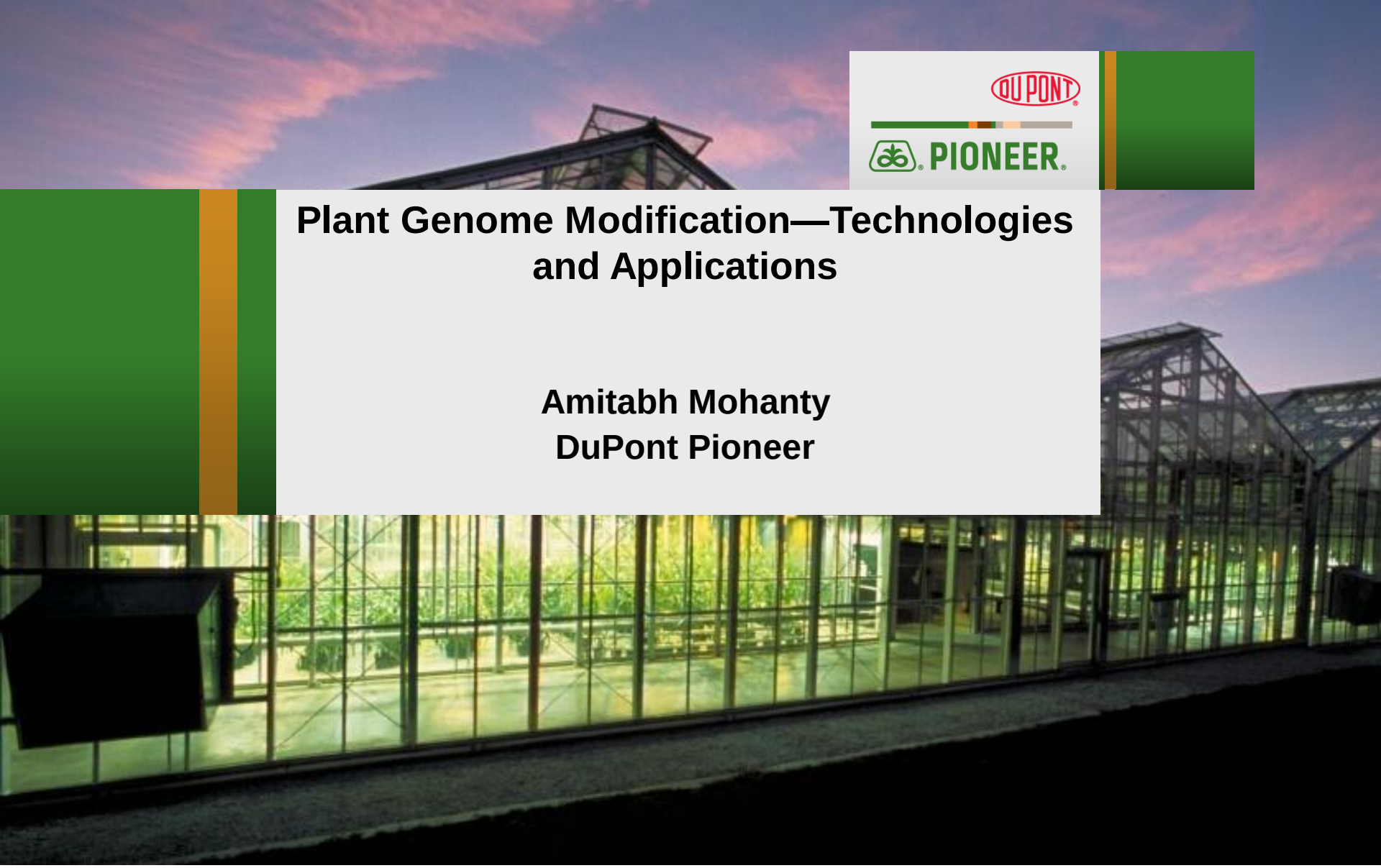


Plant Genome Modification—Technologies and Applications

Amitabh Mohanty
DuPont Pioneer

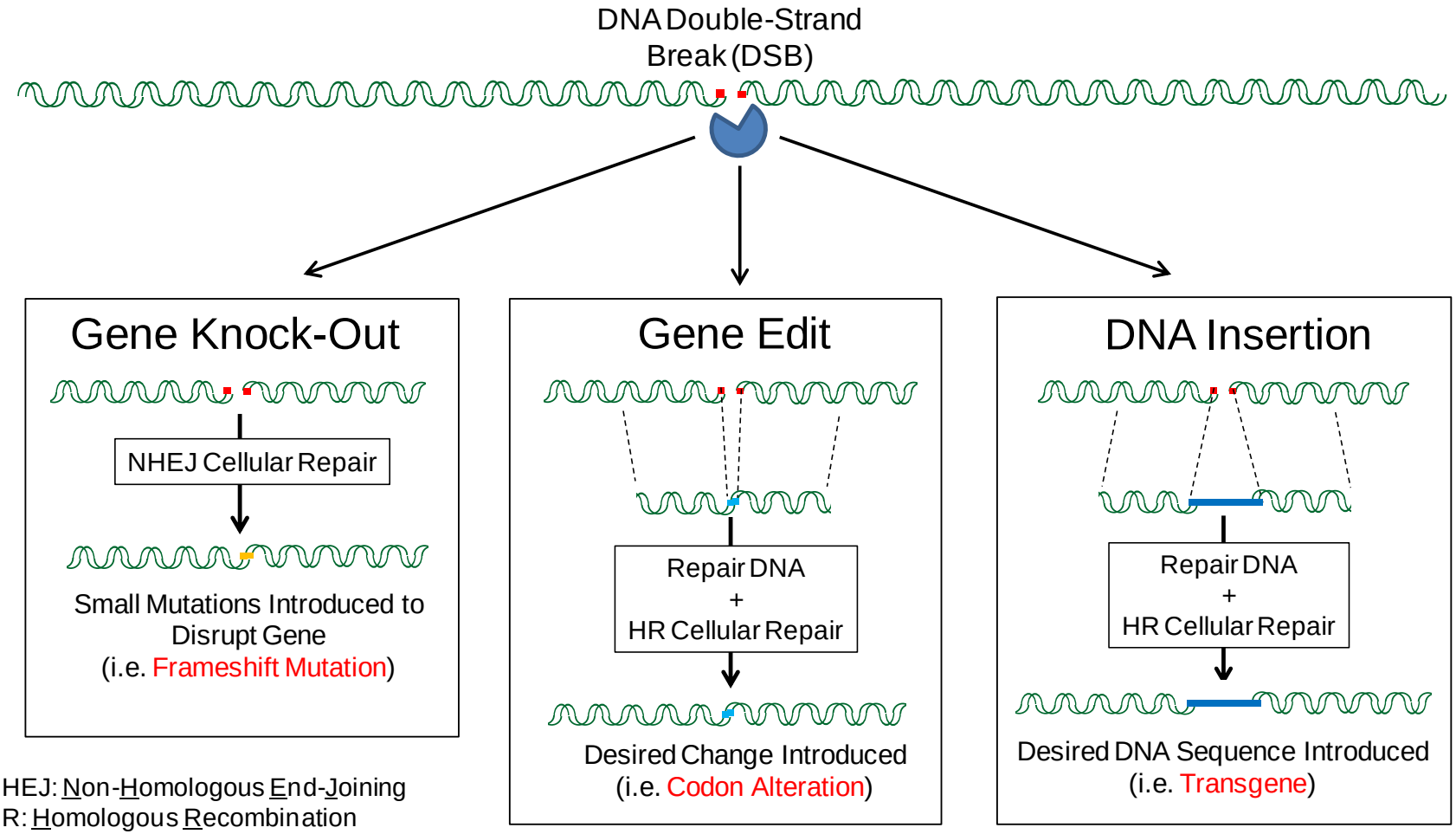
International Conference on New Plant Breeding Molecular Technologies – Technology Development And Regulation, Oct 9-10, Jaipur, India.

