New Breeding Techniques (NBTs) – Una nueva era en el mejoramiento de plantas.

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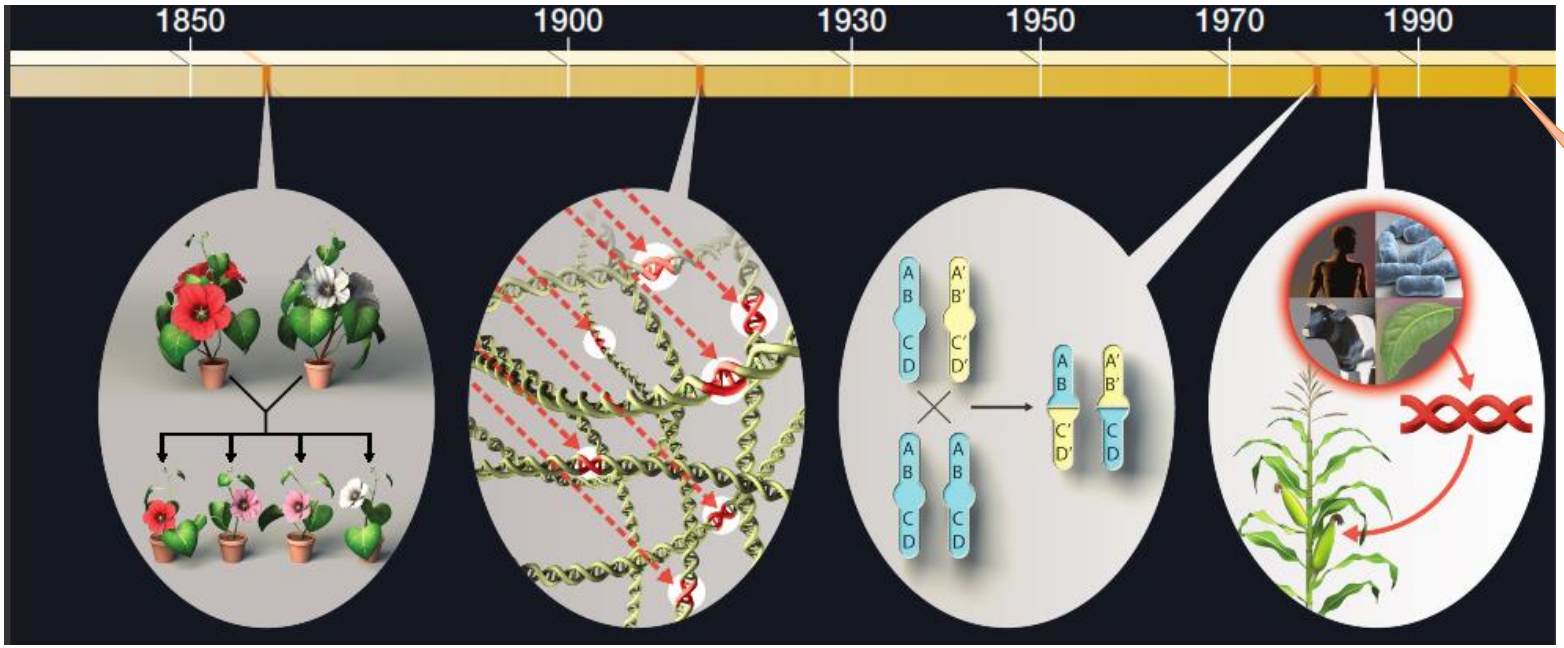
EVOLUCIÓN DEL MEJORAMIENTO GENÉTICO VEGETAL



2010:
NBTs

Cruzamientos

Mutagénesis

Selección asistida por marcadores (MAS)

Transformación genética

NBTs

- Cisgenia (e intragenia)
- Tecnología de nucleasas zinc-finger
- Mutagénesis dirigida por oligonucleótidos
- Metilación de DNA dependiente de RNA
- Agro-infiltración
- Injertación de variedades no-GM sobre portainjertos GM
- Mejoramiento Reverso
- Genómica sintética

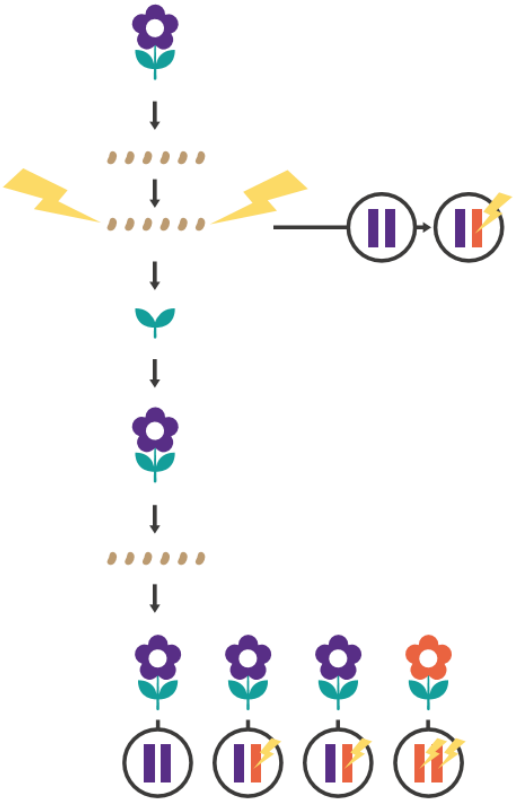
Principales requerimientos para abordar el Mejoramiento de Precisón (Precision Breeding)

- Técnicas de cultivo *in vitro*
- Transformación genética
- Conocimiento de los genomas a trabajar
  - El mejorador trabaja de forma directa y específica con variaciones moleculares que están vinculadas a (o que generan) fenotipos de interés agronómico.
  - Información Clave: Los genomas.

