Bayer Russia Plant Biotechnology Conference:

Day 6	Plant Care in CE & Intro to Molecular Assays
Day 7	Molecular Assays & Gene Editing Technology
Day 8	Molecular Assays & Model Systems
Day 9	Protoplast Systems

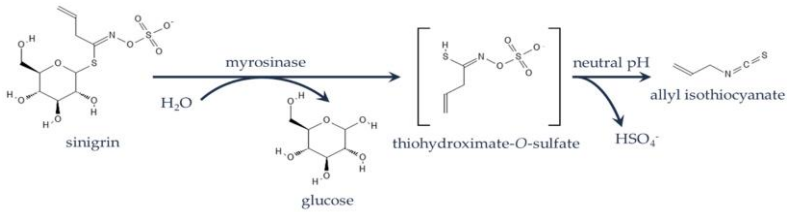
Gene Editing Technology in Plants

Bayer Russia Biotechnology Conference

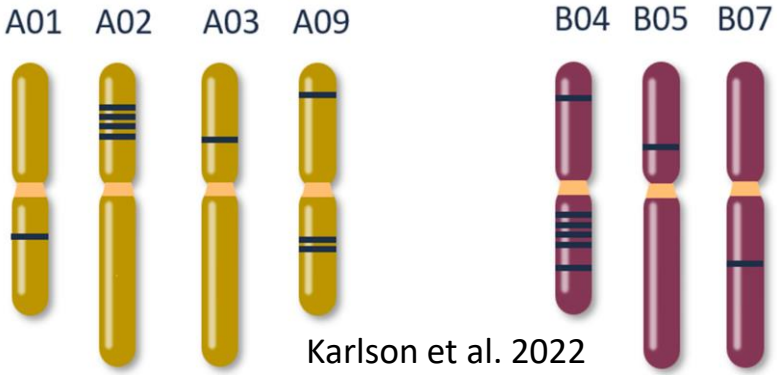
Ervin D Nagy

July 2023

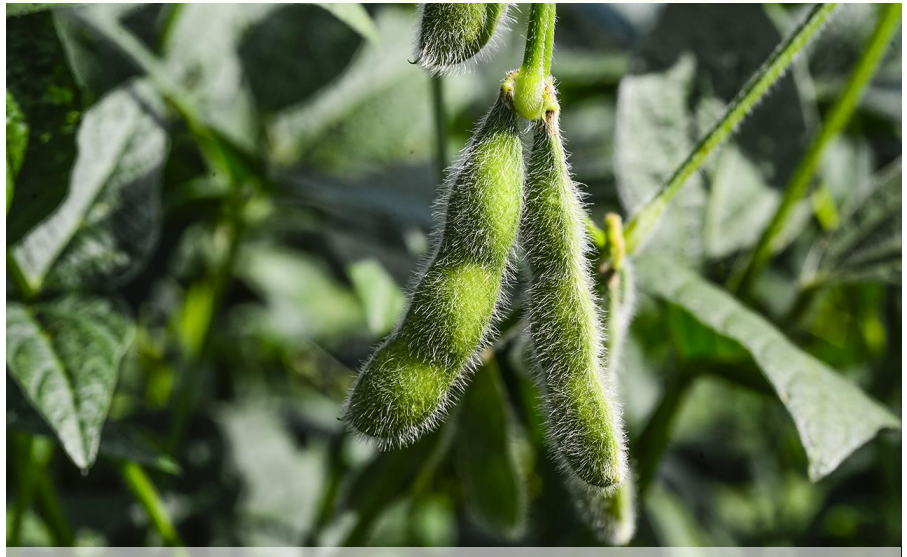
Genome editing is a powerful tool to develop new traits



Low-pungency mustard



Karlson et al. 2022 Plants

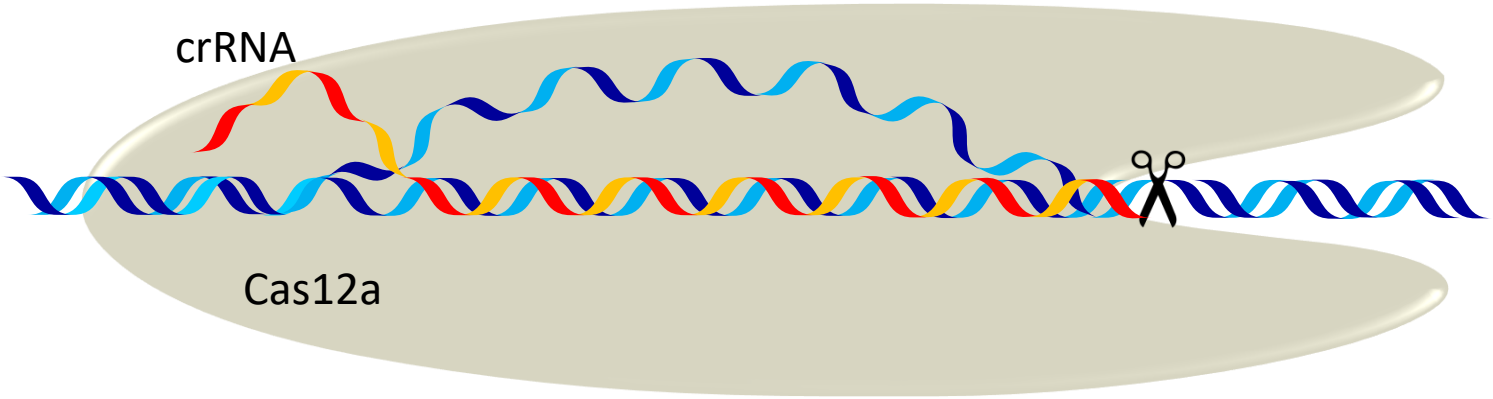


High-oleic, low-linoleic soy

- // **Reduced crop loss**
- // **Precise applications**
- // **Improved sustainability**

Genome editing – molecular mechanism of the core technology

Cas12a endonuclease is directed to chromosomal sites
by the non-coding crRNA.



