Agricultural Biotechnology Research Institute of Iran

Economic Cooperation Organization

ECOABN

Workshop on

Genetically Modified Organisms (GMOs) Bio-Safety and Regulation

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New technologies in genetic engineering of plants

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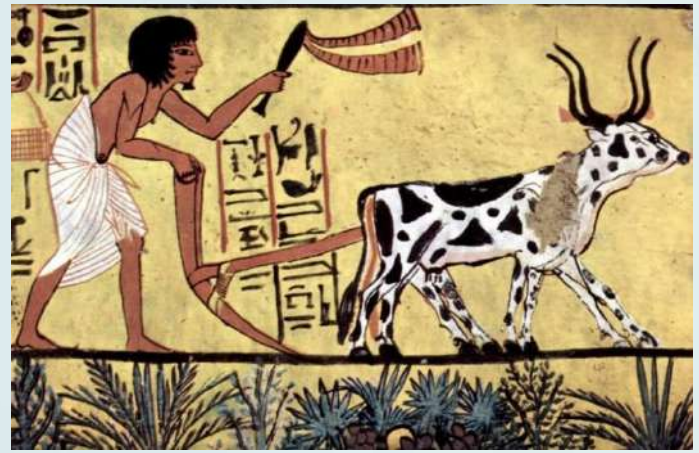
This presentation, covers the following topics:

- **The background of plant breeding, mutation breeding and genetic engineering**
- **The foundations of site-directed mutagenesis, especially CRISPR-Cas9 system and their applications**
- **Concerns and regulations about gene edited crops**

History of plant genetic manipulation

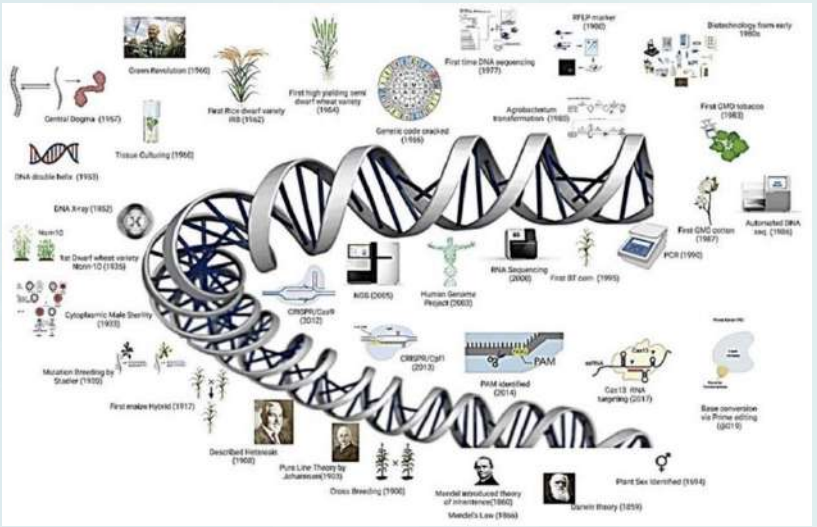
➢ **For thousands of years:**

- Human have developed many crops by "**Trial and error**"
- The main method for crop improvement was the '**repeated cycles of selecting superior varieties'**



➢ **From 1900**

- Many methods and tools for plant breeding were developed
- Alongside the expansion of irrigation infrastructure, synthetic fertilizers, pesticides, etc. ⟹ **'Green Revolution (1950 and 1984)'**



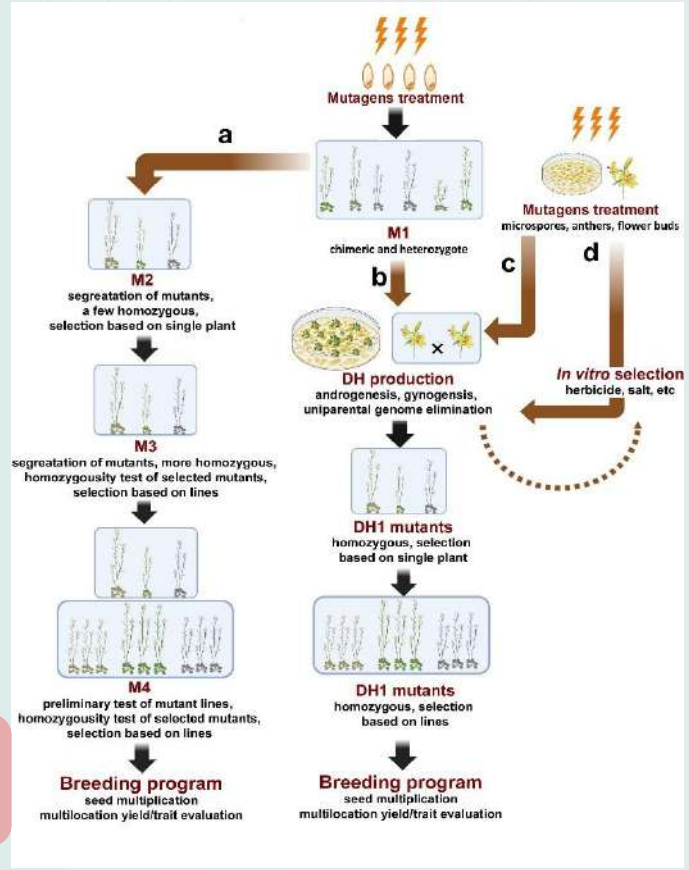
➢ **Over the last decades**

- Development of methods and tools for genetic transformation
- Progress of system biology, genome sequencing and precise genome editing ⟹ **Gene Revolution?**