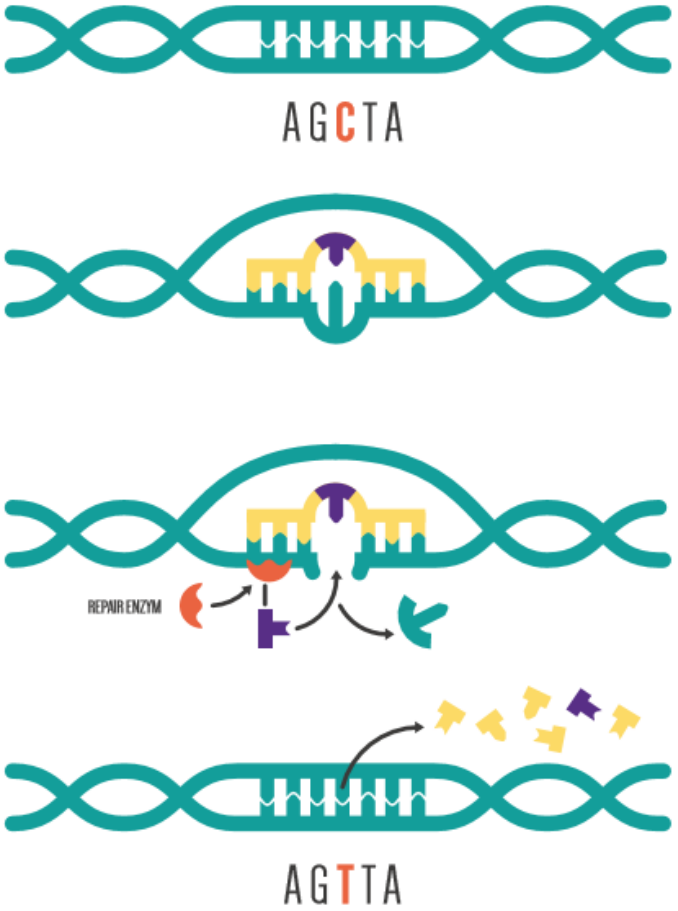
Mutagénesis por oligonucleótidos




Mutagénesis



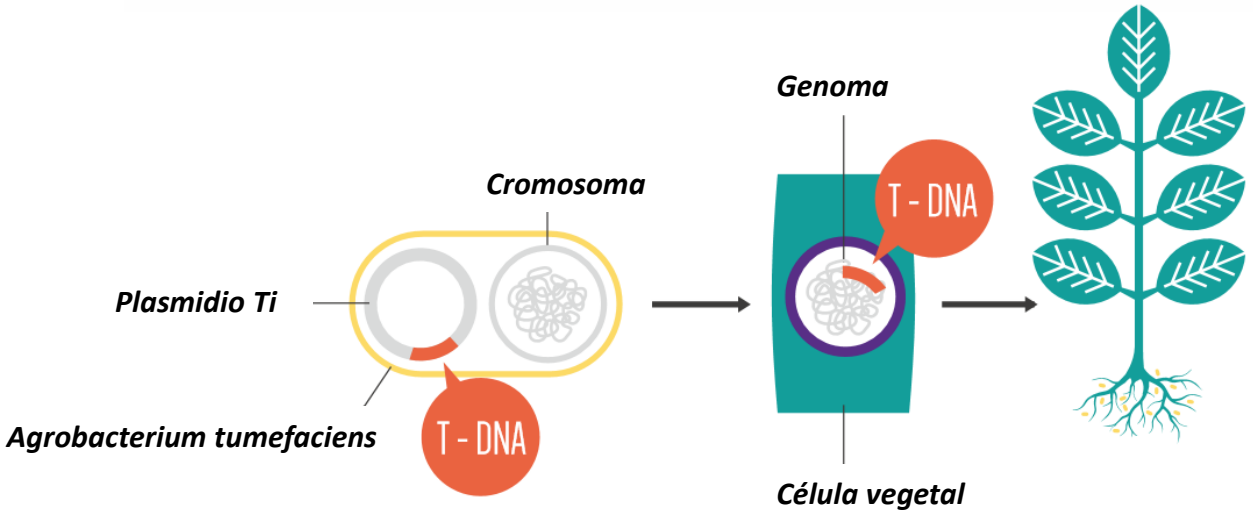
Mutagénesis dirigida por óligos

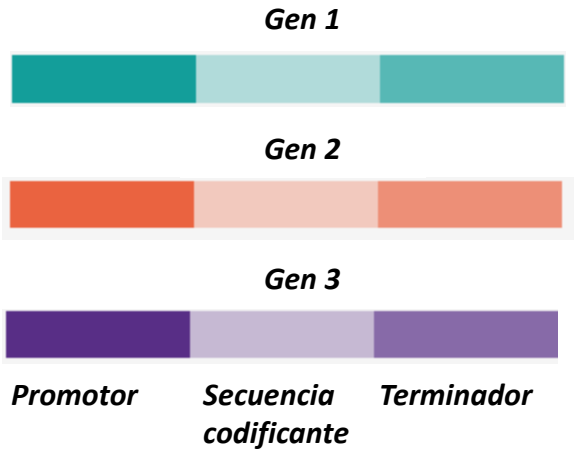


Cisgenia

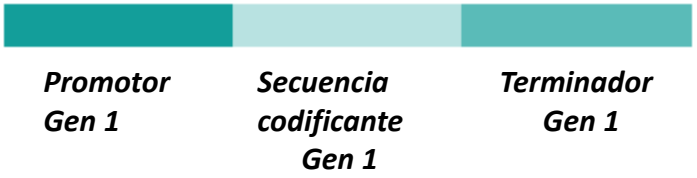



Transgenia

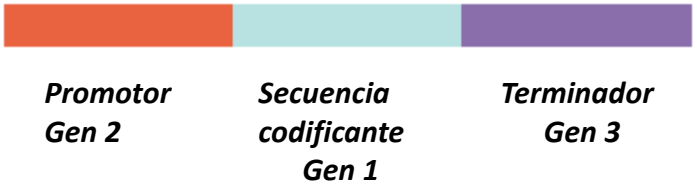




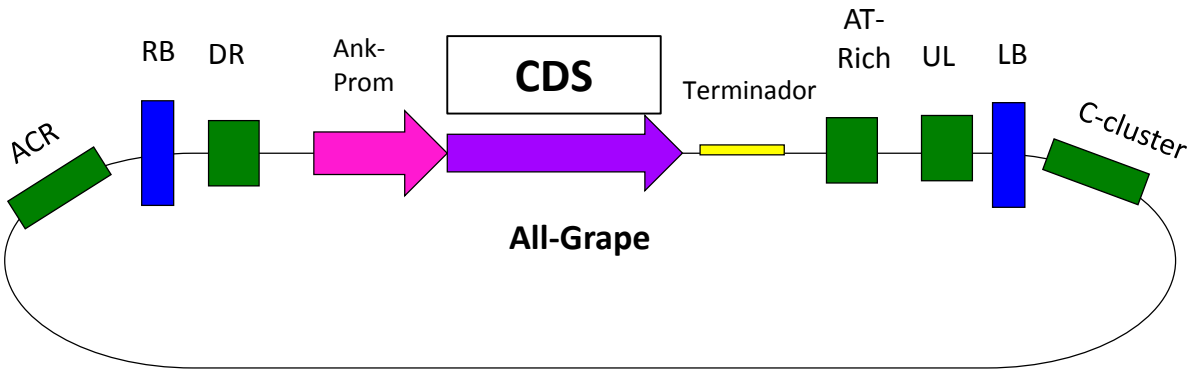
Cisgenia



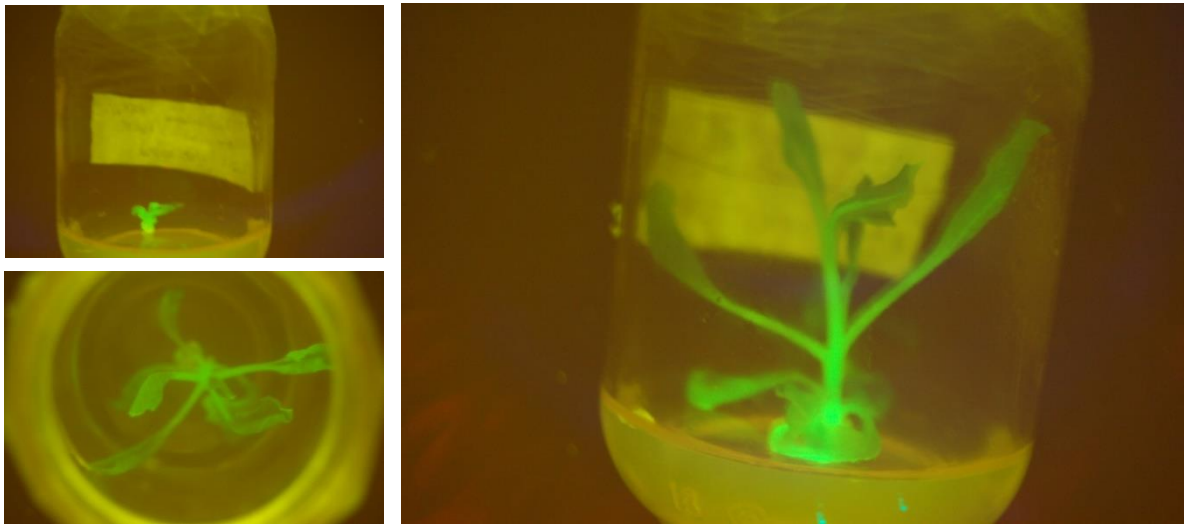
Intragenia

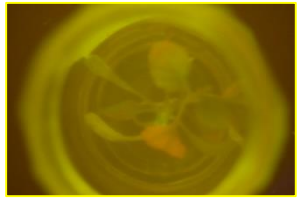


Plant derived Transfer DNAs

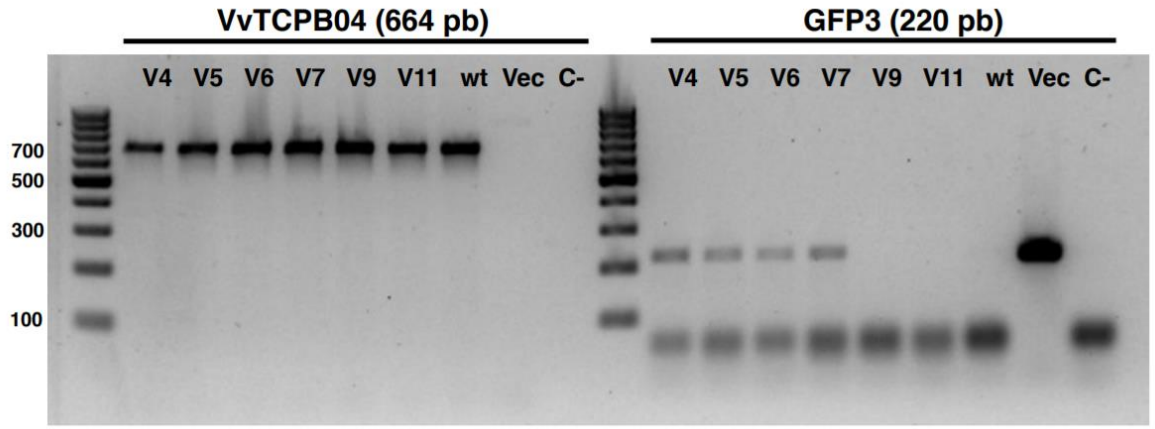


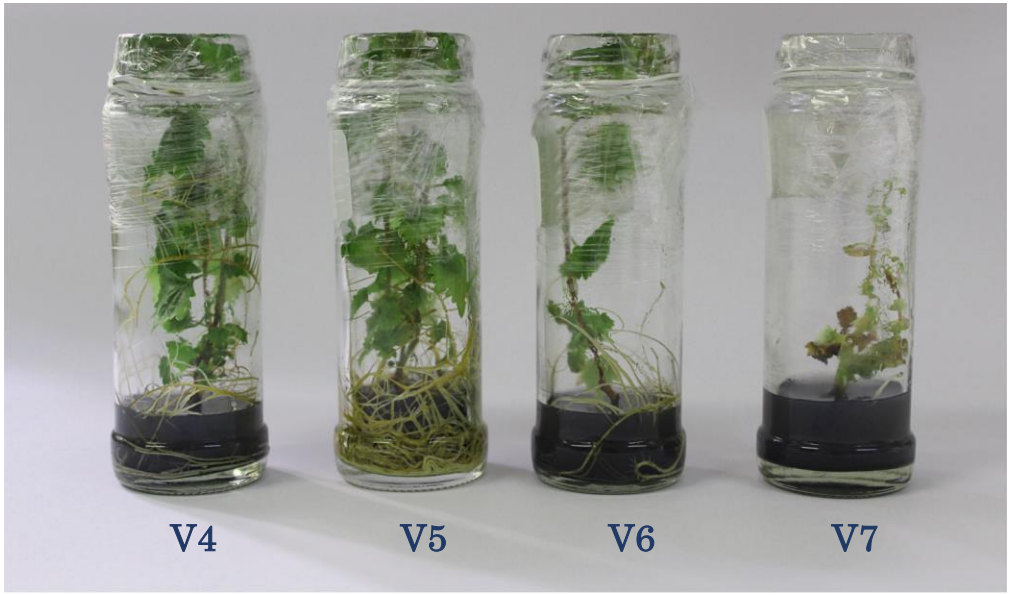
TG *N. benthamiana* + *ALL-G*





WT *N. benthamiana*

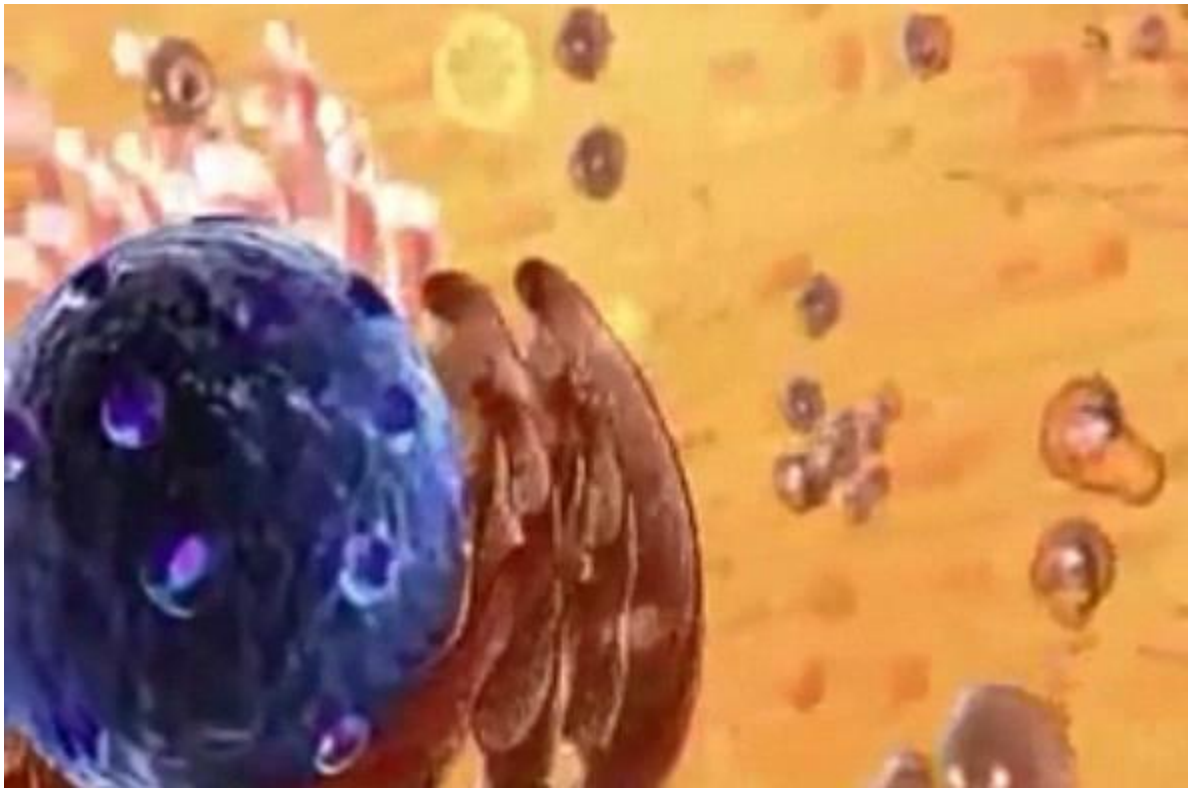

VvTCPB04 (664 pb)
GFP3 (220 pb)
V4 V5 V6 V7 V9 V11 wt Vec C-
V4 V5 V6 V7 V9 V11 wt Vec C-
700
500
300
100


V4
V5
V6
V7

RNA interferente y técnicas asociadas

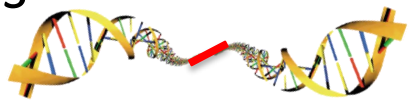



RNA interferente (RNAi): control de la expresión de un gen



siRNAs (plants)

