

## 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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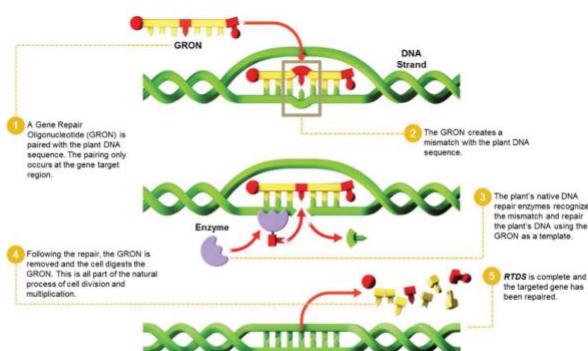


Figure 1. RTDS™ (Rapid Trait Development System) gene conversion process based on ODM (Oligonucleotide Directed Mutagenesis) technology pioneered by Cibus.

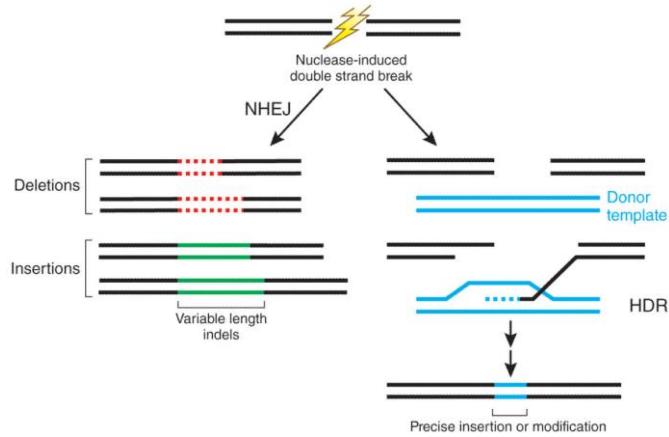
Songstad et al. 2017. Genome Editing of Plants. Critical Reviews in Plant Sciences, 36(1), 1-23.

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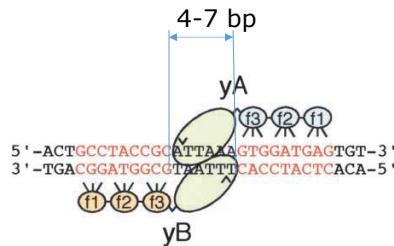
Homology directed repair  
<https://www.youtube.com/watch?v=86JCMM5kb2A>

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 okeanokoites*).

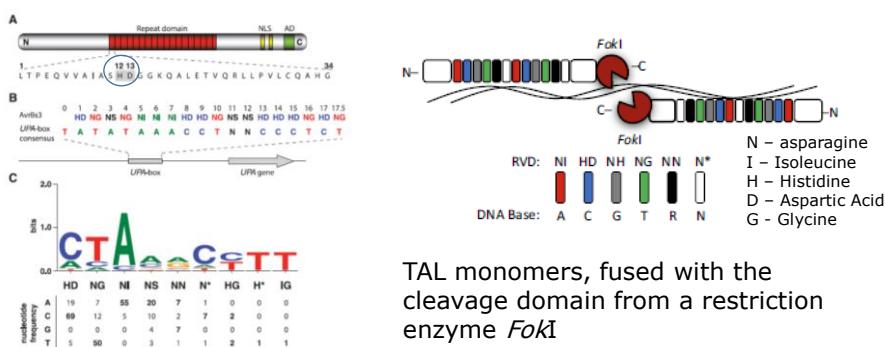
### Structure

Bibikova, M., Golic, M., Golic, 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



TAL monomers, fused with the cleavage domain from a restriction enzyme *FokI*

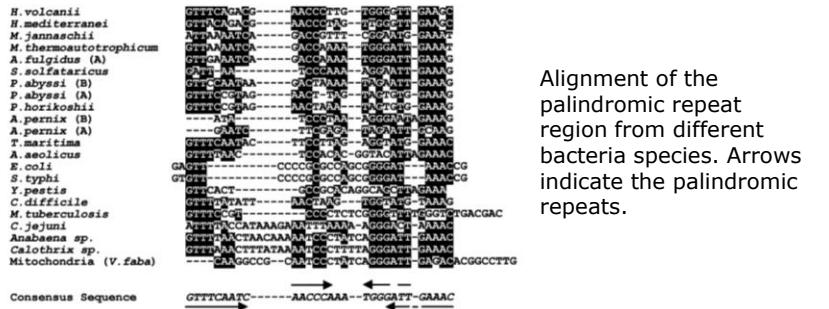
### Model for DNA-target specificity of TAL effectors

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.



