slide, eject, or restructure nucleosomes, thereby altering the accessibility of DNA to transcription factors and other regulatory proteins. Epigenome editing strategies may involve targeting these complexes to specific genomic regions to modulate gene expression.

RNA-Mediated Epigenome Editing: Non-coding RNAs, such as microRNAs and long non-coding RNAs, can influence gene expression by recruiting epigenetic modifiers to specific genomic loci. Epigenome editing techniques can involve the use of synthetic RNA molecules (e.g., guide RNAs in CRISPR systems) to target epigenetic modifiers or non-coding RNA scaffolds to modify chromatin states at specific genomic sites.

Base Editing: While not strictly an epigenome editing technique, base editing technologies (e.g., CRISPR base editors) can induce precise nucleotide changes in the genome without introducing double-strand breaks. Base editors can be engineered to convert specific DNA bases (e.g., cytosine to thymine) without altering the surrounding epigenetic landscape. However, unintended epigenetic changes may occur as a result of base editing, and careful consideration is needed to minimize off-target effects.

These mechanisms represent the current strategies for epigenome editing, each with its advantages and limitations. The precise manipulation of epigenetic marks holds promise for understanding fundamental biological processes and developing novel therapeutic approaches for various diseases.

Methods of Epigenome editing:

Epigenome editing involves manipulating the chemical modifications and protein structures that regulate gene expression without altering the underlying DNA sequence. Here's a step-by-step overview of some common methods used in epigenome editing:

Design Guide RNA or Targeting Molecule:

- For CRISPR-based epigenome editing, design guide RNA (gRNA) sequences that specifically target the desired genomic locus.
- Alternatively, design synthetic DNA-binding proteins (e.g., zinc finger proteins, TALEs) or programmable epigenetic effectors (e.g., fusion proteins containing DNA-binding domains and epigenetic modifiers) that recognize and bind to the target region.

Delivery of Epigenome Editing Components:

- Introduce the designed guide RNA or targeting molecule along with the effector domain (e.g., histone modifier, DNA methyltransferase) into the target cells.
- Delivery methods vary depending on the system used and the target cell type, including transfection, viral vectors, or physical methods like electroporation.

Localization to Target Locus:

- Ensure proper localization of the epigenome editing components to the target genomic region.
- For CRISPR-based systems, guide RNA directs the CRISPR effector protein (e.g., Cas9) to the specific DNA sequence, while other systems rely on the DNA-binding specificity of engineered proteins.

Modifying Epigenetic Marks:

- Activate or suppress gene expression by introducing or removing specific epigenetic marks (e.g., DNA methylation, histone modifications) at the target locus.
- For DNA methylation editing, recruit DNA methyltransferases (DNMTs) or demethylases (e.g., TET enzymes) to add or remove methyl groups from cytosine residues.
- For histone modification editing, recruit histone-modifying enzymes (e.g., histone acetyltransferases, histone methyltransferases) or histone demethylases to modify histone tails.

Validation and Characterization:

- Validate the efficacy and specificity of epigenome editing at the target locus.
- Analyze changes in gene expression levels using techniques such as RT-qPCR, RNA sequencing, or protein expression assays.

- Assess alterations in epigenetic marks using chromatin immunoprecipitation (ChIP) followed by sequencing (ChIP-seq) or bisulfite sequencing for DNA methylation changes.

Off-Target Analysis:

- Evaluate potential off-target effects of epigenome editing by analyzing unintended modifications at genomic loci similar to the target site.
- Perform bioinformatics analyses, such as whole-genome sequencing or off-target prediction algorithms, to identify potential off-target sites.
- Validate off-target effects experimentally using targeted sequencing or functional assays.

Iterative Optimization:

- Refine the epigenome editing strategy based on the results obtained.
- Optimize guide RNA or targeting molecule design, delivery methods, and effector domain selection to enhance specificity, efficiency, and safety.

