The CRISPR/Cas9 rEvolution

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Breedtech

Question we will try to answer these two hours

1. What is genome editing?
2. What is CRISPR/Cas9 technology? From where does it come from?
3. How does CRISPR/Cas9 work?
4. Are there possible downsides of the system?
5. What are the potential applications in plant science?
6. How can we apply this technology to plants?
7. Application of CRISPR/Cas9 technology in real world…
8. CRISPR/Cas9 perspectives…

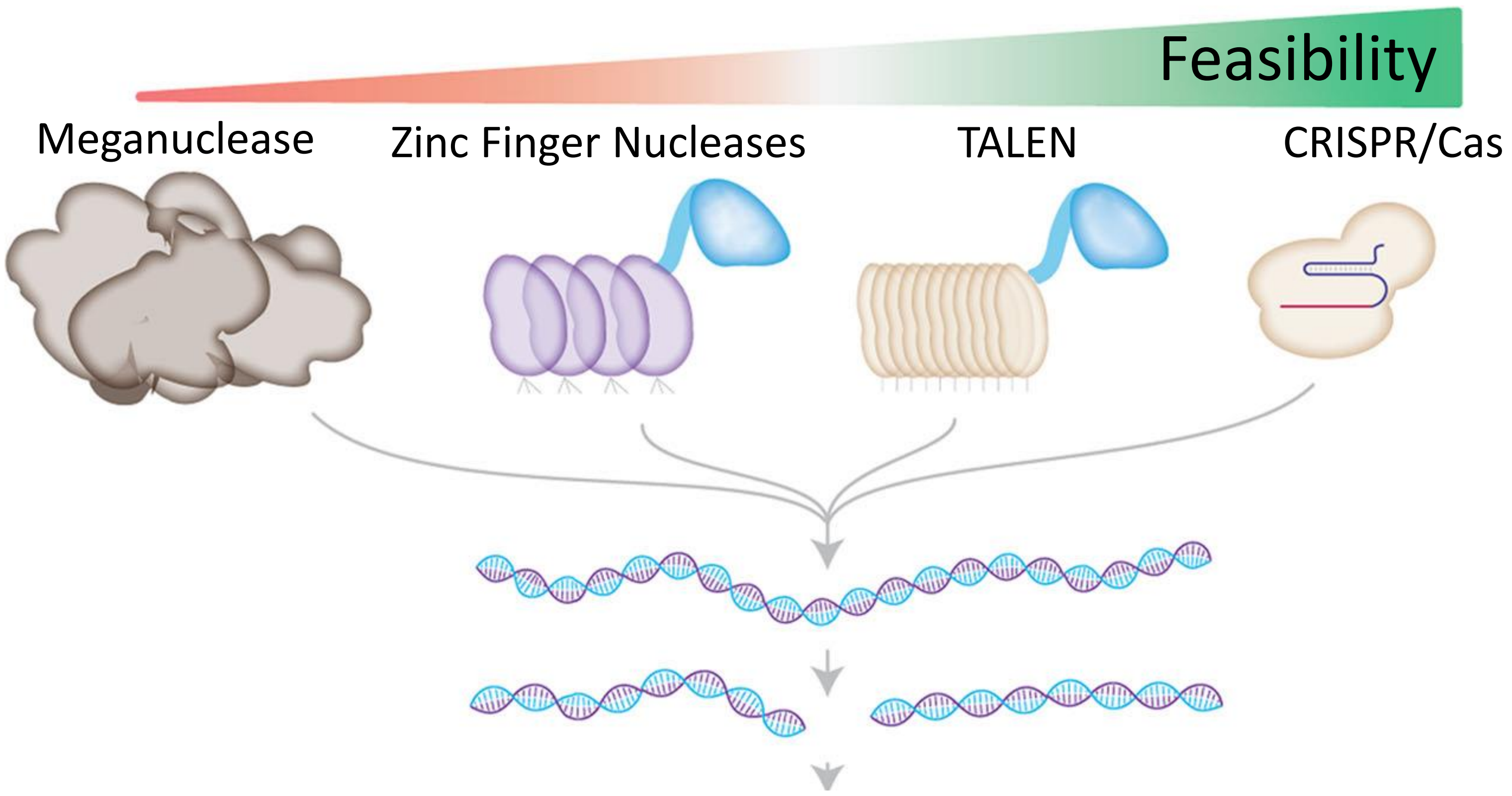
GENOME EDITING

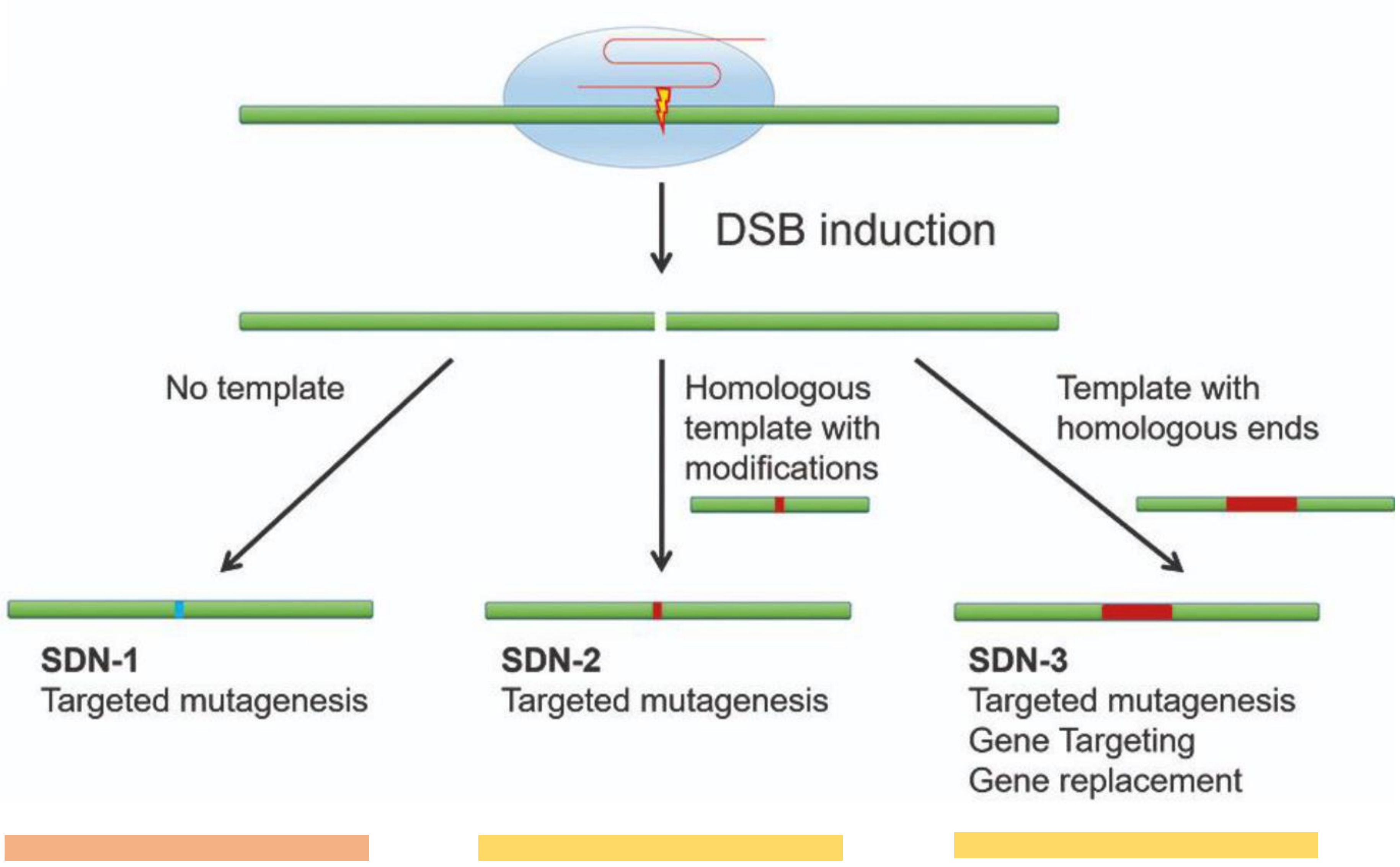
- Oligonucleotide Directed Mutagenesis (**ODM**)
- Homing Endonucleases: e.g. **Meganucleases**
- Zinc Finger Nucleases **(ZFN)**
- Transcription Activator Like Effector Nucleases (**TALEN**)
- **CRIPR-Cas**

What do they all have in common?

Precise DNA targeting

GGTAGATGCGATGCTAGCCAGATGGTGCAGAATGCTGGGCACGAAGTGTAGGCAGTGTGTAGA
GGTAGATGCGATGCTAGCCAGATGGTGCAGA-TGCTGGGCACGAAGTGTAGGCAGTGTGTAGA


Feasibility
Meganuclease
Zinc Finger Nucleases
TALEN
CRISPR/Cas



Source: https://analyticalscience.wiley.com/do/10.1002/gitlab.15844/full/

What is CRISPR/Cas9 technology? From where does it come from?

The Discovery of CRISPR loci: we need to go back 30 years ago

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CTGCAGTCTG CTCTCCATCC TACAGTTCTT CATATCCGCC GCAAAGCACT CGATGTGCAG     60
GGCTGCGAGT ATTCCGAGCA ACTAACTCAA CGACCTACTC GCTTCTCAAA CTGGGTTACC    120
TATAGTAGCG TTTGCAAGTC ATTGCACACT CGATCGACCG CTATCGTGCG ATTGGCTGTG    180
TCACCAGTTG CAAACGCTAC TATAGACAGT GCCAGTCTAA GAGTGTCATT GGAGCCCAGA    240
TCTGAAAAAC TGAAGAATAA TCACAGACCA AAGAAACAGA CCCAAGCCAT AGCAGATTAA    300
AATAATTCGA GTACTGTCCG TTCCAGATGT TGATGGTAAG TGAATATTCC CAACCACTCC    360
TCTGCCAATG GCTTCTTTTT CCTGGACGCC TTCACTAGTC ACATTCGTCC TTCCTGCTTG    420
ACCACCACCG AGAGCTACCA ACACGACCCC GATAATTGCG GTCAGGACGA GAATATAACC    480
CAGAGTGTGT CGTTAGATAG ATAGCACCGA CAGTAATGAT TCCCGCGAGA ATCGTATAGA    540
TGAGGACTGT TACTGCGACT ACTCGCTTGT CCATGTCATA AGTAGAACGA CAGTTAGTTT    600
AATTTTTATC ATTTTGAGGA ATTGGTGTAT CGCGCGTCCC GGTGTTCTCG GAAGTCCGTT    660
ACGTGGGTCT TGACCTGAAT TTCCGTCGAC CCCCCGGGGG GTTGGGGGGT ATTG          714
GGGGTCGACG GAAACT GTT GAGTGGGAGT AGTGTGTAGG AGGCTGTATA CCCTCGAATC GGGCATG    780
GTTACAGACG AACCCTAGTT GGGTTGAAGC GAACAGGATG GCGAACCGGT GTCTGCACCA GTT    843
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GTTACAGTCG AACCCTAGTT GGGTTGAAGC TCGGTAATCT GGGAAGGCGT CAGTCTCGGC CGAGTAATC    1897
GTTACAGACG AACCCTAGTT GGGTTGAAGC CTCGCCATCG CCGCGAACTC GGTCCTCCTC GGGGTG    1963
GTTACAGACG AACCCTAGTT GGGTTGAAGC AAGCCTTGAG AGTGTCTGTT GGTATGATGA ATGTT    2028
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GTTACAGACG AACCCTAGTT GGGTTGAAGC ACGATGATCT CGCCAGTCTG CAGCGTTACA TTGG    2156
GTTACAAACG AATCTTTTCT CGTGAGGACT TCCGAAACTA ACCTCTTCCC GGAACTAGTC    2216
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Studying halophilic bacteria

Sequencing parts of their genomes
Finds loci with particular architecture

identical sequences alternating with **variable sequences**

Short Regulary Spaced Repeats (SRSR)

Mojica et al. 1993 & 1995, *Mol Microbiol*

Short Regulary Spaced Repeats (SRSR)

Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria

Table 1. Main features of the SRSRs.

Organism	SRSR size (bp)	Spacing (bp)	Number of clusters	SRSR units per cluster	Reference
Archaea					
H. volcanii	30	ND	≥2	ND	Mojica *et al.* (1995) *Mol Microbiol* **9**: 13–21
H. mediterranei	30	33–39	3	21/ ND / ND	Mojica *et al.* (1995) *Mol Microbiol* **9**: 13–21
M. jannaschii	28–30	31–51	$7^A + 6^B + 1^C$	4–25	Bult *et al.* (1996) *Science* **273**: 1058–1073 and this work
M. thermoautotrophicum	30	34–38	2	124/47	This work
A. fulgidus	$37^A/30^B$	≈ 37	$1^A + 2^B$	$42^A/48^B/60^B$	This work
S. solfataricus	25	≈ 40	≥2	94/102	Sensen *et al.* (1998) *Extremophiles* **2**: 305–312
P. abysii	$29^A/30^B$	26–43	$1^A + 2^B$	$7^A/22^B/27^B$	This work
P. horikoshii	29	34–58	3	18/26/66	Kawarabayasi *et al.* (1998) *DNA Res* **5**: 55–76
A. pernix	$24^A/23^B$	37–52	$2^A + 1^B$	$19^A/27^A/42^B$	Kawarabayasi *et al.* (1999) *DNA Res* **6**: 83–101
Bacteria					
T. maritima	30	39–40	8	2–40	Nelson *et al.* (1999) *Nature* **399**: 323–329
A. aeolicus	29	36–38	1	6	This work
E. coli	29	32–33	3	2/7/13	Nakata *et al.* (1989) *J Bacteriol* **171**: 3553–3556 and this work
S. typhi	29	32	≥1	6	This work
C. jejuni	36	30	1	5	This work
Y. pestis	28	32–33	2	6/9	This work
C. difficile	29	36–38	$4^A + 2^B$	5–17	This work
M. tuberculosis	36	38–40	1	Variable	Hermans *et al.* (1991) *Infect Immun* **59**: 2695–705
Calothrix sp.	37	35–41	>1	5	Masepohl *et al.* (1996) *Biochim Biophys Acta* **1307**: 20–36
Anabaena sp.	37	32–43	>1	17	Masepohl *et al.* (1996) *Biochim Biophys Acta* **1307**: 20–36
Mitochondria					
V. faba	40	20–35	1	6	Flamand *et al.* (1992) *Plant Mol Biol* **19**: 913–923

A,B, Types of SRSRs distinct (more than 3 bp differences) within the same microorganism. ND, Not determined.

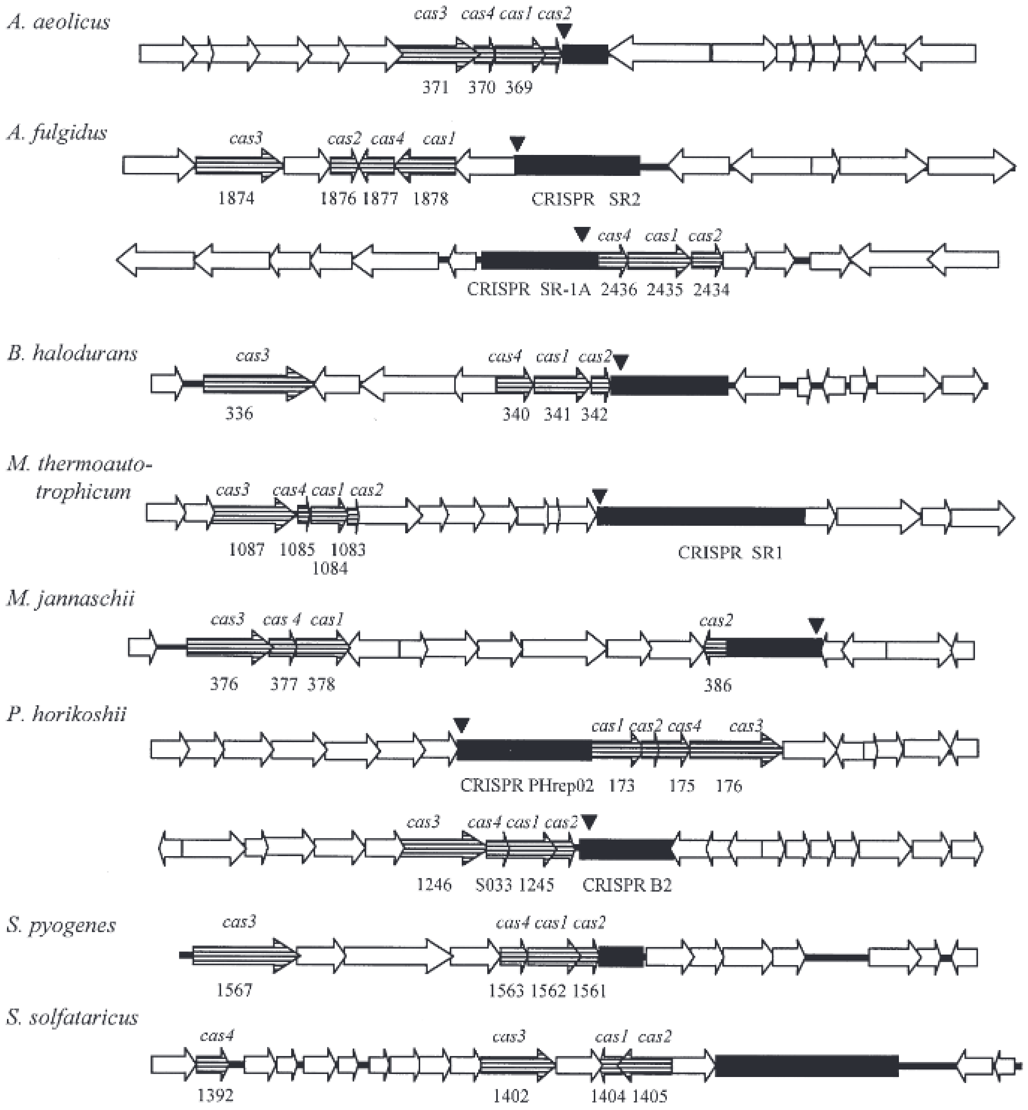
Mojica et al. 2000, *Mol Microbiol*

Identification of genes that are associated with DNA repeats in prokaryotes

~~**Short Regulary Spaced Repeats (SRSR)**~~

from 21 to 37 bp, interspaced by similarly sized non-repetitive sequences. To appreciate their characteristic structure, we will refer to this family as the clustered regularly interspaced short palindromic repeats (CRISPR). In most species with two or more CRISPR loci, these loci were flanked on one side by

Jansen et al. 2002, *Mol Microbiol*



Q: What are CRISPR loci? What is their function?...

