Targeted genome editing

Genome editing:

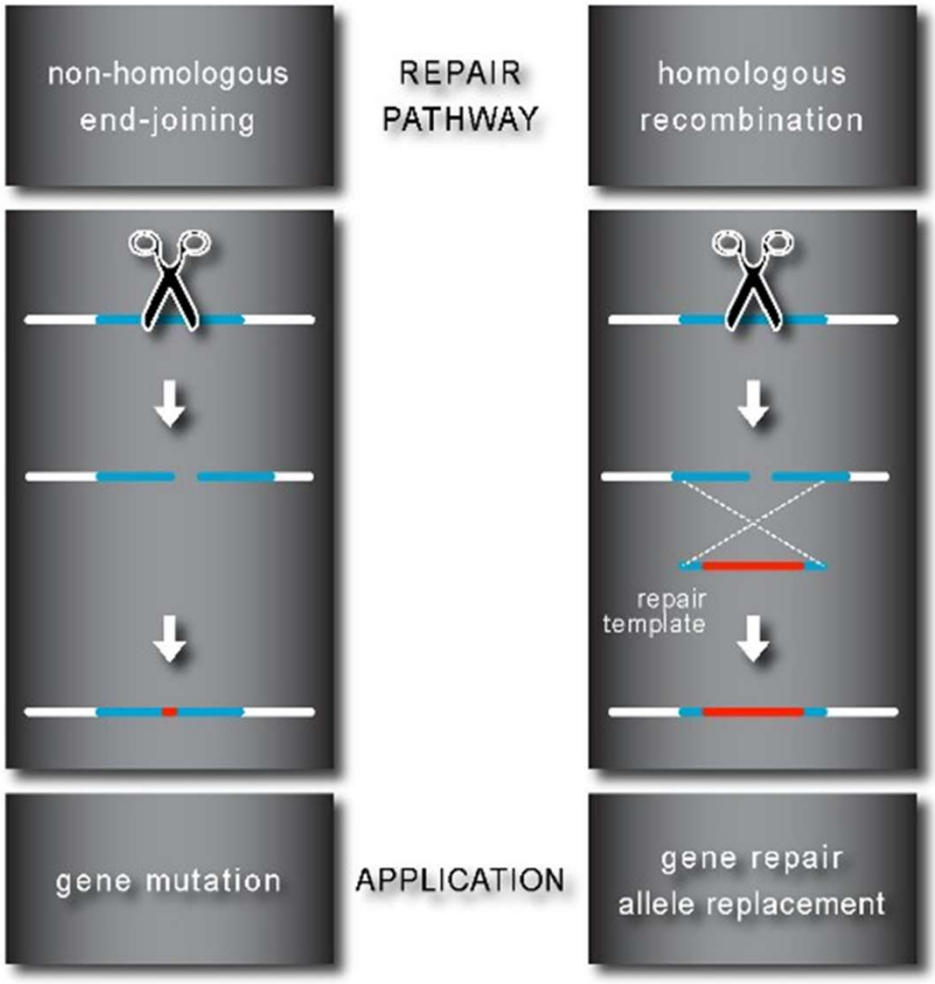
Basis:

At the site of double-stranded breaks (DSBs) within the genome, mutations are introduced by either imprecise repair of DSBs mediated by non-homologous end-joining (NHEJ) or by insertion of a donor DNA into the break by homologous recombination (HR).

For genome editing, the most successful strategy relies on the possibility to create DSBs at specific sites.

Repair without DNA insertion: autonomous repair often results in local deletion

- Repair with DNA insertion: presence of DNA template can direct specific site mutagenesis by homologous recombination



Frequency of homologous recombination

(after insertion of homologous sequence)

Ratio between homologous/non-homologous recombination

Mammals 10^{-2} -10^{-5}

Lower eucaryots (yeast, protists, filamentous fungi) > 10%

Moss *Physcomitrella patens* about 90%

- affected by sequence length, ploidy, cell type, cell cycle phase, …

Higher plants 10^{-3} - 10^{-6}

(high frequency of non-homologous recombination prevents the homologous one)

Strategies for obtaining efficient HR in plants

1. Over-expression of *E. coli* genes involved in HR: [Bernd Reiss et al. PNAS | March 28, 2000 | vol. 97 | no. 7 | 3358-3363]

Over-expression of RecA: stimulated sister chromatid exchanges, increased intra-chromosomal recombination but not the frequency of **gene targeting** (GT, defined as recombination between a foreign gene carrying homologous sequences and the complementary genomic sequences).

*Over-expression of RuvC (*involved in the resolution of Holliday junction) [Shalev et al. PNAS Vol. 96, Issue 13, 7398-7402 1999]

Increased intra-, extra-, and inter-chromosomal recombination but not GT frequency.

2. Modification of plant HR machinery:

RAD50 mutant: RAD50 may be a common factor of illegitimate recombination and HR. RAD50 is dispensable for HR. *rad50* loss-of-function mutants may have suppressed illegitimate recombination. Intra-chromosomal recombination indeed goes up in *rad50* mutants. GT frequency is yet to be demonstrated in RAD50 mutants.

INEFFICIENT

Site-specific recombination systems of prokaryotes and lower Eukaryotes (recombinase/recognized target site – **must be introduced into the genome in advance)**

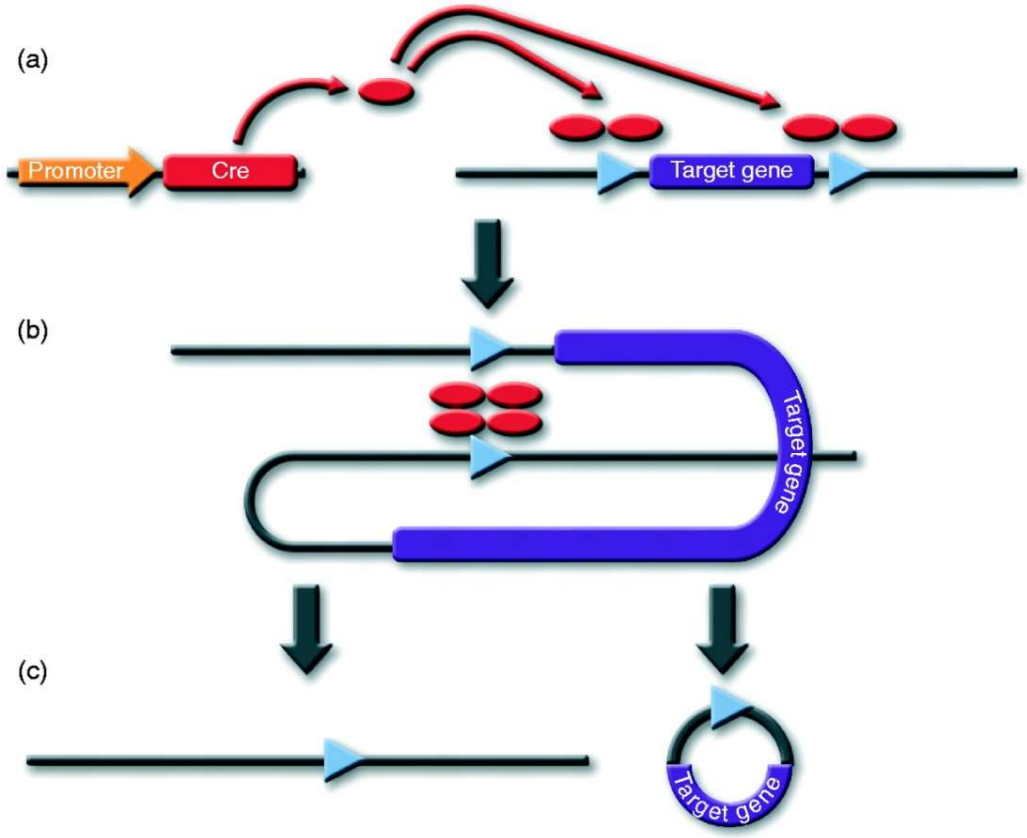
Cre/*lox*	**bacteriophage P1**
Flp/*frt*	*Saccharomyces cerevisiae*
R/*RS*	*Zygosaccharomyces rouxii*

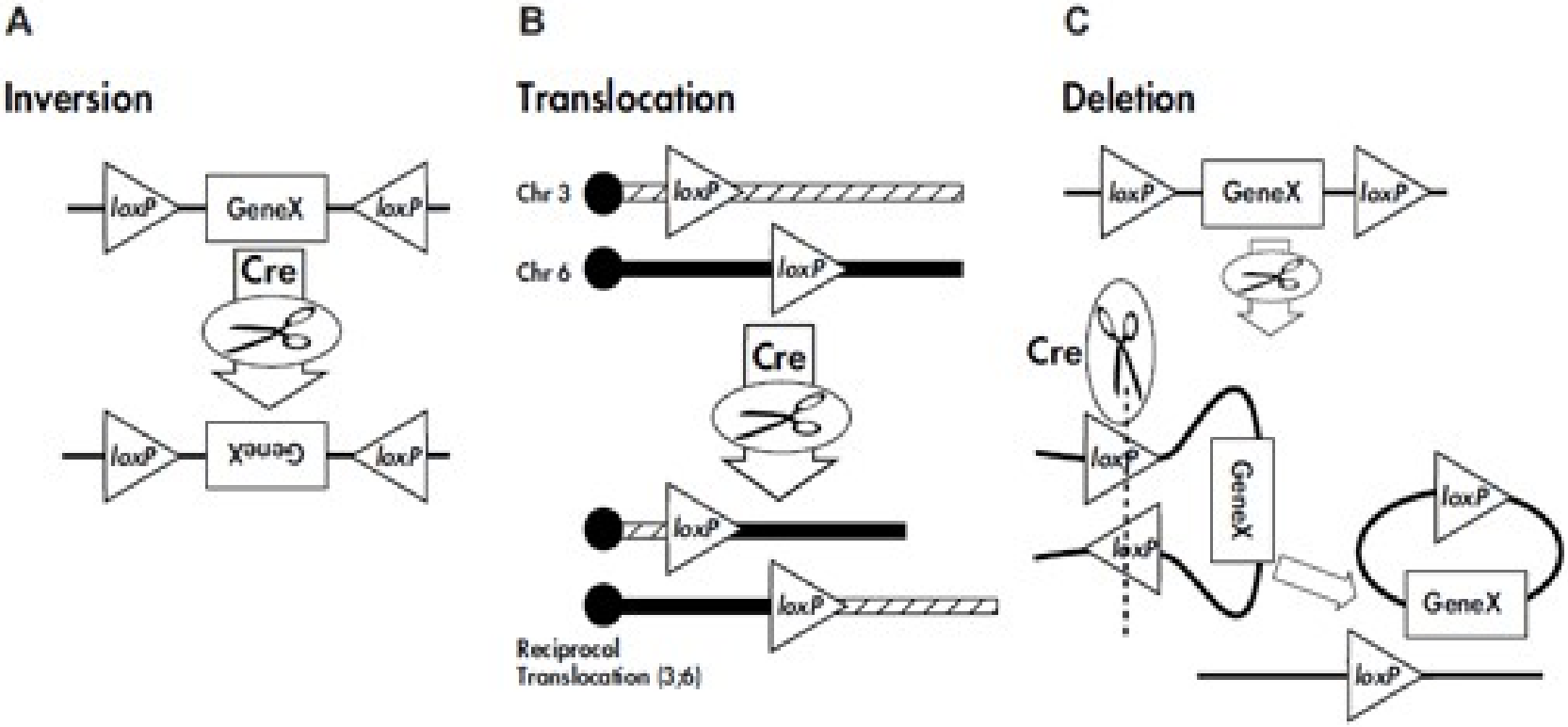
Cre/loxP

- Site-specific recombinase technology
- Widely used to carry out deletions, insertions, translocations and inversions at specific sites.
- DNA modification can be targeted to a specific cell type or be triggered by a specific external stimulus.
- Both in eukaryotic and prokaryotic systems.

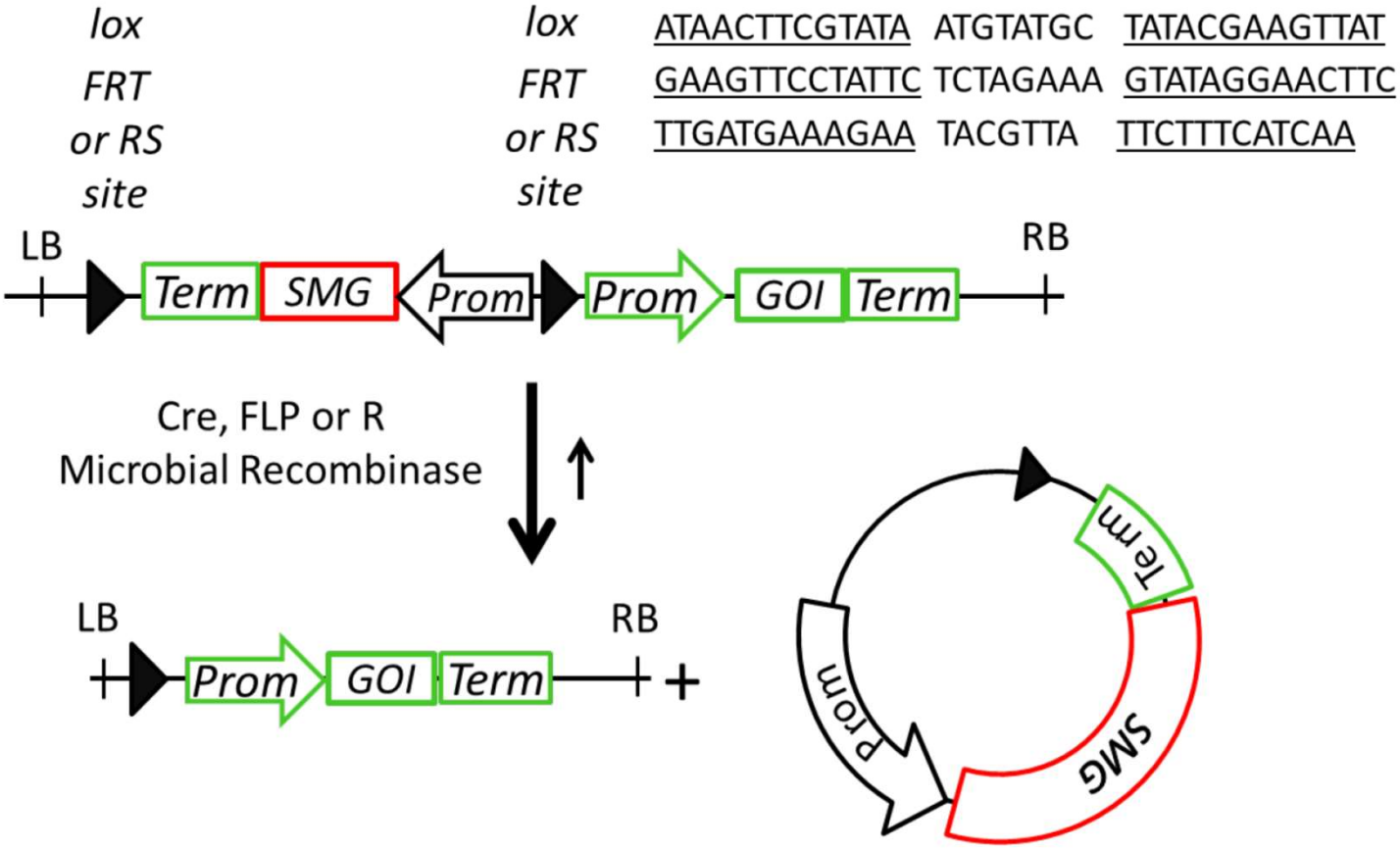
Cre/loxP

The system consists of a single enzyme, **Cre recombinase**, that recombines a pair of short target sequences called ***Lox* sequences**. This system can be implemented without inserting any extra supporting proteins or sequences. The Cre enzyme and the original *Lox* site called *LoxP* are from bacteriophage P1.

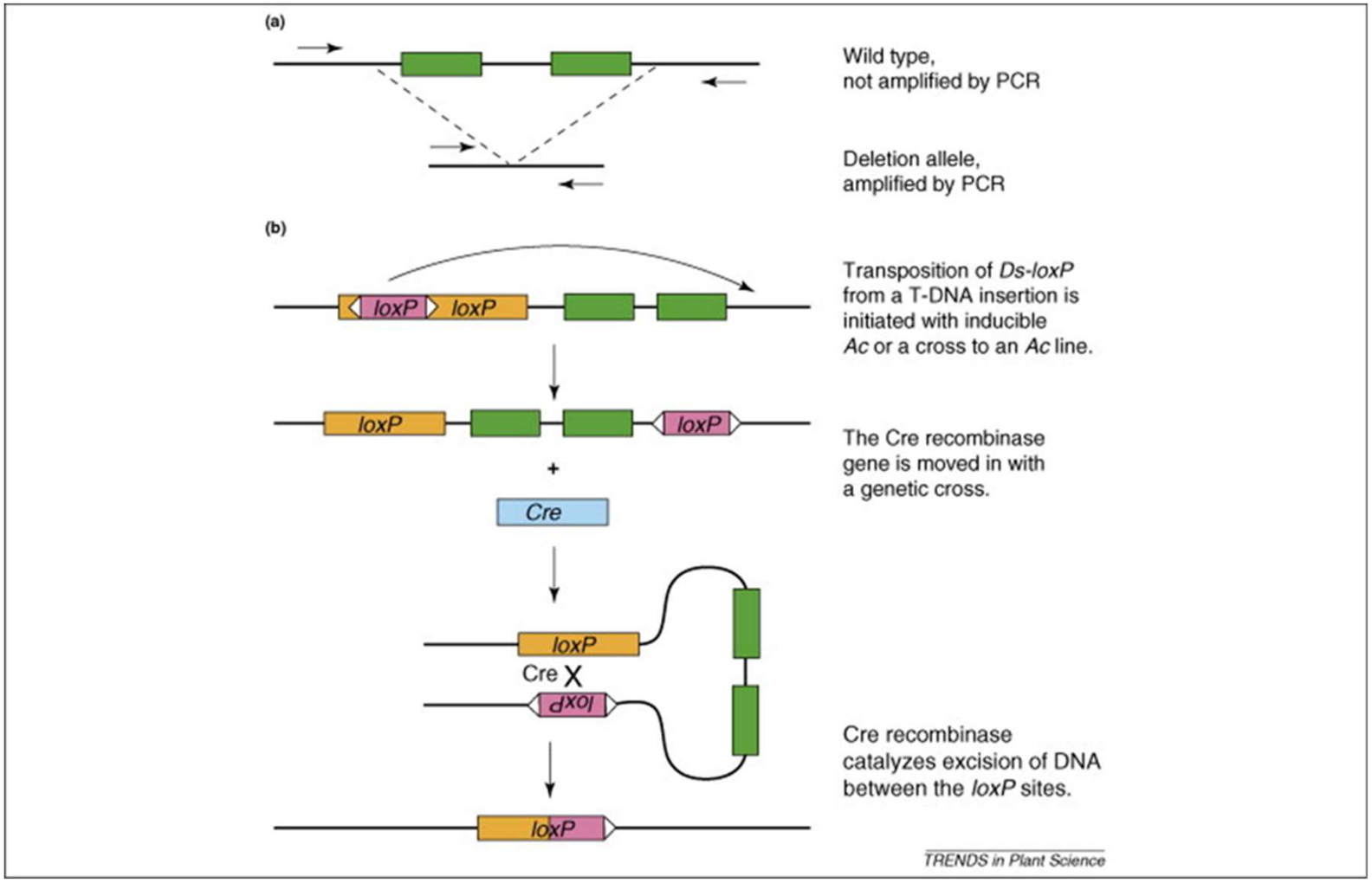



A
Inversion
loxP
GeneX
loxP
Cre
loxP
GeneX
loxP
B
Translocation
Chr 3
loxP
Chr 6
loxP
Cre
loxP
loxP
Reciprocal
Translocation (3;6)
C
Deletion
loxP
GeneX
loxP
Cre
loxP
loxP
GeneX
loxP
GeneX
loxP

Piante transgeniche prive di marker di selezione tramite ricombinasi sito-specifiche



Chromosomal deletions through localized transposition by the maize *Dissociation* (*Ds*) element and site-specific DNA excision by the bacteriophage Cre-*loxP* recombination system.



