Ministry of Agriculture, Livestock and Irrigation
Department of Agriculture
Division of Horticulture

Enhancing Nutritional Content in Rice (*Oryza sativa* L.) Using Gene Editing Technique

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Introduction

- Plant breeding – an ancient practice of crossing, selecting, and improving crops for traits of value to humans
- Necessary to develop resistance to diseases and pests, to drought and temperature extremes, and to improve quality factors
- Helping adapt crops to new locations throughout the world - improving food security and supporting local and regional food systems
- Conventional breeding – sexually crossing between two parents, transferring many traits along with traits of interest and germplasm availability limitation
- Modern breeding – newer techniques discovered in the 20th century including DNA-based selection strategies and advanced statistical models
- Plant breeders – a vital link in the chain between farmers and consumers

Plant Breeding History

- Four parts

  - Pre-Mendelian Era (before 1900) – domestication, interspecific hybridization, artificial hybridization in fruit crops, Mendel's Plant Hybridization Experiment

  - Mendelian Era (1900 – 2000) – pureline theory, selection methods, heterosis

  - Post Mendelian Era (1921 – 1950) – mutation breeding, CMS, Dominance Hyothesis, transformation principle

  - Modern Era (after 1950) – multilines, green revolution, first transgenic tobaco and cotton (genetic engineering), new breeding techniques (gene editing, reverse breeding, etc.)

GMOs vs CRISPR Gene Editing

- GMOs – a foreign gene insertion into the DNA strand and improved characteristics associated with the new gene as well as dectecion on the genetic modification
- Gene Editing – gene cut and its DNA modification and changing crop’s DNA and not identification on the mutants (similar as natural mutation)

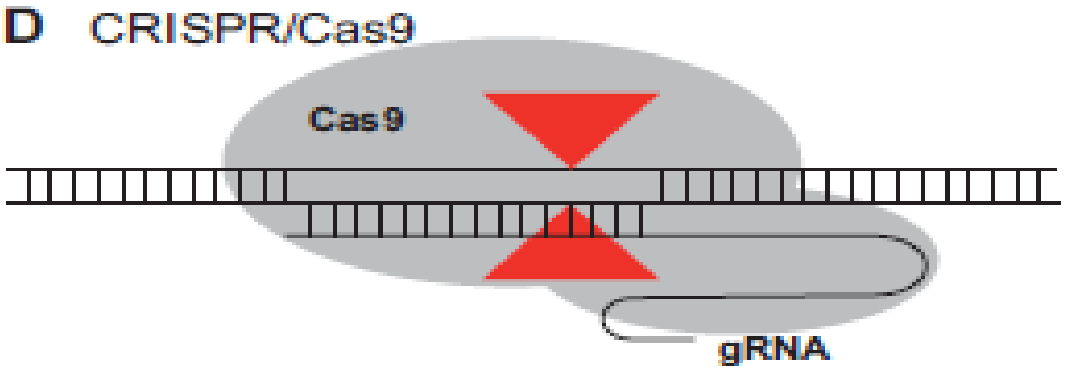
Genome Editing

- Genome editing technologies enable scientists precisely modify the target DNA of many organisms, including plants, bacteria, and animals.
- These technologies act like molecular scissors, cutting the DNA at a specific spot, then scientists can remove, add, or replace the DNA where it was cut.
- It can be manipulated for various purposes including treating disease such as gene therapies and generation of improved cultivars.

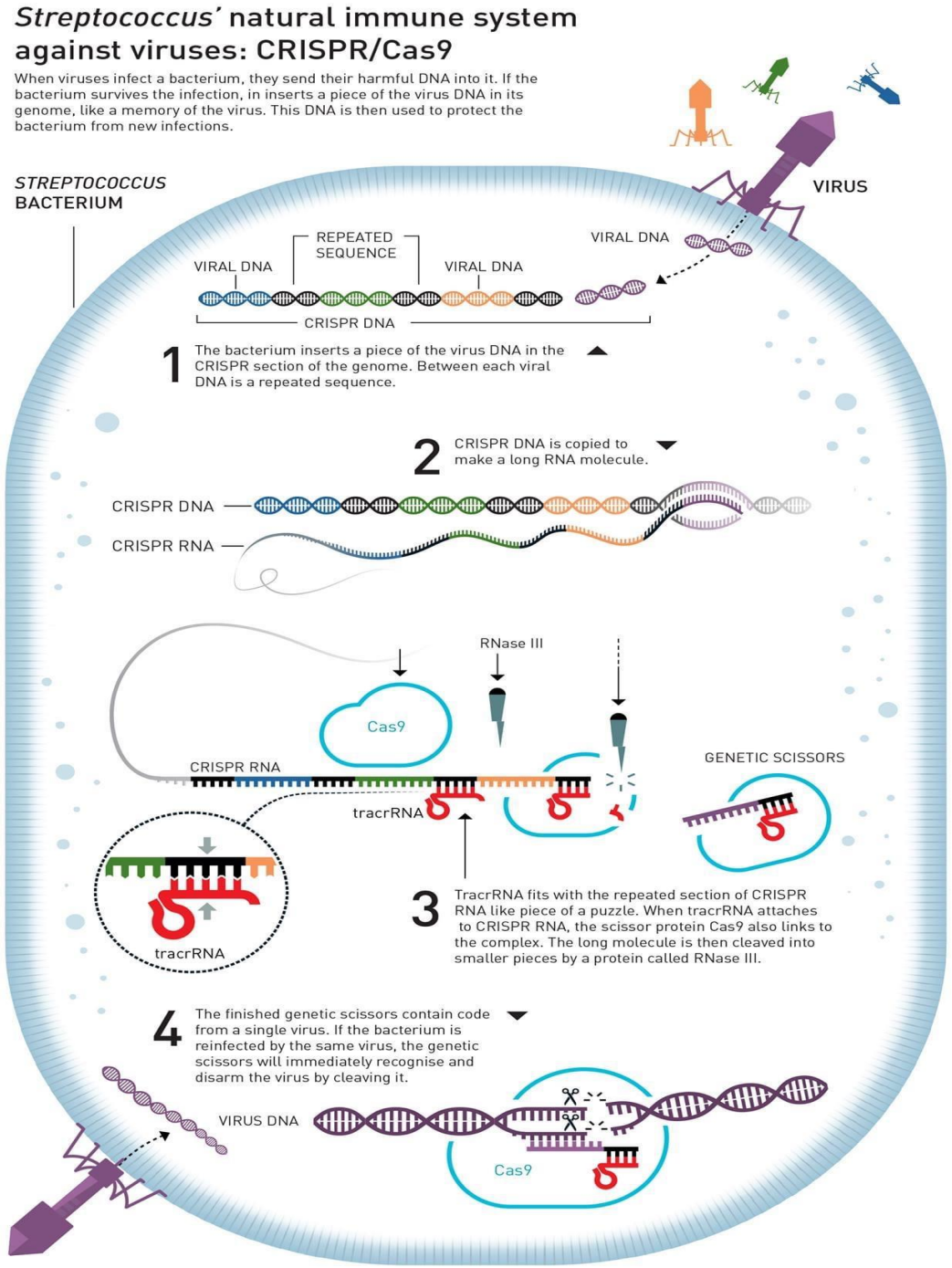
Four Gene Editing Techniques

- Meganucleases
- Zinc Finger Nucleases (ZFN)
- Transcription activator-like effector nucleases
- Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9 (CRISPR/Cas9)