By following these steps, researchers can precisely manipulate the epigenome to modulate gene expression patterns, providing insights into gene regulation mechanisms and potential therapeutic interventions for various diseases.

Applications of epigenome editing in sustainable agriculture and livestock

Epigenome editing holds great promise for applications in sustainable agriculture and livestock breeding by offering novel strategies to improve crop yields, enhance stress tolerance, and optimize livestock traits. Here are some potential applications:

Crop Improvement:

Stress Tolerance: Epigenome editing can be used to enhance stress tolerance in crops, such as drought, salinity, or temperature stress. By modulating the expression of stress-responsive genes through targeted epigenetic modifications, crops can be engineered to thrive in challenging environments.

Nutrient Utilization: Manipulating epigenetic regulation can optimize nutrient uptake and utilization in crops, leading to improved nutrient efficiency and yield.

Disease Resistance: Epigenome editing can bolster plant immunity by activating defense-related genes or suppressing susceptibility factors, thereby enhancing resistance to pathogens and reducing the need for chemical pesticides.

Crop Domestication and Evolution:

Epigenome editing can recapitulate the changes that occurred during crop domestication, accelerating the breeding process to develop varieties with desirable traits, such as increased yield, improved flavor, or reduced toxicity.

By modulating epigenetic marks associated with flowering time, plant architecture, or fruit development, researchers can tailor crops to specific agricultural needs, contributing to sustainable farming practices.

Livestock Breeding:

Disease Resistance: Epigenome editing can enhance disease resistance in livestock by modulating the expression of immune-related genes or targeting susceptibility factors. This approach reduces the reliance on antibiotics and improves animal welfare.

Productivity and Efficiency: Manipulating epigenetic regulation can optimize growth rates, feed conversion efficiency, and reproductive performance in livestock species, leading to increased productivity and reduced environmental impact per unit of output.

Quality Traits: Epigenome editing can improve the quality of animal products, such as meat, milk, and wool, by modulating genes involved in nutrient composition, flavor, and texture.

Environmental Adaptation:

Epigenome editing offers opportunities to enhance environmental adaptation in livestock by increasing resilience to climate change-related stressors, such as heat stress or infectious diseases.

By targeting genes involved in thermoregulation, immune response, or metabolic efficiency, researchers can develop livestock breeds better suited to diverse environmental conditions, contributing to the resilience and sustainability of agricultural systems.

Epigenetic Conservation and Preservation:

Epigenome editing can aid in the conservation and preservation of endangered plant and animal species by mitigating the effects of inbreeding depression, enhancing genetic diversity, and promoting adaptive responses to changing environmental conditions.

Overall, epigenome editing has the potential to revolutionize agricultural and livestock breeding practices by providing innovative solutions to enhance sustainability, productivity, and resilience in the face of global challenges. However, careful consideration of ethical, regulatory, and societal implications is essential to ensure responsible and equitable deployment of these technologies.

Random Mutagenesis with Radiation or Chemicals: Concept, Mechanisms, Methods, and Applications

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Introduction

Mutagenesis refers to the process of inducing genetic mutations, which are changes in the DNA sequence of an organism. **Random mutagenesis** is a technique widely used in genetics, biotechnology, and evolutionary studies to create genetic diversity. Two of the most common approaches to inducing random mutations are the use of **radiation** and **chemical mutagens**. These methods cause DNA damage or alteration, leading to changes in gene function that can be harnessed for various applications, including genetic research, strain improvement, and therapeutic development.

This document explores the **concept, mechanisms, methods, and applications** of random mutagenesis using radiation and chemicals in detail.

Concept of Random Mutagenesis

Definition

Random mutagenesis is the process of introducing **uncontrolled** genetic mutations in an organism's genome through external mutagenic agents. Unlike site-directed mutagenesis, which targets specific DNA sequences, random mutagenesis affects multiple sites across the genome unpredictably.