Genome Modification Objectives

- Direct trait genes to any site in genome
- Specifically modify any gene or pathway in a genome
- Rapidly stack traits and products
- Reduce time to market
- Reduce regulatory costs

Genome Modification Tools

Zinc Finger Nucleases, Meganucleases, TALENs, Cas9/gRNA

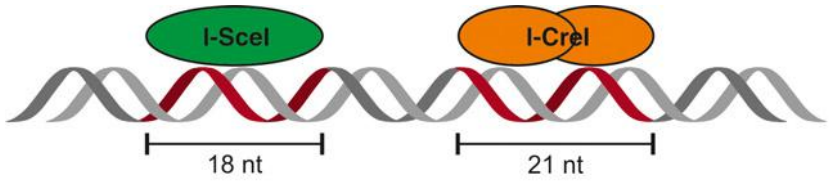


NHEJ: Non-Homologous End-Joining
HR: Homologous Recombination
DSB: Double-Strand Break

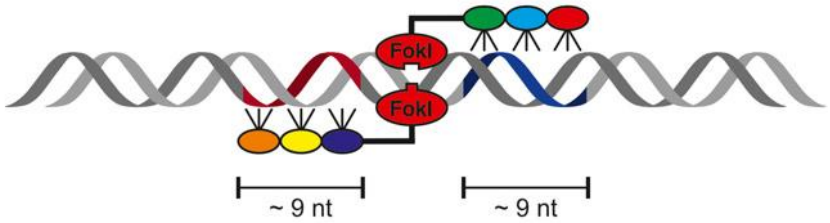
After Podevin et al., Trends in Biotech., 2013, 6:375

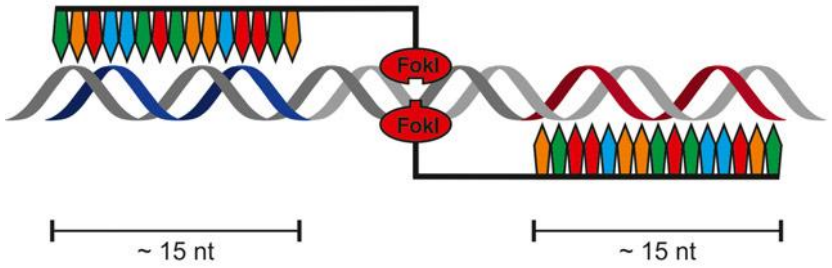
Various tools to induce DSBs

Meganuclease

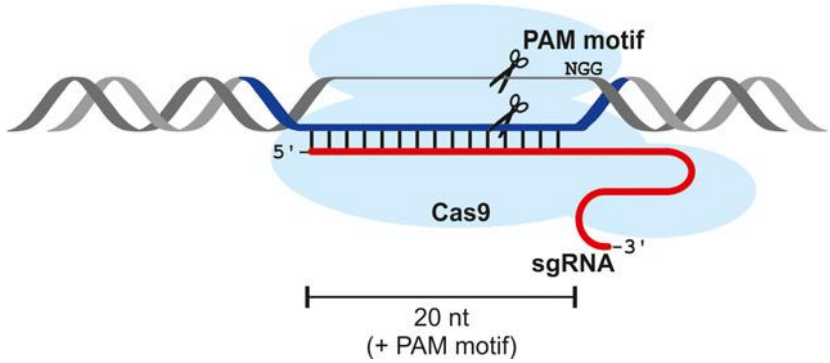


Zinc finger nuclease





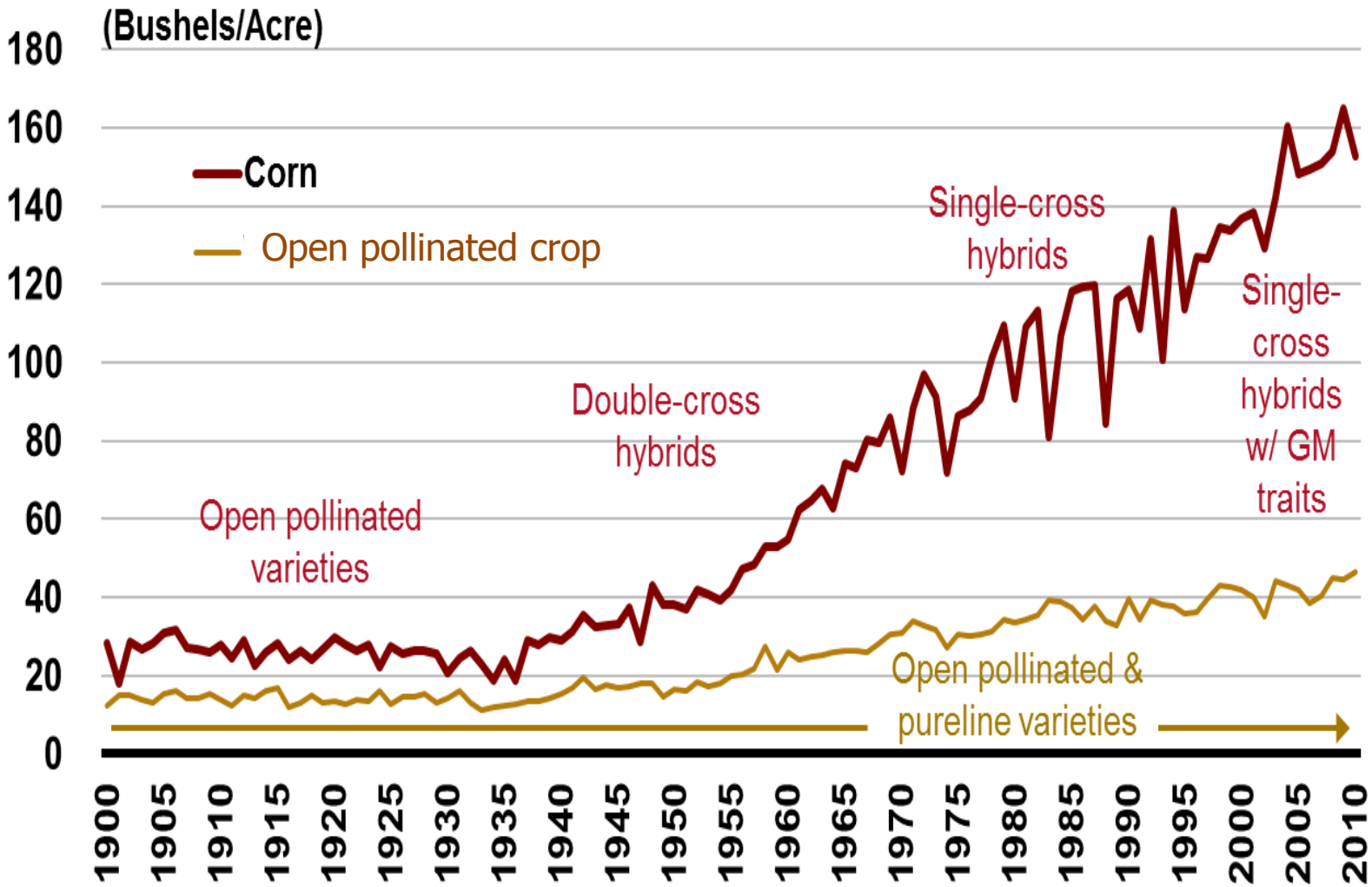
TALENs



CRISPR/Cas9

Components- MN, Zn Finger, TALEN & CRISPR (Puchta & Fauser, Plant J, 78, 727-741, 2014)