Nucleus



RDR6 or RDR1 // RNA pol II

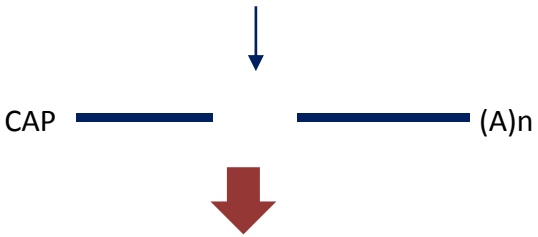
DCL2 or DCL4

HEN1

Cytoplasm

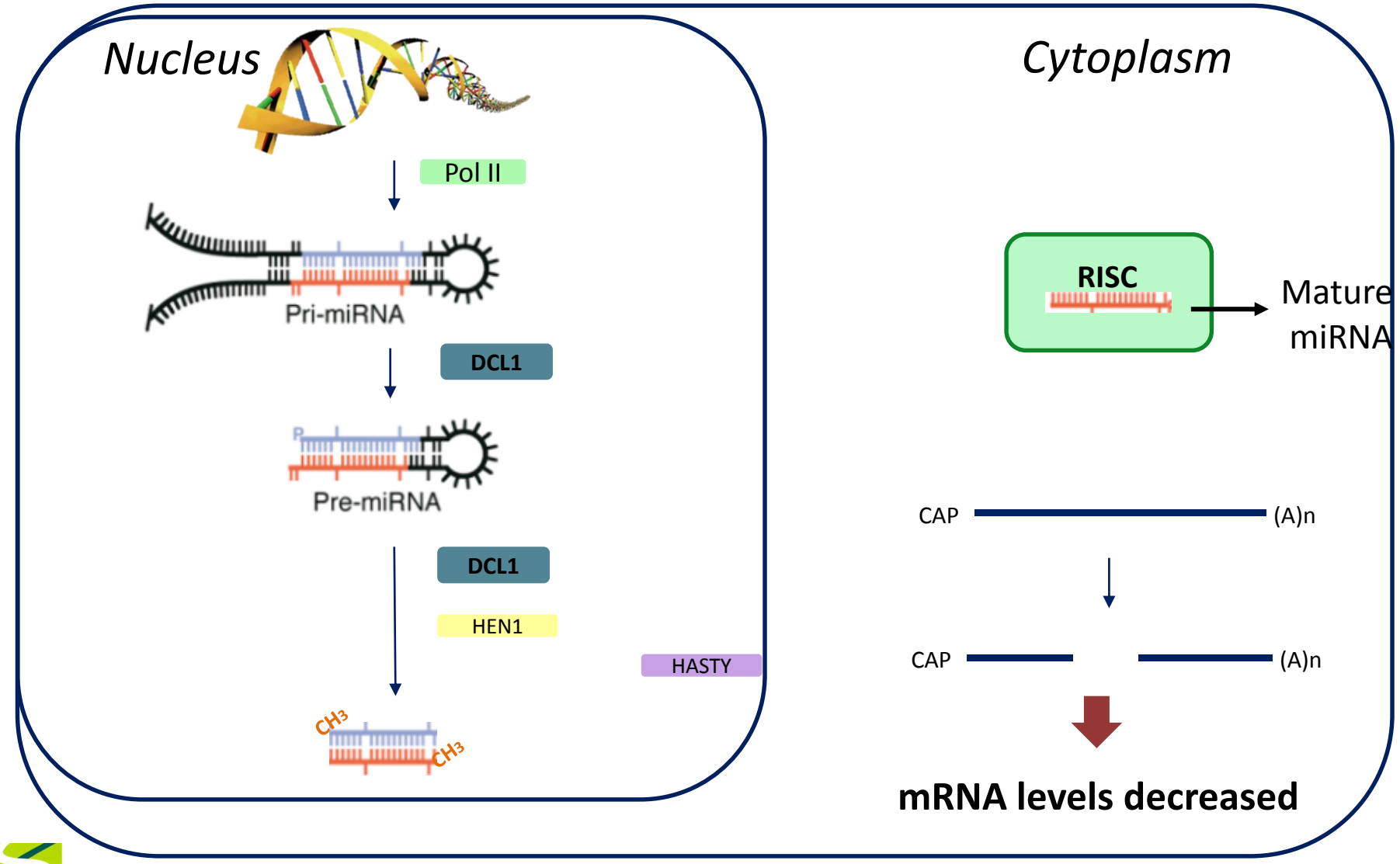
21- or 22-nt

AGO1 **RISC**

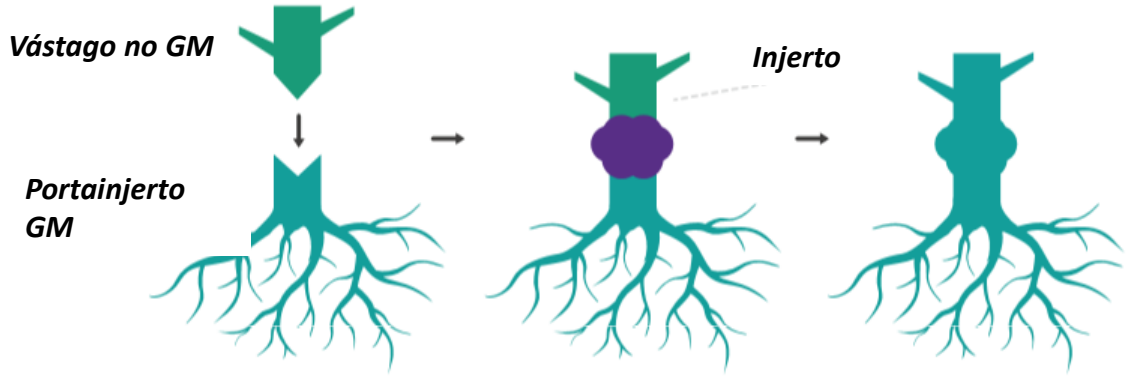


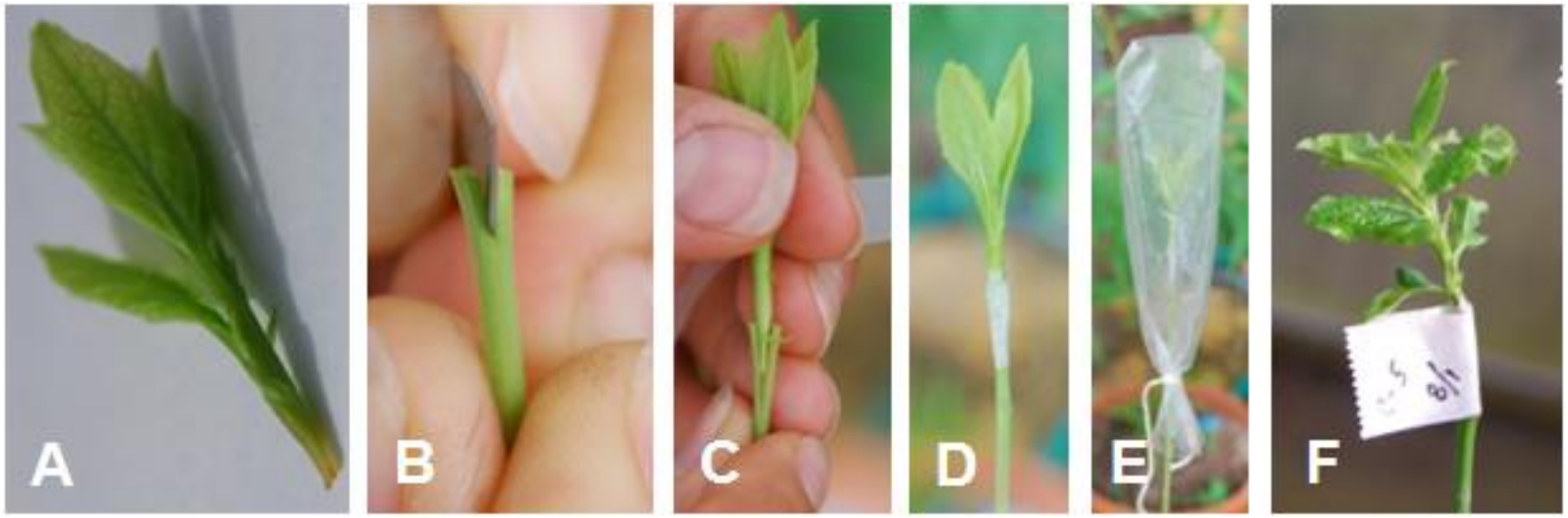
mRNA levels decreased

miRNAs


Nucleus
Pol II
Pri-miRNA
DCL1
Pre-miRNA
DCL1
HEN1
HASTY
CH3
CH3
Cytoplasm
RISC
Mature miRNA
CAP
(A)n
CAP
(A)n
mRNA levels decreased

