ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

New Breeding Techniques - an update

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ISTA Seminar: From Biodiversity to Diversification: resources, tools and technologies to meet new challenges.

Verona, IT 29 May 2023.

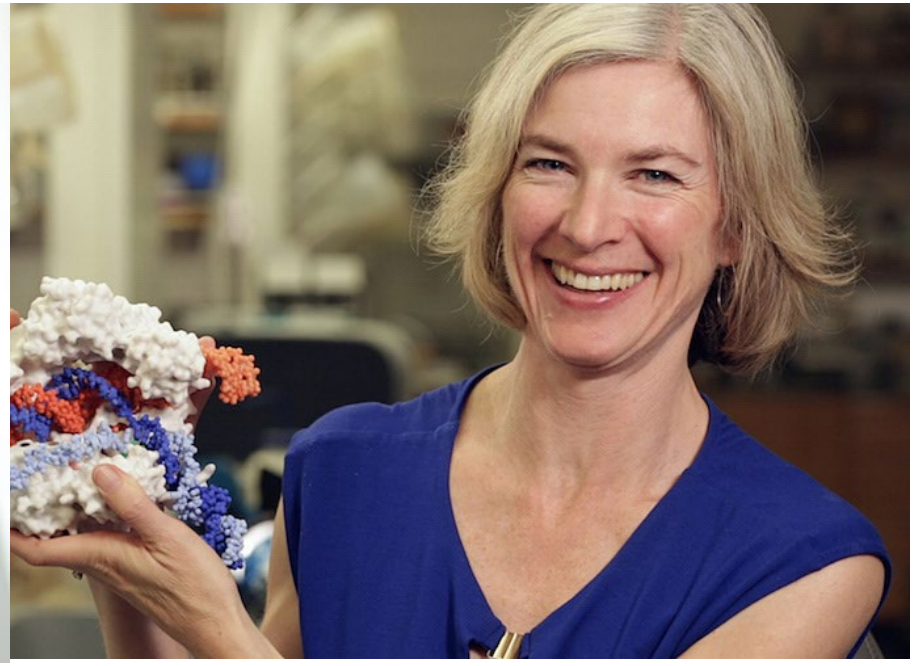
Gene editing methods

Methods based on hybrid proteins, or protein-RNA complexes, able to target specific DNA regions where they induce mutations

- **MEGANUCLEASES:** enzymes with long target recognition sequences (14-40 nt) and high DNA cleavage specificity, derived from transposon-like elements from mitcochondria, chloroplast and bacteria genomes
- **ZFN (Zinc-Finger Nucleases**): DNA binding Zinc-finger motif is associated with the nuclease motif of *FokI*
- **TALEN (Transcription Activator-Like Effector Nucleases**): DNA binding motif of a transcription activator from *Xanthomonas* associated with *FokI*.
- **CRISPR-Cas9 (Clustered-regularly interspaced short palindromic repeats – Crisp associated protein9).**

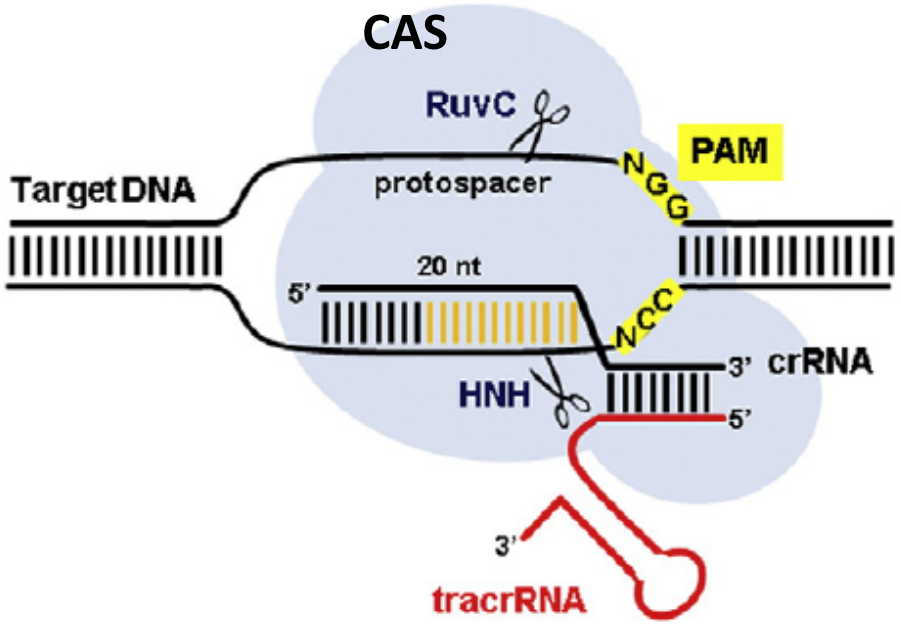
Nobel Prize for Chemistry (2020)

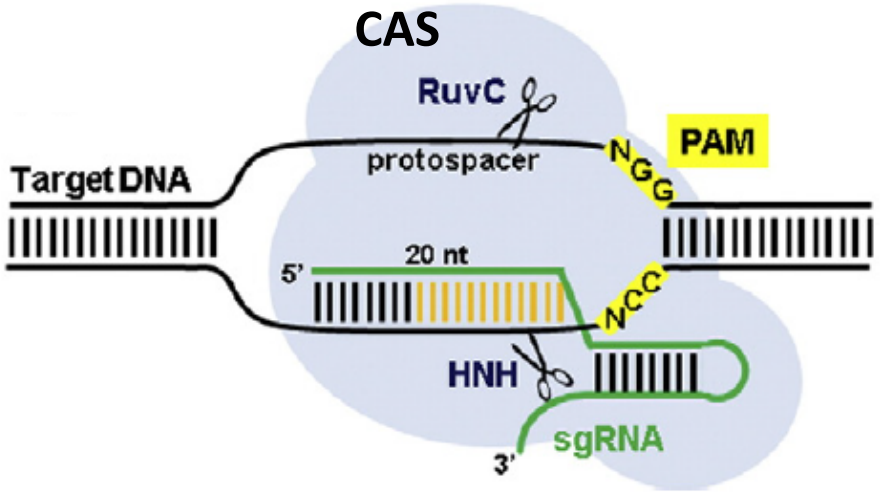
Emmanuelle Carpentier, Umea University, Svezia
(now at Max Planck Institute for Infection Biology)



Jennifer Doudna,
University of Berkeley

CRISPR-CAS main components





crRNA = CRISPR-RNA
tracrRNA = transactivating crRNA
CAS = CRISPR-Associated nuclease