Hybrids Drive Yield Gain

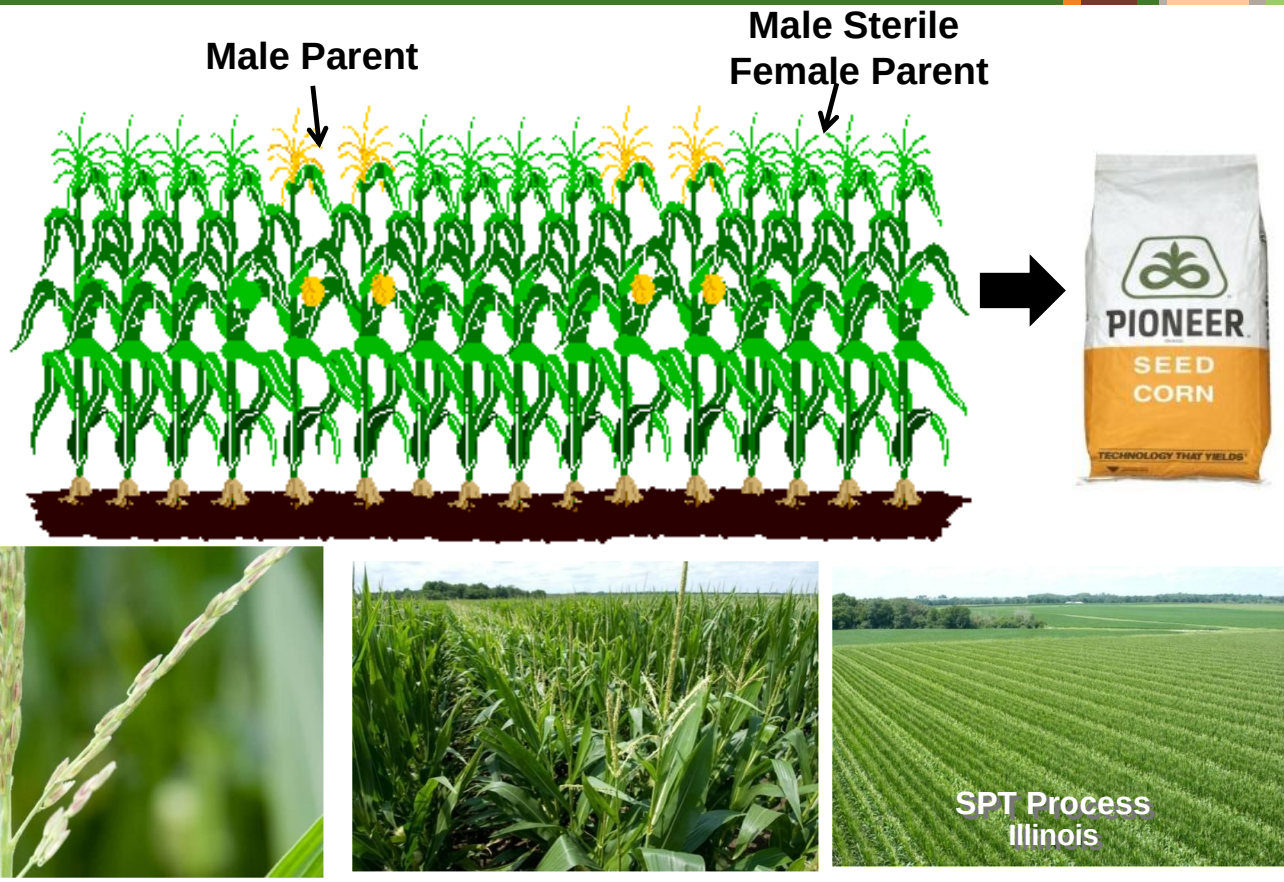


DUPONT PIONEER

How Hybrid Seed Corn is Made Today

DUPONT PIONEER

Seed Production Technology (SPT) Process



- New Pioneer technology to more efficiently produce hybrid seed corn
- SPT process provides an alternative method of hybrid seed production
- SPT process is not a commercial product or commercial trait
- Requires a nuclear recessive mutation in male fertility gene

DUPONT PIONEER

SPT Biological Components

Utilizes gene that contributes to the formation of all pollen, producing plants that do not shed pollen