1) A *Ds-loxP* T-DNA construct, containing *loxP* sites in both the *Ds* element and the T-DNA backbone is transformed into the genome
2) transposition of *Ds-loxP* is induced by crossing in *Activator* (*Ac*).
3) a second cross to bring in the *Cre* recombinase gene

-> Cre-mediated site-specific recombination between the two *loxP* sites causes the excision of the intervening DNA. Excision of a gene of interest can be detected by PCR.

A community resource of >10 000 sequenced *Ds-loxP* T-DNA insertion sites has been generated for *Arabidopsis* researchers.

Alternatives for targeted genome editing

Synthetic site-specific nucleases to generate DSBs at specific sites

1) **Zinc Finger Nucleases** (ZFN), **Transcription Activator-Like Effector Nucleases** (**TALEN)**: specificity resides in proteins (DNA binding domain)

1) **CRISPR/Cas**: specificity resides in nucleic acid (complementary RNA).

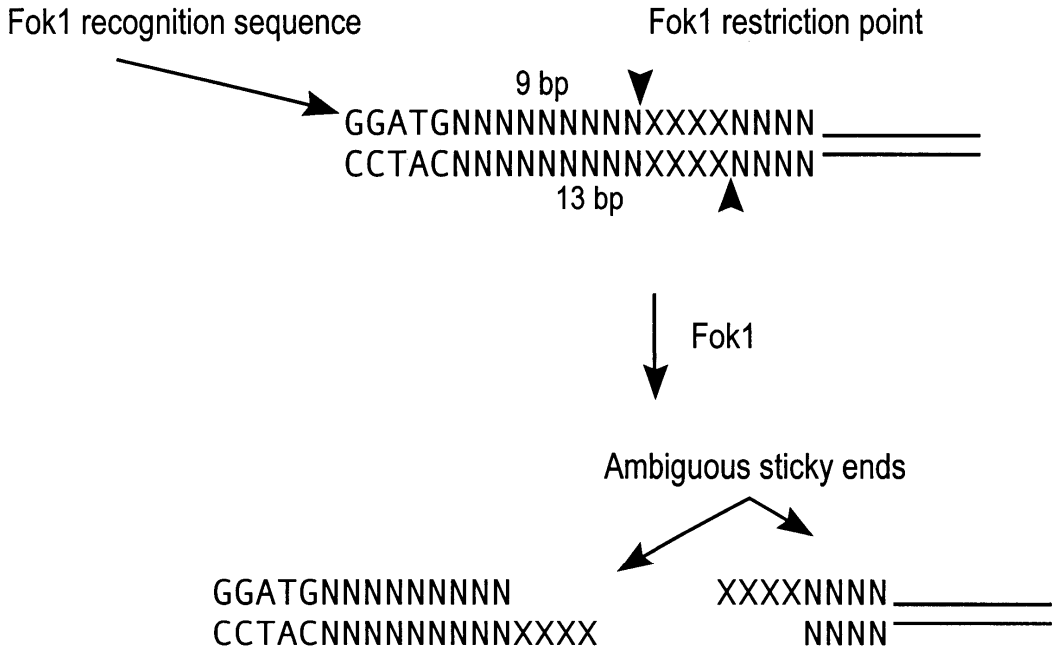
ZFN and TALEN are basically **artificial restriction enzymes** that **recognize specific stretches of bases,** consisting of :

- **DNA recognition domain (Zinc finger or TALE):** repeated protein modules that recognize specific DNA sites

- **FokI nuclease:** dimerization of nuclease domains cleaves target DNA

Type IIS enzymes: bacterial restriction enzymes that recognize an asymmetric DNA sequence and cleave a short distance from that sequence (shifted cleavage).

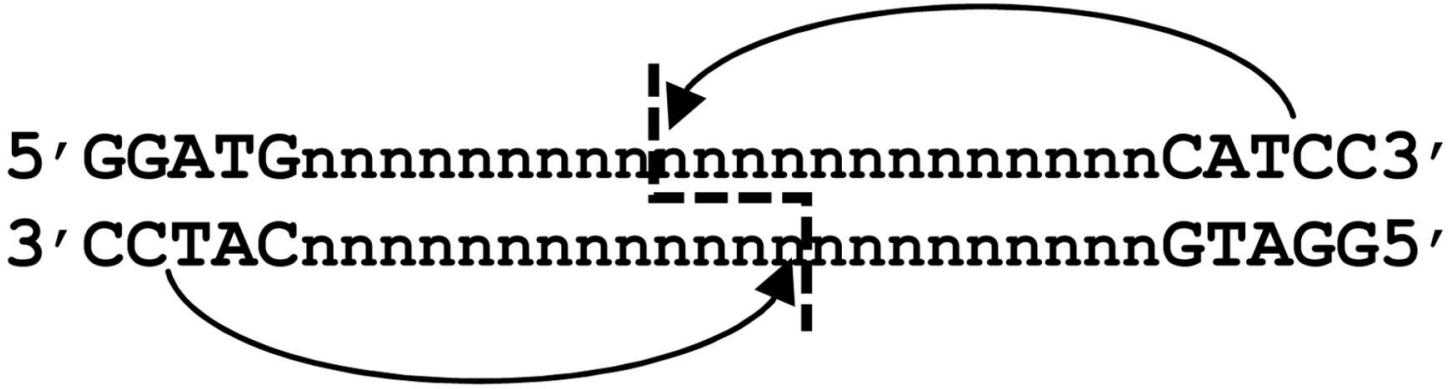
Es.: *Fok*I from *Flavobacterium okeanokoites* recognizes the non-palindromic 5'-GGATG-3':5'-CATCC-3' in duplex DNA and cleaves 9/13 nucleotides downstream of the recognition site -> **<u>two separate protein domains, one for sequence-specific recognition of DNA and the other for endonuclease activity</u>**.



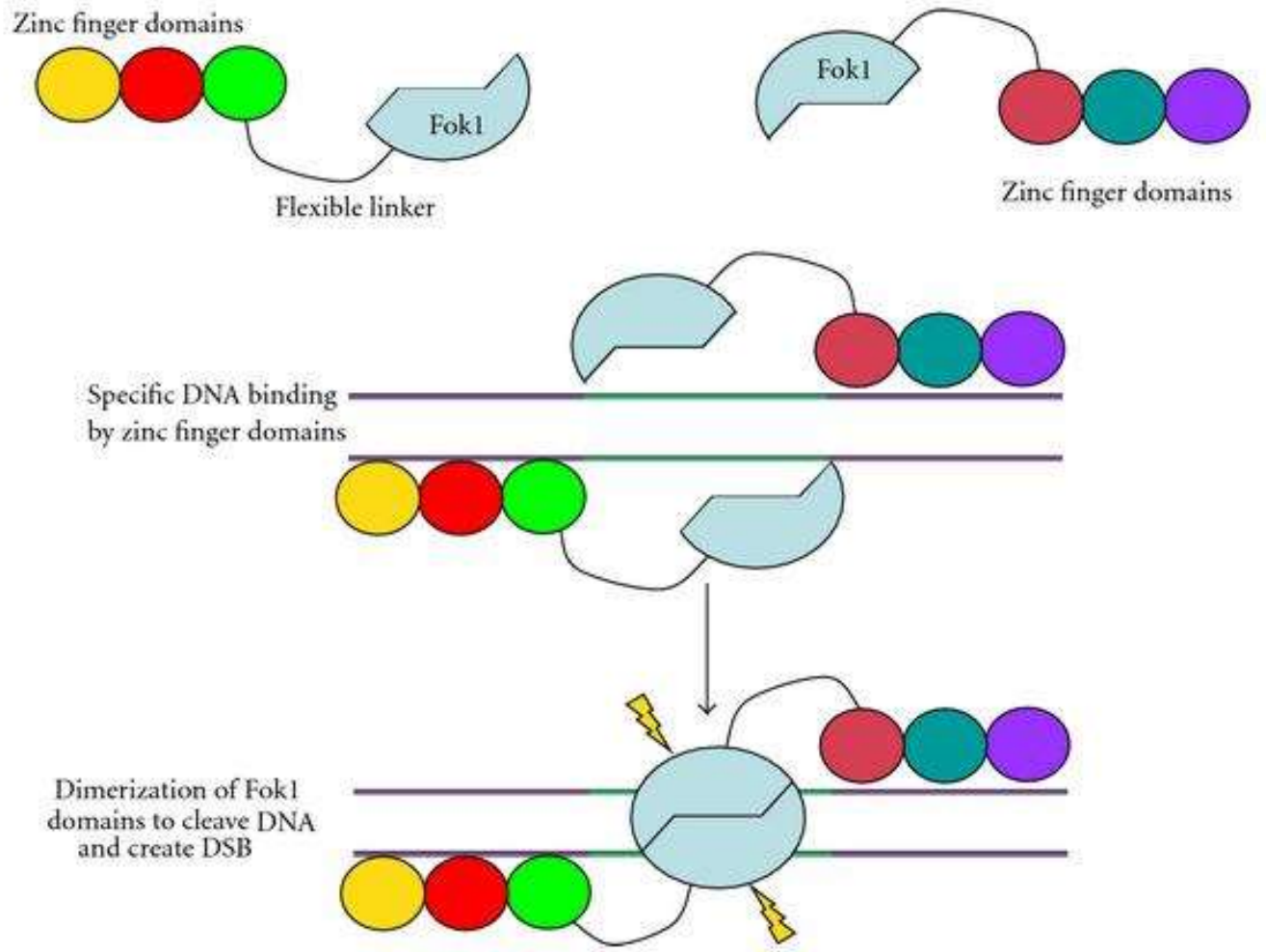
The FokI catalytic domain has only one active site so, to cut both DNA strands, the catalytic domains from two monomers associate to form a dimer.

The dimer involving a monomer at the recognition site and another from free solution is less stable than that from two proteins tethered to the same DNA. FokI thus cleaves DNA with two sites better than one-site DNA.

The two sites can be immediately adjacent, but they can alternatively be many hundreds of base pairs apart, in either inverted or repeated orientations.



It is possible to swap the recognition domain of these enzymes with other naturally occurring DNA-binding proteins which recognize longer sequences (e.g. the helix-turn-helix motif, the zinc finger motif and the basic helix-loop-helix protein containing a leucine zipper motif).

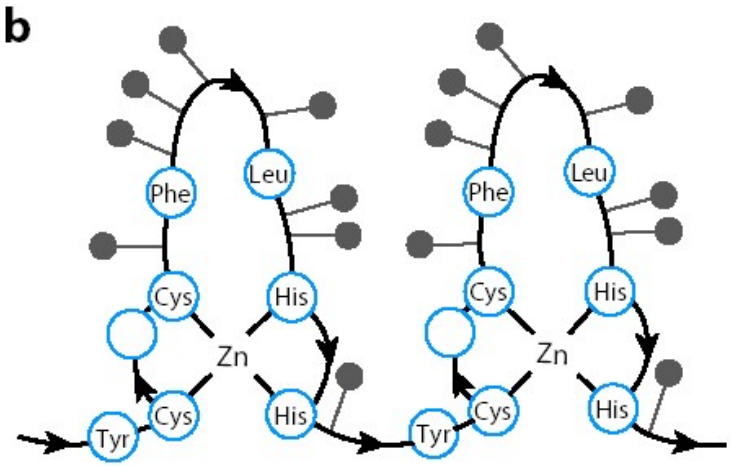


Zinc-Finger Nuclease (ZFN) Technology
(S. Chandrasegaran)

The Cys2-His2 zinc finger proteins are a class of DNA-binding proteins that contain sequences of the form (Tyr, Phe)-Xaa-Cys-Xaa_{2-4}-Cys-Xaa_3-Phe-Xaa_5-Leu-Xaa_2-His-Xaa_{3-5}-His, usually in tandem arrays. Xaa represents an unspecified amino acid.

Each of these sequences binds a Zinc(II) ion to form the structural domain termed a zinc finger. These proteins bind to DNA by inserting an α-helix into the major groove of the double helix.

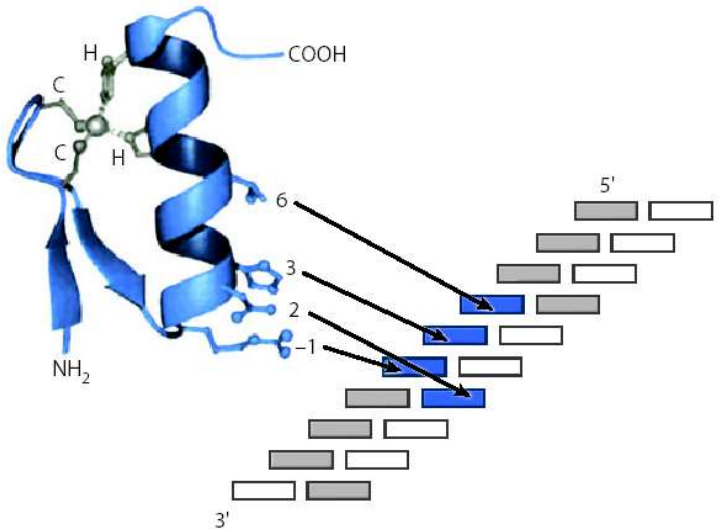
Zn-finger domains – designed for specific sequence

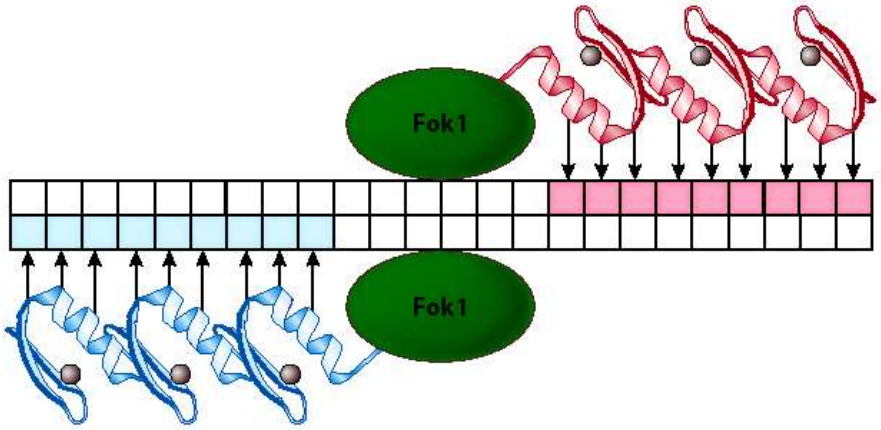


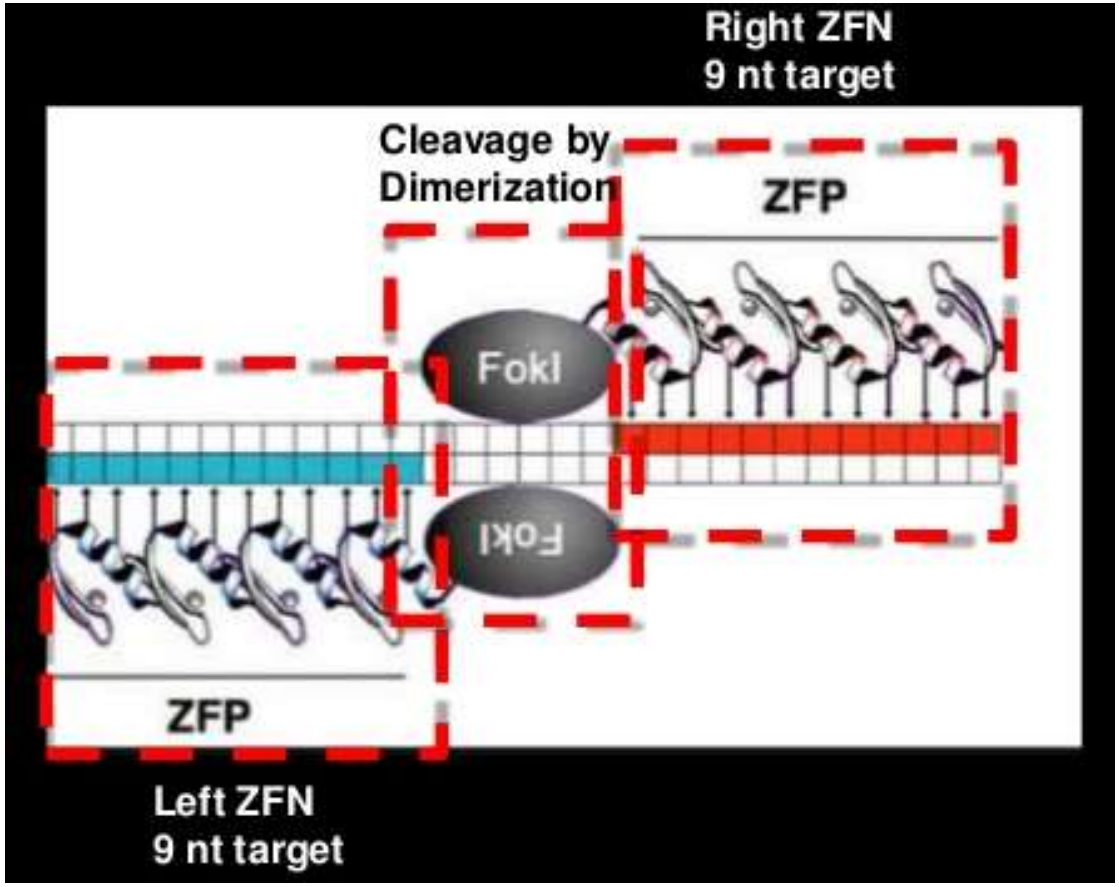
- conserved aminoacids marked
- black circles – interaction with DNA

DNA sequence recognition per finger:

3(-4) nt/finger






Right ZFN
9 nt target
Cleavage by
Dimerization
ZFP
FokI
FokI
ZFP
Left ZFN
9 nt target

- To recognize new target sequence, you should develop new zinc-finger DNA binding domain
  - Modular assembly from previously generated array
  - Selection using Phage Display/One Hybrid

