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on
“Hands-on Training of CRISPR-Cas9 for Crop Improvement”

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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.

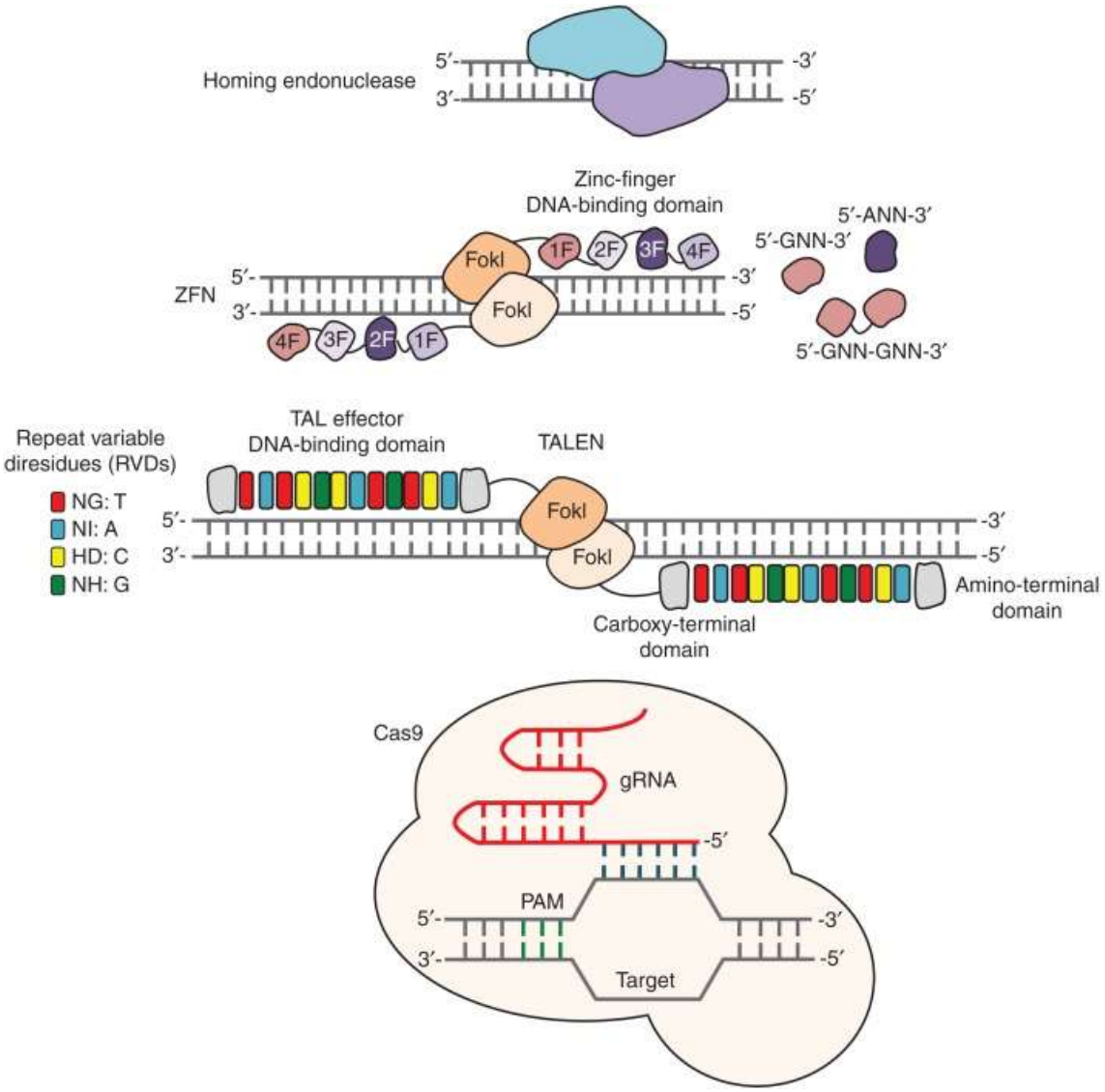


Figure 1. Genome-editing technologies. Cartoons illustrating the mechanisms of targeted nucleases. From *top* to *bottom*: homing endonucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector (TALE) nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (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.

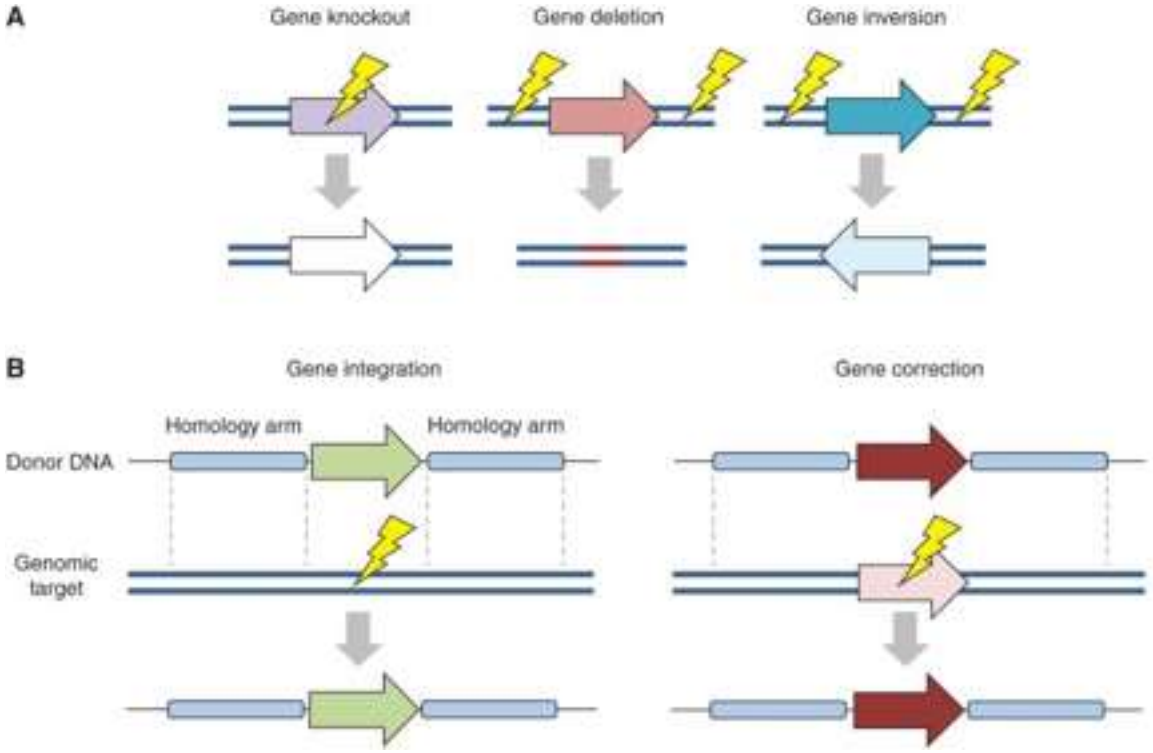


Figure 2. Genome-editing outcomes. Targeted nucleases induce DNA double-strand breaks (DSBs) that are repaired by nonhomologous end joining (NHEJ) or, in the presence of donor template, homology-directed repair (HDR). (*A*) In the absence of a donor template, NHEJ introduces small base insertions or 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.

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

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:**

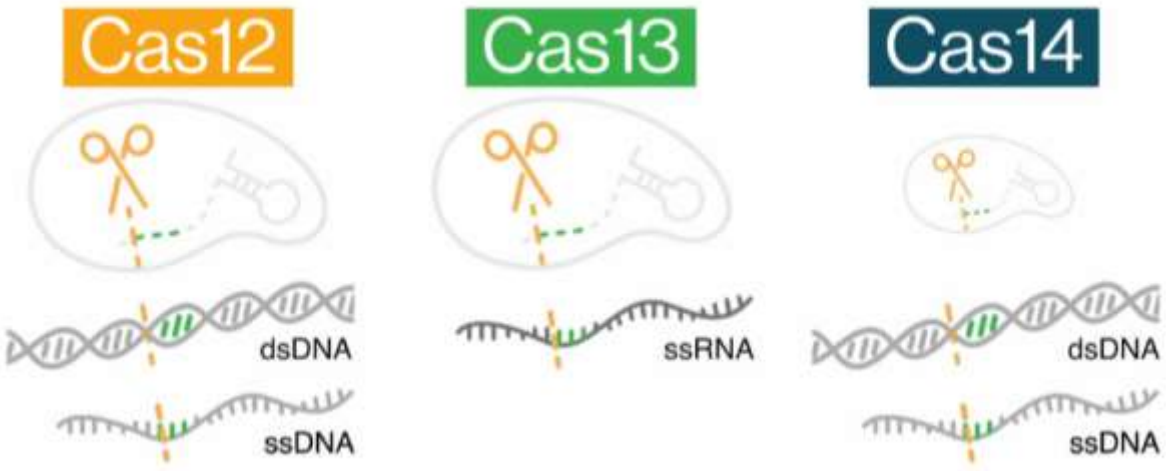
In certain types of CRISPR systems (like Type I-B, I-C, and I-D), the Cas11 protein is produced through a process called "internal translation," meaning it is encoded within another gene, like Cas8, which can be challenging to express in other organisms like human cells.

- **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.

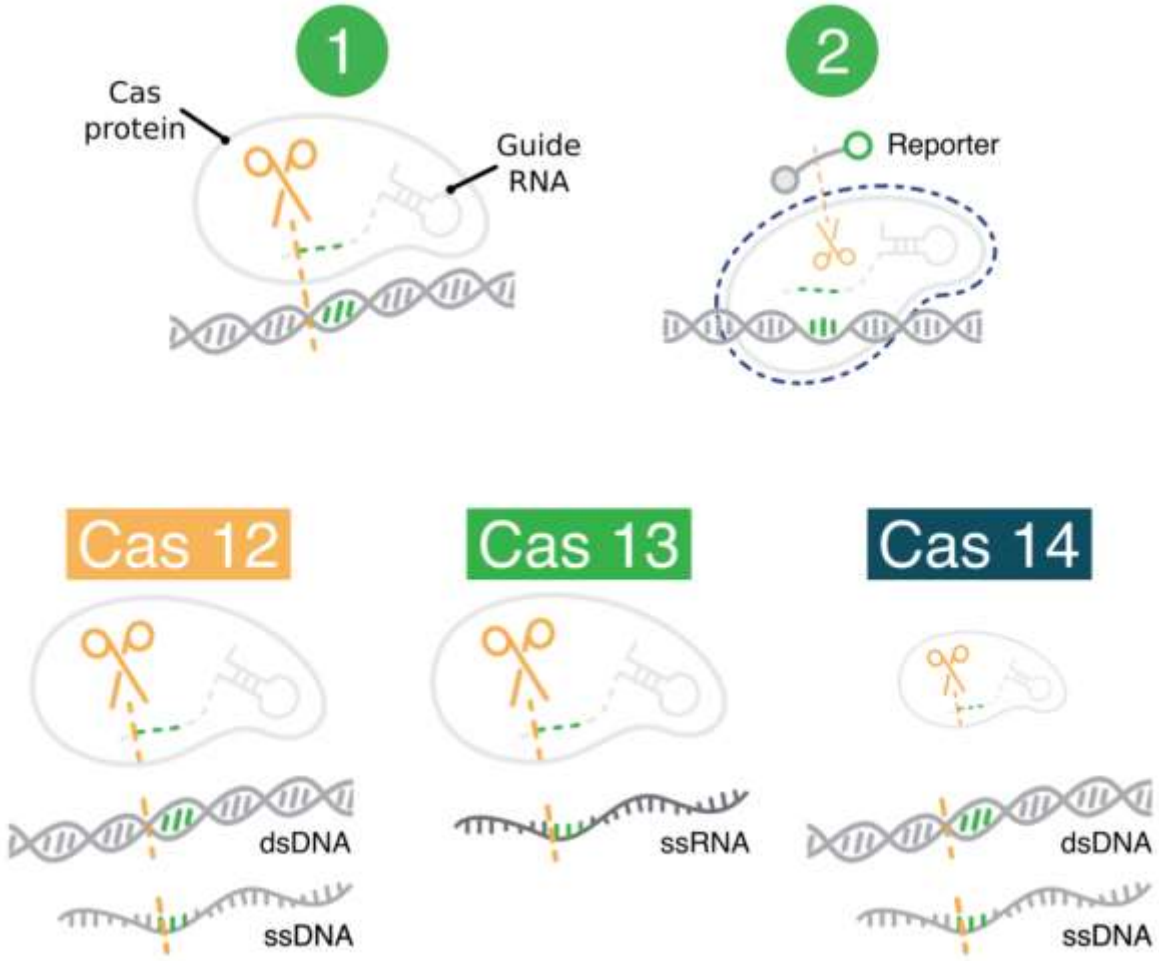


CRISPR diagnostics make use of non-specific cutting

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.

Recent Advances of CRISPR-Based Genome Editing for Enhancing Staple Crops

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1. Introduction CRISPR-based genome editing has revolutionized agricultural biotechnology by offering precise and efficient genetic modifications. The application of CRISPR-Cas systems in staple crops has enabled improvements in yield, stress tolerance, disease resistance, and nutritional quality. This report highlights recent advancements in CRISPR-mediated genome editing for enhancing staple crops, with a focus on rice, wheat, maize, and other key food sources.