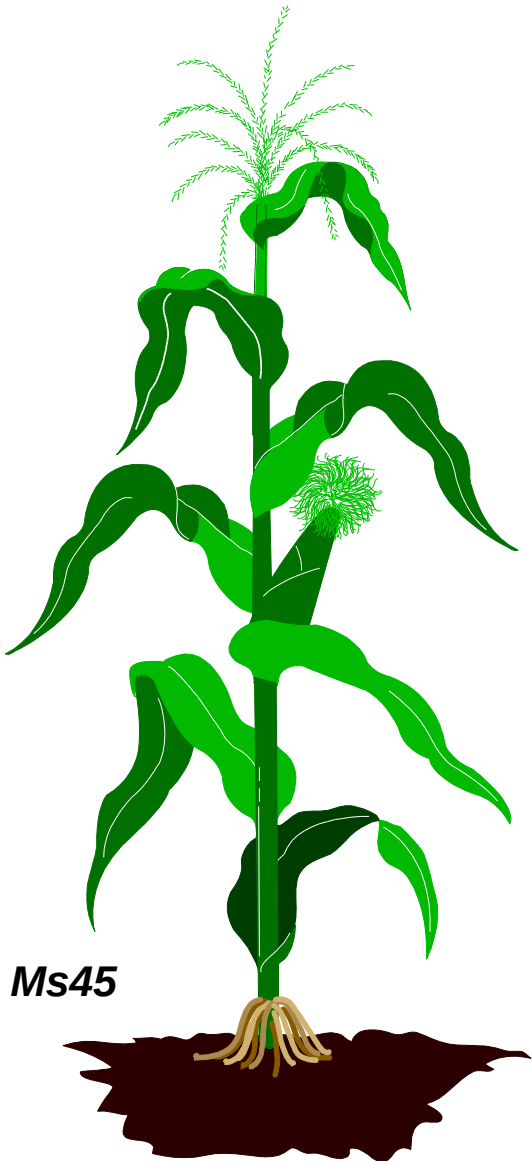
-genetic male sterile mutation

-cloned male fertility gene

- Ms26
- Ms45

Wild type

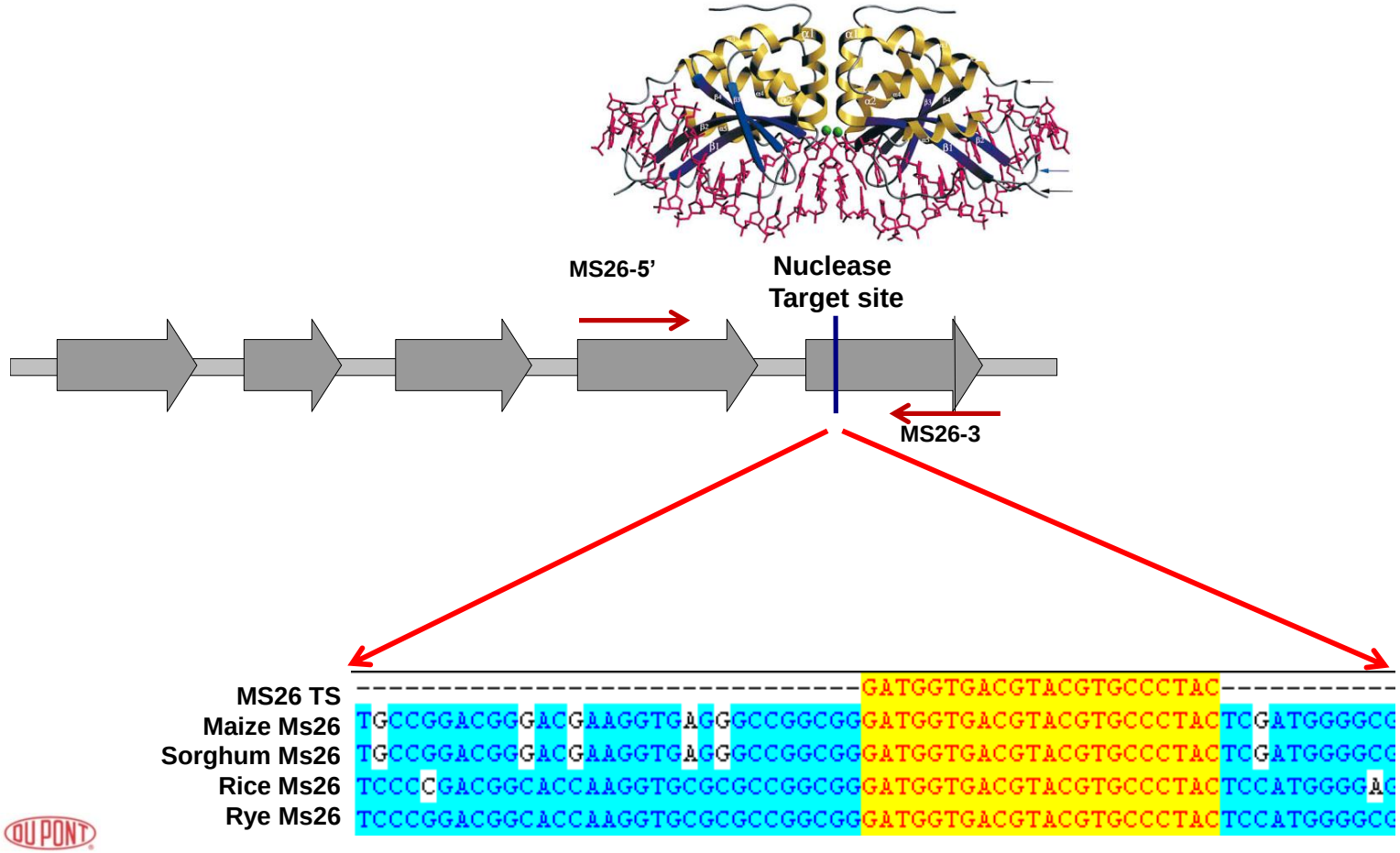
ms/ms



Pioneer SPT process in corn is based on the natural mutation in Ms45

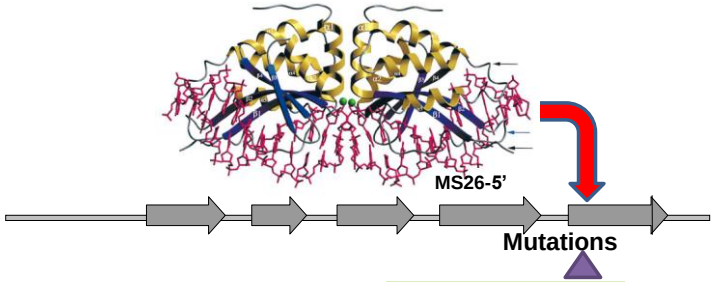
Monocot Male Fertility Gene Ms26

- **Encodes P450 involved in pollen wall development**
- **Homozygous recessive mutations confer male sterility in maize and rice**
- **Highly conserved single-copy gene in maize, rice and sorghum**
- **Contains a unique target site**



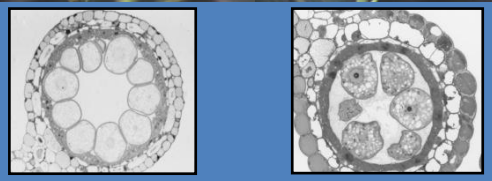
Djukanovic et al., Plant J 2013

Targeted Mutagenesis: Ms26



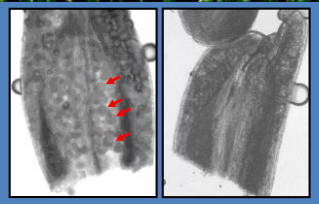

```
MS26WT GTGCTCCCCGACGGCACCAAGGTGCGCGCCGGCGGATGGTGACGTACGTGCCCTACTCCATGGGGAGGATGGAGTACAACTGGGGCCCCGACGCGGCGA
MS26.1 GTGCTCCCCGACGGCACCAAGGTGCGCGCCGGCGGGATGGTG   ACGTGCCCTACTCCATGGGGAGGATGGAGTACAACTGGGGCCCCGACGCGGCGA
MS26.2 GTGCTCCCCGACGGCACCAAGGTGCGCGCGGGCGGGATGGTGA  GCGTGCCCTACTCCATGGGGAGGATGGAGTACAACTGGGGCCCCGACGCGGCGA
MS26.3 GTGCTCCCCGACGGCACCAAGGT                GTACGTGCCCTACTCCATGGGGAGGATGGAGTACAACTGGGGCCCCGACGCGGCGA
MS26.4 GTGCTCCCCGACGGCACCAAGGTGCGCGCCGGCGGGATGGTGA                GGATGGAGTACAACTGGGGCCCCGACGCGGCGA
```

MAIZE

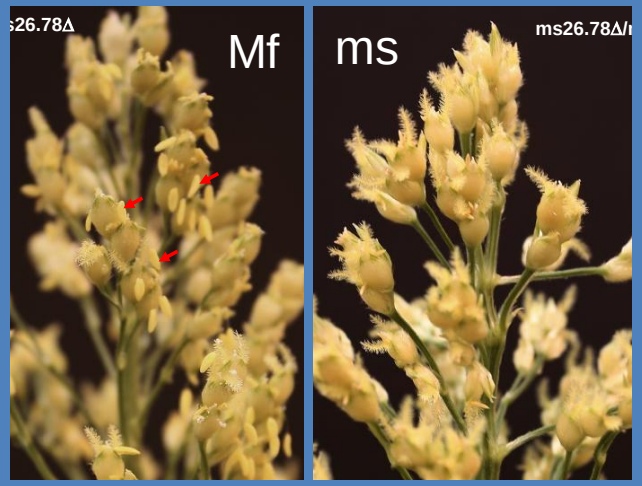


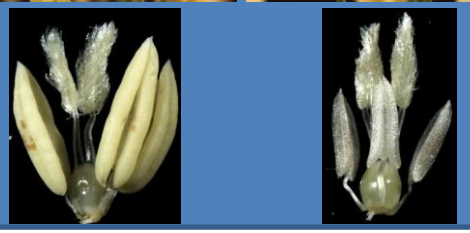
Djukanovic et al Pl Journal 2013

RICE



SORGHUM





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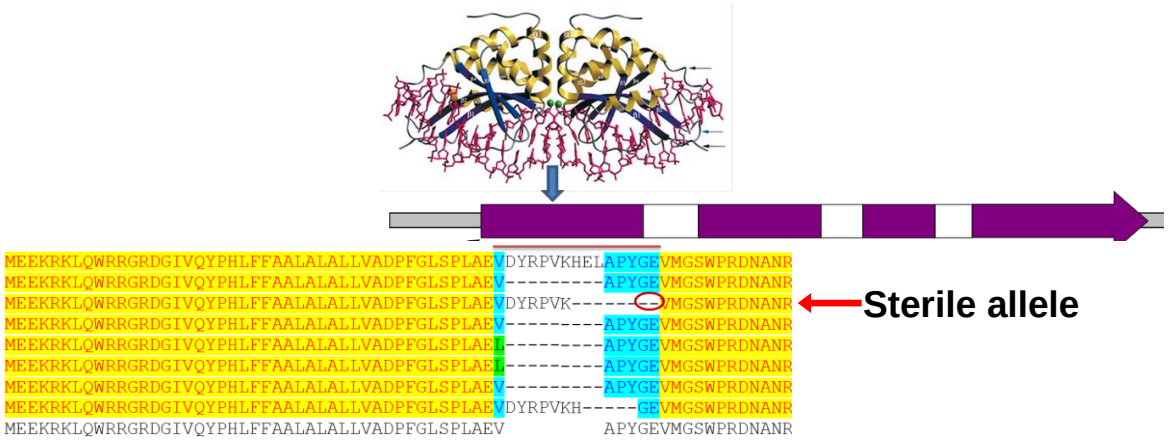
Ms45 Meganuclease