CRISPR/Cas9



- a guide RNA, complementary with a specific chromosomal target sequence
- Cas9 nuclease cleavage to RNA/DNA complex
- widely and preferentially uses in many laboratory for genome editing

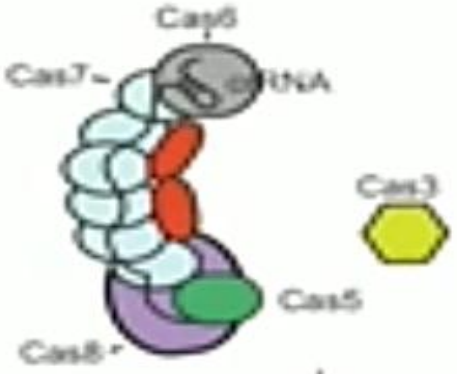


Developed from adaptative immune system of bacteria

- **Spacer acquisition**
  - Certain region of viral DNA incorporation into the CRISPR section of bacterial genome.
- **Biogenesis of CRISPR-RNAs (CrRNA)**
  - CRISPR DNA containing viral target sequence transcription into CrRNA
- **Interference**
  - CrRNA guides Cas9 to the invading viral DNA to cleave it

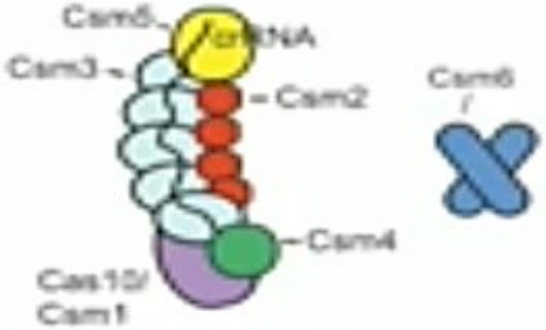
Class 1

Type I Cascade



No intrinsic nuclease activity in Cascade; recruits Cas3 to cleave DNA.

Type III Csm/Cmr



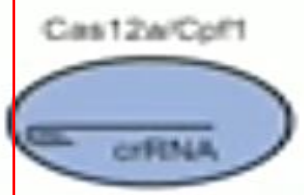
Csm cleaves DNA (transcription-dependent) and RNA; Csm6 is an auxiliary RNase.

Class 2

Type II Cas9

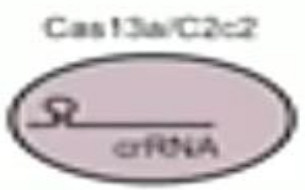
Cleaves dsDNA

Type V Cas12



Cleaves dsDNA

Type VI Cas13

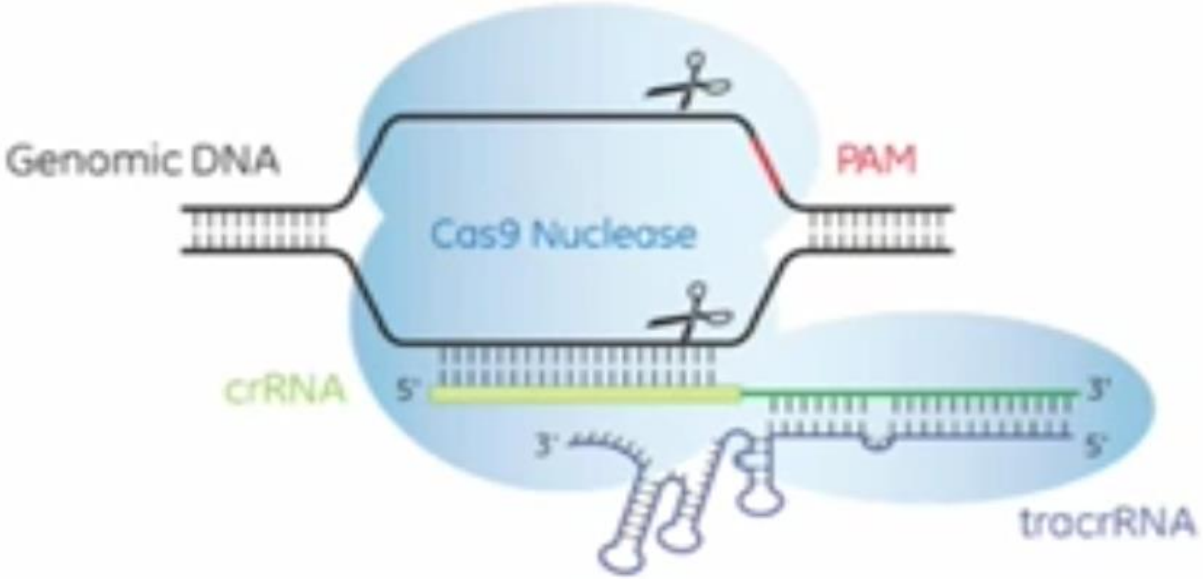


Cleaves ssRNA

The *S. pyogenes* CRISPR-Cas9 system

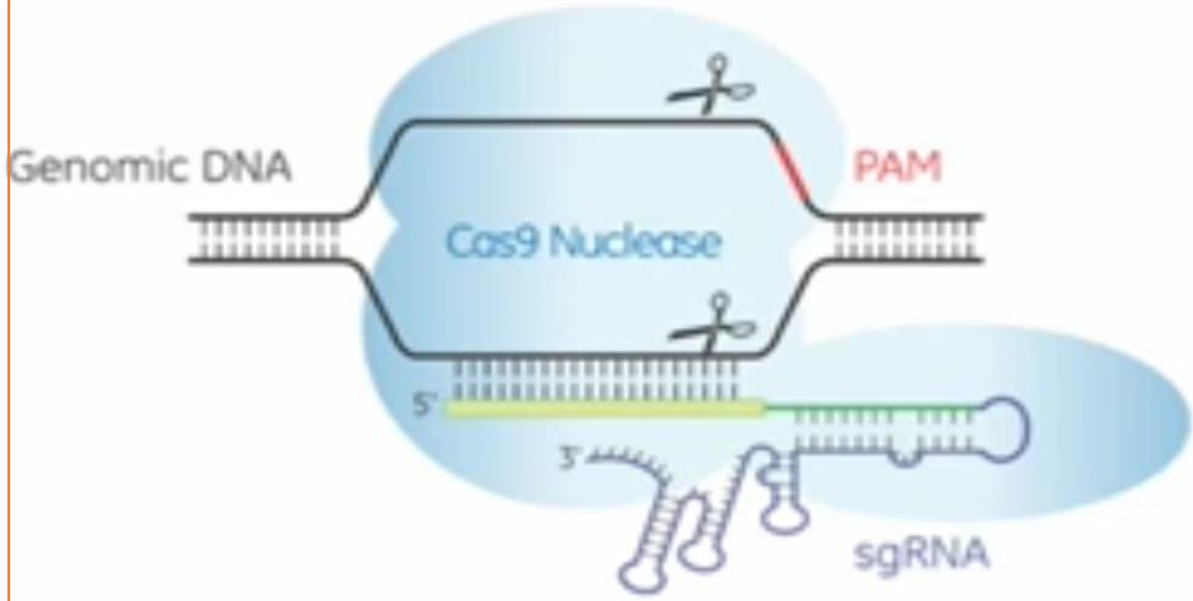
Introduction into mammalian cells to induce a double-strand break