2. CRISPR-Cas Systems in Crop Improvement The CRISPR-Cas9 and CRISPR-Cas12 systems are widely utilized for targeted genome modifications. These systems enable gene knockout, base editing, and gene regulation in crops. Recent developments include prime editing and multiplex genome editing, further expanding the scope of genetic improvements in staple crops.

3. Key Applications in Staple Crops

- **Rice (Oryza sativa)**:
  - Increased yield through the modification of genes such as GS3, Gn1a, and IPA1.
  - Enhanced resistance to bacterial blight by targeting SWEET genes.
  - Improved salinity and drought tolerance via gene edits in OsNAC and OsHKT.
- **Wheat (Triticum aestivum)**:
  - Development of disease-resistant varieties by editing mildew resistance genes (MLO).
  - Improvement in grain quality and yield by modifying TaGW2 and TaVRN genes.
  - Enhanced drought tolerance through modifications in ABA-responsive genes.
- **Maize (Zea mays)**:
  - Increased kernel size and starch biosynthesis by targeting GW2 and Sh2 genes.
  - Resistance to pests such as fall armyworm through disruption of susceptibility genes.
  - Improvement in nitrogen-use efficiency via gene modifications in NRT1.1B.
- **Other Staple Crops**:
  - **Potato (Solanum tuberosum)**: Resistance to late blight by modifying StMLO.
  - **Soybean (Glycine max)**: Improved oil content through FAD2 gene editing.
  - **Cassava (Manihot esculenta)**: Enhanced virus resistance by modifying the eIF4E gene.

4. Advances in CRISPR Technologies for Crop Improvement

- **Base Editing**: Allows precise single-nucleotide changes without double-strand breaks, reducing off-target effects.
- **Prime Editing**: Offers precise insertions and deletions, expanding genome editing capabilities.
- **CRISPR-Directed Epigenetic Modifications**: Enables gene regulation without altering DNA sequences.
- **High-Throughput Screening**: CRISPR libraries facilitate the identification of genes critical for crop resilience and productivity.

5. Challenges and Future Prospects Despite its potential, CRISPR-based genome editing faces challenges such as regulatory hurdles, off-target effects, and public acceptance. Future research should focus on improving editing efficiency, integrating multi-gene editing strategies, and enhancing delivery methods for field applications. The development of non-transgenic genome editing approaches will further facilitate regulatory approvals and commercialization.

6. Conclusion CRISPR-based genome editing holds immense promise for enhancing staple crops by improving yield, stress tolerance, disease resistance, and nutritional quality. Continuous advancements in CRISPR technologies will pave the way for sustainable agriculture and global food security.

Lecture Notes: Screening and Validation of CRISPR-Induced Mutations

1. Introduction CRISPR-Cas genome editing has transformed plant breeding by enabling precise modifications in crop genomes. However, successful genome editing requires rigorous screening and validation of induced mutations to ensure specificity, efficiency, and functional relevance. This lecture covers key methodologies used to detect, confirm, and evaluate CRISPR-induced mutations in staple crops.

2. Methods for Screening CRISPR-Induced Mutations Several molecular, biochemical, and phenotypic screening methods are employed to identify and validate mutations introduced by CRISPR-based genome editing.

2.1 Genotyping and Molecular Analysis

- **Polymerase Chain Reaction (PCR)-Based Methods**:
    - **T7 Endonuclease I (T7E1) Assay**: Detects insertions and deletions (indels) by cleaving mismatched DNA strands.
    - **Cleaved Amplified Polymorphic Sequences (CAPS) and Derived Cleaved Amplified Polymorphic Sequences (dCAPS)**: Uses restriction enzyme digestion to identify mutations.
    - **High-Resolution Melting (HRM) Analysis**: Detects small sequence variations by analyzing melting curves of DNA fragments.
- **Sanger Sequencing**:
    - Used for small-scale mutation analysis and validation of edited regions.

- **Next-Generation Sequencing (NGS)**:
    - Enables deep sequencing of target sites to detect mutations with high accuracy and sensitivity.

2.2 Phenotypic Evaluation

- **Morphological and Agronomic Trait Analysis**:
    - Field and greenhouse assessments to study plant height, leaf morphology, yield, and resistance to biotic/abiotic stresses.
- **Microscopic and Biochemical Assays**:
    - Examines cellular or biochemical changes associated with targeted mutations.
    - Example: Chlorophyll content analysis in drought-resistant CRISPR-edited plants.

2.3 Off-Target Mutation Analysis

- **Whole-Genome Sequencing (WGS)**:
    - Provides a comprehensive assessment of unintended mutations in non-target regions.
- **In Silico Prediction Tools**:
    - Software tools such as Cas-OFFinder and CRISPResso help predict potential off-target sites and analyze sequence integrity.
- **CRISPR-Cas Variants with Enhanced Specificity**:
    - Engineered Cas9 variants (e.g., Cas9-HF1, eSpCas9) reduce off-target effects.

2.4 Inheritance and Stability Studies

- **Segregation Analysis in Successive Generations**:
    - Confirms stable transmission of CRISPR-induced mutations.
    - Utilizes Mendelian inheritance patterns in segregating populations.
- **Cas9-Free Edited Lines**:
    - Identification of transgene-free plants using PCR or Southern blot analysis.
    - Ensures regulatory compliance and facilitates commercialization.

3. Challenges and Considerations in Screening CRISPR Mutations

- **Efficiency of Mutation Detection**:
    - Sensitivity of assays affects mutation detection, especially for low-frequency edits.
- **False Positives/Negatives**:
    - PCR-based methods may not detect all mutations, necessitating sequencing validation.
- **Regulatory and Biosafety Concerns**:
    - Differentiation between transgenic and non-transgenic CRISPR-edited crops.

4. Future Perspectives

- **Development of High-Throughput Screening Methods**:
  - CRISPR-based barcoding for rapid mutation screening.
- **Application of AI and Machine Learning in Mutation Analysis**:
  - Predicting editing efficiency and analyzing large-scale sequencing data.
- **Adoption of Non-Transgenic Genome Editing Approaches**:
  - Regulatory-friendly genome editing without foreign DNA integration.

5. Conclusion Screening and validation of CRISPR-induced mutations are essential for ensuring precise, efficient, and stable genome modifications in crops. Advances in sequencing technologies, molecular assays, and computational tools are improving the reliability of mutation analysis. Future research will focus on refining high-throughput screening methods and addressing regulatory challenges for broader adoption in agriculture.

Slide 1

Genome Editing Research in Bangladesh: Challenges and Opportunities

Dr. Md. Abdus Salam
Member Director (Crops)
Bangladesh Agricultural Research Council

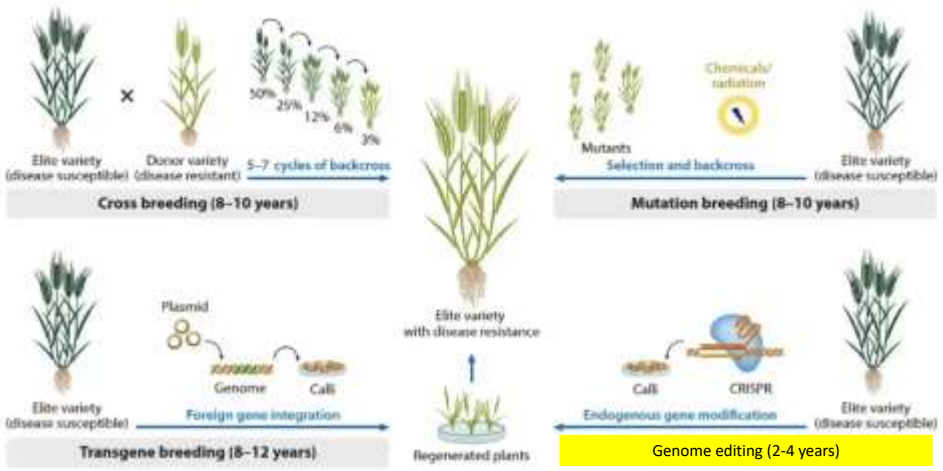
Slide 2

Genome editing and it's use

- Genome editing, also called gene editing, is one of the latest developments in crop improvement technologies.
- Genome editing is being applied to crops to introduce traits, such as withstanding climatic factors, resistance to pathogens, improved nutritional value, and even reduced wastage in the post-harvest stage in multiple crops.
- This technology is more precise and efficient than conventional breeding and, hence, can reduce the time needed to develop new plant varieties.
- Extensive research efforts are underway for over 70 crops in about 60 countries.
- Genome edited plants are already moving beyond the laboratory, and its products, such as high GABA tomato, high oleic soybean, milder flavored mustard, etc., are already on the market in Japan and the USA.

Slide 3

Why Genome editing is required?

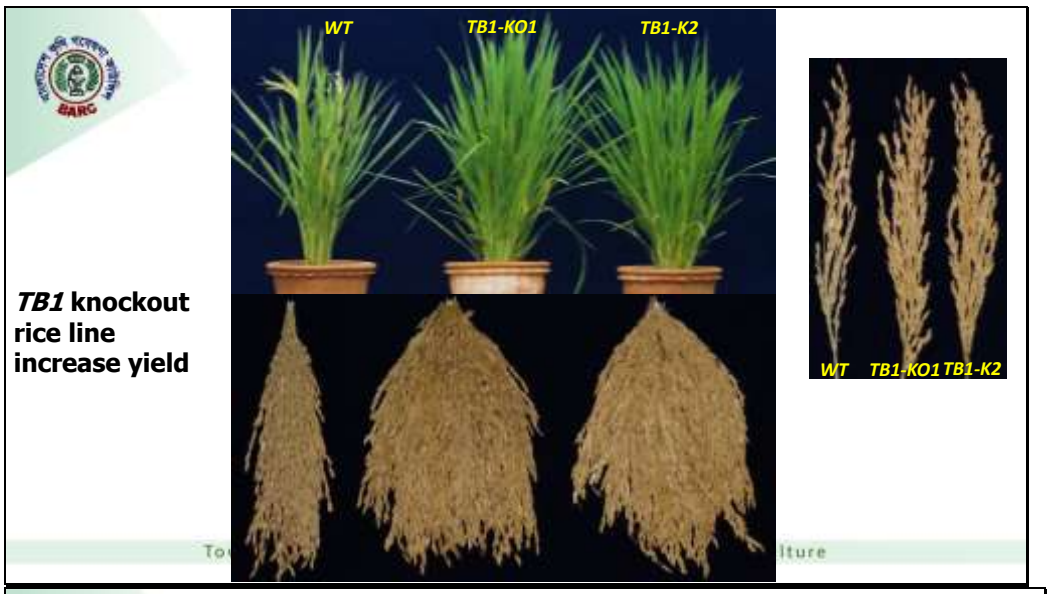


Slide 4

TB1 gene knockout rice line increase yield

Towards Building Advanced Intelligent System for Agriculture

Slide 5



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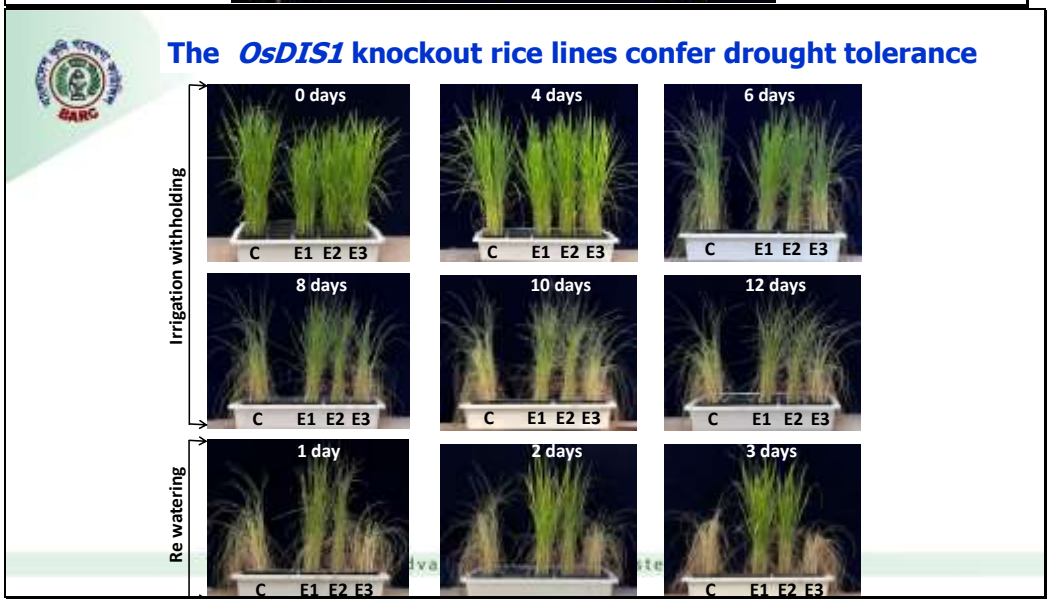