Deletions in Ms45 coding region result in male sterile maize

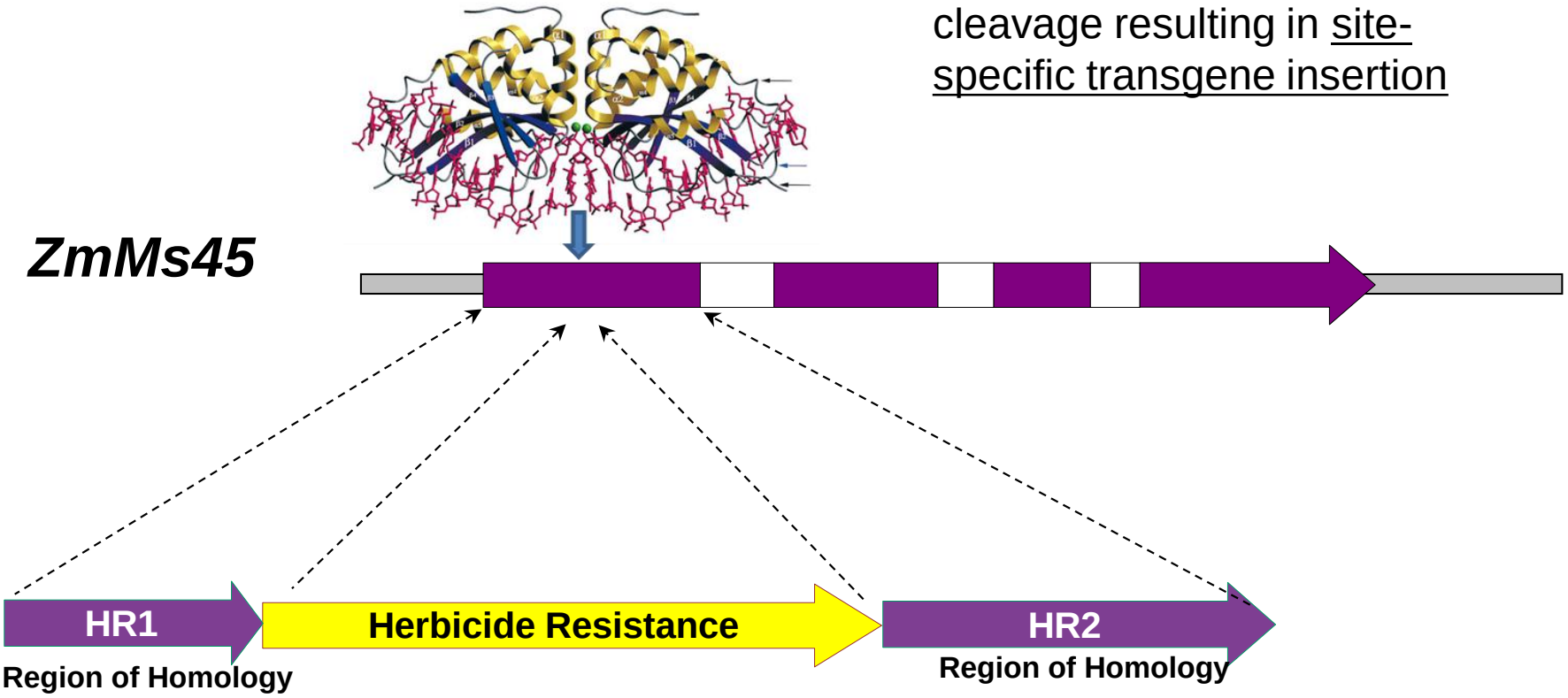


Gene Targeting to MS45

Site-Specific Insertion of Transgenes

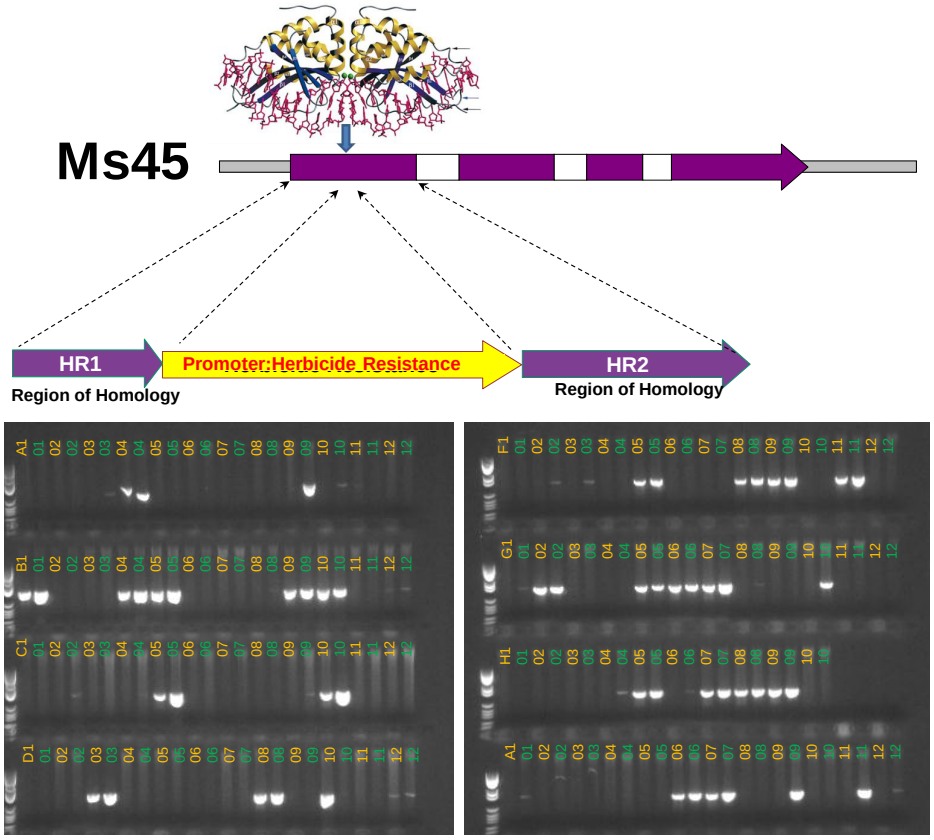
Homology Directed gene targeting

Cell uses flanking sequence homology to repair the site of cleavage resulting in site-specific transgene insertion



Gene Targeting Frequency

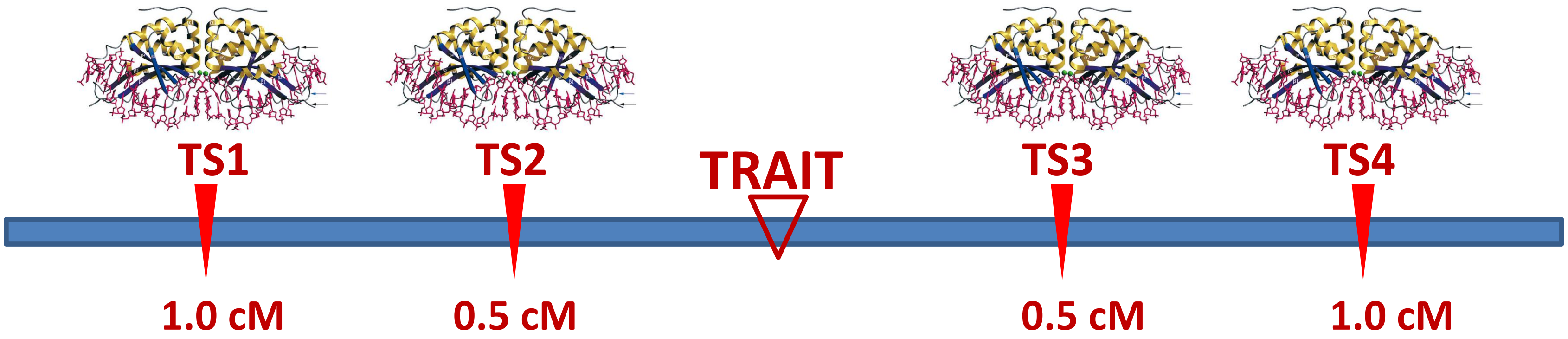
Targeted Insertions in Ms45 coding region also results in male sterile maize



MGN	Events Recovery	TS Mut Rate	Insert Rate (HR1+HR2)
MS45(1-2.36)	160	92%	13% (22)
LIG34 Meganuclease*	288	4%	0.7% (2)