Mutation breeding

First direct modification of plant genome

- Started in 1920 with the radiation of plants to induce genetic variations
- The first mutant cultivar “Chlorine” was developed in tobacco in 1927
- Due to development of molecular biology methods, new methods for random mutagenesis are introduced



The current list of method for random mutagenesis:

- **Physical mutagens:** gamma radiation, UV radiation
- **Chemical mutagens:** Alkylating mutagens (MNU, EMS)
- **Tissue culture mediated variation:** somaclonal and gametoclonal
- **Biological mutagenesis:** Transposons, RNAi/transgenic/mutated plants with defects in precision DNA repair, ZFN, TALENs, and CRISPR

Applications of mutation breeding

- **Developing new variants:** resistant/tolerant to biotic and abiotic stresses, herbicide resistance, improved quality and yield, etc.
- **Forward and reverse genetics studies:** to relate a specific trait to the genetic factors, for example the discovery of the role of ALS gene mutation in herbicide resistance

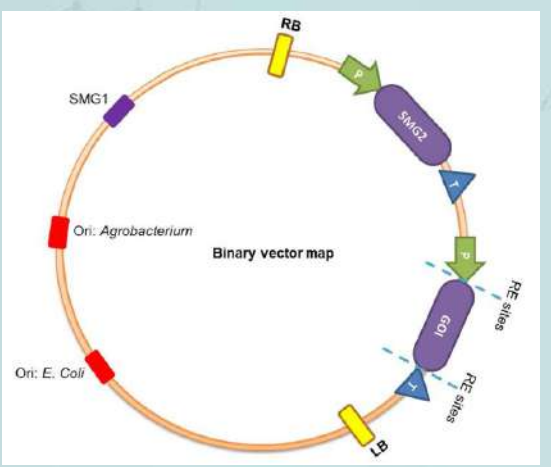
Since the method was developed before the introduction of recombinant DNA methods

Plants developed using mutation breeding are excluded from GMO rules

- ✓ The Joint FAO/IAEA MVD Program (2023) lists greater than 3,432 registered mutant lines
- ✓ There is no report about adverse effects of mutant lines on environment or human health

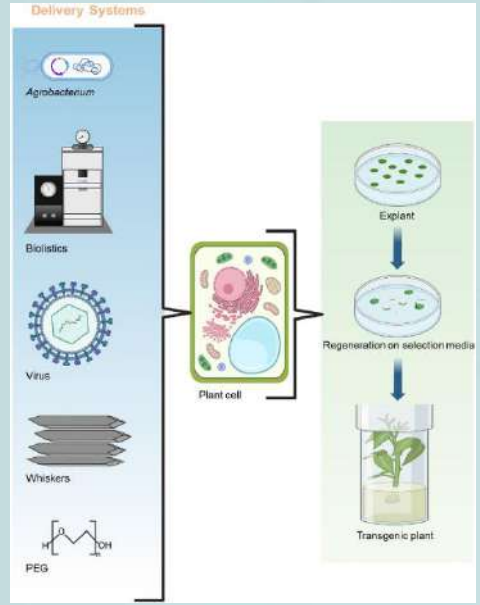
Plant transformation era

- In 1977, The natural capacity of Agrobacterium to integrate Ti plasmid DNA (T-DNA) into the DNA of recipient cell of plant was identified
- In 1983, the first in vitro plant transformation using Agrobacterium
- Development of other methods (Biolistic, PEG, Virus, and Nanotubes, etc) for plant and plastid transformation
- In 1994, the commercial release of the first genetically engineered plant (FlavrSavr Tomato)



More than 525 genetic transfer events in 32 crops commercialized until 2019 (ISAAA database 2019).