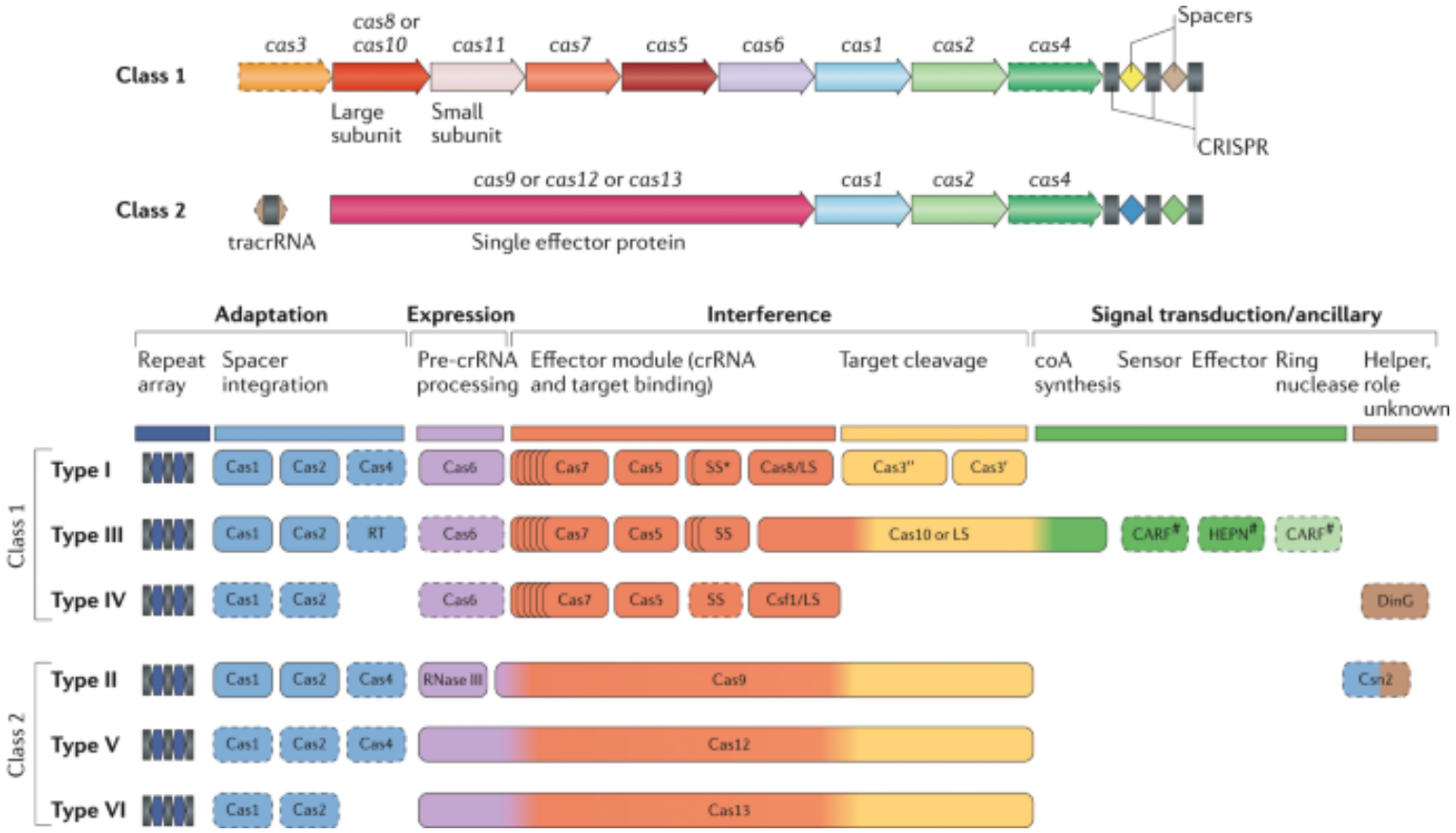
Target site is recognized by RNA/DNA complementarity.

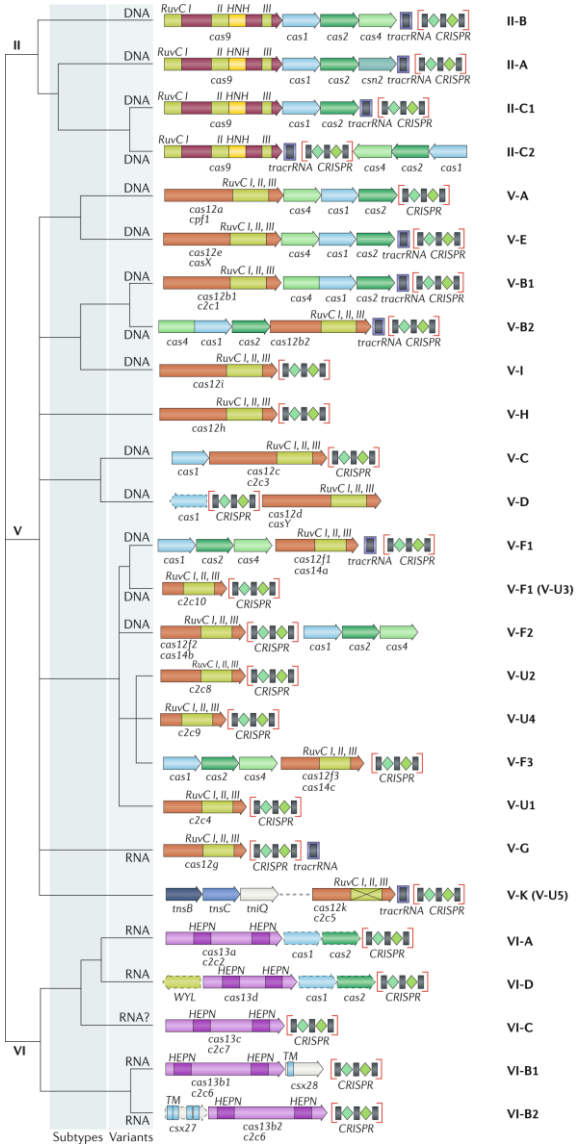
Typical molecular constructs for genome editing:

Enzyme - Pol II cassette: P-Zm.Ubi | L-Zm.Ubi | I-Zm.Ubi | NLS1 | CR-Lb.Cas12a | NLS2 | T-Os.LTP

crRNA - Pol III cassette: P-Pol III. | Rep | TS1 | Rep | TS2 | Rep | T-T7

Cas9 and Cas12a belong to Class 2 CRISPR–Cas systems





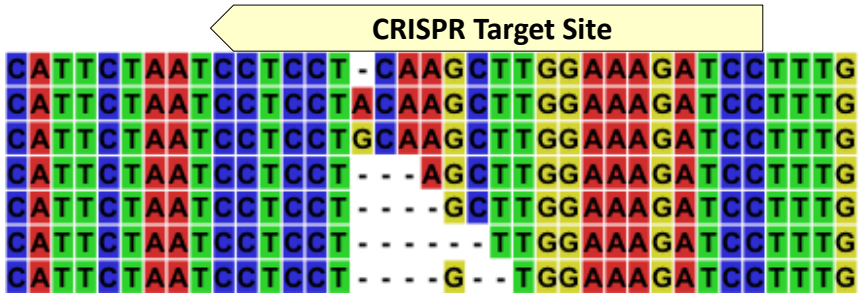
Makarova et al. 2020 Nature Reviews Microbiology

Objectives of genome editing dictate the means to achieve them

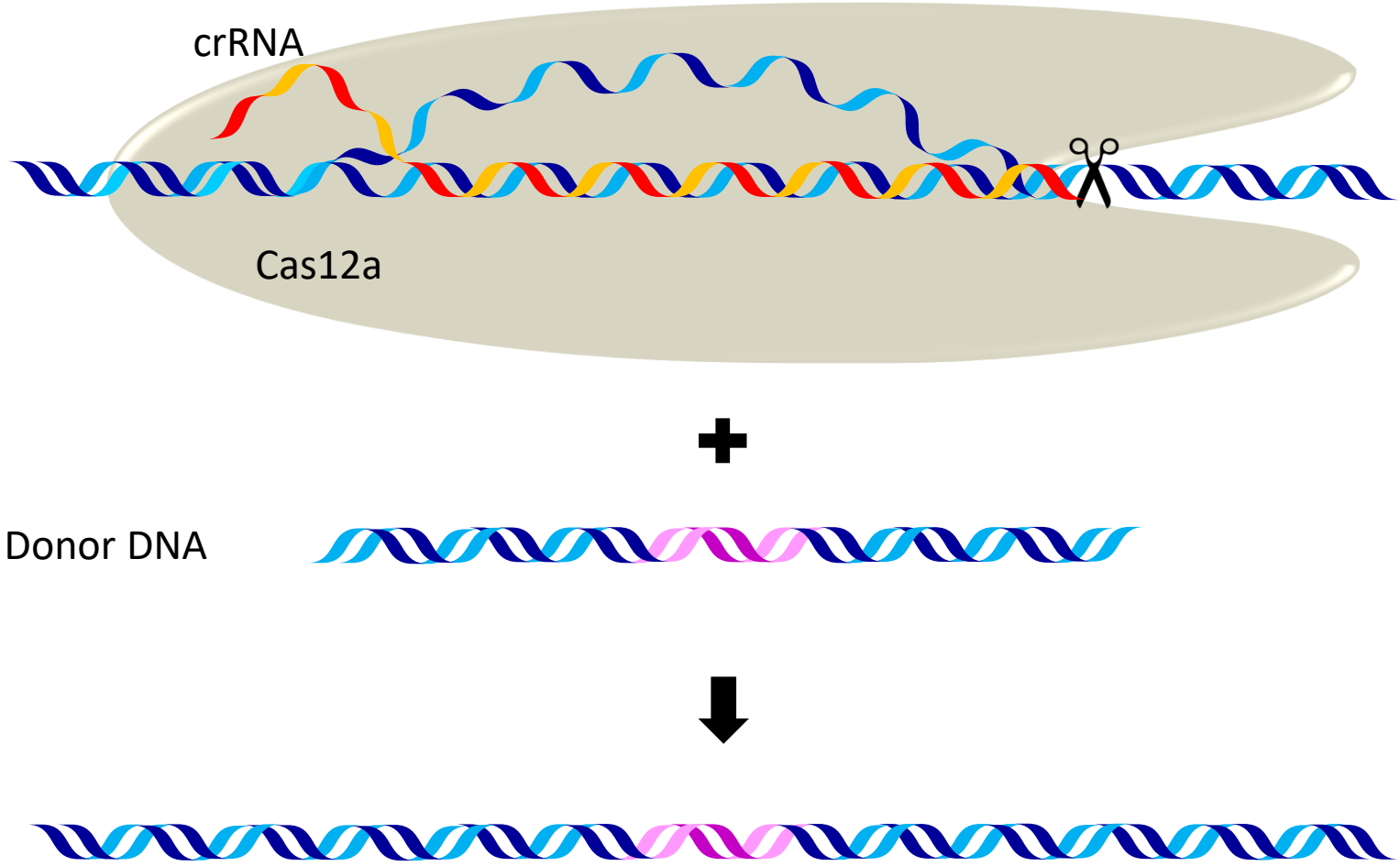
Objectives	Types of genome edits
Frameshift of open reading frames (ORFs)	Short (1-10bp) insertions or deletions (InDels)
Allelic variation in trait genes	Templated edit by endogenous homology-directed repair (HDR)
Alter gene expression level	Base editing by Cas12a fusion proteins (C-to-T or A-to-G)
Identifying causal genes	Prime editing/REDRAW editing by Cas12a fusion proteins
Gene knock-out	SDI by endogenous repair
Site-directed gene integration (SDI)	SDI by transposons
Alter gene copy number	Chromosomal recombination
Linking/de-coupling QTLs	Multiplex editing