*Near liguleless locus
Gao et al P Journal 2010

Complex Trait Loci



TS1

```
AATATGTGTACCTCATGCTATAGTTTTGGTTAAATCTGAC : : : AATCTGTTTGGAATTGAAAAACAAGTGCTTC
AATATGTGTACCTCATGCTATAN:TTTTGGTTAAATCTGACGT: : : : : : : : : : : : : : :TGAAAAACAAGTGCTTC
AAT: : : : : : : : : : : : : : : : : : : : : : : : : : : CGTGAT : : : : : :   : : : :::::::::::::::::: :::::::AAACAAGTGCTTC
AATATGTGTACCTCATGCTATAGTTTTGGTTAAATCTG: : : : : : : : : : : TTTGGAATTGAAAAACAAGTGCTTC
AATATGTGTACCTCATGCTATAGTTTTGGTT: : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :::: : : : : : : : C
AATATGTGTACCTCATGCTATAGTTTTGGTTAAATCTGA : : : : : ATCTGTTTGGAATTGAAAAACAAGTGCTTC
AATATGTGTACCTCATGCTATAGTTTTGG: : : : : : : : : : : : : : : : : : : : : : : : : :::AATTGAAAAACAAGTGCTTC
AATATGTGTACCTCATGCTATAGTTTTGGTTAAATCTGACG : GAATCTGTTTGGAATTGAAAAACAAGTGCTTC
AATATGTGTACCTCATGCTATAGTTTTGGTTAAATCTGACGTGAATCT:::::::::::::::::::::::::::::::::::::::::::::::::
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::TTTGGAATTGAAAAACAAGTGCTTC
```

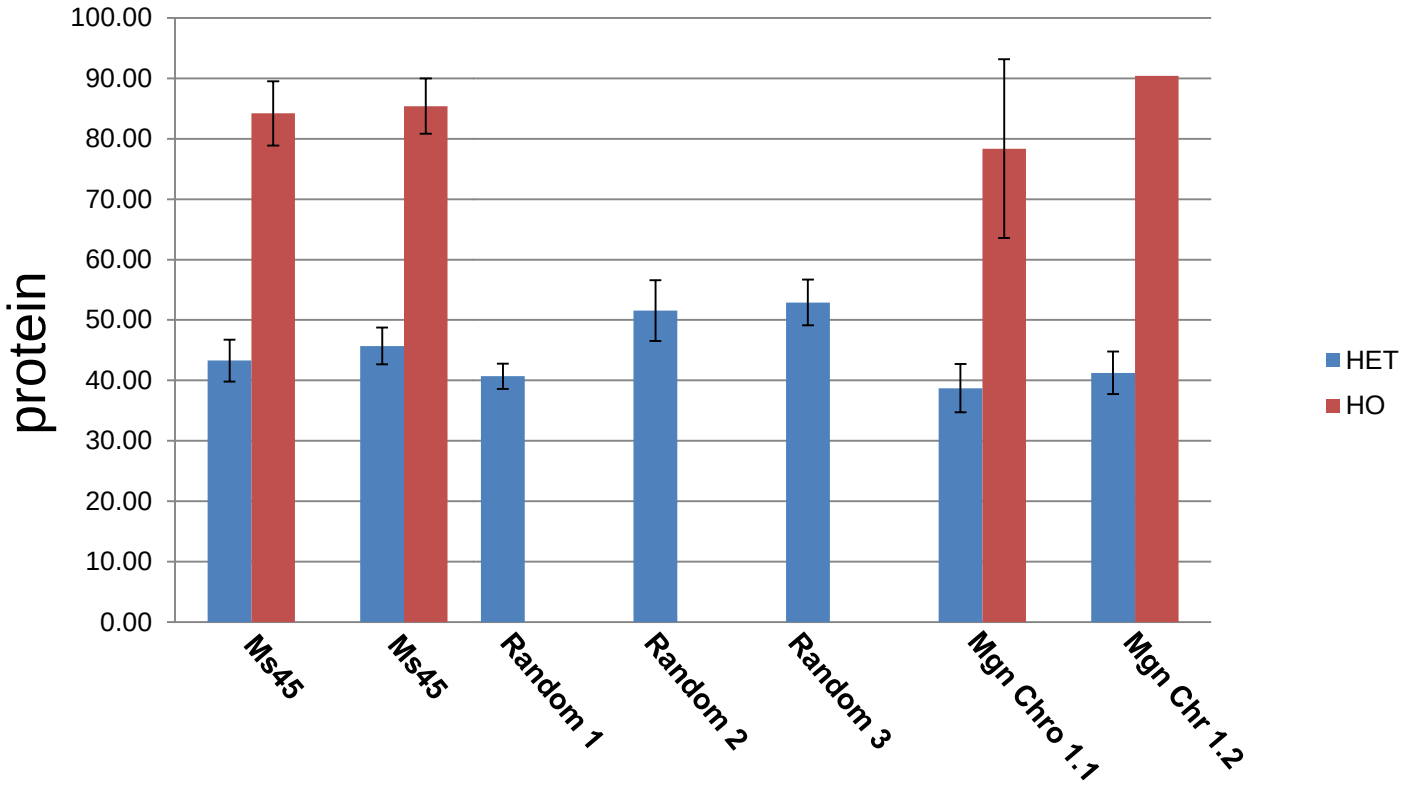
TS2

```
AAATGAGTAGCAGCGCACGTATATATACGCGTACGCGTACGTG : : AGGTATATATATCCTCCGCCGGGG
AAATGAGTAGCAGCGCACGTATATATACGCGTACGCGTACGT : : GAGGTATATATATCCTCCGCCGGGG
AAATGAGTAGCAGCGCACGTATATATAC: : : : : : : : : : : : : : : : : : : : : : : : : : : : :     CCTCCGCCGGGG
```

DUPONT
PIONEER

Uniformity of Transgene Expression

Protein expression of transgene is similar when placed either within Ms45 or other directed or random insertions



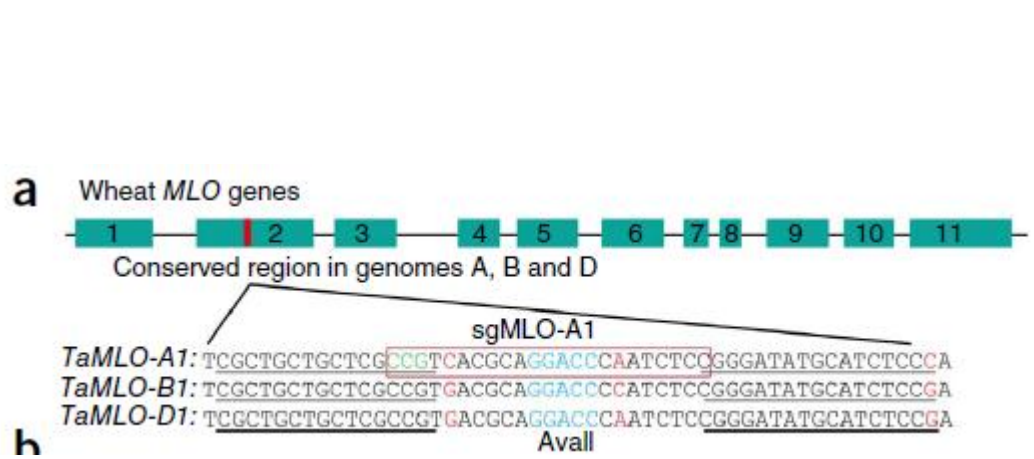
Powdery Mildew resistance in wheat by TALEN/CRISPR mediated DSB

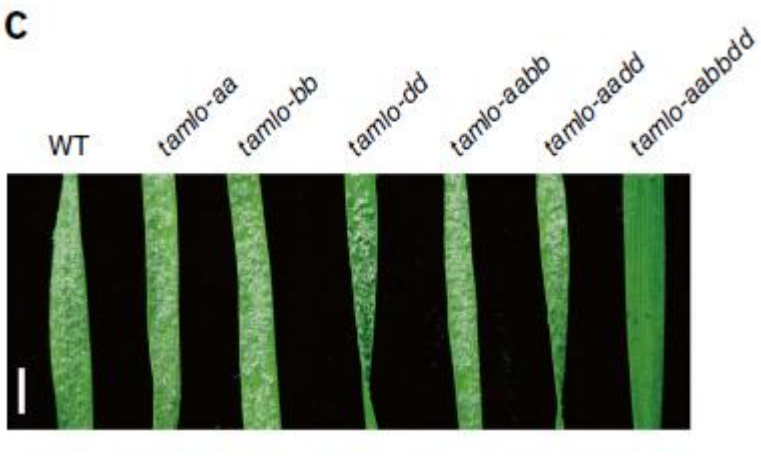
Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew

Yanpeng Wang[1,3], Xi Cheng[2,3], Qiwei Shan[1], Yi Zhang[1], Jinxing Liu[1], Caixia Gao[1] & Jin-Long Qiu[2]

nature biotechnology

doi:10.1038/nbt.2969





Wang et al. demonstrated the TALEN mediated simultaneous deletions in all the 3 homeologs in bread wheat results in broad spectrum resistance against powdery mildew. They also demonstrated deletion of one gene by CRISPR.