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
	Type III	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>	Boilotin et al., 2005; Barrangou et al., 2007; Saprauskas 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; Cpf1 single-RNA guided	Cpf1	<i>F. novicida</i>	Zetsche et al., 2015

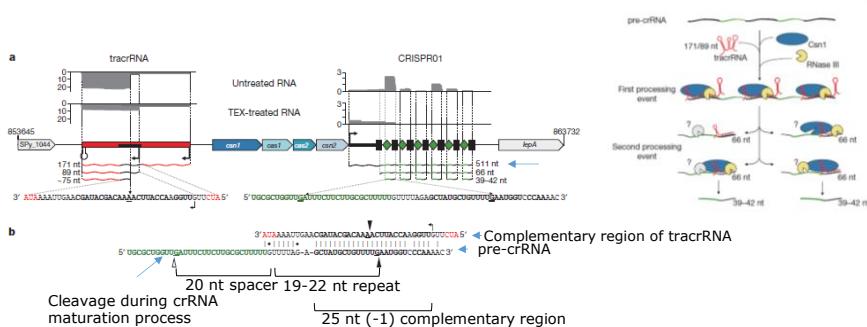
CRISPR systems are currently organized into two overarching classes: Class 1, which contain multi-subunit effectors, and Class 2, which contain single protein effectors. These classes are subdivided into five types (Makarova et al., 2015), with type IV remaining a putative type within Class 1. Although 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.

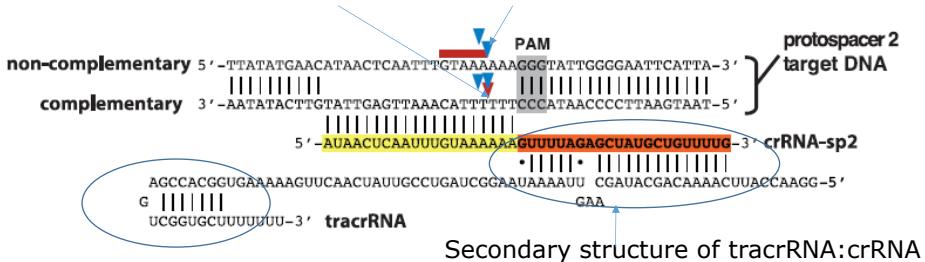


Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., . . . Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*, 471, 602.

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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.

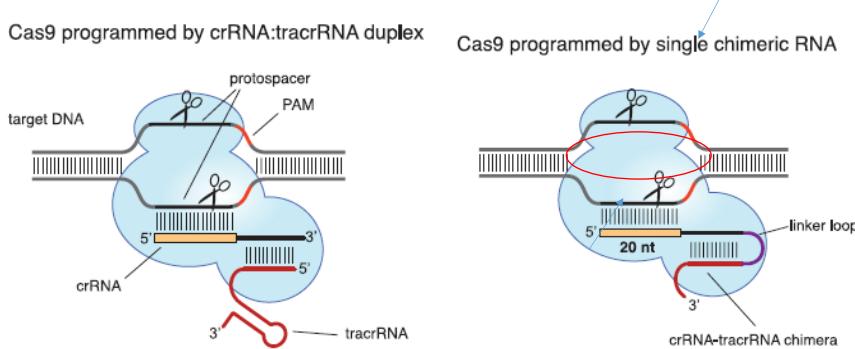
Regions of crRNA complementarity to tracrRNA (orange) and the protospacer DNA (yellow) are represented. The PAM sequence is shown in gray; cleavage sites are represented by blue arrows (C), a red arrow [(D), complementary strand], and a red line [(D), noncomplementary strand].