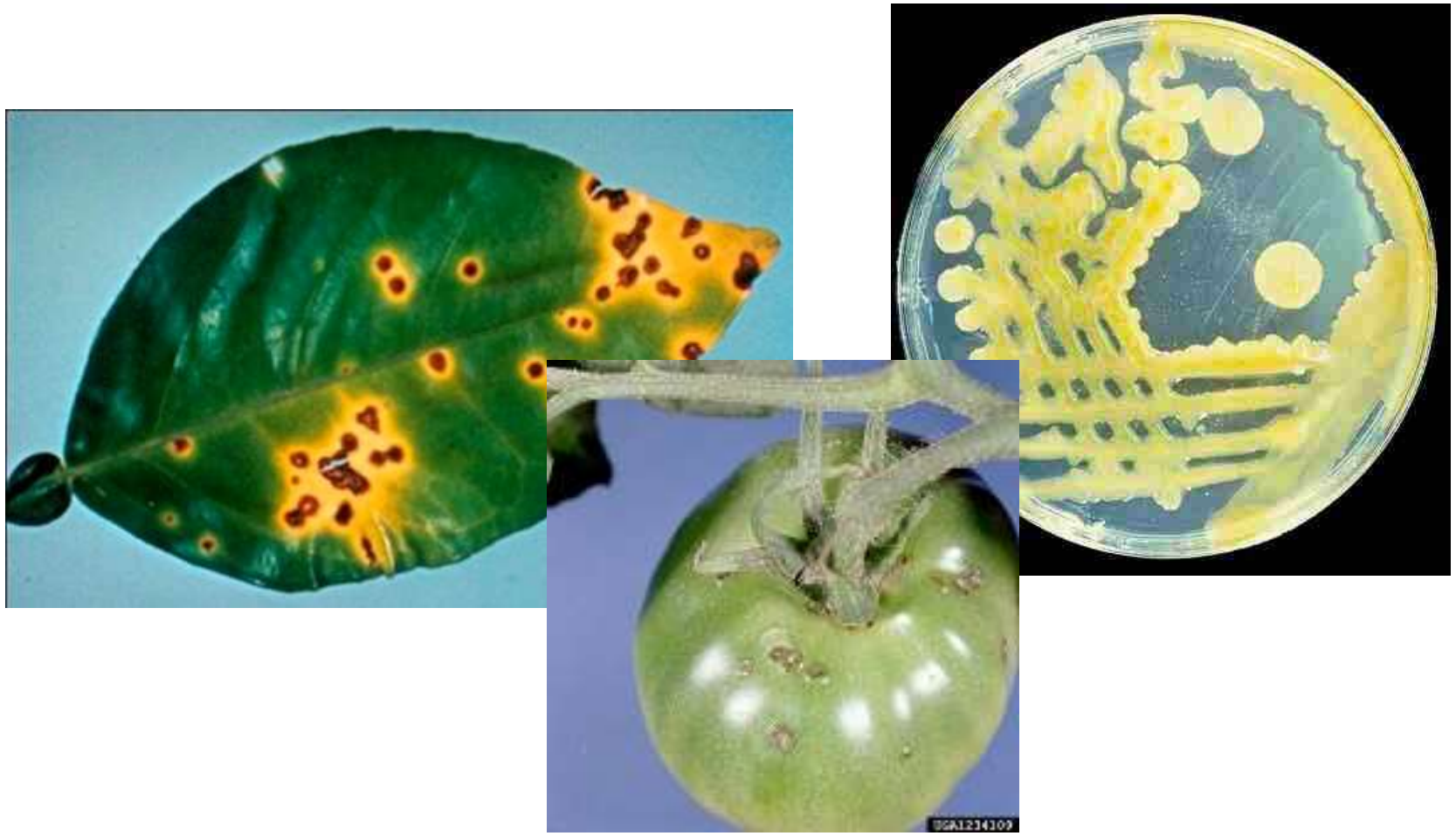
- Time consuming for the proper ZFP sets
- Failure rate is very high
- Off-target effects are very high

TALEN

Derived from TALEs

Transcriptional Activator-Like Effectors

Batteri del genere Xanthomonas manipolano l'espressione genica del loro ospite



Xanthomonas species

Monocotyledonous hosts 124

Vascular pathogens

Xanthomonas albilineans
(Leaf scald of sugarcane)

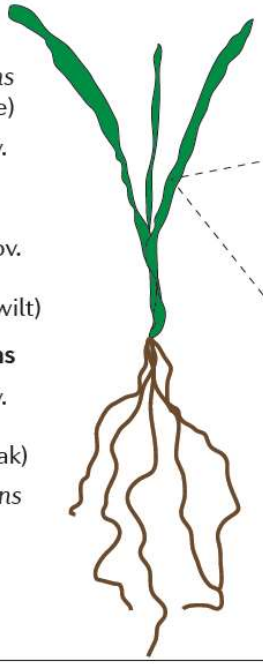
Xanthomonas oryzae pv. *oryzae*
(Rice bacterial blight)

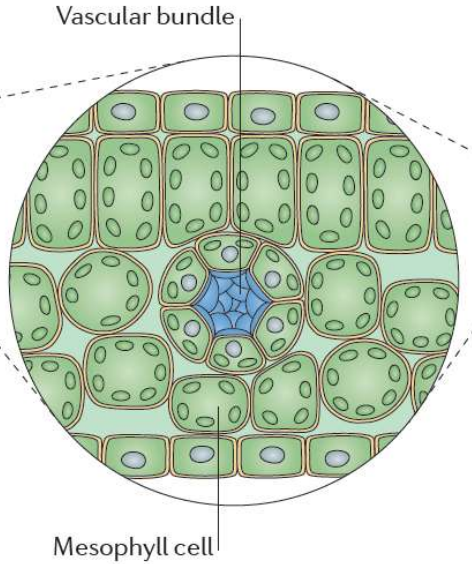
Xanthomonas vasicola pv. *musacearum*
(Banana *Xanthomonas* wilt)

Mesophyllic pathogens

Xanthomonas oryzae pv. *oryzicola*
(Rice bacterial leaf streak)

Xanthomonas translucens
(Bacterial leaf streak in cereals)





268

Dicotyledonous hosts

Vascular pathogens

Xanthomonas campestris pv. *campestris*
(Black rot of crucifers)

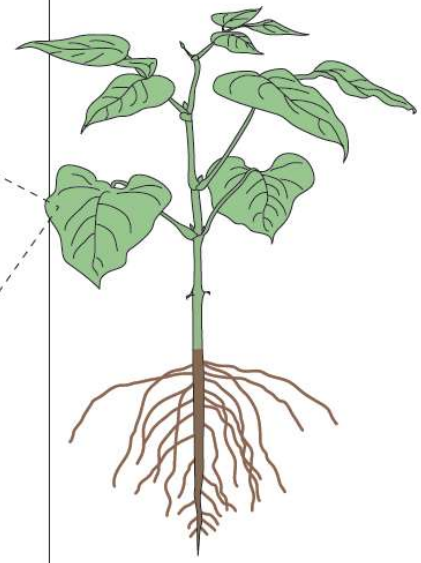
Xanthomonas manihotis
(Cassava bacterial blight)

Mesophyllic pathogens

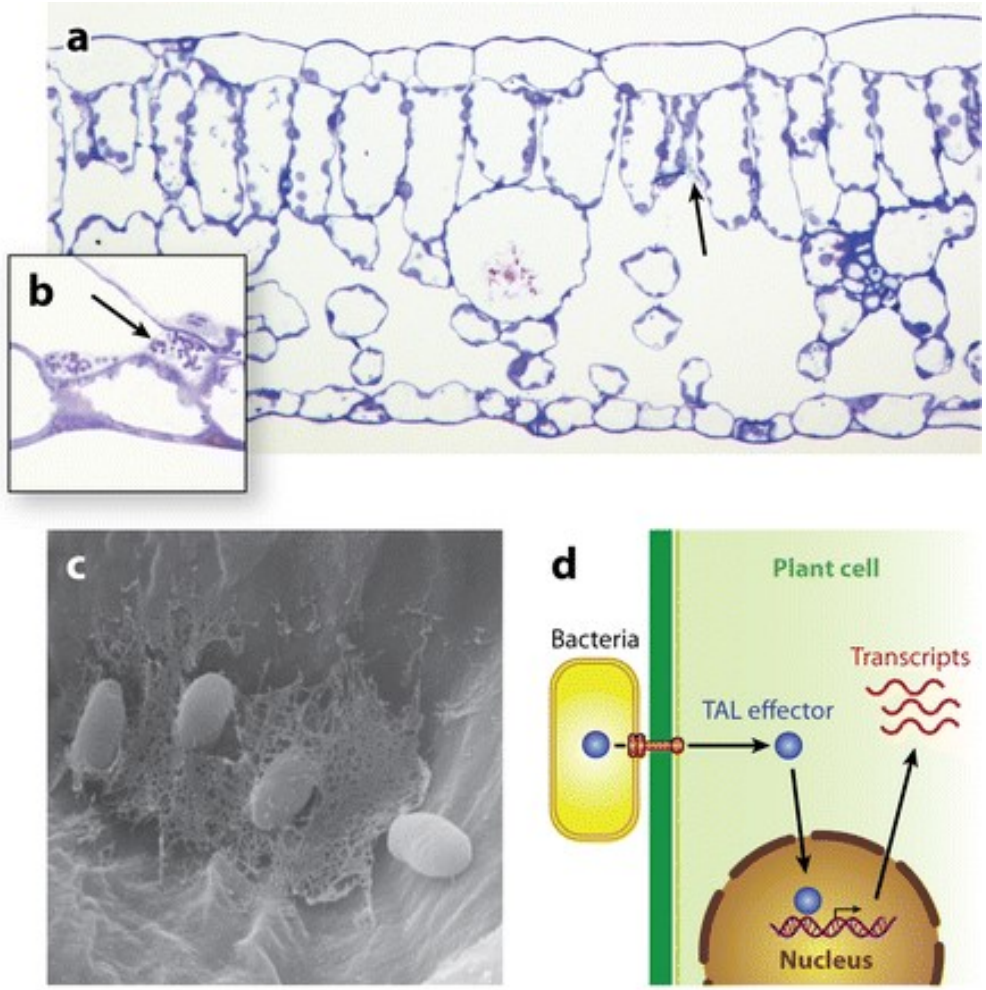
Xanthomonas campestris pv. *armoraciae*
(Leaf spot of crucifers)

Xanthomonas citri pv. *citri*
(Citrus canker)

Xanthomonas euvesicatoria
(Bacterial spot of pepper and tomato)



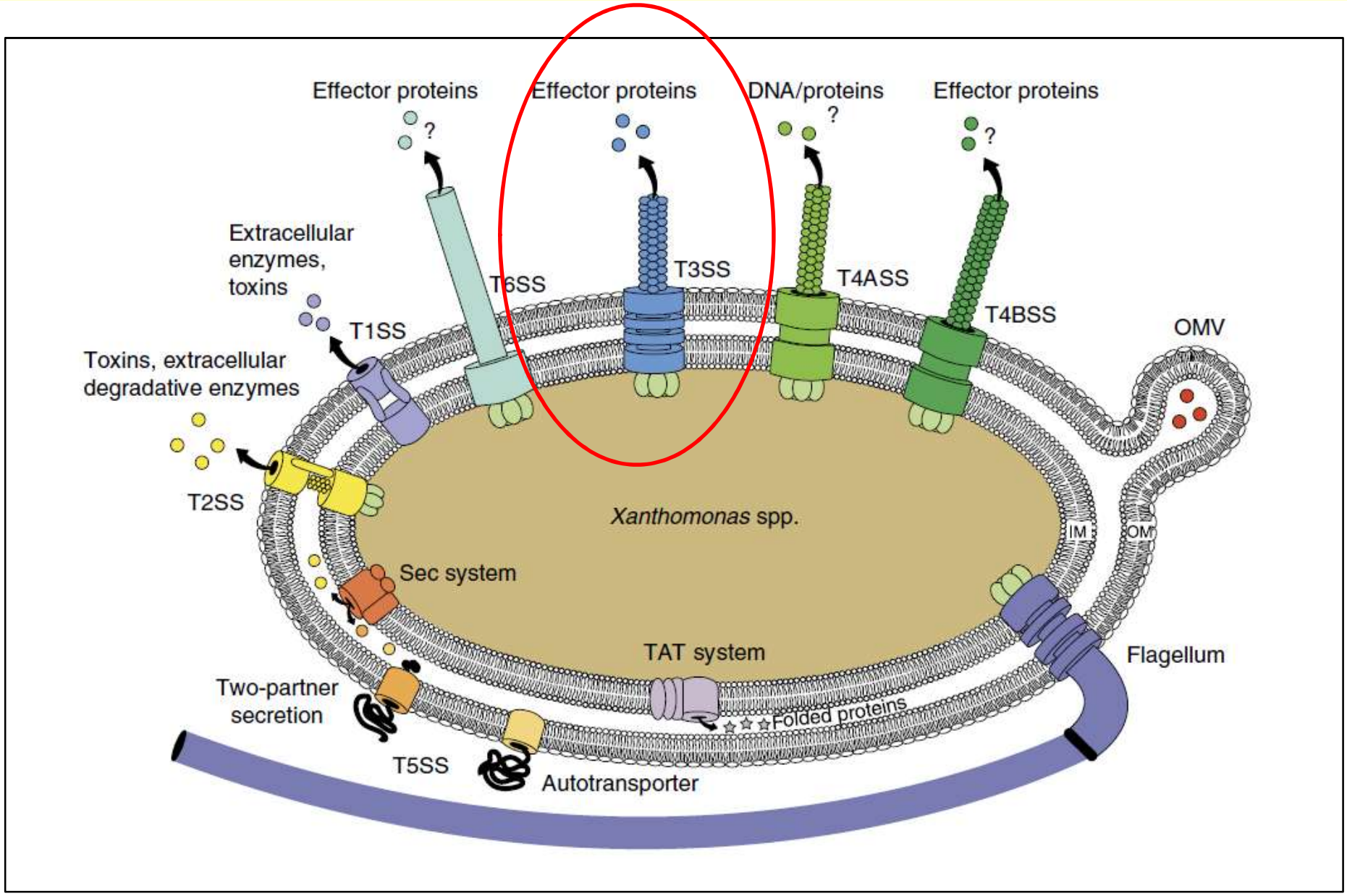
Effettori TAL (**T**ranscription **a**ctivator-**l**ike)



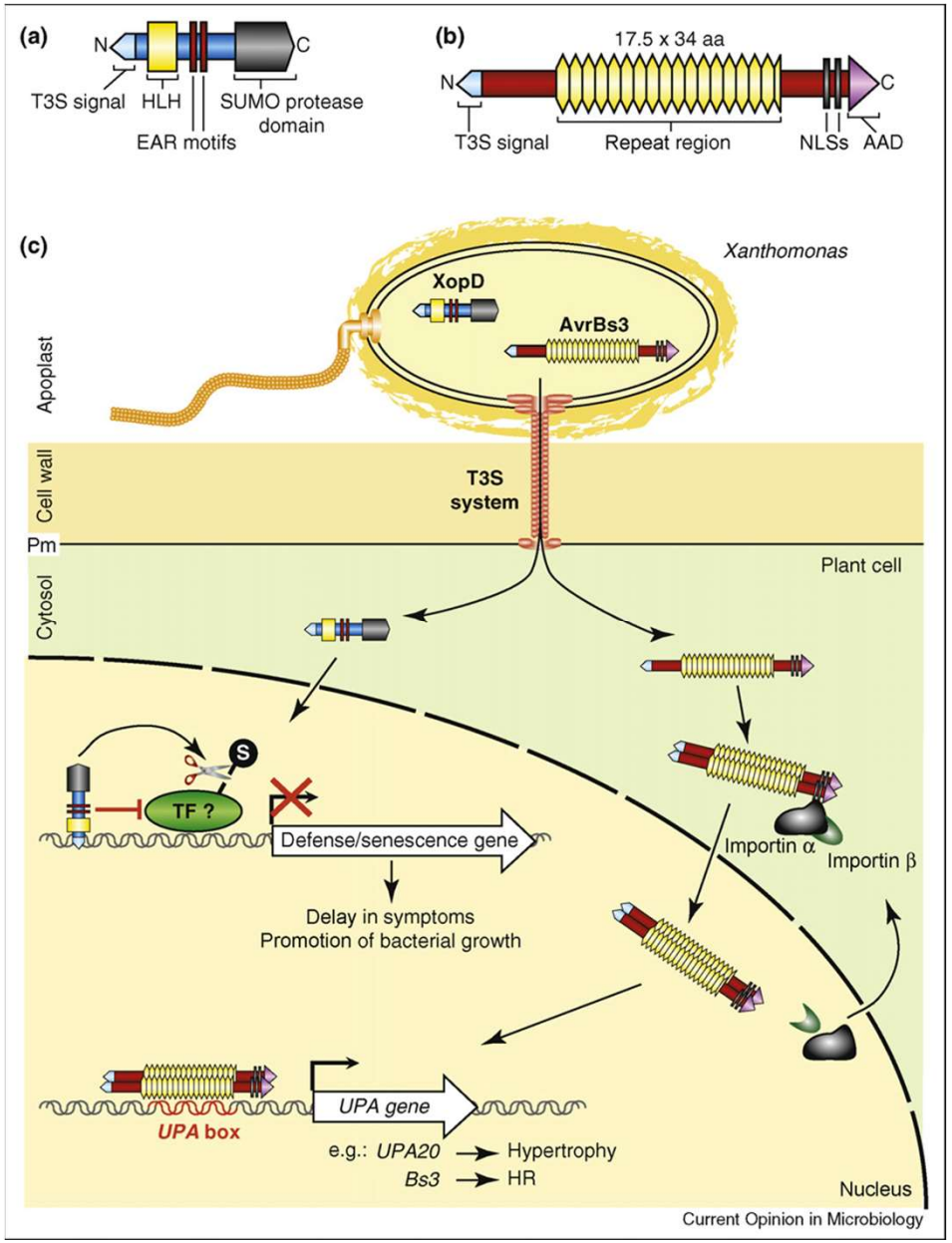
Boch J, Bonas U. 2010.
Annu. Rev. Phytopathol. 48:419–36

- Sono traslocati nelle cellule dell'ospite durante l'infezione
- Entrano nel nucleo e legano il promotore di geni vegetali, inducendone l'espressione
- L'espressione di questi geni ha effetti benefici sull'infezione