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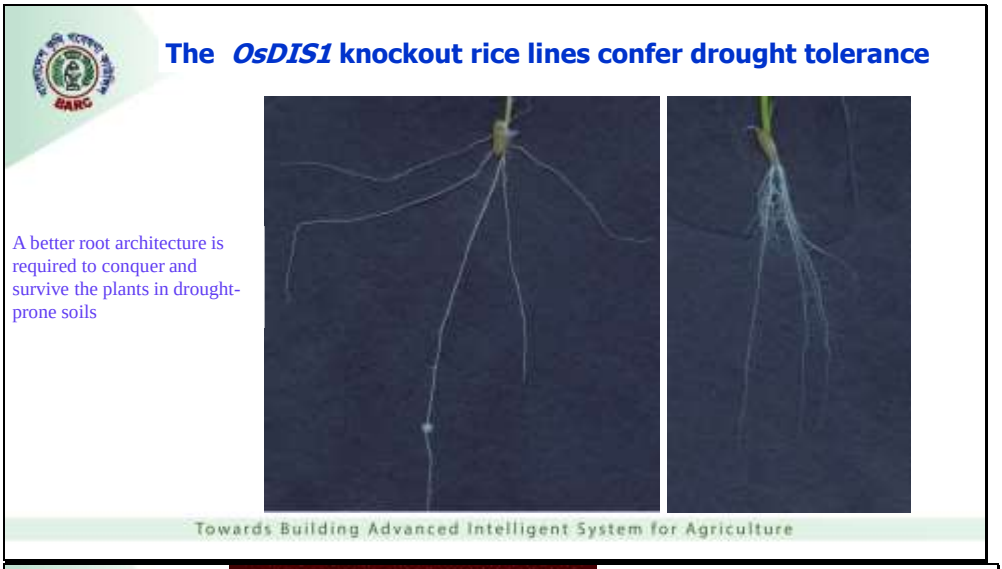

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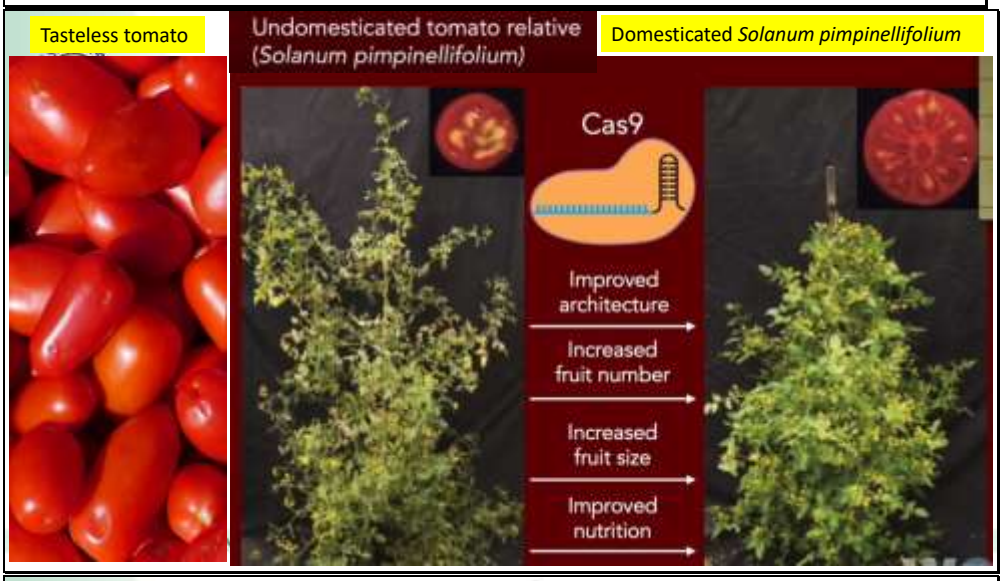

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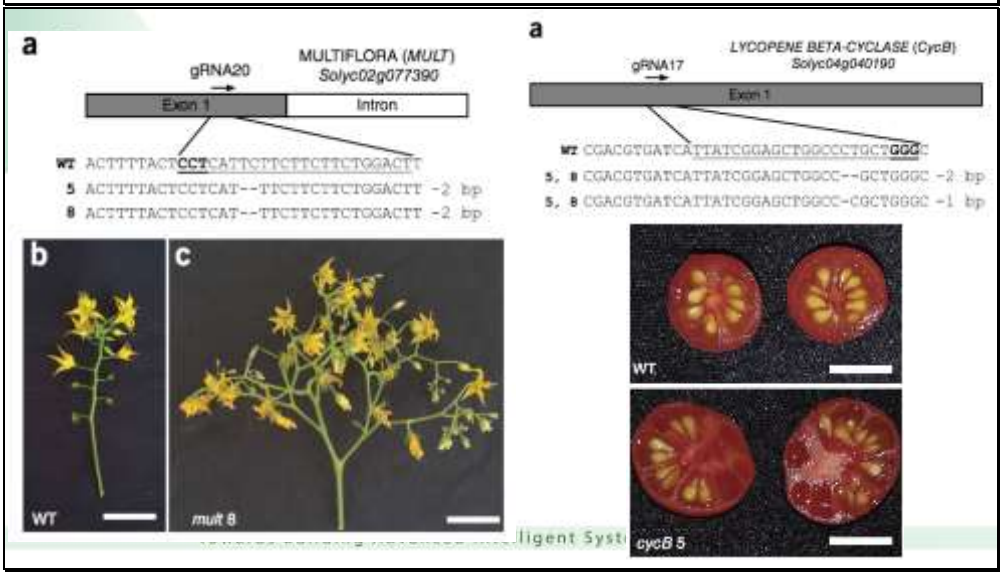

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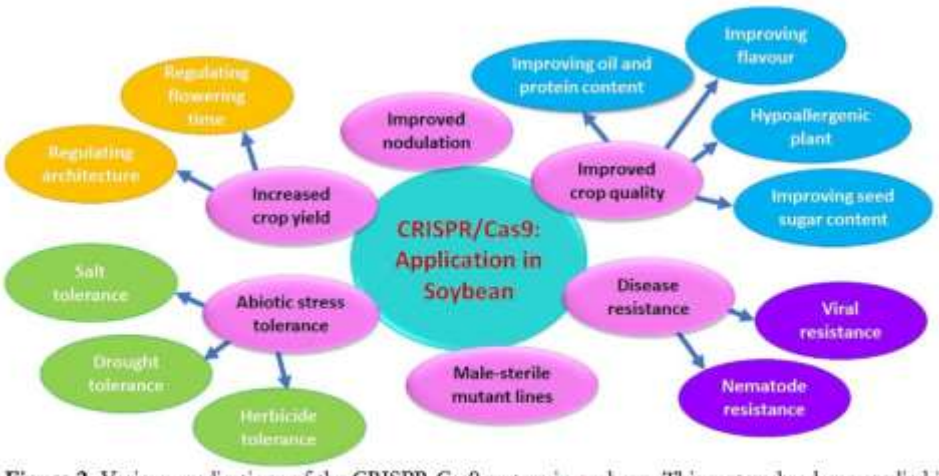
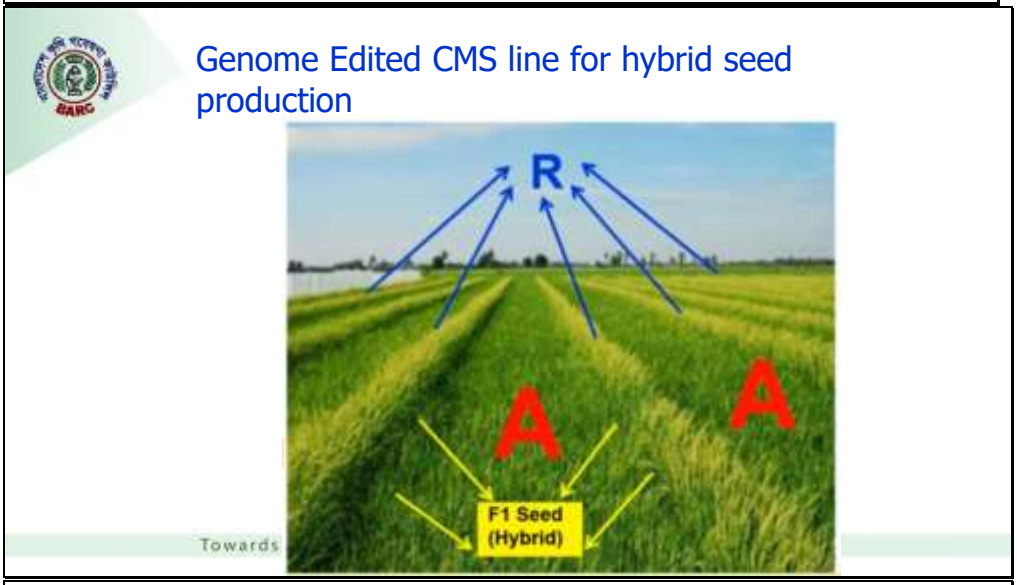


Figure 2. Various applications of the CRISPR-Cas9 system in soybean. This system has been applied in soybean for the improvement of yield, quality, nodule formation, male-sterile lines as well as biotic and abiotic stress tolerance.

Slide 11



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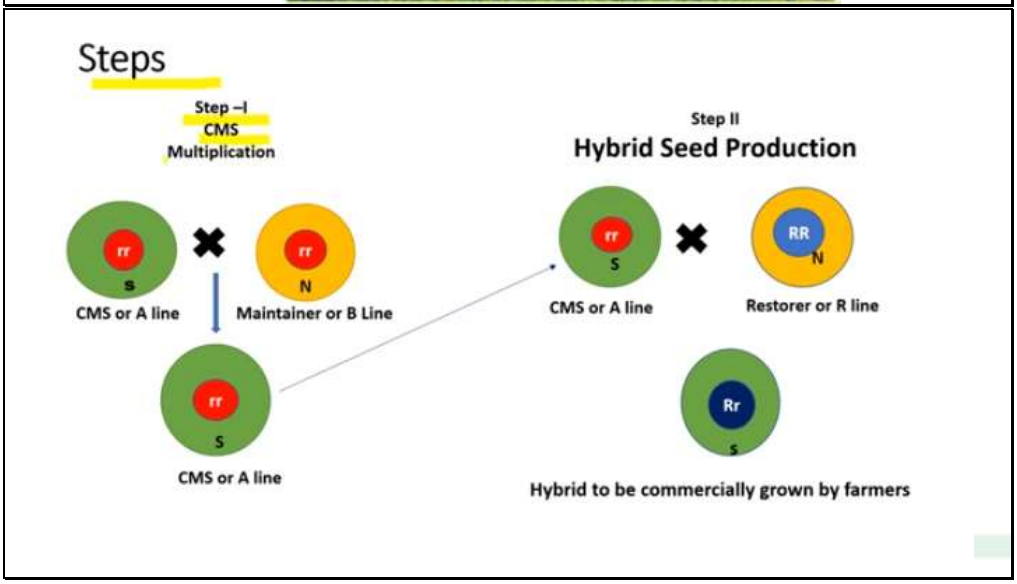


Slide 13

Engineering crop plants to herbicide resistance: For effective weed management in agriculture

Manual weed control over large areas is not feasible from the point of labor availability and its associated monetary costs

If the crop plants are engineered for herbicide resistance, then non-selective herbicides can be used to kill all types of weeds without causing any injury to crop plant

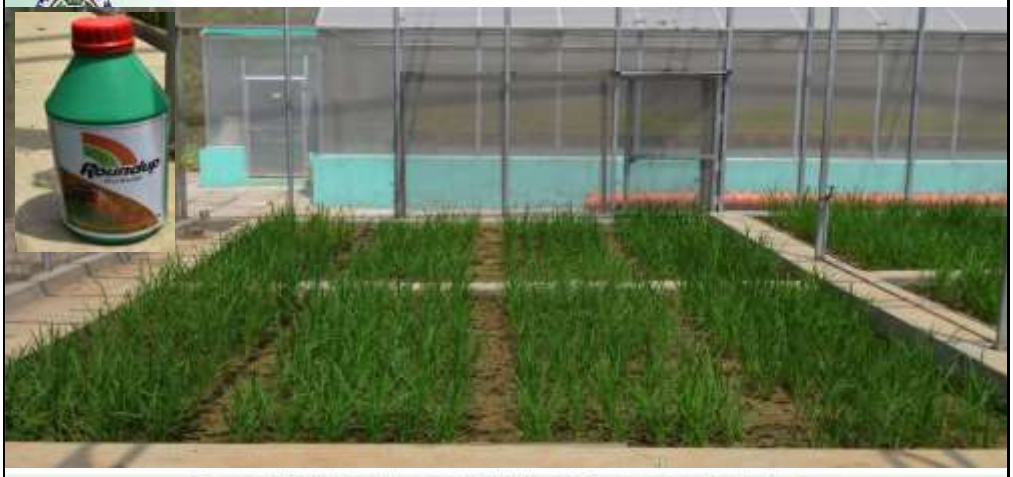
Spending billions of $ to control weeds

$30 billion in crop loss due to weed infestation

Herbicide resistant cultivars a promising future

Slide 14

Rice transgenic events grown in the field prior to application of glyphosate



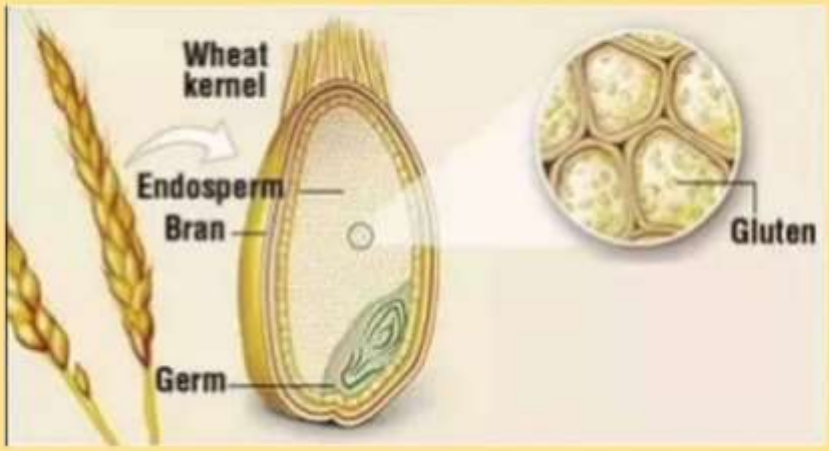
Towards Building Advanced Intelligent System for Agriculture