Precise and imprecise genome editing by endogenous DNA repair pathways

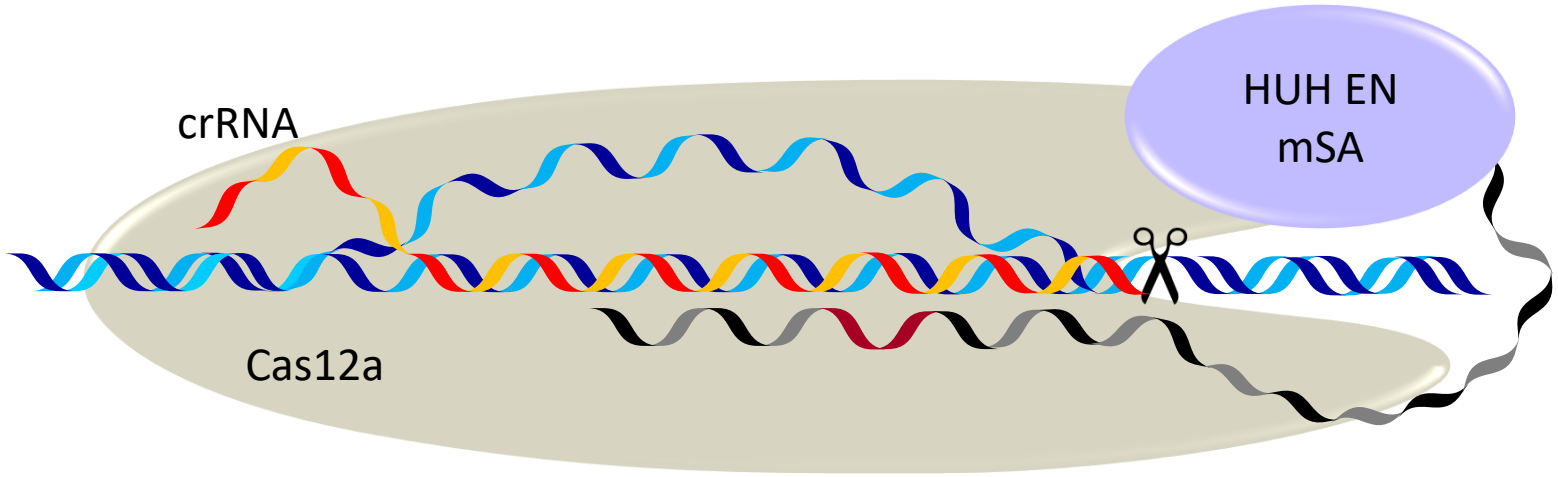
Imprecise genome editing by non-homologous end joining (NHEJ)



Precise genome editing by homology-directed repair (HDR)



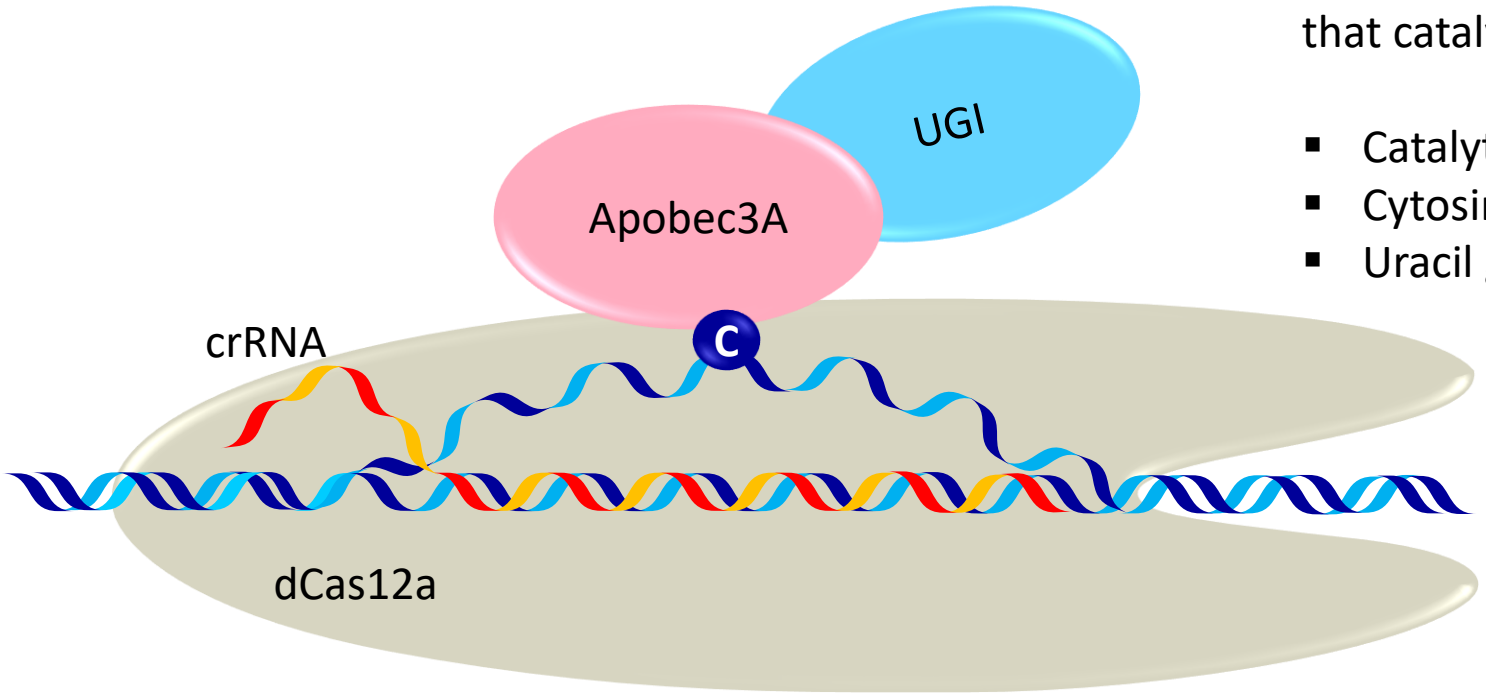
Increasing HDR frequency by molecular tethers



Cas proteins fused to DNA-binding proteins can tether the donor DNA to the CRISPR machinery.

HUH endonucleases (HUH Ens) and monomeric streptavidine (mSA) are examples of tethers.