Native

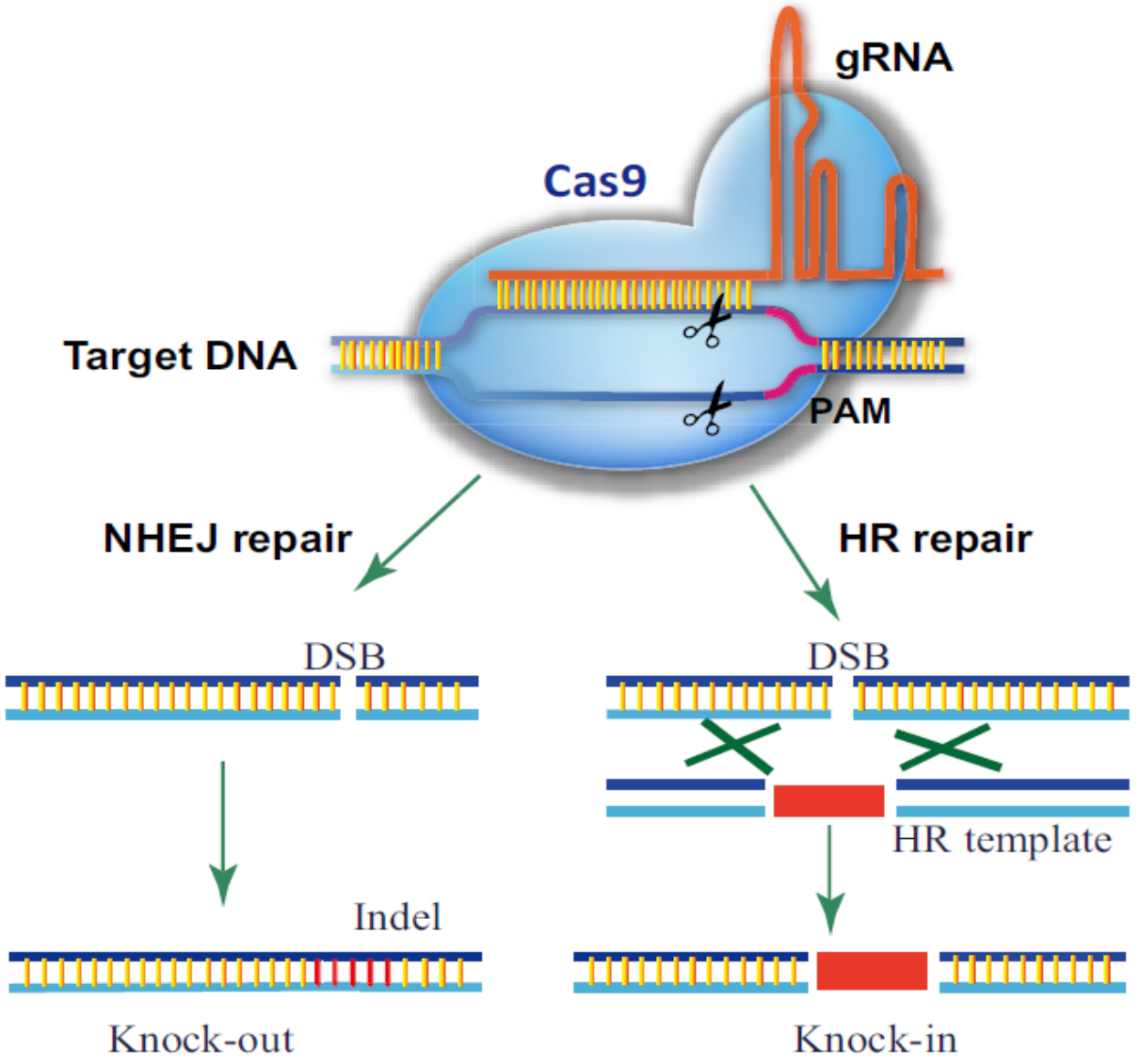
sgRNA = sigle guide RNA

Modified as gene editing tool

Mechanisms of targeted mutagenesis with CRISPR-CAS

NHEJ (non-homologous end joining)

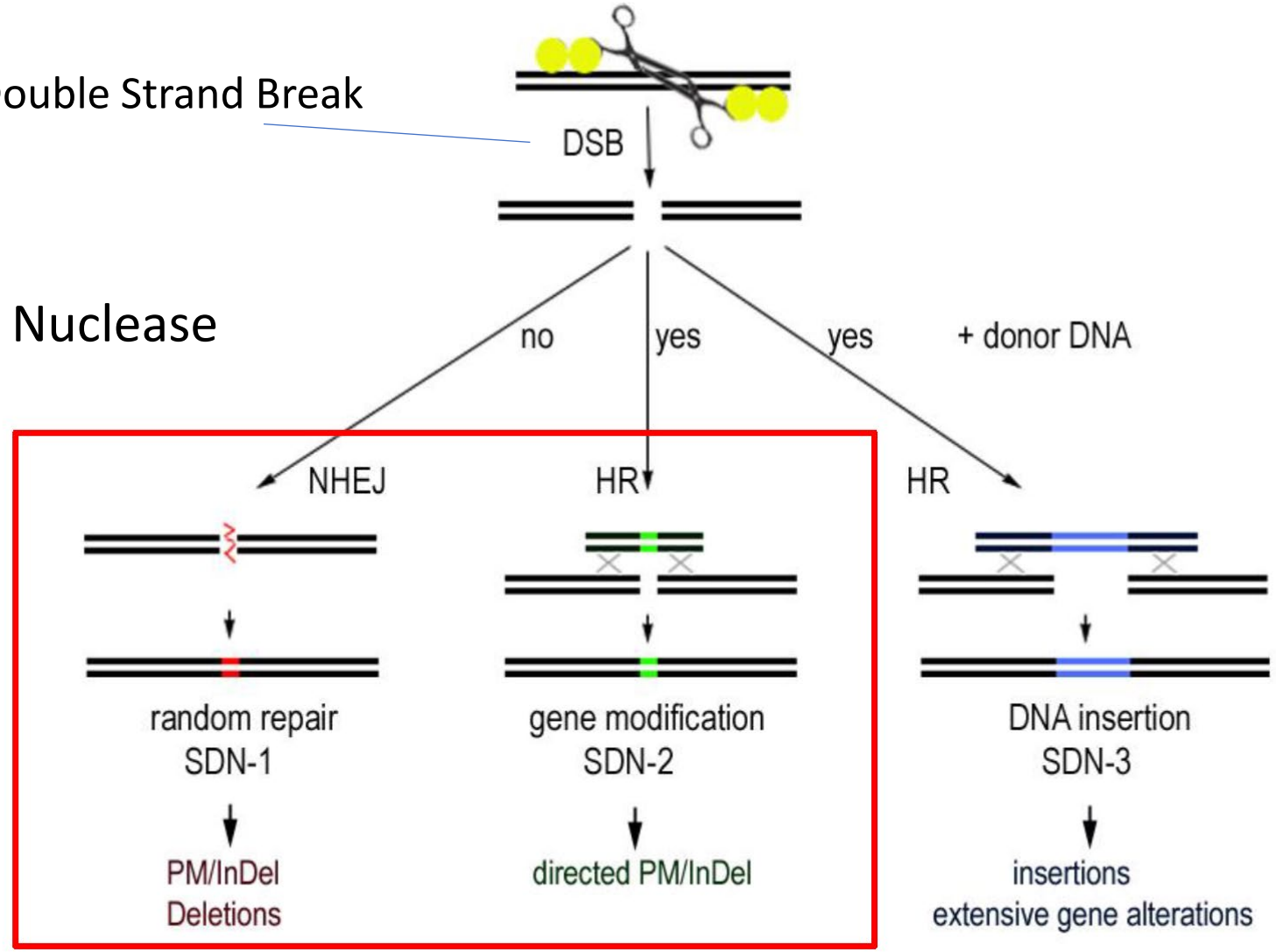
HR (homology-directed repair)