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)



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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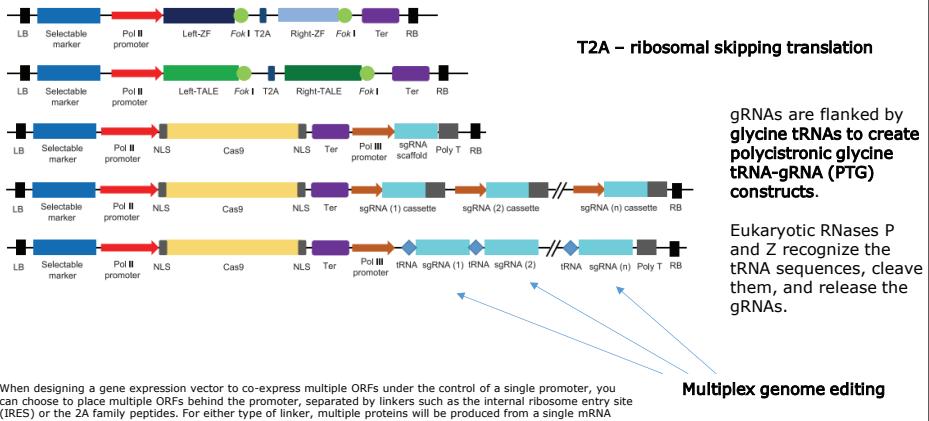
## Cas9 Species/Variants and PAM Sequences

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

AddGene: CRISPR 101: A Desktop Resource, 2<sup>nd</sup> edition

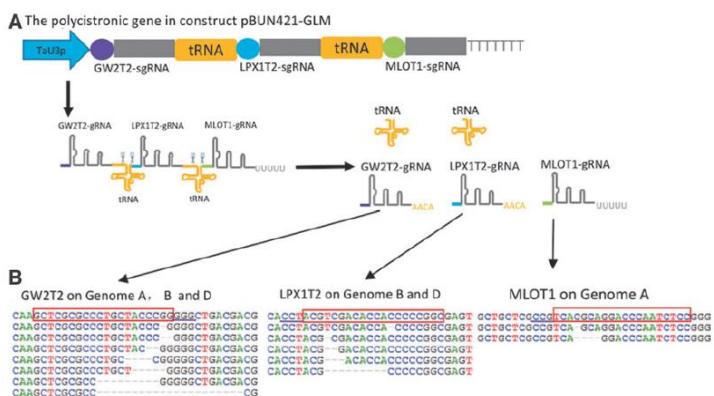
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Construct design for ZFNs, TALENs and CRISPR/Cas9 when Agrobacterium is used as the delivery method.



Ran, Y., Liang, Z., & Gao, C. (2017). Current and future editing reagent delivery systems for plant genome editing. *Science China Life Sciences*, 60(5), 490-505.

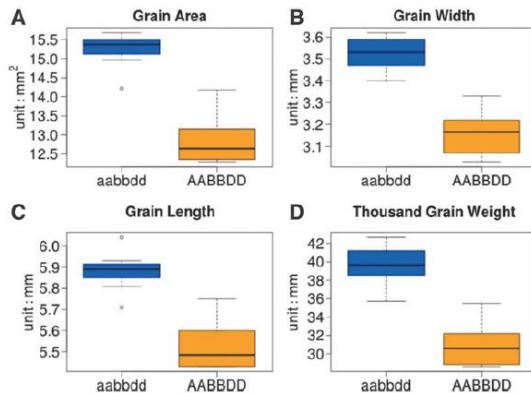
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**FIG. 1.** CRISPR-Cas9-based multiplex editing in hexaploid wheat using gRNAs processed through the endogenous tRNA-processing system. (A) Schematic of tRNA-based processing of a polycistronic gene transcript. The polycistronic gene containing three tRNA-gRNA blocks was driven by a TaU3 promoter in a MGE construct (pBUN421-GLM). Guide sequences GW2T2, LPX1T2, and MLOT1 are shown in purple, blue, and green, respectively. The GW2T2-gRNA, LPX1T2-gRNA, and MLOT1-gRNA are released after the tRNA processing. (B) The representative next-generation sequencing (NGS) results obtained for three genomic regions targeted by the pBUN421-GLM construct. The wild-type sequences are shown on the top. The target sequences are shown in the red rectangles; the PAM sequences are underlined; the deletions are shown by dashed lines.