Characteristics

1. **Induced DNA Alterations**: Mutagens induce various types of mutations such as point mutations, deletions, insertions, and chromosomal rearrangements.
2. **Diverse Mutagenic Agents**: Includes radiation (X-rays, UV, gamma rays) and chemical agents (EMS, MNNG, alkylating agents).
3. **Non-Specific Targeting**: Mutations occur randomly across the genome rather than at specific loci.
4. **Useful for Genetic Improvement**: Applied in microbial strain development, crop improvement, and cancer research.
5. **Potentially Harmful Effects**: May cause harmful mutations, leading to carcinogenesis or loss of function.

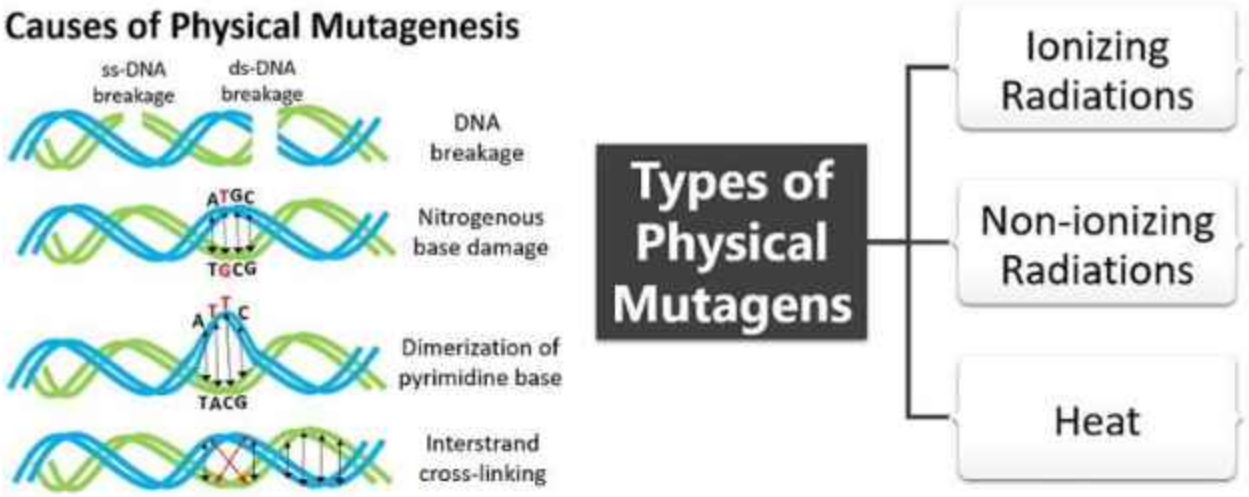
Mechanisms of Random Mutagenesis

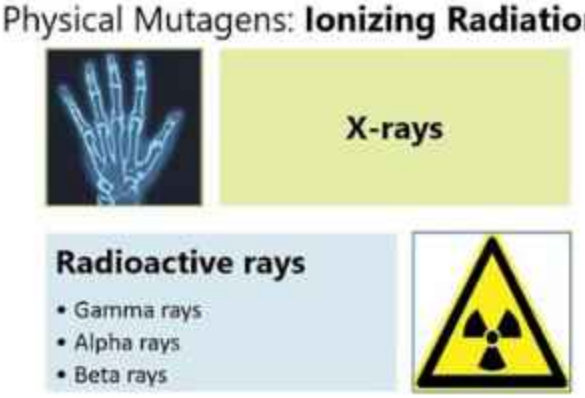
1. Radiation-Induced Mutagenesis

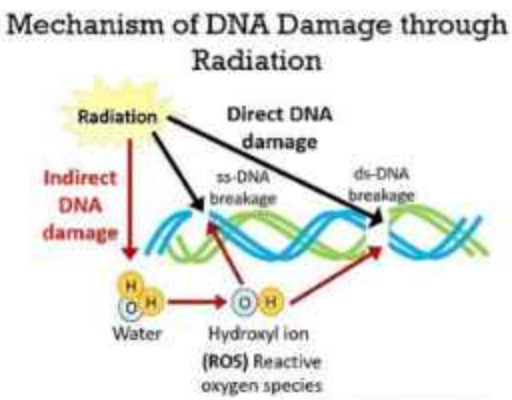
Radiation induces mutations by causing direct DNA damage or generating reactive molecules that alter DNA.