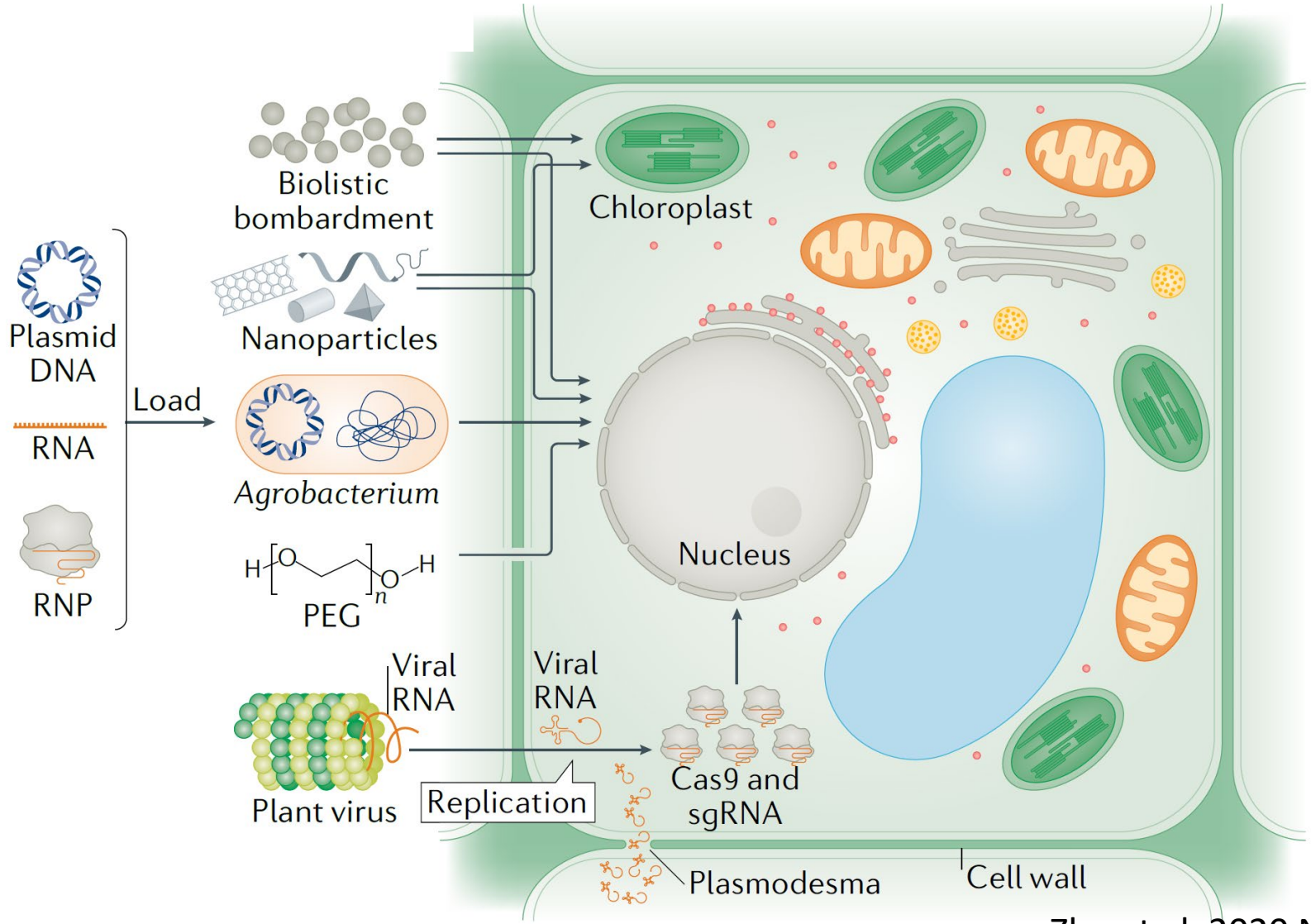
The ‘SDN’ system to classify edited events