2-part guide RNA system (crRNA:tracrRNA)



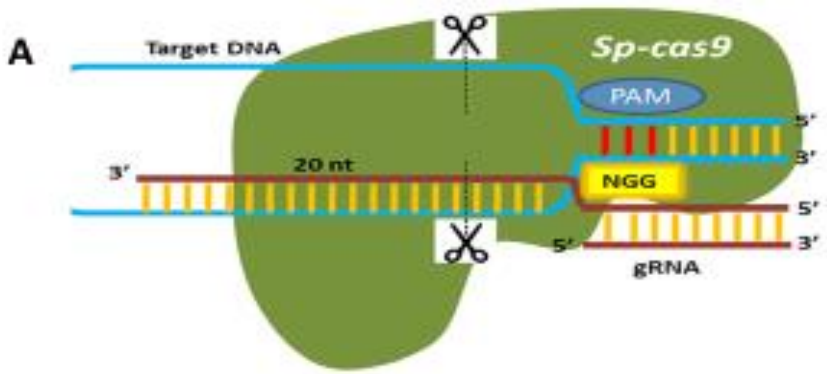
- crRNA plus tracrRNA is the natural configuration of guide RNA

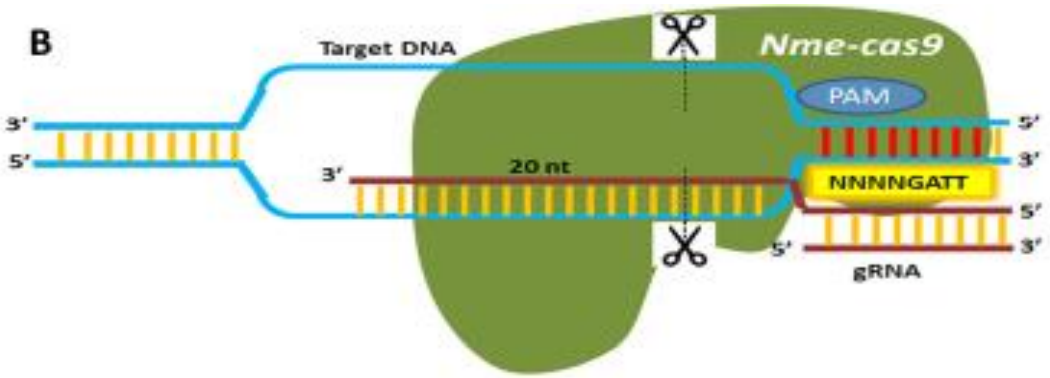
Chimeric single guide RNA (sgRNA)

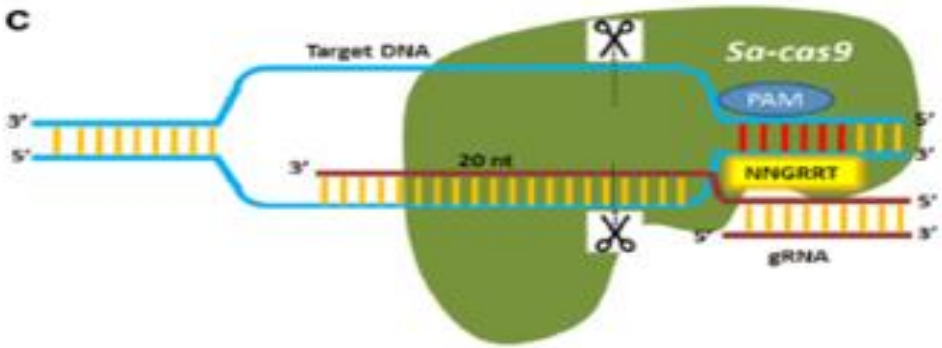


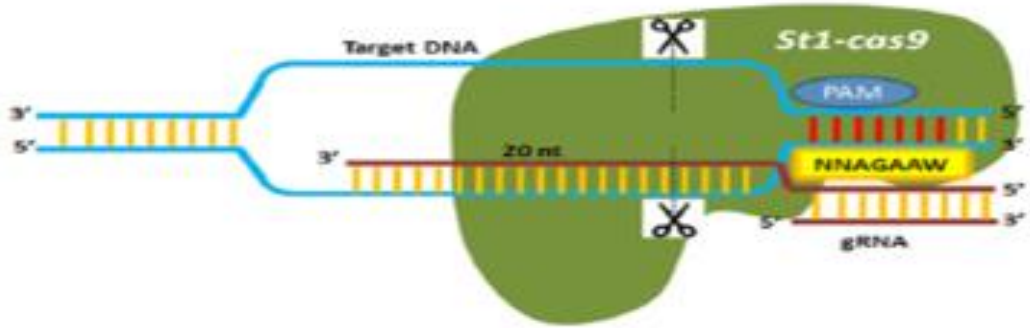
- Synthetic sgRNA
- Lentiviral sgRNA particles
- Lentiviral plasmid sgRNA