Table 5. Features of the sequences most similar to CRISPR spacers from *S. pyogenes*

Spacer	Gene	Prophage[a]	Activity	Alignment[b]
4-1	*spyM3_1239*	315.4	Unknown	gctgtgacattgcgggatgtaatcaaagtaaaaa gctgtgacattgcggaatgtaatcaaagcaaaaa
4-2	*spyM3_0941*	315.2	Capside protein	taaagcaaacctagcagaagcagaaaatgac taaagcgaacctagtagaagcagaaaacgac
4-3	*spyM18_0741*	Φ_{speC}	Methyltransferase	ctgatgtaattggtgattttcgtgatatgcttt ctgatgtaattggtgattttcgtgatatgcctt
7-1	*spyM3_1215*	315.4	Endopeptidase	gcgctggttgatttcttcttgcgcttttt gcgctggttgatttcttcttgcgcttttt
7-2	*speM*	Φ_{speLM}	Exotoxin	tatatgaacataactcaatttgtaaaaaa tatatgaacataactcaatttgtaaaaaa
7-3	*spyM18_0742*	Φ_{speC}	Methyltransferase	aggaatatccgcaataattaattgcgctct aggaatatccgcaataattaattgcgctct
7-4	*hylP*	315.3	Hyaluronidase	agtgccgaggaaaaattaggtgcgcttggc agtgccgaggaaaaattaggtgcgcttggc
7-5	*spyM3_1347*	315.5	Unknown	aaatttgtttagcaggtaaaccgtgcttt aaatttgtttagcaggtaaaccgtgcttt

[a]Prophages 315.2-5 are integrated into *S. pyogenes* MGAS315. Φ_{speC} and Φ_{speLM} are integrated into *S. pyogenes* MGAS8232.
[b]CRISPR-spacer sequence (top line) and best-match homologous sequence (bottom line).

When the **viral sequence** is present in the bacterial genome, **resistance can be observed**

CRISPR is postulated for the first time as an:
Acquired Resistance Against Viruses

Mojica et al. 2005, *J Mol Evol*

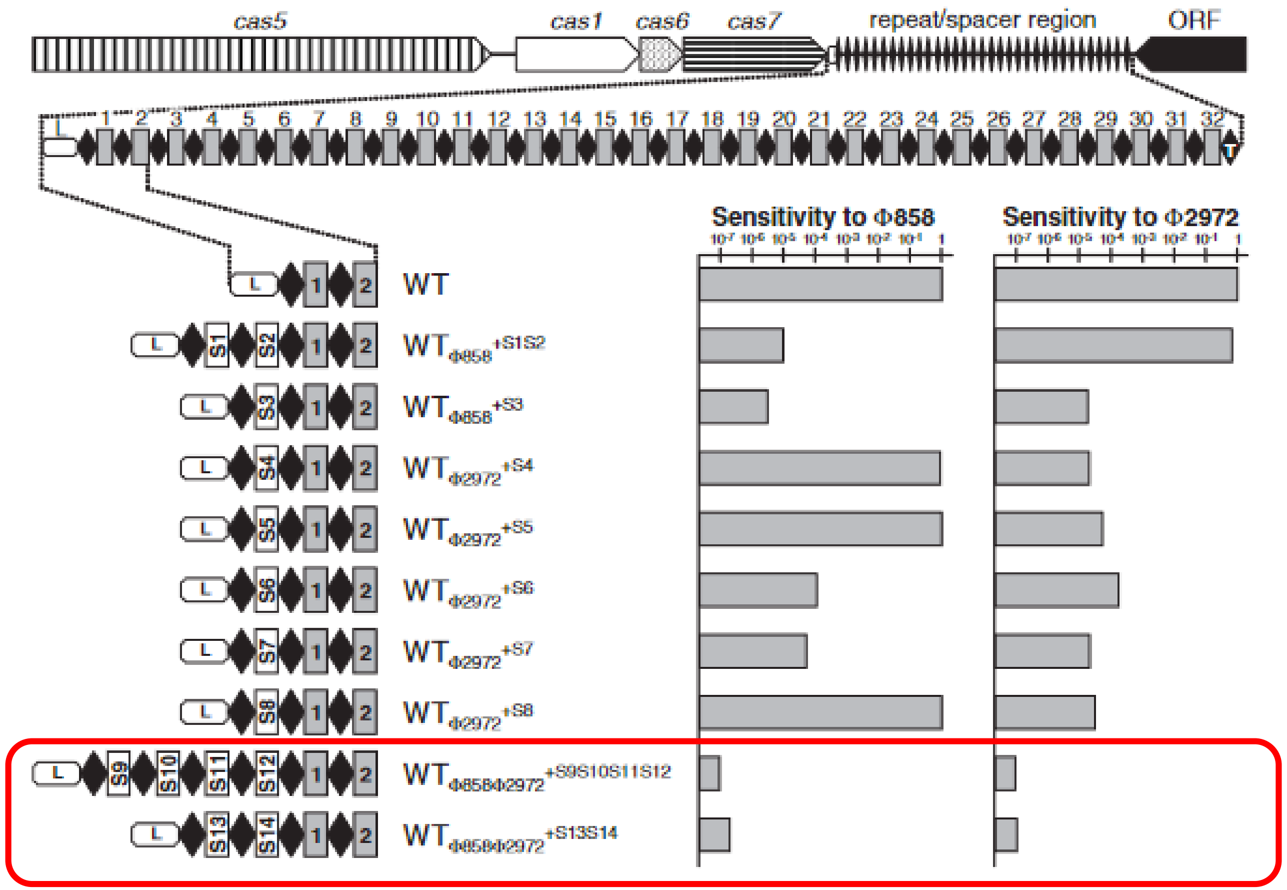
CRISPR loci have extrachromosomal origin and are components of an adaptive immune system

When plasmids containing CRISPR loci are cloned into a strain *S. thermophilus,* resistance can be induced

For the first time

bacterial immunity is engineered

Breakthrough



Barrangou et al. 2007, *Science*

Engineering the CRISPR locus of *S. thermophilus* fundamental fuctions of the adaptive immune system

When some Cas elements are not cloned, the system stops working

No **Cas9** (here called cas5)

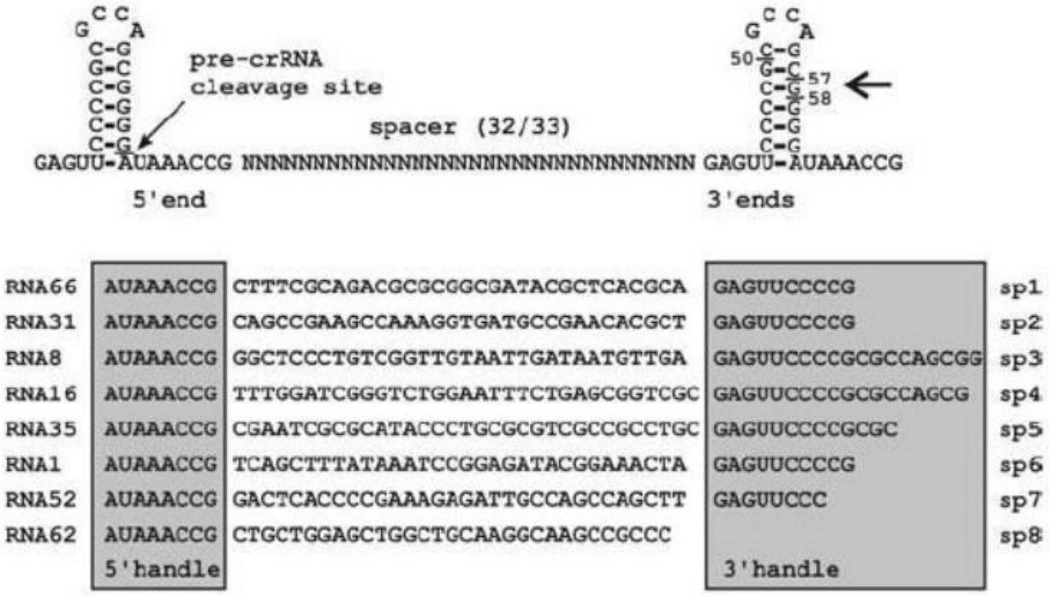
=

high sensitivity to bacteriophages

Barrangou et al. 2007, *Science*

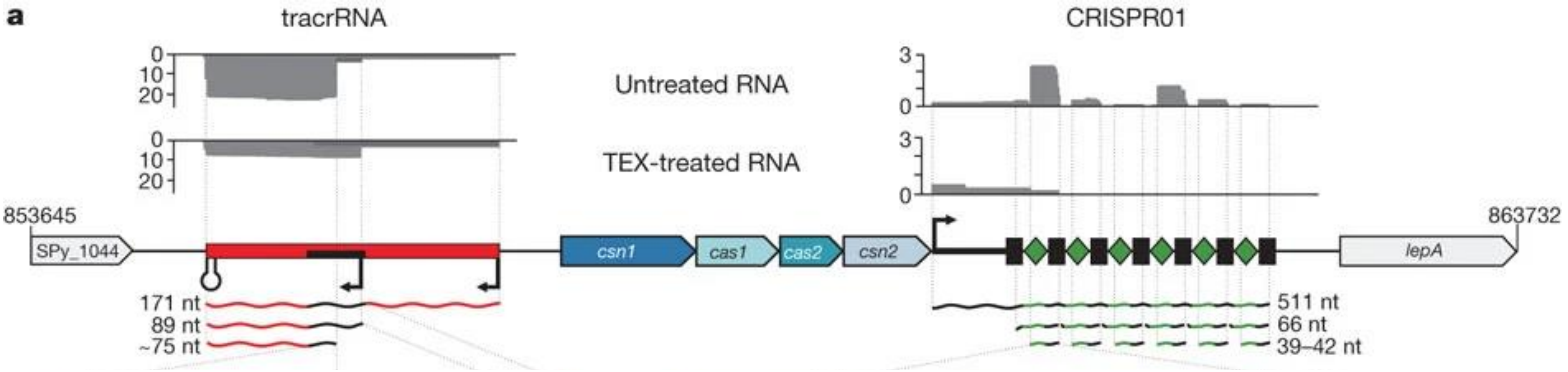
1- The CRISPR RNA (crRNA) is processed by Cas proteins into shorter fragments

CRISPR loci are transcribed and then processed into 50 b fragments where the spacer sequence is specific, and the flanking sequences are conserved (by a complex termed *Cascade*)