DSB = Double Strand Break

SDN = Site-Directed Nuclease technologies, 1-3



Delivering CRISPR/Cas reagents



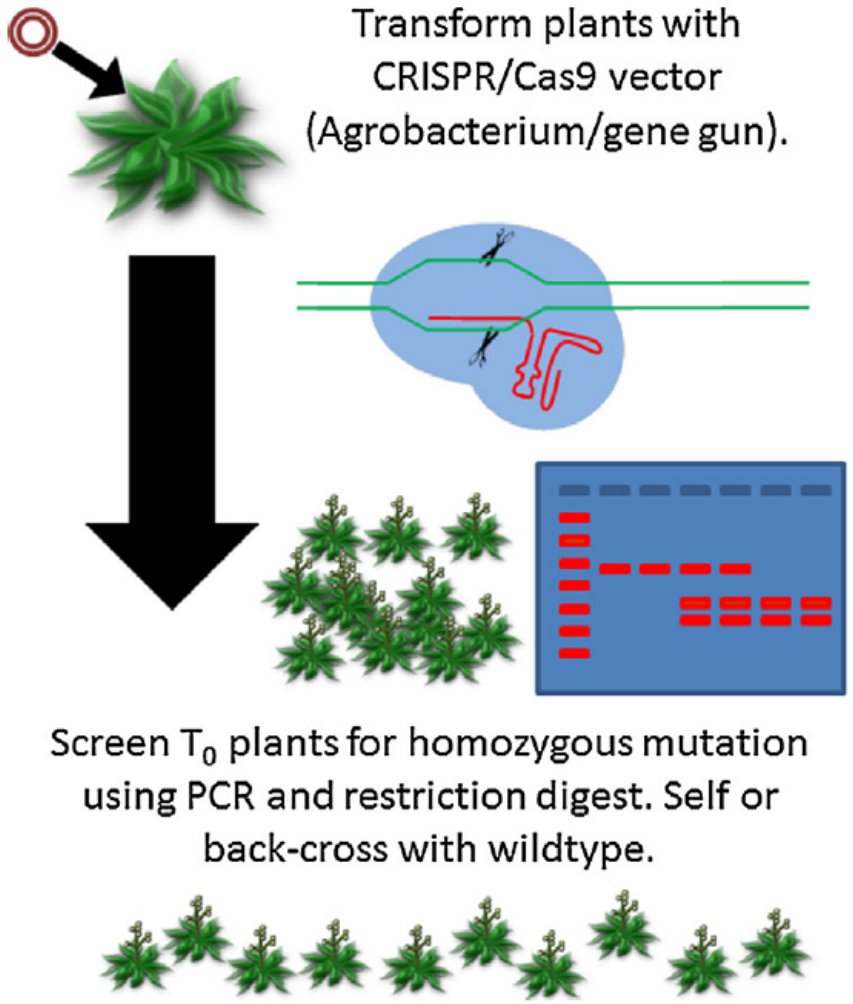
Zhu et al. 2020 Nat Rev Mol Cell Biol

Delivering CRISPR/Cas reagents

- Conventional delivery methods are still *Agrobacterium*-mediated transformation and particle bombardment

  however new methods seem promising
- Particle bombardment (or PEG-mediated transfection) using RNP
  - Boosted with regeneration, or meristem, inducers (*BBM1* and *WUS2*, etc)
- Viral vectors-mediated delivery system
- Nanoparticle-based transformation
- Grafting-based systems

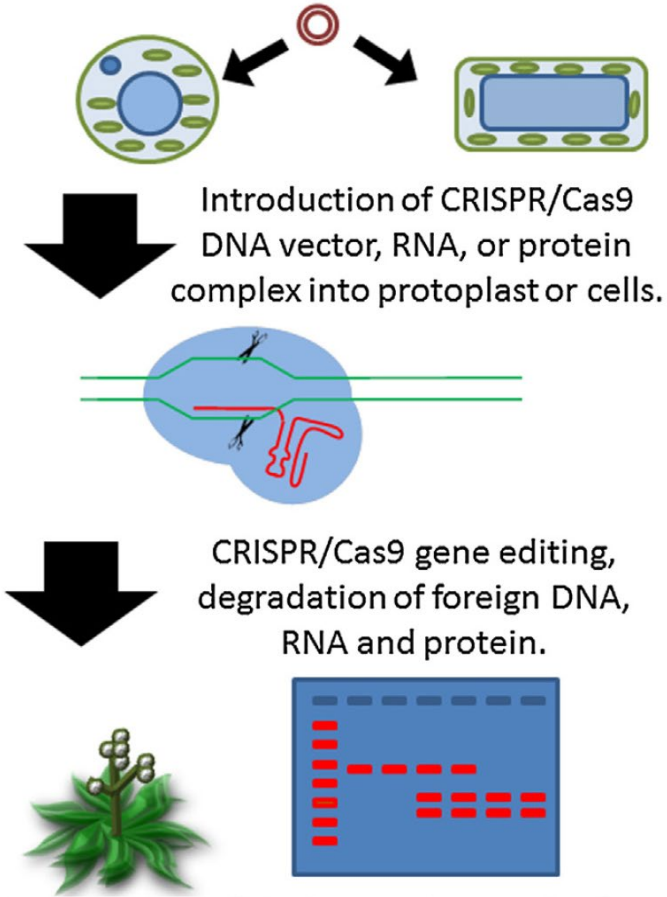
CRISPR-CAS editing traditionally goes through transgenics



T-DNA with gRNA-CAS9 construct is expected to land on a different chromosome from the one carrying the target gene, so it can be segregated off in subsequent generations

Schaeffer and Nakata, 2015, Plant Science

RNP-based delivery may produce edits without transgenics



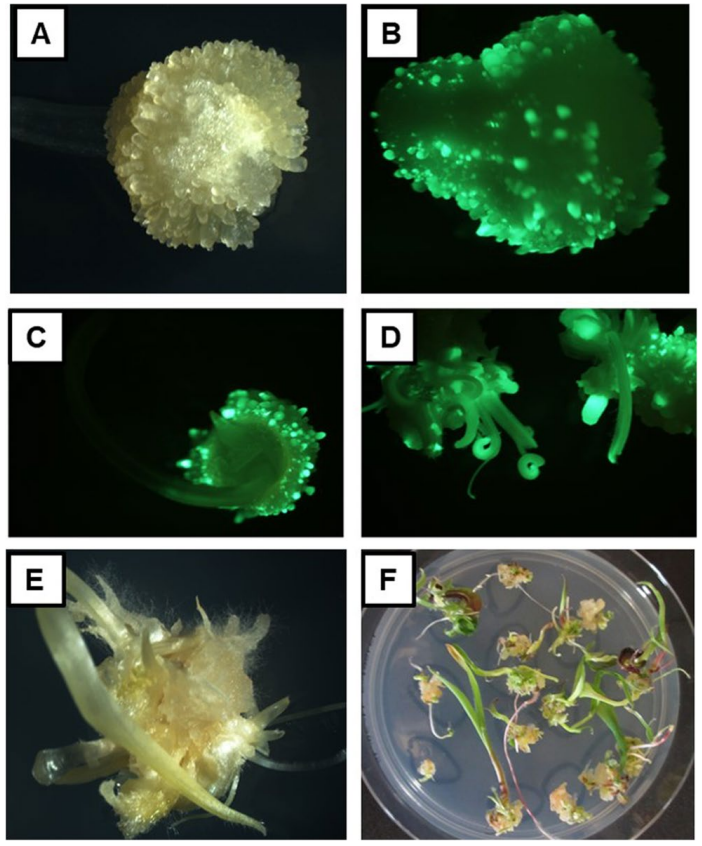
Regeneration T1/T2 plants and screen for mutation