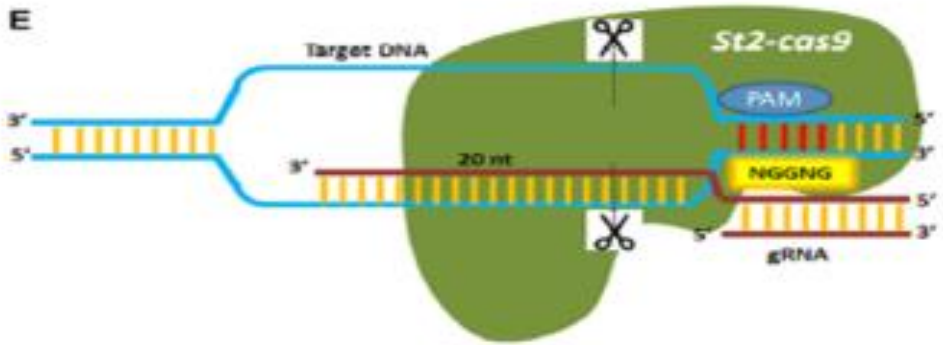
Cas9 variants

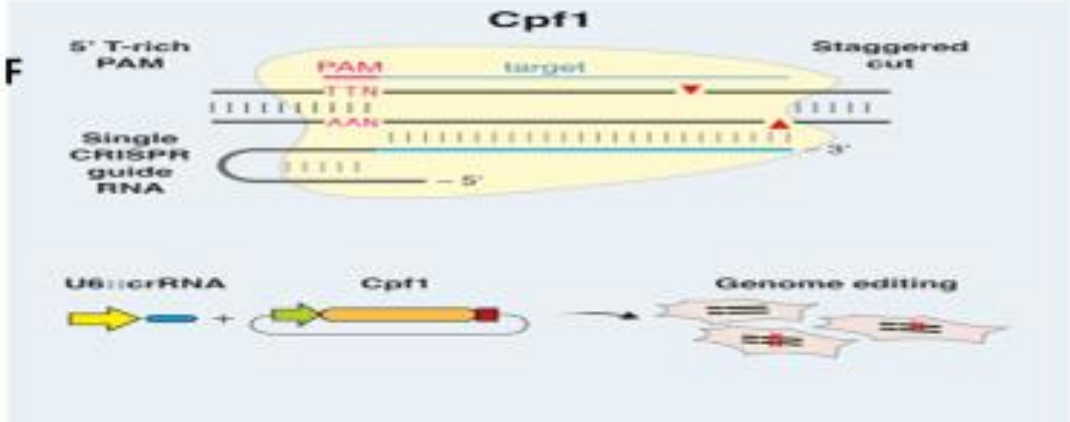






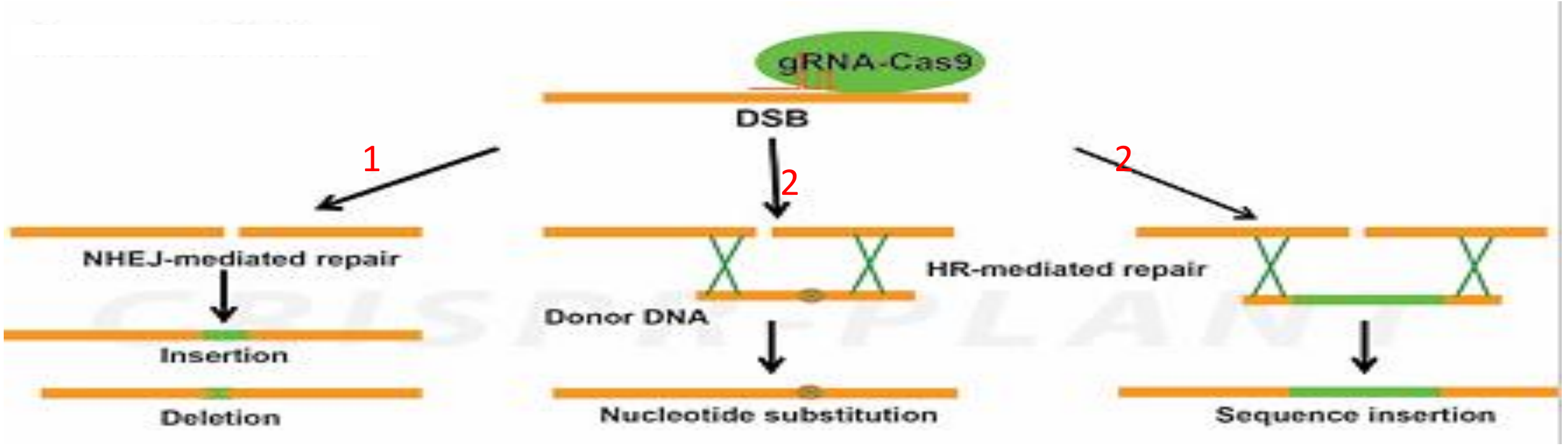






Genetic Modification via CIRSPR/Cas9

- DSB induction in the target DNA → fixing the DSB by (endogenous repaired system – Non-homologous end joining (NHEJ) or Homologous recombination (HR) in the presence of donar DNA template

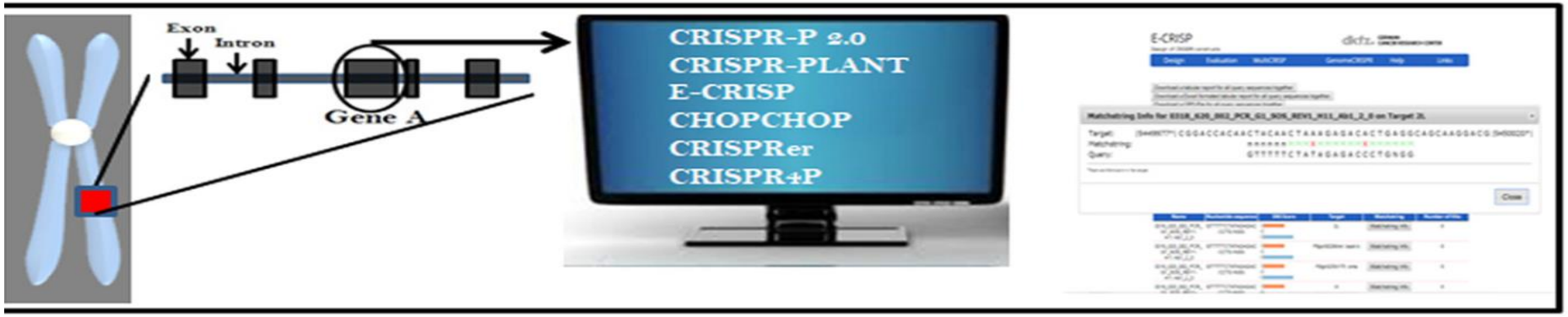


NHEJ - small indels into the DSB and resulting as frame-shift mutations or premature stop codons, knocking out the target gene

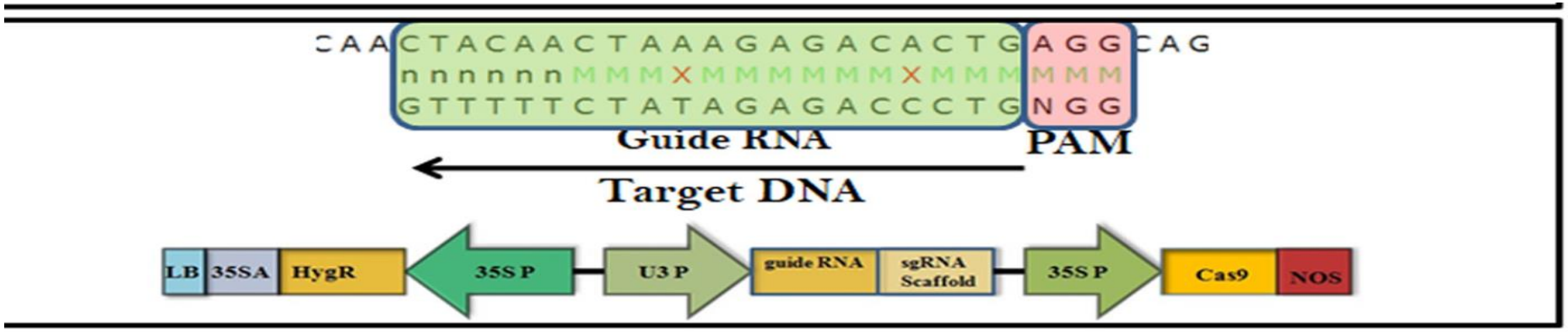
In the presence of a homologous donor DNA spanning the DSB, HR >>> cause nucleotide substitution, gene replacements and insertions in the target region of the chromosome.