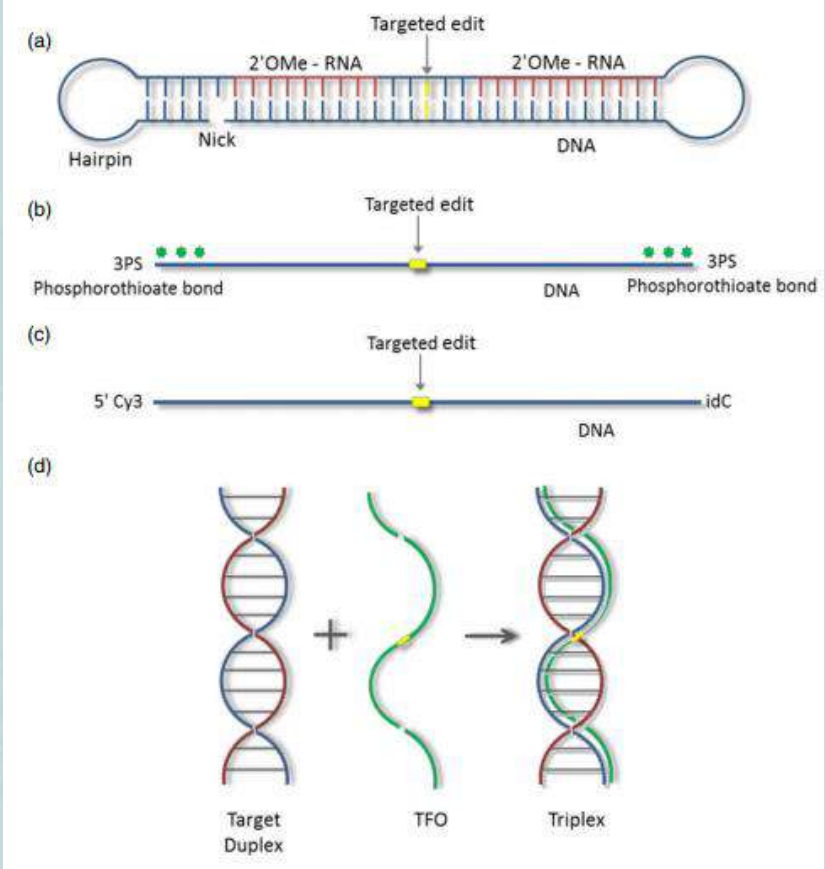
Approximately 190 million hectares of genetically modified crops were cultivated globally in 2019



Site-Directed Mutagenesis

➢ 1. Oligonucleotide-directed mutagenesis (ODM)

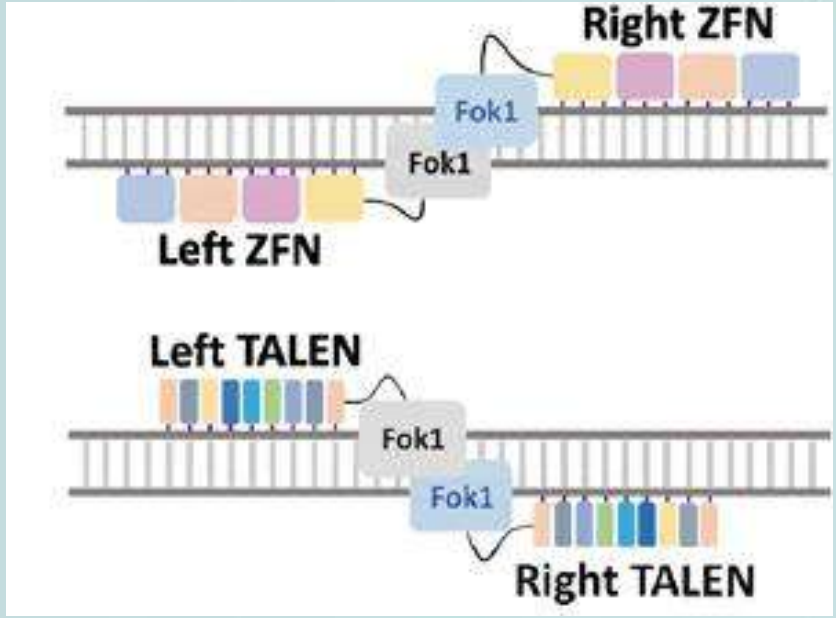
- Transferring a short DNA sequence into cells, to induce a site-specific mutation using DNA repair system
- The ODM method has been practical for plasmid mutation since 1978
- Applied in plants since 1999 using protoplast and cell bombardments
- Its low efficiency hindered its application in plants



➢ 2. Zinc Finger Nucleases (ZFNs)

➢ 3. Transcription Activator-Like Effector Nucleases (TALENs)

- Both ZFNs and TALEN identify their targets through protein-DNA interactions and rely on artificial restriction enzymes, Fok1, to create DSBs
- Both require the synthesis of new gene sequences that encode a particular protein for every novel site in the DNA

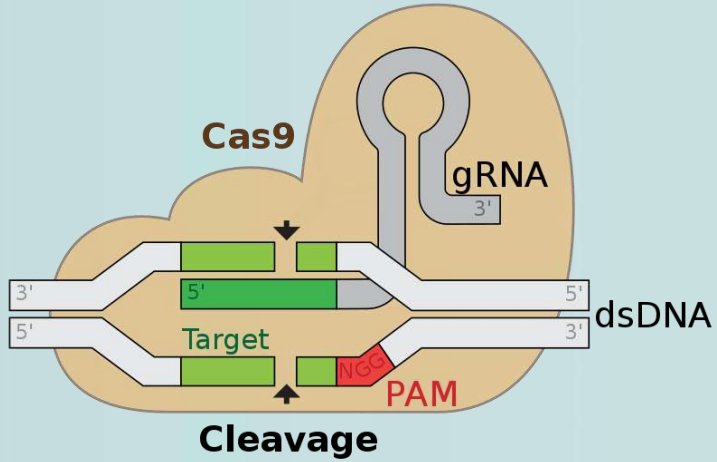


The complicated and prolonged assembly procedure of ZFNs and TALENs, hinder their practical applications

CRISPR-CAS9 system

➢ Clustered, Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR associated protein 9 (Cas9)