Wei, W., Qianli, P., Fei, H., Alina, A., Shiao, C., Harold, T., & Eduard, A. (2018). Transgenerational CRISPR-Cas9 Activity Facilitates Multiplex Gene Editing in Allopolyploid Wheat. *The CRISPR Journal*, 1(1), 65-74.

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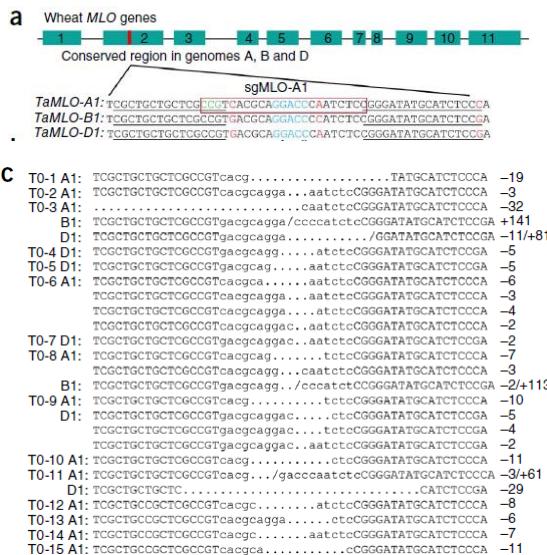


**FIG. 3.** Phenotypic effects of CRISPR-Cas9-induced mutations in the *TaGW2* gene. Box and whisker plots are used to show (A) grain area, (B) grain width, (C) grain length, and (D) thousand grain weight (TGW) of *gw2* knockout (*aabbdd*) and wild-type plants (*AABBD*).

Wei, W., Qianli, P., Fei, H., Alina, A., Shiaoman, C., Harold, T., & Eduard, A. (2018). Transgenerational CRISPR-Cas9 Activity Facilitates Multiplex Gene Editing in Allopolyploid Wheat. *The CRISPR Journal*, 1(1), 65-74.

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Figure 1 Targeted knockout of TaMLO genes using the TALEN and CRISPR-Cas9 systems. (a) Sites within a conserved region of exon 2 of wheat TaMLO homoeologs targeted by the TALEN and CRISPR-Cas9 systems. (c) TALEN-induced mutant TaMLO alleles identified by sequencing 15 representative transgenic wheat plants. The numbers on the right show the type of mutation and how many nucleotides are involved, with “-” and “+” indicating deletion or insertion of the given number of nucleotides, respectively.

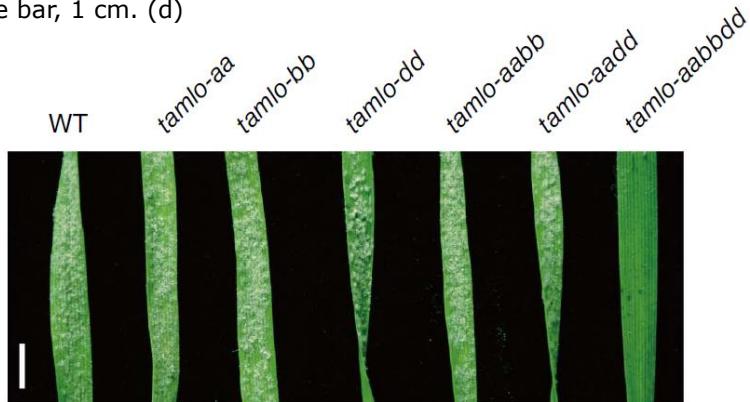


Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., & Qiu, J.-L. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature Biotechnology*, 32, 947.

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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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