Types of Radiation and Their Effects

- **Ultraviolet (UV) Radiation**:
  - Causes **pyrimidine dimers** (T-T or C-C), leading to replication errors.
  - Leads to transitions and transversions during DNA replication.
- **X-Rays and Gamma Rays**:
  - Induce **double-strand breaks (DSBs)**, causing deletions, insertions, and chromosomal rearrangements.
  - Generate **free radicals**, which chemically modify DNA bases.
- **Ionizing Radiation**:
  - Causes **oxidative stress**, damaging nucleotide structures.
  - Leads to large genomic instabilities and translocations.







2. Chemical-Induced Mutagenesis

Chemical mutagens alter the chemical structure of DNA bases, leading to incorrect base pairing during replication.

Common Chemical Mutagens and Their Effects

- **Alkylating Agents (e.g., EMS, MNNG, MMS)**:
  - Add alkyl groups to nucleotides, causing mispairing.
  - Can lead to **transition mutations** (e.g., G→A, C→T changes).
- **Base Analogues (e.g., 5-Bromouracil, 2-Aminopurine)**:
  - Mimic normal bases but increase the probability of mispairing.
  - Lead to point mutations during DNA replication.
- **Intercalating Agents (e.g., Ethidium Bromide, Proflavine)**:
  - Insert between DNA bases, causing frameshift mutations.
  - Lead to insertions or deletions during replication.
- **Deaminating Agents (e.g., Nitrous Acid, Hydroxylamine)**:
  - Convert cytosine to uracil or adenine to hypoxanthine.
  - Cause GC→AT transitions, leading to base substitution mutations.

Methods of Random Mutagenesis

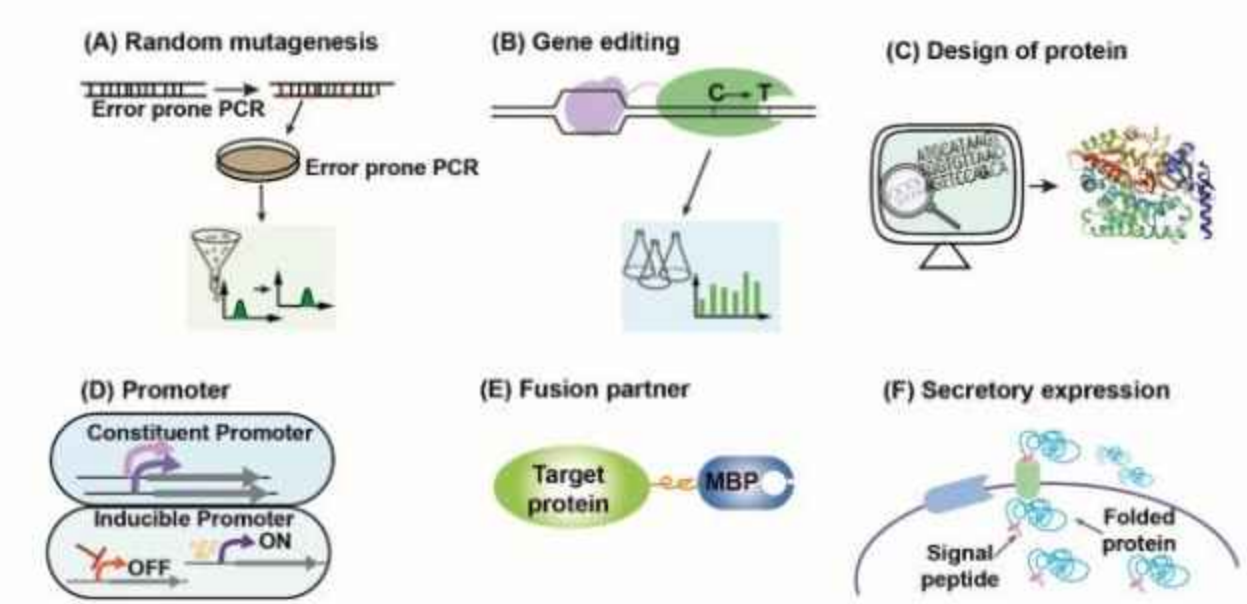
1. Radiation-Induced Mutagenesis Techniques