- First discovered in 1987 by microbiologists
- In 2007, proved as an immune system of bacteria and archaea
- Its application for genome editing began in 2012
- Cas enzyme screens the Protospacer Adjacent Motif (PAM) sequence in the genome, and single guide RNA (sgRNA) identifies the desired loci and turns the endonuclease function of Cas on



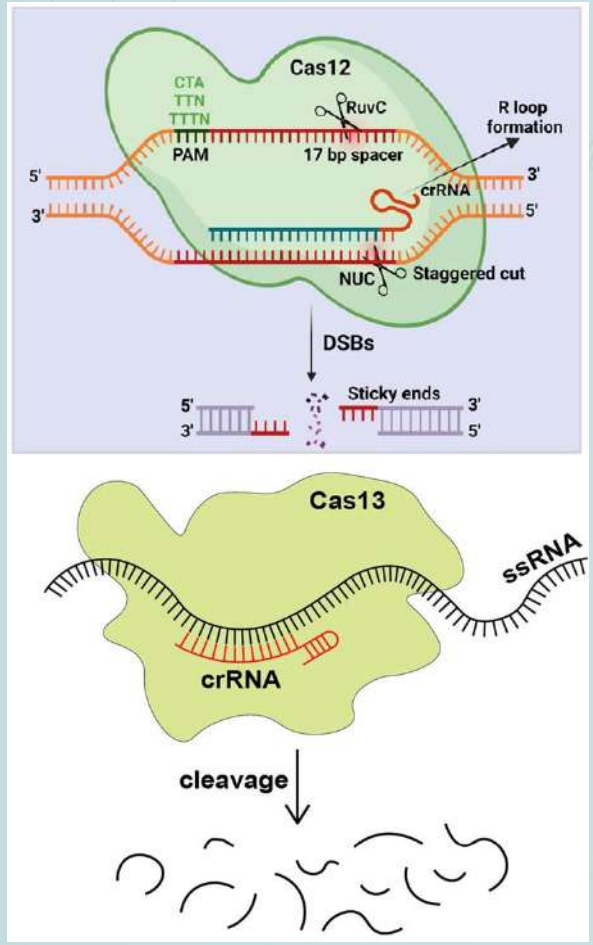
➢ CRISPR-CAS9 advantages over ZFNs and TALENs

- 1. The CRISPR/Cas9 system has a higher degree of specificity
- 2. Relies on RNA-based recognition rather than protein-based which makes its design and vector construction much easier
- 3. Capacity to carry out editing at multiple sites by co-expressing different gRNA molecules

Cas Classification and variants

- **Class-I** use a multiprotein complexe to cleave nucleic acids and it is not used for genomes editing
- **Class-II** (three types, II, V, and VI) employs only one protein effector domain to cleave the nucleic acids

- Cas 9 (type II) and Cas 12 (type V) have the RNA-guided DNA endonuclease activity
- Cas 13 (type VI) possesses the targeting activity of RNA and the cleavage activity
- Cas14 (Type V) without needing (PAM) and performs transcriptional repression and base-editing
- ……

