

Genome editing techniques

Sequence specific nucleases (SSNs), which induce double strand breaks.



knock-out (KO) or knock-in (KI) edits

- Meganucleases (e. g. I-SceI, I-CreI)
- Zinc finger nucleases, ZFNs
- Transcription activator-like effector nucleases, TALENs
- clustered regularly-interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) 9 (CRISPR/Cas9)

Oligonucleotide Directed Mutagenesis (ODM)

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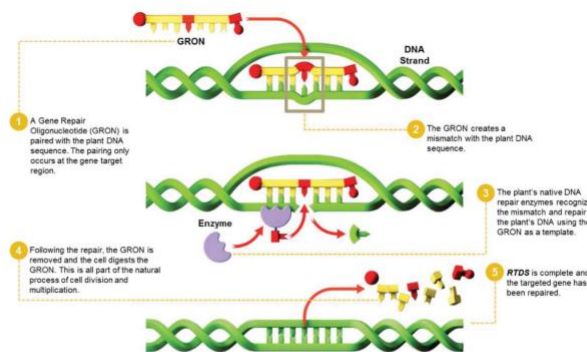


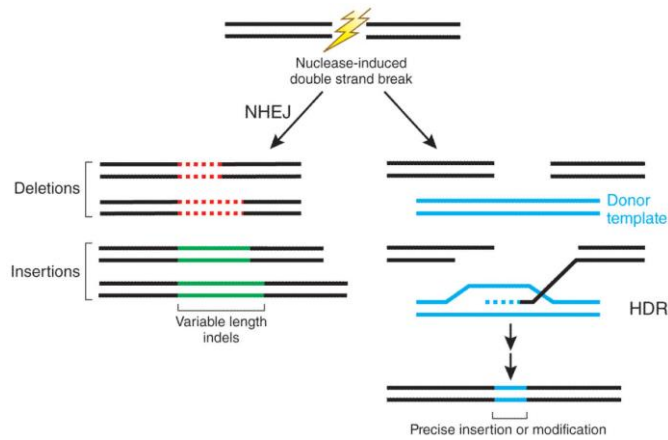
Figure 1. RTDS™ (Rapid Trait Development System) gene conversion process based on ODM (Oligonucleotide Directed Mutagenesis) technology pioneered by Cibus.

Songstad et al. 2017. Genome Editing of Plants. Critical Reviews in Plant Sciences, 36(1), 1-23.

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Two mechanisms to repair DSBs

- *non-homologous end joining, NHEJ*
- *homology directed repair, HDR*



Nuclease induced genome editing
(Vir: Sander, J. D., & Joung, J. K., 2014).

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Homology directed repair

<https://www.youtube.com/watch?v=86JCMM5kb2A>

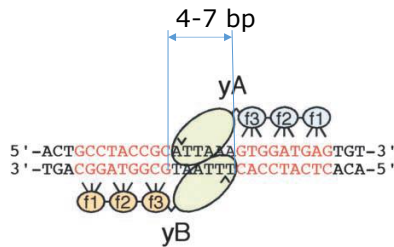
Non-homologous end joining

<https://www.youtube.com/watch?v=31stiofJjYw>

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Zinc-finger nucleases

- Monomer consists of two functional domains: a DNA-binding domain and a DNA cleavage domain comprised of the nuclease domain of *FokI*



Each finger contacts three consecutive base pairs of DNA. When both sets of fingers are bound, the cleavage domain can dimerize to form an active nuclease (obtained from *Flavobacterium okeanoikoites*).

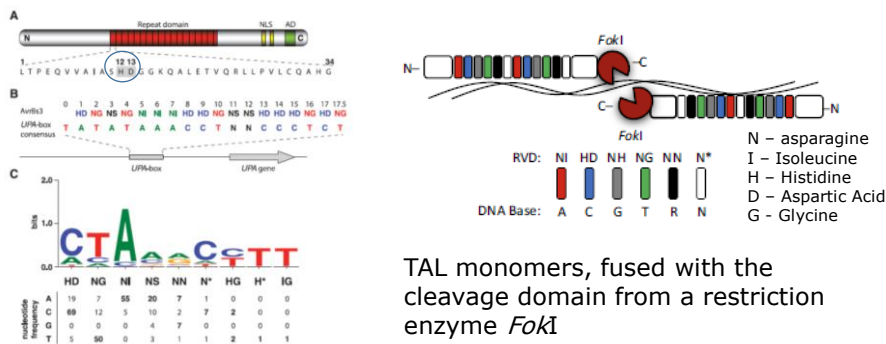
Structure

Bibikova, M., Golik, M., Golik, K. G., & Carroll, D. (2002). Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics*, 161(3), 1169-1175.