- **UV Radiation Exposure**:
  - Bacteria, fungi, and plant cells can be exposed to controlled UV doses.

- Cells with beneficial mutations are selected based on phenotype.
- **Gamma or X-Ray Irradiation**:
  - Used in **microbial strain improvement** and **crop breeding**.
  - Exposed organisms undergo screening for desirable traits.

2. Chemical Mutagenesis Techniques

- **Chemical Treatment**:
  - Cells are incubated with specific mutagens (e.g., EMS, MMS) under controlled conditions.
  - After treatment, surviving colonies are screened for advantageous mutations.
- **Adaptive Evolution by Serial Passaging**:
  - Cells are exposed to increasing mutagen concentrations over generations.
  - Beneficial mutations accumulate over time.



3. Combined Approaches

- **Radiation and Chemical Mutagens Together**:
  - Used in mutagenesis studies where multiple mutation types are desired.
- **Error-Prone PCR**:
  - A form of in vitro mutagenesis using polymerases with low fidelity.
  - Generates a high frequency of mutations in specific gene regions.

Applications of Random Mutagenesis

1. Biomedical Research and Drug Development

- **Cancer Studies**:
  - Induced mutations help model cancer-related genetic changes.
- **Antibiotic Resistance Studies**:
  - Used to study microbial resistance mechanisms and discover new drugs.

2. Agricultural and Crop Improvement

- **Mutagenesis in Plants**:
  - Radiation and chemical mutagens create crops with enhanced resistance to pests and stress.
- **Hybrid Seed Development**:
  - Generates genetic diversity for plant breeding programs.

3. Microbial Strain Improvement

- **Industrial Biotechnology**:
  - Random mutagenesis enhances the production of antibiotics, enzymes, and biofuels.
- **Probiotic Development**:
  - Used to generate more resilient probiotic strains.

4. Evolutionary Biology and Genetic Engineering

- **Experimental Evolution Studies**:
  - Helps understand how organisms adapt to new environments.
- **Directed Evolution for Protein Engineering**:
  - Generates enzyme variants with improved activity and stability.

5. Gene Function Studies and Pathway Analysis

- **Loss-of-Function Screens**:
  - Identifies genes essential for survival under specific conditions.
- **Pathway Engineering**:
  - Helps map metabolic and regulatory pathways in organisms.

Conclusion

Random mutagenesis using radiation or chemicals is a powerful tool for genetic research, crop improvement, and biotechnology applications. Despite its **non-specific nature**, it has been widely adopted for studying gene function, evolutionary adaptation, and strain improvement. With advancements in genome sequencing and synthetic biology, researchers can now analyze and harness mutagenesis more efficiently, leading to new discoveries in genetics, medicine, and industry. However, careful selection and validation of beneficial mutations are necessary to mitigate harmful genetic alterations.

Optogenetics-Based Genome Editing: Concept, Mechanisms, Methods, and Applications

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Introduction

Optogenetics-based genome editing is an innovative approach that combines **optogenetics** (the use of light to control biological processes) with **genome editing techniques** to precisely regulate gene expression and genetic modifications. This technology enables researchers to **spatially and temporally** control gene-editing processes using light, making it highly useful for biological research and therapeutic applications.

This document explores the **concept, mechanisms, methods, and applications** of optogenetics-based genome editing in detail.