Steps in the workflow of CRISPR/Cas9-based genome editing

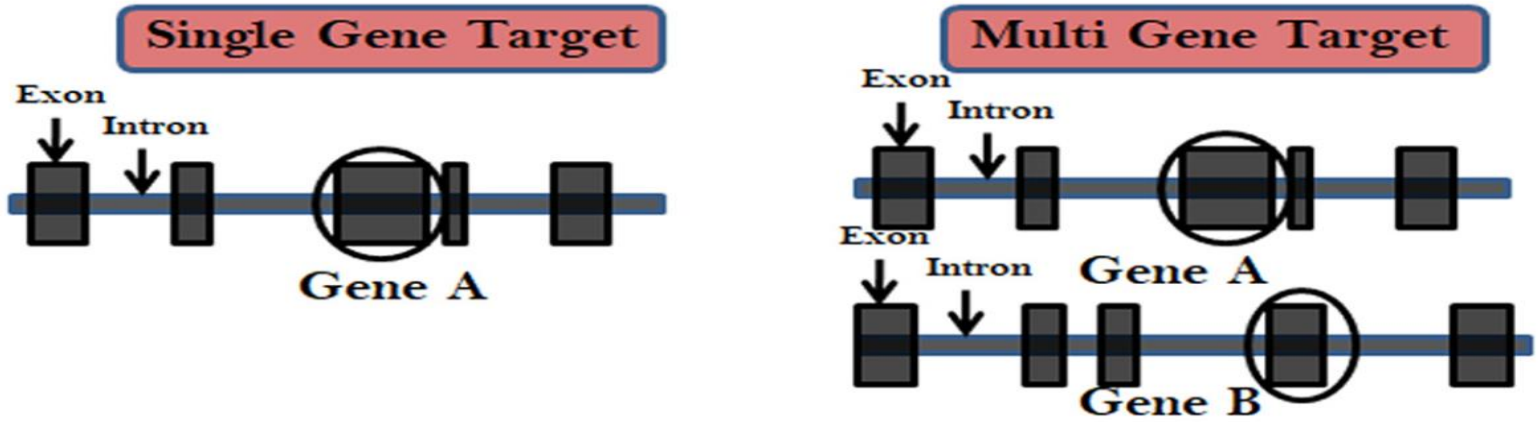
1. Gene Targeting and SgRNA Designing



2. SgRNA Synthesis and Cloning



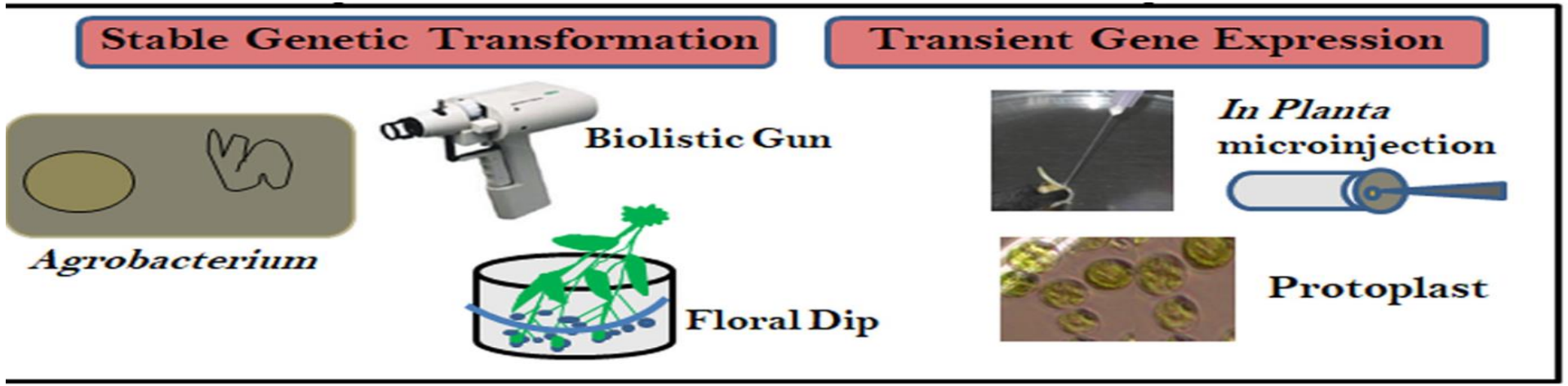
3. Single and Multiplex Gene Target



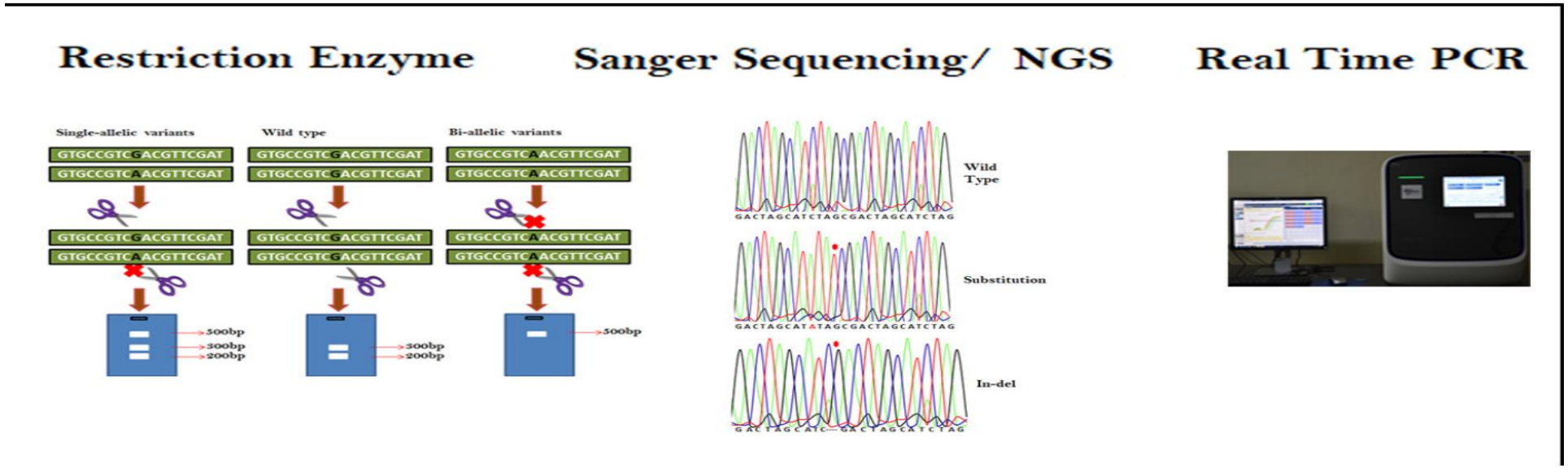
Jaganathan et al., 2018

Jaganathan et al., 2018

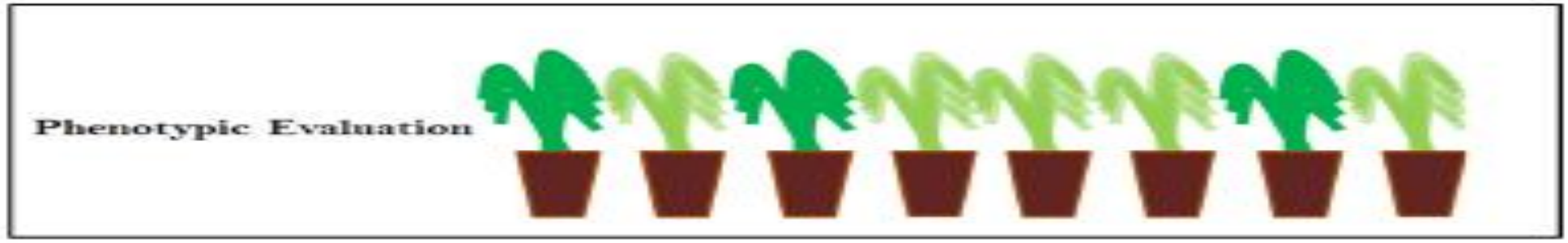
4. Delivery Method for Host System



5. Screening and Confirmation of Transgenics