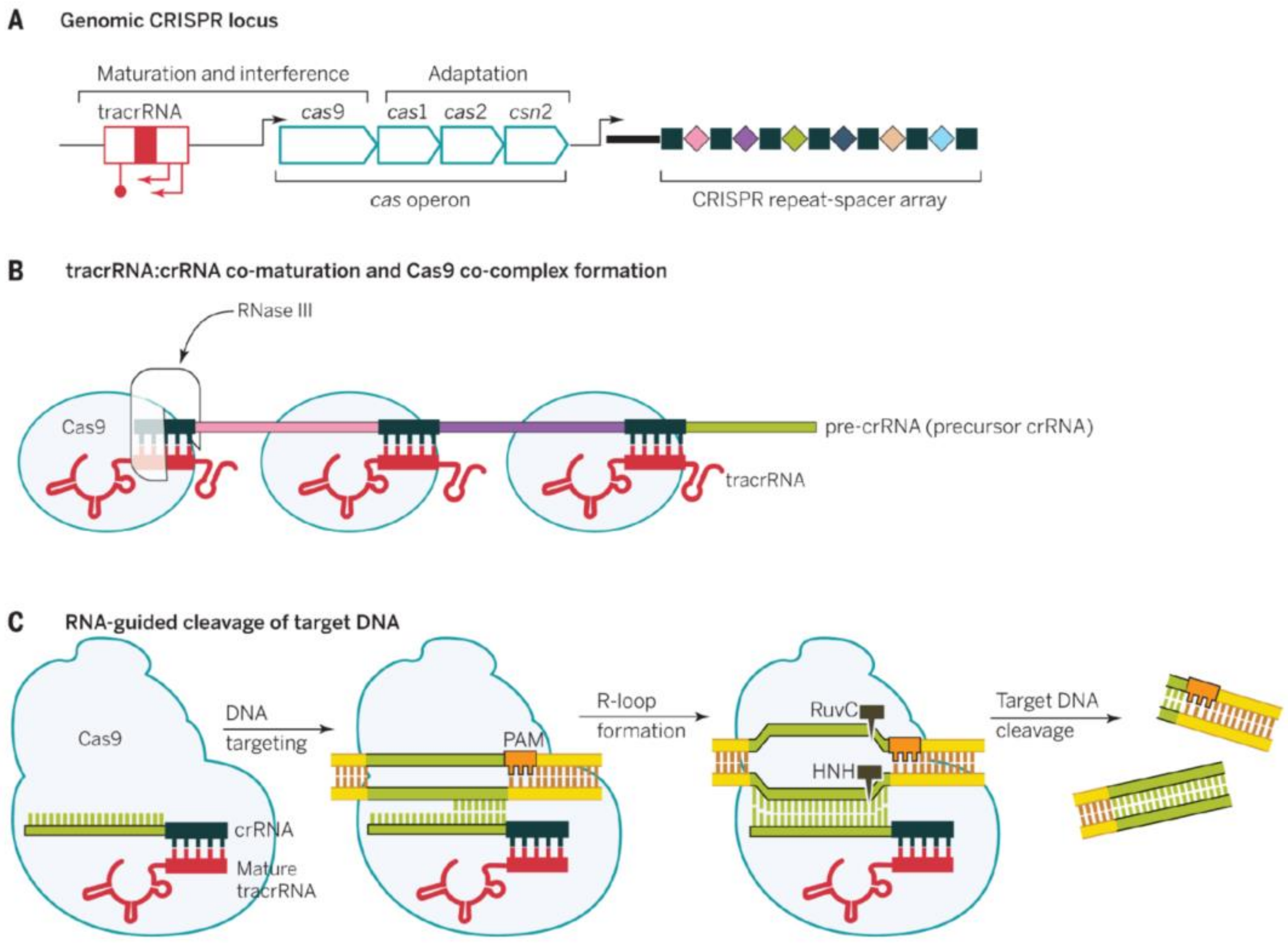
Brouns et al. 2008, *Science*

CRISPR loci produce crRNAs and tracrRNAs, required for acquired immunity

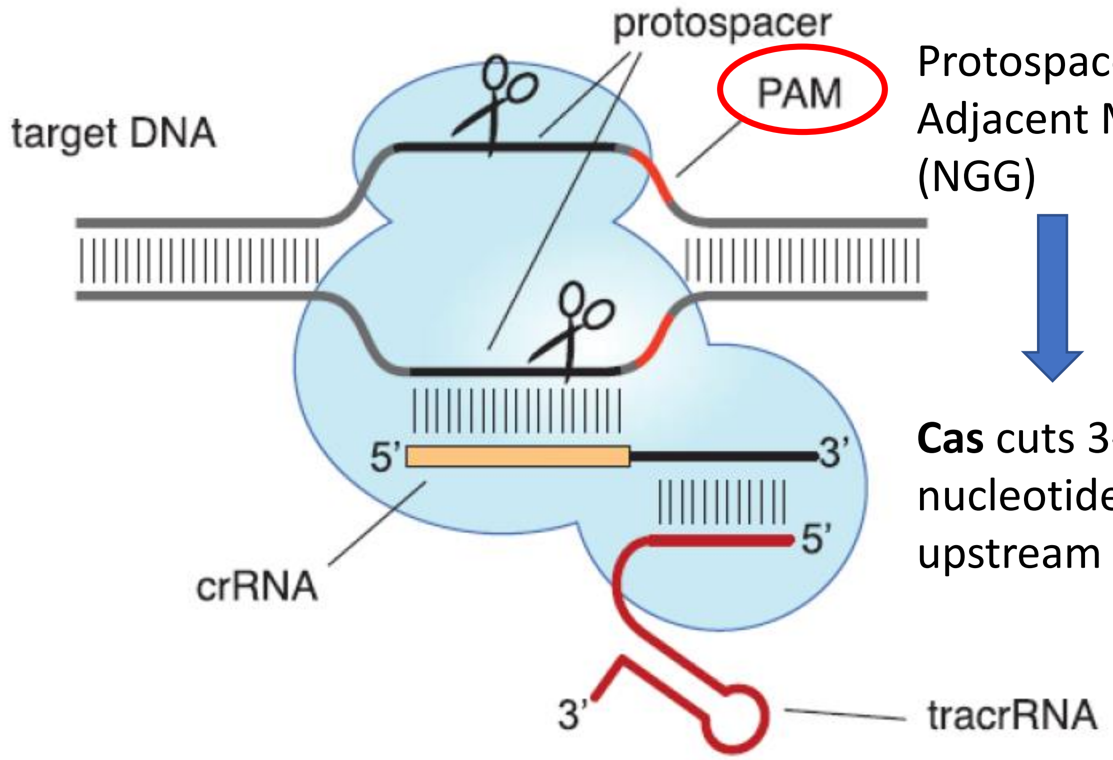


Deltcheva et al. 2011, *Nature*

Summary of the CRIPR/Cas9 system



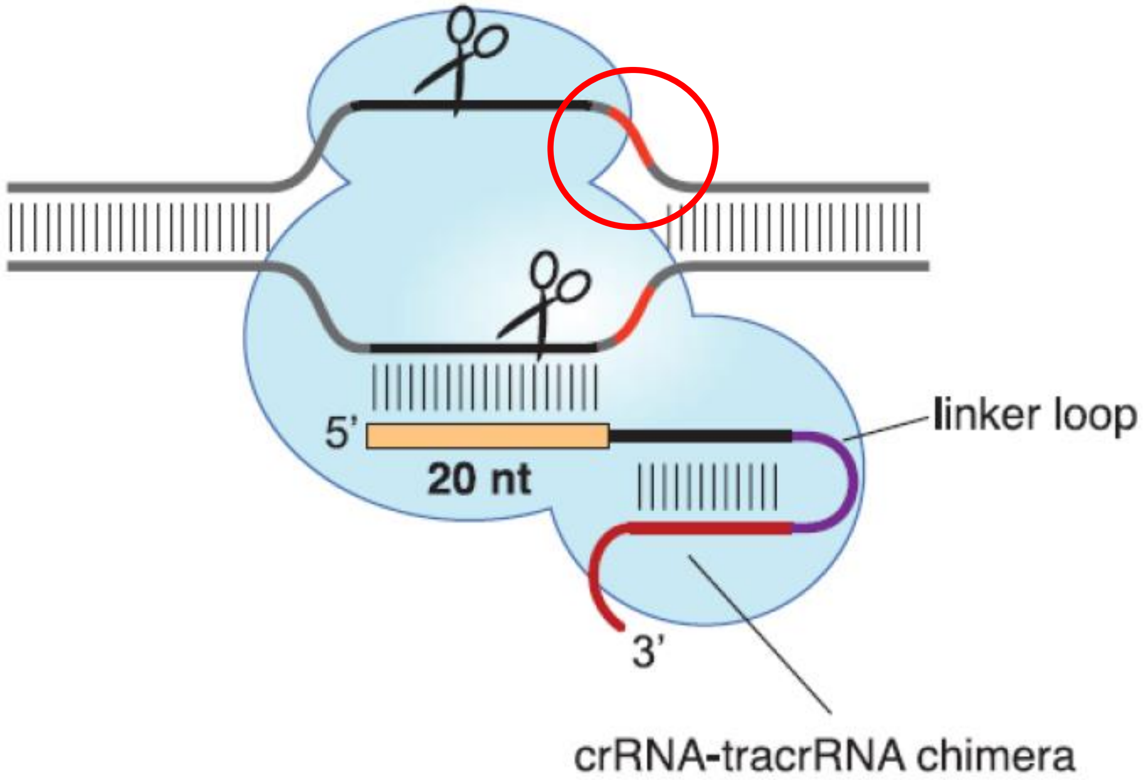
Cas9 programmed by crRNA:tracrRNA duplex



Protospacer Adjacent Motif (NGG)

Cas cuts 3-4 nucleotides upstream of it

Cas9 programmed by single chimeric RNA



…. the CRISPR/cas9 system is programmable!

Jinek et al. 2012, *Science*

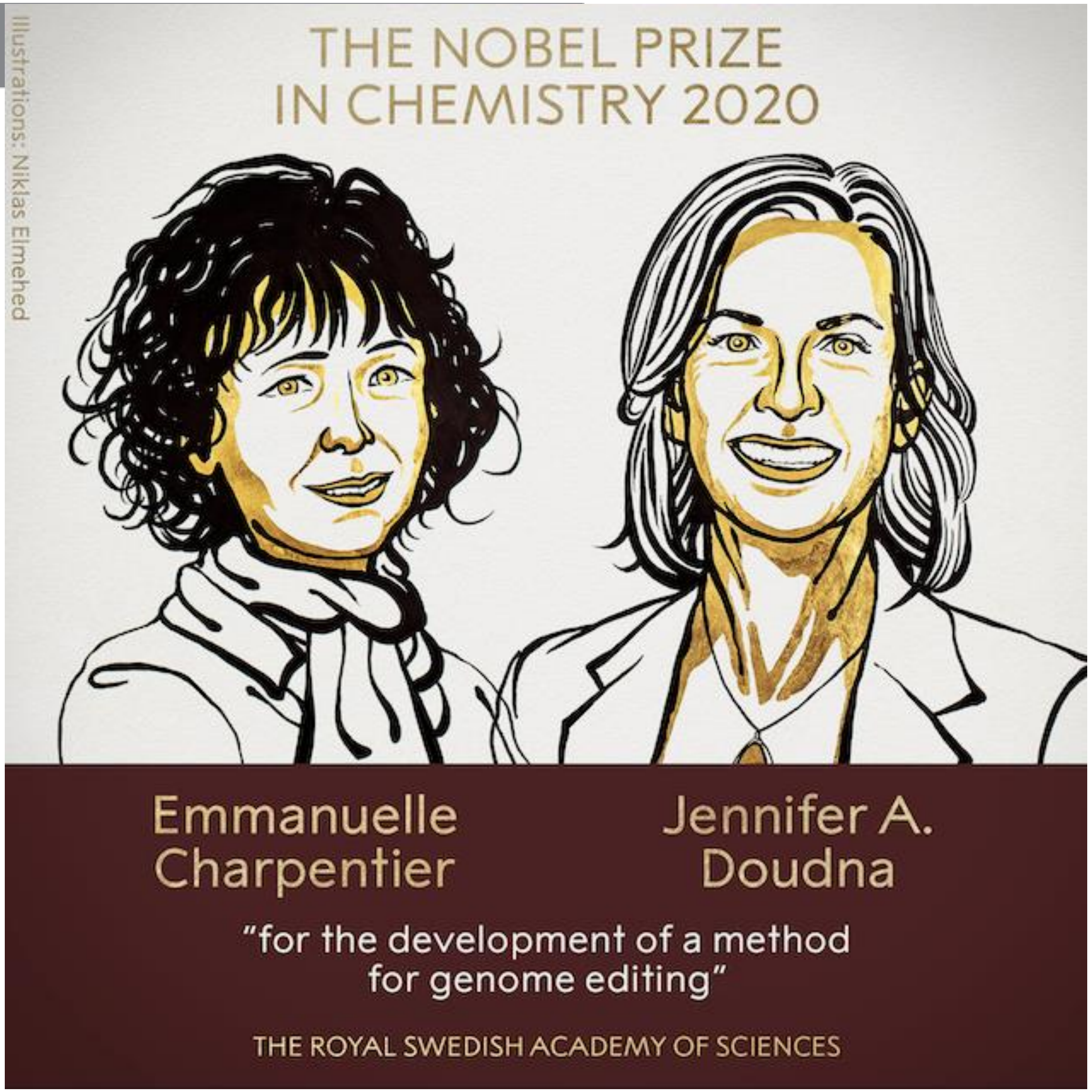
RESEARCH ARTICLE

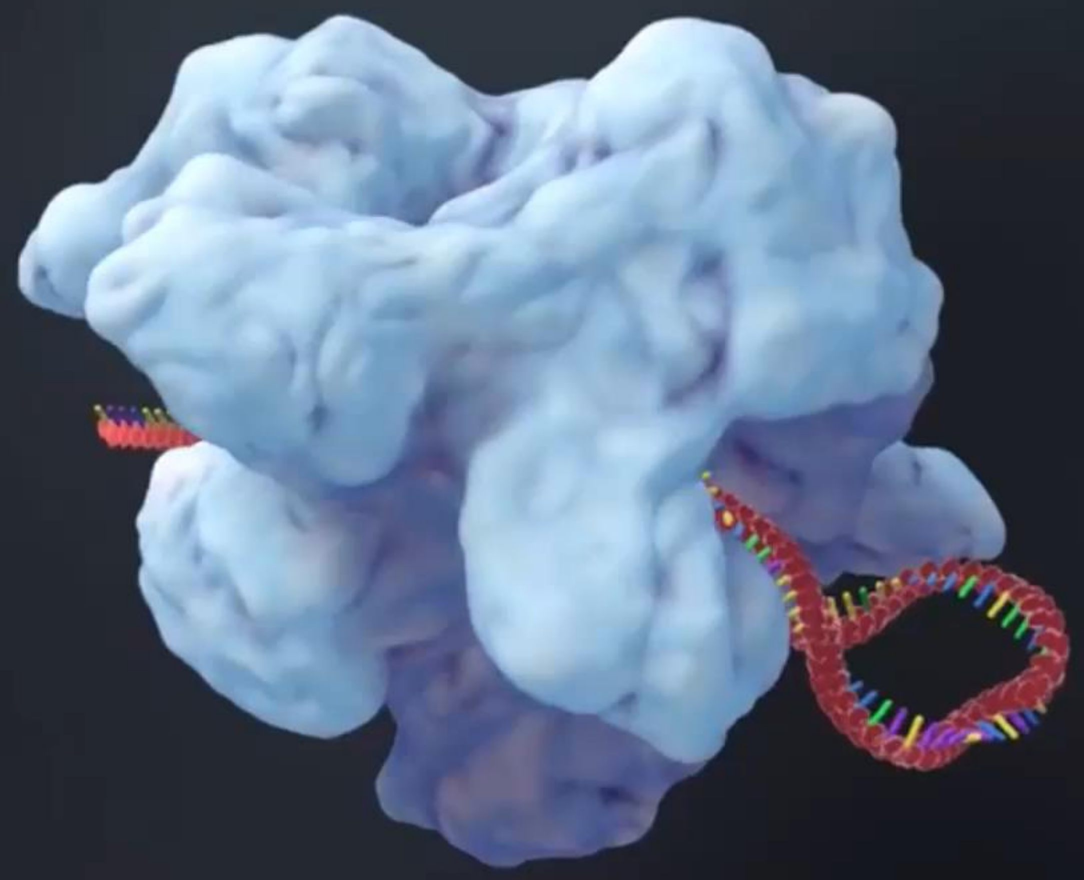
A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

Martin Jinek,[1,2]* Krzysztof Chylinski,[3,4]* Ines Fonfara,[4] Michael Hauer,[2]†
Jennifer A. Doudna,[1,2,5,6]‡ Emmanuelle Charpentier[4]‡

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. We show here that in a subset of these systems, the mature crRNA that is base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA structure that directs the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA. At sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the noncomplementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.

n. of citations may 7th 2022 = 13,658

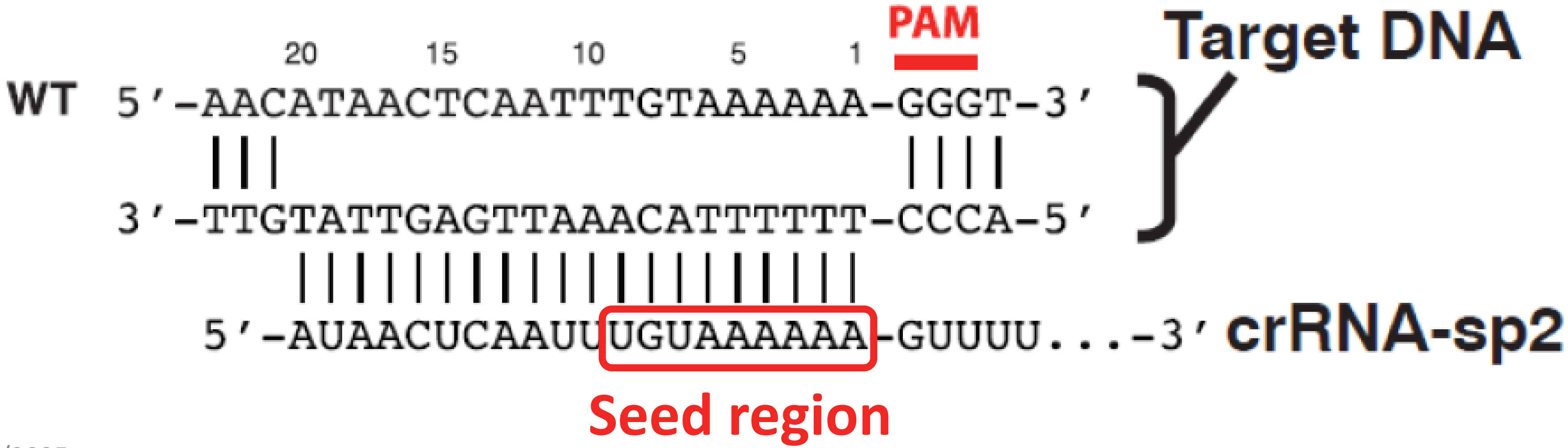




Precision of the system: off-targets

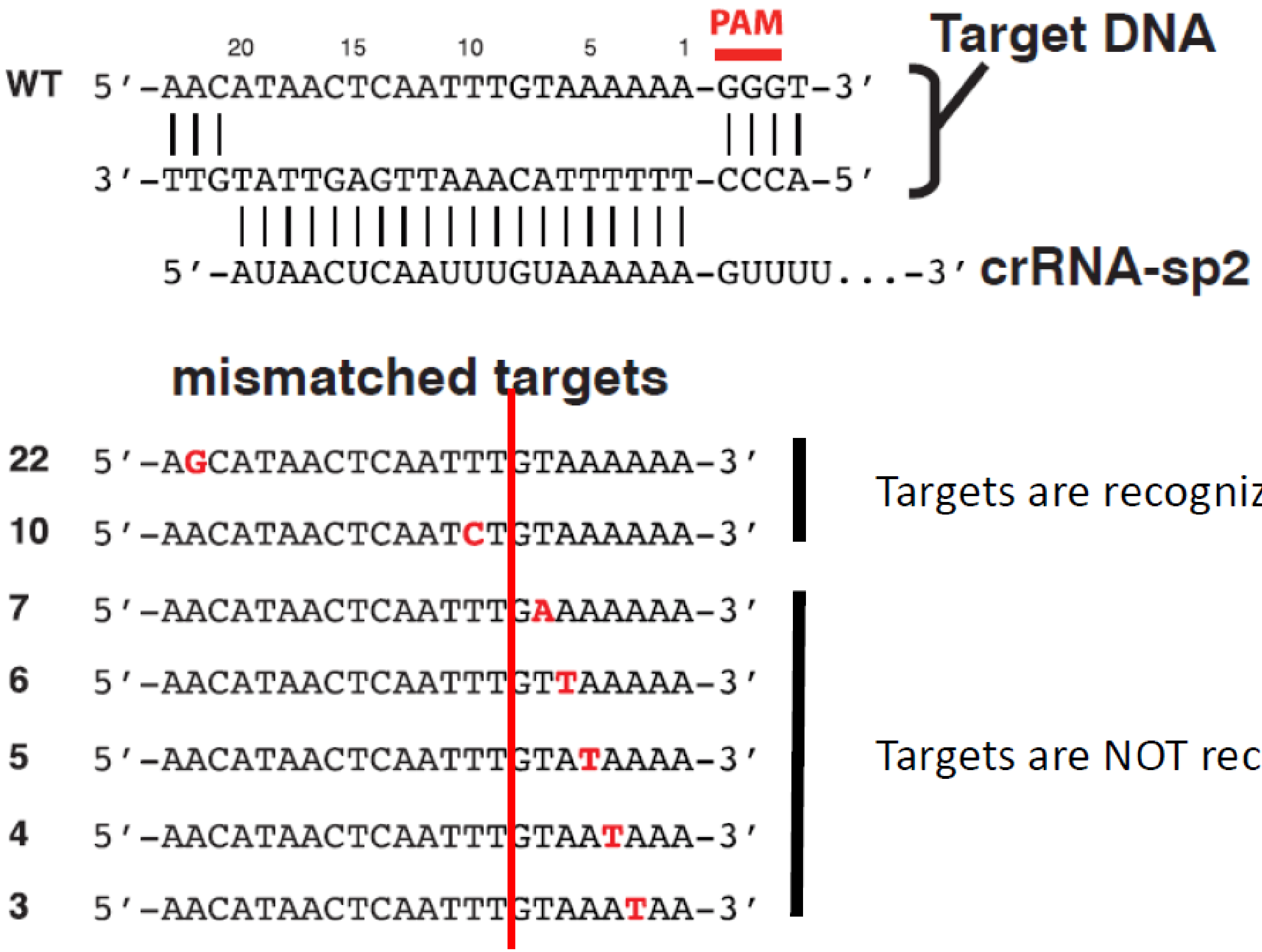
- Off-target:

  “unintended cleavage and mutation at untargeted genomic sites showing a similar but not an identical sequence to the target site”



Jinek et al. 2012, *Science*

The *seed* region of the gRNA is fundamental for target recognition and Cas9 cleavage



Targets are recognized

Targets are NOT recognized

Seed region =
8-10 bases at the 3’ end of the gRNA targeting region

Jinek et al. 2012, *Science*

The best strategy to avoid off-targets is good design of sgRNAs

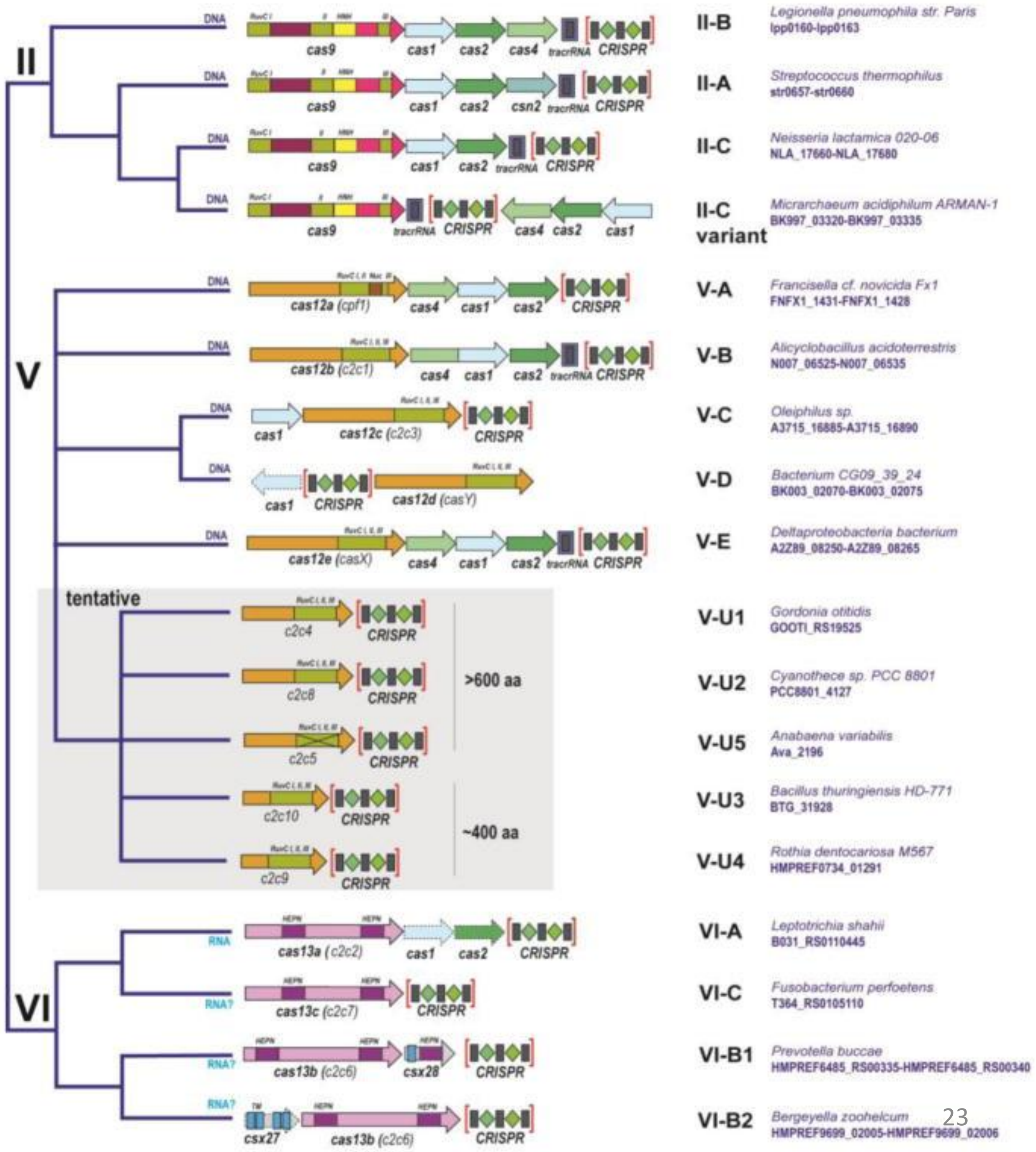
Online tool	Website	References
Cas-Offinder	http://www.rgenome.net/cas-offinder/	Bae et al. (2014)
Chop-Chop	http://chopchop.cbu.uib.no/index.php	Labun et al. (2016)
CRISPOR	http://crispor.tefor.net/	Haeussler et al. (2016)
E-CRISP	http://www.e-crisp.org/E-CRISP/	Heigwer et al. (2014)
CRISPR-P 2.0	http://crispr.hzau.edu.cn/CRISPR2/	Liu et al. (2017)
CCTop	https://crispr.cos.uni-heidelberg.de/	Stemmer et al. (2015)
Benchling	https://benchling.com/crispr	http://www.benchling.com
CRISPR-GE	http://skl.scau.edu.cn/	Xie et al. (2017)

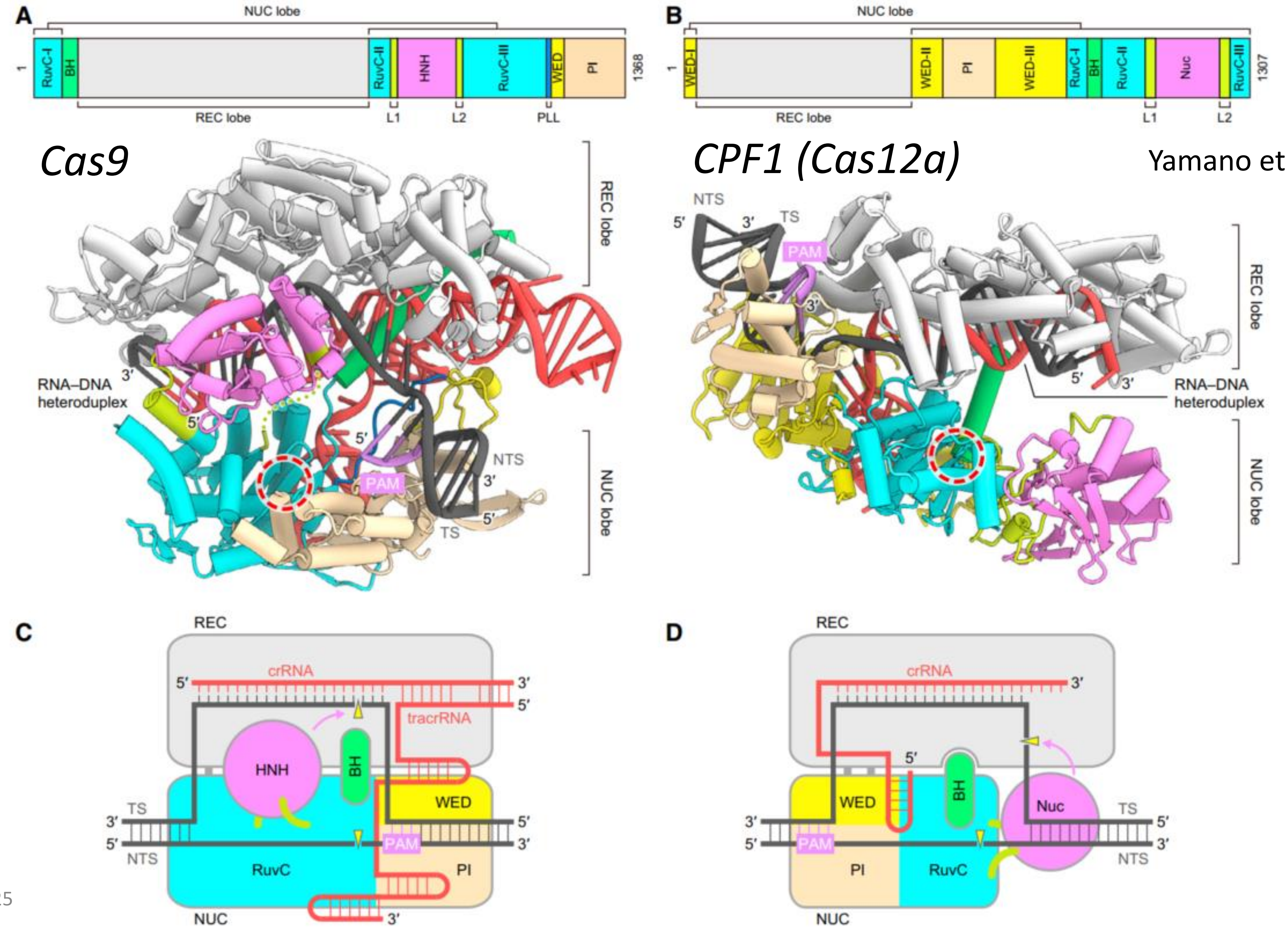
Hahn and Nekrasov 2019, *Plant Cell Reports*

Cas9 is the most versatile and easy-to-use programmable nuclease

	Zinc Finger Nuclease	TALEN	Cas9	Meganuclease
Recognition site	Typically 9-18 bp per ZFN monomer	Typically 14-20 bp per TALEN monomer	20bpguide RNA sequence+ 3bp PAM	14-40 bp
Specificity	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated (not in seed region though)	Small number of positional mismatches tolerated
Targeting constraints	Difficult to target non-G rich sequences	5' targeted base must be a T for each TALEN monomer	Targeted sequence must precede a PAM	
Dimerization required	Yes	Yes	No	No
Ease of engineering	difficult	Moderate (complex cloning methods)	easy	difficult
Ease of multiplexing	Low	Low	High	Low

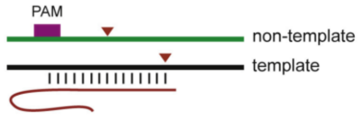
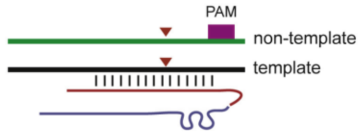
Cas9 is not alone





Yamano et al. 2016, *Cell*

Cas12a vs. Cas9

	Cas12a	Cas9
Size of protein	~1300 amino acids	~1000-1600 amino acids
RNA	 Single RNA molecule	 Two RNA molecules
Nuclease sites	Single nuclease site RuvC-Nuc	2 nuclease domains HNH and RuvC
Type of cut	 Staggered ends	 Blunt ends
PAM requirements	Recognises 5' T-rich PAM sequences of 3-4 nt	Recognises 3' G-rich PAM sequences of 3-5 nt
precrRNA processing	possesses intrinsic RNase activity to process precr-RNA	requires host RNase III and tracrRNA

Bijoya et al. 2020, *Biomed J* (review)

The CRISPR/Cas9 rEvolution

PART II

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Question we will try to answer these two hours

1. What is genome editing?
2. What is CRISPR/Cas9 technology? From where does it come from?
3. How does CRISPR/Cas9 work?
4. Are there possible downsides of the system?
5. What are the potential applications in plant science?
6. How can we apply this technology to plants?
7. Application of CRISPR/Cas9 technology in real world…
8. CRISPR/Cas9 perspectives…

Applications of CRISPR/Cas9 platforms for genome editing in plant

For REVERSE GENETICS approches alternative to:

- **Random mutagenesis** (undesirable mutations + large scale screening is costly… and tedious)
- Antisense RNA or virus-induced **gene silencing**
- **RNA interference**

} Can interrupt functions of specific genes by repressing the corresponding mRNAs

CRISPR/Cas9 provides a more efficient platform to perform:

- **Gene knock-out**
- **Gene Knock-in**
- **Disruption of cis-regulatory elements**
- **Suppression of virus infection**

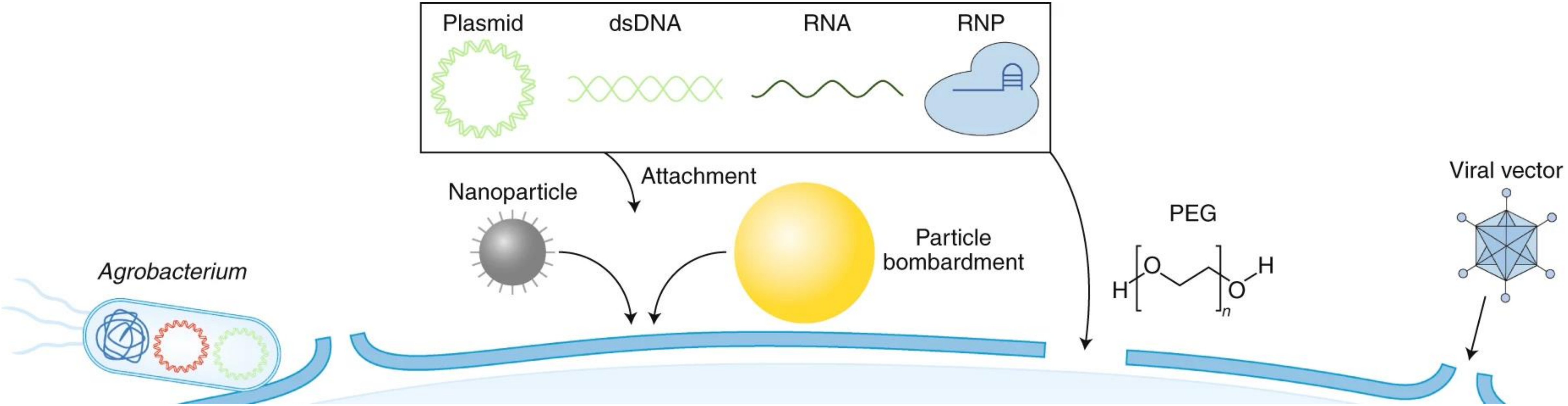
Delivery of CRISPR/Cas9 into plant cells