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TALENs

- DNA binding domain of AvrBs3 TAL effector from *Xanthomonas campestris* contains 17.5 repeats and each repeat region consists of 34 amino acid repeat units that are nearly identical (12 and 13 aa are hypervariable)
- Monomers constructed for genetic engineering consist of 15 to 20 repeats (length of the target sequence larger than 30 bp)
- One unit (repeat) pairs with specific base in the target DNA
- Larger compared to meganucleases and ZFN: 950 aa or 1900 aa for both monomers



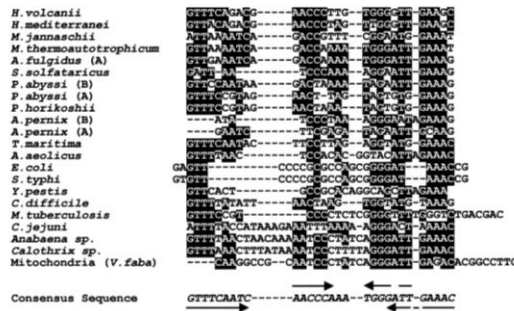
Model for DNA-target specificity of [TAL effectors](#)

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., . . . Bonas, U. (2009). Breaking the Code of DNA Binding Specificity of TAL-Type III Effectors. *Science*, 326(5959), 1509.

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CRISPR

- CRISPR arrays were first identified in the *E. coli* genome in 1987 (Ishino et al.)
- 1993 – Francisco Mojica worked on *Haloferax mediterranei*, an archaeal microbe with extreme salt tolerance. Mojica found a curious structure—multiple copies of a near-perfect, roughly palindromic, repeated sequence of 30 bases, separated by spacers of roughly 36 bases—that did not resemble any family of repeats known in microbes (Mojica et al., 1993).
- 2003 (2005) – Mojica realized that CRISPR loci must encode the instructions for an adaptive immune system that protected microbes against specific infections.



Alignment of the palindromic repeat region from different bacteria species. Arrows indicate the palindromic repeats.

Mojica, F. J. M., Díez-Villaseñor, C., Soria, E., & Juez, G. (2000). Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Molecular Microbiology*, 36(1), 244-246.

Lander. 2016. The Heroes of CRISPR.

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- 2005 – Cas9 necessary for phage resistance (in the early CRISPR literature, the now-famous cas9 gene was called cas5 or csn1) (*S. thermophilus*)
- 2008 – observation that cleavage is done 3 nt upstream of the protospacer adjacent motif (PAM)

Table 1. Classification and Examples of CRISPR Systems

Class	Type	Subtype	Hallmarks	Example effector	Example organism	Studies Cited
Class 1	Type I		multisubunit effector complex; Cas3	Cascade	<i>E. coli</i>	Brouns et al., 2008
		III-A	multisubunit effector complex; Csm effector module; DNA targeting	Cas10-Csm	<i>S. epidermidis</i>	Marraffini and Sontheimer, 2008
		III-B	multisubunit effector complex; Cmr effector module; RNA targeting	Cmr	<i>P. furiosus</i>	Hale et al., 2009
Class 2	Type II		single protein effector; tracrRNA	Cas9	<i>S. thermophilus</i>	Bolotin et al., 2005; Barrangou et al., 2007; Sapranaukas et al., 2011; Gasiunas et al., 2012
					<i>S. pyogenes</i>	Deltcheva et al., 2011; Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013
	Type V		single protein effector; single-RNA guided	Cpf1	<i>F. novicida</i>	Zetsche et al., 2015

