Modern Strategies in Plant Breeding
"New" Genomic Techniques

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“New” Genomic Techniques

- NGTs are a set of methodologies that allow changing the characteristics of crop varieties in a molecularly accurate way, to increase their productivity and tolerance to environmental factors.

- They all rely in the use of recombinant DNA technology in a certain phase of the process.
- (I am assuming you know a bit of the traditional plant genetic modification techniques)

Managing crop genomes

This group of techniques (and others that will surely follow) allow us to **manage** the genomes of valuable crops, introducing new characteristics and adjusting the expression/function of different genome elements precisely.

The accuracy of the sequence of the genomes to be managed is essential. In the near future resequencing will allow us to take into consideration the variation within a specific crop/cultivar enabling the management/modification of variants.

"New" Genomic Techniques

- Site-Directed Nucleases
- RNAi & RNAe (enhancer) *
- Oligo-Directed Mutagenesis
- Agro-infiltration **
- Cisgenesis
- Grafting on GM rootstock
- Reverse Breeding
- RNA-directed DNA Methylation
- Target Mimicry for MicroRNA regulation

* After end slides
**Agro-infiltration will be not addressed in this talk

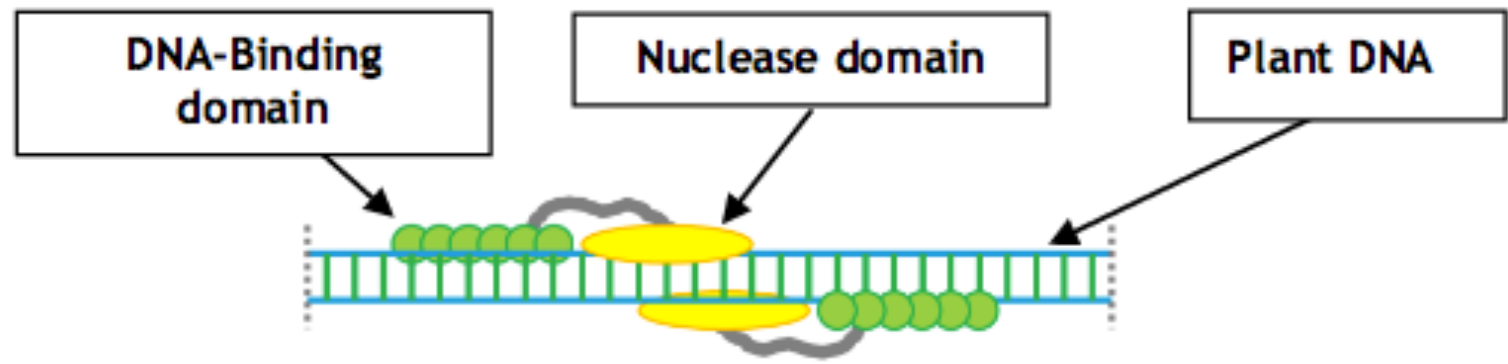
In vitro regeneration

When talking about plant genome modification there is a tendency to forget this:

- Exception made for agro-infiltration, all genomic techniques imply an in vitro regeneration system.

- In some cases, regeneration from protoplasts or from haploid cells is needed.

Site-Directed Nucleases



To produce double-strand breaks in a specific site in the DNA

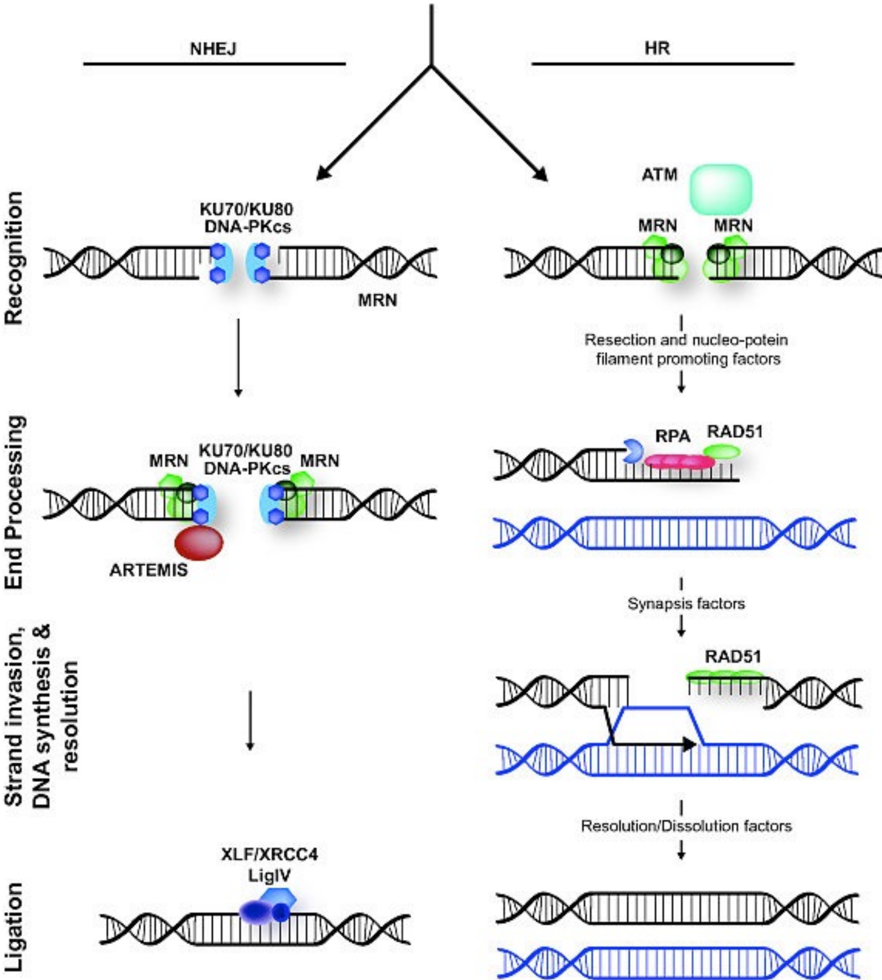
Double Strand Breaks

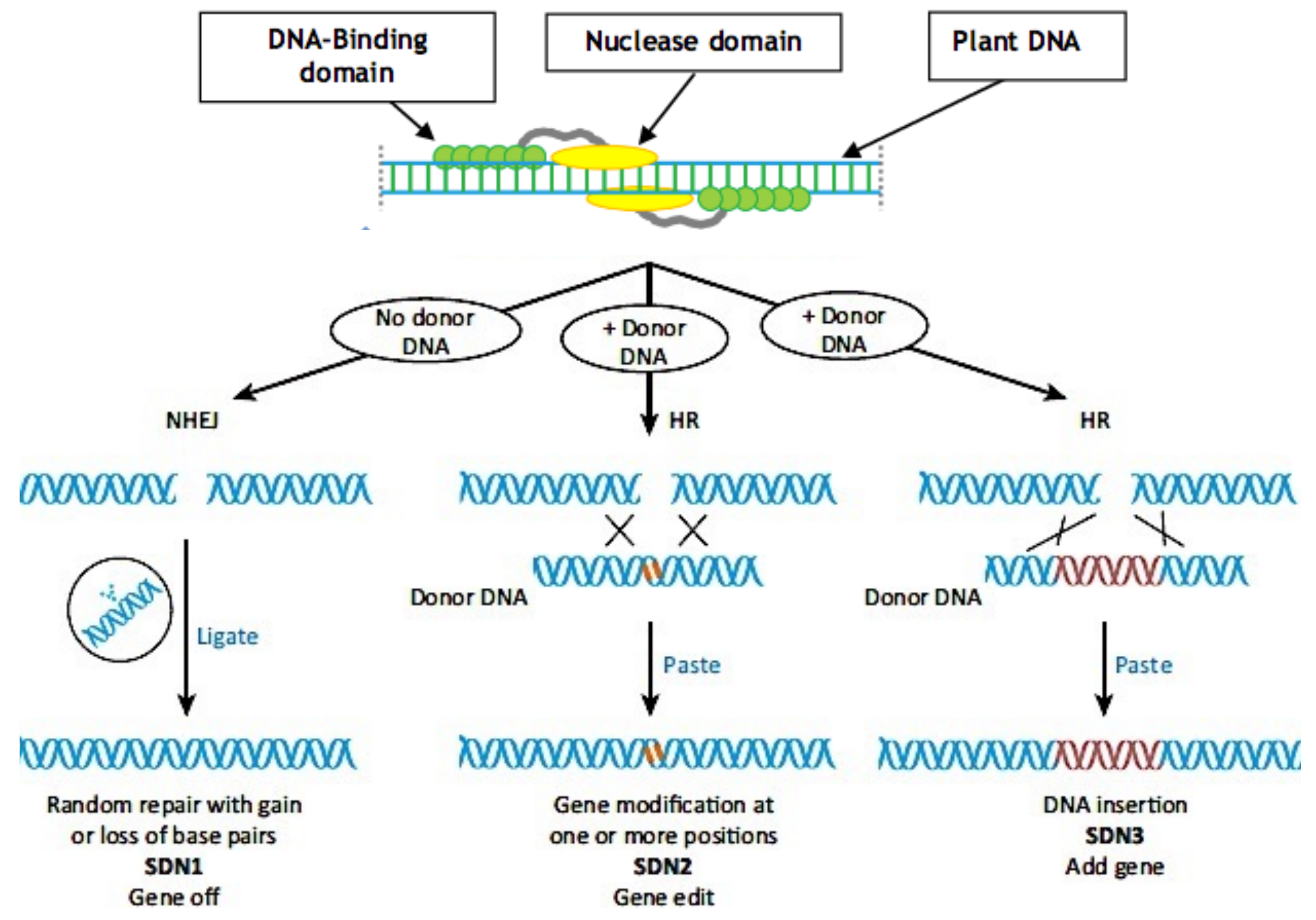
- Internal factors may produce around 10 DSB per day, per cell.
- External factors (like exposure to γ rays) may be responsible for 20 to 40 DSB per day, per cell.
- Cellular mechanisms do exist to repair these breaks. That's why we do not get cancer (almost) every day.

Two main repair processes

Non-homologous end Joining

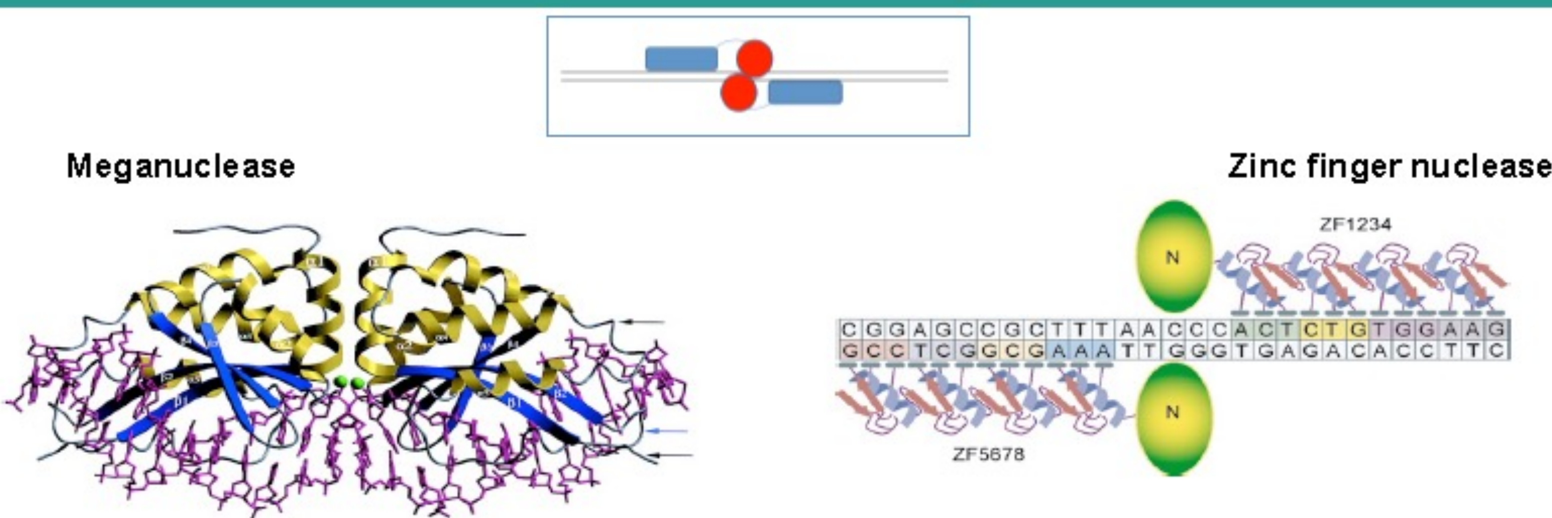
Homolougous recombination

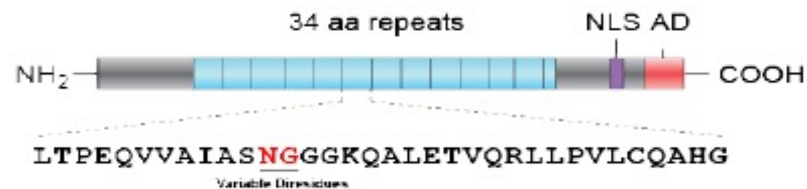


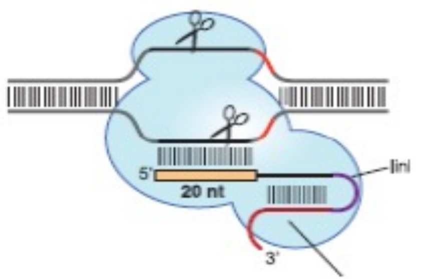


NHEJ - Non homologous end joining HR – Homologous Recombination

Four types of site directed nucleases







Transcription Activators Like effectors

- Transcription Activators Like effectors (TALE) are a family of (transkingdom) virulence factors produced by a genus of plant pathogens, *Xanthomonas spp.,*

- When injected into a host plant a TALE binds to specific host promoter sequences that regulate genes affecting the disease process, both positively and negatively.

- (Some plants have resistance genes whose promoters bind TAL effectors.)

TALEN

Nuclease domain

– The non-specific cleavage domain from the type IIs restriction endonuclease FokI is typically used as the cleavage domain in TALENs.

– This cleavage domain must dimerize in order to cleave DNA and thus a pair of TALENs are required to target DNA sites.

– Number of repeats and the sequence of the RVD determine the length and sequence of the target sequence that will be recognized.

– DNA-binding domains of individual TALENs typically contain between 12 to 30 RVDs and can thus recognise between 12 to 30 nt. In routine 2 X 15 nt are targeted.