Slide 15

Application of different doses of glyphosate in different replication plots

Slide 16

A new solution to gluten sensitivity



Slide 17

Eliminating cyanogenic compound production would require only **two** CRISPR-mediated mutations

Gene editing targets have also been identified for improving drought tolerance, shelf life, pest resistance, and yield in cassava

Sadly, international adoption of engineered cassava is currently hindered by anti-GMO activists

Shifting climate strengthens the dependence on cassava, leading to *konzo*, a neurological condition caused by cassava cyanide poisoning

Slide 18

Challenges and Opportunities in Bangladesh

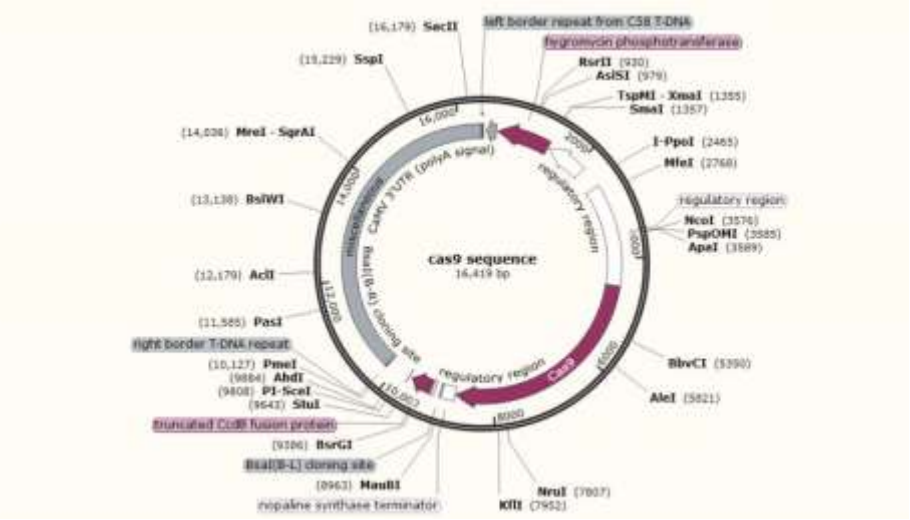
- Increase yield
- Resistance of emerging disease and pests thus reducing inputs (e.g., pesticides, herbicides and fertilizers).
- Improve resilience to increasingly extreme weather conditions, including drought.
- Respond to changing consumer demands and preferences by increasing shelf-life, nutritional value or improving flavor and appearance.
- Adapt to new agronomic systems.

- BRRI
- NIB:
- BSMI
- Dhak

Must

Primer design and multiplex CRISPR/Cas9 vector construction

ড. মোহাম্মদ শাহ আলম
উপপরিচালক
উপপরিচালকের কার্যালয়
উদ্ভিদ সংগনিরোধ কেন্দ্র
সমুদ্র বন্দর, আগ্রাবাদ, চট্টগ্রাম।



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	ATTCCACTCACTCAACCGAAGGG	+	1371	45 %
LOC_Os01g39330	Guide93	0.8205	GATGTCGGAGAGGAAGAGGAGGG	+	1038	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^{o}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 ^{o}C at 15 min

5. Two targets with U-F Primers and gR-R- Primers

   Note: PCR: 94^{o}C-2m, (94^{o}C – 10 S, 60 ^{o}C -15 S, 68 ^{o}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

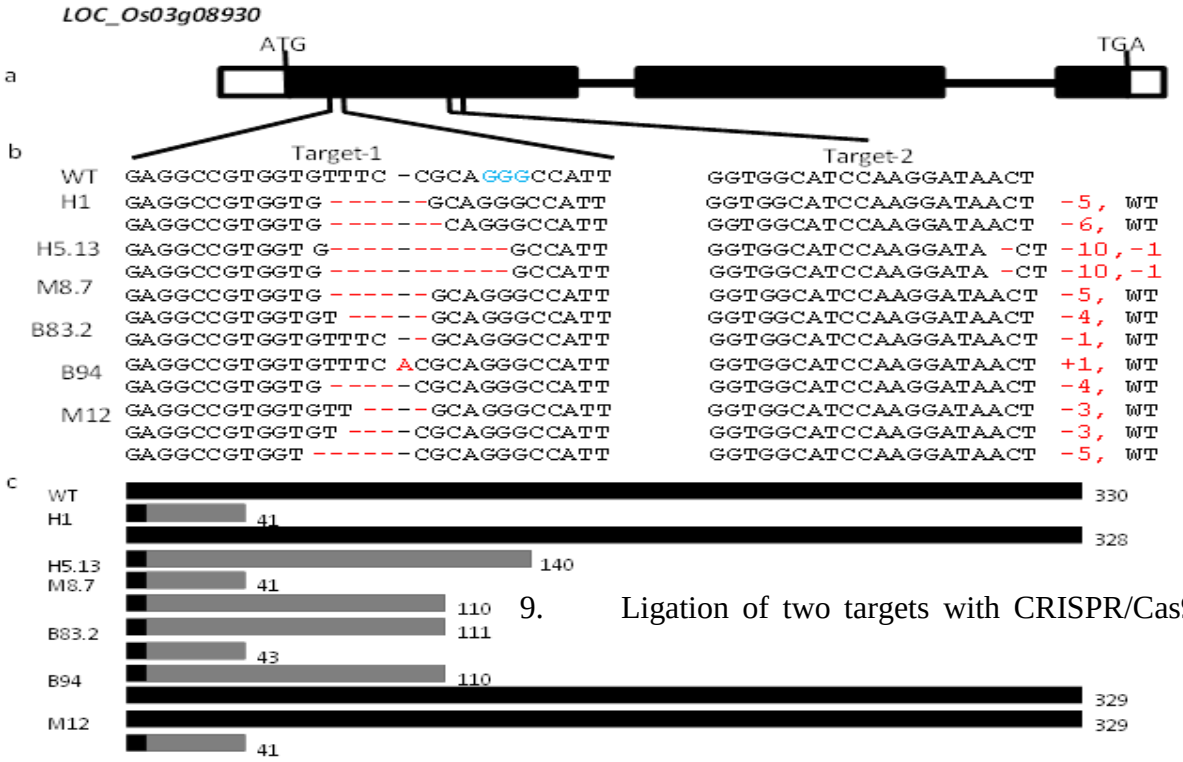
   Note: PCR: Cas 2: 95oC -2m, (95oC – 15 S, 60 oC -15 S, 68 oC -20 S) last 68 oC-1m, Cycle-17 about 40 minutes, after PCR, 3 µl 6x loading buffer for all and vortex & shake clearly. Check sequence, if sequence is good transfer into ecoli bacteria and keep in -80^{O} C

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 vector

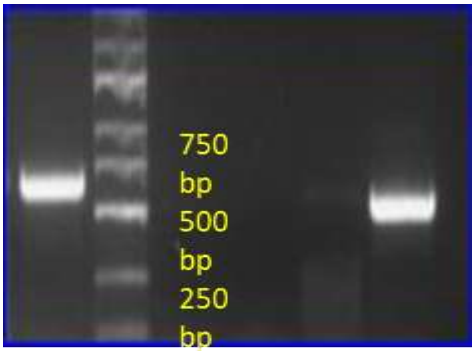
Transformation of multiplex CRISPR/Cas9 vector into plant cell

ড. মোহাম্মদ শাহ আলম
উপপরিচালক
উপপরিচালকের কার্যালয়
উদ্ভিদ সংগনিরোধ কেন্দ্র
সমুদ্র বন্দর, আগ্রাবাদ, চট্টগ্রাম।

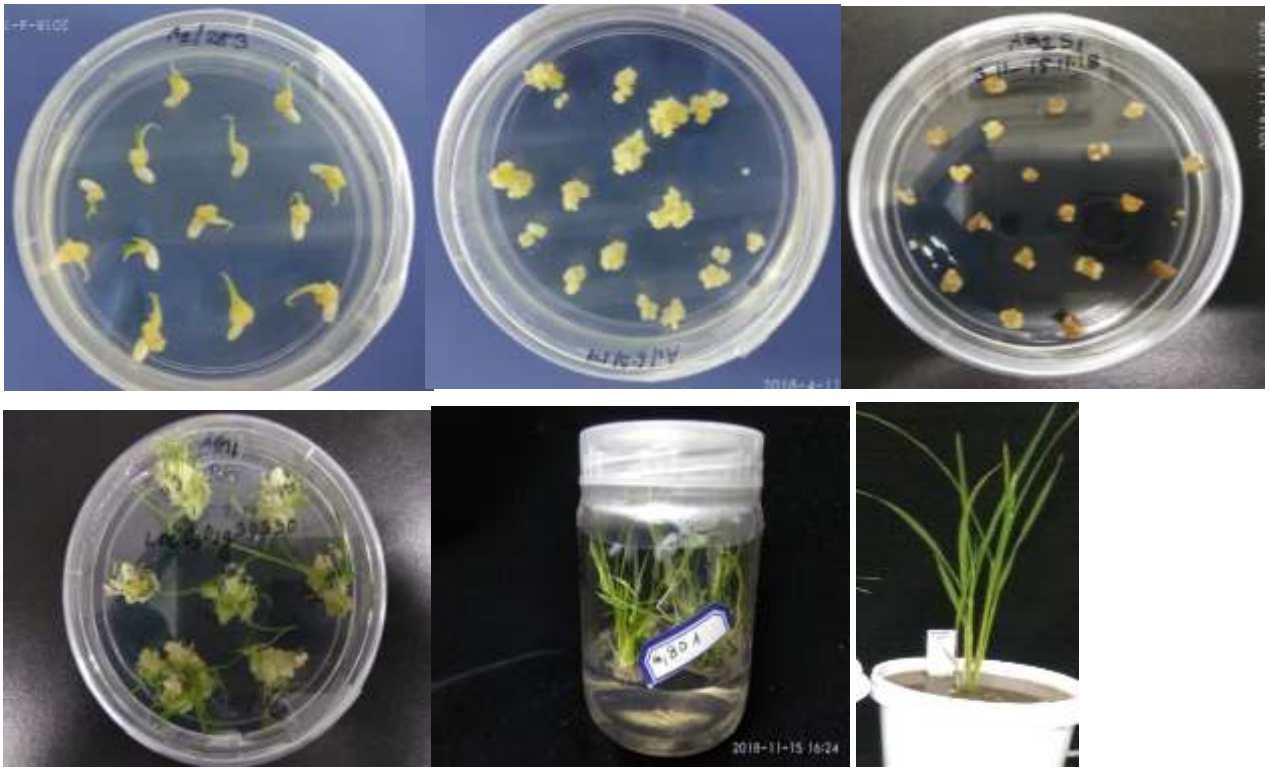
Transformation steps:

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

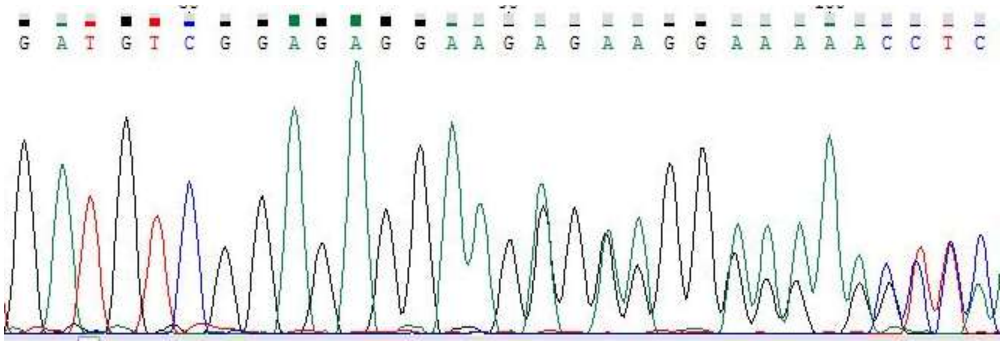
Target check in Gel-electrophoresis



Mutation Checking:



Callus to **Transgenic Plant**



Mutation detection

Allele1: ACATGATGTCGGAGAGGAAGAGGAGGGAGAAGCTC (WT)
Allele2: ACATGATGTCGGAGAGGAAG-GGAGGGAGAAGCTTA (complicated variant)
Reference:ACATGATGTCGGAGAGGAAGAGGAGGGAGAAGCTCAACGACAG CTTCCACACGCTCAGATCACTCCTCCC

Inez H Slamet-Loedin. International Rice Research Institute

CGIAR Genome Editing Initiative Lead

(islamet-loedin@cgiar.org)

Lab Protocols - DNA Extraction, Tissue Culture, and Plasmid Construction

Dr. Shahanaz Sultana
CSO (Current Charge) and Head
Biotechnology Division, BRRI, Gazipur-1701

1. Introduction Laboratory techniques such as DNA extraction, tissue culture, and plasmid construction are essential for molecular biology and genome editing research. This lecture provides an overview of key protocols used in plant biotechnology, with a focus on CRISPR-based genome editing applications.