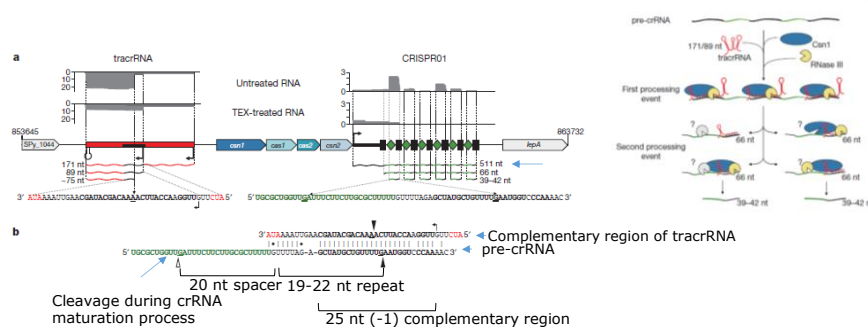
CRISPR systems are currently organized into two overarching classes: Class 1, which contain multi-subunit effectors, and Class 2, which contain single protein effectors. These classes are subdivided into five types (Makarova et al., 2015), with type IV remaining a putative type within Class 1. Although only Class 2 systems have been adapted for genome engineering, the results described in this review emerged from studying a diversity of CRISPR-Cas systems. (Type III-B systems are not discussed but represent an unusual system that targets RNA rather than DNA [Hale et al., 2009].)

Lander, E. S. (2016). The Heroes of CRISPR. *Cell*, 164(1-2), 18-28.

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2011 – discovery of tracrRNA (trans-activating CRISPR RNA) (*S. pyogenes*)

- tracrRNA – two functions:
 - a) tracrRNA directs pre-crRNA processing with Rnase III
 - b) required for cleavage of DNA together with crRNA guided Cas9 protein
- Cas9 the only Cas protein required for the production of mature crRNA and concomitant tracrRNA cleavage.

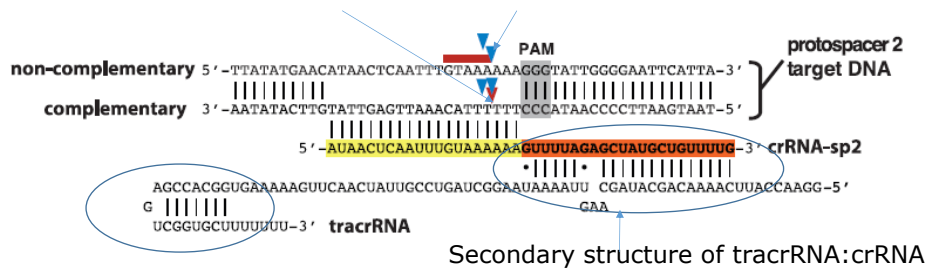


Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., . . . Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor Rnase III. *Nature* 471 602

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CRISPR/Cas9 as a system for genome editing

Cleavage sites of crRNA complementary and noncomplementary DNA



Schematic representation of tracrRNA, crRNA and complementary DNA hybridization.

Regions of crRNA complementarity to tracrRNA (orange) and the protospacer DNA (yellow) are represented. The PAM sequence is shown in gray; cleavage sites are represented by blue arrows (C), a red arrow [(D), complementary strand], and a red line [(D), noncomplementary strand].

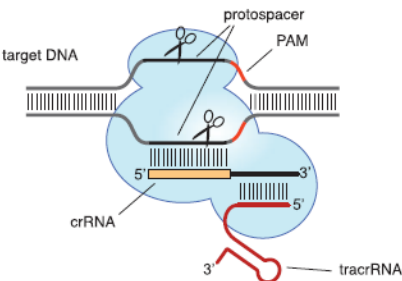
Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*, 337(6096), 816-821.