LTPEQVVAIASNGGGKQALETVQRLLPVLCQAHG

Variable Diresidues

N

N

CGGAGCCGCTTTAACCCACTCTGTGGAAG

GCCTCGGCGAAATTGGGTGAGACACCTTC

NH_2

NG = T

HD = C

NI = A

NN = G/A

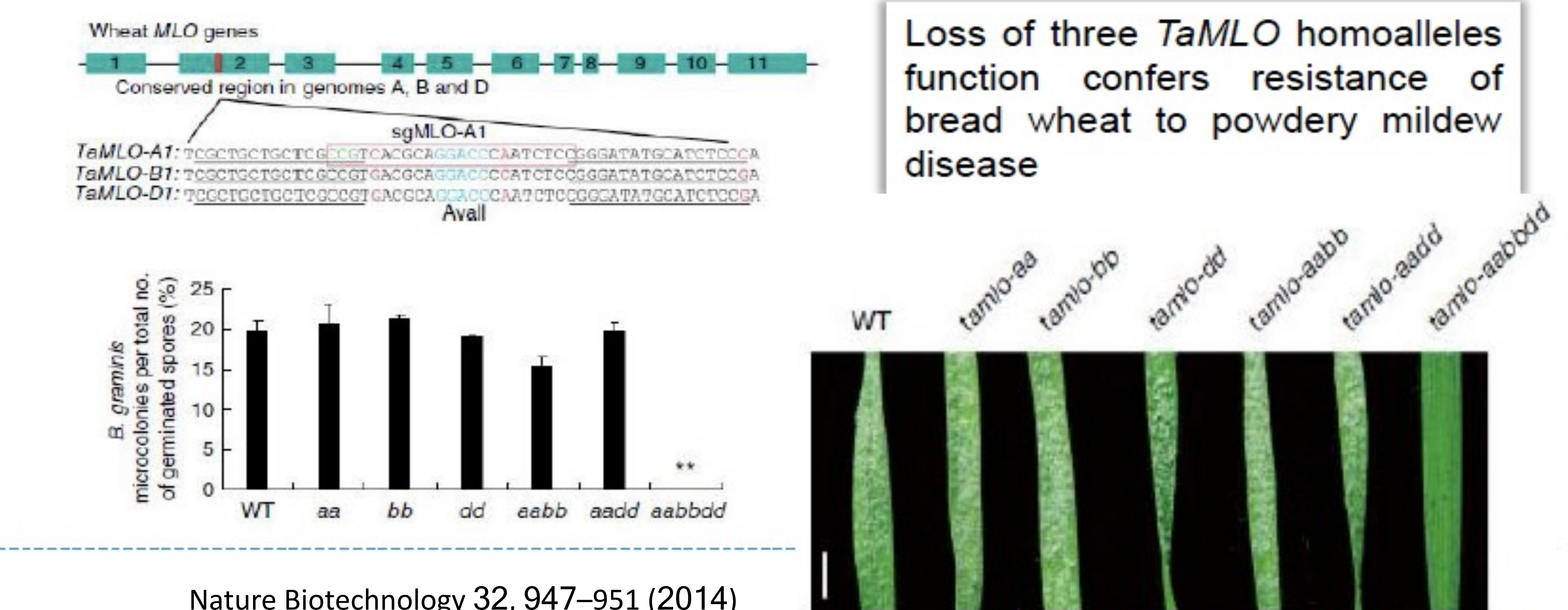
NK = G

NS = A/G/T/C

RVD – Repeat variable di-residue – each one recognise a triplet

Application of TALENs: Wheat resistance to powdery mildew

- Bread wheat (*Triticum aestivum* L.) is a major staple crop worldwide and is a allohexaploid
- In wheat, **powdery mildew** is caused by *Blumeria graminis* f. sp. *tritici* , which is one of the most **destructive plant pathogens** worldwide.
- In this study were target three *MLO* loci, which encode proteins that were shown to repress defenses against powdery mildew diseases in other plants



Nature Biotechnology 32, 947–951 (2014)

CRISPR-Cas9

Type II CRISPR (type II clustered, regularly interspaced, short palindromic repeats) systems are widespread in bacteria.

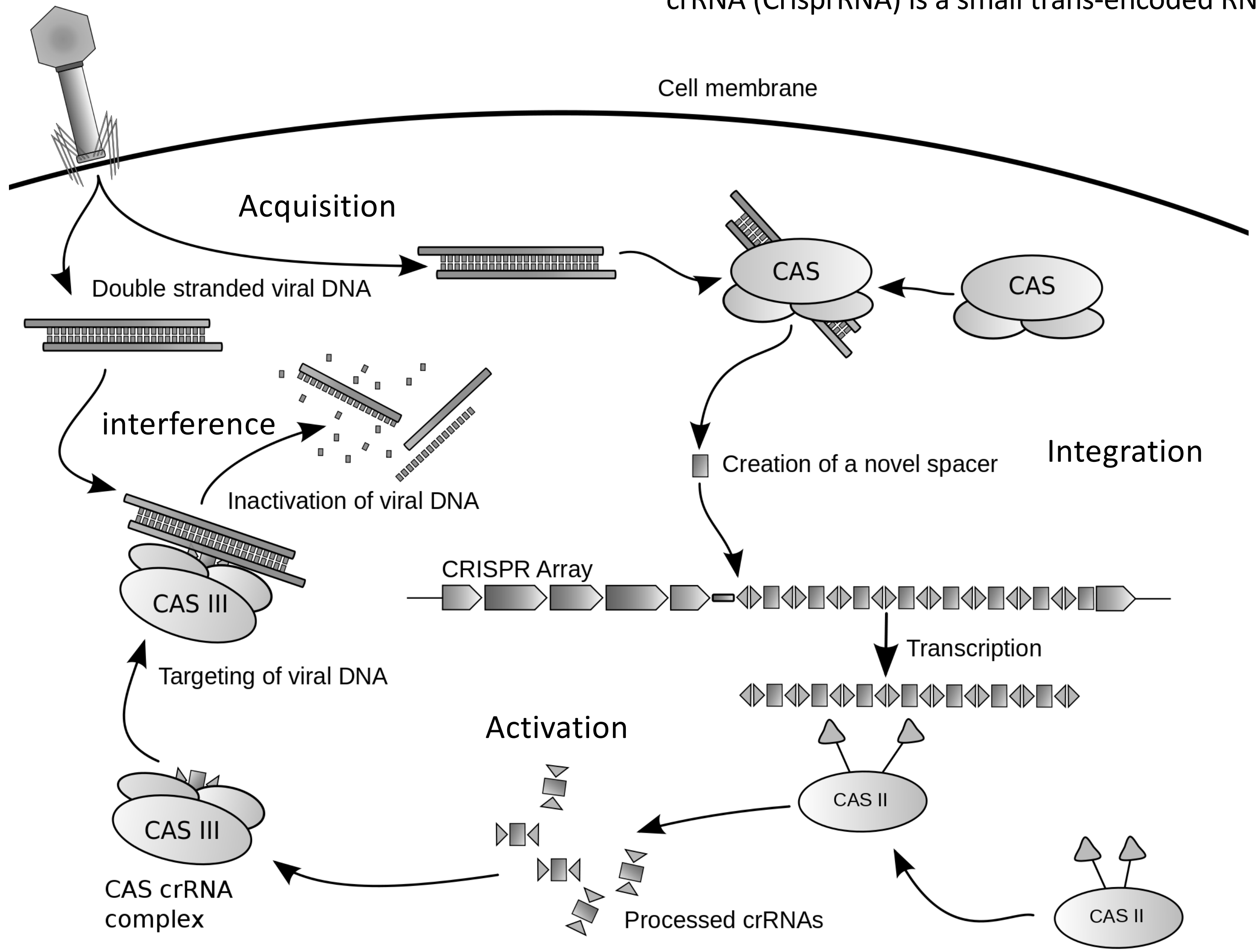
They use a single endonuclease, a CRISPR-associated protein Cas9, to provide a **defense against invading viral and plasmid DNAs.**

Cas9 can form a complex with a **synthetic single-guide RNA** (sgRNA), consisting of a fusion of CRISPR RNA (crRNA) and trans-activating crRNA (trans-activating CRISPR RNA).

The sgRNA guides Cas9 to recognize and cleave target DNA.

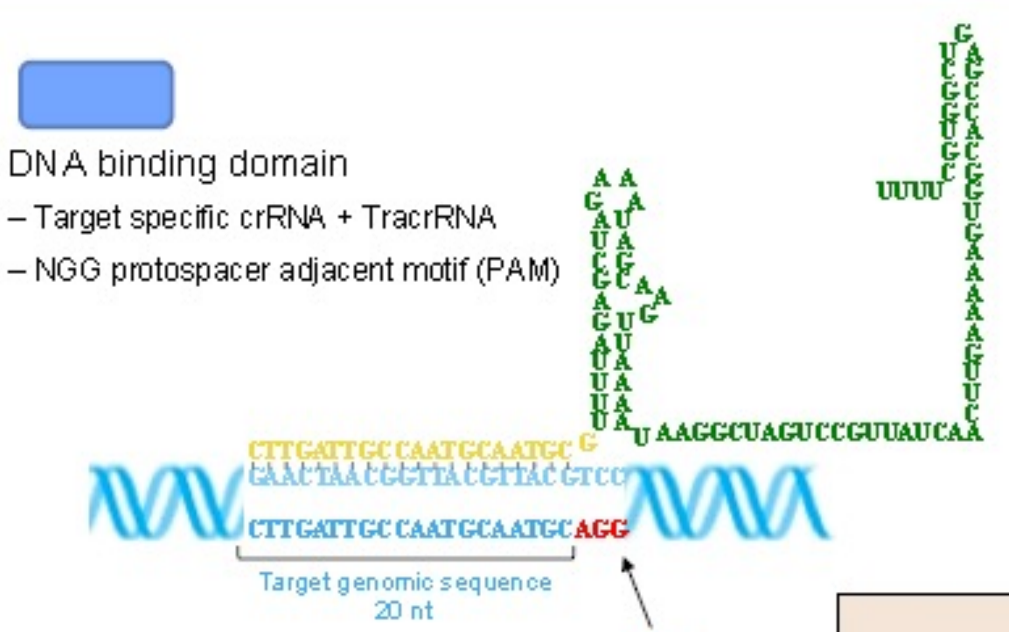
Cas9 has a **HNH nuclease domain** and a **RuvC-like domain**; each cleaves one strand of a double stranded DNA.

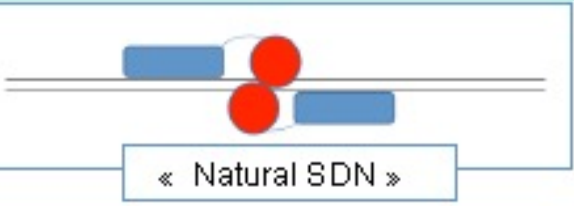
crRNA (CrisprRNA) is a small trans-encoded RNA

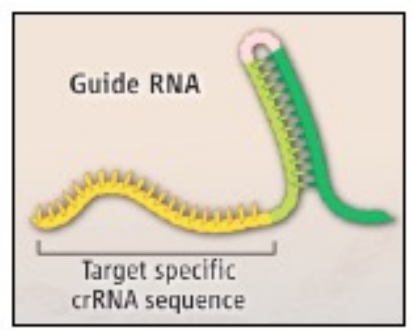


CRISPR/Cas (clustered, regularly interspaced short palindromic repeats/CRISPR-associated proteins)