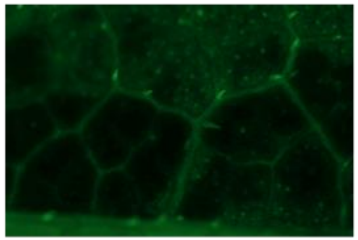
Injertación

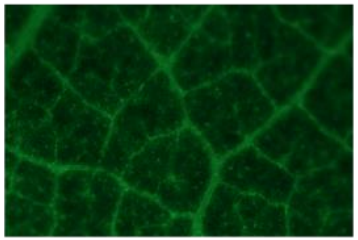


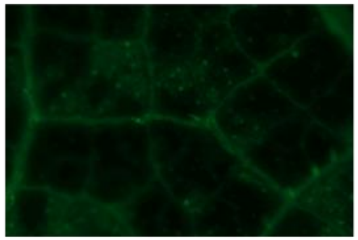


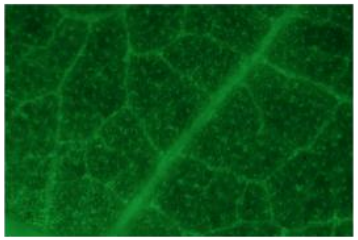
a-miRNA injerto
GFP patrón

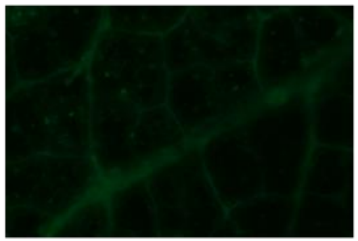
Wild Type injerto
GFP patrón

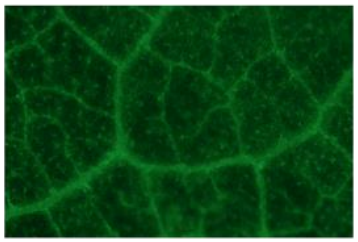










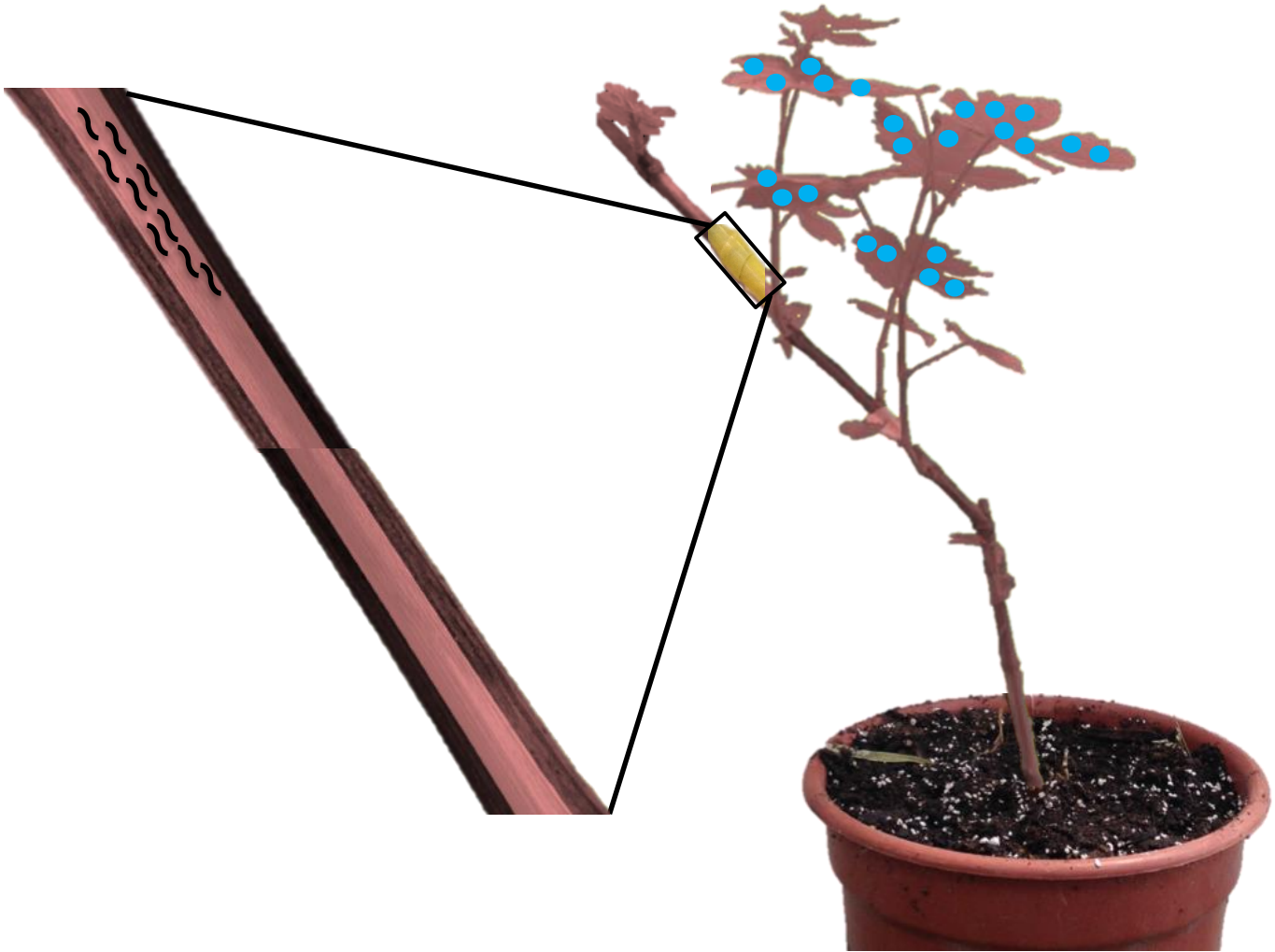


INIA

BIOFRUTALES
Genetic Fruit Improvement

Silenciamiento a larga distancia dependiente de RNA

- Virus

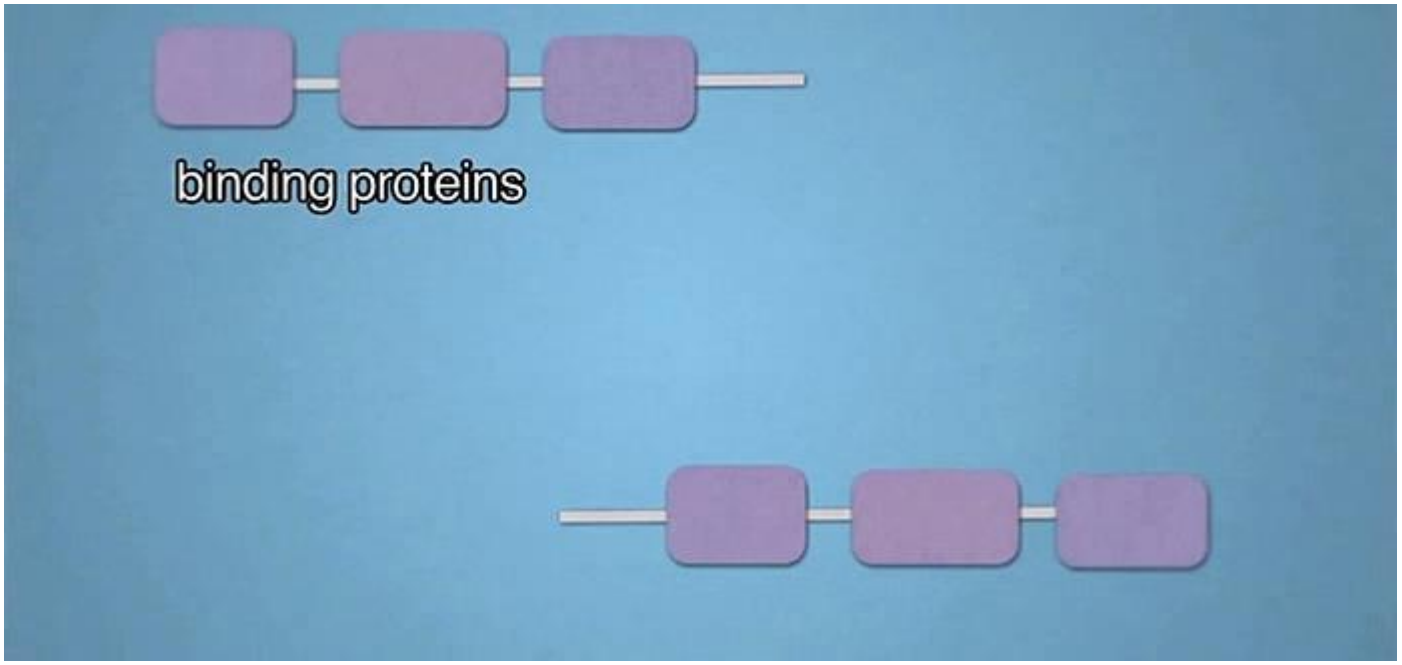


Metilación dependiente de RNA

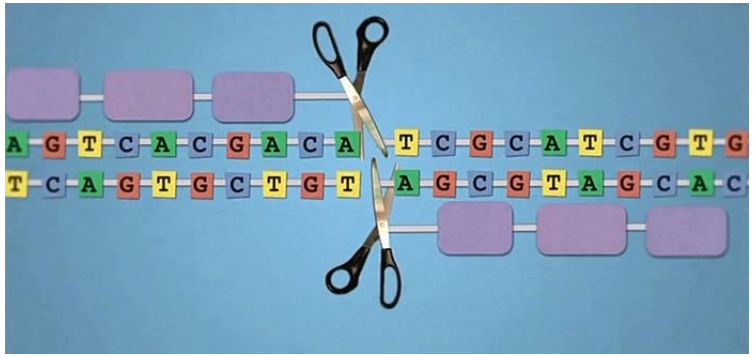
Edición de genomas por nucleasas



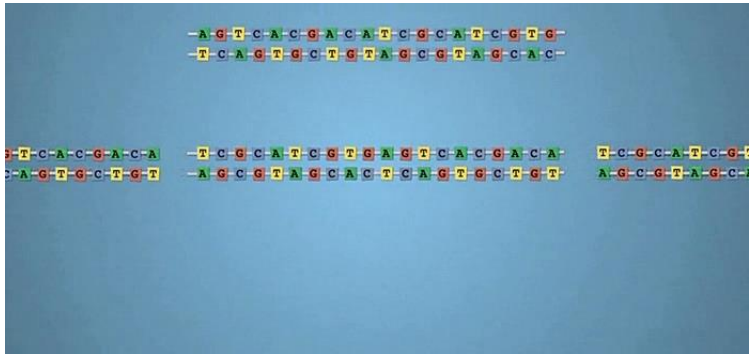

Edición de Genomas por Nucleasas