2. DNA Extraction Protocol DNA extraction is the first step in genetic analysis, allowing researchers to obtain high-quality DNA for PCR, sequencing, and genome editing.

2.1 Materials Required

- Plant leaf or root tissue
- CTAB extraction buffer (for plant samples)
- RNase A (to remove RNA contamination)
- Chloroform:isoamyl alcohol (24:1)
- Isopropanol or ethanol (for DNA precipitation)
- TE buffer (Tris-EDTA) for DNA storage

2.2 Procedure

1. **Sample Preparation:** Collect fresh plant tissue (~100 mg) and grind with liquid nitrogen.
2. **Lysis and Cell Disruption:** Add CTAB buffer and incubate at 65°C for 30 minutes.
3. **Phase Separation:** Add chloroform:isoamyl alcohol, vortex, and centrifuge.
4. **DNA Precipitation:** Transfer aqueous phase and add isopropanol. Incubate at -20°C.
5. **Pellet Washing:** Wash with 70% ethanol and air-dry.
6. **Dissolution and Storage:** Resuspend DNA in TE buffer and store at -20°C.

3. Tissue Culture Protocol Tissue culture is crucial for regenerating plants after genome editing.

3.1 Materials Required

- Murashige and Skoog (MS) medium
- Plant growth regulators (auxins, cytokinins)
- Sterile forceps and scalpels
- Laminar flow hood
- Growth chamber (25°C, 16h light/8h dark)

3.2 Procedure

1. **Sterilization:** Surface sterilize explants using 70% ethanol and 1% sodium hypochlorite.
2. **Callus Induction:** Place explants on MS medium with cytokinin/auxin.
3. **Shoot Regeneration:** Transfer callus to shoot induction medium.
4. **Rooting and Hardening:** Transfer shoots to rooting medium, then to soil.

4. Plasmid Construction Protocol Plasmid construction is a fundamental step in CRISPR genome editing.

4.1 Materials Required

- CRISPR-Cas9 vector
- Target-specific gRNA sequence
- Restriction enzymes and ligase
- E. coli competent cells
- LB medium and antibiotic selection plates

4.2 Procedure

1. **Guide RNA Design:** Use bioinformatics tools (e.g., CHOPCHOP, Benchling) to design gRNA.
2. **Vector Digestion:** Use restriction enzymes to linearize the plasmid.
3. **Ligation:** Insert gRNA sequence into the CRISPR vector using ligase.
4. **Transformation:** Introduce plasmid into competent E. coli cells via heat shock.
5. **Colony Screening:** Select recombinant colonies using antibiotic resistance and PCR.
6. **Plasmid Purification:** Extract plasmid DNA for downstream applications.

Delivery methods for CRISPR-Cas9 in plants (e.g., Agrobacterium-mediated transformation, electroporation, and nanoparticle delivery).

Dr. Shah Ashadul Islam
Senior Scientific Officer (SSO)
Agronomy Division, BRRI, Gazipur

Class Note: Delivery Methods for CRISPR-Cas9 in Plants

CRISPR-Cas9 technology has revolutionized plant genetic engineering by enabling precise genome editing. However, to effectively utilize this technology in plants, efficient delivery methods are required to introduce the CRISPR-Cas9 components (Cas9 protein and guide RNA) into plant cells. There are several methods for delivering CRISPR-Cas9 into plant cells, each with its own advantages and limitations. Here, we discuss three common delivery methods: Agrobacterium-mediated transformation, electroporation, and nanoparticle delivery.

1. Agrobacterium-Mediated Transformation

Agrobacterium-mediated transformation leverages the natural ability of *Agrobacterium tumefaciens* to transfer DNA into plant cells. This process is frequently used in plant biotechnology for generating genetically modified plants. In the context of CRISPR-Cas9, this method involves introducing both the Cas9 protein and the guide RNA (gRNA) into plant cells via Agrobacterium.

Detailed Process:

- **Plasmid Construction:** The Cas9 gene and gRNA are first cloned into a binary vector (a plasmid) that contains the necessary genes for Agrobacterium to infect plant cells. These plasmids often contain the *nopaline synthase* (NOS) promoter for constitutive expression of the Cas9 protein and the gRNA under control of a plant-specific promoter, like the *U6* promoter.
- **Agrobacterium Infection:** Once the plasmid is inserted into Agrobacterium, the bacteria are co-cultivated with plant tissues such as leaf discs, cotyledons, or callus. Agrobacterium transfers the plasmid DNA (T-DNA) into the plant cells, where it integrates into the plant genome, either stably or transiently.
- **Regeneration and Screening:** After the transformation event, plant tissues are cultured on selective media containing antibiotics or herbicides to select for transformed cells. The transformed cells are then regenerated into full plants using plant tissue culture techniques.
- **Genome Editing:** In the transformed cells, the Cas9 protein and guide RNA are expressed, targeting the specific gene of interest in the plant genome. Cas9 induces a double-strand break at the target site, leading to either non-homologous end joining (NHEJ) or homology-directed repair (HDR), depending on the presence of a donor template.

Advantages:

- **Wide Applicability:** It is one of the most efficient methods for many plant species, particularly for dicots like *Arabidopsis*, *tobacco*, and *tomato*, and has been adapted for several monocots, including *rice*, *maize*, and *wheat*.
- **Stable Transformation:** Agrobacterium-mediated transformation integrates the CRISPR components into the plant genome, allowing for stable and heritable gene editing.
- **Multiple Gene Targeting:** Agrobacterium can carry multiple CRISPR constructs, enabling simultaneous editing of several genes in a single plant.

Limitations:

- **Monocot Limitations:** Some monocots (e.g., *corn*, *barley*, and *sugarcane*) are less amenable to Agrobacterium transformation, requiring additional optimization or alternative methods.
- **Transformation Efficiency:** Some plant species have low transformation efficiency, necessitating improvements in the method for optimal use.

2. Electroporation

Electroporation involves applying an electric field to plant cells or protoplasts (plant cells without cell walls), which creates temporary pores in the cell membrane to facilitate the entry of DNA molecules, such as the CRISPR-Cas9 components.

Detailed Process:

- **Preparation of CRISPR Components:** The Cas9 gene and gRNA are either encapsulated in plasmids or directly synthesized as mRNA and protein.
- **Cell Preparation:** Protoplasts (cells without cell walls) or other plant cells are isolated from plant tissues. These cells are usually prepared by enzymatically digesting the cell wall or by physical methods.
- **Electroporation:** The protoplasts or cells are mixed with the CRISPR-Cas9 components and placed in an electroporation cuvette. A short, high-voltage electrical pulse is applied to induce membrane permeabilization.
- **Cell Recovery:** After electroporation, the cells are incubated in a growth medium to allow recovery and regeneration. The CRISPR components can now enter the cells and initiate gene editing.

Advantages:

- **Versatility:** Electroporation can be used for a wide variety of plant species, including difficult-to-transform plants such as certain monocots.

- **No Biological Vector Required:** Unlike Agrobacterium, electroporation does not rely on biological systems, making it useful in cases where Agrobacterium-mediated transformation is inefficient or unsuitable.
- **Transitory Expression:** Electroporation allows for transient expression of CRISPR-Cas9 components, which can be particularly useful for researchers studying gene function without generating stable transformants.

Limitations:

- **Cell Viability:** The application of electrical pulses can lead to high rates of cell death, especially for large, undifferentiated cells.
- **Efficiency Challenges:** Electroporation is less efficient for plant species with high cell wall content or low regeneration potential.
- **Limited Regeneration:** Protoplast-based electroporation may face issues in regenerating full plants from edited cells, especially in species that do not regenerate easily from protoplasts.

3. Nanoparticle Delivery

Nanoparticle-based delivery methods involve using nanoparticles to carry CRISPR-Cas9 components directly into plant cells. These nanoparticles can be made from a variety of materials, such as liposomes, gold nanoparticles, carbon nanotubes, and polymer-based nanoparticles. The CRISPR components (such as plasmid DNA, RNA, or proteins) are loaded onto these nanoparticles for delivery.

Detailed Process:

- **Nanoparticle Preparation:** Nanoparticles are synthesized using materials like lipids, metals (e.g., gold), or carbon-based compounds (e.g., carbon nanotubes). These particles are then loaded with the CRISPR-Cas9 components—either DNA encoding the Cas9 protein and guide RNA or pre-assembled Cas9 protein and gRNA.
- **Delivery:** The nanoparticles are applied to plant tissues, where they may enter cells through methods such as root soaking, particle bombardment (using a gene gun), or direct incubation.
- **CRISPR Expression:** Once inside the cells, the nanoparticles release the CRISPR-Cas9 components, which are then expressed in the plant cells, leading to genome editing.

Advantages:

- **Non-Viral Delivery:** Nanoparticles offer a non-viral, potentially safer method for delivering CRISPR-Cas9 components into plant cells.
- **Precise Control:** Nanoparticles can provide more controlled and localized delivery of CRISPR components, enhancing the precision of gene editing.

- **Wide Application:** Nanoparticle delivery has shown promise in a wide range of plant species, including both dicots and monocots. This makes it particularly attractive for plants that are recalcitrant to Agrobacterium-based transformation or electroporation.

Limitations:

- **Toxicity:** Some types of nanoparticles may cause cytotoxicity in plant cells, affecting plant growth and regeneration.
- **Efficiency Issues:** The efficiency of nanoparticle delivery is still under investigation, and optimizing the method for different plant species remains a challenge.
- **Nanoparticle Stability:** Nanoparticles must be stable in solution and able to release their contents inside the plant cell, which can sometimes be an issue depending on the formulation.

Comparison of Delivery Methods:

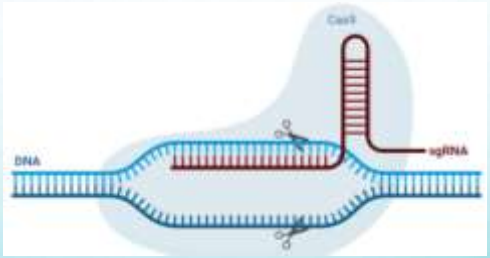
Method	Advantages	Limitations
Agrobacterium-mediated Transformation	Widely used, high efficiency in many species, stable integration of CRISPR components	Limited for some monocots, transformation efficiency issues in certain plants
Electroporation	Versatile, works for many species, no biological vector	High cell mortality, challenges in regeneration of whole plants
Nanoparticle Delivery	Non-viral, controlled delivery, potential for wide species applicability	Potential toxicity, optimization needed for different species, variable efficiency

Conclusion

The choice of CRISPR-Cas9 delivery method depends on several factors, including the type of plant, the goal of the research (stable or transient transformation), and the available infrastructure. Agrobacterium-mediated transformation remains the gold standard for many plant species, but electroporation and nanoparticle delivery are emerging alternatives with significant promise for expanding the range of editable species and improving transformation efficiency. As CRISPR-Cas9 technology continues to evolve, these delivery methods are likely to become more refined, offering exciting possibilities for plant breeding and genetic modification in agriculture.