SYNTHETIC CRISPR-Cas



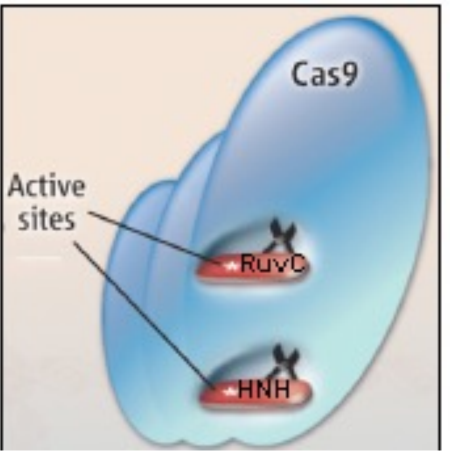




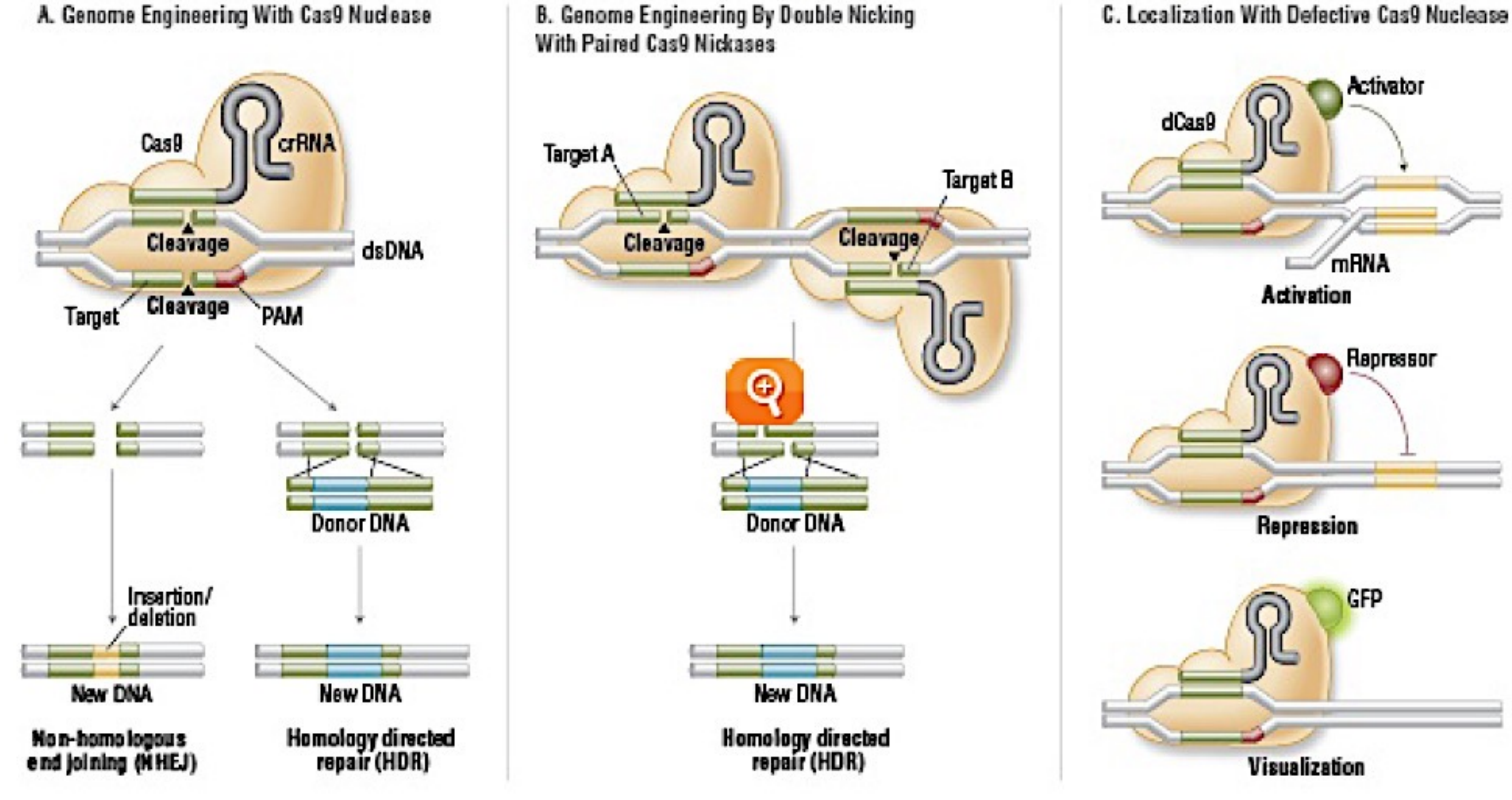
PAM = XGG

Nuclease domain

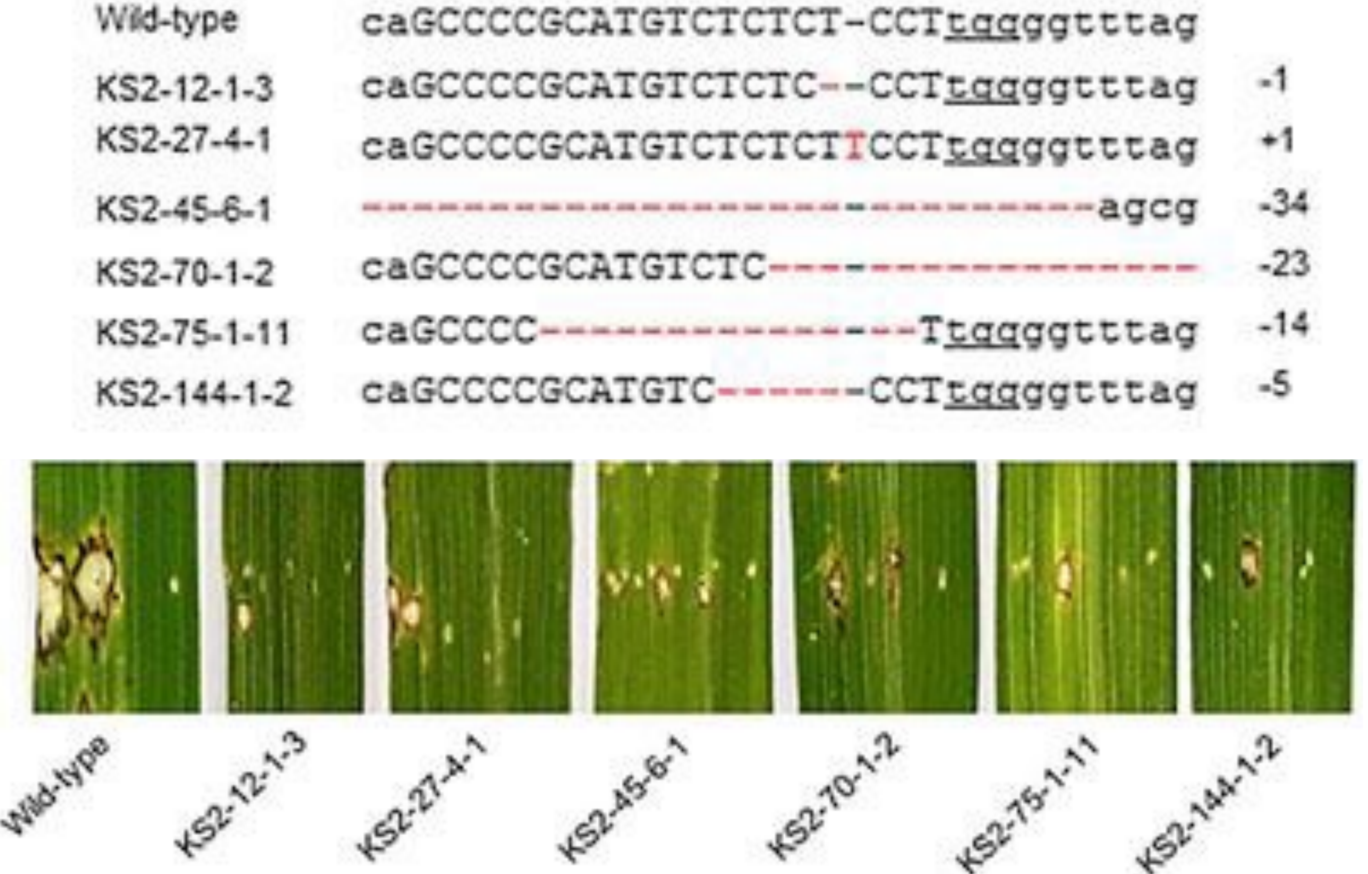
– Cas9 is a nuclease with two active cutting sites, RuvC (a Holliday junction resolvase) and HNH (an endonuclease), one for each strand of the double helix.



CRISPR/Cas9 System Applications



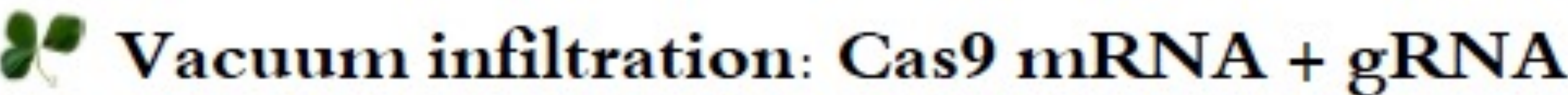
Crispr/Cas9 and rice blast



The ERF transcription factor is a negative regulator of blast resistance in Rice

Wang *et al.*, 2016 *PloS one*

Crispr/cas9 delivery without genome integration



1) In vitro-transcribed gRNA and Cas9 mRNA are joined in a Lipid complex

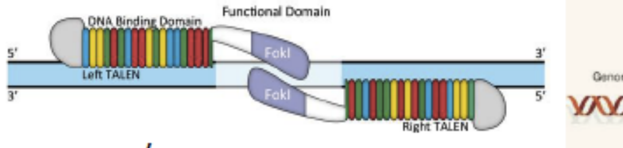
3) Inoculate lipid mixture into the M9-10a excised leafs and infiltrate by vacuum

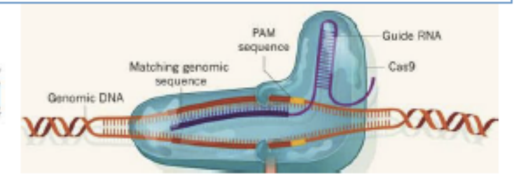
4) *Medicago truncatula* M9-10a regeneration by somatic embryogenesis

5) Mutation detection: PCR followed by restriction enzyme digestion

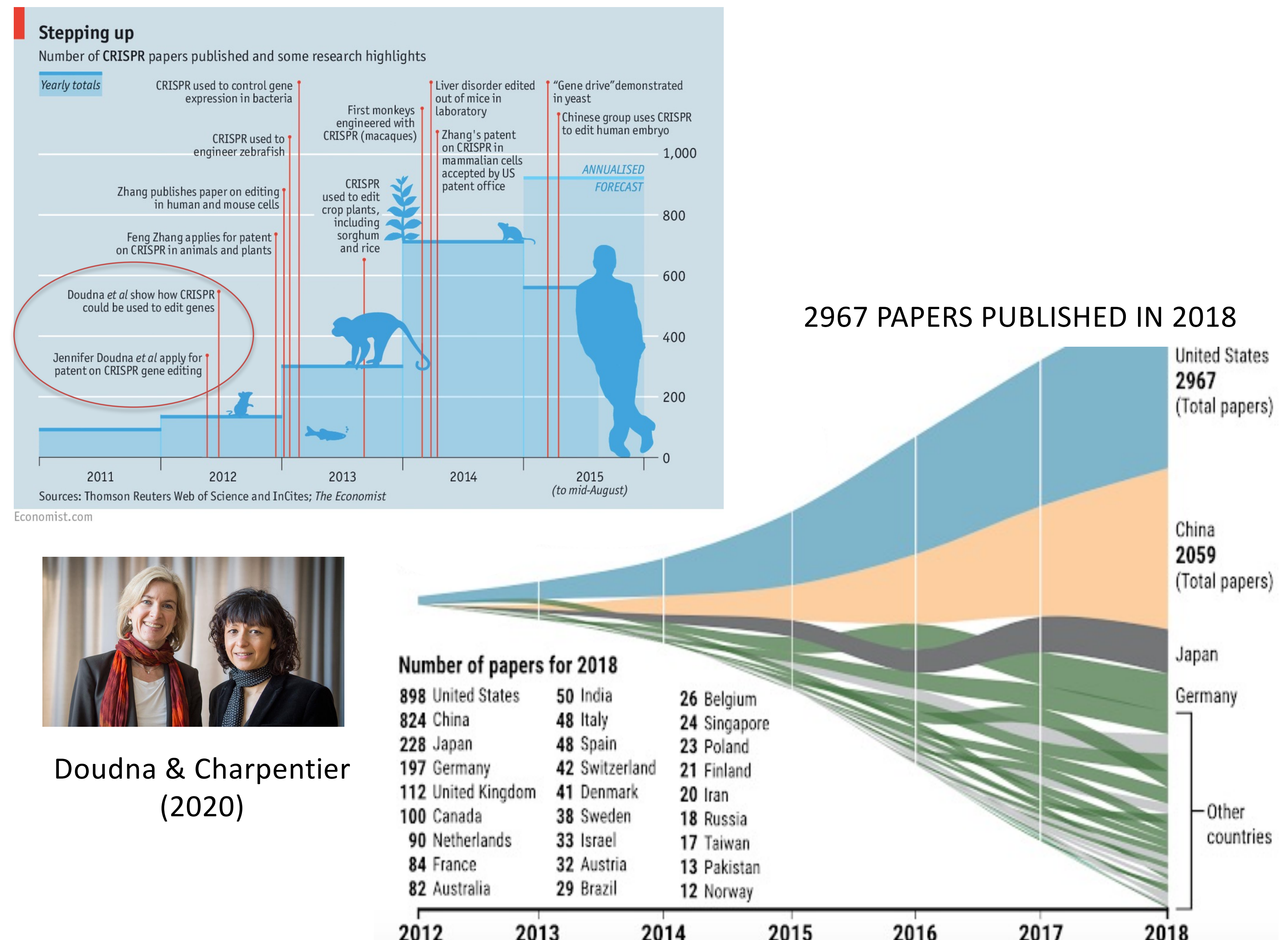
TALENs vs CRISPR/Cas9

	TALEN	CRISPR/Cas9
Origin	Plant pathogenic bacteria (*Xanthomonas sp*)	Diverse bacteria and archaea
Target-binding principle	Protein DNA specific recognition	Watson-Crick complementary rule
Working mode	TALE specifically recognizes the target DNA and dimeric Fok I makes the DSB, which is repaired by NHEJ or HR	Guide RNA specifically recognizes the target DNA and Cas9 makes the DSB, which is repaired by NHEJ or HR
Target DNA length	~2 x 17 bp + spacer (14-18 bp)	~20bp
Off-target effects	Minor effects	Moderate to high

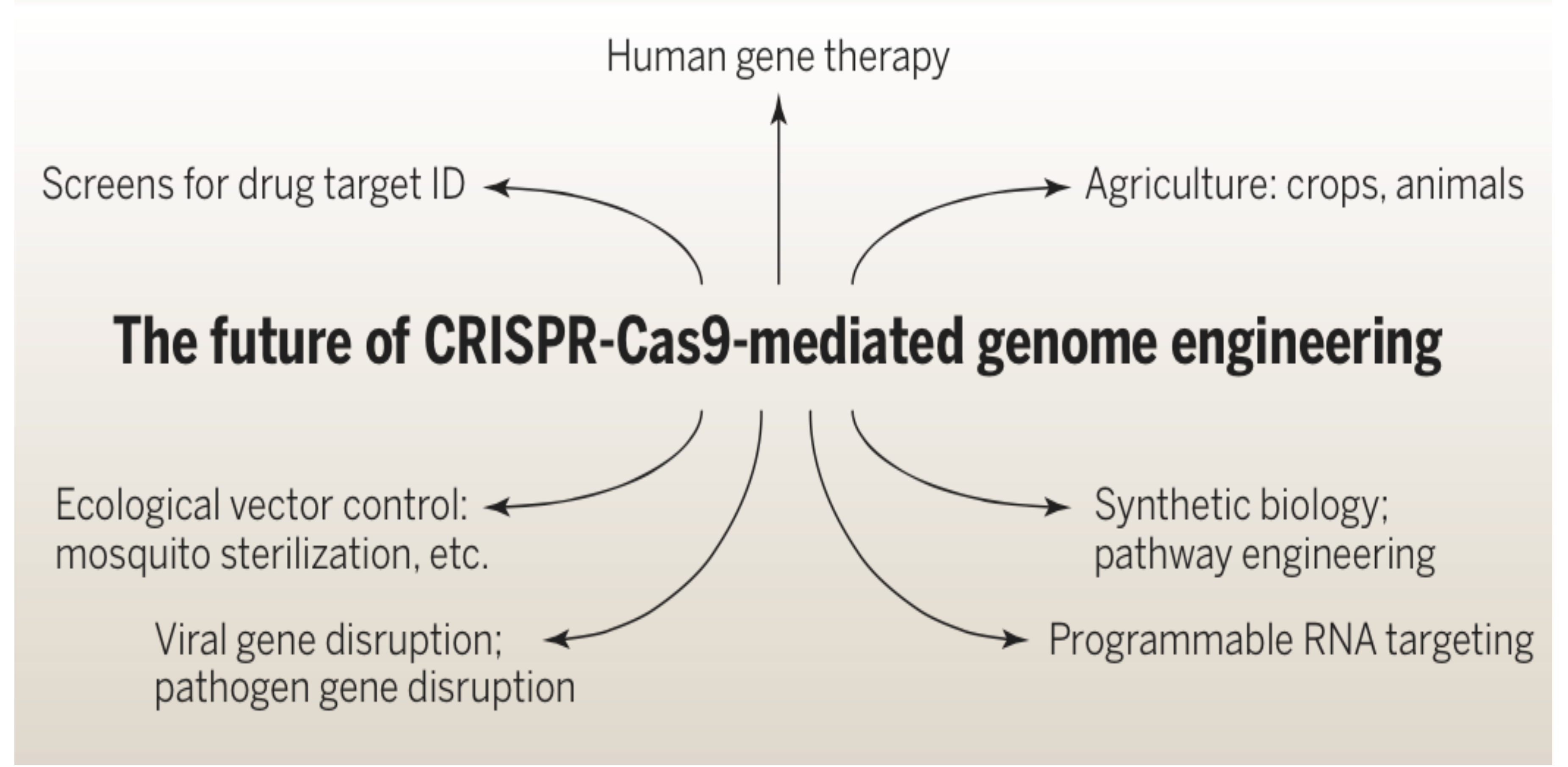




http://www.genecopoeia.com/product/talen-tal-effector/

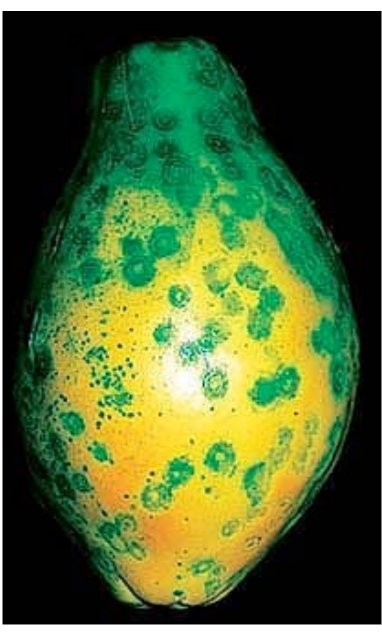


Doudna & Charpentier (2020)


Human gene therapy
Screens for drug target ID
Agriculture: crops, animals
The future of CRISPR-Cas9-mediated genome engineering
Ecological vector control:
mosquito sterilization, etc.
Synthetic biology;
pathway engineering
Viral gene disruption;
pathogen gene disruption
Programmable RNA targeting

RNA interference (RNAi)

The papaya ring spot virus history



Kapoho field trial started in 1995, showing a solid block of PRSV-resistant Rainbow growing well while the surrounding susceptible non-transgenic Sunrise is severely infected with PRSV.

http://www.apsnet.org/publications/apsnetfeatures/Pages/papayaringspot.aspx

RNA interference (RNAi)

Is a process in which ***double-stranded*** **RNA fragments** called ***small interfering RNAs*** trigger catalytically mediated gene silencing, most typically by targeting the **RNA induced silencing complex** (RISC) to bind to and degrade the mRNA or prevent translation.