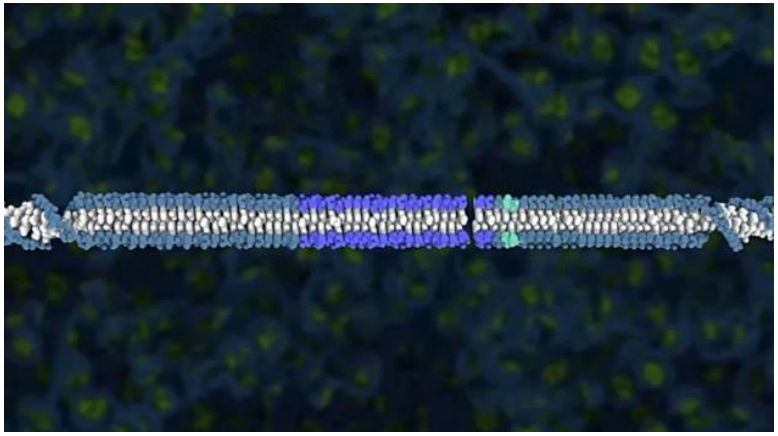
Site Directed Nuclease type 1
SDN1

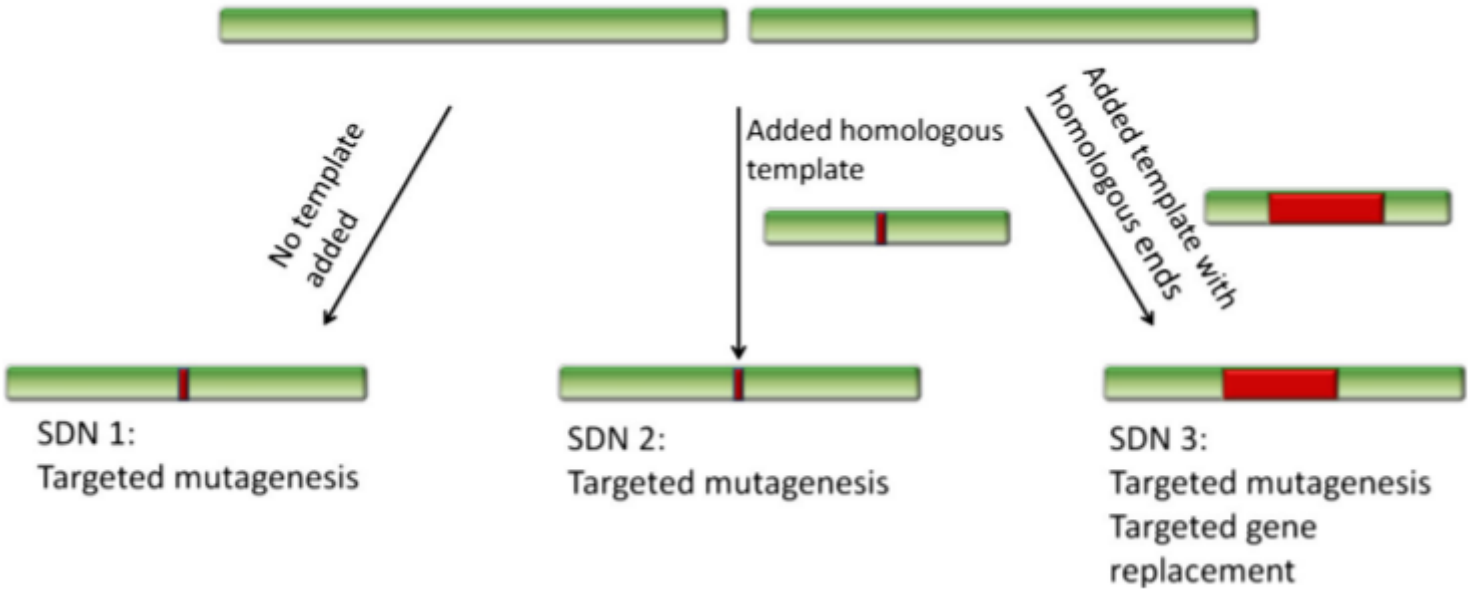


Site Directed Nuclease type 2
SDN2

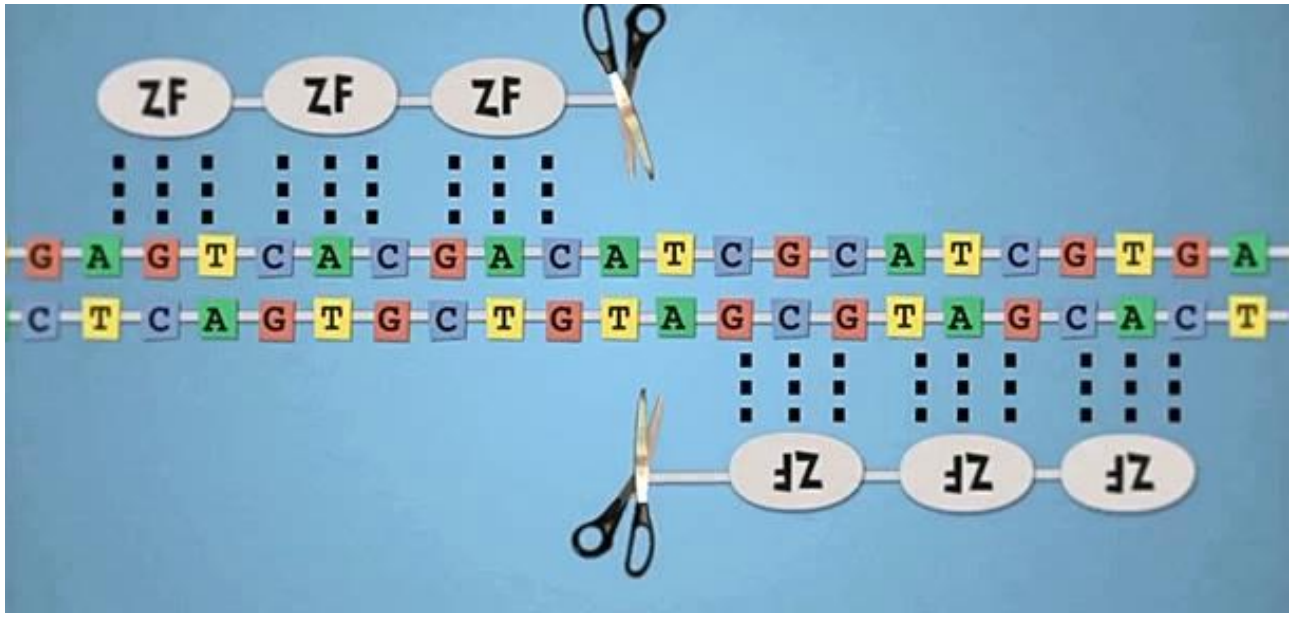


Site Directed Nuclease type 3
SDN3

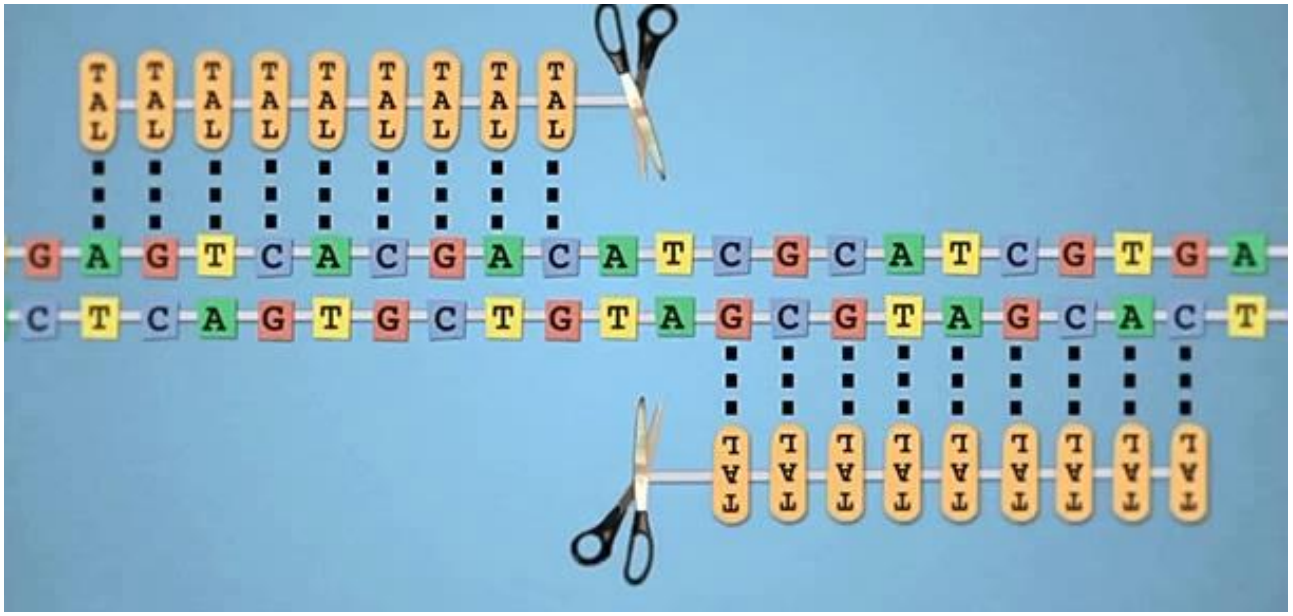



No template added
Added homologous template
Added template with homologous ends
SDN 1:
Targeted mutagenesis
SDN 2:
Targeted mutagenesis
SDN 3:
Targeted mutagenesis
Targeted gene replacement

Nucleasas de dedos de Zinc (ZFNs)

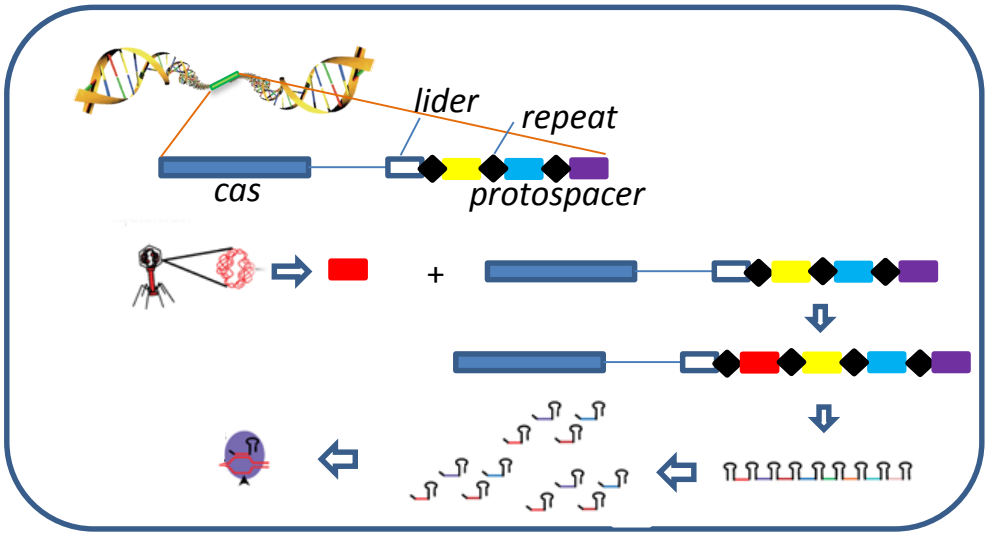


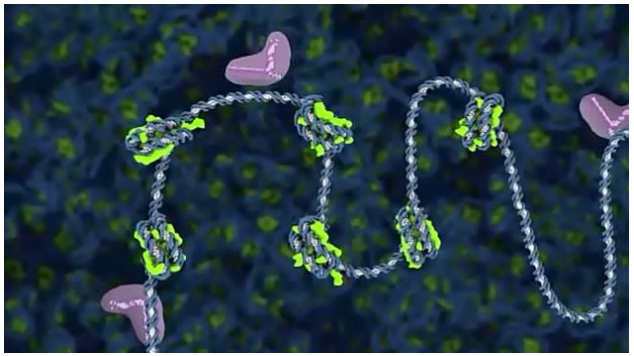
TALEN: transcription activator-like efector nucleases (nucleasas efectoras tipo activador de transcripción)