Slide 1

Hands-on Training of CRISPR/Cas9 for Crop Improvement



03.02.2025
Organised by Bangladesh Agricultural Research Council, Dhaka

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

Slide 2

Outline

A. Background of Genes' function

B. Background of Gene Editing

C. CRISPR/Cas9 Gene Editing

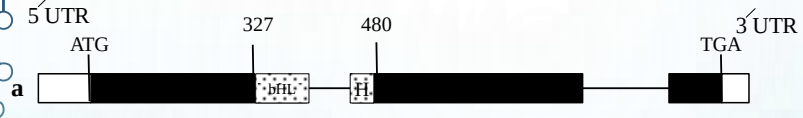
D. Primer design and multiplex CRISPR/Cas9 vector construction

E. Transformation of multiplex CRISPR/Cas9 vector into plant cell

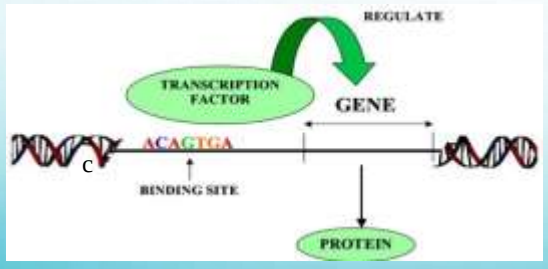
F. CRISPR/Cas9 edited OsbHLH024, OsbHLH044 and OsbHLH055

Slide 3

Background of Genes' function



OsbHLH044 (LOC_Os03g08930, Os03g0188400)



c

AP2/ERF, bHLH, bZIP, MYB, NAC, WRKY

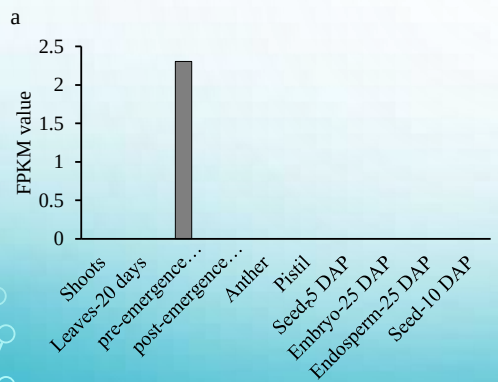
e

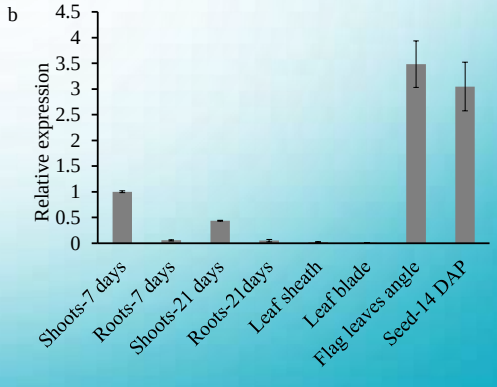
	TFs	family	bHLH
Arabidopsis	2016	56	162
Rice	2424	58	167

(Alam et al., 2021Riechmann et al., 2000; Bailey et al., 2003; Li et al., 2006a, Liang et al., 2021 and Latchman, 1997)

Slide 4

Background of Genes' function

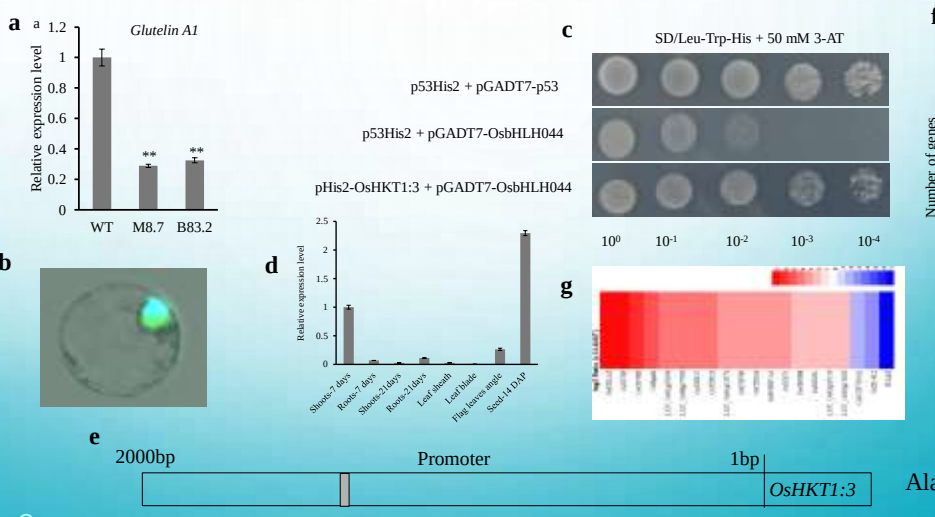




Expression level of OsbHLH055

Slide 5

Background of Genes' function



Alam et al., 2021

The function of genes OsbHLH044 and OsbHLH055

Slide 6

Background of Genes' function

Green Revolution, SD1 gene

It took almost 10,000 years for food grain production to reach 1 billion tons, in 1960, and only 40 years to reach 2 billion tons, in 2000. This unprecedented increase, which has been named the '**green revolution**', resulted from the creation of genetically improved crop varieties, combined with the application of improved agronomic practices.

GS Kush 2001-nature reviews genetics

The semi-dwarf trait in green revolution rice was originally derived from the Chinese cultivar Dee-Geo-Woo-Gen (DGWG), IR8, known as the miracle rice, was also bred by using *sd1*. *SD1* gene encodes the rice Gibberellin 20 oxidase-2 (GA20ox2). As such, the *SD1* gene is instrumental in uncovering the molecular mechanisms underlying gibberellin biosynthesis-Youlin Peng et al., 2021

CRISPR edited some examples

Drought and salt tolerance (OsDST) gene-Santosh Kumar, 2020, OsbHLH024- salt tolerance, Alam 2021, Os8N3 in rice to confer resistance to *Xanthomonas oryzae*-AK Young 2019

Slide 7

Background of gene editing

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.

I. Zinc Finger Nucleases (ZFNs)
II. Transcription Activator-Like Effector Nucleases (TALENs)
III. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

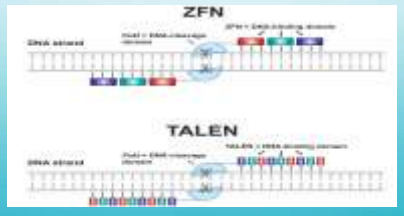
SK Gupta et al., 2017, M Pacesa et al., 2024, J McMahan et al., 2024

Slide 8

Background of gene editing

1. Zinc Finger Nucleases (ZFNs) are artificial restriction enzymes that can bind and cleave DNA at specific sequences. They are composed of two protein domains: a zinc finger DNA-binding domain and a DNA-cleavage domain. reported in 2003, though it was discovered in 1985. It sometimes targets the wrong sequence.

2. Transcription Activator-Like Effector Nucleases (TALENs) are artificial restriction enzymes that can be engineered to cut specific sequences of DNA. They are made by fusing a TAL effector DNA-binding domain to a DNA cleavage domain. First published in 2009 in Science, Germany. TALENs have a higher efficiency than ZFNs.

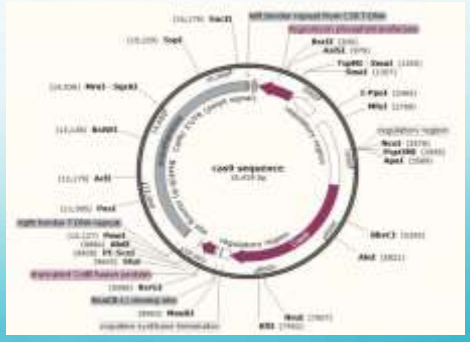


L F Clark et al., 2024

Slide 9

CRISPR/Cas9 gene editing

In 2012, it was discovered that the bacterium Streptococcus pyogenes could be adapted for gene engineering. This system consists of the "Clustered regulatory interspaced short palindromic repeat" CRISPR of RNA, which acts as a guide, and the Cas9 working as an endonuclease and enabling double-strand breaks (DSBs)



pYLCRISPRCas9Pubi-H

Slide 10

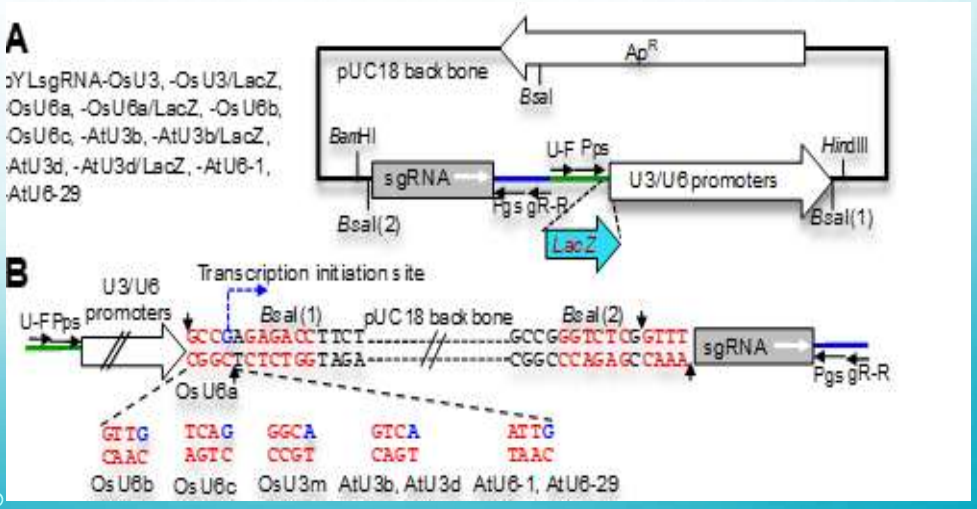
Primer design and vector construction

http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/SCORE

seq_id	sgRNA_id	Score	Sequence	strand	pos	%GC
LOC_Os03g08930	Guide9	0.7466	GAGGCCGTGGTGTTTCCGCAGGG	+	134	65%
LOC_Os03g08930	Guide38	0.6975	GGTGGCATCCAAGGATAACTCGG	+	313	50%

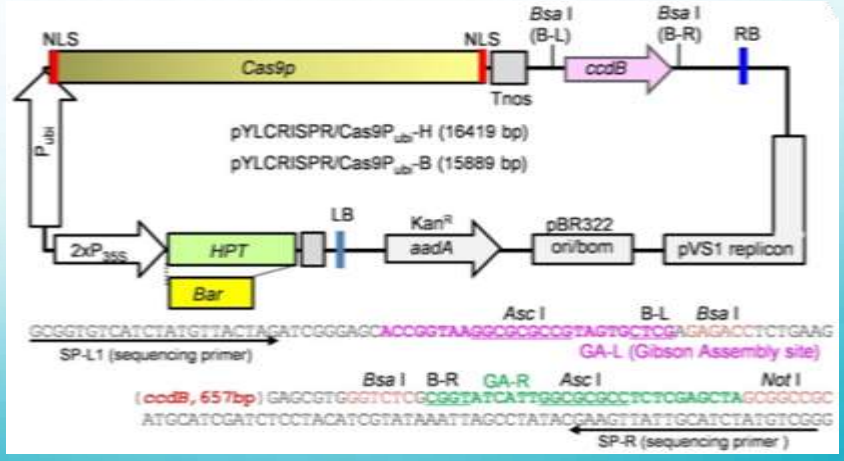
Slide 11

Primer design and vector construction



Slide 12

Primer design and vector construction

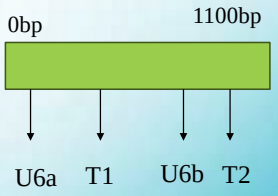


Slide 13

Primer design and vector construction

1. Preparation of Guide RNA by mixing forward (F) and Reverse (R)

Target	Volume (µl)	
Guide1 T1F	914	2 µl+ 2 µl+16 µl dd
Guide1 T1R	884	water=20 µl Target 1



2. Ligation of two targets (1 and 2) Guide RNA with site-specific promoters

Component	Volume (µl)
10XT4 DNA Ligase buffer	1 µl
10XT4 DNA Ligase	0.1 µl
u6a promoter	0.5 µl
Target 1	0.5 µl
Dd water	7.9 µl
Total	10 µl

Slide 14

Primer design and vector construction

3. Two targets with U-F Primers and gR-R- Primers

Component	Volume (µl)
KOD buffer	7.5 µl
gR-R- Primers	0.2 µl
U-F Primers	0.2µl
dNTPs	1.5 µl
Target 1	5.4 µl
KOD FX (enzyme)	0.2 µl
Total	15 µl

PCR: 94ºC-2m, (94ºC – 10 S, 60 ºC -15 S, 68 ºC -20 S) Cycle-28, Cas 1 PCR about 50 min. Adjust volume up to 100 µl

Check sequence

4. Two targets with PpS-GGL - PGS- GG2 Primers

Component	Volume (µl)
KOD buffer	15 µl
dNTPs	6 µl
Primer- PpS-GGL	0.9 µl (1T)
Primer-PGS- GG2	0.9 µl (1R)
KOD FX (enzyme)	0.6 µl
PCR (Target 1)	3 µl
DD water	3.6 µl
Total	30 µl

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

Slide 15

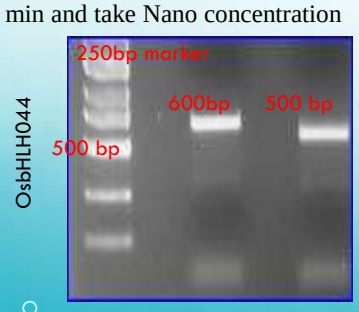
Primer design and vector construction

5. Gel Electrophoresis

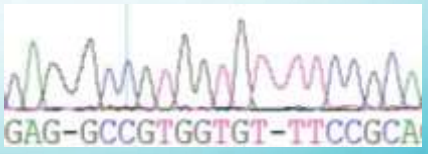
If you find clear red coloured bands with accurate sizes of U6a and U6b, cut the bands and put them in a 2 ml tube. Weight tubes and measure the accurate weight of the band (gram).

6. 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



U6a/Target 1 U6b/Target 2



U6a/Target 1

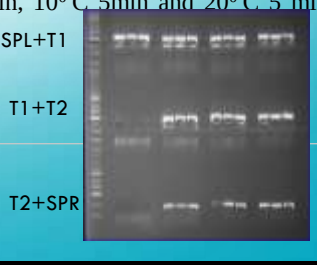
U6b/Target 2

Slide 16

Primer design and vector construction

7. Ligation of two targets with CRISPR/Cas9 vector

Component	Volume (µl)	Component	Volume (µl)
Cut smart buffer	1.5 µl	Mixture (Step A)	15 µl
Bas I-HF	0.8 µl	10XT4 DNA Ligase	0.15 (0.2 µl)
DD Water	11.3 µl	ATP	1 µl
CRISPR/Cas 9 vector	1 µl (60-80 ng/µl)	PCR-Cas3 for 4 hours (37 ^{0}C 5 min, 10^{0}C 5min and 20^{0}C 5 min, 15 cycles)	
U6a-sgRNA	0.6 µl (10-15 ng/ µl)		
U6b-sgRNA	0.6 µl (10-15 ng/ µl)		
Total	15 µl		
Shake and heat at 37 ^{0}C for 15 min.			