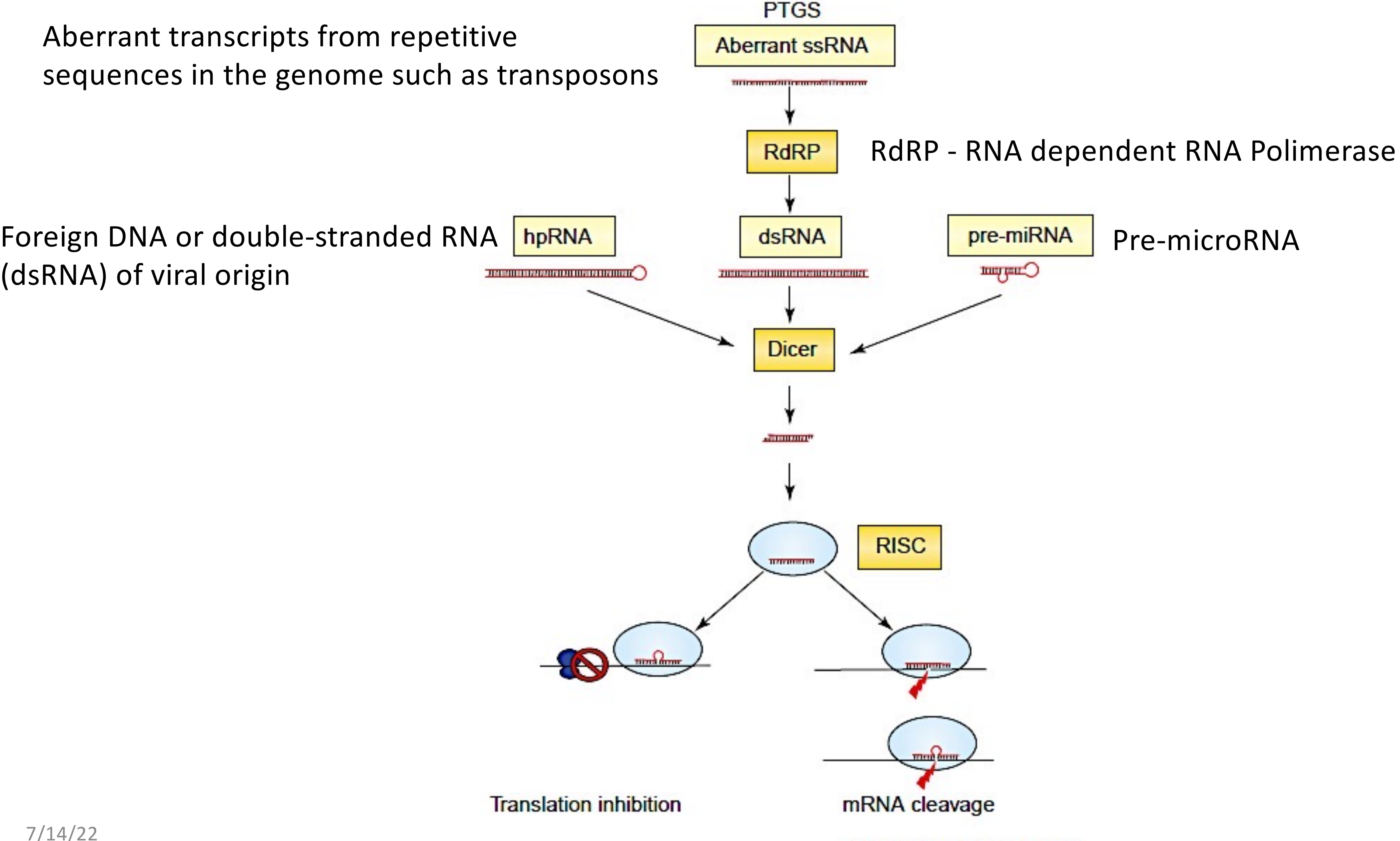
RNAi is also called Post-transcriptional gene silencing (PTGS)

Aberrant transcripts from repetitive sequences in the genome such as transposons

RdRP - RNA dependent RNA Polimerase

Foreign DNA or double-stranded RNA (dsRNA) of viral origin

Pre-microRNA

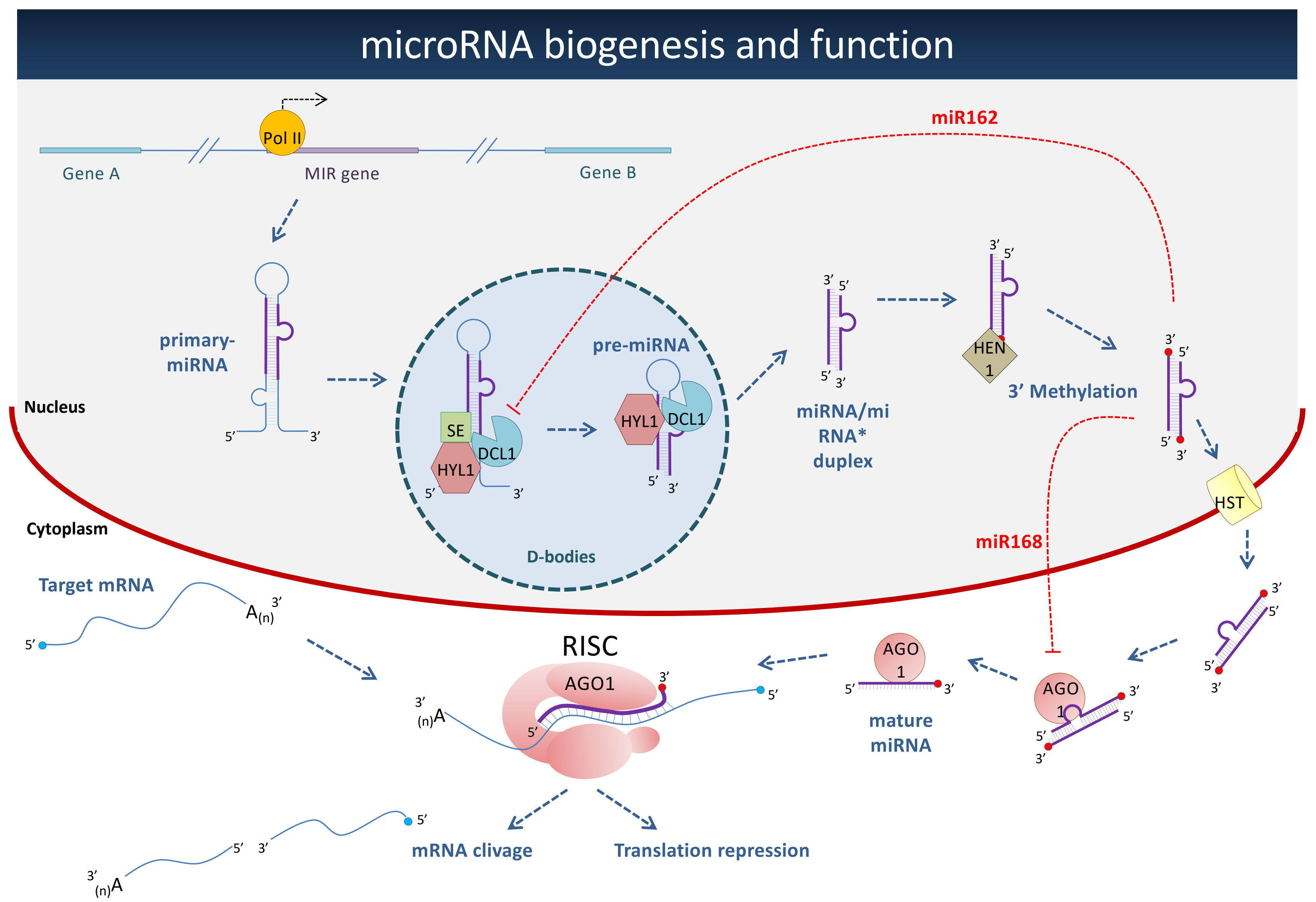


Endogenous triggers of RNAi pathway

include

a) foreign DNA or double-stranded RNA (dsRNA) of viral origin,
b) aberrant transcripts from repetitive sequences in the genome such as transposons, and
c) pre-microRNA .

In plants, RNAi forms the basis of virus-induced gene silencing (VIGS), suggesting an important role in pathogen resistance


microRNA biogenesis and function
Pol II
Gene A
MIR gene
Gene B
miR162
primary-miRNA
Nucleus
pre-miRNA
SE
DCL1
HYL1
D-bodies
miRNA/miRNA* duplex
HEN 1
3′ Methylation
HST
miR168
Cytoplasm
Target mRNA
RISC
AGO1
mature miRNA
mRNA clivage
Translation repression

Generic workflow for RNAi technology

1) Target gene identification and **carefull** sequence analysis

2) Vector development

Selectable marker

Promoter choice:

a) Constitutive
b) Tissue or organ specific
c) miRNA

Terminator choice

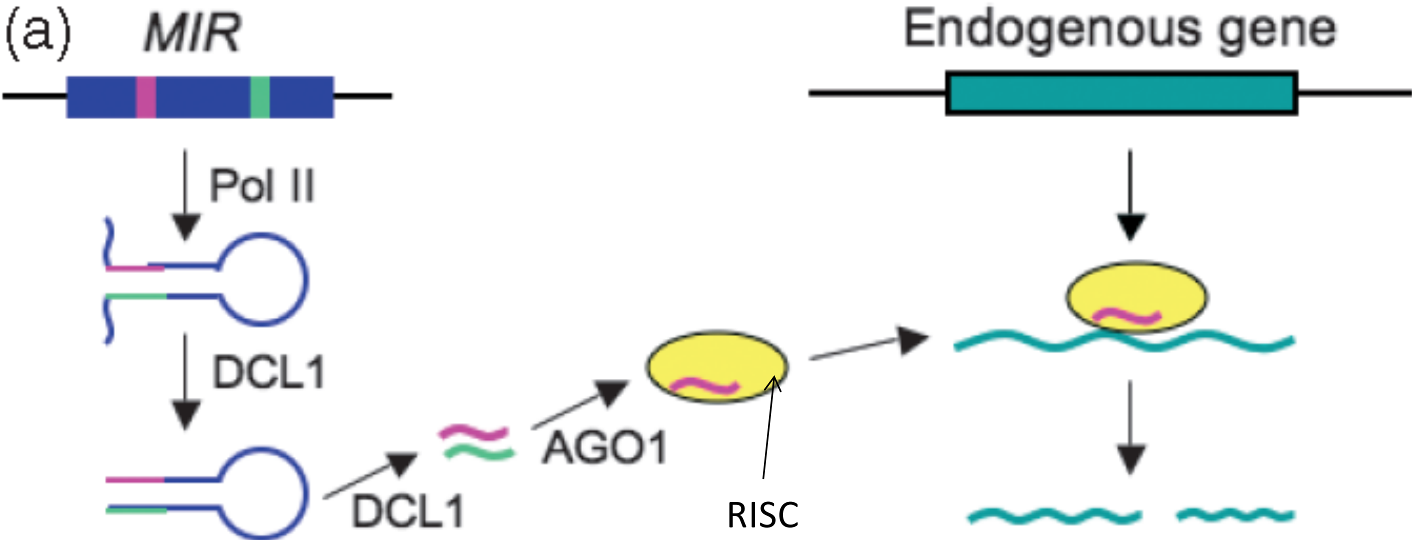
dsRNA or harpin vectors, or AmiRNA-like vectors

AmiRNA – Artificial MiRNA

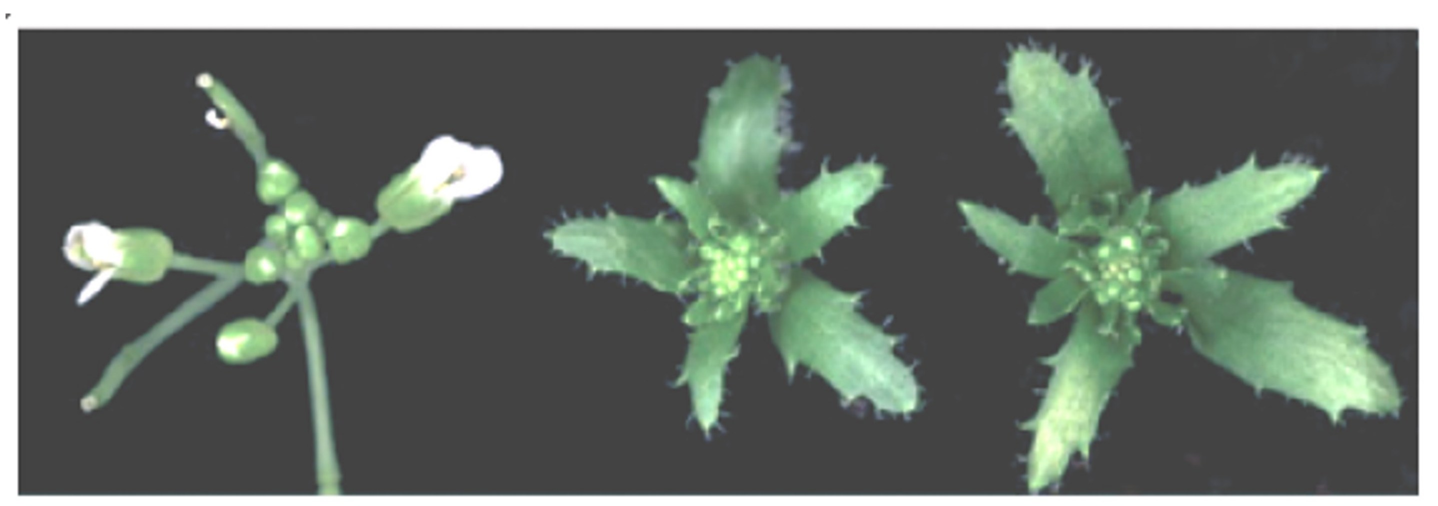
3) Delivery into plants

4) Screening and evaluation

Using an artificial miRNA



(a) A diagram of the amiRNA approach. A known *MIR* gene is manipulated such that the sequences of the mature miRNA and its antisense strand miRNA* are replaced by those of an amiRNA and its antisense strand.