Direct delivery

Agrobacterium tumefaciens-
mediated trasformation

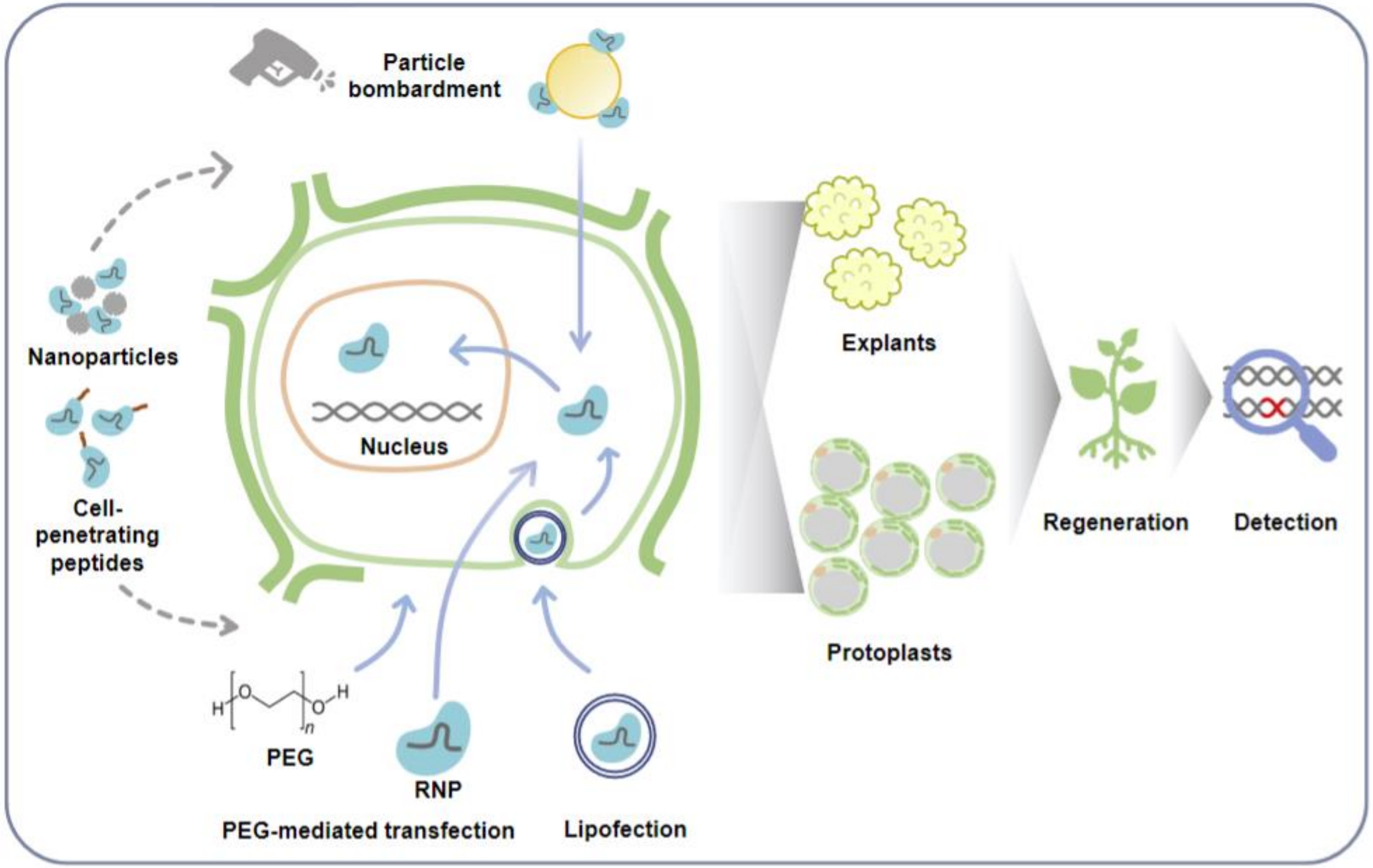
Applications of CRISPR technology in plant cells

Outside the plant cell, CRISPR reagents can be delivered as **plasmids**, **dsDNA**, **RNA** and **ribonucleoprotein** (RNP) through **PEG-mediated transformation** (polyethylene glycol), particle bombardment or nanoparticles. Plasmids can also be delivered by *Agrobacterium tumefaciens* and viral vectors.



Zhang et al. 2019, *Nature Plants*

Direct delivery: all about it



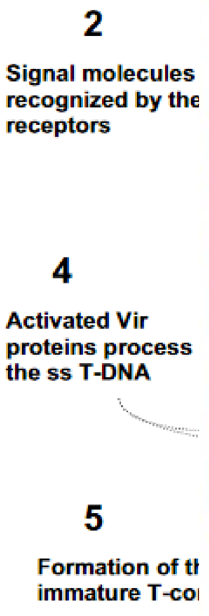
CRISPR reagents can be delivered into plant cells as RNPs.

- **Particle bombardment** can be used to deliver **plasmids** (or **RNPs)** into explants.
- **PEG-mediated transfection** and lipofection can be used to deliver plasmids (or RNPs) into protoplasts.
- **Nanoparticles** and **cell-penetrating peptides** are emerging methods for RNP delivery. Transformed cells and tissues are used for plant regeneration and edit detection.

Zhang et al. 2021, *Plant Communications*

What is *Agrobacterium tumefaciens?*

- It causes crown gall disease
- The infection process is associated with presence of the so-called *Ti* plasmid
- During the process, the a part of the plamid (T-DNA) is integrated in the host genome (random position)
- T-DNA carries information of multiple genes (eucaryotic promoter) .
- The expression of the newly acquired genes causes the tumor



Pacurar et
Plant Path

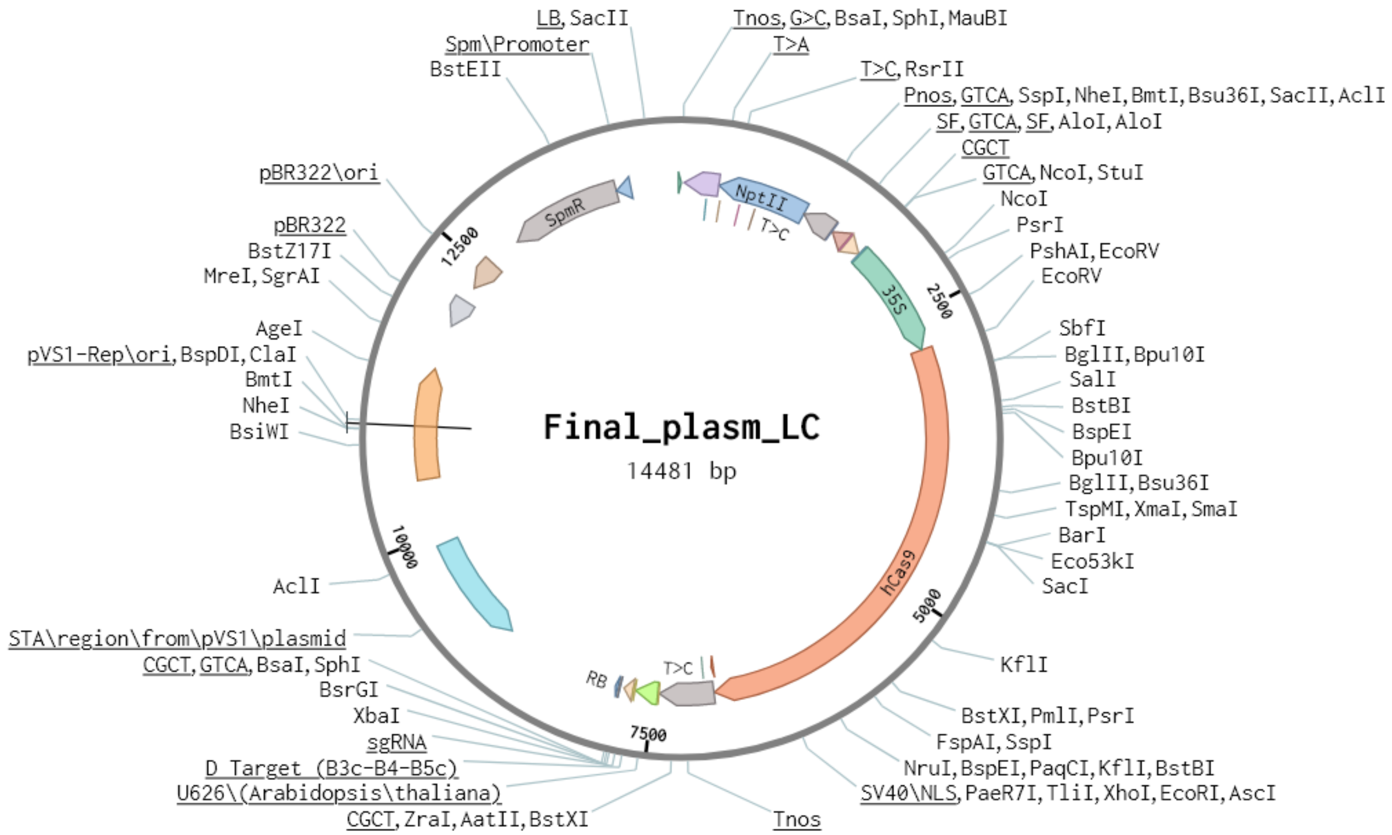
Agrobacterium tumefaciens-mediated transformation

- *Agrobacterium tumefacies* has been extensively used as 'platform' for transformation

- Principle based on the modification of the of the Ti plasmid

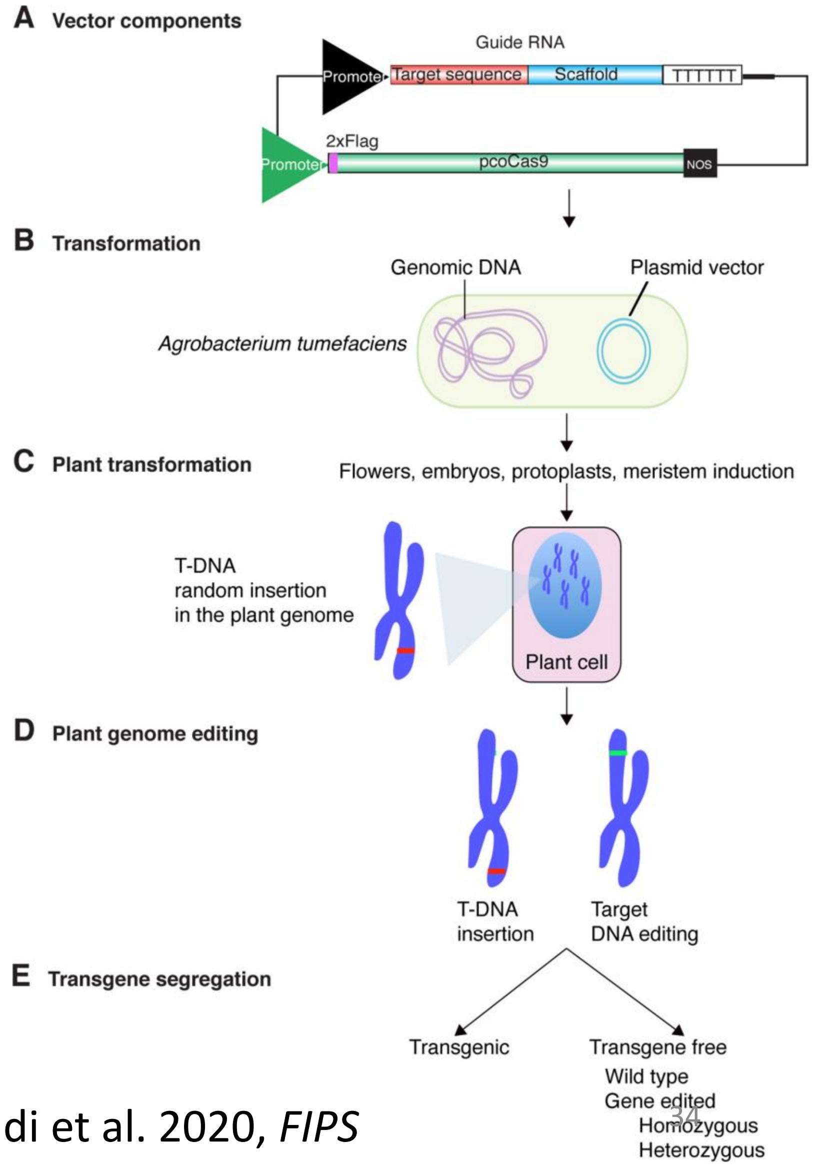
- There are several systems, a quite common one is the so-called **binary system** (it can be cloned in both *E. coli* and *A. tumefaciens*)

- The final plasmid will carry *vir* genes and the artificial T-DNA



Agrobacterium tumefaciens-mediated CRISPR-Cas

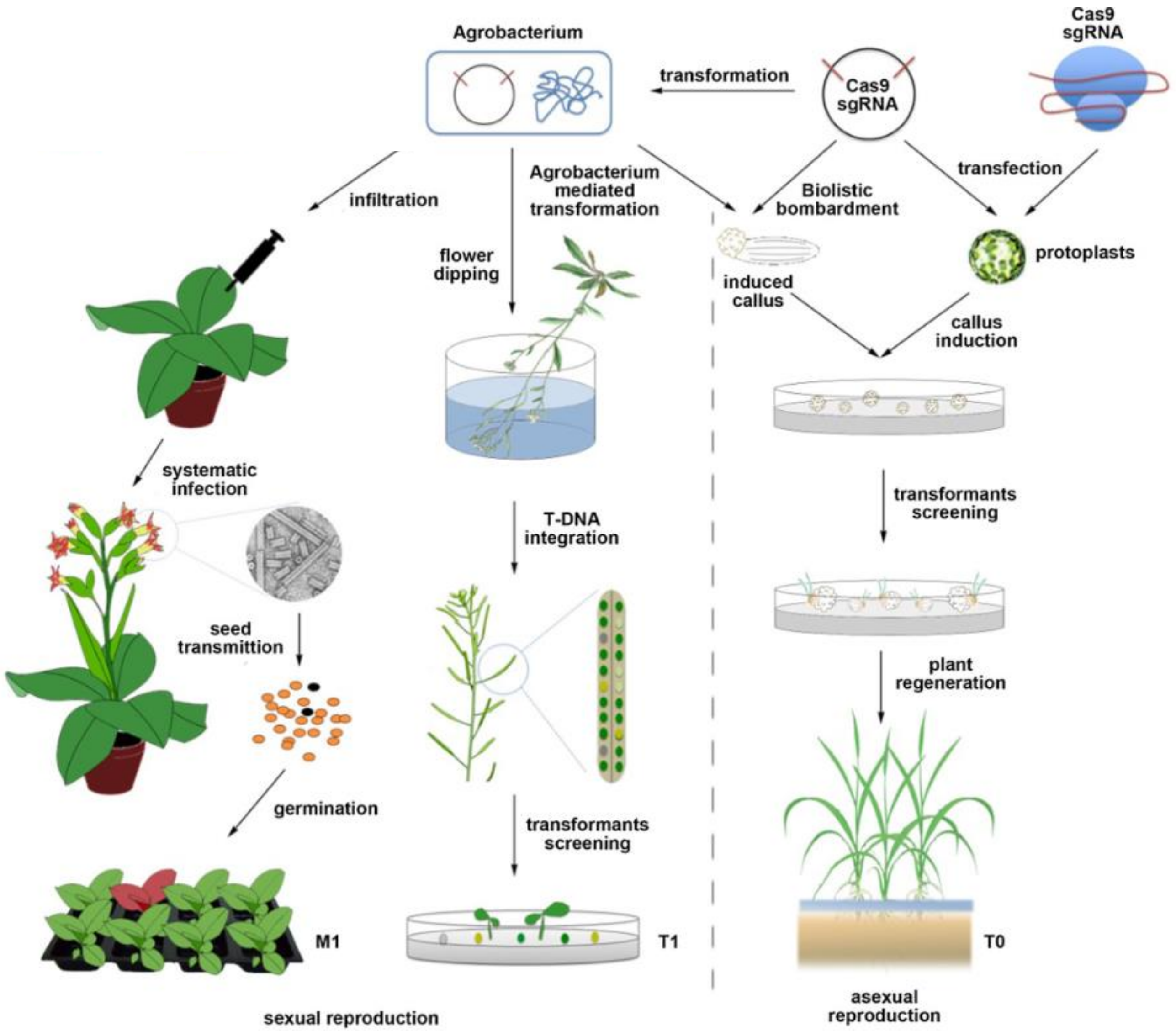
A. Assemble a plasmid containing all the information you want to carry into the plant cells.
B. *A. tumefaciens* is transformed with the plasmid vector carrying the cassette for Cas9 protein and guide RNAs
C. *A. tumefaciens* is used to **transform ovules in flowers, embryos, explants, meristematic cells** or **protoplast**. The integration is random.
D. Expression of Cas9 protein and guide RNAs lead to editing of the target DNA; insertion site and target site are likely not linked.
E. The insertion and the edited DNA can be separated through Mendelian segregation.