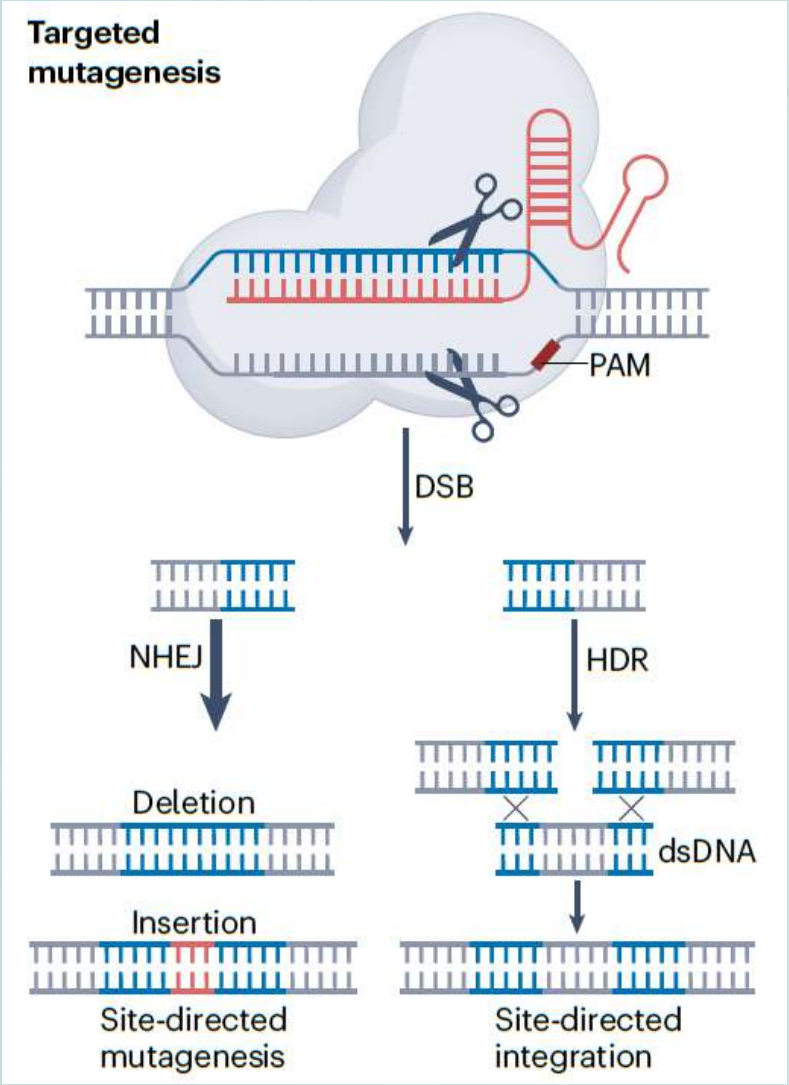


Applications of CRISPR/Cas system

Site directed mutagenesis and Site directed integration

- **The double-strand break (DSB) at the target site can be repaired by:**
- **1. Non-homologous end joining (NHEJ):**
- Often results in insertions and/or deletions that cause in-frame or out-of-frame mutation

- **2. Homology-directed repair (HDR):**
- Precise insertions (or deletions) by supplying a template DNA harboring the desired changes
- Very low efficiency in plants



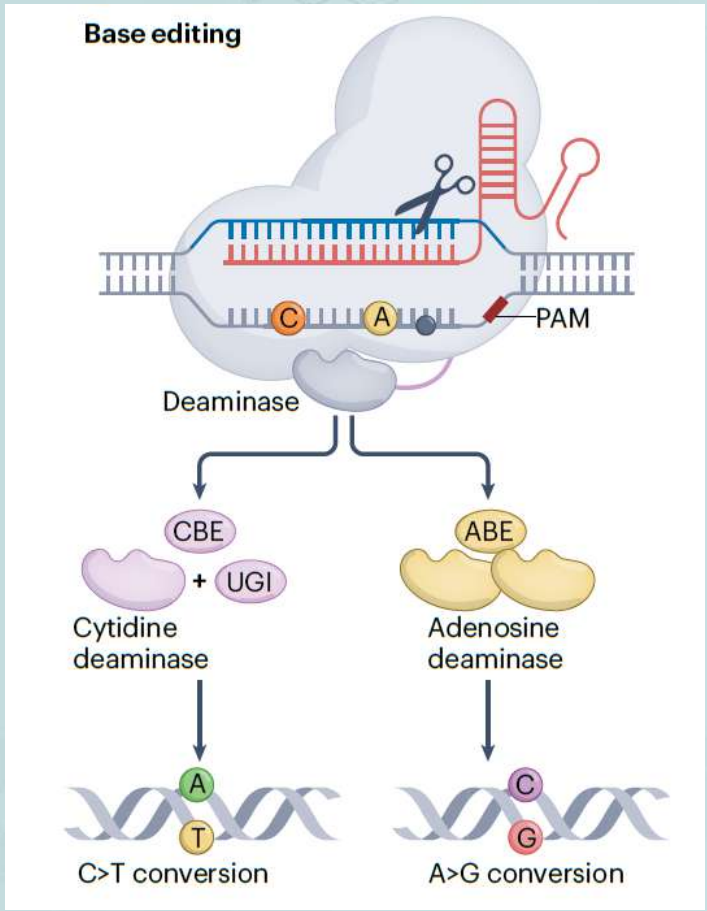
Applications of CRISPR/Cas system

Base editing

- The enzyme used for base editing is modified Cas9 (dead Cas9 -dCas9 or nCas9) which is a nuclease-deficient Cas9.

- **Adenine base editing (ABE, converts A-T to G-C)**
- Adenosine deaminase fused to dCas9

- **Cytosine base editing (CBE, converts C-G to T-A)**
- Cytidine deaminase fused to nCas9
- A uracil DNA glycosylase inhibitor (UGI) is also attached to nCas9 to inhibit excision of the uracil (formed as a result of deamination) by the repair enzyme uracil DNA glycosylase (UDG)

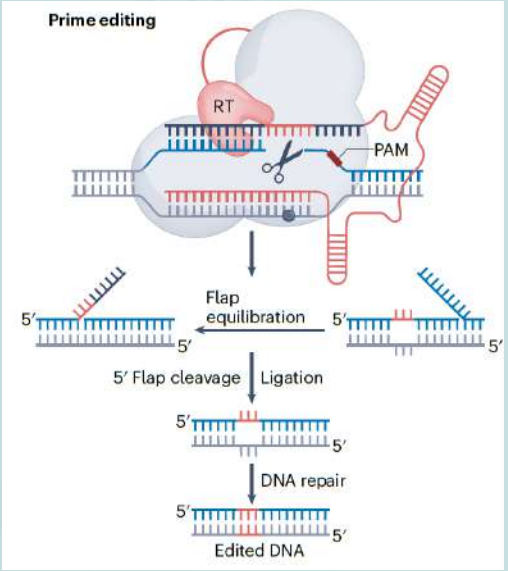


Applications of CRISPR/Cas system

Prime Editing

➢ The Prime editing system consists of

- nCas9 (H840A)
- Reverse transcriptase (RTase)
- Prime editing guide RNA (pegRNA)