(b) An example of amiRNA-based gene knock down in Arabidopsis. A wild-type plant (left) generates flowers after making a certain number of leaves, whereas a mutant in the *LEAFY* gene (middle) shows a partial conversion of flowers into leaves. A wild-type plant that harbors an amiRNA targeting *LEAFY* (right) phenocopies the *leafy* mutant (middle). The images in B were part of Figure 3A of Schwab *et al.* (2006) *Plant Cell*, **18,** 1121–1133

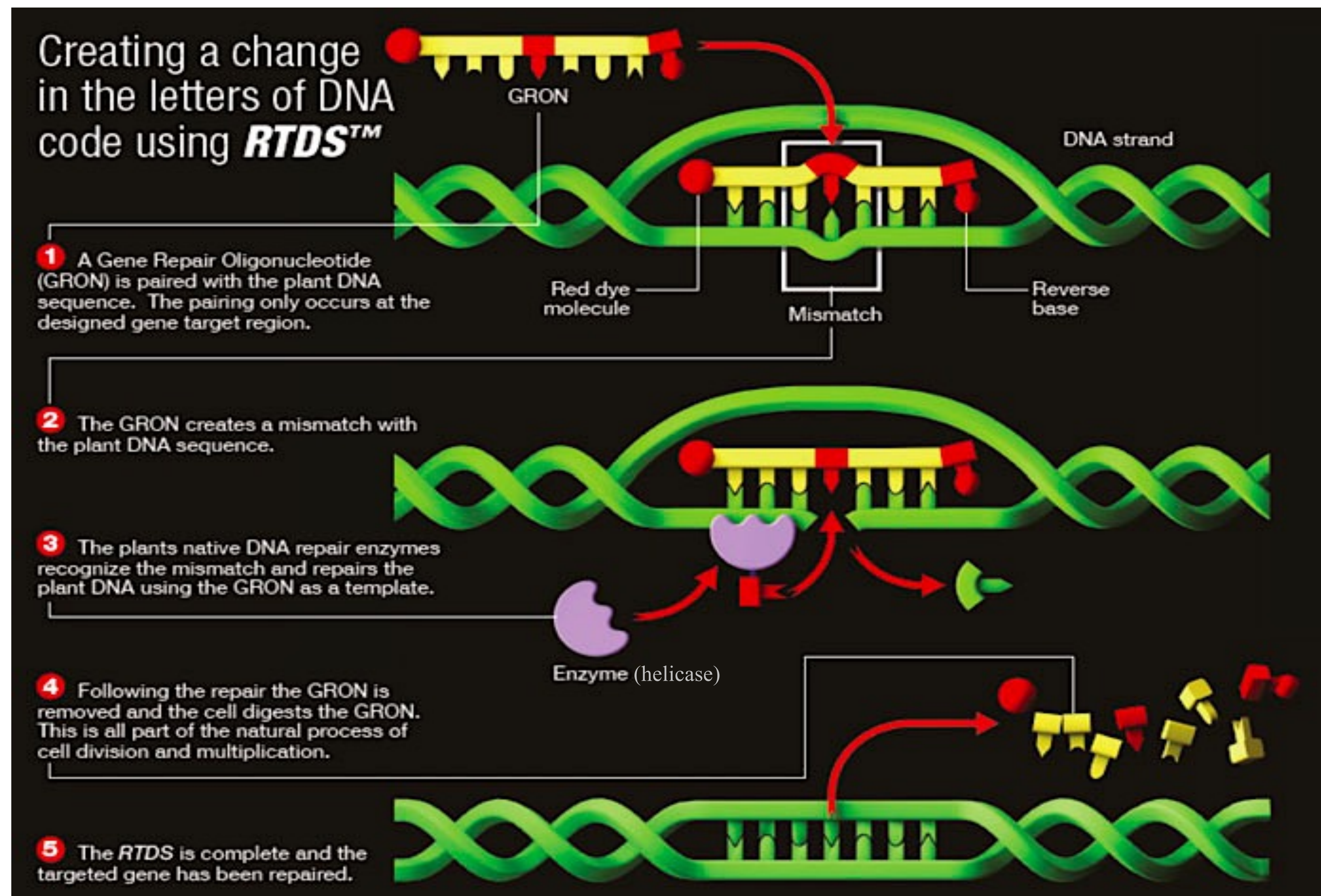
Oligo-Directed Mutagenesis

Oligo-Directed Mutagenesis (ODM) by Gene Repair Oligonucleotide (GRON)

- Oligonucleotide-directed mutagenesis (ODM) is a non-transgenic base pair-specific precision genome editing platform.

- ODM employs chemically synthesized oligonucleotides to mediate genome editing by acting as DNA templates during the editing process.

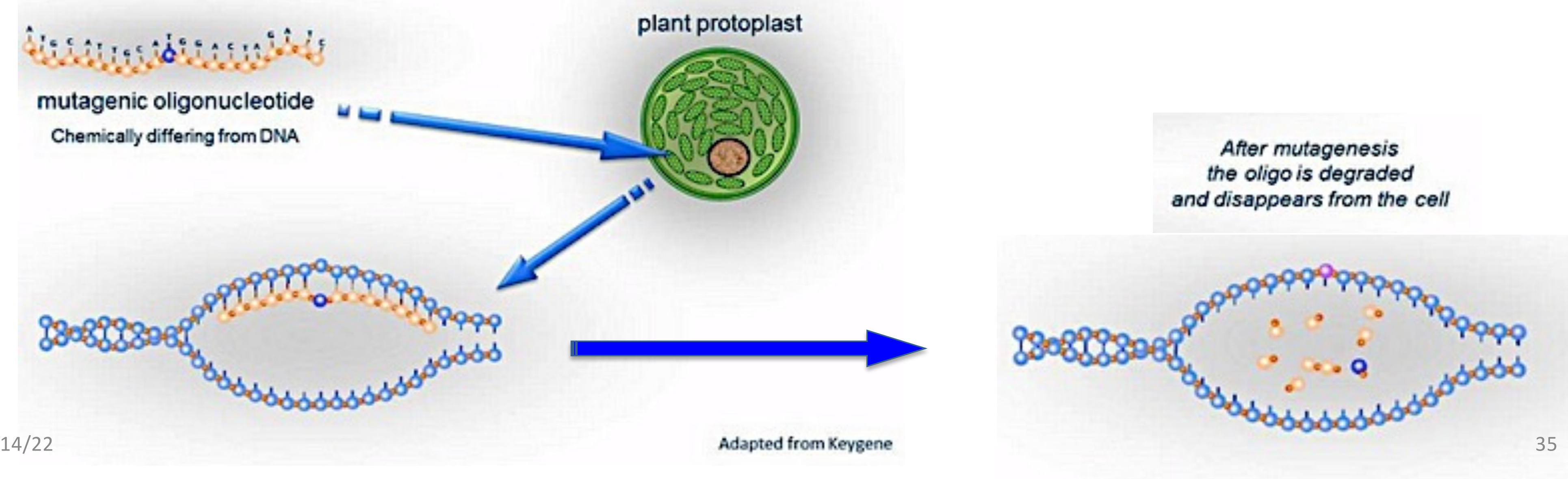
- ODM relies in the knowledge of the influence of single nucleotide polymorphisms in a specific characteristic.


Creating a change in the letters of DNA code using RTDS™
GRON
DNA strand
1 A Gene Repair Oligonucleotide (GRON) is paired with the plant DNA sequence. The pairing only occurs at the designed gene target region.
Red dye molecule
Mismatch
Reverse base
2 The GRON creates a mismatch with the plant DNA sequence.
3 The plants native DNA repair enzymes recognize the mismatch and repairs the plant DNA using the GRON as a template.
Enzyme (helicase)
4 Following the repair the GRON is removed and the cell digests the GRON. This is all part of the natural process of cell division and multiplication.
5 The RTDS is complete and the targeted gene has been repaired.

Oligo-Directed Mutagenesis

The inserted oligonucleotide is identical to part of the genetic material, except for the presence of one intended change.

The oligonucleotide acts as a template for natural DNA repair mechanisms, which detect the mismatch between the template and the endogenous genetic material and copies the intended change into DNA.



Gene Repair Oligonucleotide

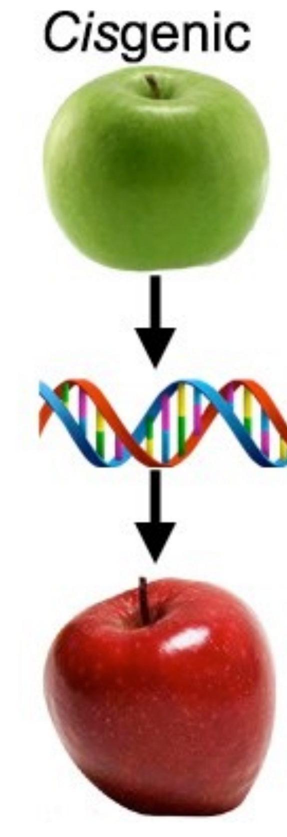
Oligonucleotide-directed mutagenesis for precision gene editing (2015)

- **Plant Biotechnology Journal - Volume 14, Issue 2** p. 496-502
- Noel J. Sauer, Jerry Mozoruk, Ryan B. Miller, Zachary J. Warburg, Keith A. Walker, Peter R. Beetham, Christian R. Schöpke, Greg F. W. Gocal
- **https://doi.org/10.1111/pbi.12496**

Cisgeneses

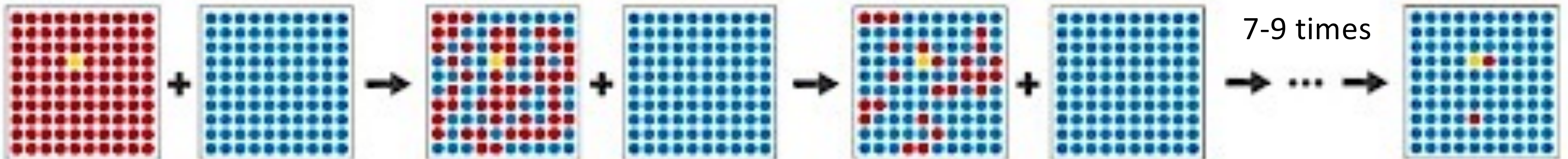
Cisgeneses

A specific trait, such as disease resistance, is transferred from a same or closely related crossable plant species to another without altering the plant's overall genetic makeup.

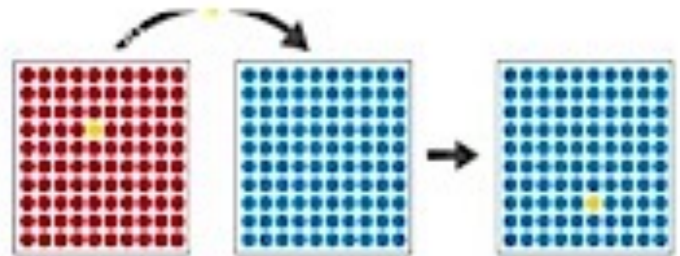


https://www.biofortified.org/2013/08/cisgenesis-new-dawn-on-food-production/

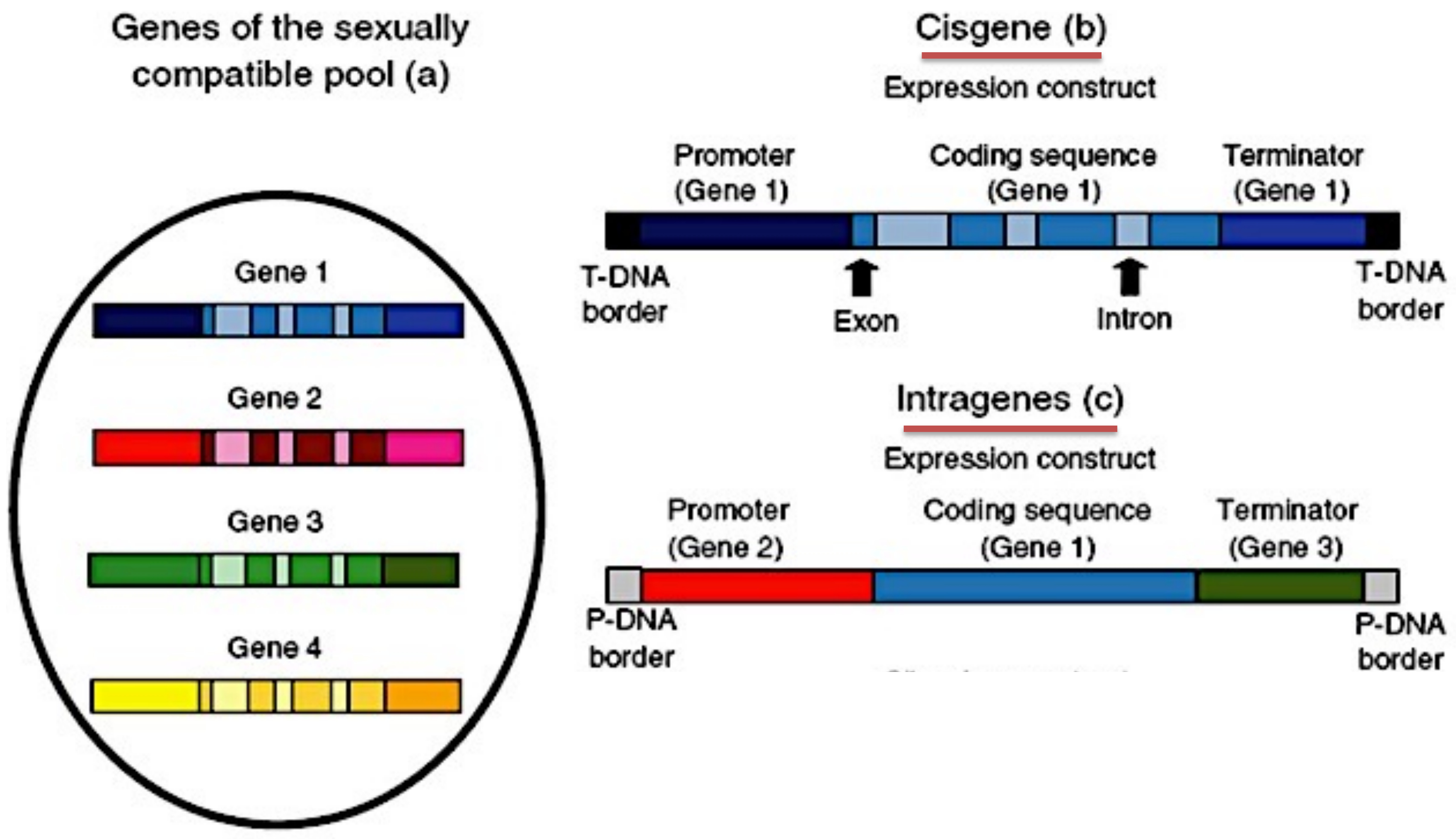
Conventional breeding



Cisgenesis



Cisgeneses and Intragenesis



Holme et al. (2013) Plant Biotechnol. J. 11:395-407

Cisgeneses and Intragenesis

CISGENESIS (Schouten et al., 2006)

Full CDS including introns of a gene originating from the sexually compatible gene pool of the recipient plant along with **gene's own promoter and terminator** are used for transformation

INTRAGENESIS (Rommens et al., 2004)

The full or **partial** CDS of genes originating from the sexually compatible gene pool of the recipient plant can be used in sense or **antisense** orientation.