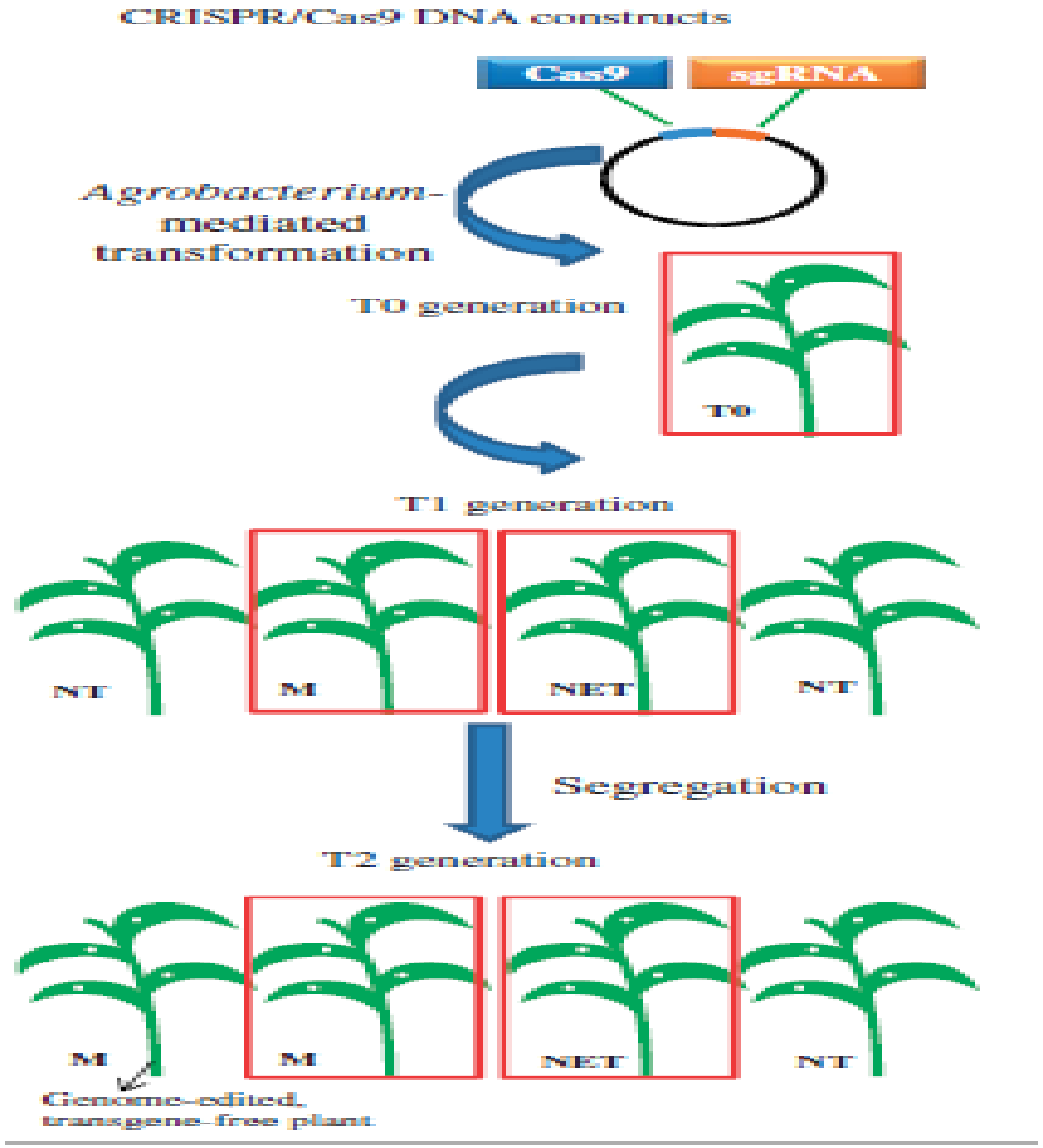


6. Evaluation for Abiotic Stress Tolerance

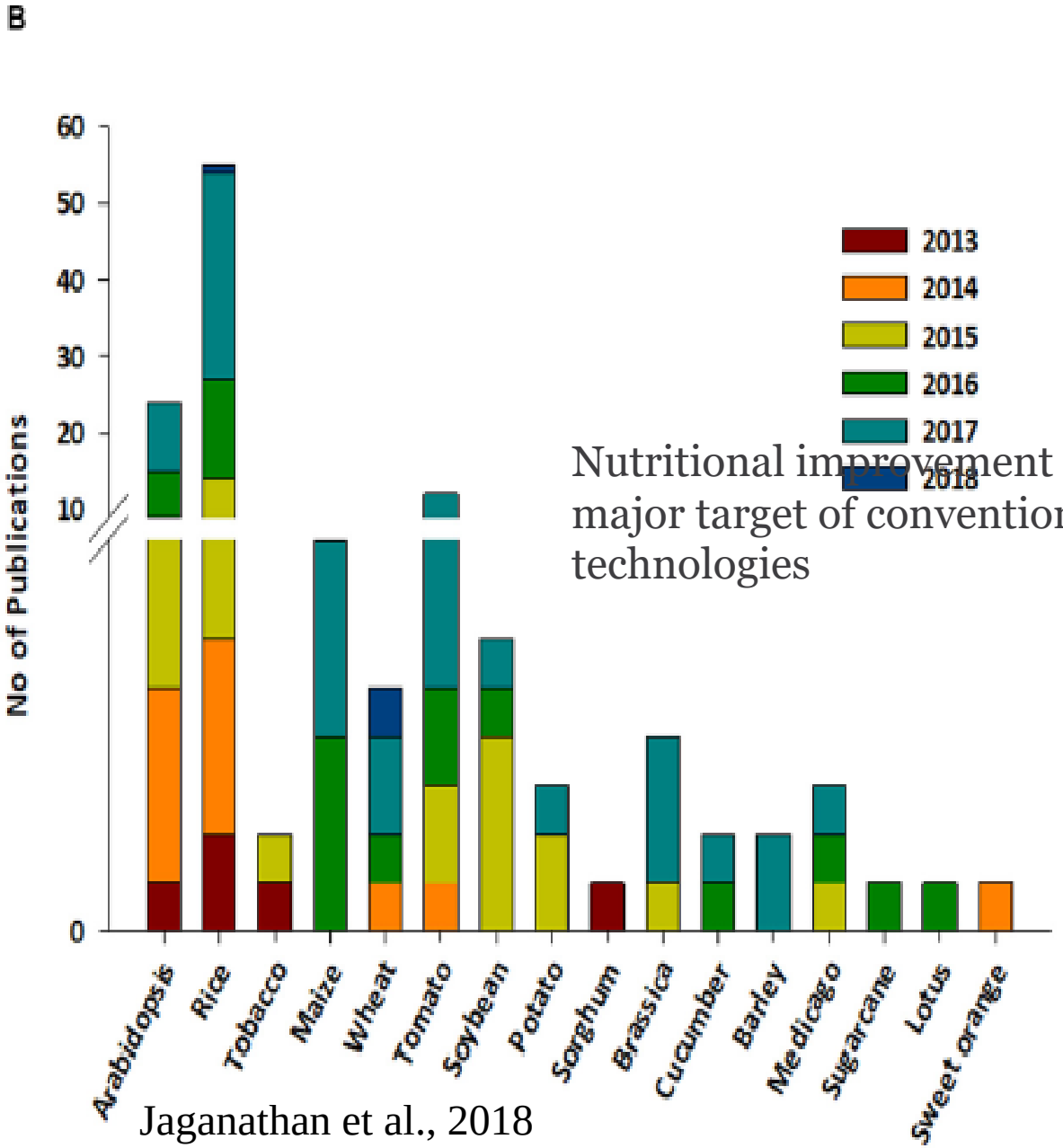


Transgene-free plants production

- Agrobacterium (CRISPR/Cas9 expression) transformation >>> the transgenic plants (T) generate in T0 generation.
- Selection of the edited transgenic plants (M) and non-edited transgenic plants (NET) in the T1 generation to produce the T2 generation, while throw non-transgenic plants (NT)