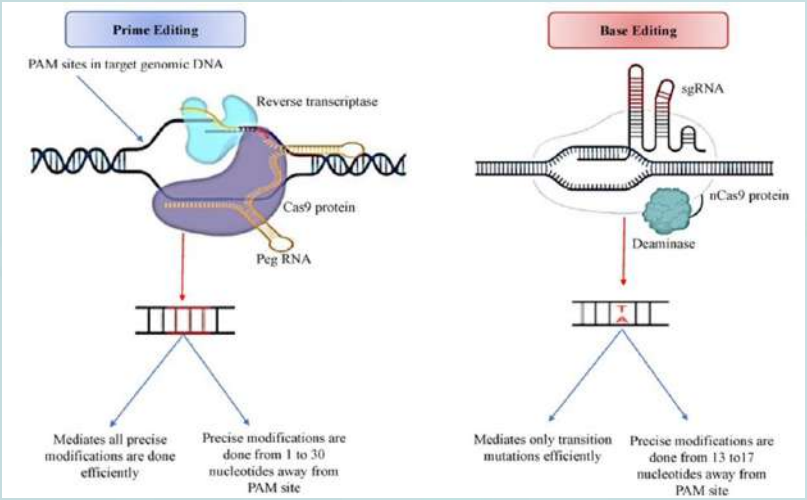
➢ Base Editing vs Prime Editing

• Prime editing

- Mediates all 12 types of nucleotide modifications
- Cover 1-30 nucleotides away from the PAM sequence

• Base editing

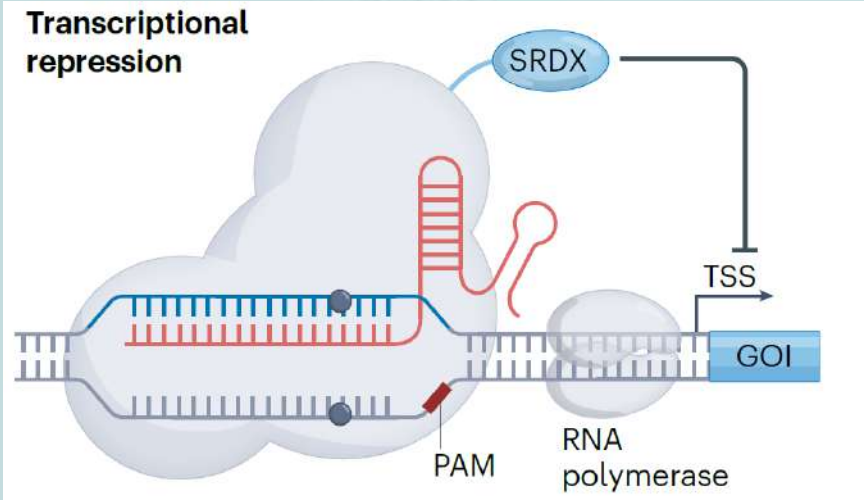
- Mediates 4 types of nucleotide modifications
- Cover 13-17 nucleotides away from the PAM sequence



Transcriptional activation/repression

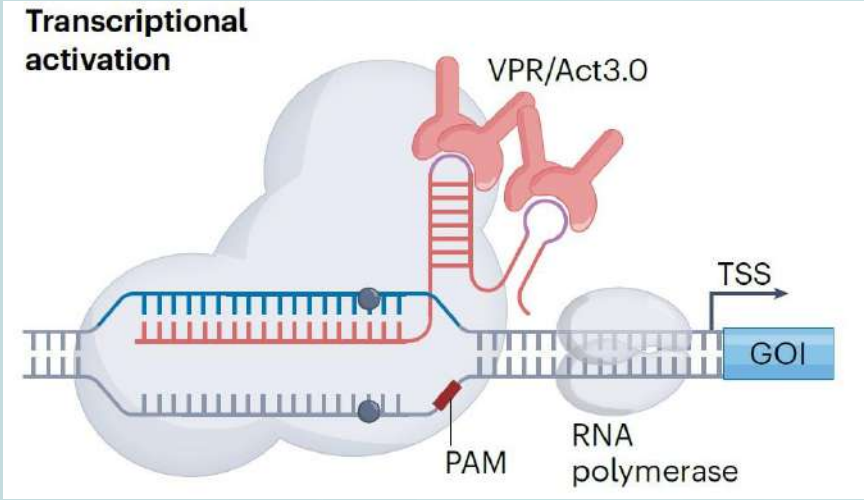
➢ Transcriptional activation

- Various types of activators attached to dCas help to recruit the transcriptional machinery