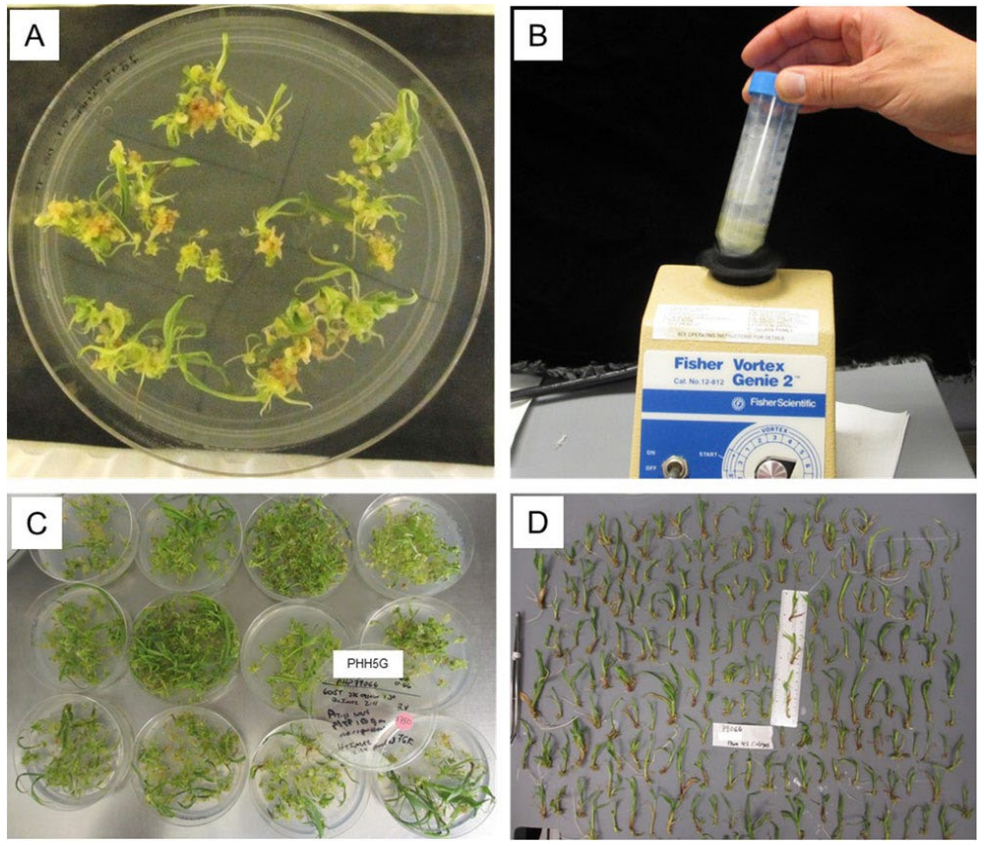
RNP = Ribonucleoprotein = gRNA + CAS9 (protein or DNA or RNA)

Schaeffer and Nakata, 2015, Plant Science

Use of morphogenetic regulators to boost regeneration

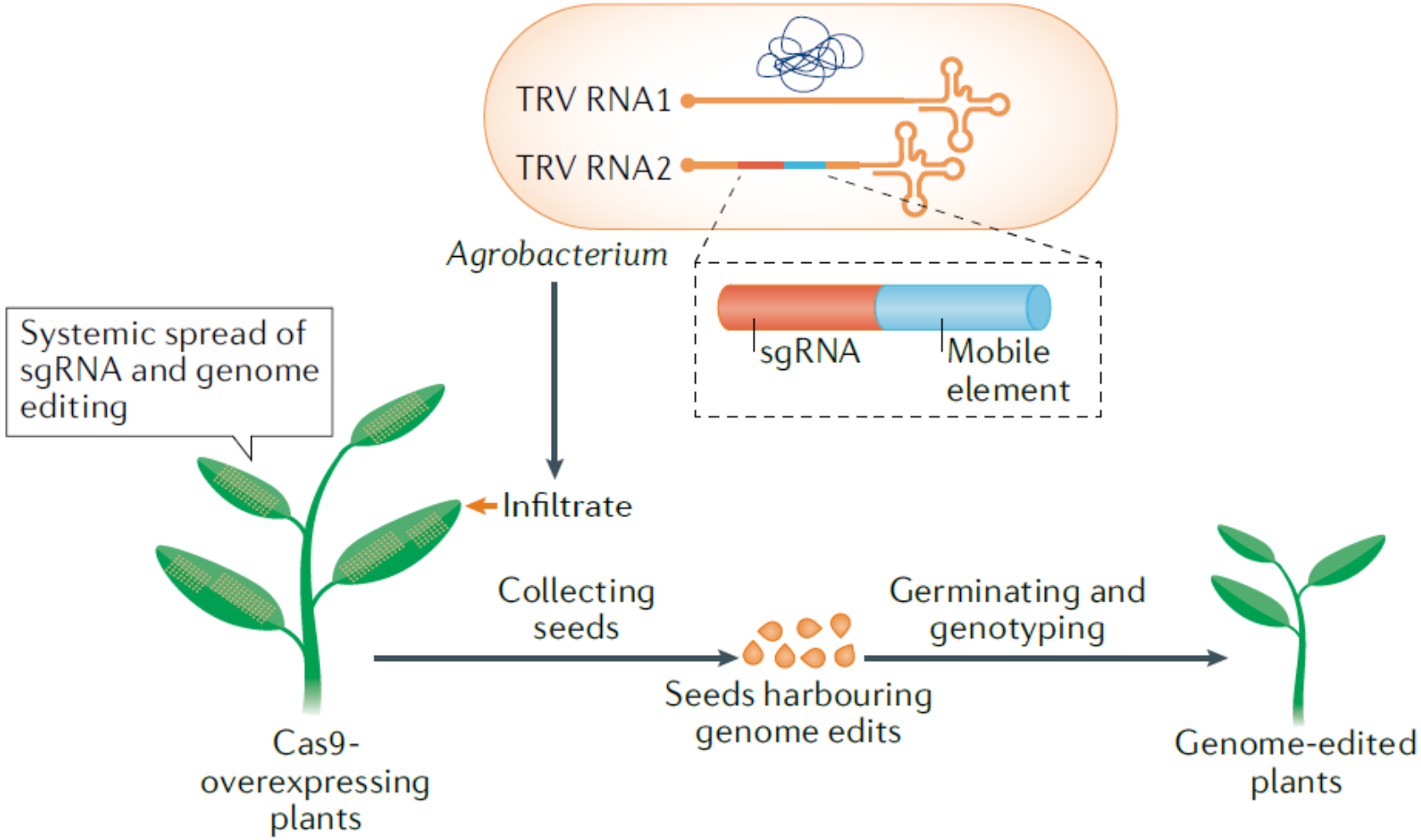
BABY BOOM 1 (*BBM1*) and *WUS2 in co-transformation or assembled in RNP*