Precise editing by CRISPR/Cas12a fused to deaminases – Base Editing (BE)



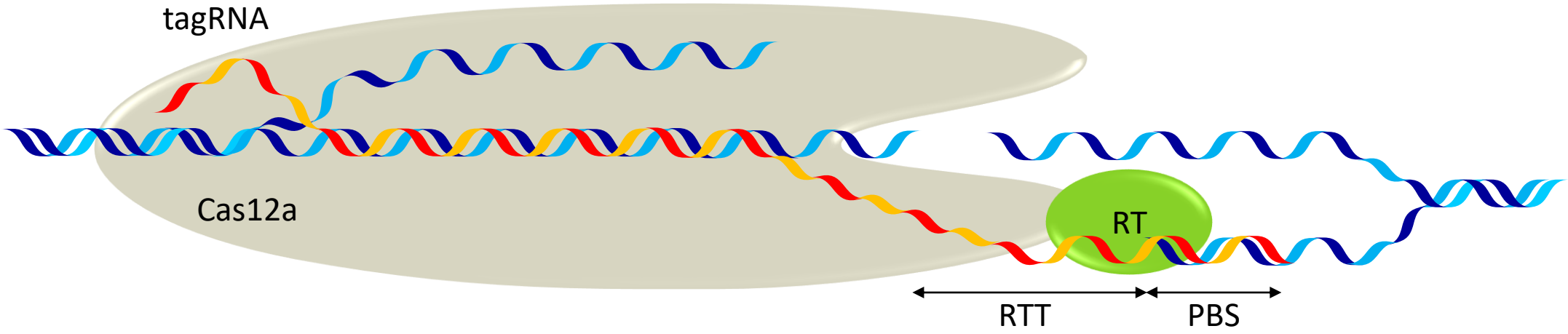
Cytosine Base editors (CBE) are tripartite fusion proteins that catalyze **C-to-T** transitions on the non-target strand:

- Catalytically deactivated Cas12a (dCas12a)
- Cytosine deaminase (CBE, Apobec3A)
- Uracil glycosilase inhibitor (UGI)

Adenosine Base editors (ABE) include the TadA* adenosine deaminase and catalyze **A-to-G** transitions on the non-target strand.

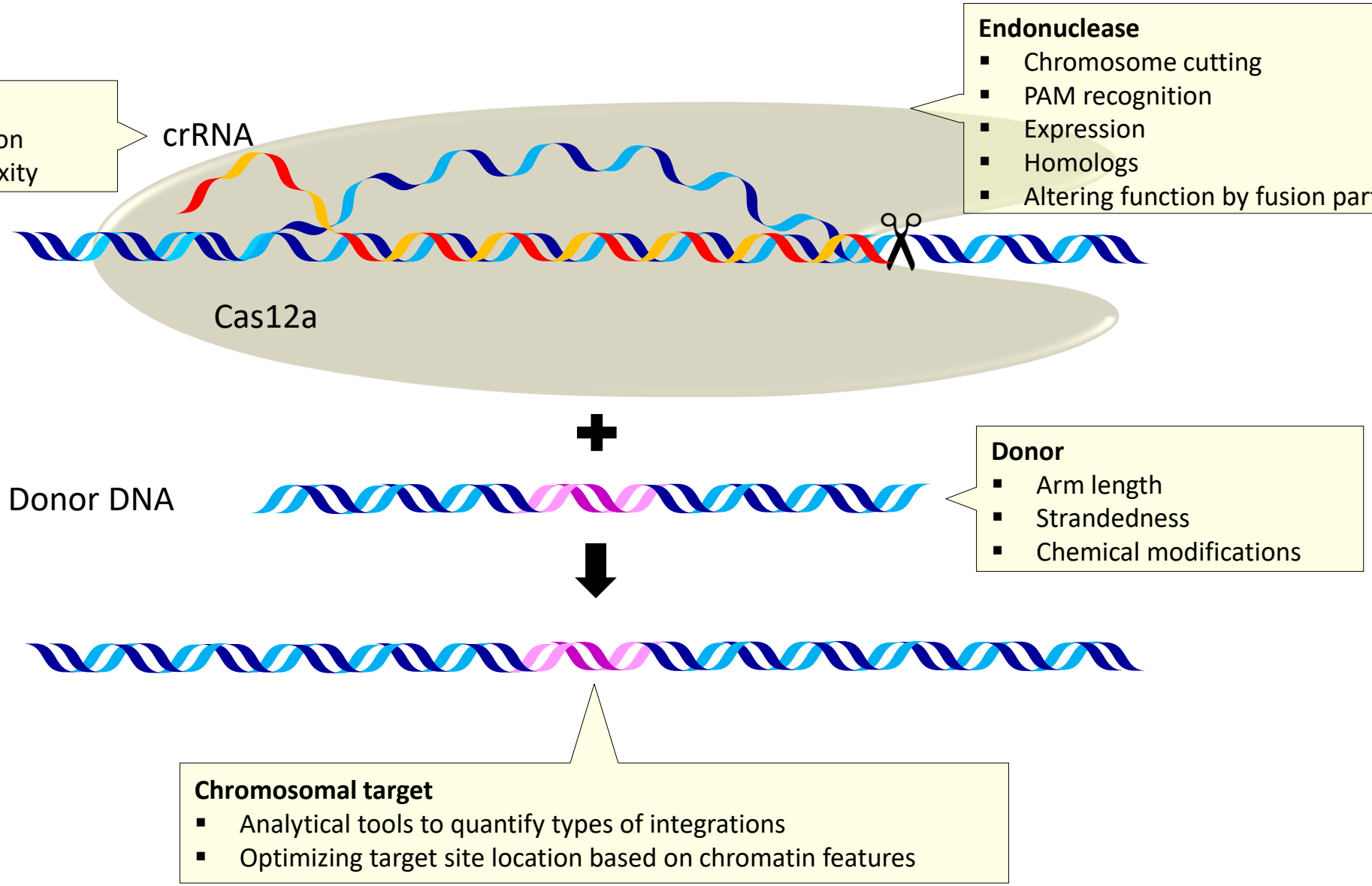
Precise editing by CRISPR/Cas12a fused to RT – Prime Editing/REDRAW

In **REDRAW**, the target-allele guide RNA (**tagRNA**) is extended, so that the target strand can act as a primer for reverse transcriptase (**RT**). The reverse transcript incorporates into the genomic DNA.



In **Prime Editing** the non-target strand serves as a primer for RT.