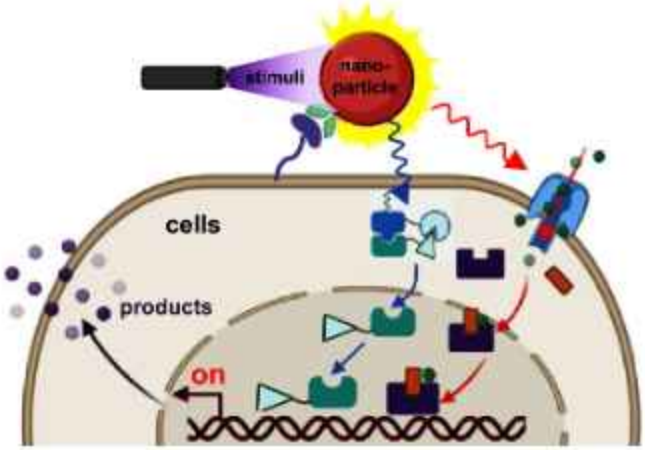
Concept of Optogenetics-Based Genome Editing

Definition

Optogenetics-based genome editing refers to the use of **light-sensitive proteins** (optogenetic actuators) to regulate the activity of genome-editing tools such as CRISPR-Cas9, TALENs, or zinc-finger nucleases. This approach allows researchers to precisely control genetic modifications with high spatiotemporal resolution.

Characteristics

1. **Light-Driven Control**: Uses specific wavelengths of light to activate or inhibit genome-editing enzymes.
2. **High Precision**: Allows gene editing with **temporal and spatial** accuracy.
3. **Reversible Modifications**: Some optogenetic systems enable reversible genetic modifications.
4. **Minimally Invasive**: Light activation reduces the need for chemical inducers or external modifications.
5. **Applications in Research and Medicine**: Applied in developmental biology, synthetic biology, and therapeutic gene editing.



Mechanisms of Optogenetics-Based Genome Editing

Step 1: Introduction of Optogenetic Actuators

- Cells or organisms are genetically engineered to express **light-sensitive proteins** that control genome-editing enzymes.
- These optogenetic actuators include **photoreceptors** that respond to light exposure.

Step 2: Activation of Genome Editing Enzymes

- Upon exposure to specific wavelengths of light, the **optogenetic system activates or inhibits genome-editing tools**.
- These tools include:
  - **CRISPR-Cas9**: Light controls the activity of Cas9, allowing precise DNA cleavage.
  - **TALENs and Zinc-Finger Nucleases**: Optogenetic systems regulate these nucleases to enable precise editing.

Step 3: Target DNA Modification

- The activated genome-editing enzymes **introduce genetic modifications** such as:
  - **Insertions**
  - **Deletions**
  - **Base modifications**
  - **Epigenetic changes**

Step 4: Deactivation and Control

- The editing process **stops when light exposure ceases**, preventing off-target effects.
- Some optogenetic systems also allow **reversible gene regulation**, ensuring controlled modifications.

Methods of Optogenetics-Based Genome Editing

1. Optogenetic Control of CRISPR-Cas9

- **Light-Activated Cas9 (photoactivatable Cas9, or paCas9)**:
  - Uses **light-sensitive domains** (e.g., CRY2-CIB1) to regulate Cas9 activity.
  - Enables light-dependent activation of DNA cleavage.

2. Photoactivatable Base Editing

- Light-sensitive base editors enable **precise single-base modifications** upon illumination.

3. Optogenetic Regulation of Transcription Factors

- Light-controlled transcription factors regulate gene expression **without permanent genome modification**.

4. Light-Inducible Epigenetic Modifications

- Uses optogenetic tools to **add or remove epigenetic marks** (e.g., histone modifications, DNA methylation) in response to light stimuli.

5. Temporal Control Using Light Pulses

- Short or long **light pulses** determine the duration and extent of genome editing activity.