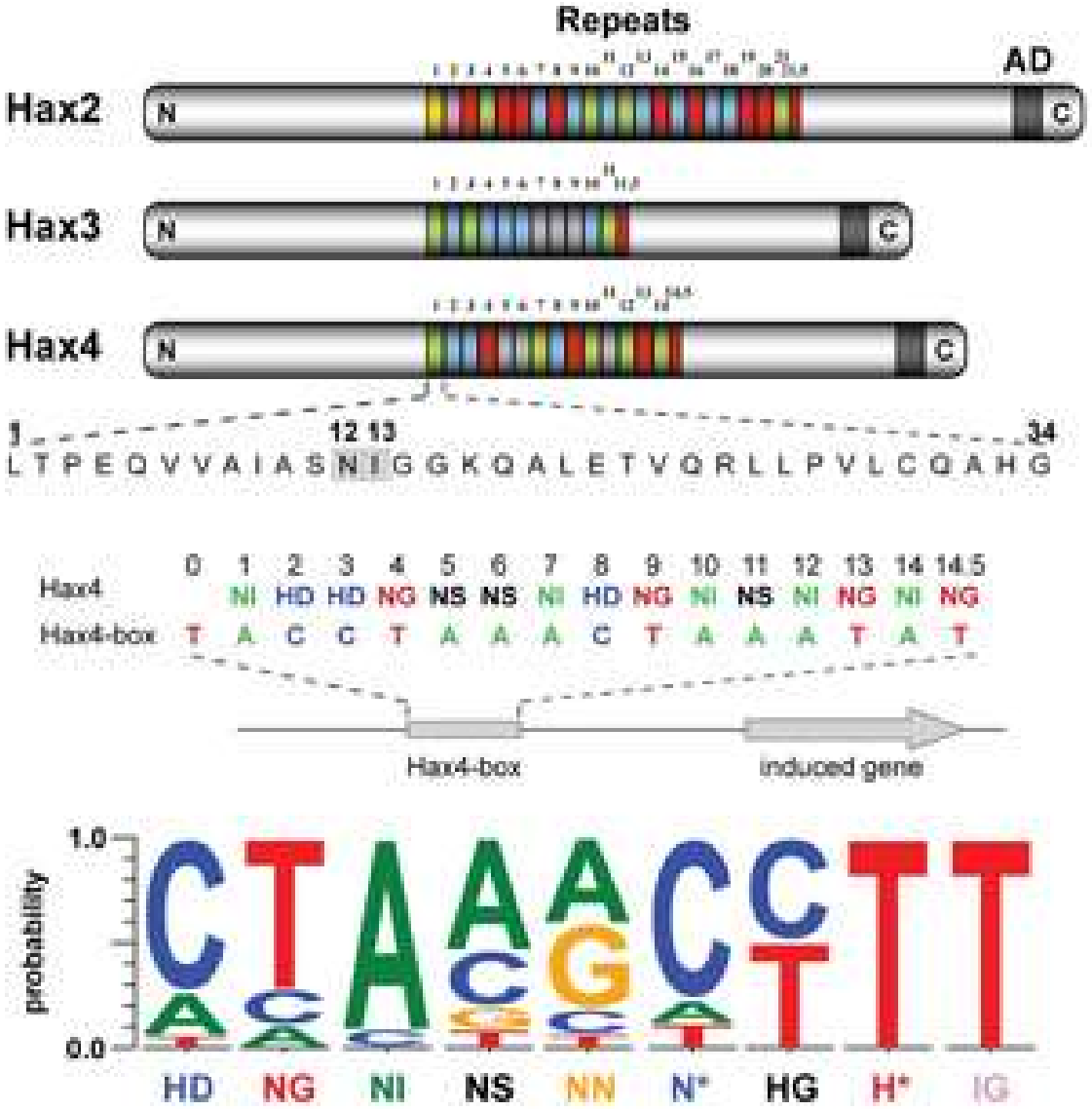
Sistemi di secrezione di Xanthomonas



Gli effettori XopD e **AvrBs3** di tipo III di Xanthomonas



Il riconoscimento da parte dell'effettore TAL delle sequenze sui promotori da attivare è mediato da un dominio ripetitivo centrale

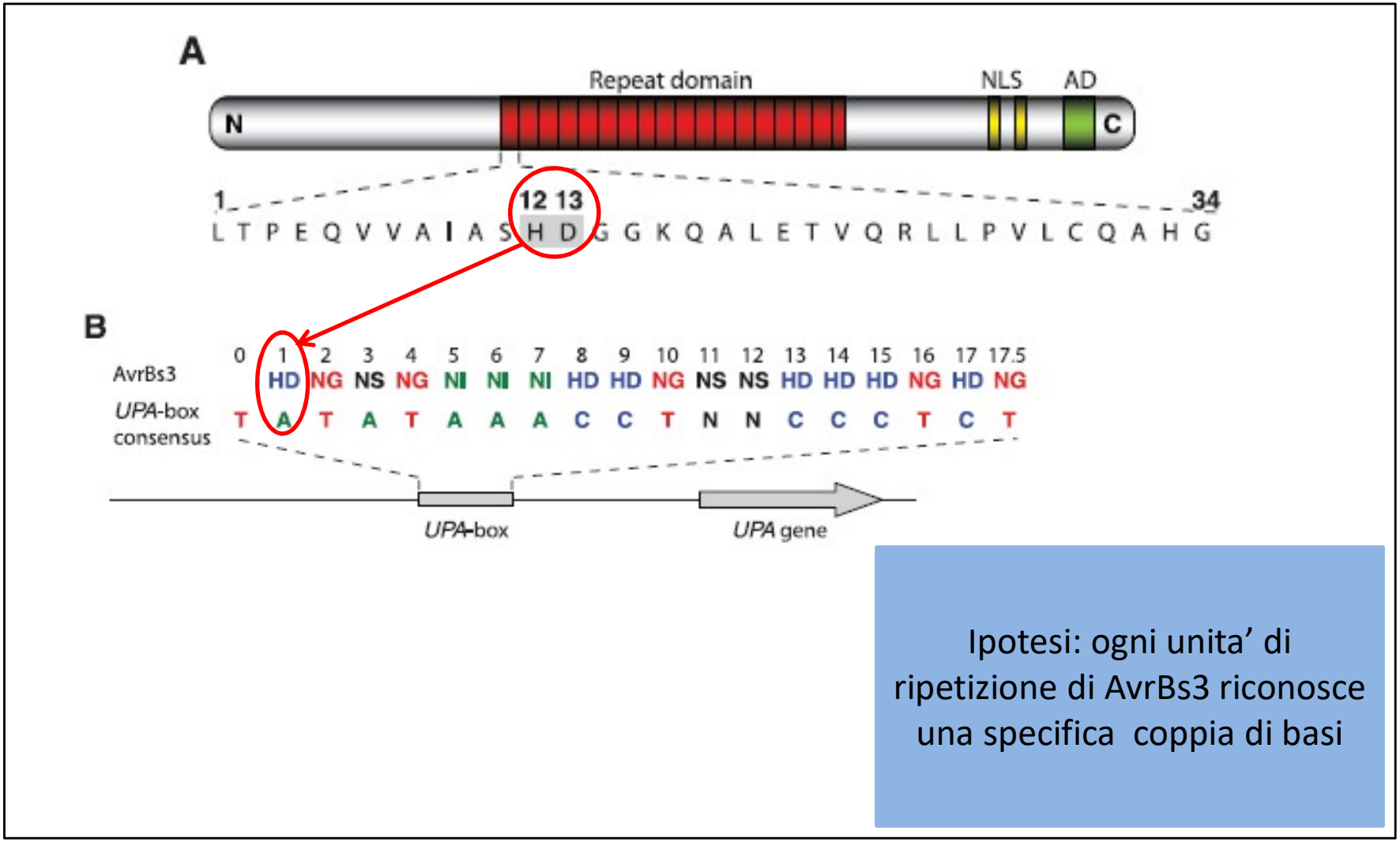


Science
AAAS

Breaking the Code of DNA Binding Specificity of TAL-Type III Effectors

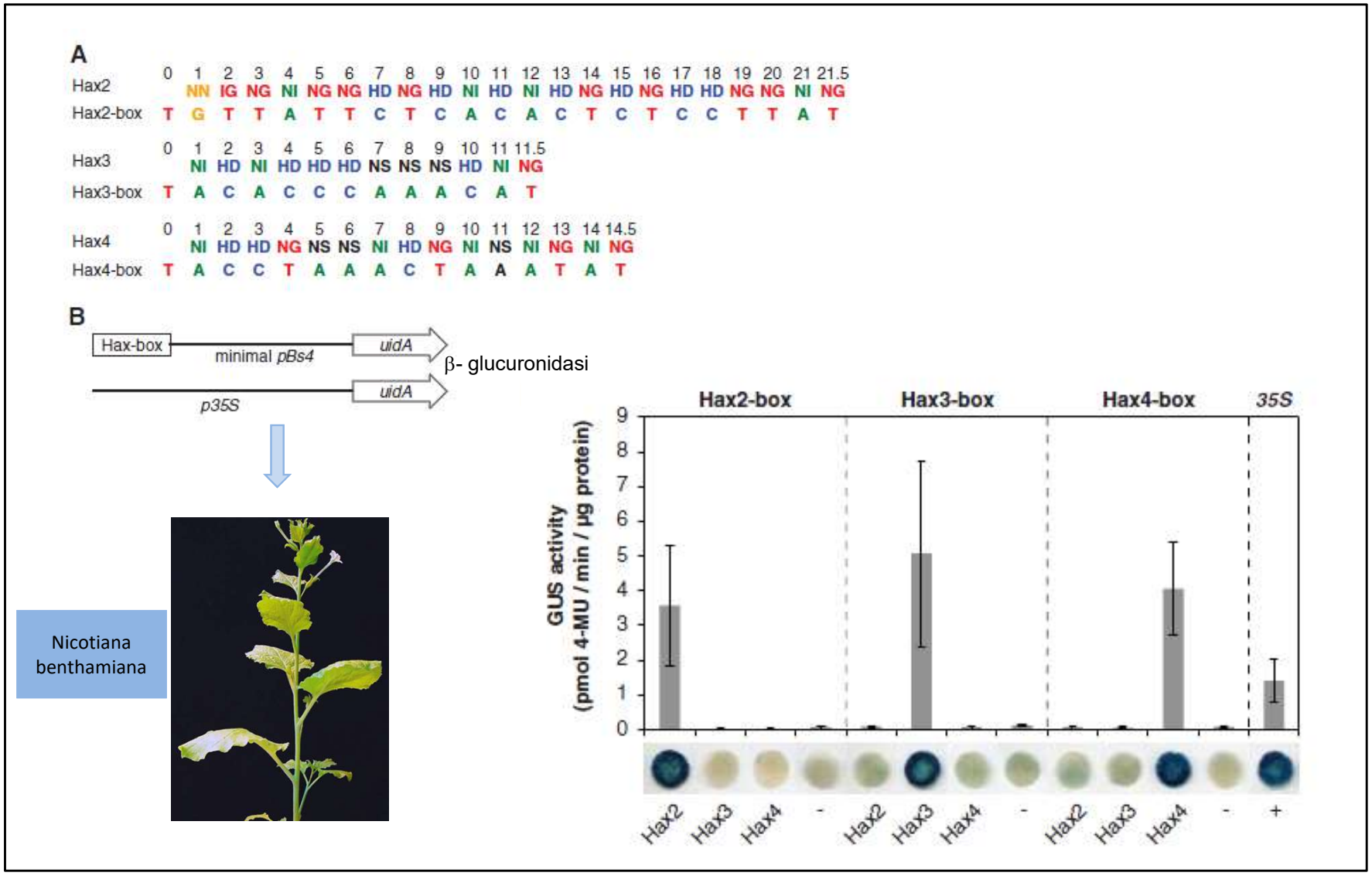
Jens Boch,* Heidi Scholze, Sebastian Schornack,† Angelika Landgraf, Simone Hahn, Sabine Kay, Thomas Lahaye, Anja Nickstadt,‡ Ulla Bonas

2009

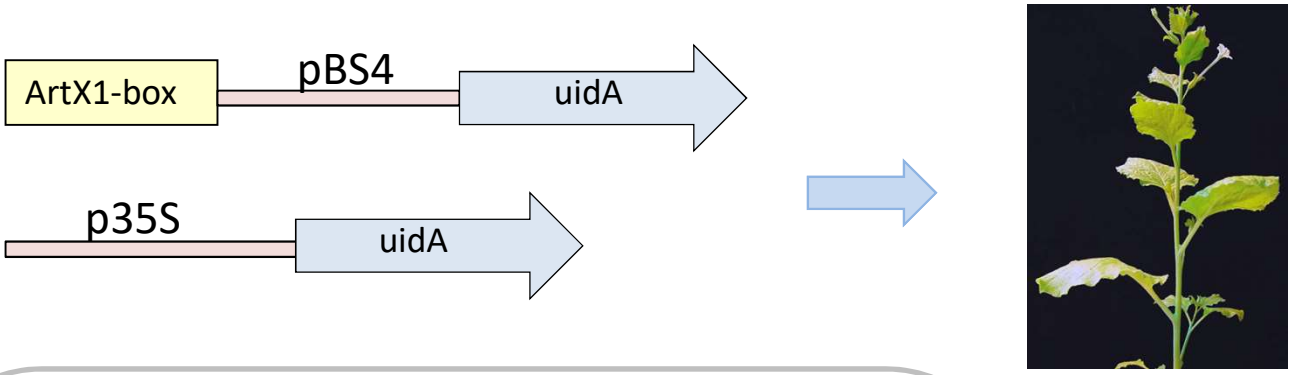


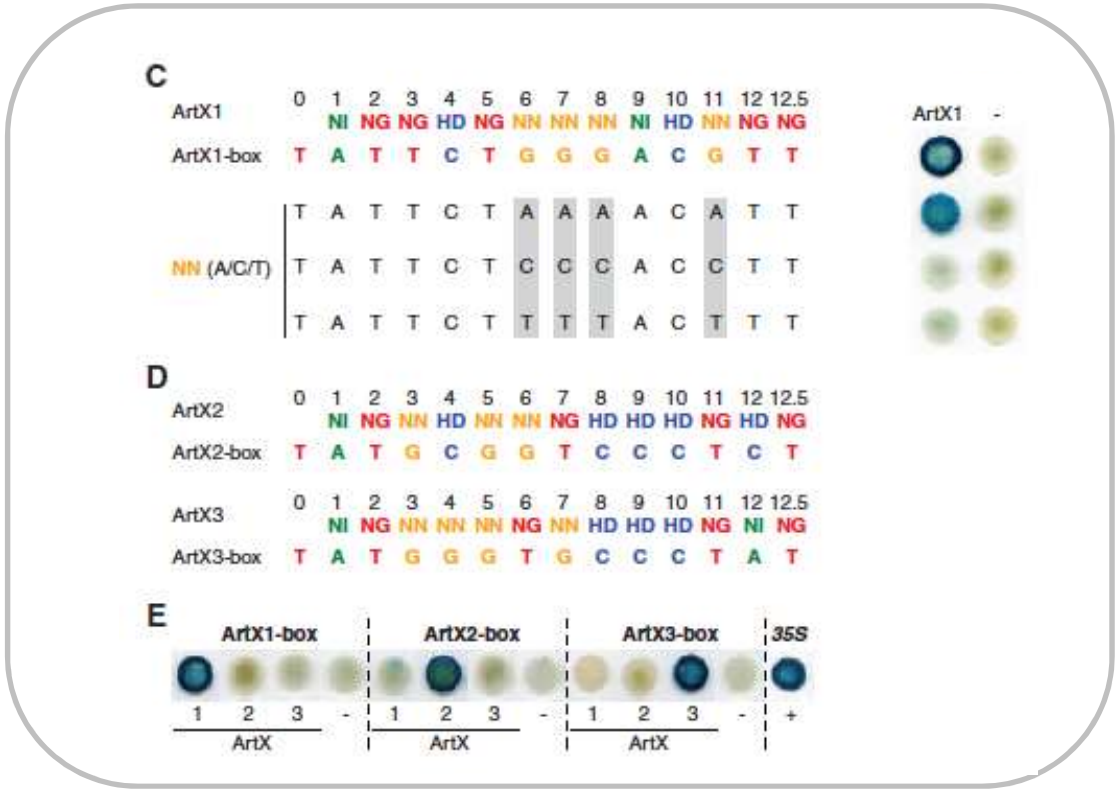
Ipotesi: ogni unita' di ripetizione di AvrBs3 riconosce una specifica coppia di basi

Conferma sperimentale dell'ipotesi

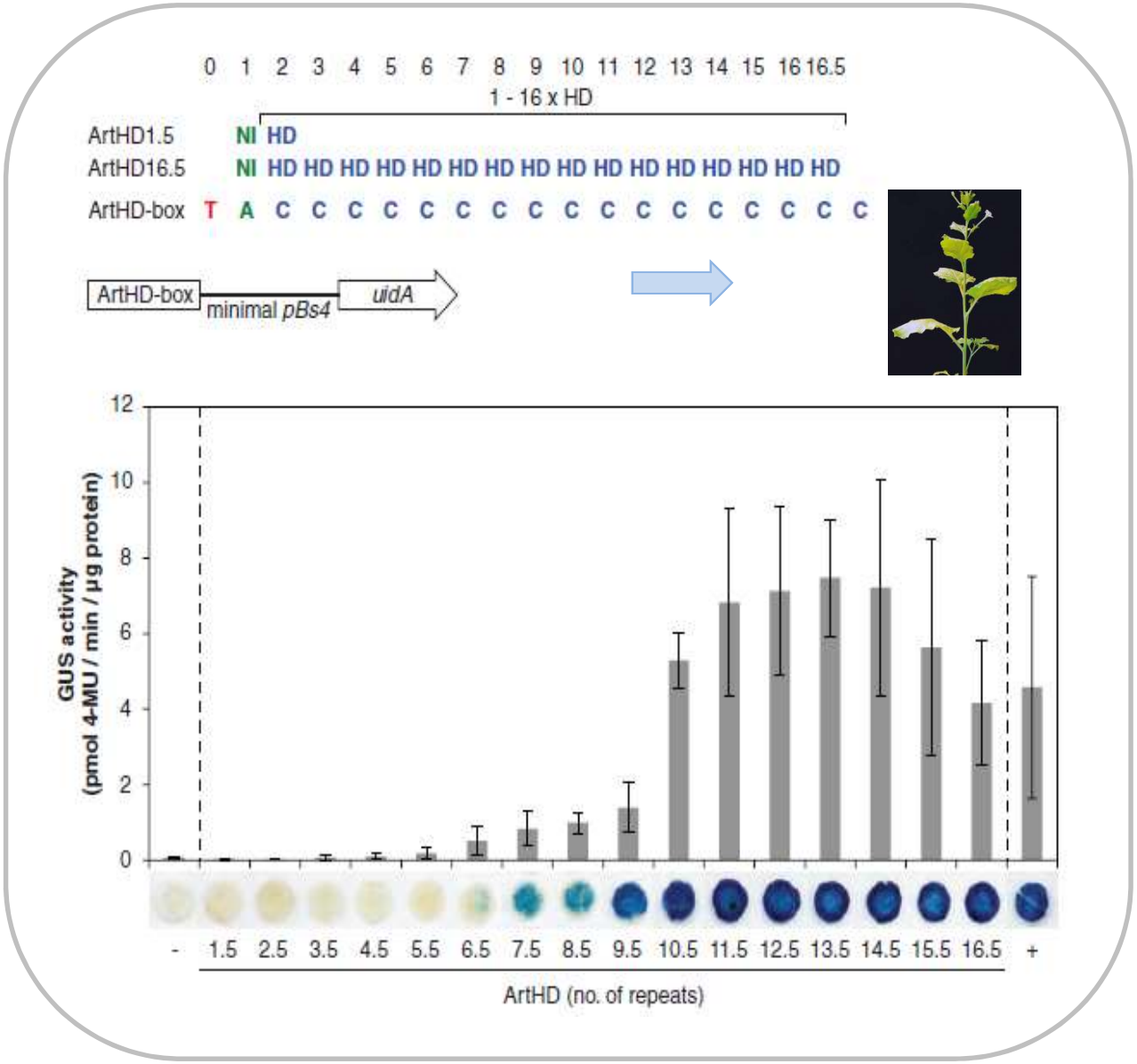


ArtX: Artificial TALEs





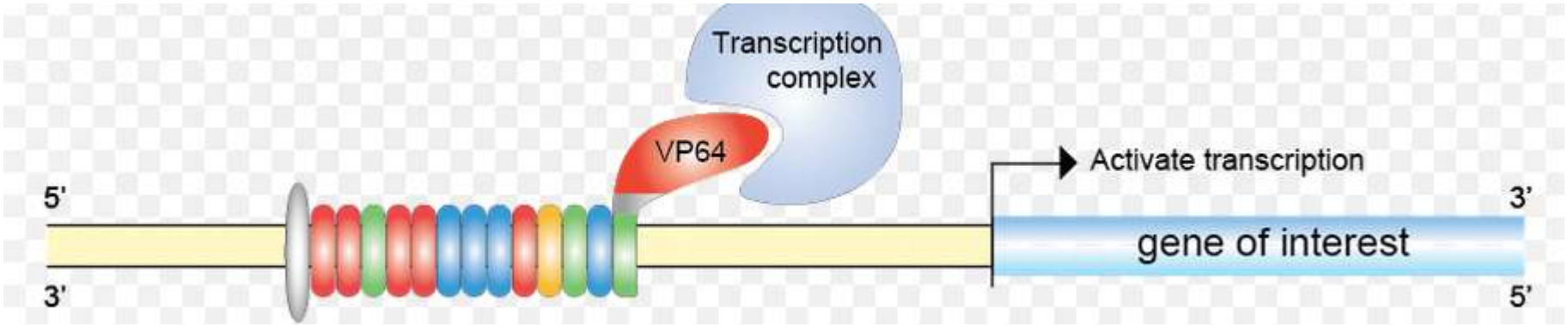
Come il numero di ripetizioni influenza l'espressione dei geni target.