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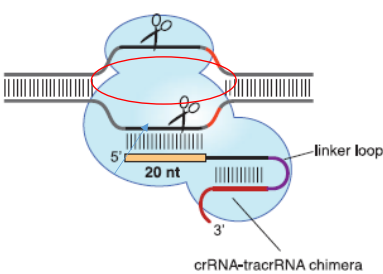
CRISPR/Cas9 as a system for genome editing

- Each Cas9 nuclease domain cleaves one DNA strand. Cas9 contains domains homologous to both HNH (cleavage of complementary strand) and RuvC endonucleases (cleavage of noncomplementary strand).
- crRNA and tracrRNA engineered as a single transcript > guide RNA (gRNA, sgRNA, synthetic guide RNA)

Cas9 programmed by crRNA:tracrRNA duplex



Cas9 programmed by single chimeric RNA



Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*, 337(6096), 816-821.

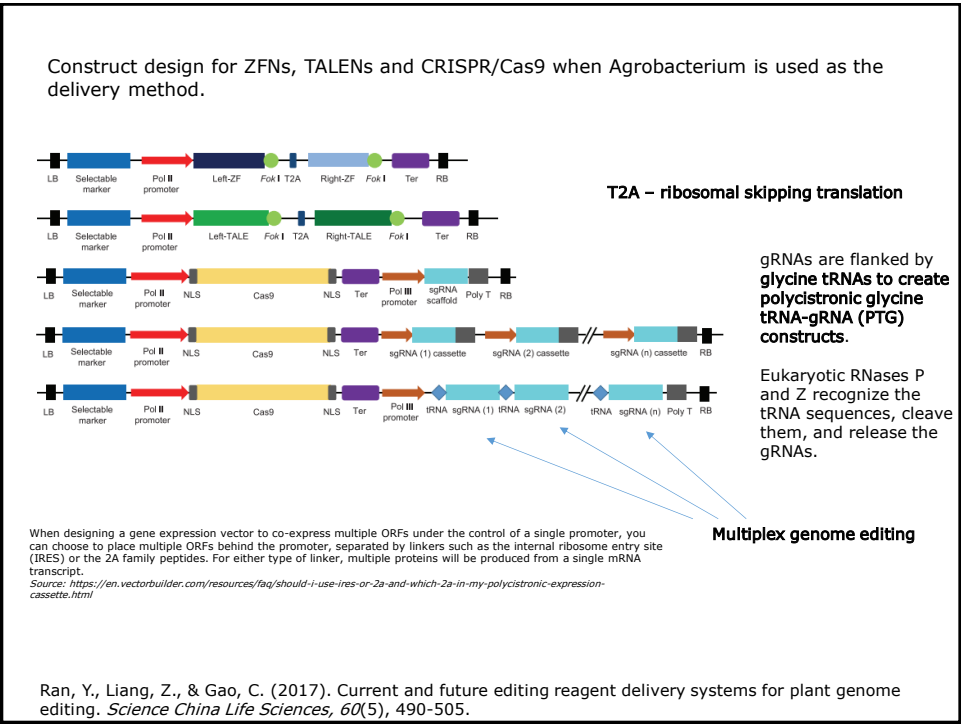
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Cas9 Species/Variants and PAM Sequences