The promoter and terminator could originate from sexually compatible gene pool of the recipient plant (not necessarily from the 'cisgene' itself).

Table 1. Intragenic/cisgenic crops developed or currently under development.

	Intra-/Cis-genesis	Type	Gene	Trait	References
	Crops with commercially widespread clones				
A	Potato (Intra)	Silencing	*GBSS**	High amylopectin	4
B	Potato (Intra)	Silencing	*Ppo*	Preventing black spot bruise	5
C	Potato (Intra)	Silencing	*Ppo, R1, PhL*	Limiting degradation of starch. Limiting acryl-amide formation	6
D	Potato (Intra)	Silencing	*StAs1,StAS2*	Limiting acrylamide formation	7
E	Potato (Intra)	Silencing	*StAs1*	Limiting acrylamide formation	8
F	Potato (Cis)	Genes from related species	*R-genes*	Late blight resistance	9
G	Apple (Cis)	Gene from related species	*HcrVf2*	Scab resistance	10
H	Strawberry (Intra)	Overexpression	*PGIP*	Gray mould resistance	11
I	Grapevine (Cis)	Gene from related species	*VVTL-1****	Fungal disease resis-tance	12

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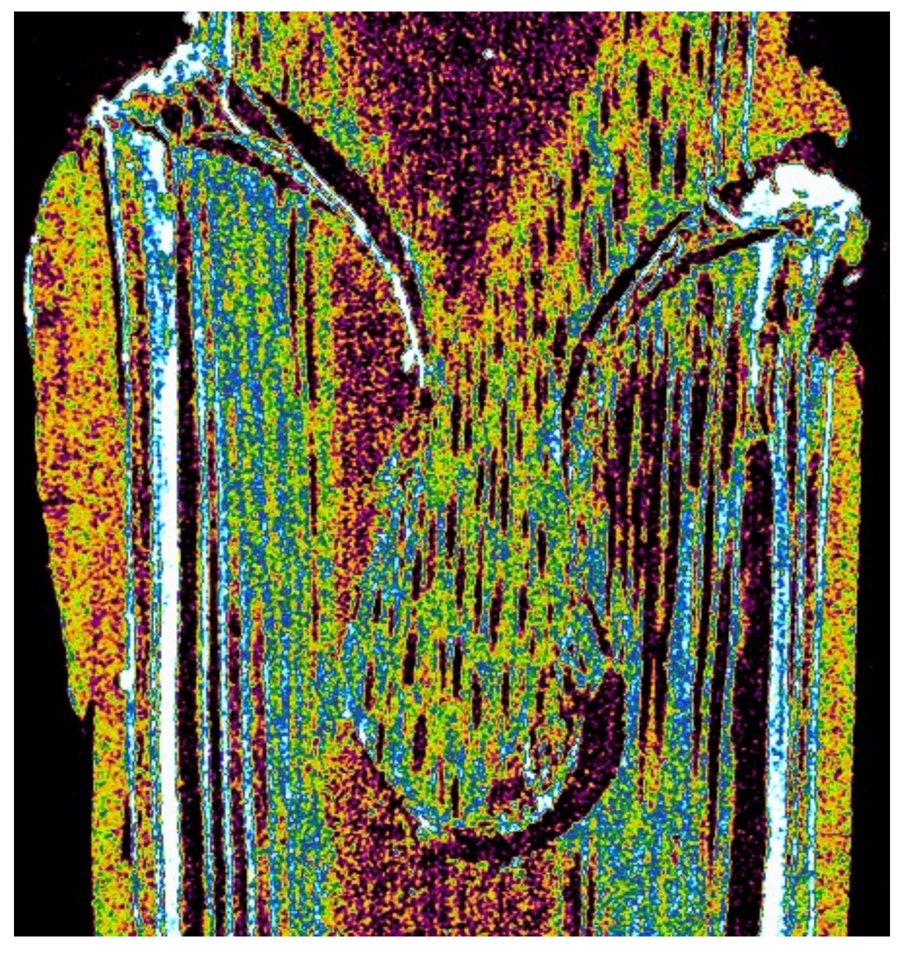
Grafting on GM Rootstocks



X-ray computerized microtomography of a *Vitis* graft

Table 2. Application of transgenic rootstocks in grafting woody plants.

Nontransgenic scion	Transgenic rootstock	Trait	Movement direction	Experimental evidence	Reference
Five apple cultivars	Transgenic apple rootstock 'M26'-rolB and 'M9'-rolB1	The rolB transgenic rootstocks showed increased rooting ability and reduced plant size. The rolB transgenic rootstocks altered growth and development of nontransgenic scion cultivars.	*RolB* mRNA was not detected in the tissues of nontransgenic scion cultivars.	Reverse transcription polymerase chain reaction (RT-PCR)	Welander et al., 1998; Zhu et al., 2000; Zhu et al., 2001; Smolka et al., 2010
Not applicable (NA)	Transgenic common pear 'BP10030' containing the *rolB*	The rolB transgenic rootstocks showed the increased rooting ability.	NA	NA	Zhu et al., 2003
NA	Transgenic apple rootstock 'Alnarp 2' overexpressing the arabidopsis gibberellic acid insensitive gene (*gai*)	Transgenic 'Alnarp 2' showed reduced plant size and rooting ability.	NA	NA	Zhu et al., 2008
Nontransgenic scion grape 'Chardonnay'	Transgenic lines of the grapevine rootstocks '41B' (*Vitis vinifera* × *V. berlandieri*) expressing the coat protein gene of *Grapevine fanleaf virus* (GFLV)	Nontransgenic scions on three out of 16 independent transgenic rootstock lines showed GFLV resistance.	Rootstock-to-scion transfer of the overexpressing coat protein of the GFLV	NA	Vigne et al., 2004
Walnut (*Juglans regia*) 'Chandler'	Walnut hybrid (*Juglans hindsii* × *J. regia*) rootstock expressing *rolABC*	Transgenic rootstock showed phenotype changes but did not affect the phenotype of the scion.	NA	NA	Vahdati et al., 2002
NA	Transgenic citrange (*Citrus sinensis* × *Poncirus trifoliata*) rootstock 'Carrizo' overproducing proline	Transgenic plants had proline accumulation but did not show morphological alterations.	NA	NA	Molinari et al., 2004
Grapevine 'Cabernet Sauvignon'	Transgenic european grapevine 'Thompson Seedless' expressing the Shiva-1 lytic peptide gene	Presence of the Shiva-1 peptide was detected in xylem sap of the nontransgenic scion.	Rootstock-to-scion transfer of the Shiva-1 peptide	Enzyme-linked immunoabsorbent assay (ELISA)	Dutt et al., 2007
Sweet orange 'Tarocco Nucellare'	Transgenic citrange 'Troyer' over-expressing *rolABC* genes of agrobacterium (*Agrobacterium rhizogenes*)	Transgenic rootstock showed increased rooting ability and reduced plant size. Nontransgenic scion grafted on transgenic rootstock had reduced plant size and altered hormone levels.	NA	NA	La Malfa et al., 2011
Sweet cherry 'Emperor Francis'	Transgenic cherry rootstocks 'Gisela 6' and 'Gisela 7' expressing short hairpin RNAs of genomic RNA3 of *Prunus necrotic ringspot virus* (PNRSV-hpRNA)	PNRSV resistance in transgenic rootstock as well as nontransgenic scion.	Rootstock-to-scion transfer of hpRNA-derived PNRSV-specific siRNAs	Small RNA sequencing and PNRSV tolerance	Song et al., 2013a; Zhao and Song, 2014

J. AMER. SOC. HORT. SCI. 140(3):203–213. 2015.

Reverse Breeding

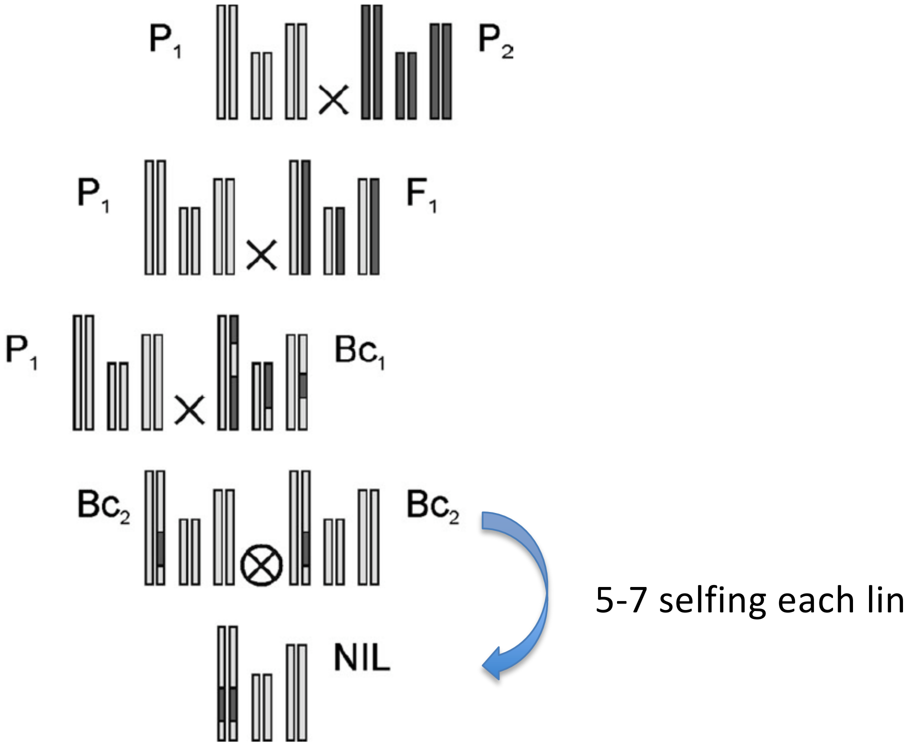
Reverse Breeding

- Reverse breeding (RB) is a novel plant breeding technique designed to directly produce near isogenic parental lines for any heterozygous plant, one of the most sought-after goals in plant breeding.

- The method is based on reducing genetic recombination in a selected heterozygote by eliminating meiotic crossing over.

Hans de Jong and Erik Wijnker
Laboratory of Genetics, Wageningen University, Wageningen, the Netherlands

Near isogenic lines



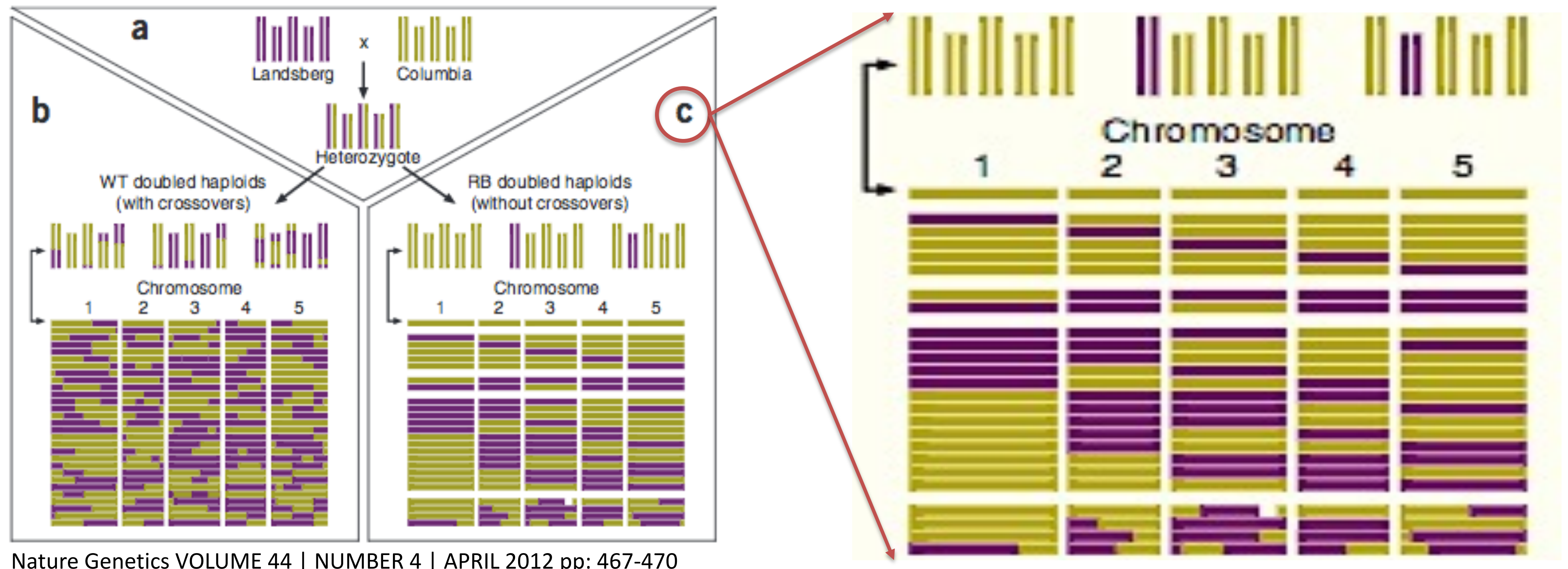
The construction of NILs through repeated backcrossing. Crossing two genetically distinct parental lines results in a heterozygous offspring. By backcrossing the heterozygote to the recipient parent, the proportion of donor parental genome is reduced with 50%. In recurrent backcrosses, heterozygosity is further reduced to a small introgression followed by selfing or sibling mating to obtain a near isogenic line (NIL).

Scott A. Rifkin (ed.), Quantitative Trait Loci (QTL): Methods and Protocols, Methods in Molecular Biology, vol. 871

Reverse breeding

Reverse breeding comprises three essential steps:

- the suppression of crossover recombination in a selected plant;
- auto pollination and;
- regeneration of double-haploids from spores containing non-recombinant chromosomes.