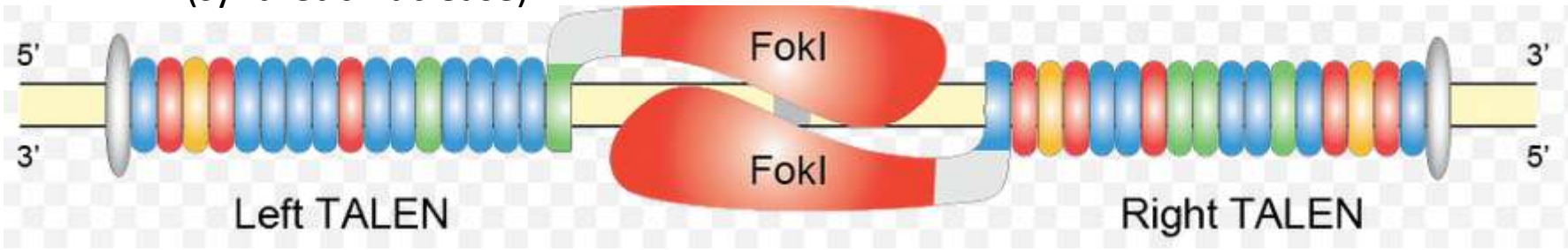
Sommario

- La specificita' di legame al DNA e' basata su un motivo di 2 aa che riconoscono una coppia di basi
- Una T in posizione 0 contribuisce all'attivazione di promotori indotti da TAL effectors
- Alcuni tipi di ripetizioni favoriscono il riconoscimento di coppie di basi specifiche, mentre altri sono piu' flessibili
- Si possono prevedere con successo geni bersaglio per i TAL effectors alcuni dei quali sono importanti fattori virulenti
- Un numero minimo di ripetizioni e' necessaria per riconoscere il DNA bersaglio ed attivare efficentemente l'espressione genica.

TALE



TALEN (synthetic nuclease)



Altre applicazioni…

✓ per guidare modificazioni genetiche nelle cellule umane pluripotenti (embryonic stem cells –ESC- and induced pluripotent stem cells -iPSC-)

Nat Biotechnol. 2011 Jul 7;29(8):731-4. doi: 10.1038/nbt.1927.

Genetic engineering of human pluripotent cells using TALE nucleases.

Hockemeyer D, Wang H, Kiani S, Lai CS, Gao Q, Cassady JP, Cost GJ, Zhang L, Santiago Y, Miller JC, Zeitler B, Cherone JM, Meng X, Hinkley SJ, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R.

The Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA.

✓ per ottenere modelli knock-out (rat, worm, zebrafish)

Nat Biotechnol. 2011 Aug 5;29(8):695-6. doi: 10.1038/nbt.1940.

Knockout rats generated by embryo microinjection of TALENs.

Tesson L, Usal C, Ménoret S, Leung E, Niles BJ, Remy S, Santiago Y, Vincent AI, Meng X, Zhang L, Gregory PD, Anegon I, Cost GJ.

✓ per attivare trascrizionalmente geni silenziati inibendo l'azione di repressori epigenetici

Nucleic Acids Research Advance Access published March 13, 2012

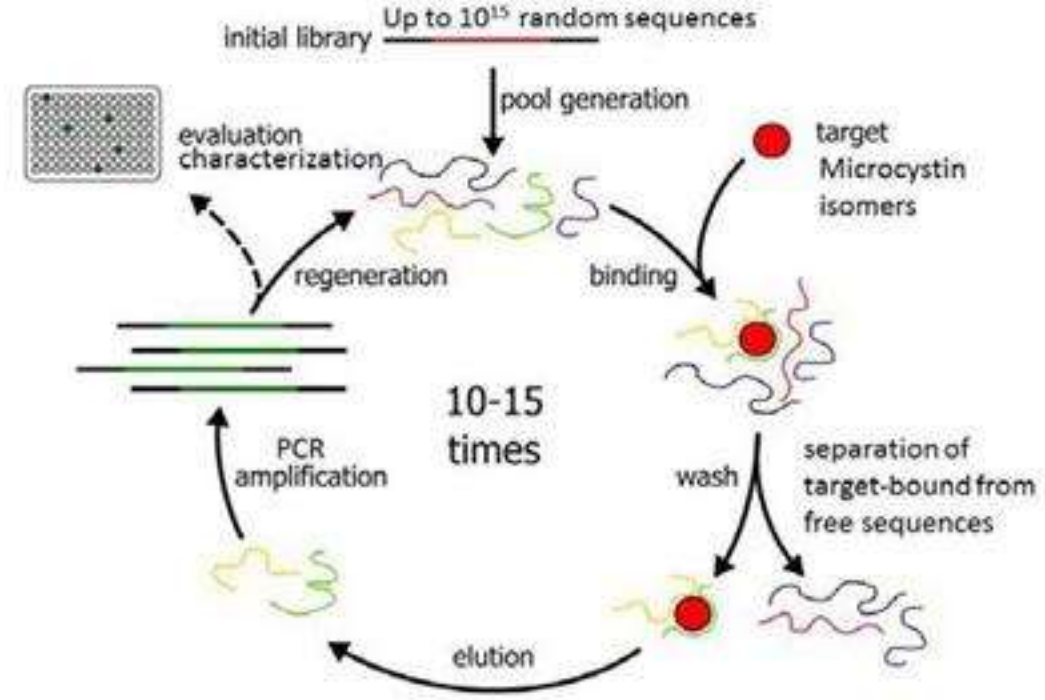
Nucleic Acids Research, 2012, 1–10
doi:10.1093/nar/gks399

Targeted transcriptional activation of silent *oct4* pluripotency gene by combining designer TALEs and inhibition of epigenetic modifiers

Sebastian Bultmann[1], Robert Morbitzer[2], Christine S. Schmidt[1], Katharina Thanisch[1], Fabio Spada[1], Janett Elsaesser[2], Thomas Lahaye[2,*] and Heinrich Leonhardt[1,*]

Come ingegnerizzare un TALE naturale

- Scelta di un TALE naturale in grado di esibire un alto tasso di specificità di legame con sequenze target
- Amplificazione tramite PCR delle sequenze codificanti per diversi TALE dal DNA genomico di ***X. axonopodis* pv *citri***. I cloni ottenuti mancano di circa 152 residui nel N-ter importanti per il trasporto nelle piante ma non per altre funzioni.
- Le proteine ottenute vengono analizzate per **SELEX** (*Systematic Evolution of Ligands by Exponential Enrichment*).



Tecniche high throughput per la produzione di TALEs

- Le TALEN sono uno strumento per l'editing genomico
- Altri tipi di TALE di fusione (es. VP64) possono trovare usi ancora più ampi
- Le TALEN si sono dimostrate altamente efficienti anche nella produzione di animali KO
- A differenza delle ZNF, il codice è noto ed i costrutti sono disponibili in AddGene

È desiderabile un protocollo universale che permetta la produzione di costrutti codificanti TALE che riconoscano elementi di sequenza e lunghezza qualsiasi.

Una tecnica del genere deve rispondere a criteri di economicità, efficienza e velocità.

CRISPR/Cas9

Journal of Experimental Botany, Vol. 66, No. 1 pp. 47–57, 2015
doi:10.1093/jxb/eru429 Advance Access publication 4 November, 2014

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

The CRISPR–Cas system for plant genome editing: advances and opportunities

Vinay Kumar and Mukesh Jain*

Functional and Applied Genomics Laboratory, National Institute of Plant Genome Research (NIPGR), New Delhi-110067, India

* To whom correspondence should be addressed. E-mail: mjain@nipgr.ac.in

Received 14 July 2014; Revised 22 September 2014; Accepted 23 September 2014

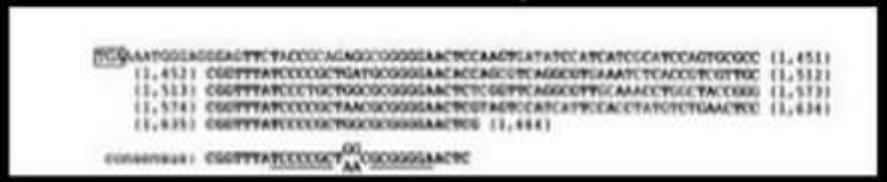
Abstract

Genome editing is an approach in which a specific target DNA sequence of the genome is altered by adding, removing, or replacing DNA bases. Artificially engineered hybrid enzymes, zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), and the CRISPR (clustered regularly interspaced short palindromic repeats)–Cas (CRISPR-associated protein) system are being used for genome editing in various organisms including plants. The CRISPR–Cas system has been developed most recently and seems to be more efficient and less time-consuming compared with ZFNs or TALENs. This system employs an RNA-guided nuclease, Cas9, to induce double-strand breaks. The Cas9-mediated breaks are repaired by cellular DNA repair mechanisms and mediate gene/genome modifications. Here, we provide a detailed overview of the CRISPR–Cas system and its adoption in different organisms, especially plants, for various applications. Important considerations and future opportunities for deployment of the CRISPR–Cas system in plants for numerous applications are also discussed. Recent investigations have revealed the implications of the CRISPR–Cas system as a promising tool for targeted genetic modifications in plants. This technology is likely to be more commonly adopted in plant functional genomics studies and crop improvement in the near future.

Key words: CRISPR, Cas9, genome editing, mutation, protospacer adjacent motif, sgRNA.

Humble Beginning as Exotic Repeat Sequences in Bacterial Genome

- Found as 'exotic junk DNA' with unknown function



Ishino et al., J.Bacteriol (1987)

- Widespread presence in Archeae and Bacteria
  Jansen et al, Mol. Microbiol (2002)

- Named as..

Clustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeat

- **C**RISPR **As**sociated protein (Cas)
  Family of genes associated with CRISPR

- Sequence similarity between phage

http://www.slideshare.net/suknamgoong/crisprcas9-101

CRISPR as bacterial immune system against bacteriophagy

CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes

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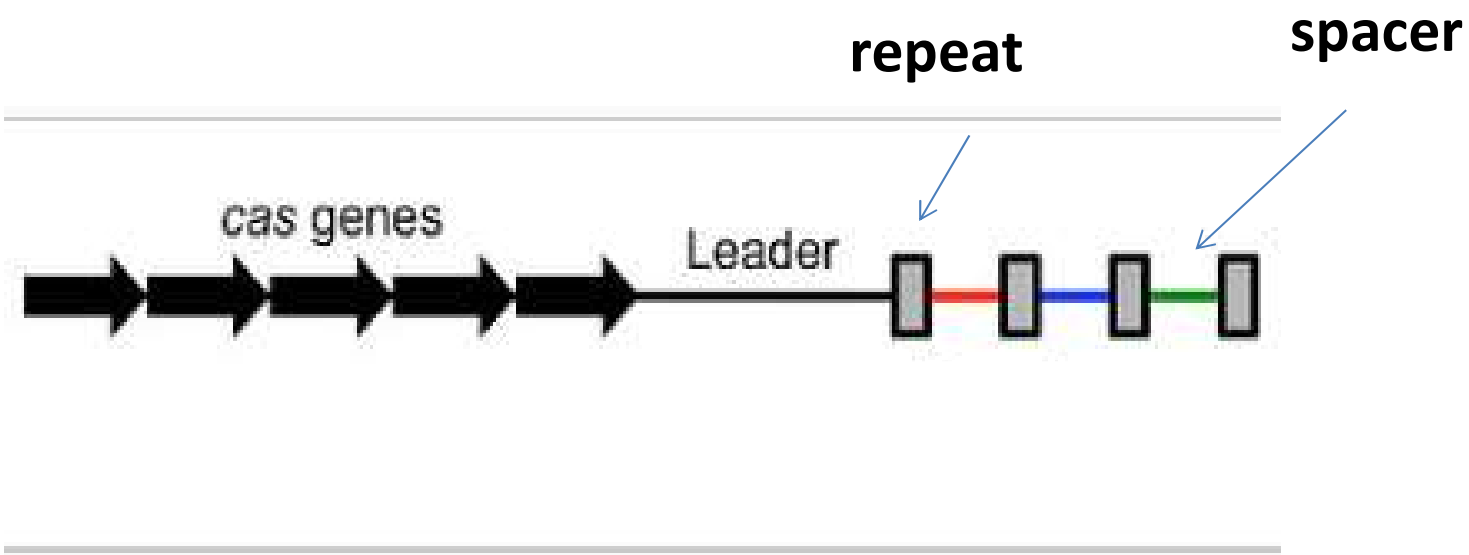
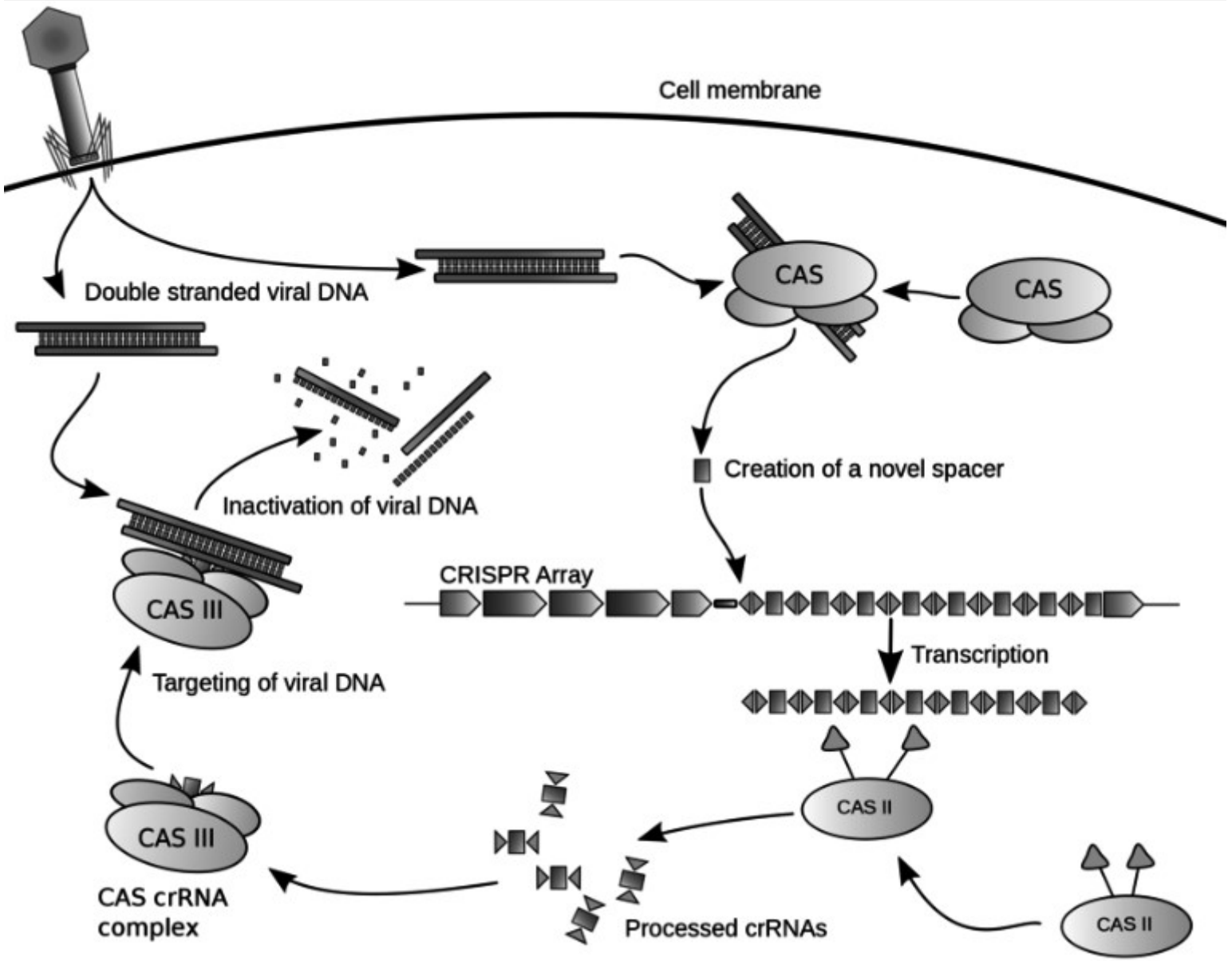
Clustered regularly interspaced short palindromic repeats (CRISPR) are a distinctive feature of the genomes of most Bacteria and Archaea and are thought to be involved in resistance to bacteriophages. We found that, after viral challenge, bacteria integrated new spacers derived from phage genomic sequences. Removal or addition of particular spacers modified the phage-resistance phenotype of the cell. Thus, CRISPR, together with associated *cas* genes, provided resistance against phages, and resistance specificity is determined by spacer-phage sequence similarity.