Applications of Optogenetics-Based Genome Editing

1. Biomedical Research and Disease Modeling

- Used to study **gene functions** in real-time with precise temporal control.
- Helps create accurate **disease models** by inducing mutations under controlled conditions.

2. Gene Therapy and Regenerative Medicine

- Enables precise genome editing for **correcting genetic disorders**.
- Used to regulate stem cell differentiation for regenerative medicine.

3. Synthetic Biology and Biocomputing

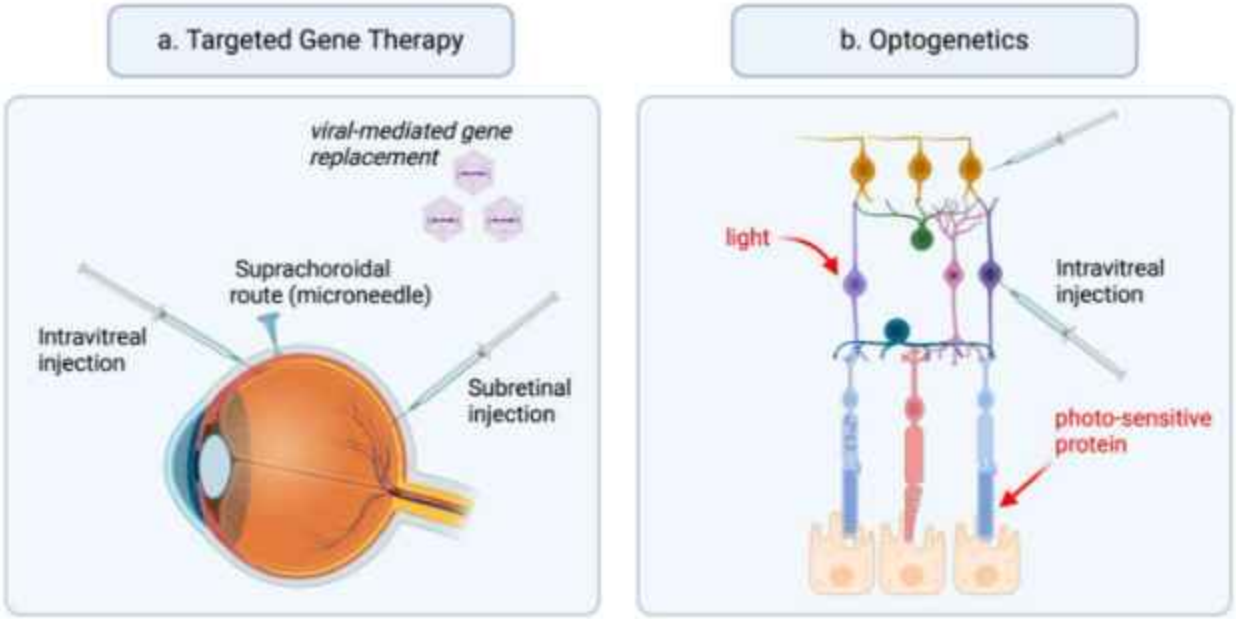
- Enables the design of **programmable biological systems** with light-controlled gene circuits.
- Used in biocomputing to create **light-controlled biosensors** and logic circuits.

4. Plant and Agricultural Biotechnology

- Allows **precise gene editing in crops** for improved traits.
- Enhances agricultural research by **modulating plant gene expression** under controlled conditions.

5. Neuroscience and Brain Research

- Optogenetic genome editing can **regulate neural genes** with light stimulation.
- Used in neurodevelopmental studies to investigate **gene function in brain circuits**.



Conclusion

Optogenetics-based genome editing is a revolutionary technology that enables **precise, light-controlled gene modifications**. It enhances traditional genome-editing methods by providing **spatiotemporal control**, reducing off-target effects, and expanding applications in biotechnology, medicine, and synthetic biology. Continued advancements in this field will likely **improve therapeutic strategies, enable complex gene regulation systems, and expand our ability to manipulate genomes with unprecedented precision**.