Actually, *A. tumefaciens* is not the one only option, the same principles can be apllied using *Rhizobium rhizogenes*

El-Mounadi et al. 2020, *FIPS*

Brief recap on the most common approaches



…producing 'edited' plants is complex

Several limiting factors

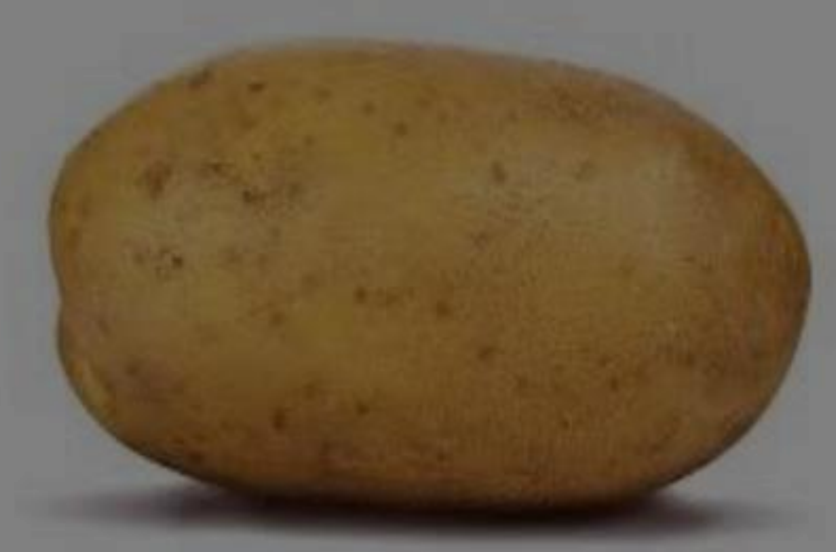
1. Many species (or genotypes within species) are recalcitrant to in vitro culture
2. The efficiency of most approches is still low
3. Policy-makers are behind

Edited organisms are reaching the table

Non-browning mushrooms

Low phytate maize

Powdery mildew resistant tomato



Altered starch quality in potato (low acrylamide)

High oleic soybean

Stunning application of CRISPR/Cas technology in plant science

LETTER

nature biotechnology

De novo domestication of wild tomato using genome editing

Agustin Zsögön[1,7], Tomáš Čermák[2,6,7], Emmanuel Rezende Naves[1], Marcela Morato Notini[3], Kai H Edel[4], Stefan Weinl[4], Luciano Freschi[5], Daniel F Voytas[2], Jörg Kudla[4] & Lázaro Eustáquio Pereira Peres[3]

So… what is domestication?

definition: a sustained multi-generational relationship in which humans assume a significant degree of control over the reproduction and care of another group of organisms to secure a more predictable supply of resources from that group

500 ***Angiospermae*** species (250.000)

20 animal species (5.000)

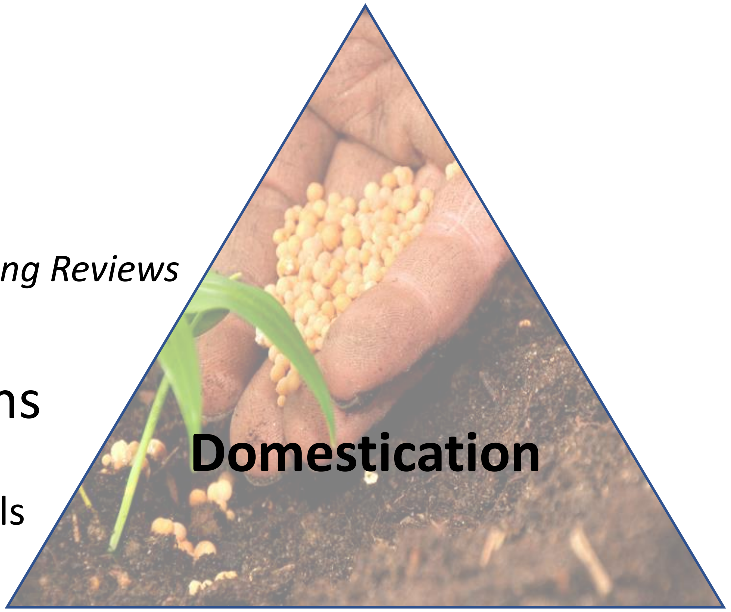
e.g. domestication and evolution of a species under domestication did not generally lead to the creation os new species.
(exception : *Triticum aestivum*)

Results of interacion of 3 factors

Plants or Animals

- Morphology
- Behaviour
- Genetics

P. Gepts, 2004; *Plant Breeding Reviews*



Humans

Cultural development:
- Knowledge of plants and animals
- Technology

Environment

- Climate shift
- Seasonality
- Diversity of enironmental niches

Domestication of crops made agriculture possible

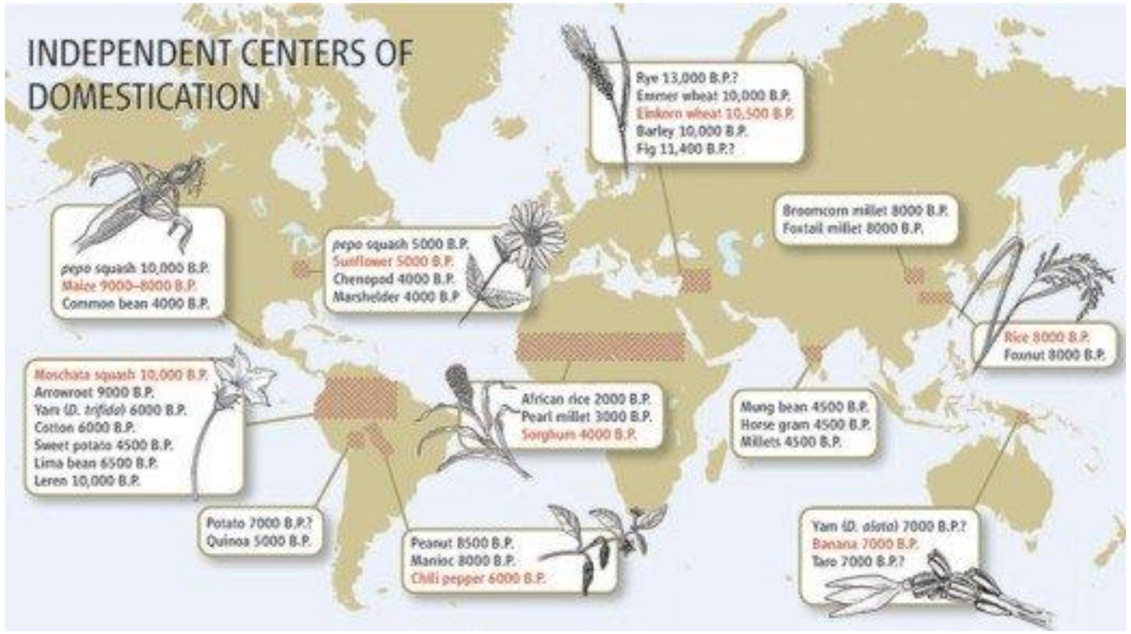
- Domestication is the genetic modification of a wild species to create a new form that is altered to meed human needs
- The process by which humans actively interfere with nature and direct evolution
- In the beginning, an uncoscious process of selection
- Humans change the conditions in which cultivated species live and reproduce -> species adapt and evolve as a consequence
- Results in a continuum of increasing **codependance** between people and crops/livestock

Timeline for crop domestication

Table 1.2. Time frame of domestication and early spread of agriculture

Location	Crop[z]	Age (years BP)	Source
DOMESTICATION CENTERS			
Mesoamerica	Squash	10,000	Smith 1997
	Maize	6,200	Piperno and Flannery 2001
Fertile Crescent	Einkorn wheat	9,400-9,000	Willcox 1998
	Lentil[y]	9,500-9,000	Willcox 1998
	Flax[y]	9,200-8,500	Willcox 1998
	Goat[x]	10,000	Zeder and Hesse 2000
	Pig[x]	10,000	Giuffra et al. 2000
China	Rice	9,000-8,000	Zhao 1998
Eastern United States	Squash	4,300	Asch 1995, cited by Hart et al. 2002
	Sunflower	4,300	Crites 1993
SPREAD FROM DOMESTICATION CENTERS			
Lowland Mesoamerica and Central America	Cassava, *Dioscorea* yam, arrowroot, maize	7,000-5,000	Piperno et al. 2000 Pope et al. 2001
Eastern North America	Maize	1,100	Smith 1989 Hart et al. 2002
Europe	Einkorn wheat	9,000-5,000	Ammerman and Cavalli-Sforza 1984

[z] Only the earliest domesticated crop remains are listed
[y] Uncertainty as to the domestication status
[x] Additional centers of domestication for the goat (in the Indian subcontinent) and the pig (in Eastern Asia) have been postulated



Domestication of plants: traits

In particular:

- No seeds dispersal

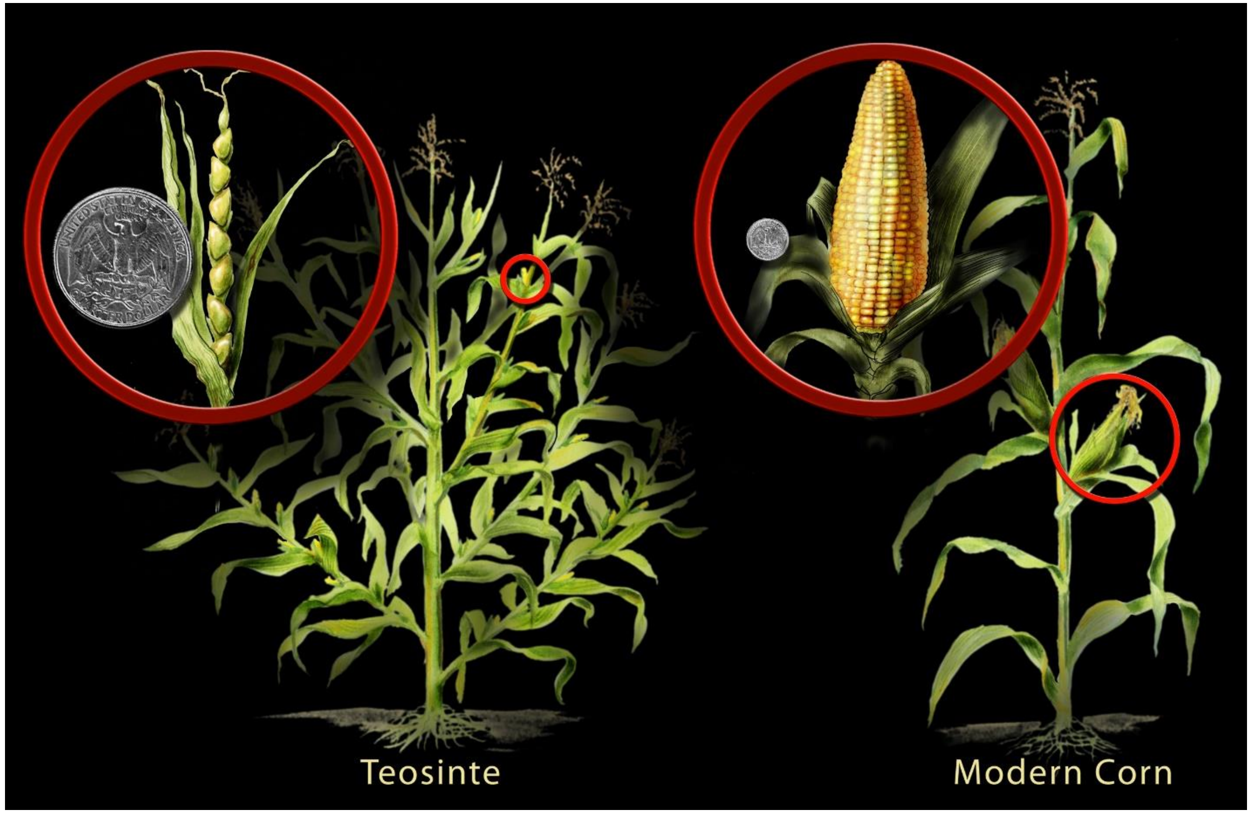
- No seed dormancy

These are taxonomic traits

… also:

- Compact habitus
- Bigger edible parts
- Increased fertility
- Increased inbreding
- Photoperiod-independent behavoiur
- Less toxic compunds
- Colours

So called Domestication Sindrome (Hammer 1984)



Domestication traits of crops

Seed shattering

- The tendency to disperd seeds; favoured in the wild (*e.g.* epizoochory), bad for harvesting
- Very few genes involved

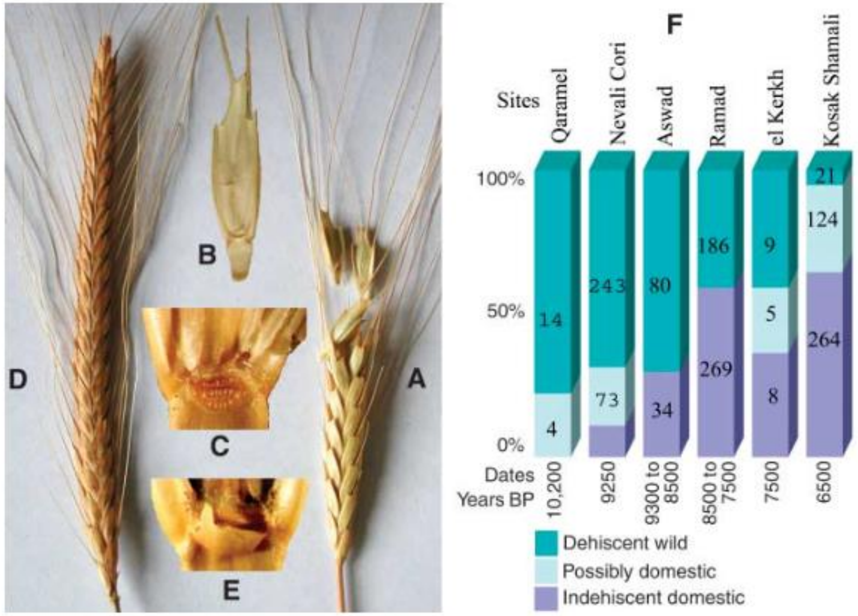
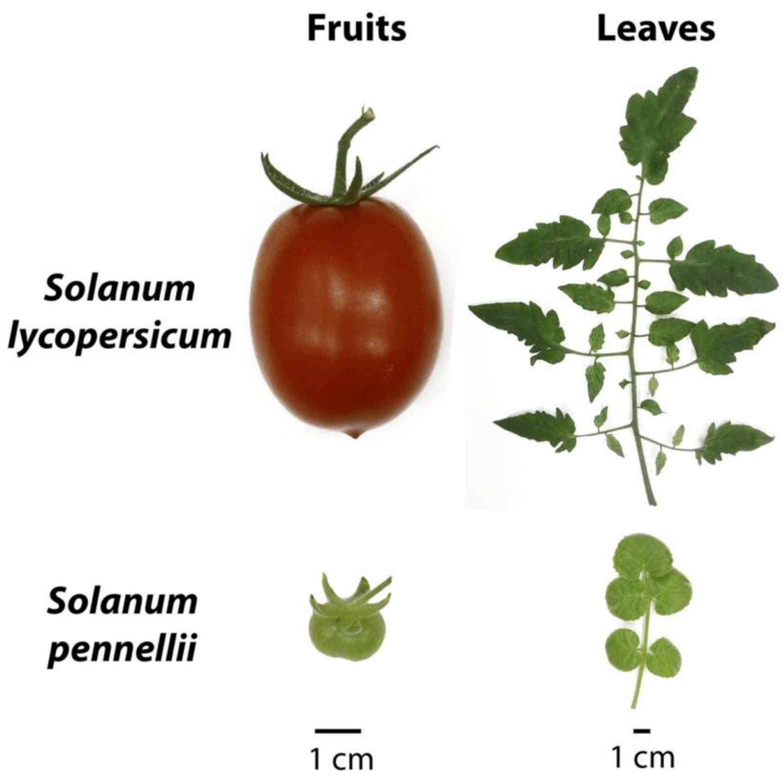
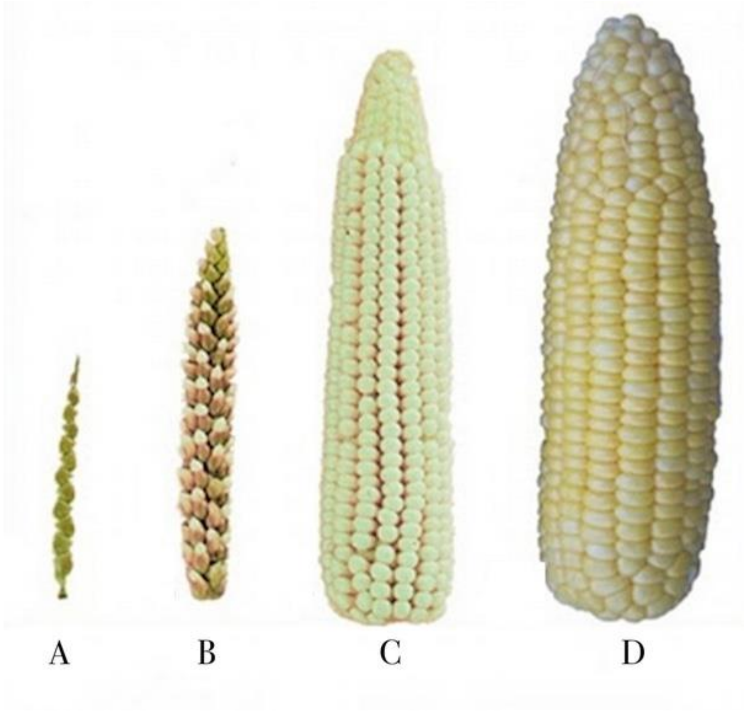


Fig. 1. Modern examples of dehiscent wild einkorn wheat ear (**A**) and spikelet (**B**). Detail of spikelet with smooth wild abscission scar (**C**), indehiscent domestic ear (**D**), and detail of spikelet with jagged break (**E**) are shown. The bar chart (**F**) gives relative frequencies of subfossil finds with the absolute figures. Records from Aswad and Ramad (*6*) are of barley; the other four sites are of wheat. For full data of both studies, see table S1.