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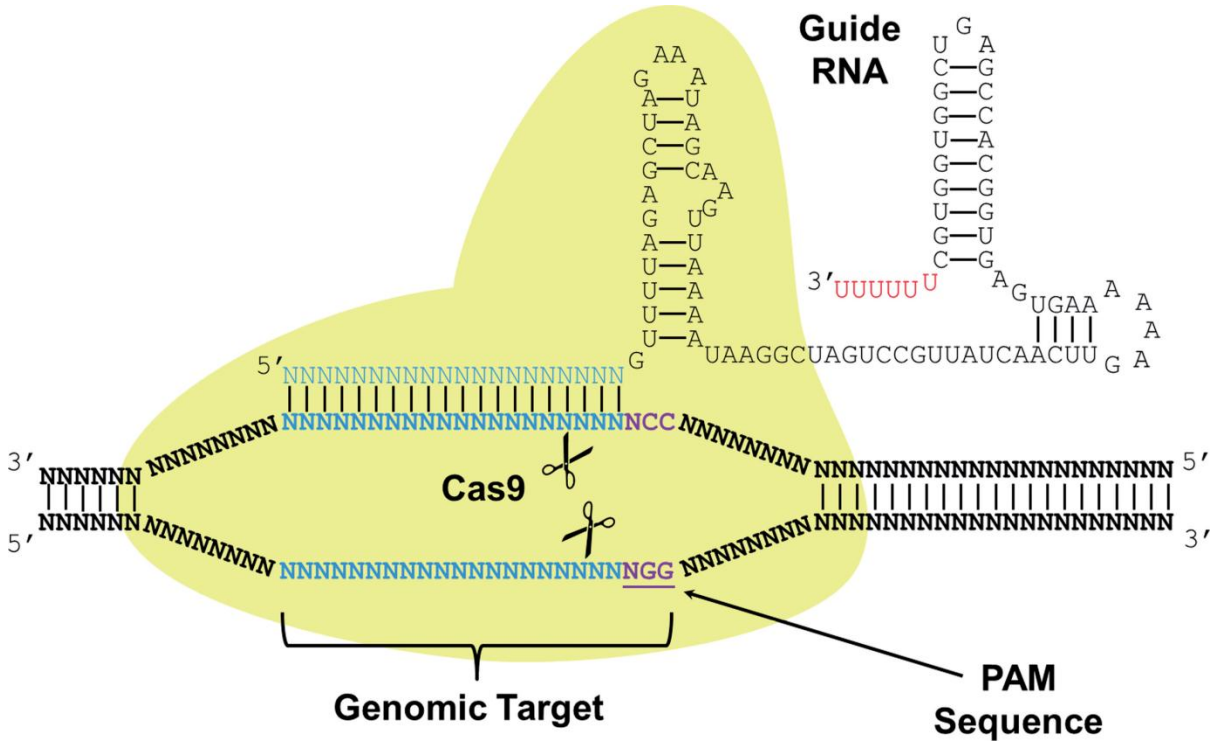
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

- Target site as coding sequence
- PAM-NGG (DeCarlo et al. Nucl Acd Res.2013)



CRISPR Components

- **2 basic components- Cas9 and guideRNA**

Cas9 cassette (Rice codon optimized; NLS1-SV40 and NLS-VIRD2)

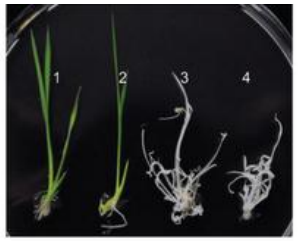
Guide RNA cassette with RNA PolIII OsU3 promoter

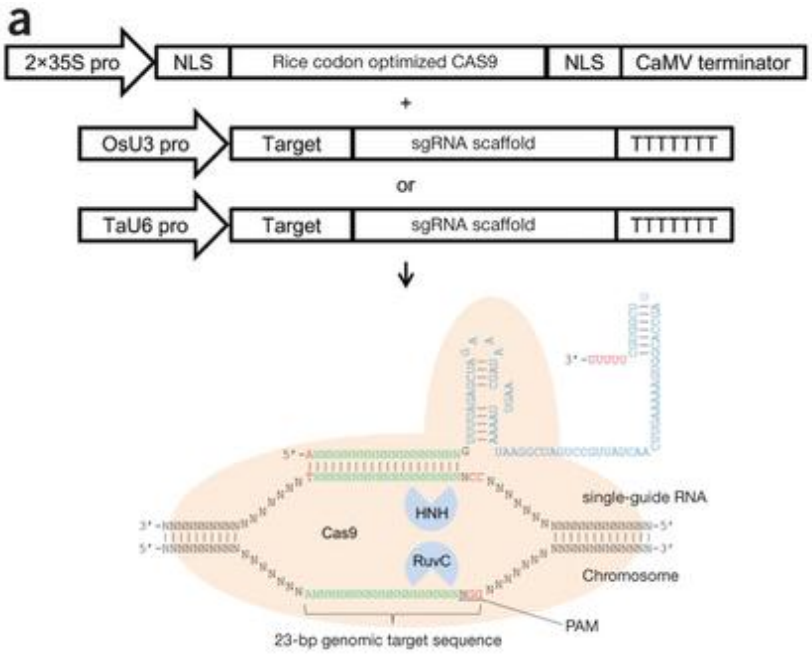
Guide RNA cassette with RNA PolIII TaU6 promoter

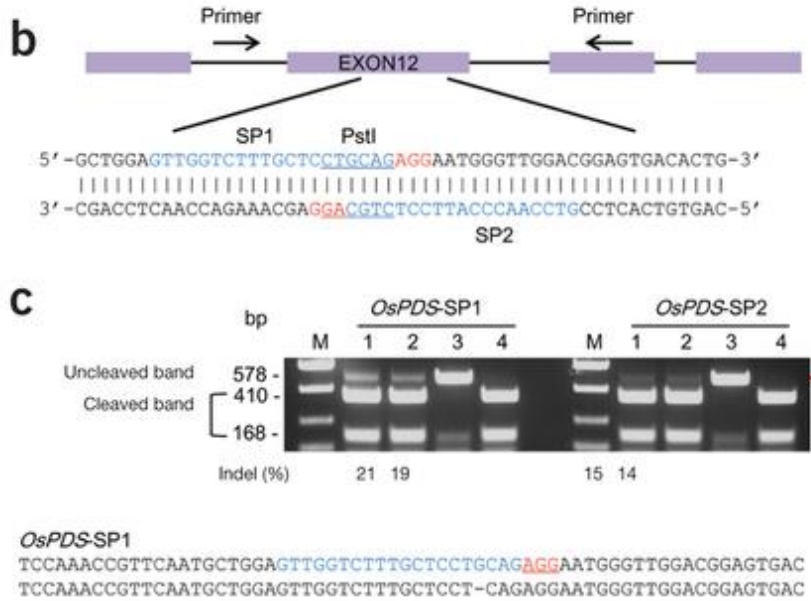
(Qin et al 2007, Shan et al. 2013)