Lowe et al 2018 In Vitro Cellular & Developmental Biology - Plant 54:240–252

Virus-induced heritable gene editing

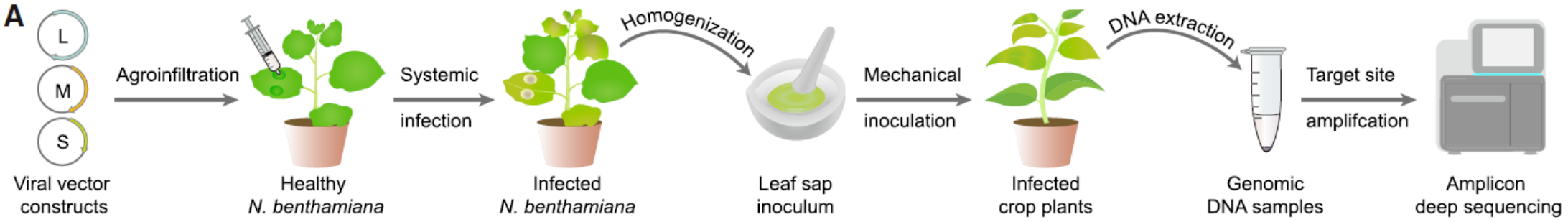


Zhu et al. 2020 Nat Rev Mol Cell Biol

Virus-based vectors: miniature cargos for CRISPR machinery

- various virus-based vectors can be utilized to deliver genome-editing reagents without risking the genetic integration of foreign DNA into the plant genome.
- tobacco rattle virus (TRV) and potato virus X (PVX), have been used as carriers for targeted mutagenesis in plants, but owing to their limited cargo capacity, only single guide RNAs (sgRNAs) have been delivered in Cas9-overexpressing (Cas9-OE) transgenic lines.
- Recently, Liu et al. reported a tomato spotted wilt virus (TSWV)-based vector for the delivery of CRISPR/Cas9 and Cas12a machinery in various crops for targeted mutagenesis

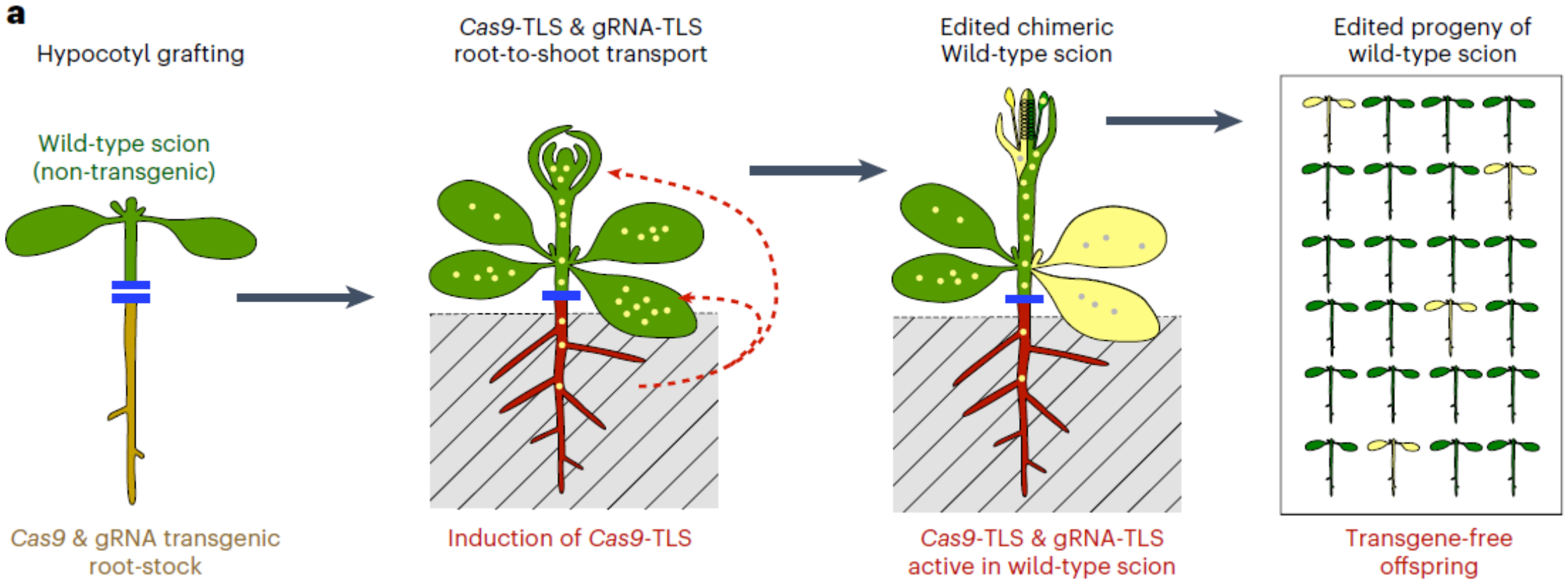
Virus-induced heritable gene editing
(‘-’RNA virus cargos with both sgRNA and CAS)



Liu et all 2023 Engineered biocontainable RNA virus vectors for non-transgenic genome editing across crop species and genotypes. Mol. Plant. 16, 616–631

CRISPR–Cas9-mediated transgene-free gene editing by grafting

Fusions of Cas9 and guide RNA transcripts to **tRNA-like sequence** motifs that move RNAs from transgenic rootstocks to grafted wild-type shoots (scions)



Yang et al. 2023 Nature Biotechnology. https://doi.org/10.1038/s41587-022-01585-8