Areas of optimization of genome editing



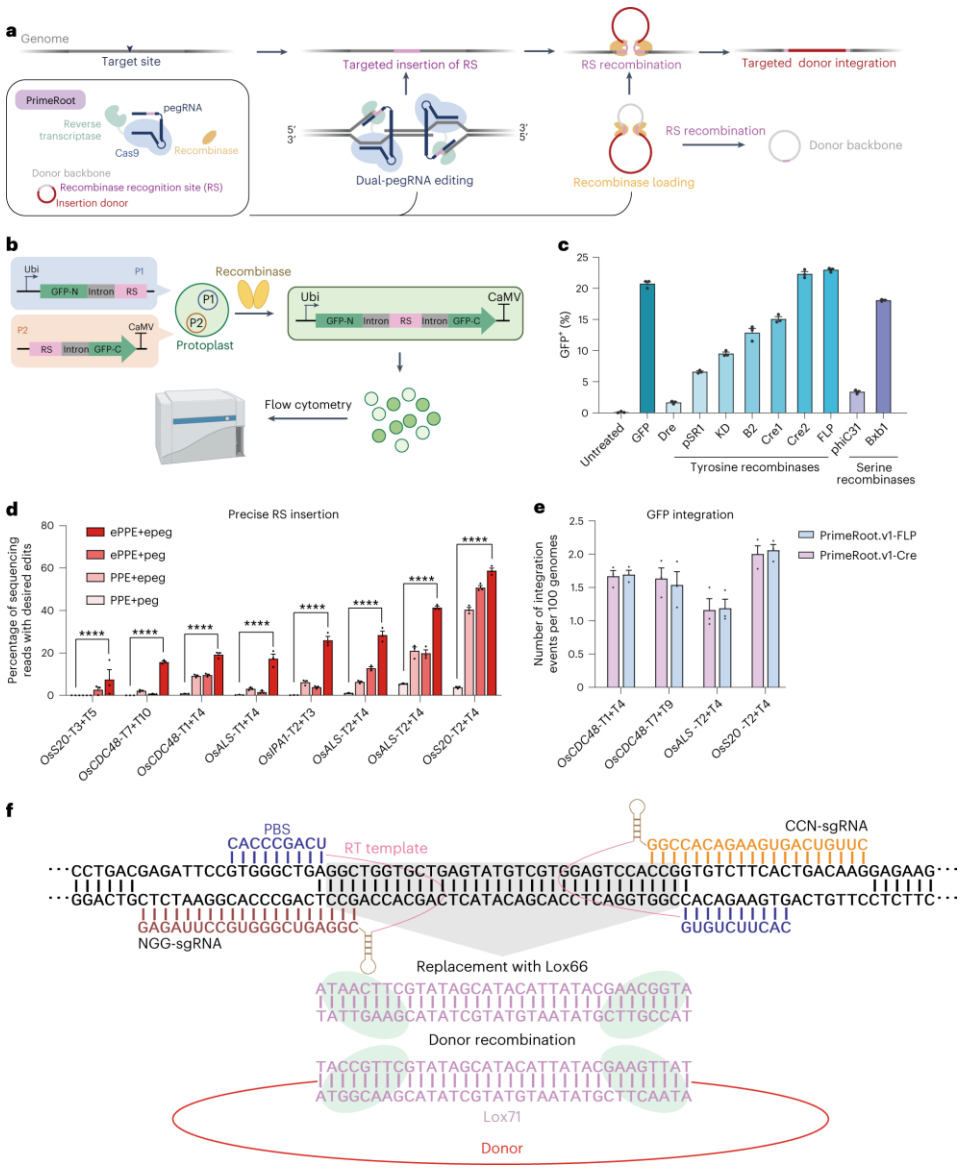
crRNA
- Expression
- Multiplexity

Endonuclease
- Chromosome cutting
- PAM recognition
- Expression
- Homologs
- Altering function by fusion partners

Donor
- Arm length
- Strandedness
- Chemical modifications

Chromosomal target
- Analytical tools to quantify types of integrations
- Optimizing target site location based on chromatin features

Examples of optimization of genome editing - PrimeRoot

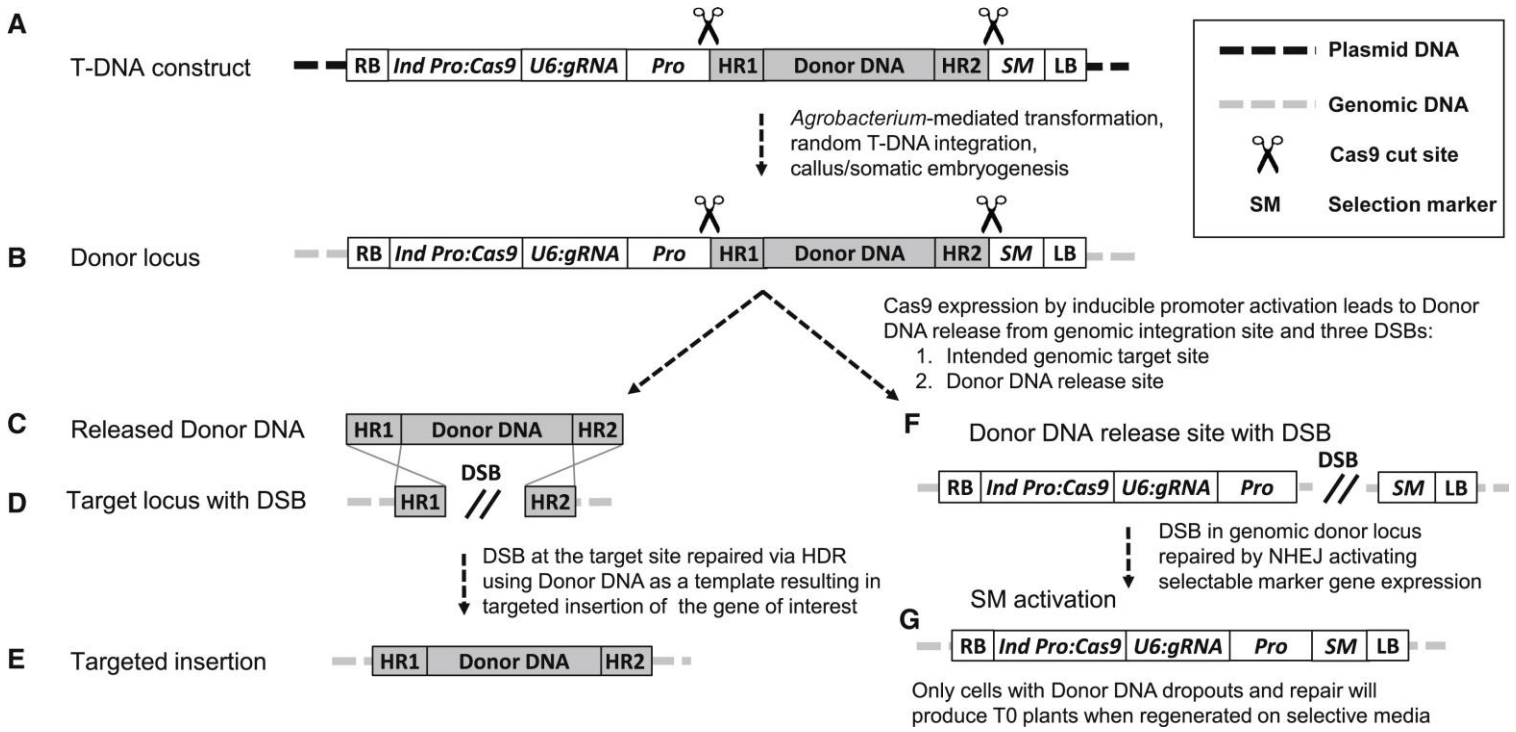


PrimeRoot combines plant-optimized recombinases and enhanced plant PE to create targeted DNA insertions.