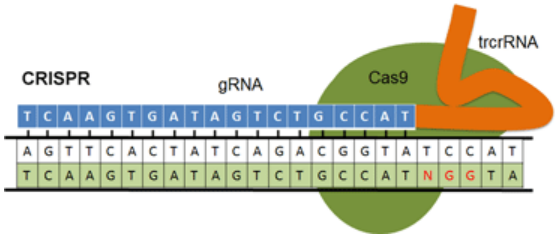


CRISPR/*cas*
clustered regularly interspaced short palindromic repeats

Un sistema (immune) adaptativo en bacterias
por ejemplo:
Streptococcus pyogenes

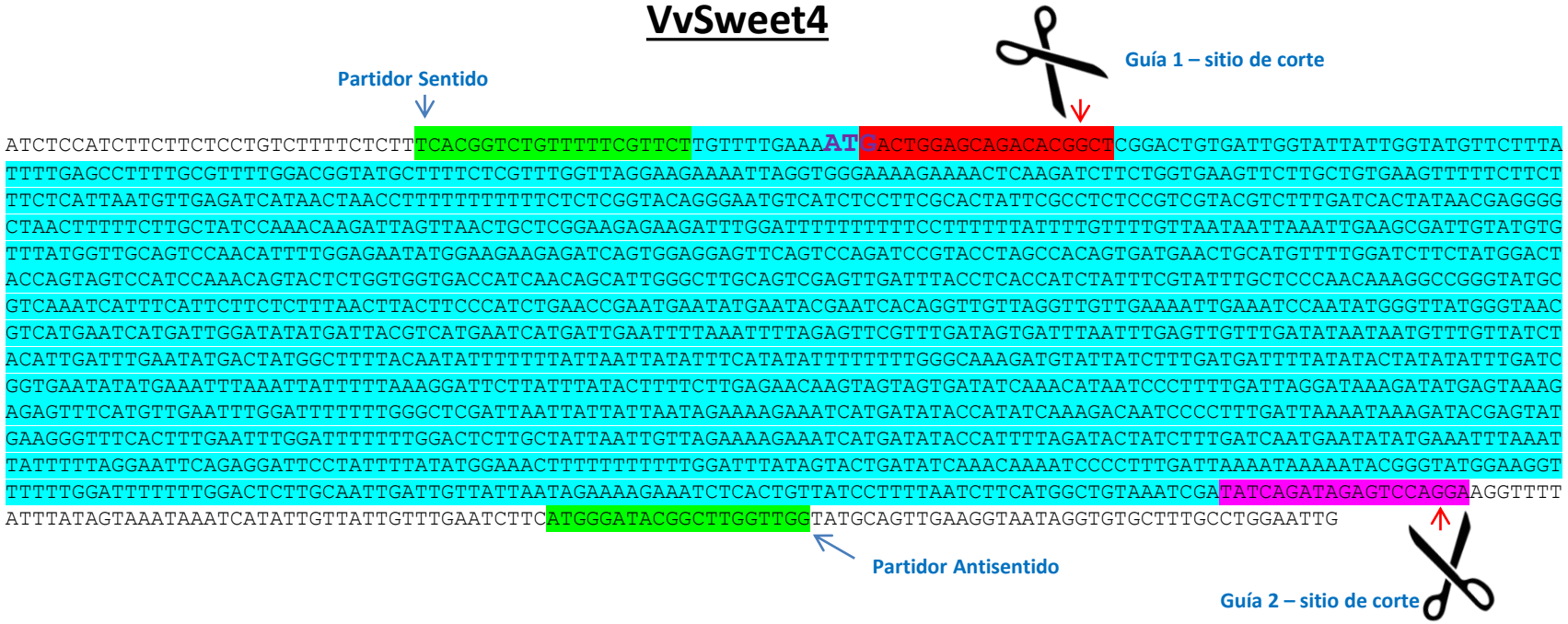






Estudio zona de edición gen SWEET vides

VvSweet4



VvSweet4 Editado

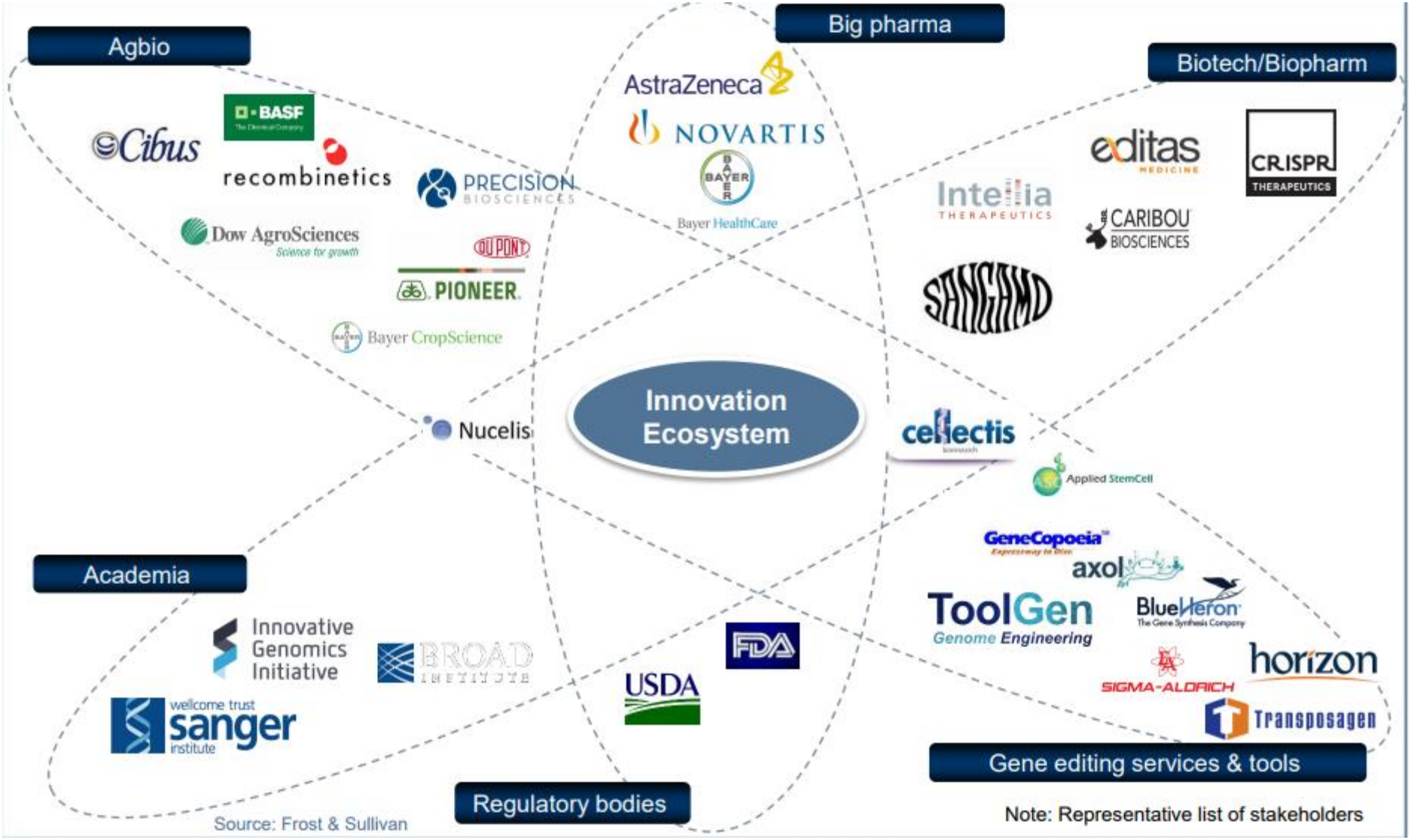
G16	TCACGGTCTGTTTTTCGTTCTTGTTTTGAAAATGACTGGAGCAGACACGGGAAGGTTTTATTTATAGTAAATAAATCATATTGTTATTGTTTGAATCTTCATGGGATACGGCTTGGTTGG
G2, G7, G9	TCACGGTCTGTTTTTCGTTCTTGTTTTGAAAATGACTGGAGCAGACACG~~G~~GAAGGTTTTATTTATAGTAAATAAATCATATTGTTATTGTTTGAATCTTCATGGGATACGGCTTGGTTGG
G14, G15	TCACGGTCTGTTTTTCGTTCTTGTTTTGAAAATGACTGGAGCAGA~~CACG~~GGAAGGTTTTATTTATAGTAAATAAATCATATTGTTATTGTTTGAATCTTCATGGGATACGGCTTGGTTGG
G18, G19, G21, G22, G23	TCACGGTCTGTTTTTCGTTCTTGTTTTGAAAATGACTGGAGCAG~~ACACGG~~GAAGGTTTTATTTATAGTAAATAAATCATATTGTTATTGTTTGAATCTTCATGGGATACGGCTTGGTTGG
G25	TCACGGTCTGTTTTTCGTTCTTGTTTTGAAAATGACTGGAGCAGACAC~~G~~AGGAAGGTTTTATTTATAGTAAATAAATCATATTGTTATTGTTTGAATCTTCATGGGATACGGCTTGGTTGG