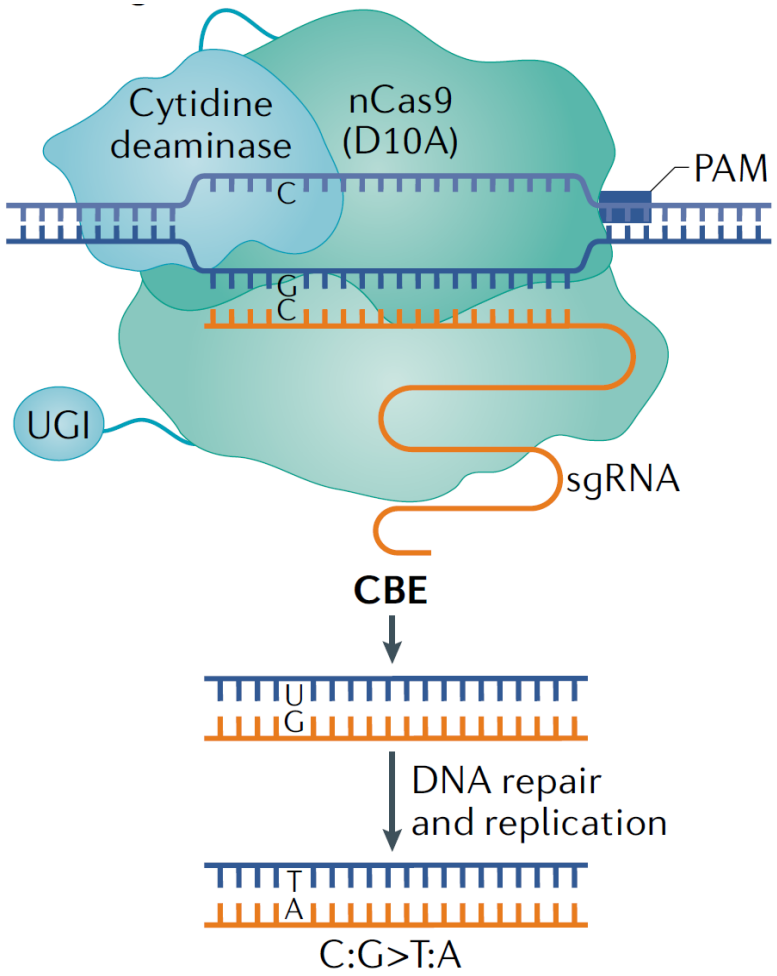
Improving the specificity of genome editing (ie. reduction of off-targets)

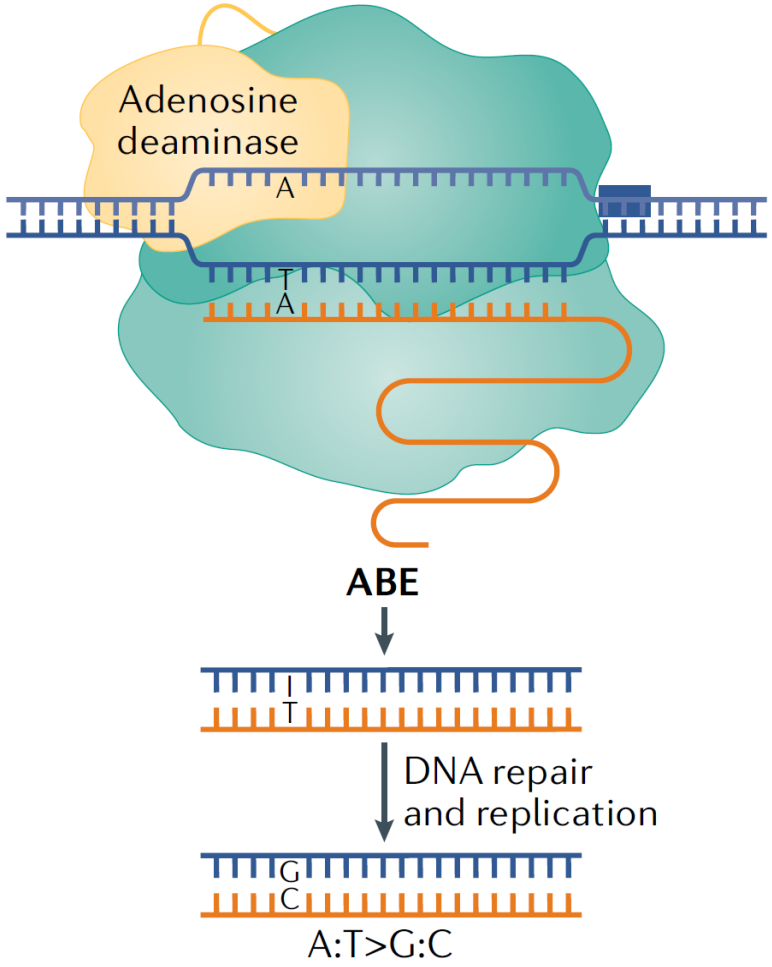
- Correct sgRNA design (eg 40-60% GC, and other constraints)
- Chemical modification of sgRNA (eg. Integration of bridges and locks)
- Use of Cas9-gRNA ribonucleoproteins (RNP)s
- Engineered precision variants of Cas9, Cas12a, and deaminases or high-fidelity Cas9 (eg. enhanced specificity SpCas9, eSp-Cas9)

Improving the range of genome edits

Cytosine and Adenine base editing

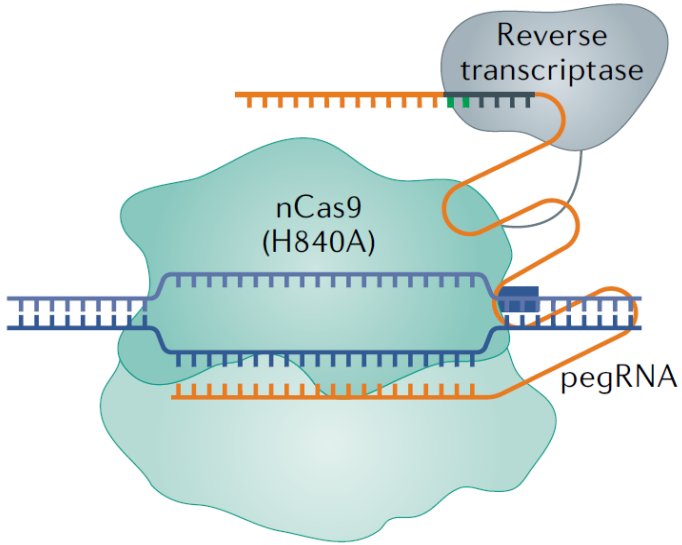
Generate base transition!