Science 2007

The research was carried at by researcher in DANISCO.Inc (acquired by DuPont at 2011)

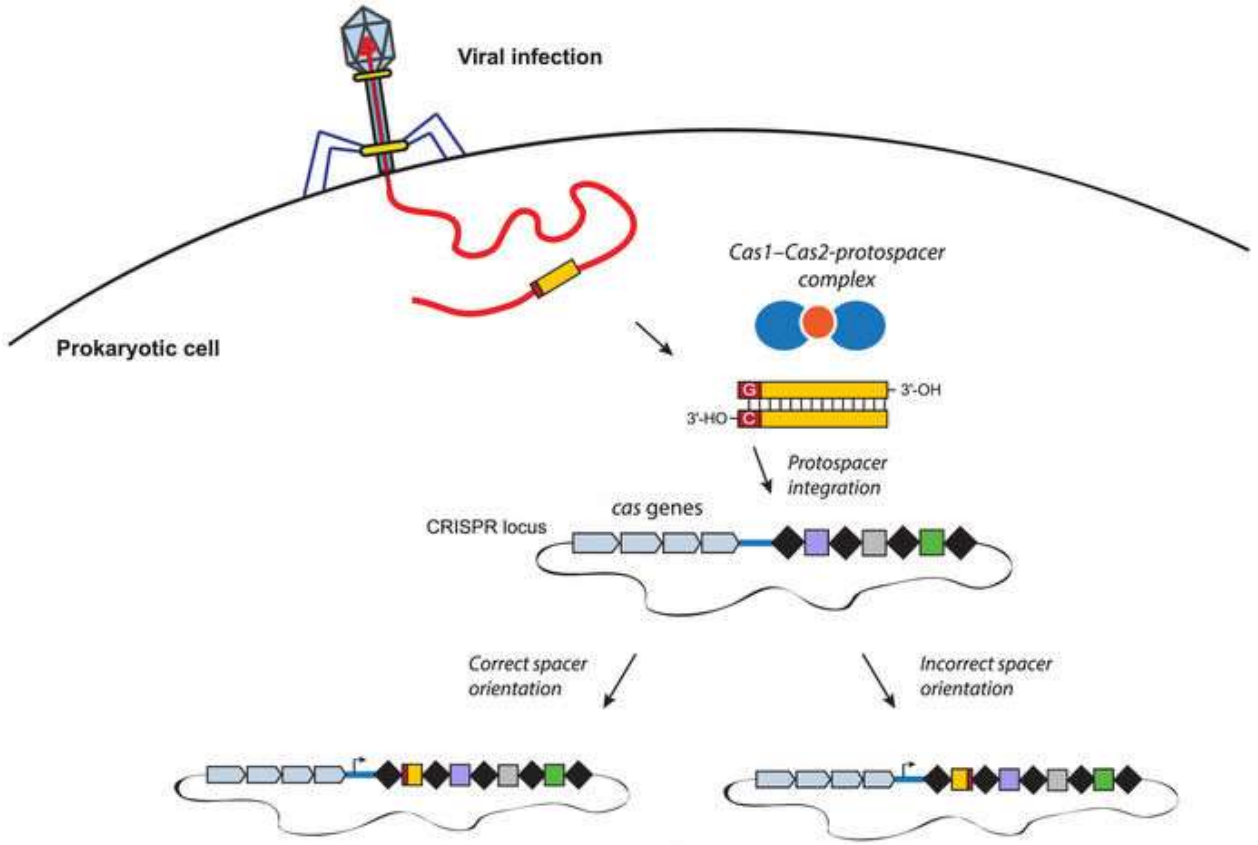

repeat
spacer
cas genes
Leader


Cell membrane
Double stranded viral DNA
CAS
CAS
Creation of a novel spacer
Inactivation of viral DNA
CRISPR Array
CAS III
Transcription
Targeting of viral DNA
CAS II
CAS III
CAS crRNA
complex
Processed crRNAs
CAS II

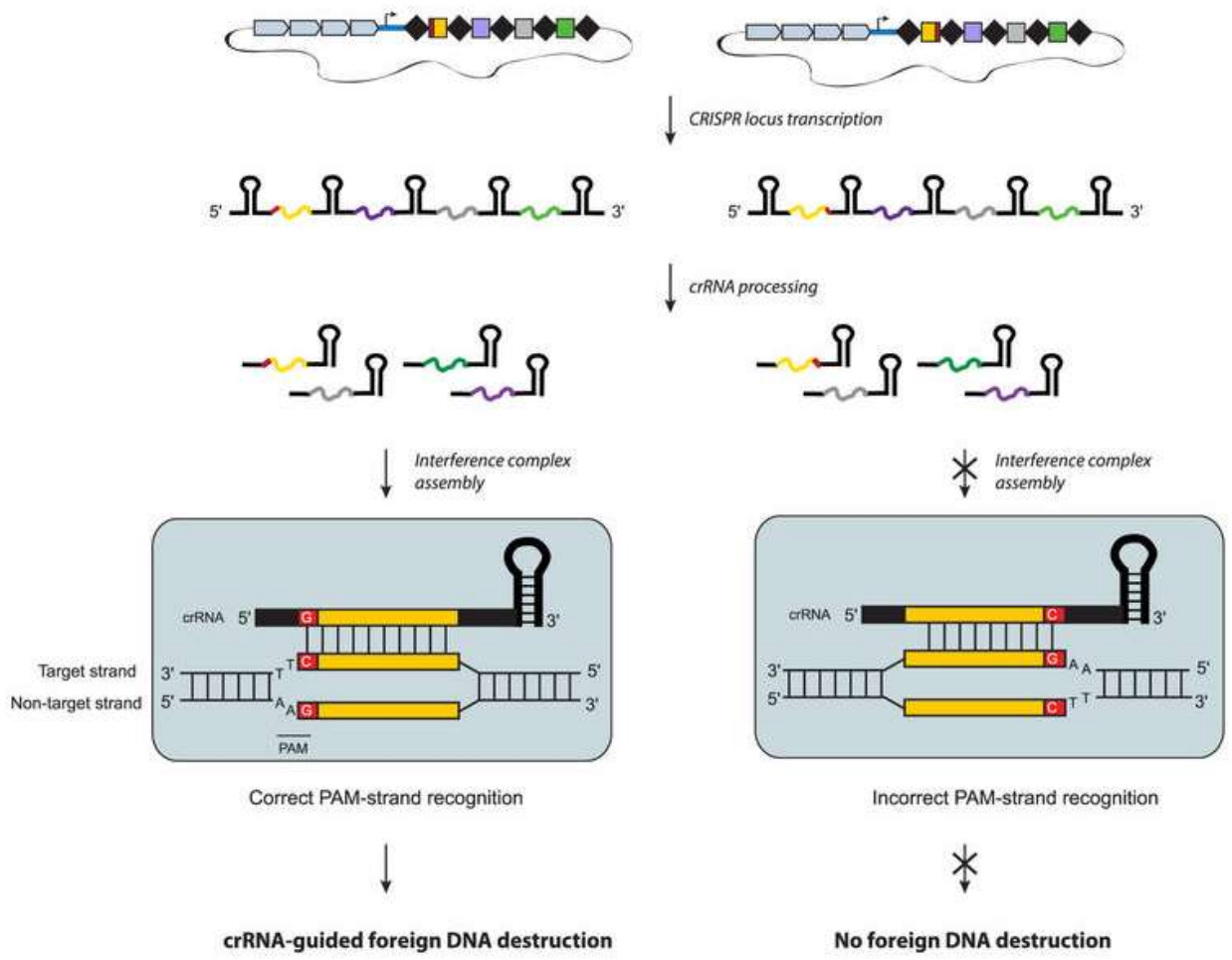
The acquisition process involves recognition and integration of **foreign DNA as a spacer** within the CRISPR locus. Regions of phage genomes that are excised as spacers (**termed protospacers**) are not randomly selected but are found adjacent to short (3 – 5 bp) DNA sequences termed protospacer adjacent motifs (PAM).

The insertion of a single copy of **spacer of approximately 30 bp occurs preferentially at the leader** side of the CRISPR array. **The first direct repeat**, **adjacent to the leader sequence is copied,** with the newly acquired spacer inserted between the first and second direct repeats.

The new spacer is always inserted on the AT-rich side of the leader sequence located before a CRISPR cassette that also contains promoter elements and landing sites for regulatory proteins



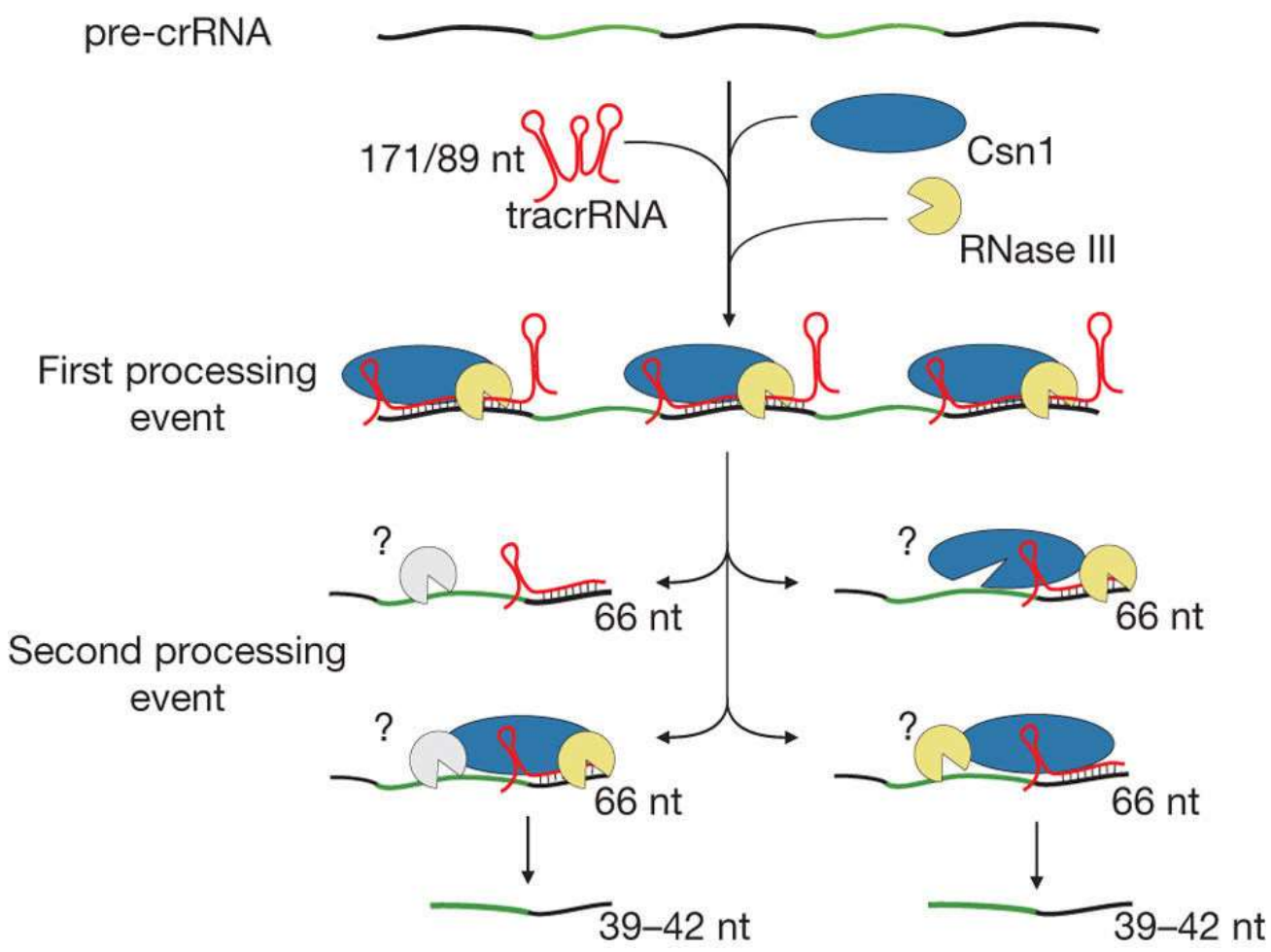
At the second stage, transcription, the entire CRISPR locus is transcribed into a **long pre-crRNA** (poly-spacer precursor crRNA).
Type II systems encode an extra small RNA that is complementary to the **repeat** sequence, known as a trans-activating RNA (tracrRNA).



The classical DNA targeting pathway, common to all type II CRISPR-Cas systems

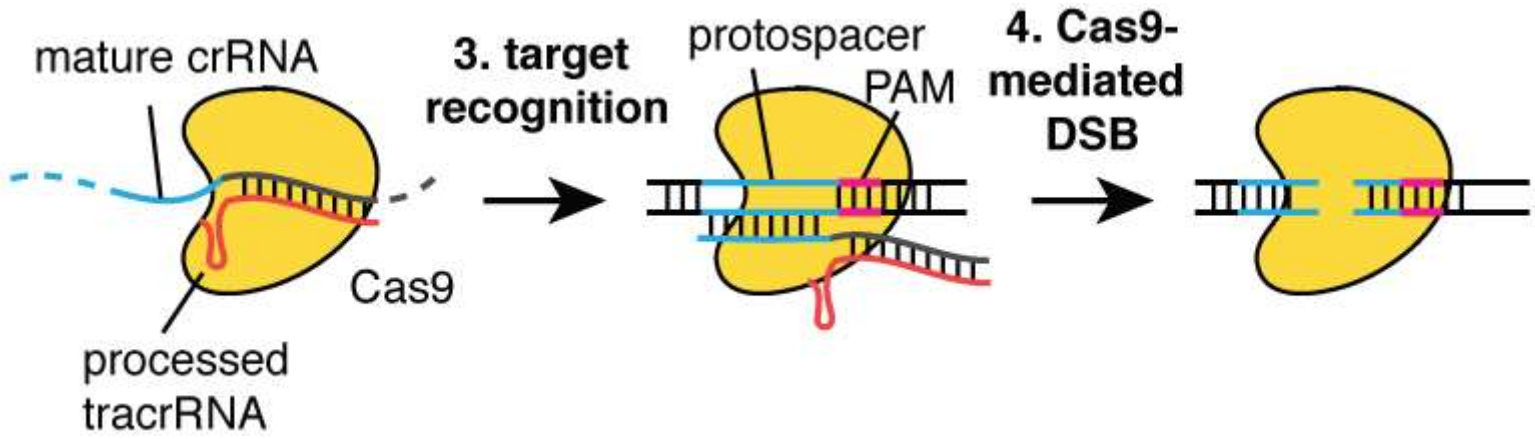
tracrRNA and primary CRISPR transcripts result in base pairing and the **formation of cas9-stabilized tracrRNA:pre-crRNA duplexes upon binding of tracrRNA anti-repeat to the pre-crRNA repeat.**

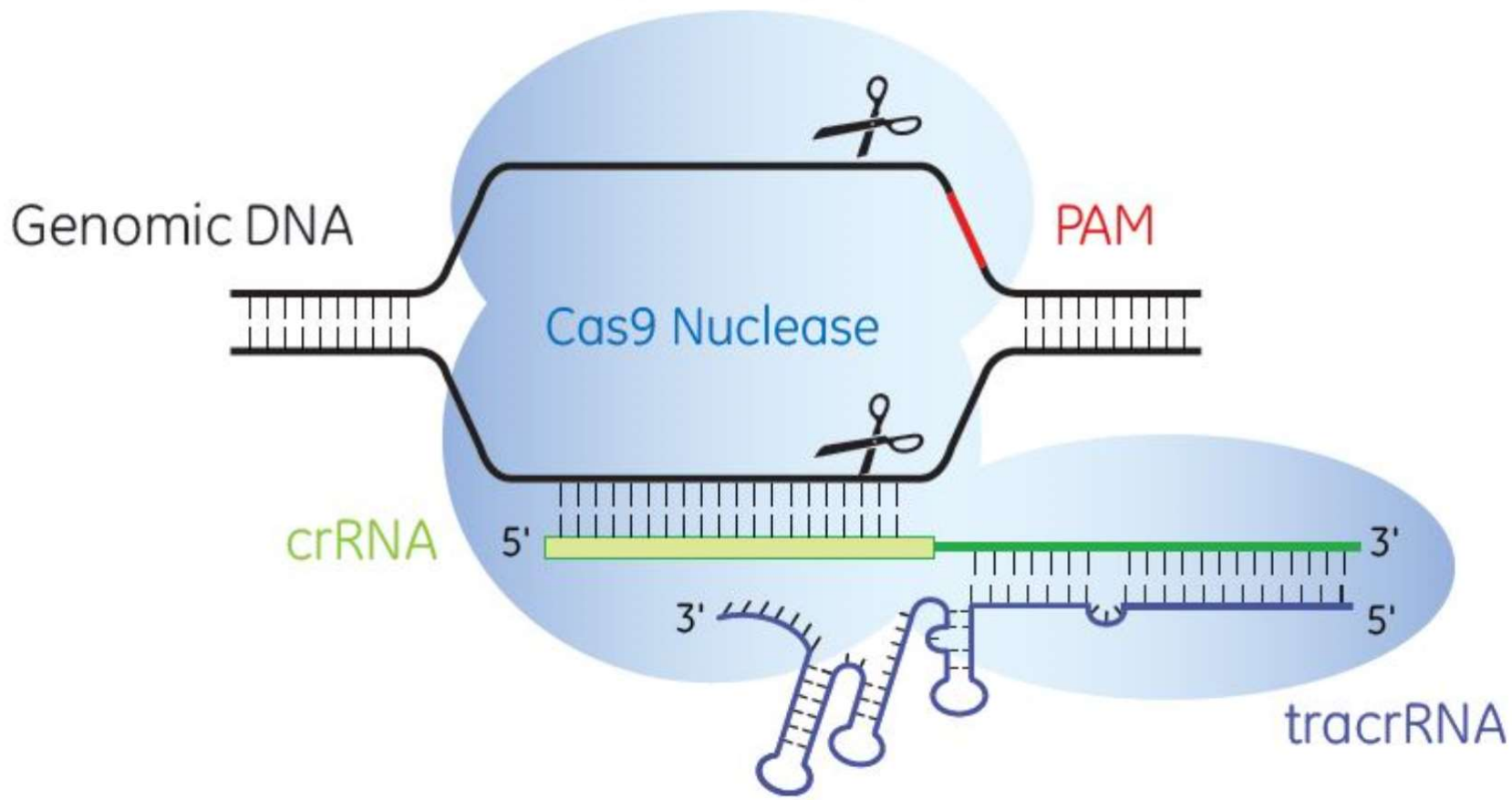
The duplexes are processed into **mature crRNA by RNase III followed by trimming (10 nt) by a yet unknown mechanism**



The classical DNA targeting pathway, common to all type II CRISPR-Cas systems

The mature tracrRNA:crRNA guides the Cas9 endonuclease to introduce site-specifically dsDNA breaks in the invading DNA.