a, Schematic overview of how PrimeRoot creates precisely targeted large DNA insertions in plants. **b**, Schematic diagram of the fluorescence reporter for evaluating the integration activity of SSRs in plant protoplasts. **c**, Percentage of GFP$^+$ plant protoplasts, reflecting recombinase activity, as measured by flow cytometry. Seven tyrosine recombinases and two serine recombinases were evaluated, and GFP was used as a positive control. Cre1 and Cre2 use different recombinase sites with the same Cre recombinase, as noted in the Supplementary Methods. Values and error bars represent means and standard errors of means for three independent biological replicates. **d**, Percentages of precise insertions of recombinase sites generated by PPE+peg, PPE+epeg, ePPE+peg and ePPE+epeg at seven endogenous sites as measured by high-throughput sequencing. Detailed editing efficiencies at each site are shown in Extended Data Fig. 2d. Values represent editing efficiencies across the seven sites, and error bars represent means and standard errors of means for three independent biological replicates. P values were obtained using the two-tailed Student's *t*-test: $^{****}P < 0.0001$. **e**, Percentages of GFP insertions across four endogenous sites induced by PrimeRoot.v1-Cre and PrimeRoot.v1-FLP measured by ddPCR. Values and error bars represent means and standard errors of means for three independent biological replicates. **f**, Scheme of PrimeRoot integration at *OsS20*, showing dual-ePPE-mediated RS insertion followed by donor recombination.

Sun et al. 2023 Nature Biotechnology

Examples of optimization of genome editing – inducible CRISPR expression



Inducible Cas9 and Selectable Marker Activation System for HR-Mediated Intra-genomic GT in Maize.
(A) Schematic description of the T-DNA construct containing Cas9 driven by an inducible promoter (*Ind Pro*), a guide RNA expressed by a U6 promoter, a donor flanked by homology arms (HR1 and HR2), and Cas9 cut sites (indicated by scissors) positioned between the selectable marker and upstream promoter such that the selectable marker is non-functional in this configuration.
(B) Stable random integration of T-DNA into the plant genome.
(C and D) Induction of Cas9 expression releases a donor DNA repair template and **(D)** simultaneously generates DSB at the target site.
(E) HR-mediated repair at the target site leads to targeted insertion of the gene of interest.
(F and G) The DSB at the donor locus repaired by NHEJ, **(G)** resulting in activation of a functional selectable marker.

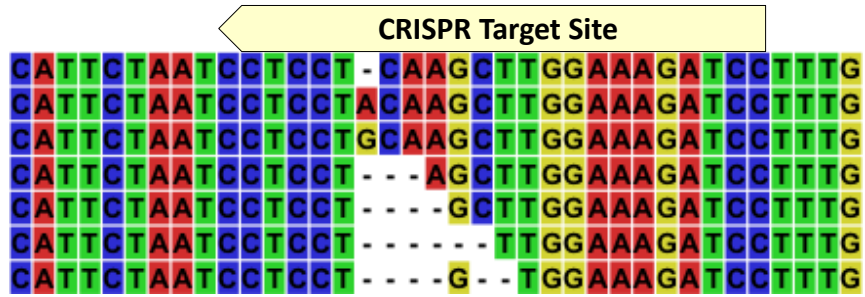
Barone et al. 2020 Molecular Plant

Genome editing testing workflow

1. Preparation of editing reagents
2. Transformation into plants
3. Molecular analysis of transformants

***In vitro RNP* assembly**

crRNA Cas protein or derivatives donor DNA



Step 1. Preparation of editing reagents

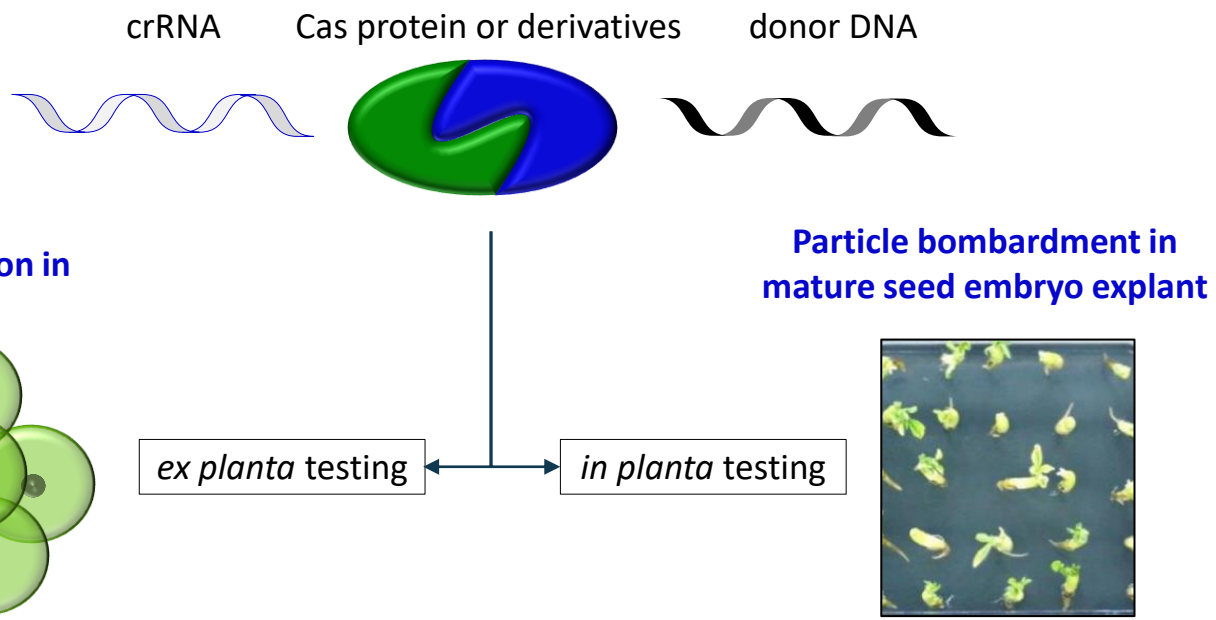
Methods:

1. **Plasmids carrying gene expression cassettes.**

Enzyme - Pol II cassette: P-Zm.Ubi | L-Zm.Ubi | I-Zm.Ubi | NLS1 | CR-Lb.Cas12a | NLS2 | T-Os.LTP

crRNA - Pol III cassette: P-Pol III. | Rep | TS1 | Rep | TS2 | Rep | T-T7

2. ***In vitro*** **assembly of Cas12a/crRNA ribonucleoprotein (RNP) complexes.**



Step 2. Transformation into plants

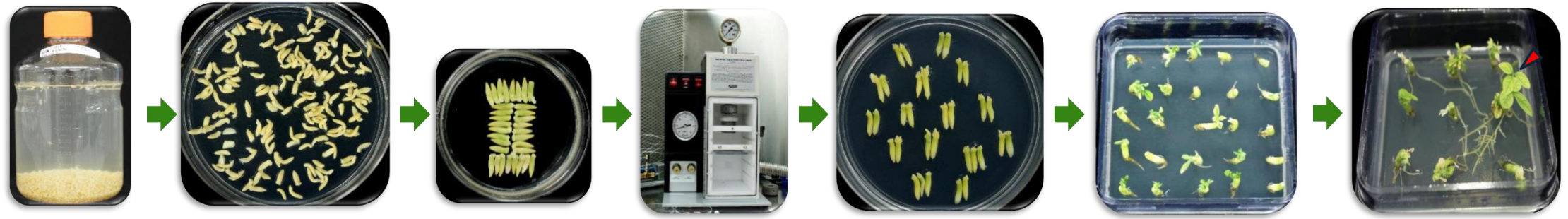
Methods:

1. Agrobacterium-mediated transformation – pipeline (HTP) solution in plants.

2. Protoplast transformation (model system).

3. Particle bombardment into plants – discovery alternative in plants.

Particle bombardment



Dafu Wang, Ashok Shrawat

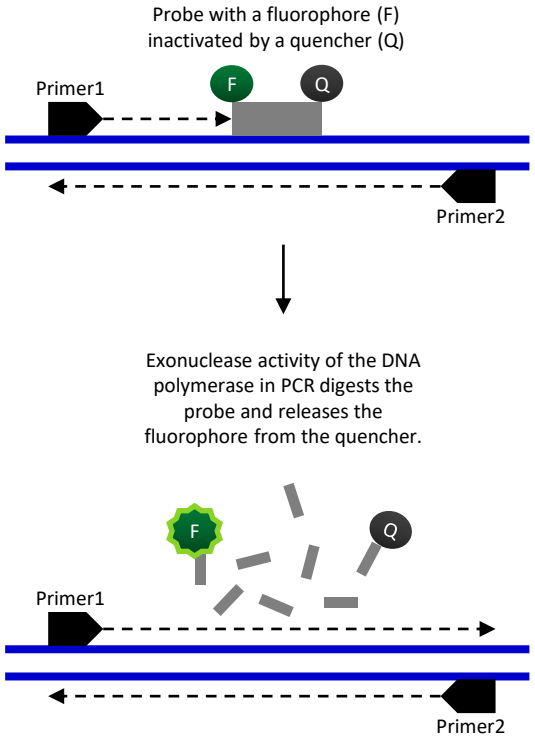
Step 3. Molecular analysis of transformants

Method:

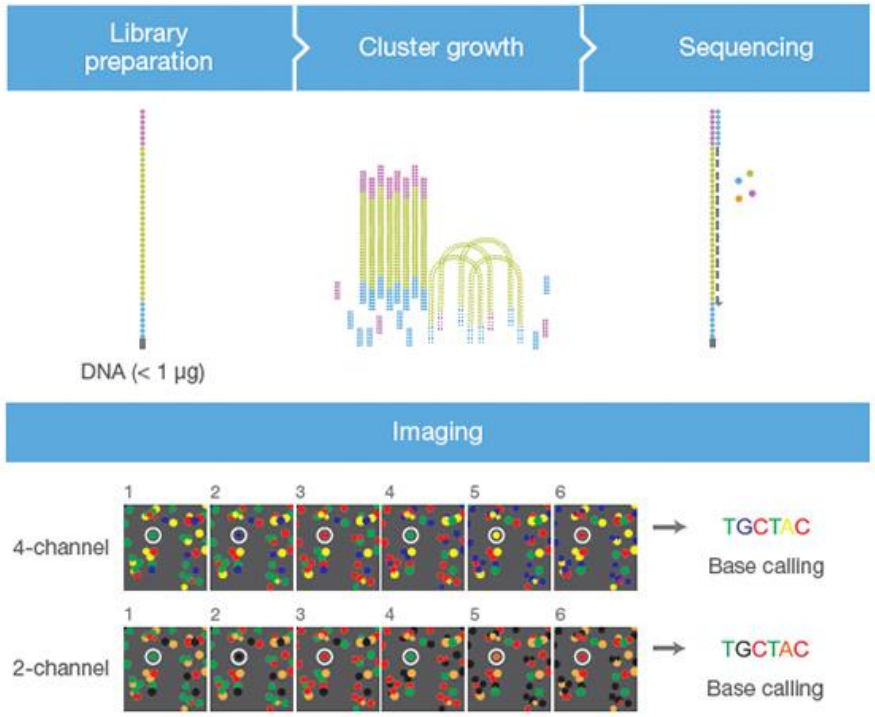
1. TaqMan-based quantitative PCR for transgene copy number.

2. Amplicon sequencing by Next Generation Sequencing (AmpSeq, NGS).

TaqMan



AmpSeq (illumina.com)



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