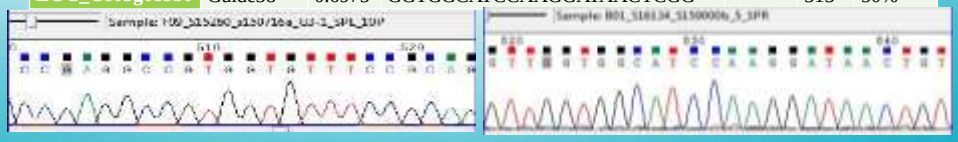


Slide 17

Transformation of multiplex CRISPR/Cas9 vector into plant cell

1. Confirmation of CRISPR/Cas9 Vector+ target genes sequences
2. Transformation of CRISPR/Cas9 Vector+ target genes sequences to *E. coli* bacteria
3. Confirmation of CRISPR/Cas9 Vector+ target genes sequences in *E. coli* 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

seq_id	sgRNA_id	Score	Sequence	strand	pos	%GC
LOC_Os03g08930	Guide9	0.7466	GAGGCCGTGGTGTTTCCGCAGGG	+	134	65%
LOC_Os03g08930	Guide38	0.6975	GGTGGCATCCAAGGATAACTCGG	+	313	50%

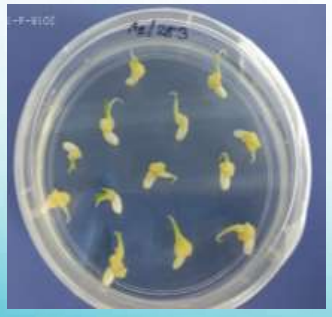


U6a/Target 1

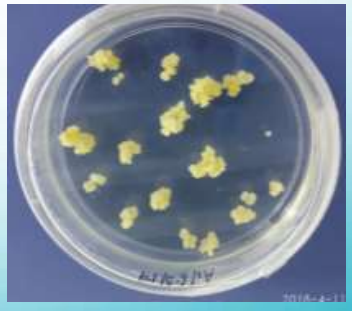
U6b/Target 2

Slide 18

Transformation of multiplex CRISPR/Cas9 vector into plant cell



20-days



30-days

Preparation of Rice Callus

Molecular Assays for Mutation Detection and Phenotypic Evaluation of Edited Plants

Dr. Shah Ashadul Islam
Senior Scientific Officer (SSO)
Agronomy Division, BRRI, Gazipur-1701

1. Introduction CRISPR-based genome editing introduces precise genetic modifications in plants, necessitating robust molecular assays for detecting mutations and evaluating resultant phenotypes. This lecture covers key molecular assays for mutation detection and phenotypic evaluation of edited plants.

2. Molecular Assays for Mutation Detection Mutation detection involves identifying and confirming CRISPR-induced genetic modifications through various molecular techniques.

2.1 Polymerase Chain Reaction (PCR)-Based Assays

- **T7 Endonuclease I (T7E1) Assay**: Detects insertions and deletions (indels) by cleaving mismatched DNA strands.
- **Cleaved Amplified Polymorphic Sequences (CAPS) and Derived Cleaved Amplified Polymorphic Sequences (dCAPS)**: Uses restriction enzyme digestion to identify sequence alterations.
- **High-Resolution Melting (HRM) Analysis**: Detects small sequence variations by analyzing DNA melting curves.

2.2 Sequencing-Based Assays

- **Sanger Sequencing**: Used for small-scale mutation confirmation by comparing edited and wild-type sequences.
- **Next-Generation Sequencing (NGS)**: Provides deep sequencing of target regions for precise mutation analysis.
- **Whole-Genome Sequencing (WGS)**: Assesses genome-wide off-target effects.

2.3 Genotyping and Off-Target Analysis

- **In Silico Prediction Tools**: Software such as Cas-OFFinder and CRISPResso for predicting potential off-target sites.
- **CRISPR-Cas Variants with Enhanced Specificity**: Engineered versions (e.g., Cas9-HF1, eSpCas9) to minimize off-target effects.

3. Phenotypic Evaluation of Edited Plants Assessing the impact of CRISPR-induced mutations on plant traits is essential for validating successful genome edits.

3.1 Morphological and Agronomic Trait Analysis

- **Growth Parameters**: Plant height, leaf shape, and biomass measurement.
- **Yield Components**: Grain weight, kernel number, and fruit size.
- **Abiotic Stress Responses**: Drought, salinity, and heat tolerance studies under controlled conditions.

3.2 Microscopic and Biochemical Assays

- **Histological Staining**: Examines cell structure alterations (e.g., GUS staining for reporter genes).
- **Chlorophyll Content and Photosynthetic Efficiency**: Measures physiological impact of mutations.
- **Enzyme Activity Assays**: Evaluates biochemical changes in metabolic pathways.

3.3 Molecular and Metabolomic Analysis

- **RT-qPCR and RNA-Seq**: Assesses changes in gene expression due to genome edits.
- **Proteomics and Metabolomics**: Identifies alterations in protein abundance and metabolic pathways.

4. Conclusion Molecular assays are critical for confirming CRISPR-induced mutations and assessing their functional consequences. Combining genotypic and phenotypic evaluations ensures the development of improved crop varieties with desirable traits.

CRISPR Experimental Design for a Selected Crop Trait

Dr. Shah Ashadul Islam
Senior Scientific Officer (SSO)
Agronomy Division, BRRI, Gazipur-1701

1. Introduction CRISPR-Cas genome editing enables precise modification of genes associated with key agronomic traits in crops. A well-designed CRISPR experiment ensures successful gene targeting, minimal off-target effects, and effective phenotypic validation. This lecture outlines the essential steps for designing a CRISPR experiment for improving a selected crop trait.

2. Selecting the Crop and Target Trait

- Choose a trait with significant agricultural importance (e.g., disease resistance, drought tolerance, yield improvement).
- Identify the target gene(s) associated with the trait using genomic databases and prior studies.

3. Guide RNA (gRNA) Design and Selection

- Use bioinformatics tools such as CHOPCHOP, CRISPR-P, or Benchling to design gRNAs targeting the selected gene.
- Select gRNAs with high on-target efficiency and minimal off-target potential.
- Optimize gRNA specificity using CRISPR-Cas variants (e.g., eSpCas9, Cas12a).

4. Plasmid Construction and Delivery

- Choose an appropriate CRISPR vector (e.g., pCas9-T, pHSE401) based on plant transformation requirements.
- Insert gRNA sequences into the CRISPR-Cas9 construct using cloning techniques (e.g., Golden Gate assembly).
- Introduce the CRISPR construct into plant cells using:
    - **Agrobacterium-mediated transformation** (for dicots like tomato, soybean).
    - **Particle bombardment (biolistics)** (for monocots like rice, maize).
    - **Protoplast transfection** (for transient assays and functional validation).

5. Regeneration and Selection of Edited Plants

- Use selectable markers (e.g., antibiotic resistance genes) for screening transformed plants.
- Regenerate whole plants via tissue culture techniques.
- Confirm T0 generation plants through molecular assays.

6. Screening and Validation of Mutations

- **PCR-Based Assays:** T7E1 assay, CAPS, or HRM analysis for mutation detection.
- **Sequencing Approaches:** Sanger sequencing and NGS to confirm precise gene edits.
- **Off-Target Analysis:** Whole-genome sequencing and in silico prediction tools.

7. Phenotypic Evaluation of Edited Plants

- Assess morphological and agronomic traits (e.g., plant height, leaf morphology, seed yield).
- Perform stress tolerance assays under controlled conditions.
- Validate functional changes through biochemical and physiological analyses.

8. Field Trials and Regulatory Considerations

- Conduct multi-location field trials to evaluate agronomic performance.
- Address biosafety regulations and intellectual property aspects for commercialization.

9. Conclusion A successful CRISPR experimental design integrates precise gene editing, efficient plant transformation, rigorous molecular screening, and thorough phenotypic assessment. This approach accelerates crop improvement and sustainable agricultural practices.

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

Dr Md Panna Ali
Principal Scientific Officer
Crops Division, Bangladesh Agricultural Research Council
New Airport Road, Farmgate, Dhaka 1215

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:

TTGATGCAATTAATTCCACATTAATTCGCTGTTTCAACATCCATCTTACTAGTTA
GAGTACTACACAAGTATACACTATCTGTCGCATTCCACACCTTGATCTCAGATA
ACGTTGATCTCTTCAACTCATGTGATAAATCTAATGAAAATCTTCAGTGCCCAA
CGTGTGCATCTACCTCTCCTCCAACCAACCACCCACTATATAAACCCTTCCCCA
CTTAACCACGGTTAGTTCATACCAACACATCTCCATTGCTGCTTAAGTTTCTTG
GACCAAACGTGCACCCCAAGTGTTCGACGATATGGAGCTCACCATGGCGTCGA
CGATGTCGCTCGCGCTGCTCGTGCTCTCCGCGGCGTACGTGTTGGTCGCGTTG
AGGAGGAGCCGGTCGTCGTCGTCAAAGCCACGGCGGCTGCCGCCGTCGCCGC
CGGGGTGGCCGGTGATCGGGCACCTCCACCTCATGTCCGGCATGCCGCACCAC
GCGCTGGCCGAGCTGGCGCGCACCATGCGCGCGCCGCTGTTCCGGATGCGGC
TGGGGAGCGTGCCGGCGGTGGTGATCTCCAAGCCGGACCTCGCCCGCGCCGC
GCTCACCACCAACGACGCCGCGCTGGCGTCGCGGCCGCACCTGCTCTCCGGCC
AGTTCCTGTCGTTCGGCTGCTCCGACGTGACGTTCGCGCCGGCGGGGCCGTAC
CACCGGATGGCGCGCCGCGTGGTGGTGTCGGAGCTCCTGTCGGCGCGTCGCG
TCGCCACGTACGGCGCCGTCAGGGTCAAGGAGCTCCGCCGCCTGCTCGCGCAC
CTCACCAAGAACACCTCGCCGGCGAAGCCCGTCGACCTCAGCGAGTGCTTCCT
CAACCTCGCCAACGACGTGCTCTGCCGCGTCGCGTTCGGCCGCCGGTTCCCGC
ACGGCGAGGGCGACAAGCTCGGCGCGGTGCTCGCCGAGGCGCAGGACCTCTT
CGCCGGGTTCACCATCGGCGACTTCTTCCCCGAGCTCGAGCCCGTCGCCAGCA
CCGTCACCGGACTCCGCCGCCGCCTCAAGAAGTGCCTCGCCGACCTCCGCGAG
GCCTGCGACGTGATCGTGGACGAACACATCAGCGGCAACCGCCAGCGCATCCC
CGGCGACCGCGACGAGGACTTCGTCGACGTCCTCCTCCGCGTCCAGAAATCCC
CCGACCTCGAGGTCCCCCTAACCGACGACAATCTCAAGGCCCTCGTCCTGGAC
ATGTTCGTCGCCGGCACGGACACCACGTTCGCGACGCTGGAGTGGGTGATGAC
GGAGCTAGTCCGCCACCCACGGATCCTCAAGAAGGCGCAGGAGGAGGTCCGG
CGAGTCGTCGGCGACAGCGGCCGCGTCGAGGAGTCCCACCTCGGCGAGCTCC
ACTACATGCGCGCCATCATCAAGGAGACGTTCCGGCTGCACCCGGCGGTGCCG
TTGCTAGTGCCGCGCGAGTCCGTCGCGCCGTGC

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

Gene		**Primer sequence (5′→3′)**	**Purpose**
	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	
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/ l 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.

Name of the media	Composition
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).

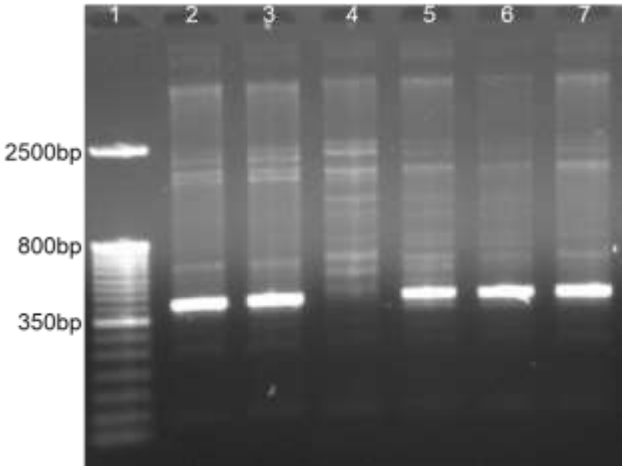


Fig. 1. Electrophoresis of PCR product amplified by target primers of CYP71A1 and Cas9 vector. Lane 1: DNA lader (50bp); lane 2 - 7: Recombinant Cas9 vector DNA.

```
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 CTTAAATTGATAAGGGGTTTTAGGGGGATAGGGTATAAATACAAGCATTCCCTTGTTGTT 300

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

CYP71A1          ---------------------------------------------------------------------------------- 20
Cas9/gRNA-CYP71A1 CTTCT ------------------------------------------------------------------------------ 365
```

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.