- The editing of target genes – not finish in one generation
- T2 generation >>> plants with desired mutation(s) and lost the Cas9/sgRNA transgenes through segregation (transgene free lines)

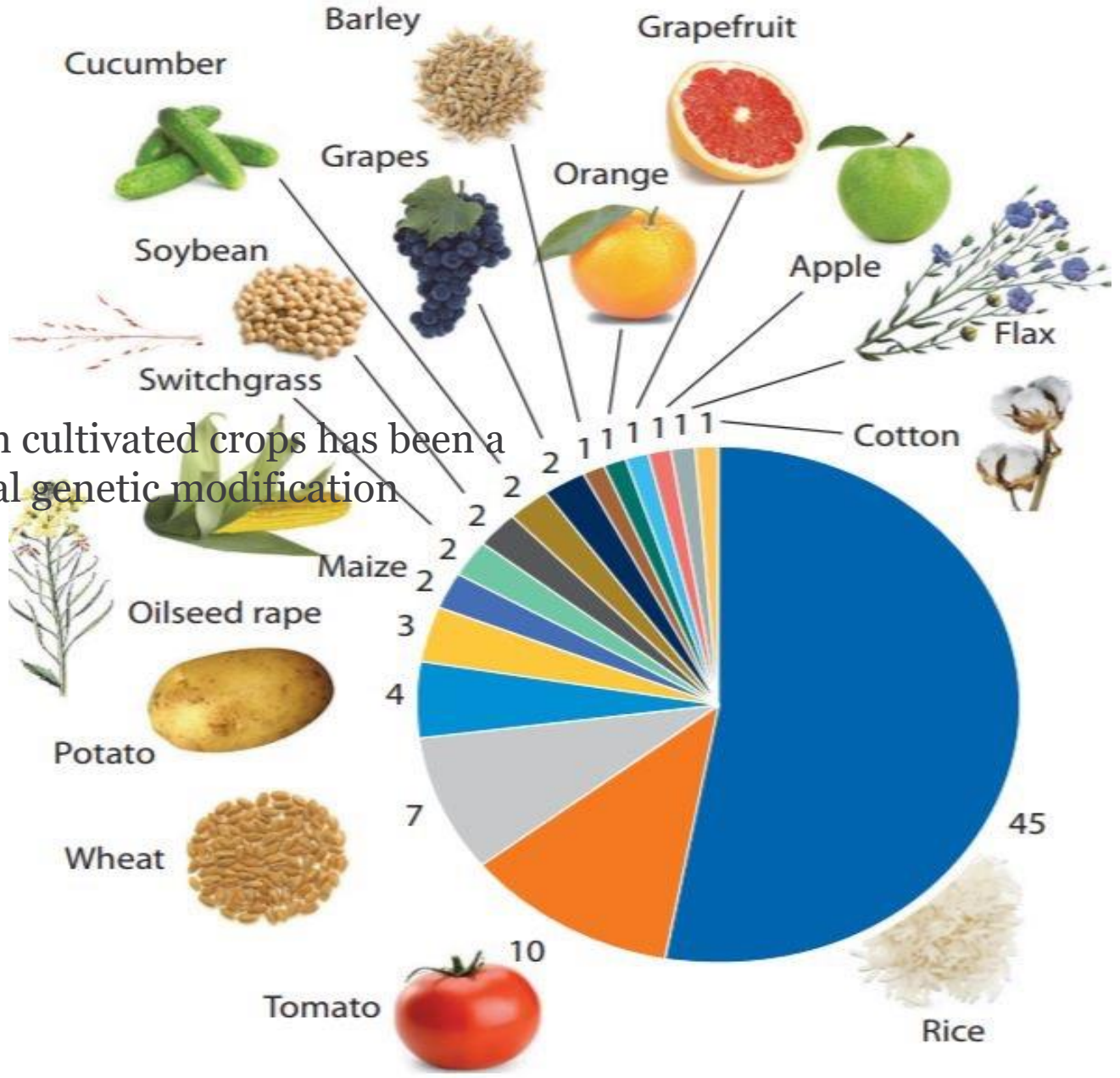


CRISPR/Cas9 Application in Agriculture



Jaganathan et al., 2018

Nutritional improvement in cultivated crops has been a major target of conventional genetic modification technologies