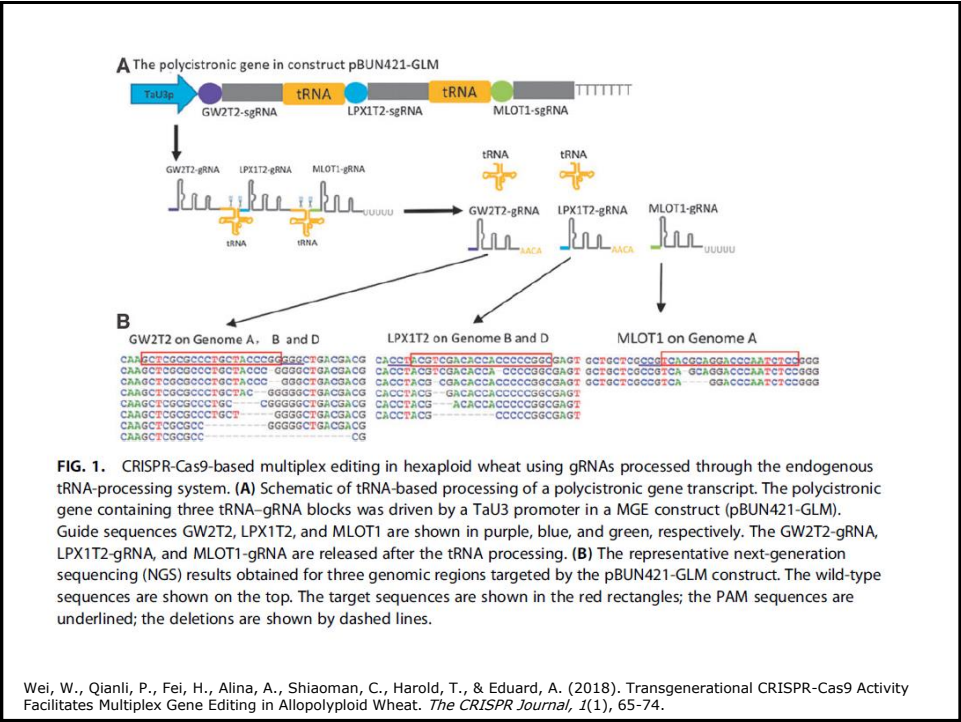
Species/Variant of Cas9	PAM Sequence
<i>Streptococcus pyogenes</i> (SP); SpCas9	NGG
SpCas9 D1135E variant	NGG (reduced NAG binding)
SpCas9 VRER variant	NGCG
SpCas9 EQR variant	NGAG
SpCas9 VQR variant	NGAN or NGNG
<i>Staphylococcus aureus</i> (SA); SaCas9	NNGRRT or NNGRR(N)
<i>Neisseria meningitidis</i> (NM)	NNNNGATT
<i>Streptococcus thermophilus</i> (ST)	NNAGAAW
<i>Treponema denticola</i> (TD)	NAAAAC
Cpf1 (from various species)	TTN
Additional Cas9s from various species	PAM sequence may not be characterized

AddGene: CRISPR 101: A Desktop Resource, 2nd edition

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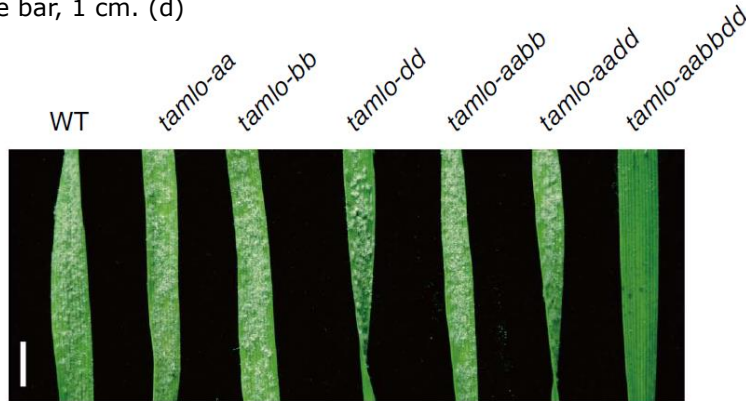
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Wheat powdery mildew (*Blumeria graminis*)

Macroscopic infection phenotypes of representative leaves of WT and the indicated mlo mutants 7 d after inoculation of detached leaves with Bgt. Scale bar, 1 cm. (d)



Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., & Qiu, J.-L. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature Biotechnology*, 32, 947.

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Addition of Multiple Introns to a Cas9 Gene Results in Dramatic Improvement in Efficiency for Generation of Gene Knockouts in Plants (Grützner et al., 2020)

Multilayered VBC score predicts sgRNAs that efficiently generate loss-of-function alleles (Michlits et al., 2020)

Genome-edited plants in the field (Metje-Sprink et al., 2020)

Heat-shock-inducible CRISPR/Cas9 system generates heritable mutations in rice (Nandy et al., 2019)

Direct detection of SARS-CoV-2 using CRISPR-Cas13a and a mobile phone (Fozouni et al., 2020)

<http://www.rgenome.net/cas-designer/>

<https://zlab.bio/guide-design-resources>

<https://innovativegenomics.org/crisprpedia/>

[CRISPR in Agriculture: 2022 in Review - Innovative Genomics Institute \(IGI\)](#)

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