Nature Genetics VOLUME 44 | NUMBER 4 | APRIL 2012 pp: 467-470

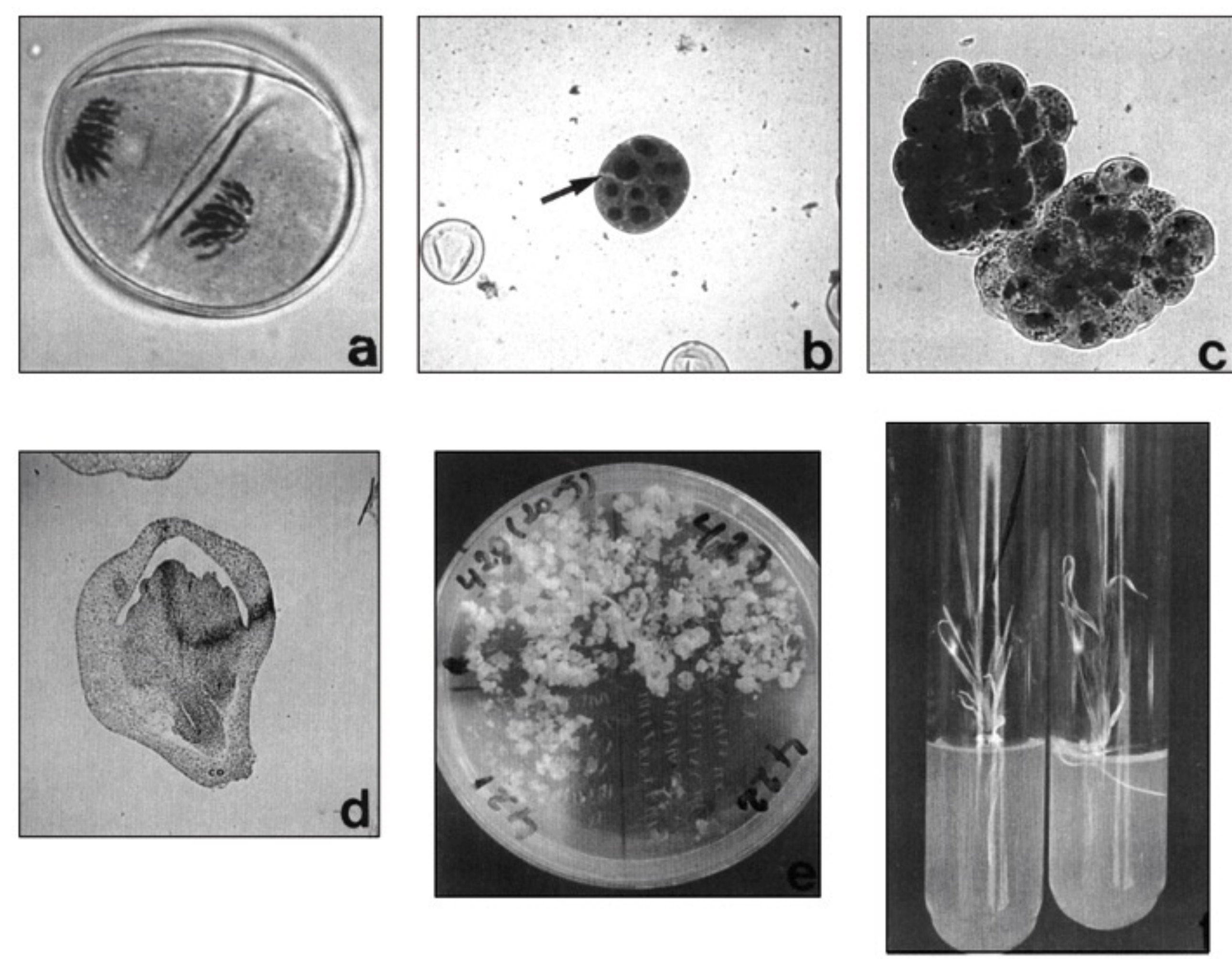
C represents 21 different genotypes in which no crossovers occurred from among 36 reverse-breeding doubled haploids.
The first row represents the genotype of one of the recovered original parents;
the next seven genotypes represent chromosome-substitution lines and the remainder are mosaics of Col and Ler chromosomes.
The last four represent genotypes of haploid offspring that showed crossovers

How to suppress cross-over

- Genes that are essential in crossover formation but leave the chromosome structure intact are particularly useful.
- Examples are the Arabidopsis ASY1 and the rice ASY1 homologue PAIR2, the mutants of which display univalents at metaphase I.
- One typical approach would be to use RNA interference (RNAi) constructs to silence the relevant genes.

What is a doubled haploid?

- A **doubled haploid** (DH) is a genotype formed when haploid cells undergo chromosome doubling.



Somatic embriogenesis in *Hordeum vulgaris* polen grains

http://dx.doi.org/10.1590/S0034-71082000000200016

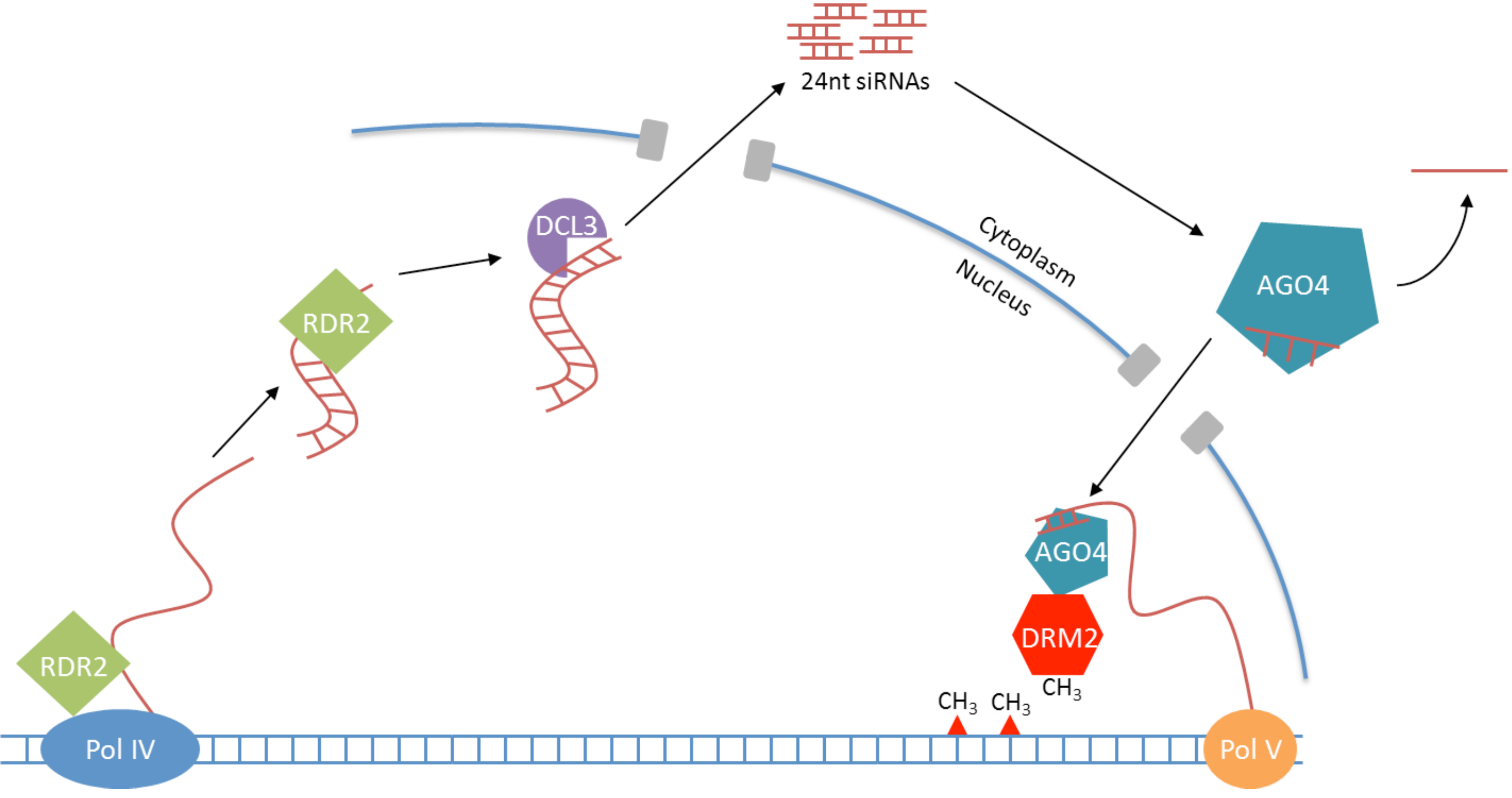
RNA-directed DNA Methylation

RNA-directed DNA Methylation

- RNA-directed DNA methylation is a pathway that results in the *de novo* methylation of genomic elements and is mediated by 24 nt siRNAs, RNA polymerases IV and V, argonaute 4, as well as many other accessory proteins.

- DNA/Histone methylation tend to result in gene silencing.

Simplified scheme of RdDM

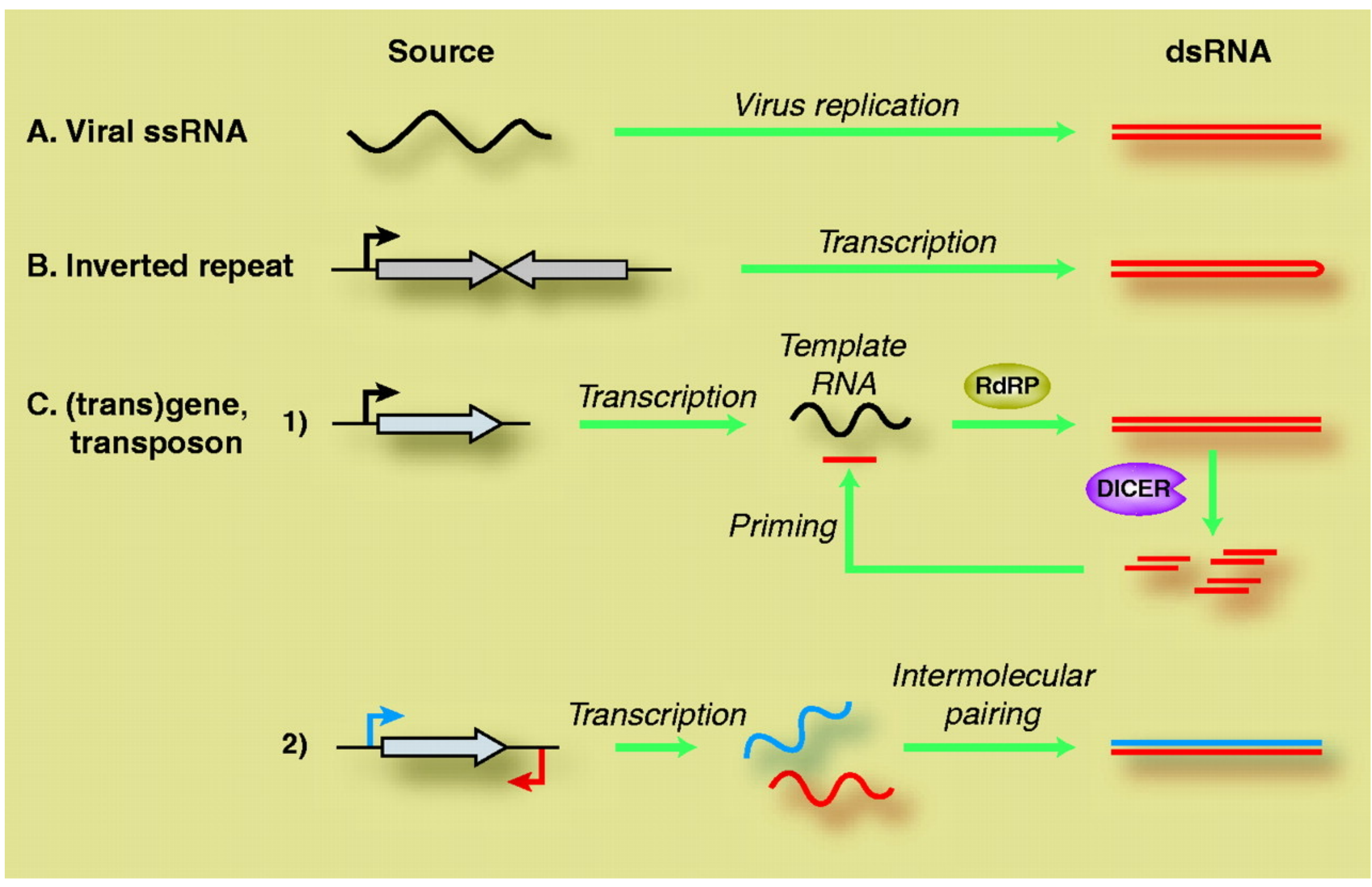


RDR2 - RNA-dependent RNA polymerase 2

DRM2 - DNA (cytosine-5)-methyltransferase

https://pods.iplantcollaborative.org/wiki/display/PLS599/Science

Mechanisms that generates dsRNA in plants



© The Company of Biologists Limited 2004

Olivier Mathieu, and Judith Bender J Cell Sci 2004;117:4881-4888

- RdDM can be used to improve a wide variety of plant traits in most plant species by the down-regulation of an endogenous gene, without alteration of the genetic material.

- Methylation events are thought to be transmittable to offspring.

Target mimicry for MiRNA regulation

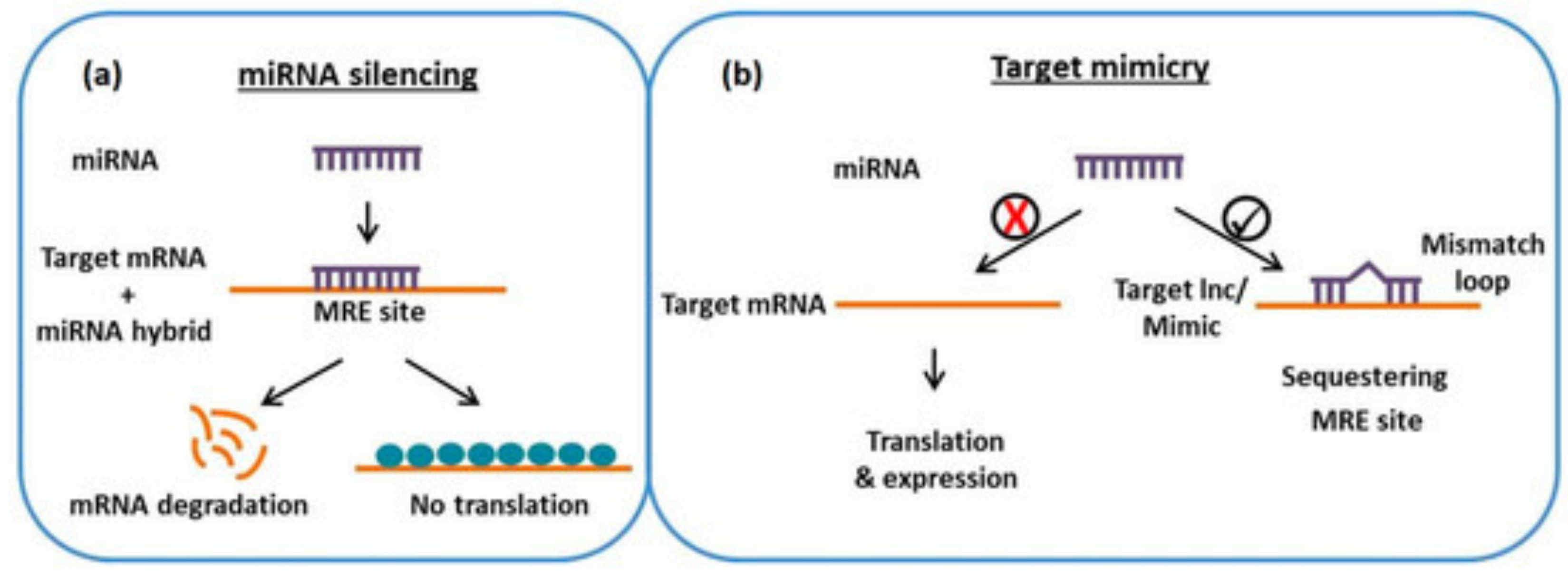
Non-protein coding gene INDUCED BY PHOSPHATE STARVATION1 (IPS1) from A. thaliana

IPS1 contains a motif with sequence complementarity to the phosphate (Pi) starvation-induced miRNA miR-399, but the pairing is interrupted by a mismatched loop at the expected miRNA cleavage site.