Nutritional Improvement in Crops Using CRISPR/Cas9

- Nutritional improvement in cultivated crops – a major target of conventional genetic modification technologies

Common name	Phenotype	Target gene	Target region	GE result on target expression or activity	GE technique	Molecular function of the target gene
Rice	increased content of resistant starch	*SBEI and SBEIIb*	CDS	DOWN	CRISPR/ Cas9	Regulate amylose contents
Rice	low cesium accumulation	*OsHAK-1*	CDS	DOWN	CRISPR/ Cas9	Cs + uptake in roots
Sorghum	increased digestibility and protein quality	*k1C gene family*	n.i.	DOWN	CRISPR/ Cas9	α-kafirins (major storage proteins)
Bread wheat	low gluten content	*sgAlpha-1 sgAlpha-2*	CDS	DOWN	CRISPR/ Cas9	the immunoreactive α-gliadin
Soybean	altered fatty acids levels	*FAD2-1A* and *FAD2-1B*	CDS	DOWN	TALEN	Fatty acid desaturase 2
Peanut	increased oleic acid content	*FAD2A* and *FAD2B*	CDS	DOWN	CRISPR/ Cas9	Converts oleic acid to linoleic acid gene coding sequences
Peanut	0.5–twofold increase in the oleic acid content	*FAD2*	CDS	DOWN	TALEN	Converts oleic acid to linoleic acid gene coding sequences
Sweet potato	decreased amylose content	*GBSSI*	CDS	DOWN	CRISPR/ Cas9	Granule-bound starch biosynthesis
Sweet potato	decreased amylopectin content; increased amylose content	*SBEII*	CDS	DOWN	CRISPR/ Cas9	Starch branching for amylopectin
Potato	decreased browning	*PP02*	CDS	DOWN	CRISPR/ Cas9	Converts phenolic substrates to quinones
Potato	decreased steroidal glycoalkaloid content	*16DOX*	CDS	DOWN	CRISPR/ Cas9	Steroidal glycoalkaloid biosynthesis
Potato	reduced levels of acrylamide	*Vinv*	CDS	DOWN	TALEN	Accumulation of reducing sugars which cause acrylamide accumulation.
Tomato	reduced concentration of γ-aminobutyric acid	*GABA-TP1, GABA-TP2, GABA-TP3, CAT9 and SSADH*	CDS	DOWN	CRISPR/ Cas9	Essential genes for the γ-aminobutyric acid (GABA) pathway
Tomato	SSC, fiber, fructose, ascorbic acid, total phenol, carotene, oxalic acid	*L1L4*	CDS	DOWN	ZFN	Metabolite pathway
Tomato/ wild tomato	high lycopene content	*cycB*	CDS	DOWN	CRISPR/ Cas9	Metabolite pathway
Eggplant	decreased browning	*PP04, PPOS, and PP06*	CDS	DOWN	CRISPR/ Cas9	Converts phenolic substrates to quinones
Grape	decreased tartaric acid content	*IdnDH*	CDS	DOWN	CRISPR/ Cas9	Tartaric acid biosynthesis Vegetables
Carrot	decreased anthocyanin content	*F3H*	CDS	DOWN	CRISPR/ Cas9	Anthocyanin biosynthesis
Brassica rapa	decreased fructose, glucose, and increase sucrose contents	*BrOG1A and BrOG1B*	CDS	DOWN	CRISPR/ Cas9	Primary metabolism
Rapeseed	increased seed oil content	*SFAR4 and SEARS*	CDS	DOWN	CRISPR/ Cas9	Oil degradation
Rapeseed	increased oleic acid content; decreased linoleic and linolenic acid contents	*FAD2*	CDS	DOWN	CRISPR/ Cas9	Fatty acid biosynthesis
Chinese kale	yellow color of Chinese kale with improved market prospects	*BoaCRTISO*	CDS	DOWN	CRISPR/ Cas9	Carotenoid biosynthesis
Lettuce	increased oxidation stress tolerance and ascorbate content	*LsGGP2*	uORF	UP	CRISPR/ Cas9	Deleted uORFs of LsGGP2 to increase the translation of mRNAs
Banana	increased F-carotene content	*LCYe*	CDS	DOWN	CRISPR/ Cas9	ß-carotene metabolism
Mush-room	decreased browning	*PPO*	CDS	DOWN	CRISPR/ Cas9	Converts phenolic substrates to quinones
Pome-granate	unique accumulation of gallic acid 3-0- and 4-0-glucosides	PgUGT84A23 and PgUGT84A24	CDS	DOWN	CRISPR/ Cas9	UDP-dependent glycosyltransferases (UGTs) enzymes with overlapping activities in ß-glucogallin biosynthesis

Research Experiences

- To enhance the content of grain Fe and Zn by promoter modification of *OsNAS2* using CRISPR/Cas9

- IR64-IRS1361-009-001-033 and IR64-IRS1361-009-001-031 (mutants) from T_2 generation >>> high grain Fe and Zn content per plant and higher yield compared to WT (IR64)

The grain Zn and Fe content per plant of candidate plants of IR64-IRS1361 events in field-bed experiment					
EVENTS	Zn Mest. (ppm)	Fe Mest. (ppm)	Yield (g)	Zn/plant (ppm)	Fe/plant (ppm)
IR64-IRS1361-009-001-031(+)	31.05	4.07	20.31	630.63 A	82.66 A
IR64-IRS1361-009-001-033(+)	28.56	4.95	15.91	454.39 C	78.76 A
IR64-IRS1361-TC1-001-031	25.42	2.69	14.87	377.95 E	40.00 G
WT (IR64)	25.82	2.65	18.57	475.65 C	47.62 F

Events	Sequence Information	Note
	REF: GGA-GTGACCATACGCGAGAAGC	
IR64-IRS1361-009-001-033	A1: GGA-GTGACCATACGCGAGAAGC	No indels/SNP
IR64-IRS1361-009-001-031	A1: GGACGTGgCCATACGCGAGgAGC	1 bp insertion + 2 SNPs

Research Experiences

- The similar result of the two fold-increases of grain Fe and Zn content in polished seed through overexpressing *OsNAS2* gene (Johnson et al., 2011, and Zhen et al., 2010)
- The introduction of 35S enhancer element in T-DNA backbone for *OsNAS3* - the content of micronutrients, Fe (2.9-fold) and Zn (2.2-fold) in mutant plants than WT grains (Lee et al., 2009)
- Longest grains – higher Fe and Zn content > WT (IR64) and TC, Grain size – determine grain Fe and Zn content; longer grain (Basmati) – high grain Fe content, narrower grains – higher Zn, Mn and P contents (Zheng et al., 2005 and Anuradha et al., 2012)
- New breeding techniques such as CRISPR/Cas9 system – crop improvement programs in both rice and other commercial crops depending on consumers' preferences and climate change

THANK YOU SO MUCH!!!