Fruit size

- More energy diverted towards fruit bodies than what would be necessary for survival alone





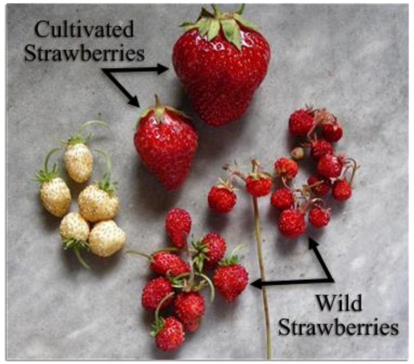
Maize Vs teosinte

Change in photoperiod sensitivity

- Alteration of seasonality
- Diffusion across different latitudes

Changes in sexual reproduction

- Induced sterility
- Lack of normal pollinating organs



Wild banana

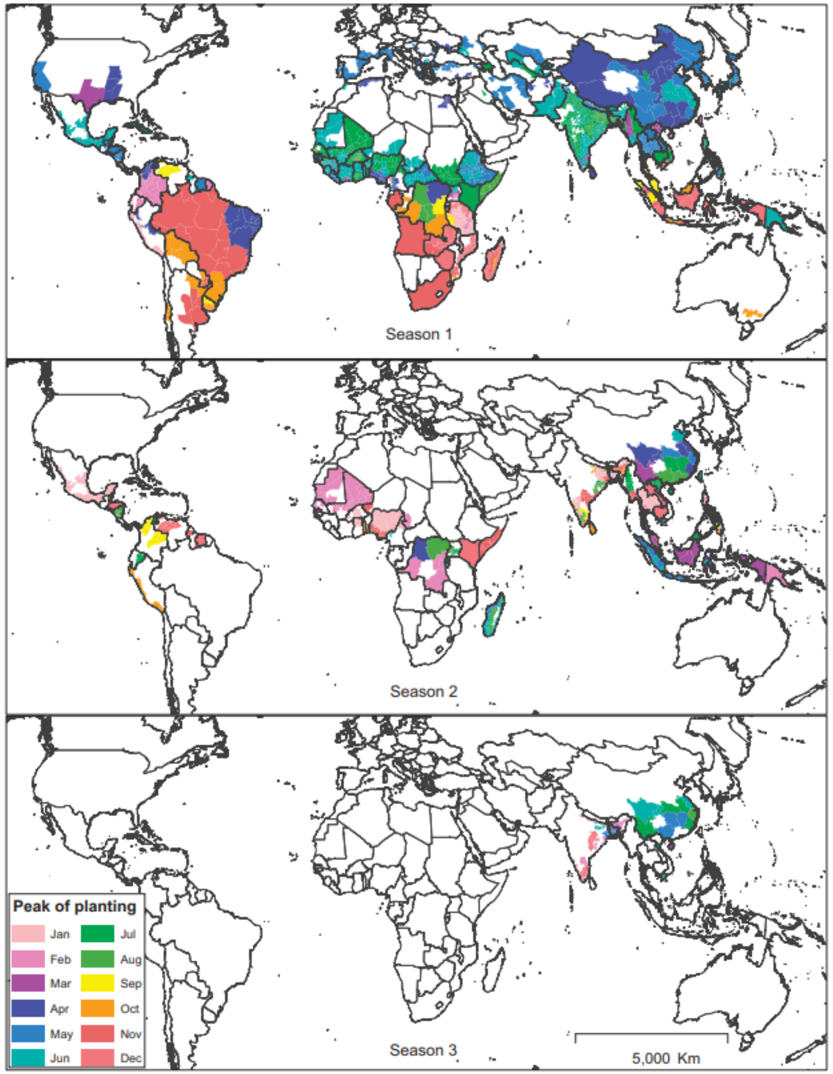


Figure 2. Peak rice planting months by season. Season 1 refers to the main rice-growing season, that is, the season with the highest rice production. Season 2 has the second highest rice production and Season 3 the least.

Increased apical dominance

- more resources in the main stem of the plant and a corresponding suppression of axillary branches

Determined growth

- reduction of vegetative growth in favor of fruiting bodies
- Switch from perenniality to annuality

Loss of seed dormancy

- Seeds immediately ready to produce plants

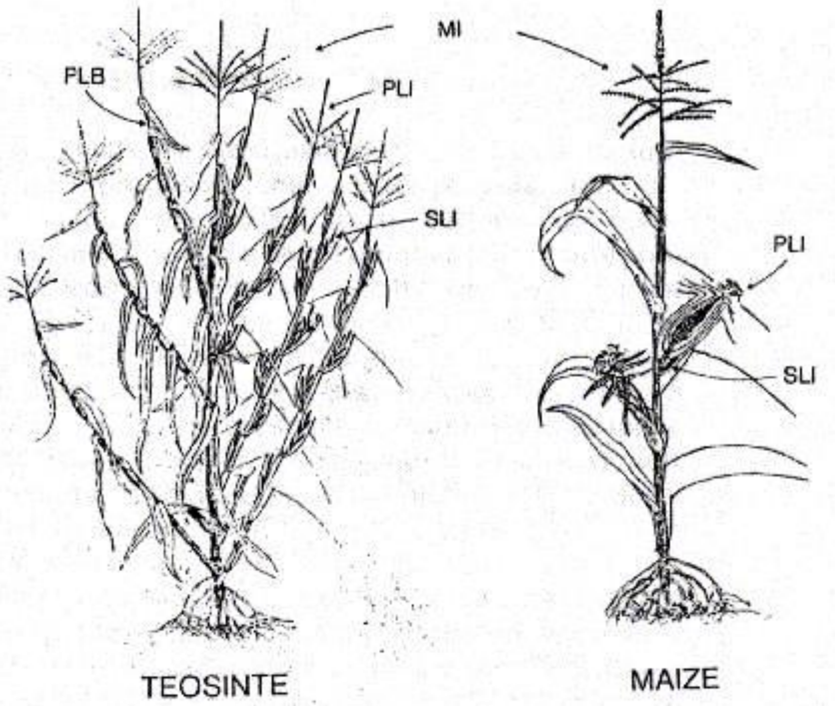


FIG. 1. Annual teosinte and maize plant architectures, adapted from Iltis (7). MI, main inflorescence; PLI, primary lateral inflorescence; SLI, secondary lateral inflorescence; PLB, primary lateral branch.

The idea behind this work

nature biotechnology
LETTER

De novo domestication of wild tomato using genome editing

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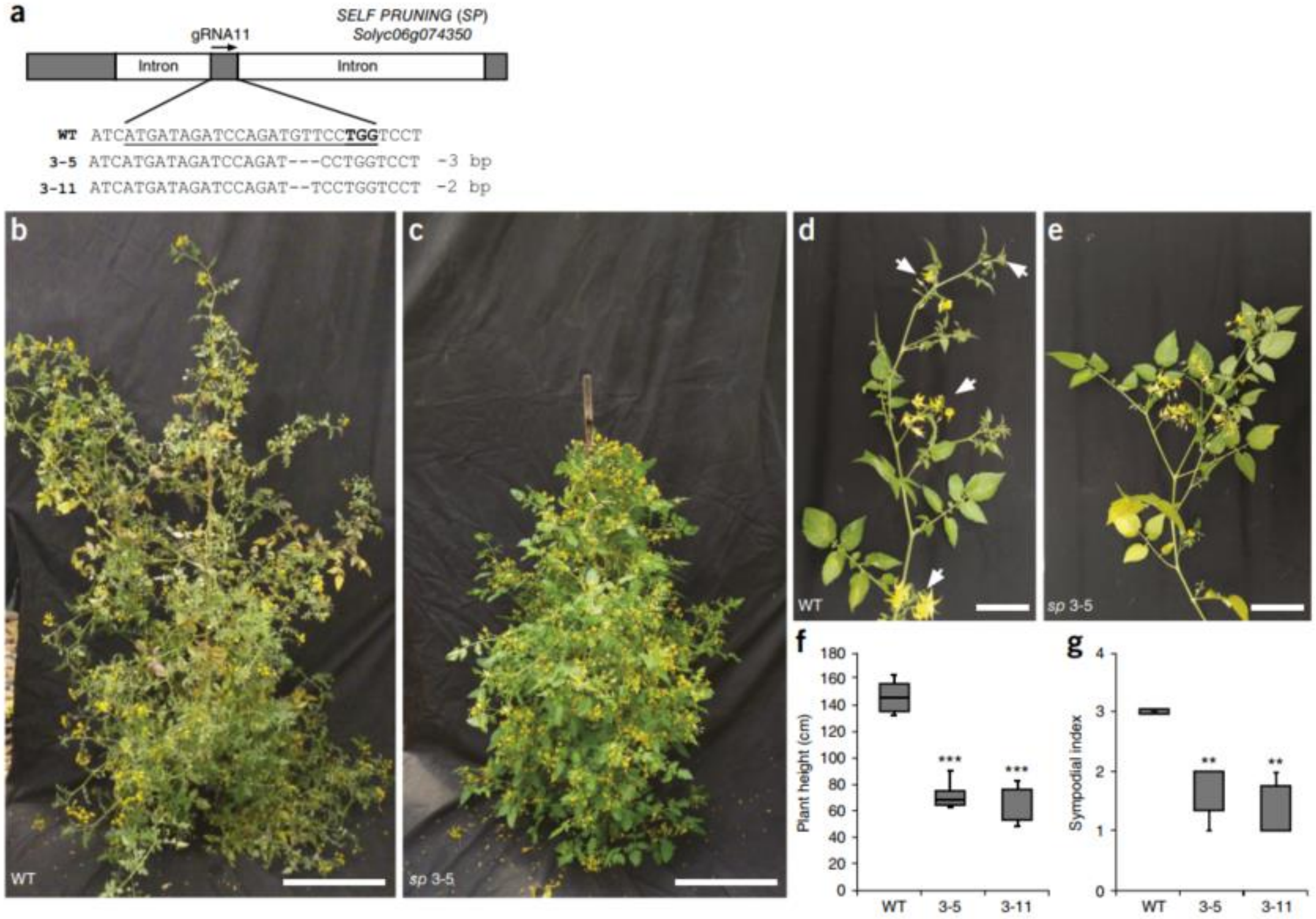
1. **Identify a wild species**
   - *Solanum pimpinellifolium*
2. **Identify a suite of key loci that have shaped morphology and agronomic potential**
   - Growth habit
   - Fruit shape and size
   - Fruit number
   - Nutritional value

   } **Forward Genetics**
3. **Target this set of genes using multiplex CRISPR-Cas9 approach to generate loss-of-function alleles**

Growth habit

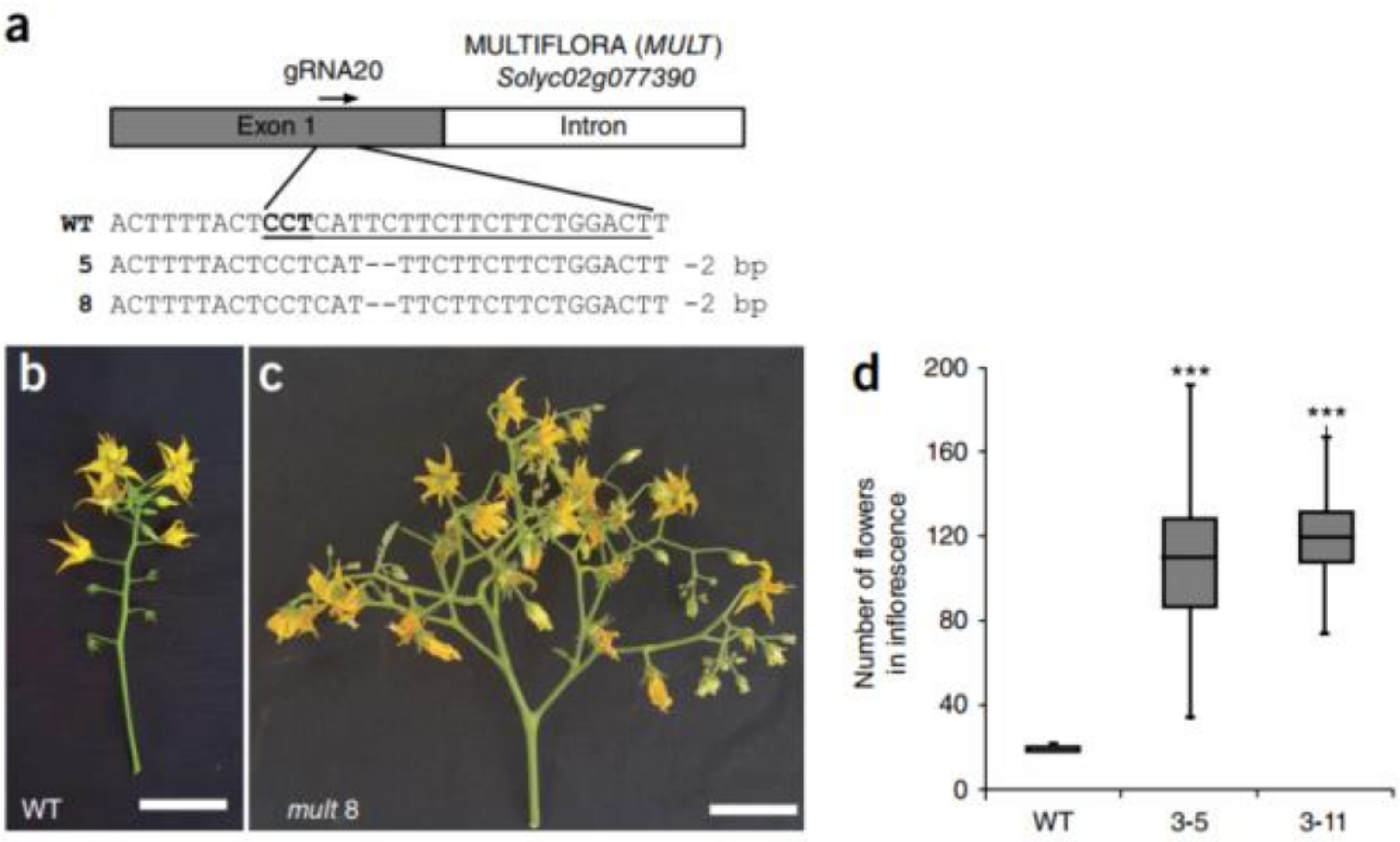
- Two events of missense mutation
- Displayed WT vs T2 plant
- Reduced plant height
- Reduced sympodial index (sympodial unit 3 leaves + flower)



Zsögön et al. 2018, *Nature biotech*

Fertility

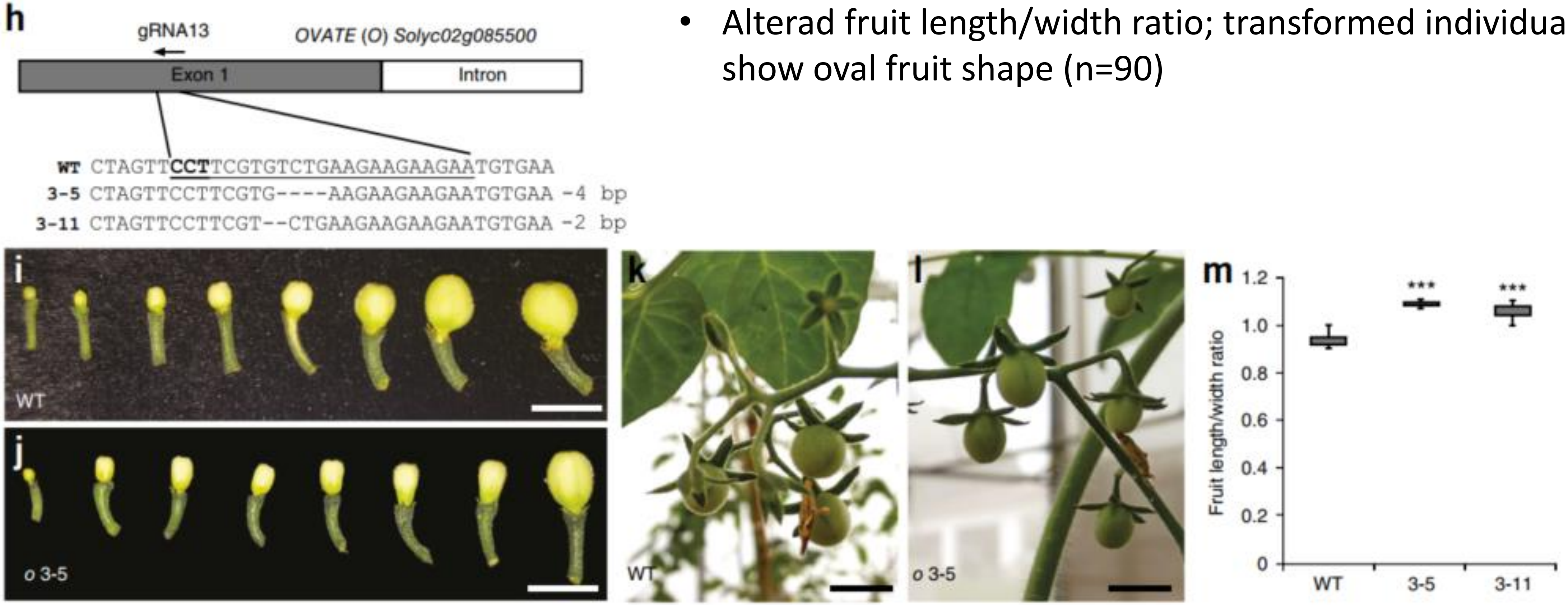
- Two events of missense mutation (identical)
- Displayed WT vs T2 plant
- Increased number of flowers per inflorescience in both events (n=6).