➢ Transcriptional repression

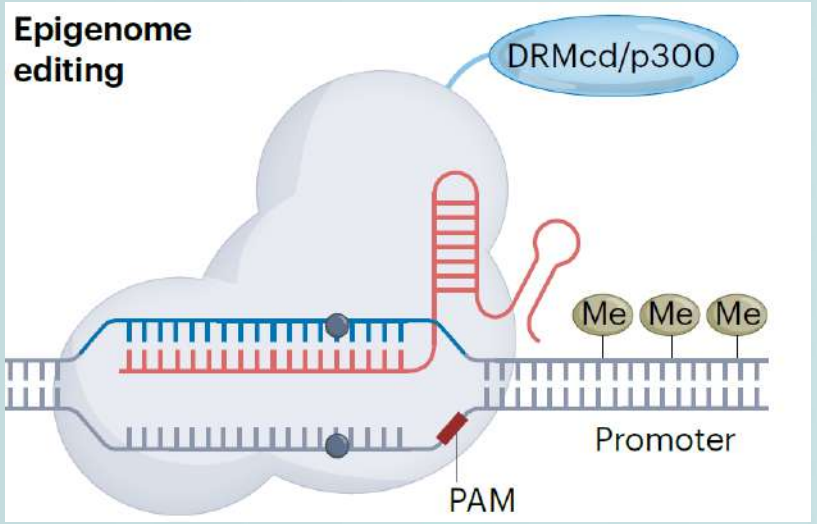
- Transcriptional repressors fused to dCas recruit other repressors and directly inhibit assembly or binding of the transcriptional machinery



Applications of CRISPR/Cas system

Epigenome modifications

- Expression of a gene can be regulated by altering its epigenetic status
- In this case, dCas is guided to the target gene promoter by an sgRNA



➢ The target gene can be silenced by:

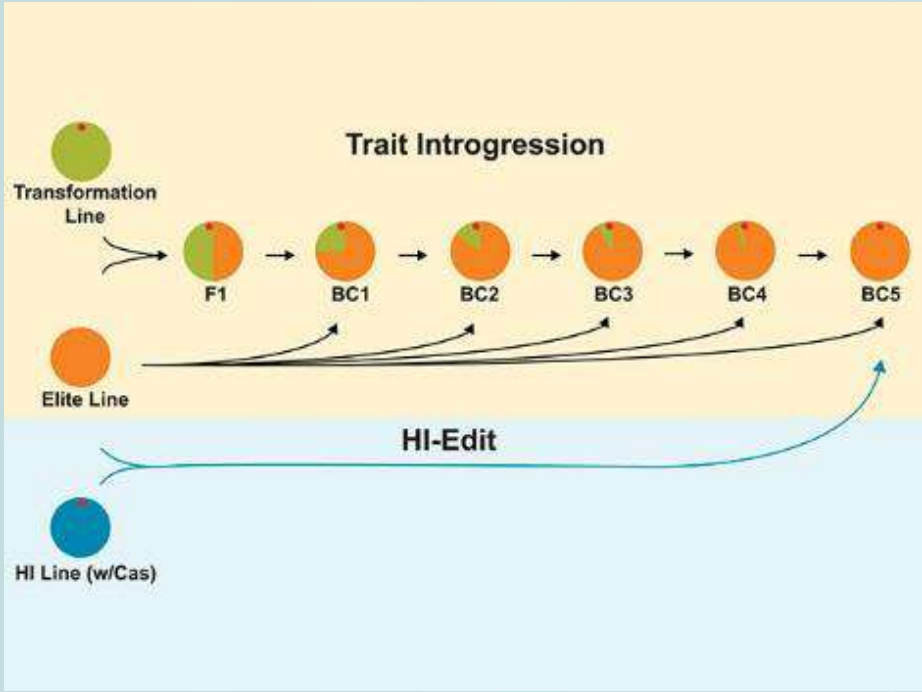
- **DNA methyltransferases** (for example, domains rearranged methylase catalytic domain (DRMcd)

➢ Or Activated by:

- **Histone acetyltransferases** (for example, p300)

Transformation procedures for genome editing

- Agrobacterium-mediated T-DNA transformation,
- Biolistic
- PEG-mediated transformations
- Virus based genome edition
- Ribonucleoprotein (RNP) complex (A purified Cas9 protein mixed with excess gRNAs
- Simultaneous haploid induction, genome edition and elimination of construct (HI edit, Syngenta)



Risks and concerns about CRISPR Technology

➢ 1. Off-Target Effects

- Unintentional mutation of DNA sequence during the activity of CRISPR/Cas machinery
- In major crop occur at low rates (<10%)

➢ 2. Epigenetic Consequences

- Chemical modification of DNA structure (e.g., methylation), histone proteins and chromatin remodeling
- Unintended effects of epigenetic modification are still poorly explored

➢ 3. Toxicity of Cas proteins for Health

- In prokaryotes and pluricellular eukaryotes, induced DSB and heterologous Cas9 protein expression can impair cell growth
- To date, no reports of Cas9-associated toxicity have been found in plants (Dey, 2021).