INIA

BIOFRUTALES
Genetic Fruit Improvement

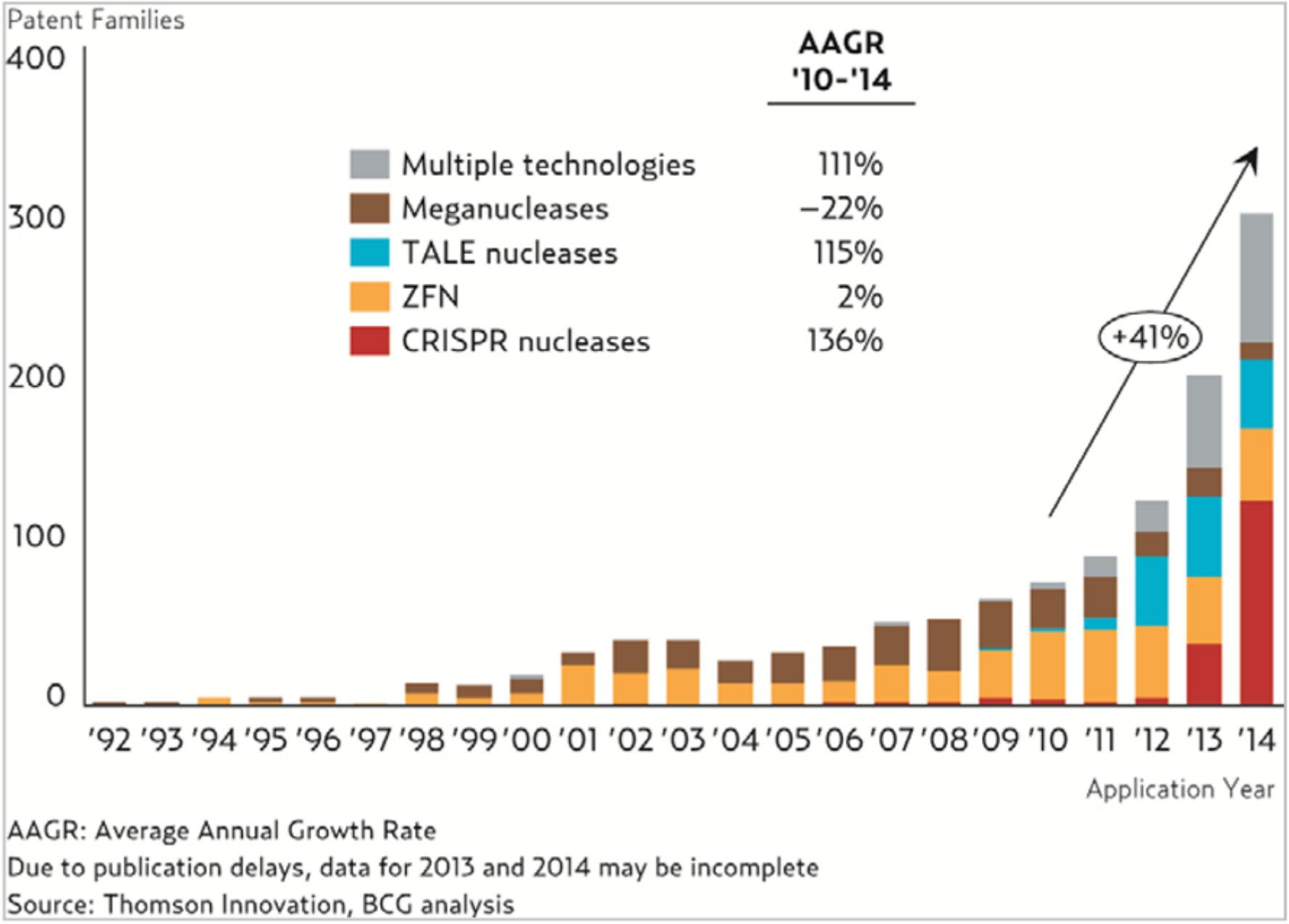
El entorno



Source: Frost & Sullivan

Note: Representative list of stakeholders

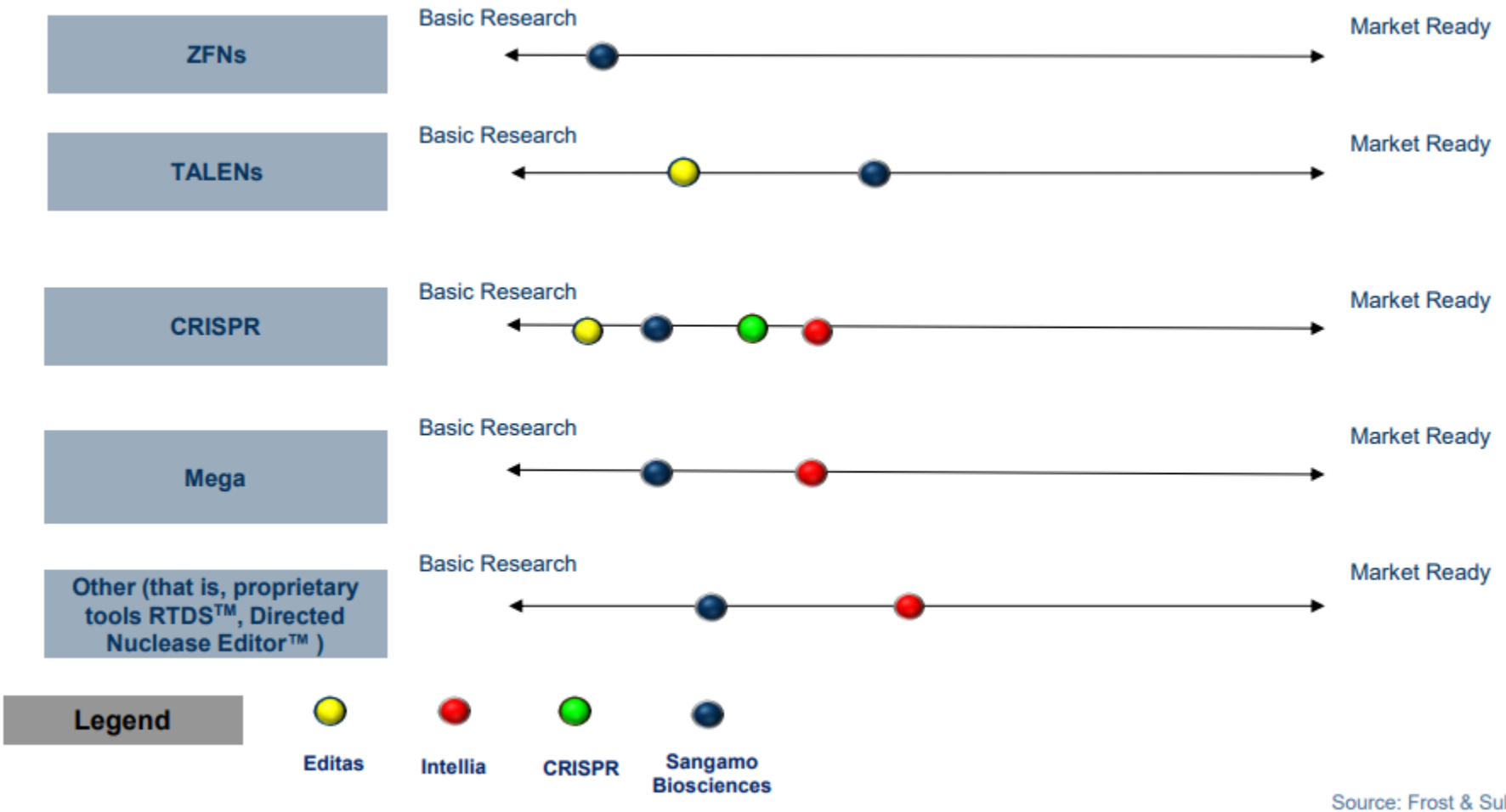


AAGR: Average Annual Growth Rate

Due to publication delays, data for 2013 and 2014 may be incomplete

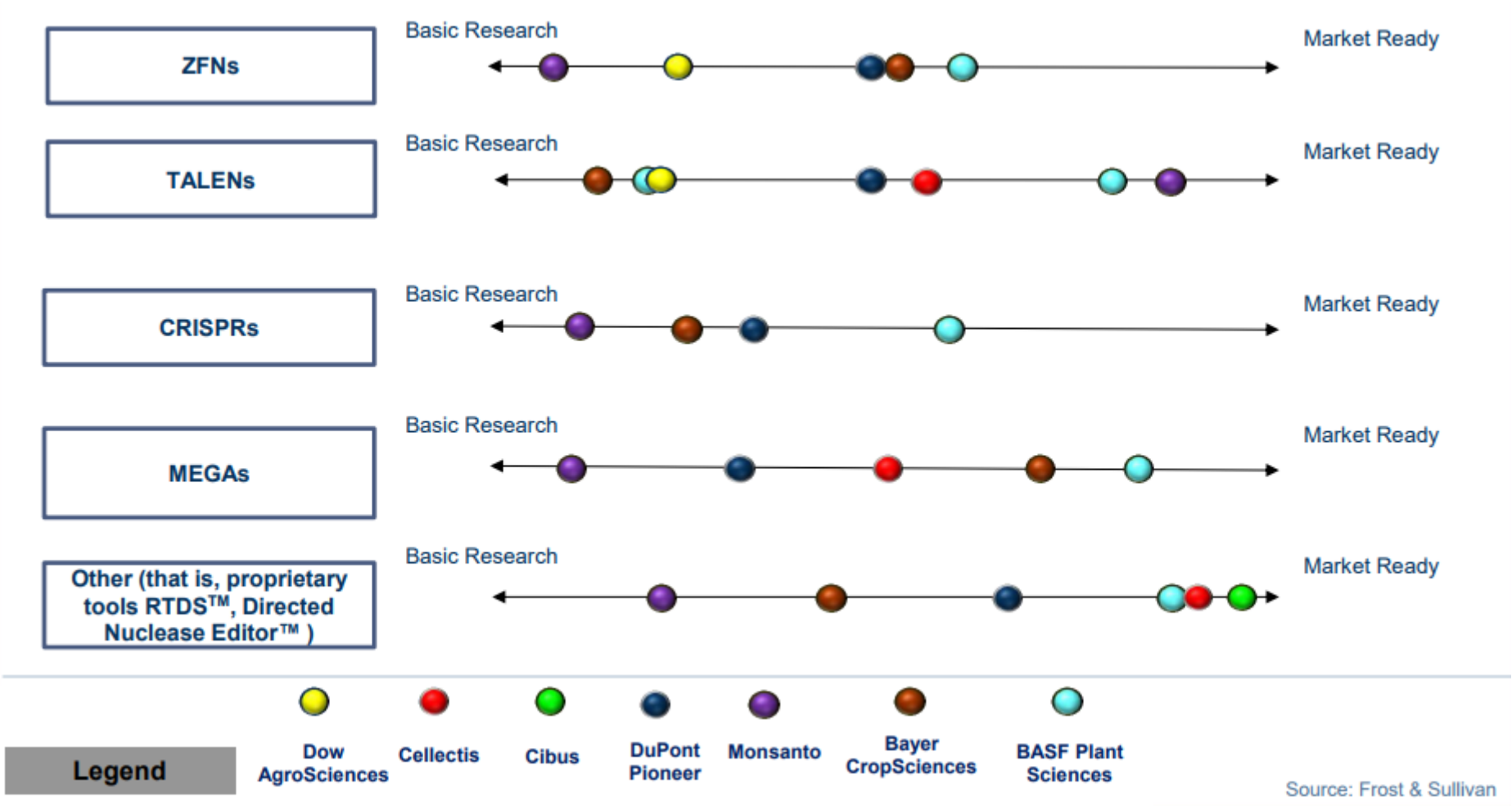
Source: Thomson Innovation, BCG analysis

Terapéutica



Source: Frost & Sullivan

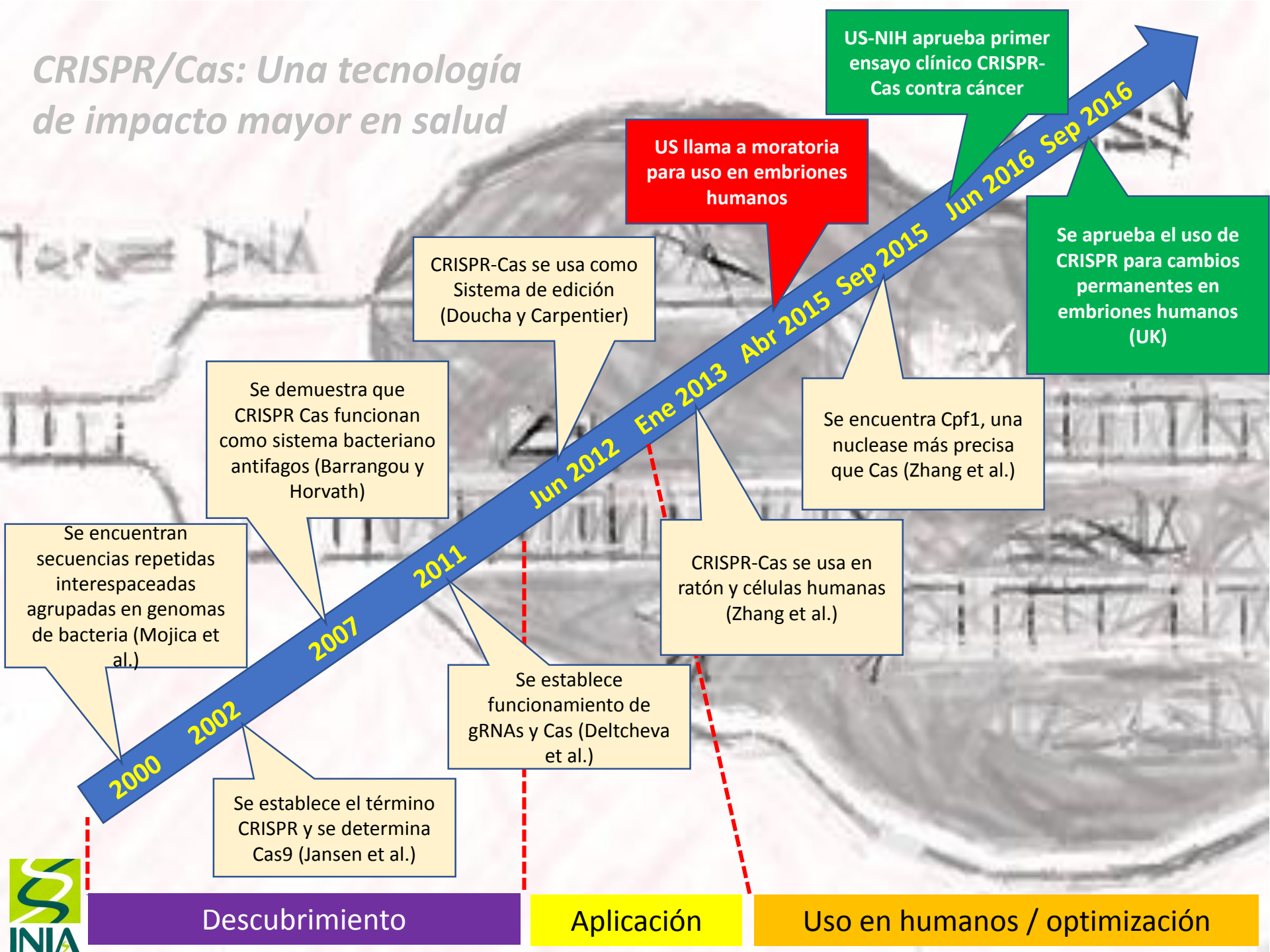
Agricultura



La perspectiva (CRISPR)




CRISPR/Cas: Una tecnología de impacto mayor en salud



Octubre 2017…

LETTER

doi:10.1038/nature17946

Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage

Alexis C. Komor[1,2], Yongjoo B. Kim[1,2], Michael S. Packer[1,2], John A. Zuris[1,2] & David R. Liu[1,2]

Current genome-editing technologies introduce double-stranded (ds) DNA breaks at a target locus as the first step to gene correction[1,2]. Although most genetic diseases arise from point mutations, current approaches to point mutation correction are inefficient and typically induce an abundance of random insertions and deletions (indels) at the target locus resulting from the cellular response to dsDNA breaks[1,2]. Here we report the development of 'base editing', a new approach to genome editing that enables the direct, irreversible conversion of one target DNA base into another in a programmable manner, without requiring dsDNA backbone cleavage or a donor template. We engineered fusions of CRISPR/Cas9 and a cytidine deaminase enzyme that retain the ability to be programmed with a guide RNA, do not induce dsDNA breaks, and mediate the direct conversion of cytidine to uridine, thereby effecting a C→T (or G→A) substitution. The resulting 'base editors' convert cytidines within a window of approximately five

the efficiency of gene correction relative to HDR without introducing an excess of random indels. Catalytically dead Cas9 (dCas9), which contains Asp10Ala and His840Ala mutations that inactivate its nuclease activity, retains its ability to bind DNA in a guide RNA-programmed manner, but does not cleave the DNA backbone[7]. In principle, conjugation of dCas9 with an enzymatic or chemical catalyst that mediates the direct conversion of one base to another could enable RNA-programmed DNA base editing.