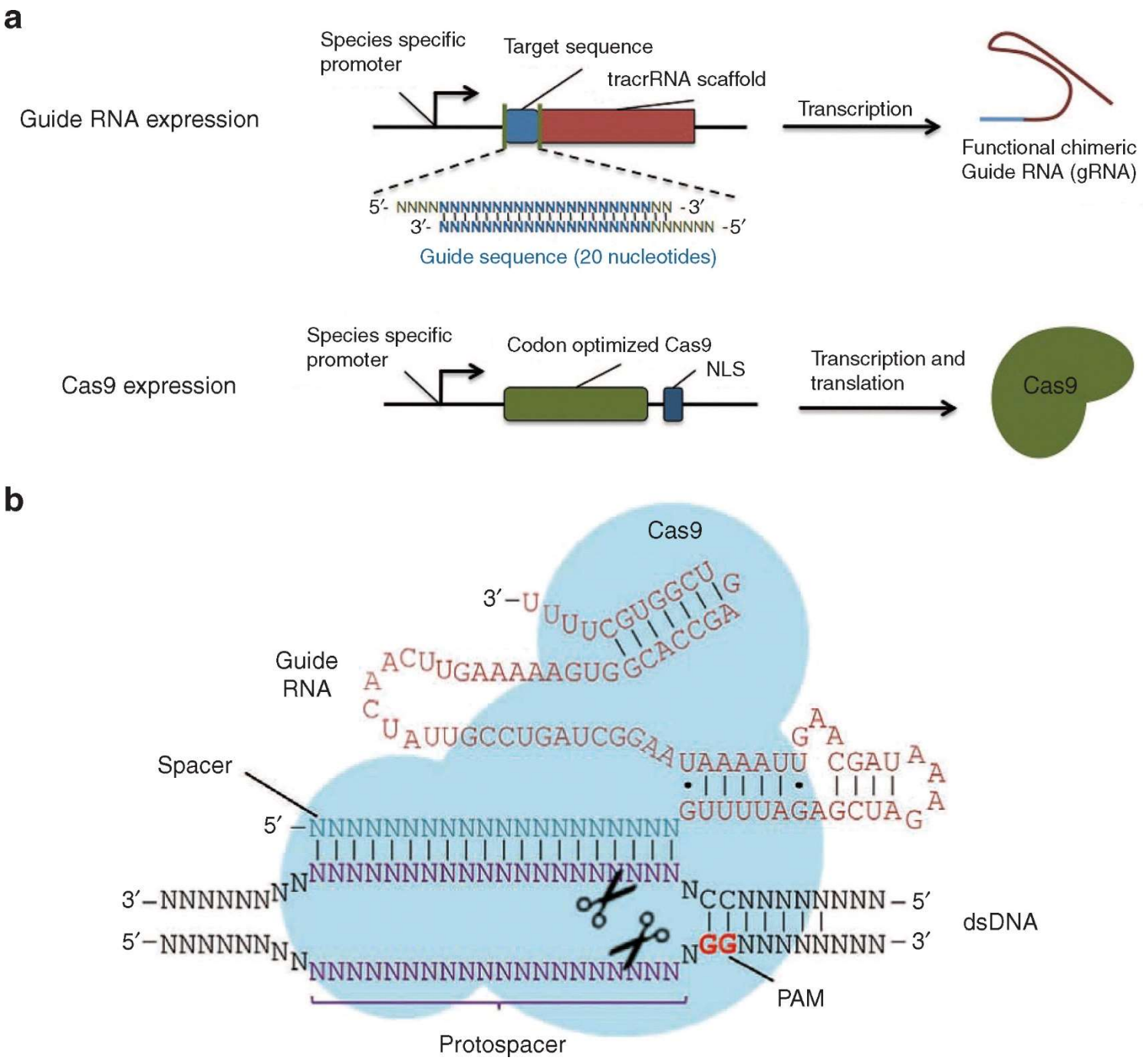


Cas9 is the nuclease guided by the crRNA and tracrRNA (or trans-activating crRNA) to cleave specific DNA sequences (**Deltcheva et al. 2011**).

For biotechnological purposes, a guide RNA (gRNA or single-gRNA) can be designed to include a hairpin that mimics the tracrRNA-crRNA complex (**Jinek et al. 2012**).

Binding specificity is based on the gRNA and a three nucleotide NGG sequence called the **protospacer adjacent motif (PAM)** sequence (**Marraffini and Sontheimer, 2010**).

For site-specific genome editing, the CRISPR/Cas9 system minimally requires the **Cas9 nuclease** and the **gRNA**.


a
Guide RNA expression
Species specific promoter
Target sequence
tracrRNA scaffold
Transcription
Functional chimeric Guide RNA (gRNA)
5′- NNNNNNNNNNNNNNNNNNNNNNNN -3′
3′- NNNNNNNNNNNNNNNNNNNNNNNN -5′
Guide sequence (20 nucleotides)
Cas9 expression
Species specific promoter
Codon optimized Cas9
NLS
Transcription and translation
Cas9
b
Cas9
Guide RNA
Spacer
5′ –NNNNNNNNNNNNNNNNNNNN
3′–NNNNNNNNNNNNNNNNNNNN
5′–NNNNNNNNNNNNNNNNNNNN
NCCNNNNNNNN – 5′
NGGNNNNNNNN – 3′
dsDNA
PAM
Protospacer

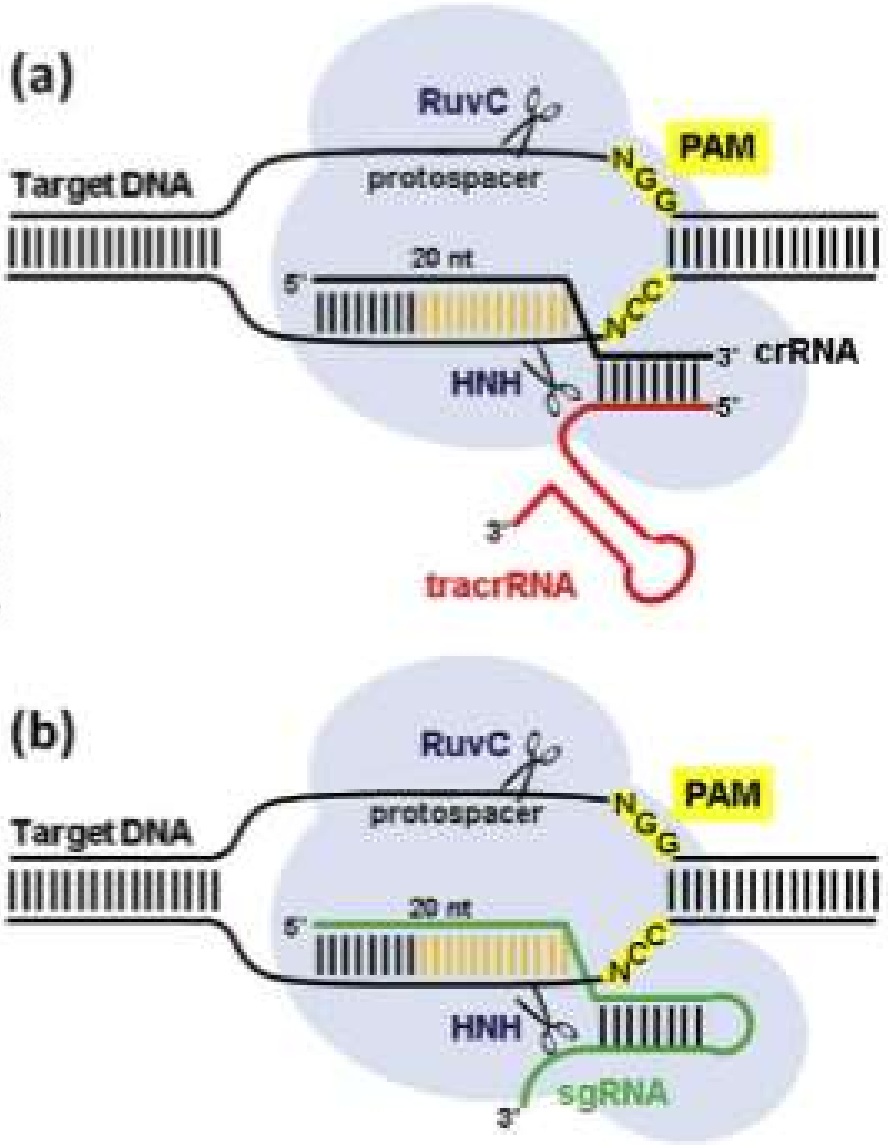


Fig. 2. RNA-guided DNA cleavage by Cas9. (a) In the native system, the Cas9 protein (light blue) is guided by a structure formed by a CRISPR RNA (crRNA, in black), which contains a 20-nt segment determining target specificity, and a trans-activating CRISPR RNA (tracrRNA, in red), which stabilizes the structure and activates Cas9 to cleave the target DNA (protospacer). The presence of a protospacer-adjacent motif (PAM, in yellow), i.e., an NGG (or less frequently NAG) sequence directly downstream from the target DNA, is a prerequisite for DNA cleavage by Cas9. Among the 20 RNA nucleotides determining target specificity, the so-called seed sequence of approximately 12 nt (in orange) upstream of the PAM is thought to be particularly important for the pairing between RNA and target DNA. (b) Cas9 can be reprogrammed to cleave DNA by a single guide RNA molecule (gRNA, in green), a chimera generated by fusing the 3′ end of the crRNA to the 5′ end of the tracrRNA.

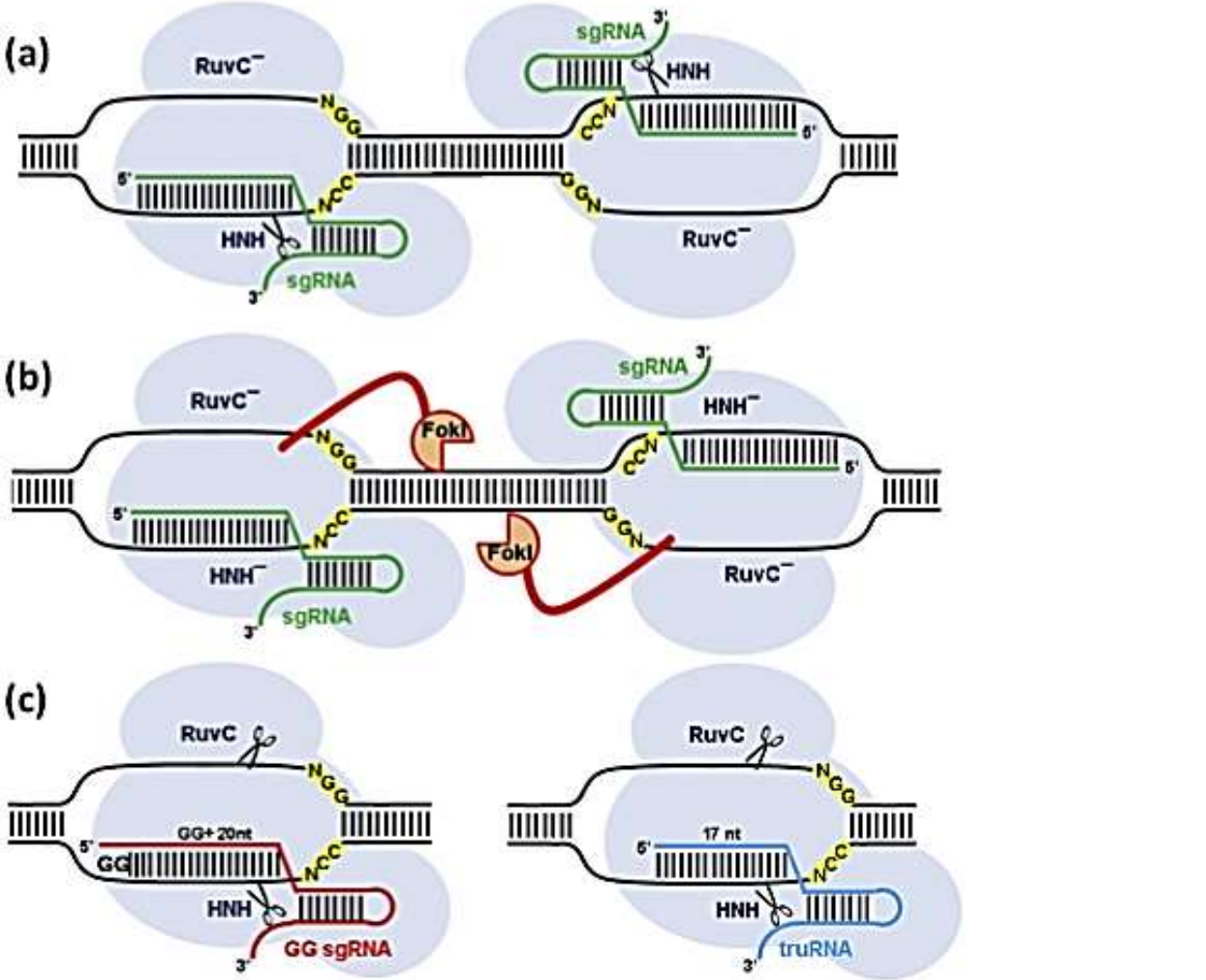


Fig. 3. Strategies to increase CRISPR/Cas9 target specificity. The most important strategy to avoid off-target effects is the design of a specific gRNA by checking for the presence of homologous sequences in the genome. Further strategies can then be employed to reduce the risk of off-target cleavage further. (a) A pair of offset Cas9 nickases. The D10A mutation inactivates the RuvC endonuclease domain so that Cas9 can cleave only the DNA strand complementary to the gRNA. The simultaneous use of two Cas9 nickases binding to sequences on opposite DNA strands generates a staggered DSB with overhangs. (b) Cas9-FokI fusion proteins. A catalytically inactive Cas9 variant, carrying a mutation in both endonuclease domains (RuvC⁻ HNH⁻), can be fused to the FokI nuclease domain. DNA cleavage by FokI is dependent on dimerization and the enzyme must bind two precisely disposed half-sites on the genome, greatly reducing the number of possible off-target sequences. (c) Altering the length of the gRNA. Both extending the gRNA by adding two guanidine residues at the 5′ end (left), or shortening it to a truncated gRNA (truRNA) of only 17 nt (right) can reduce off-target effects.

The pipeline of generating a CRISPR/Cas9-mutagenised plant line.

1. Select genomic target

a. 20 bp sequence followed by the PAM (NGG)

b. Use online tools to minimize off-targeting

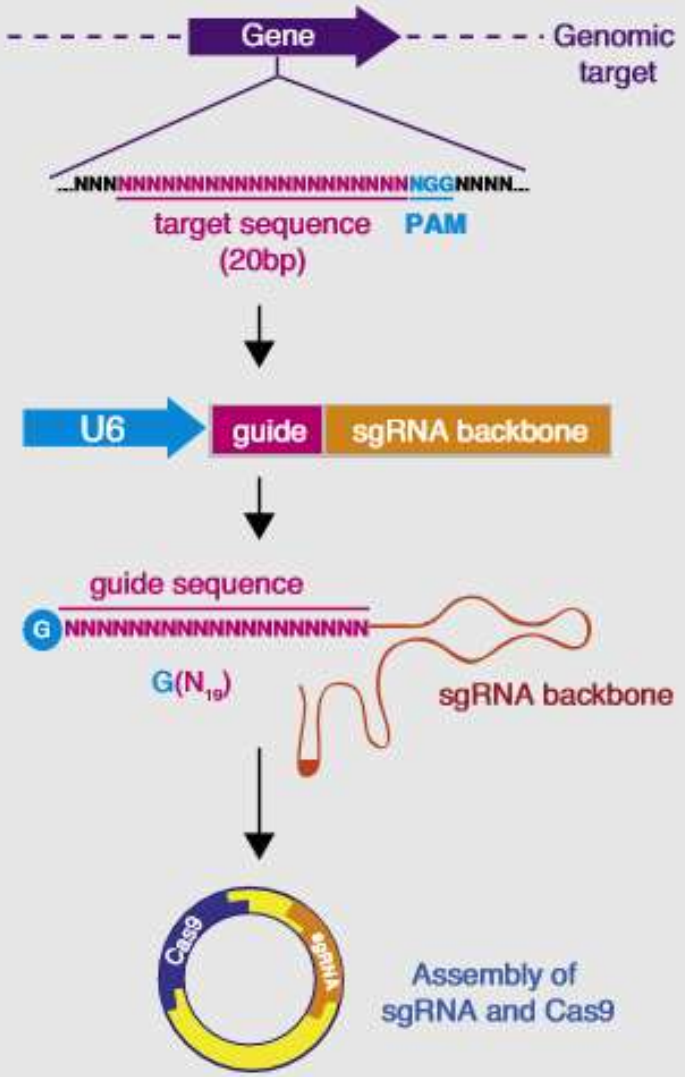
2. Design sgRNA

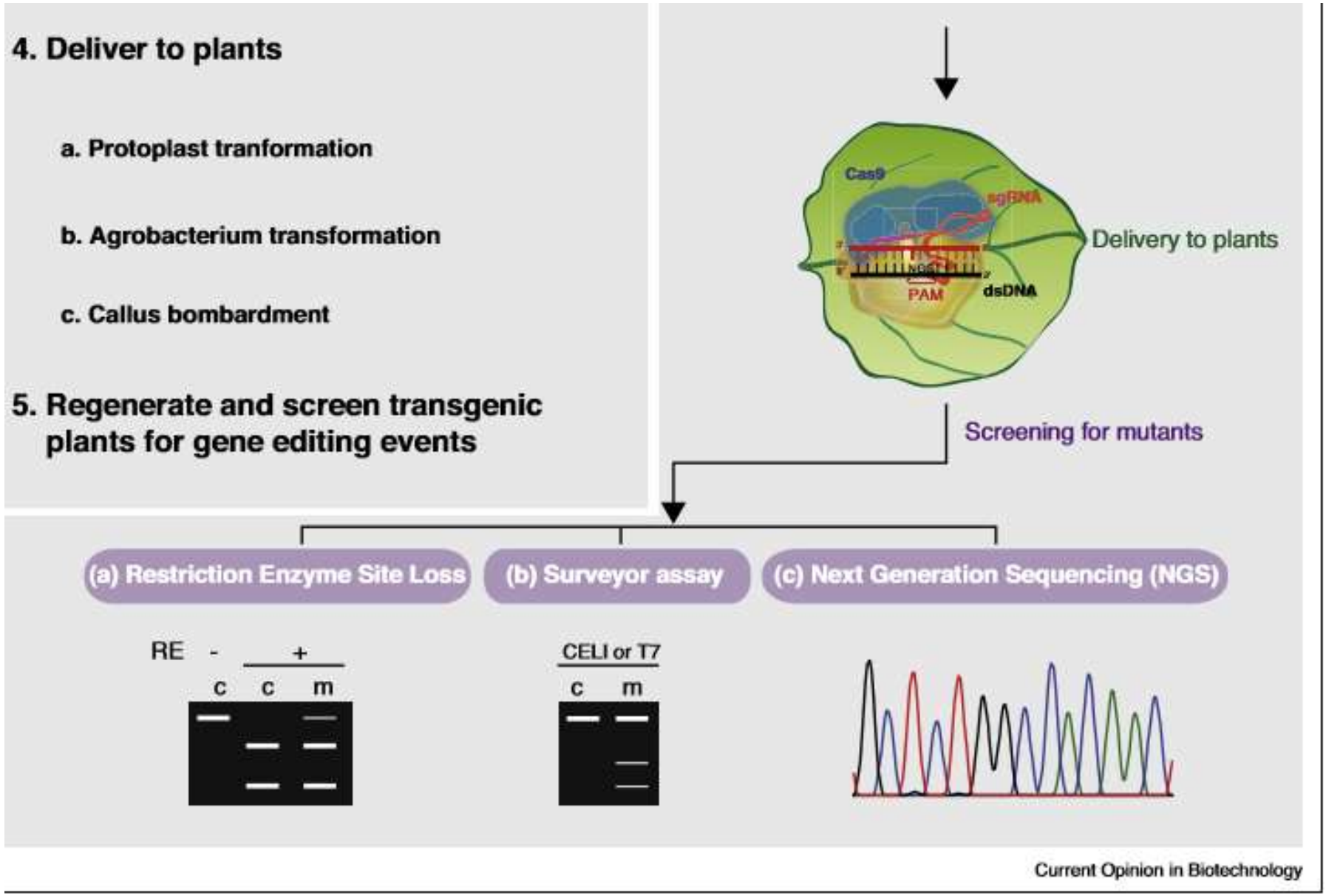
a. sgRNA is expressed using a small RNA promotor, such as U6p or U3p

b. First nucleotide in the guide sequence is a "G", if U6p is used, or an "A", if U3p is used

c. Guide sequence should match the target, except for the first nucleotide (5' G or A) that does not have to match