Zsögön et al. 2018, *Nature biotech*

Fruit Shape

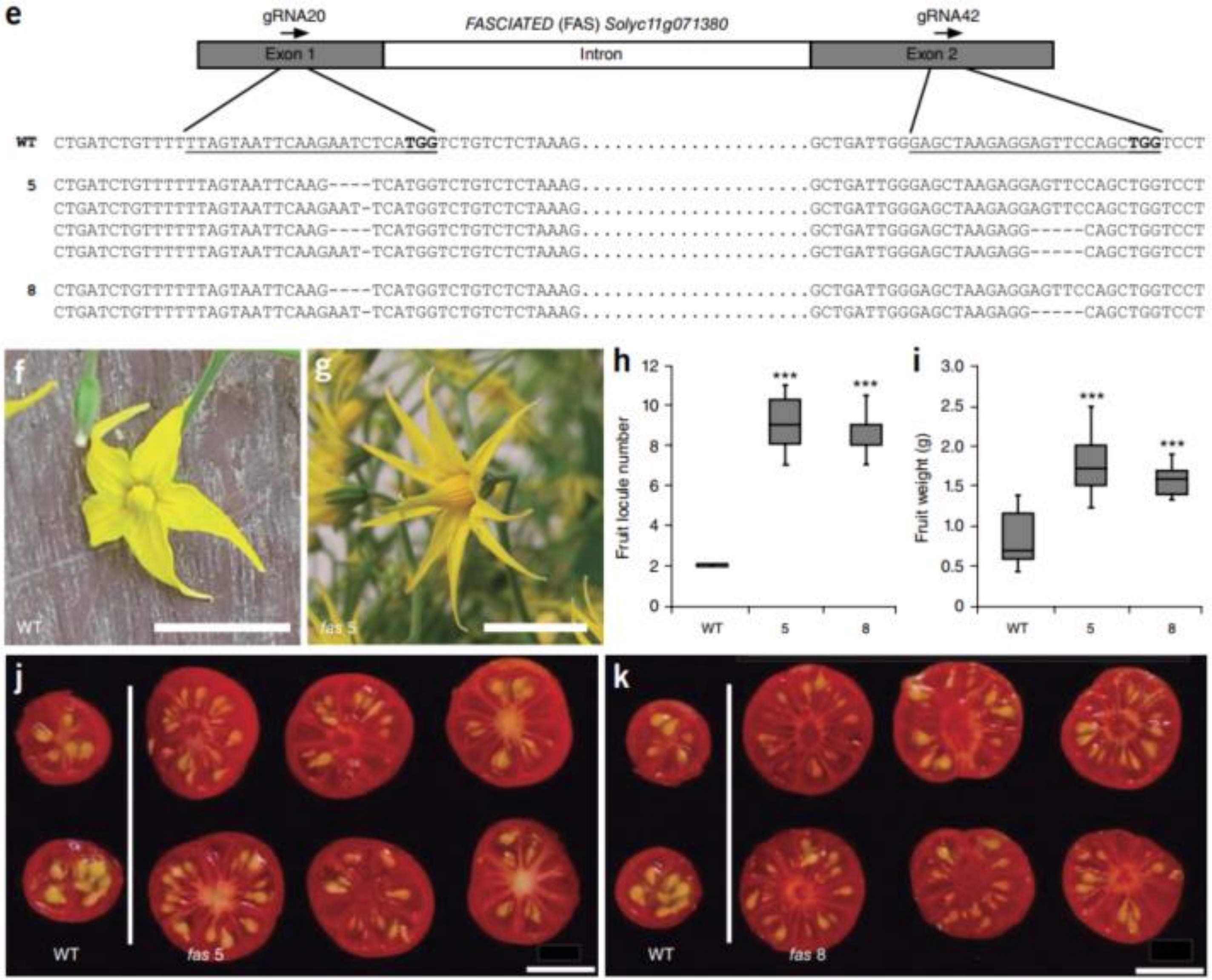
- Two events of missense mutation
- Displayed WT vs T2 plant
- Alterad fruit length/width ratio; transformed individuals show oval fruit shape (n=90)



Zsögön et al. 2018, *Nature biotech*

Fruit size

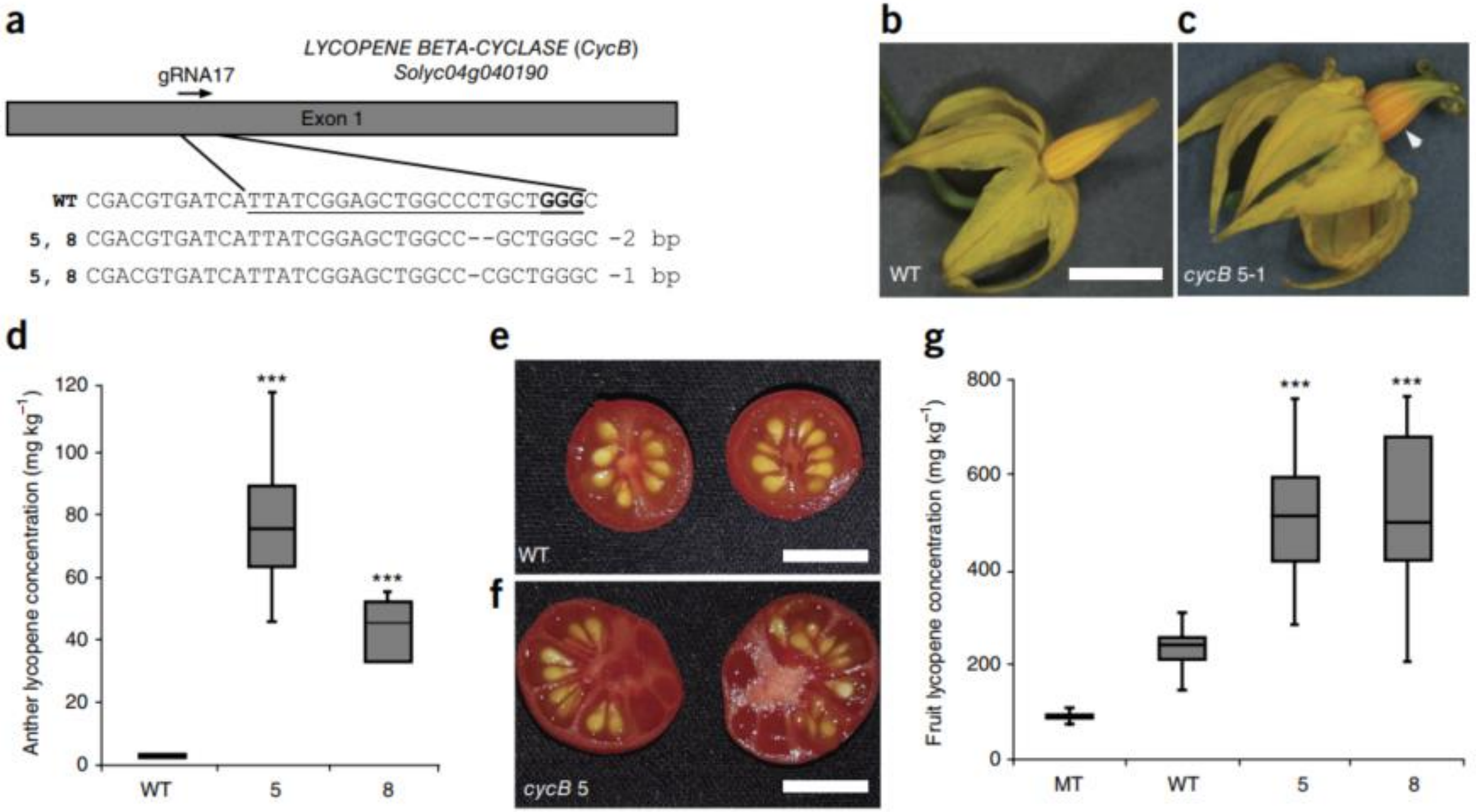
- Events of missense mutation, gRNAs terget two loci of the *fas* in CLV3; in T1 we observe **biallelic deletion in exon 1** while **heterozygous in exon 2**;
- Displayed WT vs T1 plant
- Increased number of locules per fruit (n=60)
- Increased fruit weight (n=90)



Zsögön et al. 2018, *Nature biotech*

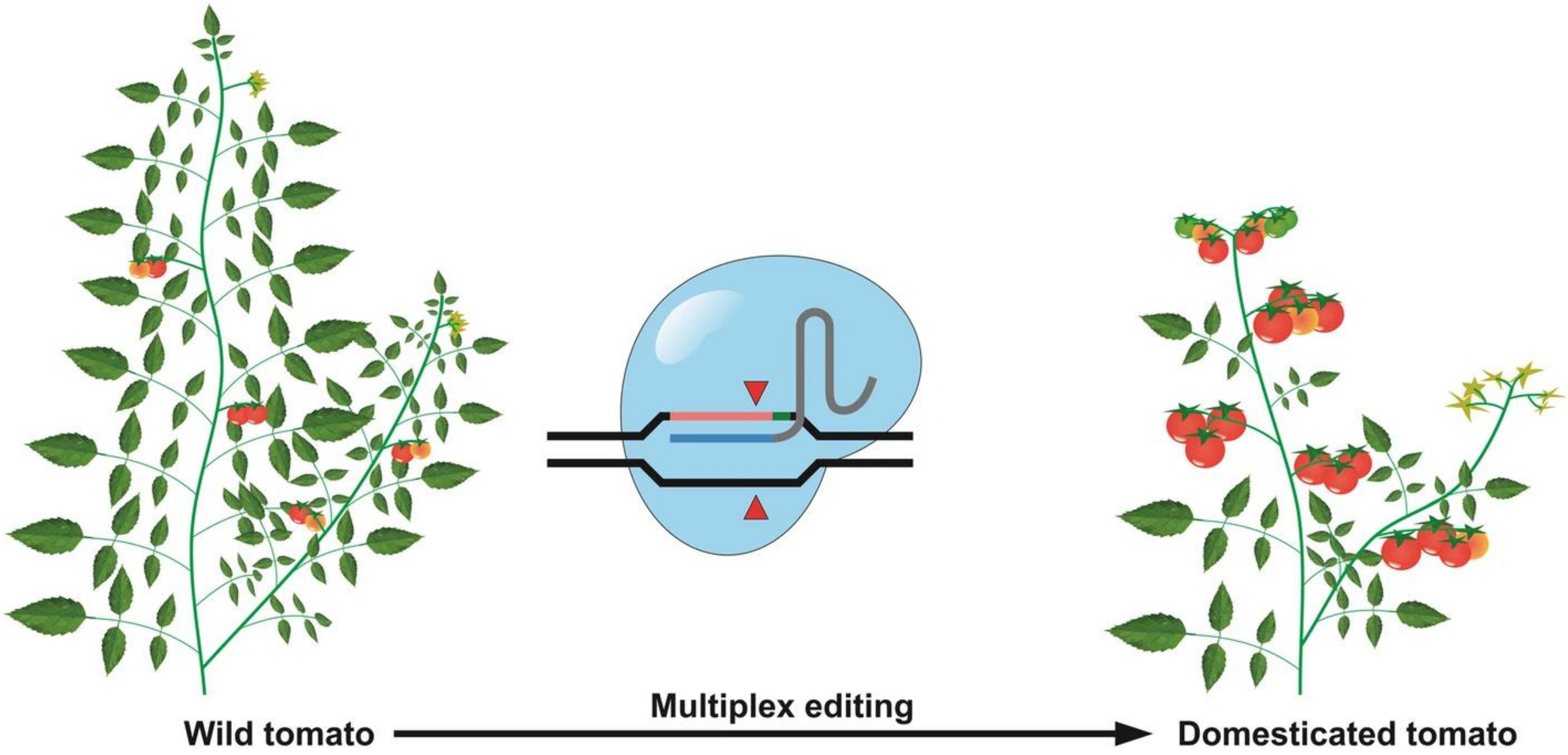
Colour

- Two events of missense mutation
- Displayed WT vs T1 plant
- Increased number of locules per fruit (n=60)
- Increased fruit weight (n=90)



Zsögön et al. 2018, *Nature biotech*

De novo domestication of tomato



Adapted from Li et al., 2018, *Nature Biotechnology*

The impact of CRIPR/Cas9 technology: a broader picture

Table 2 | **Ongoing clinical trials using CRISPR technologies to engineer immunotherapies for the treatment of human cancers**

Target and method	Cell type	Phase	Clinical trial identifier
PD1 KO	Autologous TILs	I	NCT03081715 (REF.[332])
PD1 KO	Autologous TILs	I	NCT02793856 (REF.[286])
PD1 KO	Autologous EBV CTLs	I/II	NCT03044743 (REF.[333])
PD1 KO	Autologous TILs	I	NCT04417764 (REF.[334])
PD1 and TCR KO	Allogeneic mesothelin-targeting CAR T cells	I	NCT03545815 (REF.[335])
Edited endogenous HPK1	Autologous CD19-targeting CAR T cells	I	NCT04037566 (REF.[336])
Endogenous CD5 KO	Allogeneic CD5-targeting CAR T cells	Early phase I	NCT04767308 (REF.[337])
Endogenous TCR and β_2m KO	Allogeneic CD19-targeting CAR T cells	I	NCT03166878 (REF.[338])
Insert CAR, endogenous TCR and MHC-I KO	Allogeneic CD70-targeting CAR T cells	1	NCT04502446 (REF.[339])
Insert CAR, endogenous TCR and MHC-I KO	Allogeneic BCMA-targeting CAR T cells	I	NCT04244656 (REF.[340])
Insert CAR, PD1 and endogenous TCR KO	Allogeneic CD19-targeting CAR T cells	I	NCT04637763 (REF.[341])
Insert CAR, endogenous TCR and MHC-I KO	Allogeneic CD70-targeting CAR T cells	I	NCT04438083 (REF.[342])
Insert CAR, CD52 KO	Allogeneic CD19-targeting CAR T cells	I	NCT04557436 (REF.[343])
CISH KO	Autologous CD19-targeting CAR T cells	I/II	NCT04426669 (REF.[344])

β_2m, β_2-microglobulin; BCMA, B cell maturation protein (also known as TNFRSF17); CAR, chimeric antigen receptor; CISH, cytokine-inducible SH2-containing protein; CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; HPK1, haematopoietic progenitor kinase 1; KO, knockout; MHC-I, major histocompatibility complex class I; PD1, programmed cell death protein 1; TCR, T cell receptor; TIL, tumour-infiltrating lymphocyte.

Katti et al., 2022, *Nature Cancer Reviews*

EC and the CRISPR/Cas9

On **25th of July 2018**, the **Court of Justice of the European Union** ruled that the regulatory framework for genetic engineering should be extended to the so-called **New Breeding Technologies** including recently developed methods of genome editing known as new genetic technologies (NGTs). The judgement claimed to be based on the **precautionary principle**.

Little incentive for private research

Recommended readings

- Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., … Horvath, P. (2007). **CRISPR provides acquired resistance against viruses in prokaryotes**. *Science*, *315*(5819), 1709–1712. https://doi.org/10.1126/science.1138140
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- A. Zsögön, et al., **De novo domestication of wild tomato using genome editing**. Nat. Biotechnol. (2018) https:/doi.org/10.1038/nbt.4272.

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Thanks for you attention