IPS1 RNA is not cleaved but instead sequesters miR-399.

Thus, IPS1 overexpression results in increased accumulation of the miR-399 target PHO2 mRNA and, concomitantly, in reduced shoot Pi content.

Target mimicry provides a new mechanism for regulation of microRNA activity.
Franco-Zorrilla JM, *et al*. Nat Genet, 2007 Aug. PMID 17643101


(a)
miRNA silencing
miRNA
Target mRNA
+
miRNA hybrid
MRE site
mRNA degradation
No translation
(b)
Target mimicry
miRNA
Mismatch
loop
Target mRNA
Target lnc/
Mimic
Sequestering
MRE site
Translation
& expression

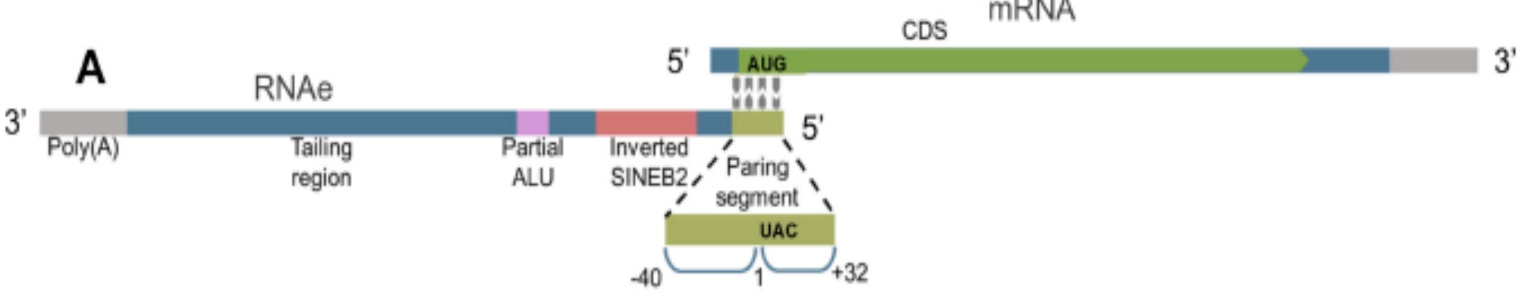
RB
CaMV35S
MIM408
t35S
Kan^R
LB

Thank you for your attention

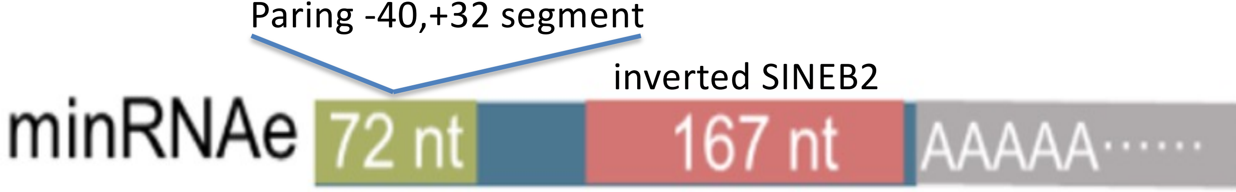
RNAe (enhancer) or SINEUPs

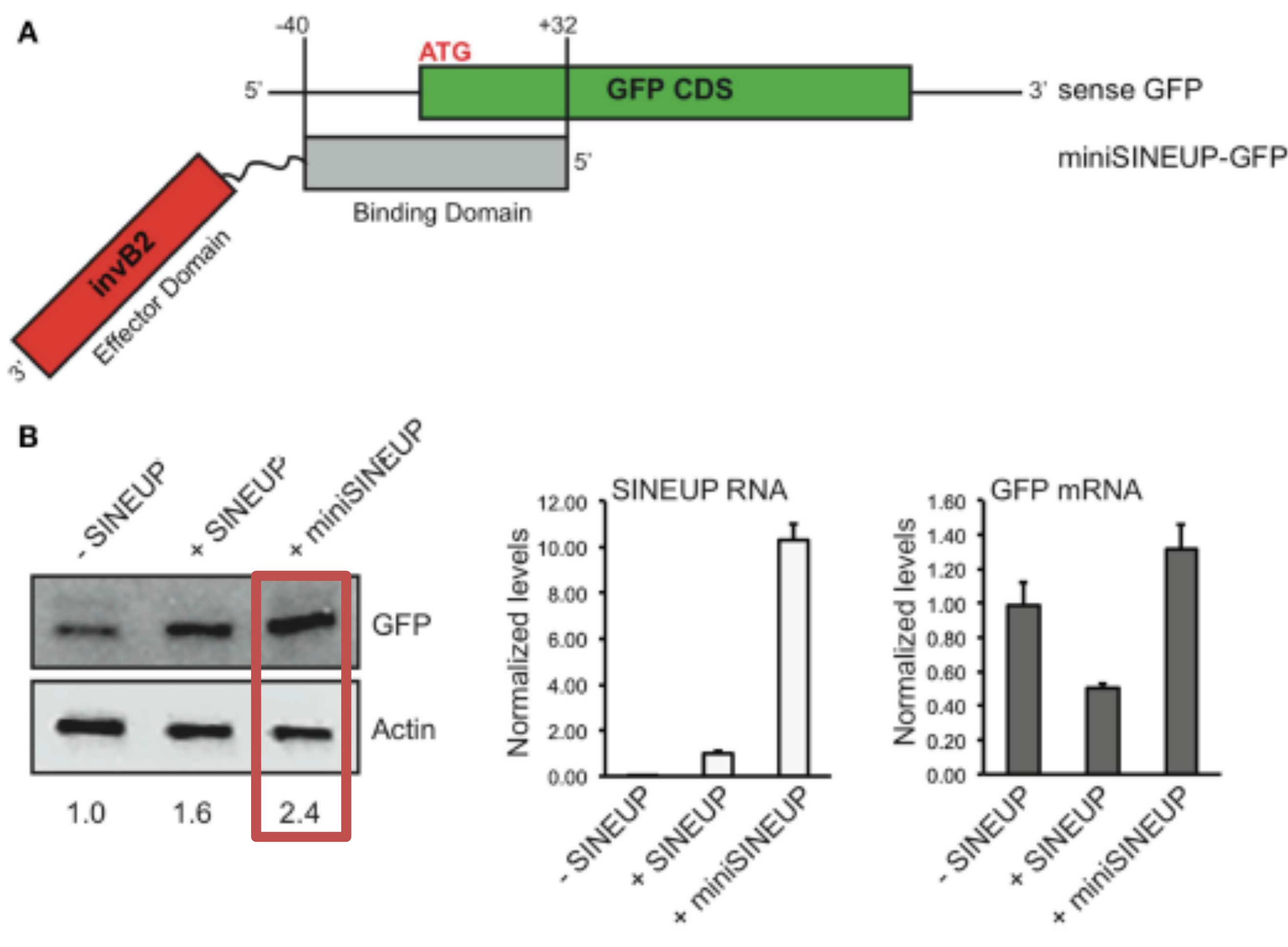
Carrieri et al. reported a new functional class of long non-coding RNAs (lncRNAs) with an overlapping antisense sequence targeting the 5' terminus of mRNAs, which enhances the translation of the corresponding protein at the post-transcriptional level, as in the case of ubiquitin carboxy-terminal hydrolase L1 (UCHL1).

Carrieri et al. (2012) Nature, 491, 454–457.

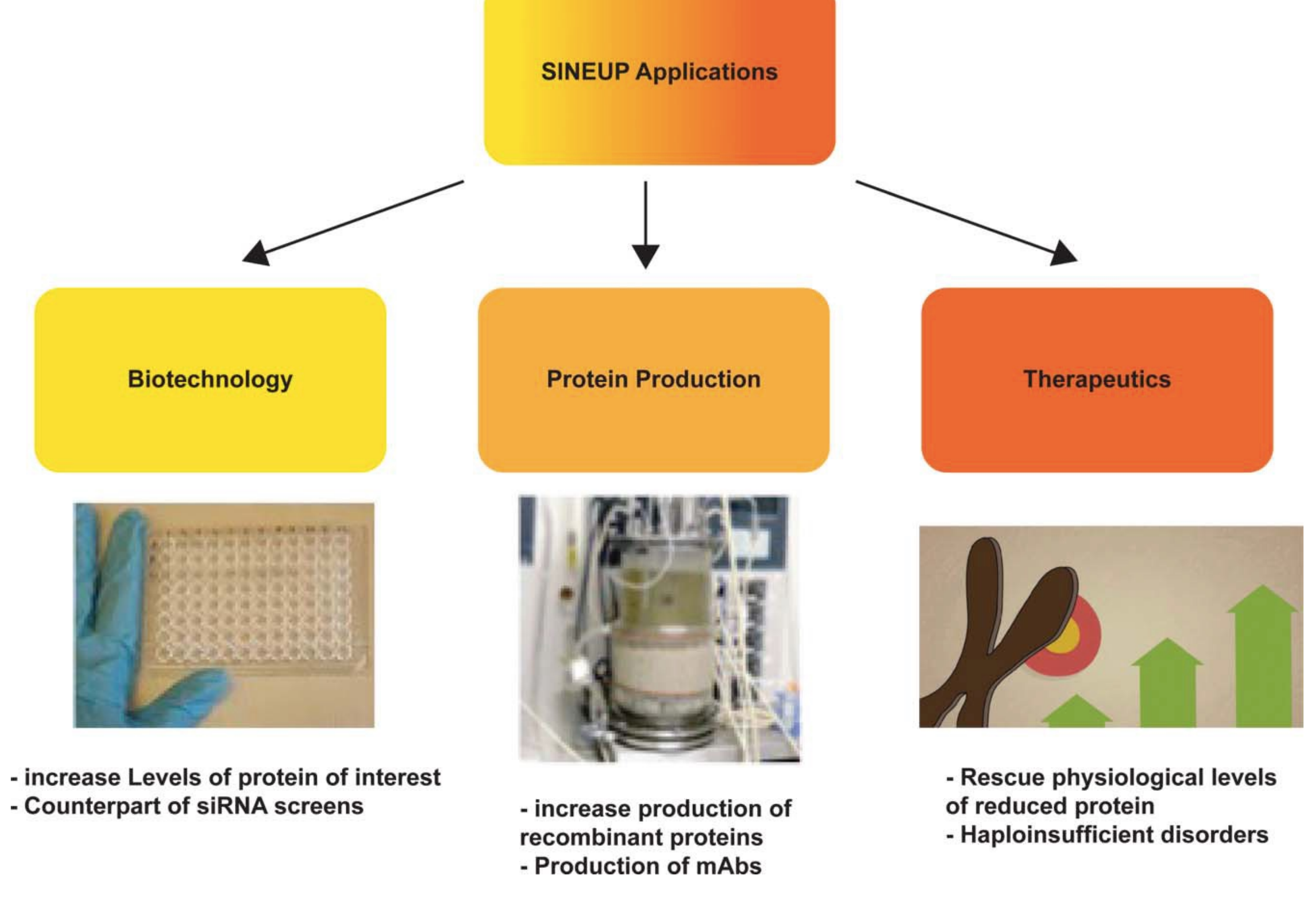


- Truncations of RNAe confirmed that the inverted SINEB2 element is sufficient for the RNAe effect
- Inverted Short interspersed element (SINE) repeat





Zucchelli et al. (2015) *Front. Cell. Neurosci. 9:174.*



Zucchelli et al. (2015) RNA Biology 12:8, 771--779; 2015

Synthetic SINEUP Design

Table 1. Flowchart for Synthetic SINEUP design

1. Target mRNA TSS analysis
⇒ Interrogate public databases to identify the correct transcription initiation site (TSS) and AUG-surrounding region of the mRNA of interest in the relevant cell(s) and tissue(s).
⇒ FANTOM5 database in Zenbu Genome Browser tool (http://fantom.gsc.riken.jp/5/) {Severin, 2014 #19}
⇒ ENCODE database in UCSC genome browser tool (http://genome.ucsc.edu/ENCODE/) (ADD REF?)
2. Design SINEUP BD
⇒ Identify the −40/+33 region: +33 bases in the coding sequence to −40 nucleotides (in annotated 5' untranslated region) before the translation start site of target mRNA,
⇒ Identify the −40/+4 around initiating AUG
⇒ Reverse complement analysis
⇒ Design −40/+33 (Long) and −40/−4 (Short) BD variants
3. Cloning SINEUP BD
⇒ Primer design
⇒ Annealing and PCR amplification
⇒ Cloning SINEUP BD upstream to SINEUP ED
⇒ Transfer to Mammalian expression vector (any eukaryotic promoter-containing vector). Vectors for retroviral and lentiviral packaging can also be efficiently used.
4. Measure SINEUP Activity
⇒ Transfect/infect target cells of interest with gene-specific SINEUP
⇒ Measure protein quantities by western blot, and/or flurescence- or luminescence-based methods
⇒ Measure target mRNA and SINEUPs RNA levels with qRT-PCR
⇒ Estimate SINEUP activity as fold changes in protein levels encoded by targeted mRNAs in the presence/absence of SINEUP with mRNA amounts kept constant ($p > 0.05$).
5. Troubleshooting⇒ Anatomy of target mRNA is not known in cells of interest: perform 5′RACE experiments to identify real TSS
⇒ 5′UTR of target mRNA is shorter than −40 nucleotides: design SINEUPs of different length spanning region around initiating AUG
⇒ Target cells are not suitable for SINEUP activity: test SINEUP-GFP activity as positive control to monitor new cellular system
⇒ Low SINEUP RNA levels: change transfection reagents, test viral-mediated infection to achieve good quantities of SINEUP RNA. Test different times after transfection, before sample collection.

Zucchelli et al. (2015) RNA Biology 12:8, 771--779; 2015