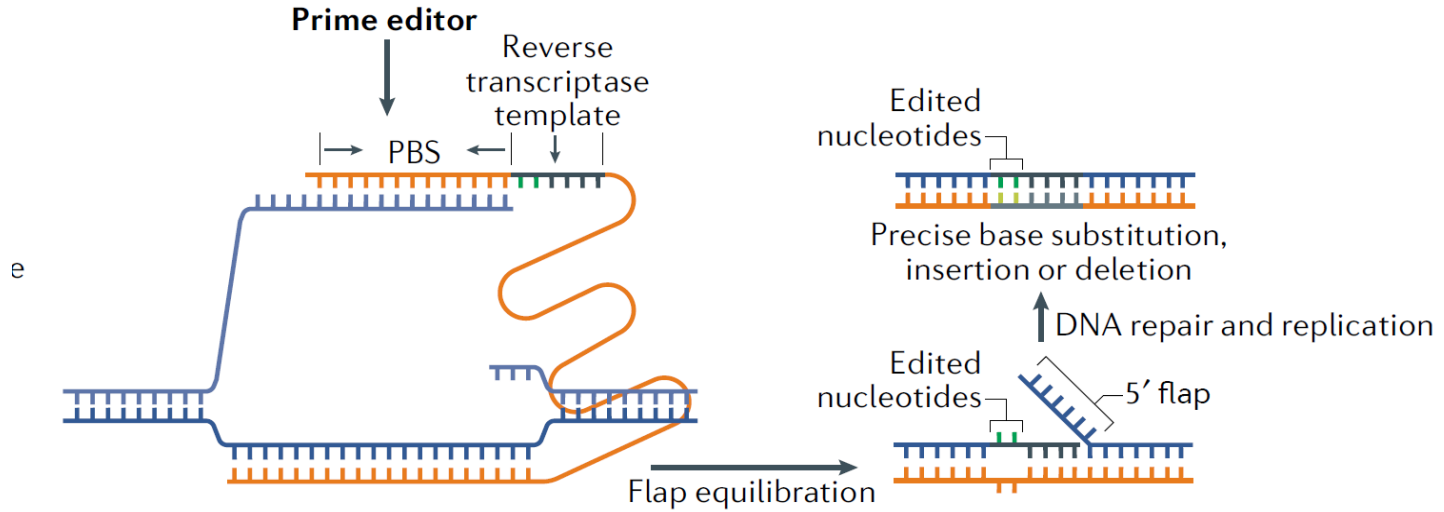




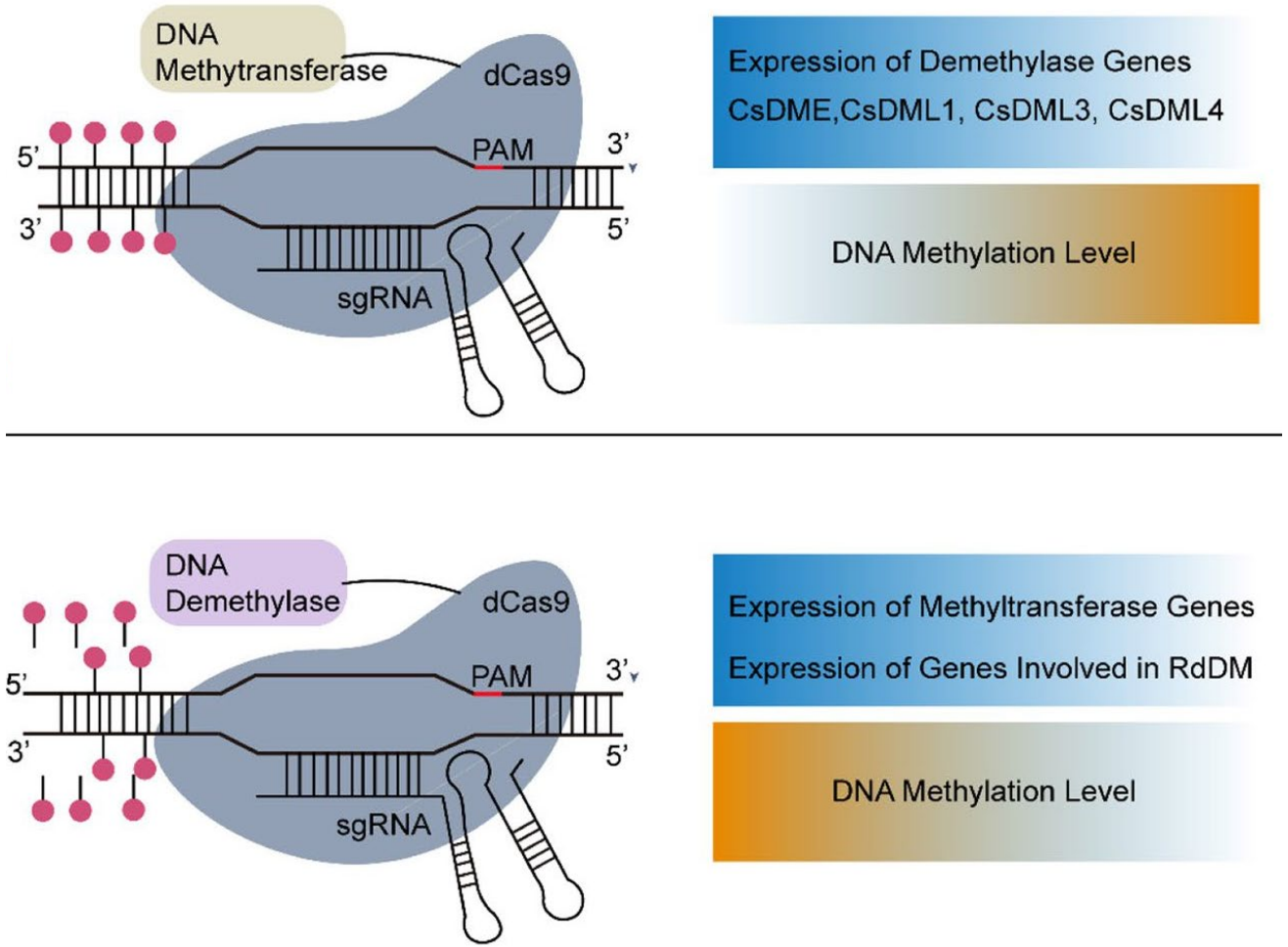
Prime editing

- Can produce all 12 kinds of base substitutions on target (at least in human cells)
- Under optimization in plants





CRISPR/dCas9-based epigenetic modifier



Ma *et al. Molecular Horticulture (2023) 3:1*

Conclusions

- Gene editing by CRISPR-CAS has already proved to be applicable to crops
- The protocols and molecular components are being further optimized, so scope, efficiency and precision will likely improve strongly in the near future
- The regulation is clearly the current main obstacle to full exploitation of this technology

Gene editing by CRISPR-CAS method