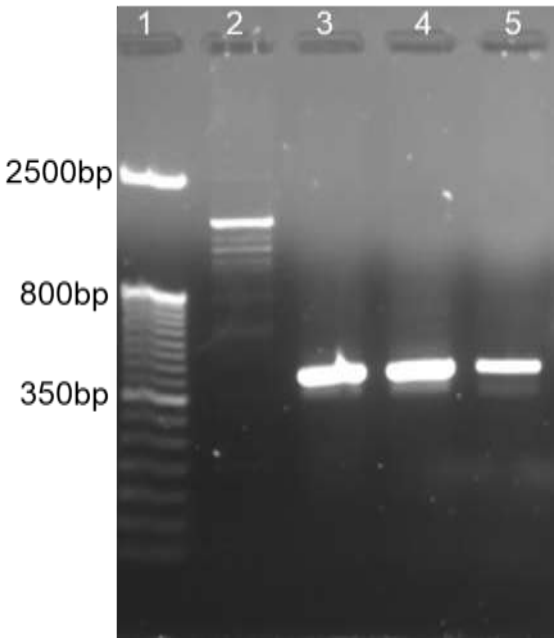


Fig. 3. Electrophoresis of PCR product amplified by target primers of CYP71A1 and Cas9 vector. Lane 1: DNA ladder (50bp); lane 2: Blank Agrobacterium LBA4404 and 3 - 5: Recombinant Agrobacterium LBA4404.

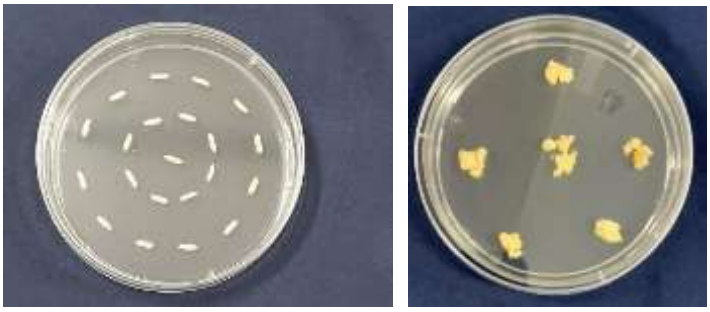


Fig. 4. Development of callus. Dehusked seeds were placed in callus induction plate (left side) and callus developed from seed culture plate (right side).

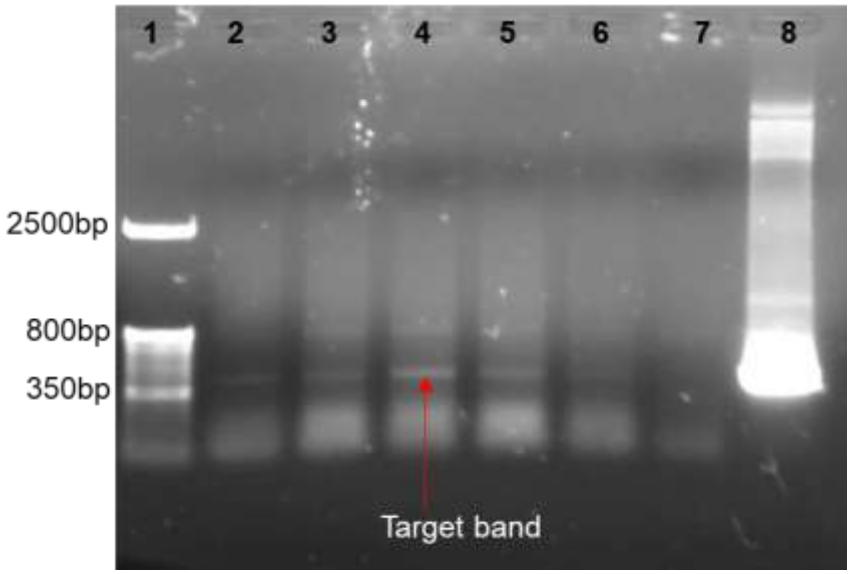


Fig. 5. Electrophoresis of PCR product amplified by SpCas9 primers. Lane 1: DNA ladder (50bp); lanes 2 - 6: CRISPR Cas9 edited plants (mutants); lane 7: BRRI dhan87 (control) and lane 8: Cas9/gRNA-CYP71A1 recombinant vector.

Ethical, Regulatory, and Biosafety Aspects of CRISPR-Edited Crops Globally

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The regulatory landscape for CRISPR-edited crops varies globally, influencing research, commercialization, and public acceptance.

9.1 Ethical Considerations

- Concerns over unintended genetic consequences and ecological impact.
- Debate over labeling requirements and consumer rights.
- Potential socio-economic implications, including farmer access to CRISPR-edited seeds.

9.2 Regulatory Frameworks

United States (USDA, FDA, EPA)

- The **USDA** does not regulate CRISPR-edited crops if no foreign DNA is introduced.
- The **FDA** evaluates the safety of CRISPR-edited crops for human consumption.
- The **EPA** regulates crops with pesticidal traits (e.g., those expressing insecticidal proteins).

European Union (EU)

- The EU classifies CRISPR-edited crops as GMOs, subjecting them to strict regulations.
- Requires extensive safety assessments, environmental impact studies, and labeling for consumer awareness.
- Ongoing debates on revising policies to distinguish CRISPR edits from traditional GMOs.

Asia

- **China:** Supports CRISPR research with newly established guidelines for crop approval.
- **Japan:** Allows CRISPR-edited crops without foreign DNA under a relaxed regulatory framework.
- **India:** Maintains strict GMO policies but is reviewing CRISPR regulations for potential adoption.

9.3 Biosafety Considerations

- Risk assessment of gene flow to wild relatives.

- Monitoring for unintended mutations and ecological effects.
- Development of regulatory guidelines to ensure safe deployment.

Lecture: Applications of CRISPR-Cas9 in Enhancing Resistance

CRISPR-Cas9 has been widely used to enhance resistance in crops against biotic and abiotic stresses by precisely modifying resistance-related genes.

Resistance to Biotic Stresses (Pathogens and Pests)

- **Example 1: Powdery Mildew Resistance in Wheat**
  - The **MLO (Mildew Locus O)** gene was knocked out in wheat using CRISPR-Cas9.
  - Mutant plants showed enhanced resistance to powdery mildew without yield penalties.
- **Example 2: Bacterial Blight Resistance in Rice**
  - The **OsSWEET13** gene was edited to prevent infection by *Xanthomonas oryzae*.
  - CRISPR-induced mutations blocked pathogen-induced gene expression, increasing resistance.

Resistance to Abiotic Stresses (Drought, Salinity, and Heat)

- **Example 3: Drought Tolerance in Maize**
  - CRISPR was used to modify the **ARGOS8** gene, which regulates ethylene response.
  - Edited maize plants exhibited improved drought tolerance and better yield stability under water stress.
- **Example 4: Salt Tolerance in Tomato**
  - The **SlHKT1;2** gene was knocked out to improve ion homeostasis under saline conditions.
  - Resulted in enhanced salt tolerance without negative effects on growth.

Insect Resistance

- **Example 5: Pest Resistance in Rice**
  - Editing the **Bph3** gene increased resistance to *Nilaparvata lugens* (brown planthopper).
  - CRISPR-based modifications improved plant defense mechanisms.

Future Prospects

- Expanding CRISPR applications to more staple crops (e.g., potato, cassava, sorghum).
- Combining CRISPR with other breeding techniques for durable resistance.
- Addressing regulatory hurdles for global acceptance and commercialization.

Conclusion

A successful CRISPR experimental design integrates precise gene editing, efficient plant transformation, rigorous molecular screening, and thorough phenotypic assessment. Furthermore, ethical, regulatory, and biosafety considerations play a crucial role in determining the adoption and commercialization of CRISPR-edited crops worldwide. The ability of CRISPR-Cas9 to enhance resistance to biotic and abiotic stresses represents a major breakthrough in sustainable agriculture.

Hands-on Training on CRISPR-Cas9 for Crop Improvement

Organized by : Crops Division, Bangladesh Agricultural Research Council (BARC)
Date and time : 03-05 February 2025, Time: 9.30 AM - 5.00 PM
Venue : Conference room-2 (3rd floor), Main Building, BARC, Farmgate, Dhaka.
Participants : 30 Scientists from NARS institutes, University and private sectors.
Course Director : Dr. Md. Abdus Salam, Member Director (Crops), BARC.
Course Coordinator : Dr. Md. Panna Ali, Principal Scientific Officer, Crops Division, BARC.

Day 1 (03.02.2025)		
Time	**Topics**	**Facilitators**
09.00-09.30	**Registration**	**Dr. Md. Jamal Uddin** Principal Scientific Officer (Crops), BARC
09.30-10.00	**Inaugural program**	**Welcome address: Dr. Md. Panna Ali** Principal Scientific Officer, Crops Division, BARC **Chief Guest: Dr. Nazmun Nahar Karim** Executive Chairman, BARC **Chair: Dr. Md. Abdus Salam** Member Director (Crops), BARC
10.00-10.30	**Tea break**	
10.30-11.30	Recent advances of CRISPR-based genome editing for enhancing staple crops	**Dr. Md. Abdus Salam** Member Director (Crops), BARC
11.30-12.30	Introduction to CRISPR-Cas9 Technology (biology, mechanisms, and relevance)	**Dr. Md. Panna Ali** Principal Scientific Officer (PSO) Crops Division, BARC, Dhaka
13.00-14.00	**Lunch and prayer break**	
14.00-15.00	Primer design and multiplex CRISPR/Cas9 vector construction	**Dr. Shah Alam** Deputy Director, Plant Quarantine Station, Seaport, Chattogram
15.00-16.00	Transformation of multiplex CRISPR/Cas9 vector into plant cell	**Dr. Shah Alam** Deputy Director, Plant Quarantine Station, Seaport, Chattogram
16.00-17.00	Screening and validation of CRISPR-induced mutations.	**Dr. Shah Alam** Deputy Director, Plant Quarantine Station, Seaport, Chattogram
Day 2 (04.02.2025) (BRRI)		
Time	**Topics**	**Facilitators**
09.30-10.30	Lab protocols: DNA extraction, tissue culture, and plasmid construction.	**Dr. Shahanaz Sultana** CSO (Current Charge) and Head Biotechnology Division BRRI, Gazipur-1701
10.30-11.00	**Tea break**	
11.00-12.00	Lab protocols: DNA extraction, tissue culture, and plasmid construction.	**Dr. Shahanaz Sultana** CSO (Current Charge) and Head Biotechnology Division

		BRRI, Gazipur-1701
12.00-13.00	Lab protocols: DNA extraction, guide RNA design, and plasmid construction.	**Dr. Shahanaz Sultana** CSO (Current Charge) and Head Biotechnology Division BRRI, Gazipur-1701
13.00-14.00	**Lunch and prayer break**	
14.00-15.00	**CRISPR-Cas9 Workflow** Delivery methods for CRISPR-Cas9 in plants (e.g., Agrobacterium-mediated transformation, electroporation, and nanoparticle delivery).	**Dr. Shah Ashadul Islam** Senior Scientific Officer (SSO) Agronomy Division, BRRI, Gazipur
15.00-15.30	**Tea break**	
15.30-16.30	**CRISPR-Cas9 Workflow** Molecular assays for mutation detection and phenotypic evaluation of edited plants	**Dr. Shah Ashadul Islam** Senior Scientific Officer (SSO) Agronomy Division, BRRI, Gazipur
Day 3 (05.02.2025) (BRRI)		
Time	**Topics**	**Facilitators**
09.30-10.30	CRISPR experimental design for a selected crop trait.	**Dr. Md. Panna Ali** Principal Scientific Officer (PSO) Crops Division, BARC, Dhaka
10.30-11.00	**Tea break**	
11.00-12.00	Screening and validation of CRISPR-induced mutations.	**Dr. Shah Ashadul Islam** Senior Scientific Officer (SSO) Agronomy Division, BRRI, Gazipur
12.00-13.00	Gene selection, guide RNA design, and recombinant plasmid construction.	**Dr. Md. Panna Ali** Principal Scientific Officer (PSO) Crops Division, BARC, Dhaka
13.00-14.00	**Lunch and prayer break**	
14.00-15.00	Ethical, Regulatory, and Biosafety Aspects of CRISPR-edited crops globally (e.g., USA, EU, Asia).	**Dr. Shah Ashadul Islam** Senior Scientific Officer (SSO) Agronomy Division, BRRI, Gazipur
15.00-16.00	Applications of CRISPR-Cas9 in Enhancing Resistance	Dr. Md. Panna Ali Principal Scientific Officer (PSO) Crops Division, BARC, Dhaka
16.00-17.00	**Closing & Certificate giving ceremony**	**Chief Guest: Dr. Nazmun Nahar Karim** Executive Chairman, BARC **Chair: Dr. Md. Abdus Salam,** Member Director (Crops Division), BARC