The deamination of cytosine (C) is catalysed by cytidine deaminases[8] and results in uracil (U), which has the base-pairing properties of thymine (T). Most known cytidine deaminases operate on RNA, and the few examples that are known to accept DNA require single-stranded (ss) DNA[9]. Recent studies on the dCas9–target DNA complex reveal that at least nine nucleotides (nt) of the displaced DNA strand are unpaired upon formation of the Cas9–guide RNA–DNA 'R-loop' complex[10]. Indeed, in the structure of the Cas9 R-loop

Cas 9 modificada para cambiar residuos AT a GC (Oct 4) // Tecnología "Base Editing"

Cas 13: edición de RNA por reemplazo de residuos A por I (Oct 25) // Tecnología "REPAIR";

Cas 13 modificada para edición de RNA (Oct 25)

Science

RESEARCH ARTICLES

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RNA editing with CRISPR-Cas13

David B.T. Cox,[1,2,3,4,5,6]* Jonathan S. Gootenberg,[1,2,3,4,7]* Omar O. Abudayyeh,[1,2,3,4,6]* Brian Franklin,[1,2,3,4] Max J. Kellner,[1,2,3,4] Julia Joung,[1,2,3,4] Feng Zhang[1,2,3,4]†

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*These authors contributed equally to this work.

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Nucleic acid editing holds promise for treating genetic disease, particularly at the RNA level, where disease-relevant sequences can be rescued to yield functional protein products. Type VI CRISPR-Cas systems contain the programmable single-effector RNA-guided RNases Cas13. Here, we profile Type VI systems to engineer a Cas13 ortholog capable of robust knockdown and demonstrate RNA editing by using catalytically-inactive Cas13 (dCas13) to direct adenosine to inosine deaminase activity by ADAR2 to transcripts in mammalian cells. This system, referred to as RNA Editing for Programmable A to I Replacement (REPAIR), which has no strict sequence constraints, can be used to edit full-length transcripts containing pathogenic mutations. We further engineer this system to create a high specificity variant and minimize the system to facilitate viral delivery. REPAIR presents a promising RNA editing platform with broad applicability for research, therapeutics, and biotechnology.

Precise nucleic acid editing technologies are valuable for studying cellular function and as novel therapeutics. Current editing tools, based on programmable nucleases such as the prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR)-associated nucleases Cas9 (*1–4*) or Cpf1 (*5*), have been widely adopted for mediating targeted DNA cleavage which in turn drives targeted gene disruption through non-homologous end joining (NHEJ) or precise gene editing through template-dependent homology-directed repair (HDR) (*6*). NHEJ utilizes host machineries that are active in both dividing and post-mitotic cells and provides

Cas13 enzymes have two Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) endoRNase domains that mediate precise RNA cleavage with a preference for targets with protospacer flanking site (PFS) motifs observed biochemically and in bacteria (*10*, *11*). Three Cas13 protein families have been identified to date: Cas13a (previously known as C2c2), Cas13b, and Cas13c (*12*, *13*). We recently reported that Cas13a enzymes can be adapted as tools for nucleic acid detection (*14*) as well as mammalian and plant cell RNA knockdown and transcript tracking (*15*). Interestingly, the biochemical PFS was not required for RNA interference with

LETTER

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RNA targeting with CRISPR–Cas13

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RNA has important and diverse roles in biology, but molecular tools to manipulate and measure it are limited. For example, RNA interference[1–3] can efficiently knockdown RNAs, but it is prone to off-target effects[4], and visualizing RNAs typically relies on the introduction of exogenous tags[5]. Here we demonstrate that the class 2 type VI[6,7] RNA-guided RNA-targeting CRISPR–Cas effector Cas13a[8] (previously known as C2c2) can be engineered for mammalian cell RNA knockdown and binding. After initial screening of 15 orthologues, we identified Cas13a from *Leptotrichia wadei* (LwaCas13a) as the most effective in an interference assay in *Escherichia coli*. LwaCas13a can be heterologously expressed in mammalian and plant cells for targeted knockdown of either reporter or endogenous transcripts with comparable levels of knockdown as RNA interference and improved specificity. Catalytically inactive LwaCas13a maintains targeted RNA binding activity, which we leveraged for programmable tracking of transcripts in live cells. Our results establish CRISPR–Cas13a as a flexible platform for studying RNA in mammalian cells and therapeutic development.

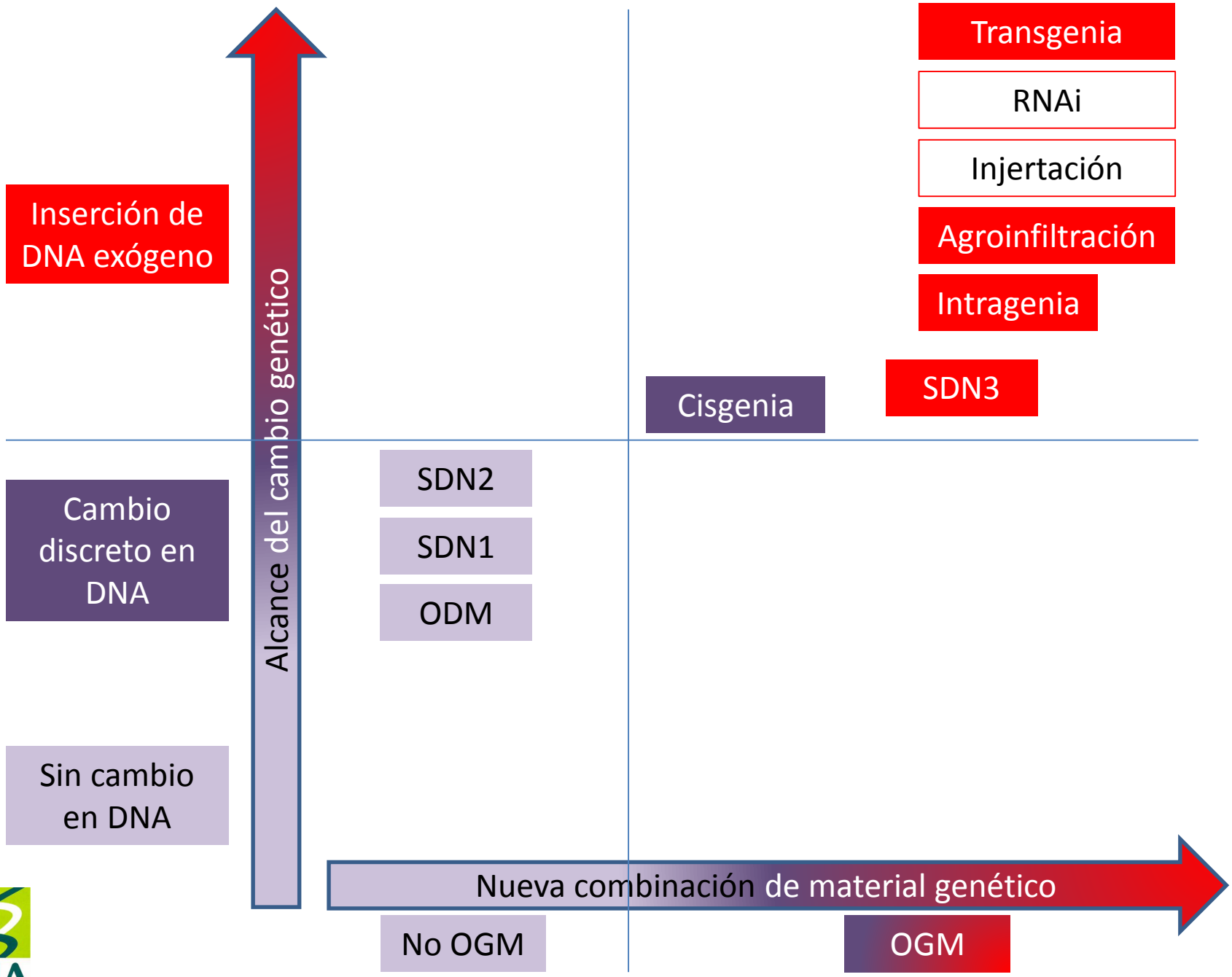
To achieve robust Cas13a-mediated RNA knockdown, we first evaluated 15 Cas13a orthologues for protospacer flanking site (PFS) preference and activity using a previously described ampicillin-resistance assay[8] (Fig. 1a and Extended Data Fig. 1a). This assay

corresponding pre-crRNA transcript (Extended Data Fig. 2h). We also explored the crRNA constraints on LwaCas13a cleavage by truncating the spacer, finding that LwaCas13a retained *in vitro* cleavage activity with spacer lengths as short as 20 nt (Extended Data Fig. 2i). Although guide lengths less than 20 nt no longer support catalytic activity, the LwaCas13a–crRNA complex may still retain binding activity, providing an opportunity for orthogonal applications with a single enzyme[10].

We next evaluated the ability of LwaCas13a to cleave transcripts in mammalian cells. We cloned mammalian codon-optimized LwaCas13a into mammalian expression vectors with msfGFP fusions on the C or N terminus and either a dual-flanking nuclear export sequence or nuclear localization sequence (NLS) and evaluated expression and localization (Fig. 1d). We found that msfGFP-fused LwaCas13a constructs expressed well and localized effectively to the cytoplasm or nucleus according to the localization sequence. To evaluate the *in vivo* cleavage activity of LwaCas13a, we developed a dual-luciferase reporter system that expressed both *Gaussia* luciferase (Gluc) and *Cypridinia* luciferase (Cluc) under different promoters on the same vector, allowing one transcript to serve as the LwaCas13a target and the other to serve as a dosing control (Fig. 1e). We then designed guides against Gluc and cloned them into a $tRNA^{Val}$ promoter-driven guide expression vector. We transfected the LwaCas13a expression vector, guide vector, and dual-luciferase construct into HEK293FT cells and

Regulaciones





Transgenia
RNAi
Injertación
Agroinfiltración
Intragenia
SDN3
Cisgenia
Inserción de DNA exógeno
Alcance del cambio genético
SDN2
SDN1
ODM
Cambio discreto en DNA
Sin cambio en DNA
Nueva combinación de material genético
No OGM
OGM

Regulación NBTs

Technique / Country	ZFN1	ZFN2	ZFN3	ODM	Cisgenesis	RdDM	Reverse Breeding	Grafting	Agro-infiltration
Argentina	#	#	#	#	#	#	#	#	#
Australia		#	#	#	#			#	#
Brazil									
Canada	#	#	#	#	#	#	#	#	#
China									
India	#	#	#	#	#	#	#	#	#
Japan									
Rep of Korea									
New Zealand	#								
Russia									
South Africa									
Switzerland									
USA	#	#	#	#	#	#	#	#	#

Legend

White	Information not available
Blue	Non-regulated or exempted from applicable GM legislation
Black	Regulated under applicable GM legislation
#	Case-by-case review

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Regulación NBTs (enfoque Chile)

Categoría 1: Introducción **transitoria** del ADN durante un paso intermedio del desarrollo del producto final.

El producto final es similar y no se distingue de los productos desarrollados por mejoramiento convencional.

Categoría 2: Introducción **estable** del ADN durante un paso intermedio del desarrollo del producto final.

El producto final es similar y no se distingue de los productos desarrollados por mejoramiento convencional.

Categoría 3: **Integración estable** del ADN

El producto final es similar a un transgénico, salvo el caso del uso de genes de la misma especie (cisgenia)

Foco en:

Country	Legislative/Regulatory Focus±
EU	Product/Process
Argentina	Product/Process
Australia	Process
Brazil	Process
Canada	Product
China	Process
India	Product/Process
Japan	Product/Process
New Zealand	Product/Process
Rep of Korea	Product/Process
Russia	Unknown
South Africa	Process
Switzerland	Product/Process
USA	Product

INIA

Muchas gracias!