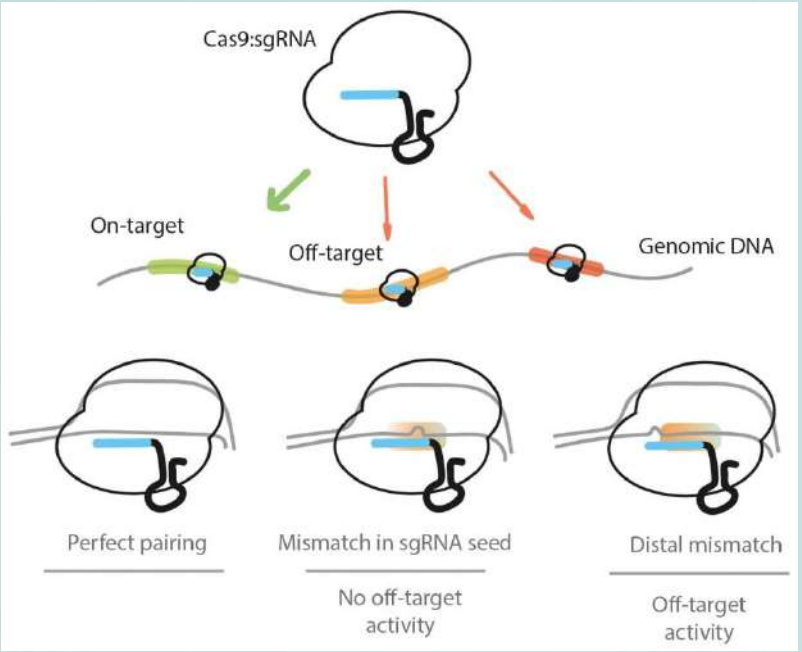
Off targets
The main issue of Gene Editing

Types of Off-targets

- 1) Sequences sharing high similarities to the target
- 2) Irrelevant genomic off-target sites

Some methods to detect off-target mutations:

- Digenome-seq (Kim D. et al., 2015)
- GUIDEseq (Tsai et al., 2014)
- SITE-seq (Cameron et al., 2017)
- CIRCLEseq (Tsai et al., 2017)
- DISCOVER-seq (Wienert et al., 2019)



Gold Standard: Genomewide NGS for the identification of the potential off-targets

Strategies to avoid off-Targets and Cas Toxicity in Plants

➢ 1. Optimizing the gRNA design,

- Web-based software's predict potential off-target sites in plant genomes including Cas-OFFinder, CHOPCHOP, CRISPOR

➢ 2. Cas Protein and gRNA Variants

- The use of Cas9 orthologs
- The use of Cas9 protein modifications
- CRISPR GUARD uses shortened guide RNAs

➢ 3. Alternative CRISPR Component Formats

- Pre-assembled RNPs
- The Use of Viral Vectors and Nanomaterials

Regulations of Gene-Edited Crops

- **Approach 1: GMO regulations are applied**
- Prior safety assessment
- Approval by the government are required
- **Approach 2: Simplified GMO regulations**
- Simplified safety review
- Simplified approval procedures

- **Approach 3: GE products are exempt from GMO regulations.**
- Confirmation by the government is required before placing on the market
- **Approach 4: Genome-edited products are exempt from GMO regulations**
- Prior confirmation is not required by the government

Approaches	How the product is treated under the regulation: GMO or non-GMO	Applied Regulatory Oversight	Country or authority	
Approach 1	GMO	GMO Regulation as it is	EU, NZ (EPA)	
Approach 2	GMO	Simplified GMO regulations	UK*, FSANZ*, China	
Approach 3	non-GMO	Exempted but with confirmation by regulatory authority	Argentina and South America, Japan, India, Philippines	
Approach 4	non-GMO	Confirmation not required by regulatory authority	US (USDA), Australia (OGTR)	

The reasons of the convergence in regulations (Tachikawa and Matsuo, 2023):

- There are high expectations for the potential of genome-editing technologies
- Countries are not only engaged in research and development but also regulatory competition
- Most countries have tried to adopt regulations through administrative procedures rather than legal revisions, which makes the process of decision more active and up to date
- Finally, to facilitate international trade and cooperation, comprehensive and relatively similar regulations are useful

Approach	2014	2015	2016	2017	2018	2019	2020	2021	2022
Approach 1	New Zealand (EPA)				EU				
Approach 2								Australia & New Zealand (FSANZ)*	China (MARA), UK (Defra)*
Approach 3		Argentina		Chile Israel	Brazil	Japan			Canada (Health Canada), India, Philippines
Approach 4						Australia (OGTR)	US (USDA)		

An asterisk (*) indicates that it is under consideration.

Genome-edited products available in the market or awaiting approval

Product	Target genes	Method	Phenotype	Market status	Company
Maize[35,189]	*GBSSI*	CRISPR–Cas	High-yield waxy	Pre-commercial	Corteva
Tomato[52]	*GAD3*	CRISPR–Cas	High GABA	Released, 2021	Sanatech Seed
Soybean[54,190]	*FAD2-1A*, *FAD2-1B*	TALEN	High oleic acid	Released, 2019	Calyxt
Red sea bream[157]	Myostatin	CRISPR–Cas	More muscle mass	Released, 2021	Regional Fish Institute
Tiger puffer fish[157]	Leptin receptor	CRISPR–Cas	Increased appetite	Released, 2021	Regional Fish Institute
Cattle[183]	Prolactin receptor	CRISPR–Cas	Heat-tolerant slick coat	FDA-approved	Recombinatics
Pennycress[191]	NA	CRISPR–Cas	Reduced erucic acid and fibre	Pre-commercial FDA-approved	CoverCress
Lettuce (GreenVenus)[192]	NA	CRISPR–Cas	Non-browning	Pre-commercial, expected release, 2023	Intrexon
Mustard greens[193]	Myrosinase	CRISPR–Cas	Reduced pungency	FDA-approved, expected release, 2023	Pairwise

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In a viewpoint:

GM and GE crops are ‘natural steps’ in the long history of plant breeding innovations