3. Assemble Cas9 / sgRNA construct



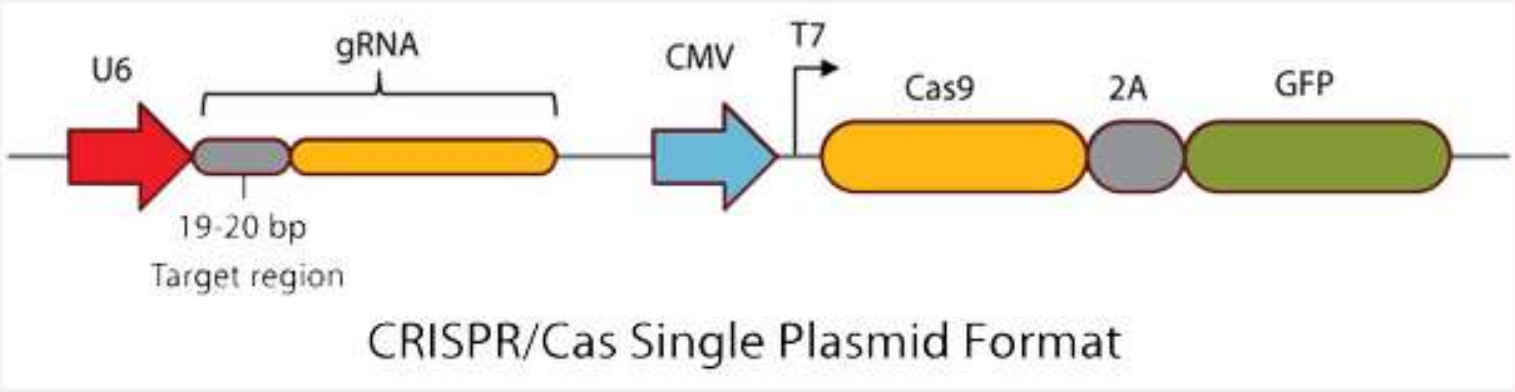


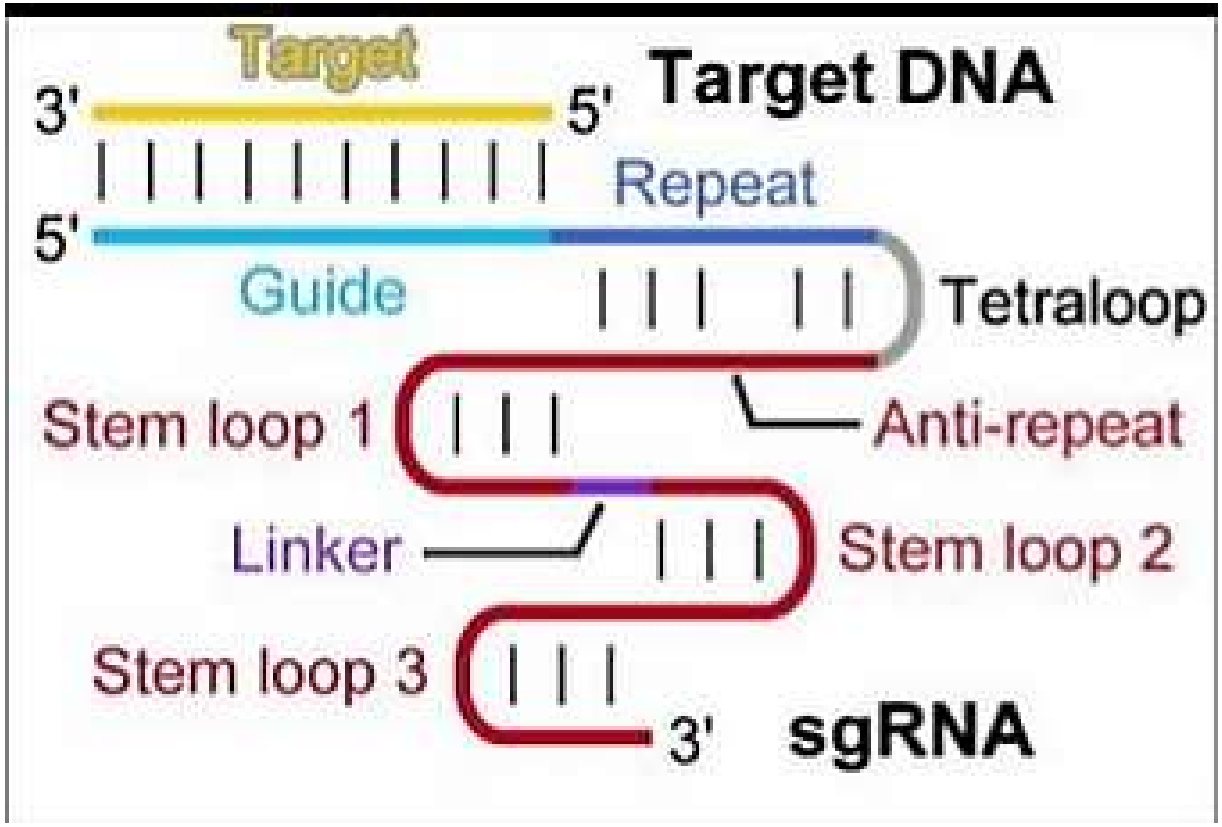
The pipeline of generating a CRISPR/Cas9-mutagenised plant line. c, control; m, mutagenized; RE, restriction enzyme. CELI and T7 are DNA endonucleases used in the surveyor assay.

Table 1

Available bioinformatic tools for selecting optimal CRISPR/Cas9 target sites and predicting off-targets

Site	Purpose	Reference
http://www.genome-engineering.org Directly at: http://crispr.mit.edu/	CRISPR/Cas9 design tool to find target sites within an input sequence. *A. thaliana* genome is available.	[56,58]
http://eendb.zfgenetics.org/casot	Open-sourced tool that is used locally, designed to identify potential off-target sites in any user-specified genome.	[66]
http://plants.ensembl.org/info/website/ftp/index.html	Download link to access 38 plant genomes.	
http://www.e-crisp.org/E-CRISP/designcrispr.html	Web-based tool to design sgRNA sequences for genome-library projects or individual sequences. Target site homology is also evaluated to predict off-targets. 5 plant genomes are available.	[67]
www.genome.arizona.edu/crispr	8 representative plant genomes are available to predict sgRNAs with low chance of off-target sites.	[68]
https://chopchop.rc.fas.harvard.edu/	Online tool for accurate target sequence selection and prediction of off-target binding of sgRNAs. Includes the design of target-specific primers for PCR genotyping. The only plant genome available is *A. thaliana*.	[69]
http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design	Online tool for designing highly active sgRNAs.	[64•]


U6
gRNA
19-20 bp
Target region
CMV
T7
Cas9
2A
GFP
CRISPR/Cas Single Plasmid Format


Target
3'
5'
Target DNA
Repeat
5'
Guide
Tetraloop
Stem loop 1
Anti-repeat
Linker
Stem loop 2
Stem loop 3
3'
sgRNA

Advantage of CRISPR/Cas9 over TALEN or ZFN (1)

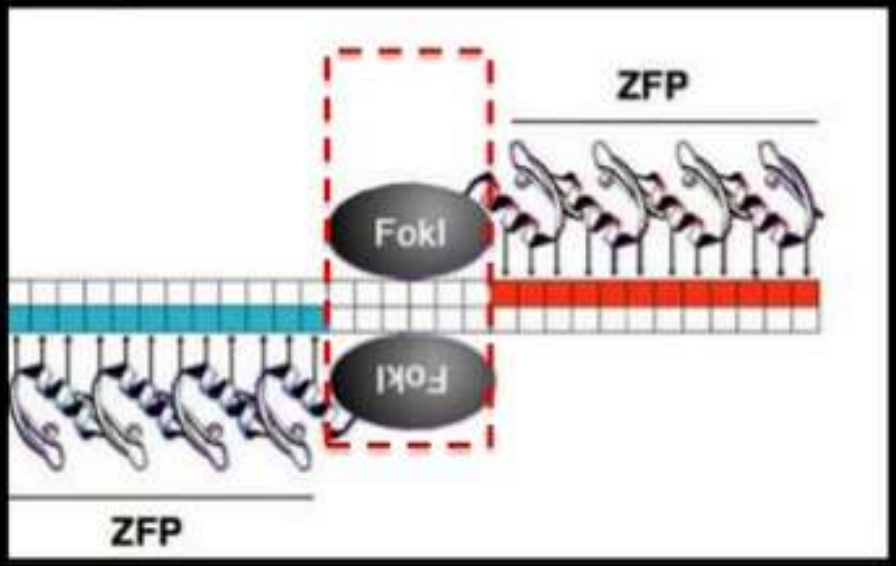
TALEN or ZFN
Artificial protein gene recognizing the target sequences are required

X 2

Synthesis of TALE gene is not trivial due to repeated nature of TALE

Advantage of CRISPR/Cas9 over TALEN or ZFN (2)

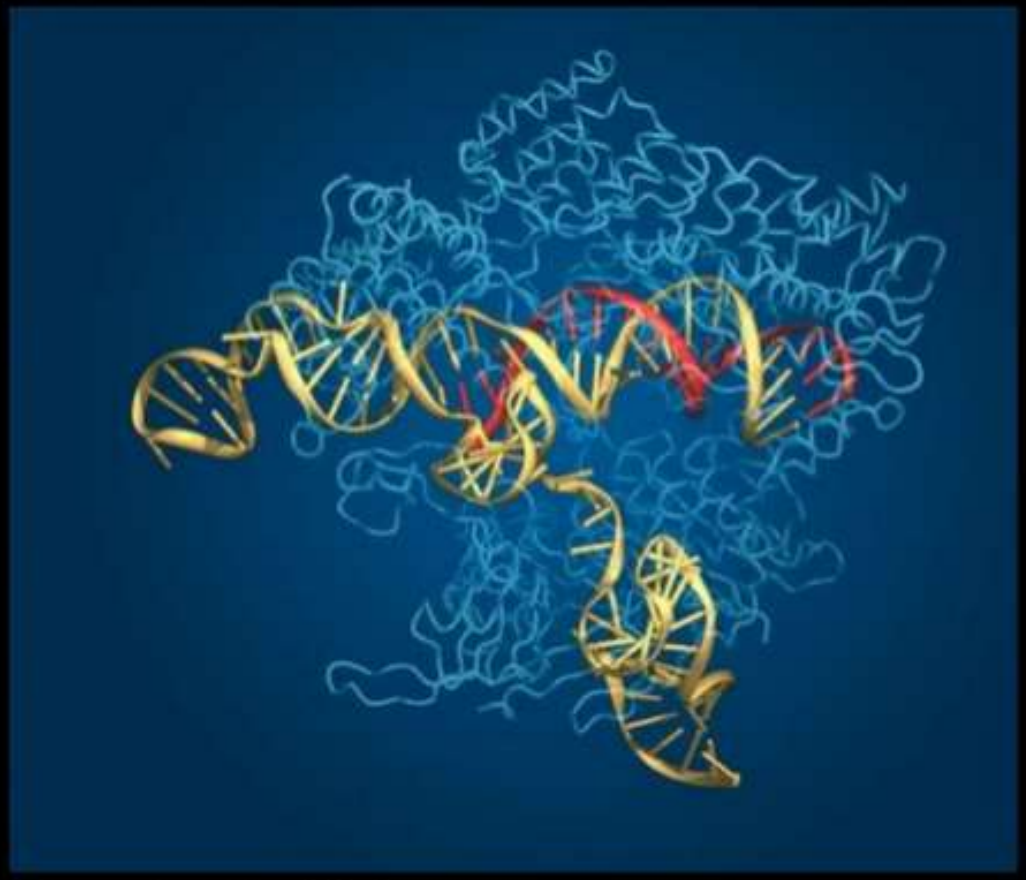
- Not as optimal compared with bona-fide endonuclease?



Dimerization of FokI cleavage domain is essential for DNA cleavages

If binding affinity of one of ZFN/TALEN pair is less than other, cleavage efficiency is lo

Cas9 is *bona fide* RNA-dependent DNA endonuclease by itself



- Higher catalytic efficiency
- Evolved to cleave Phage DNA after injection ASAP.

Table 2: Examples of agronomic traits modified through the application of *genome editing* approaches in various crops

Crop	Trait	Gene	Technology	References
Gene knockout (SDN-1)[1]				
Rice	Resistance to bacterial blight	*OsSWEET14*; *OsSWEET13*	TALEN; CRISPR/Cas9	Li et al. (2012); Zhou et al. (2015a)
	Fragrance	*OsBADH2*	TALEN	Shan et al. (2015)
Bread wheat	Resistance to powdery mildew	*TaMLO-A1, TaMLO-B1, TaMLO-D1*	TALEN	Wang et al. (2014)
Maize	Phytate biosynthesis	*IPK1*	ZFN	Shukla et al. (2009)
	Leaf epicuticular wax composition	*glossy2 (gl2)*	TALEN	Char et al. (2015)
	Leaf development; Male fertility; Herbicide resistance	*LIG1; Ms26, Ms45; ALS1, ALS2*	CRISPR/Cas9	Svitashev et al. (2015)
Soybean	Profile and unsaturation level of seed fatty acids	*FAD2-1A* and *FAD2-1B*	TALEN	Haun et al. (2014)
Poplar	Lignin content; Condensed tannin content	*4CL1*; *4CL2*	CRISPR/Cas9	Zhou et al. (2015b)
Potato	Accumulation of reducing sugars after cold storage and acrylamide after high-temperature processing	*VInv*	TALEN	Clasen et al. (2015)
	Accumulation of steroidal glycoalkaloids	*StSSR2*	TALEN	Sawai et al. (2014)
Tomato	Plant development	*PROCERA (PRO)*	TALEN	Lor et al. (2014)
	Leaf development	*ARGONAUTE7 (SlAGO7)*	CRISPR/Cas9	Brooks et al. (2014)
Gene editing (SDN-2)				
Maize	Herbicide resistance	*ALS2*	CRISPR/Cas9	Svitashev et al. (2015)
Soybean	Herbicide resistance	*ALS1*	CRISPR/Cas9	Li et al. (2015)
Tobacco	Herbicide resistance[2]	*ALS SuRA* and *SuRB*	ZFN	Townsend et al. (2009)
Gene replacement/stacking (SDN-3)				
Maize	Phytate production/herbicide resistance	*IPK1/PAT*	ZFN	Shukla et al. (2009)
	Herbicide resistance[2]	*PAT*	CRISPR/Cas9	Svitashev et al. (2015)

[1]SDN, site-directed nuclease.
[2]Tested *in vitro*.

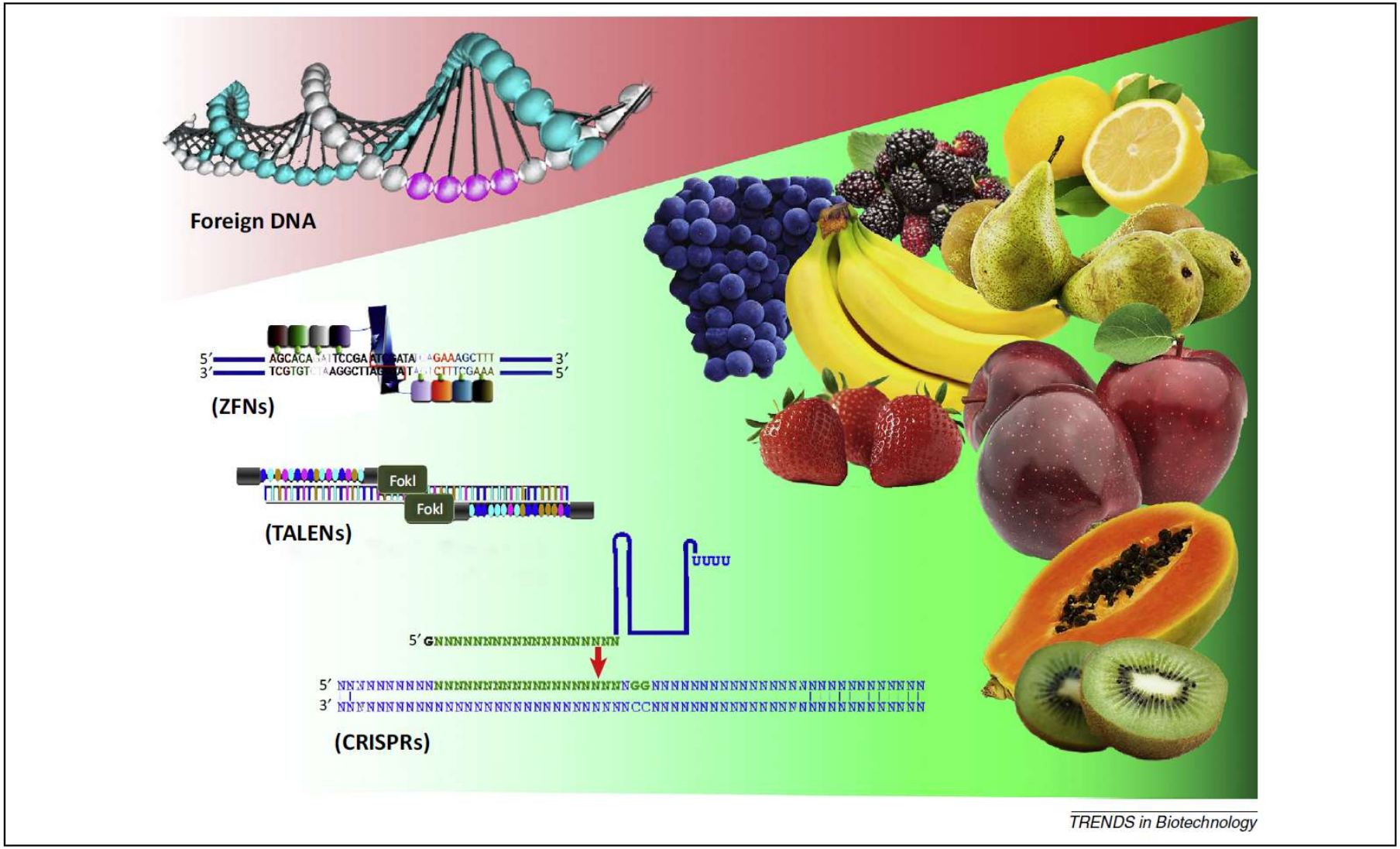


Figure 1. Comparison between genetic modification technologies. Top left, introduction of foreign DNA. Bottom left, emerging technologies such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases. Right, fruits for which genes have already been modified to fight biotic and abiotic stress [16]. Red background: not generally acceptable to the public. Green: background: more acceptable to the public.