Targeted Knock Out: Rice PDS (Phytoene desaturase) gene

- Disruption of PDS gene results in albino and dwarf phenotypes in Arabidopsis by impairing chlorophyll, carotenoid, and gibberellin biosynthesis (Qin et al 2007)
- PDS knock out by CRISPR (Shan et al. 2013)

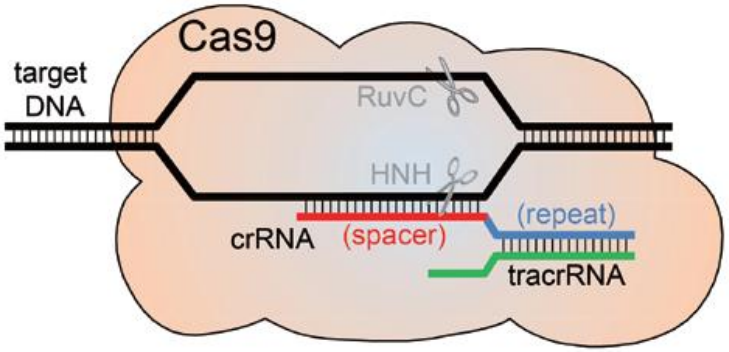






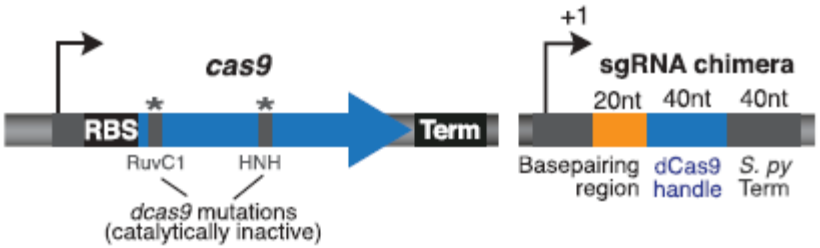
Engineered Cas9 Variants

- Wild Type Cas9- DNA DSB
- Mutant Cas9 (dCas9)- Transcription Interference

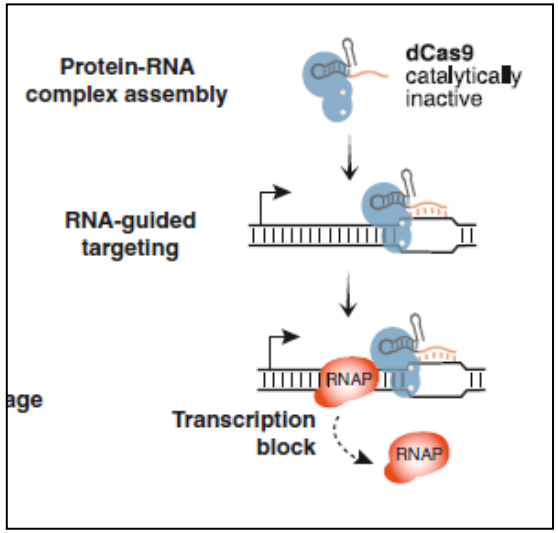


Knock Out

P. Horvath & R. Barrangou, Cell Res.2013



Qi et al. Cell. 2013



Knock down

Qi et al .2013. Cell

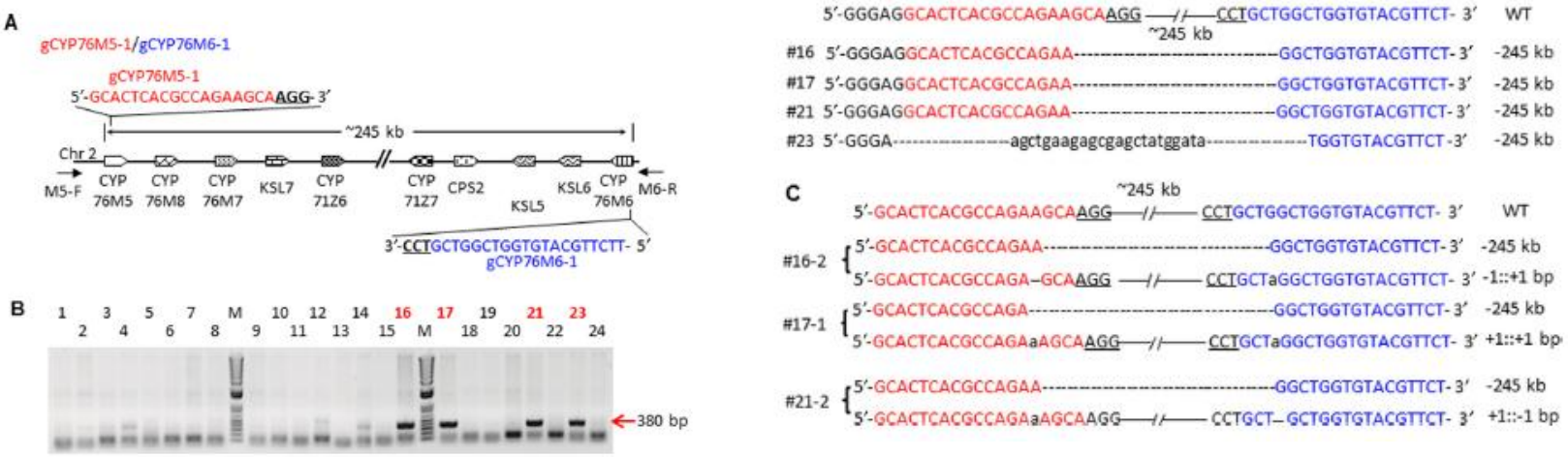
Large chromosomal deletions using CRSISPR/Cas9 system in rice

Nucleic Acids Research Advance Access published September 8, 2014

Nucleic Acids Research, 2014 1
doi: 10.1093/nar/gku806

Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice

Huanbin Zhou[1], Bo Liu[1], Donald P. Weeks[2], Martin H. Spalding[1] and Bing Yang[1,*]



Deletion of 245 kb region in rice. Authors also demonstrated creation of mutations in all 4 SWEET genes in rice

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DSB Technology Comparison

	Zinc Finger Nuclease	Redesigned Meganuclease	TALEN	Cas9/gRNA
Year	2001-3	2006-7	2009	2012
Time to engineer	Weeks to months	Months	Weeks	Days
Quality of reagents	???	-- to +++	-- to ++	-- to ++++
Cost/reagent ($)	???	Several thousand	Several thousand	<$100
Binding	Protein-DNA	Protein-DNA	Protein-DNA	RNA-DNA
Flexibility in selecting target sites	+	+	++	++++
% of successful reagents	???	++	++	++++

DUPONT PIONEER

USDA "Am I Regulated" Website

Inquiry	USDA Response
Bioglow Arabidopsis	Not Regulated
Drought Tolerant Switchgrass	Not Regulated
Null Segregants from Transgenic Plants	Not Regulated
Dwarf, Glyphosate Tolerant St. Augustines Turfgrass	Not Regulated
Dwarf, Glyphosate Tolerant Kentucky Bluegrass	Not Regulated
Increased Biomass, Increased Sugar Switchgrass	Not Regulated
Increased Anthocyanin Seedles Grapes	Not Regulated
Low Phytate Corn	Not Regulated
Products Created by Targeted Deletion	Not Regulated
Altered Color Baby's Breathe	Not Regulated
Targeted Deletion via Meganuclease	Not Regulated
Gene Edit via Meganuclease	Case by Case
Null Segregant – Doubled Haploid from GEM GMO	Not Regulated
Null Segregant – from Faster Flowering Time GMO	Not Regulated
Glyphosate Tolerant Kentucky Bluegrass	Not Regulated

Not Regulated means that the USDA has decided that the product as described to USDA would not be regulated by APHIS under 7 CFR part 340

Global Status of NBT Products

EMBO reports

science & society

Transgenic or not? No simple answer!

New biotechnology-based plant breeding techniques and the regulatory landscape

Nancy Podevin, Yann Devos, Howard Vivian Davies & Kaare Magne Nielsen

EMBO reports

outlook

Renegotiating GM crop regulation

Targeted gene-modification technology raises new issues for the oversight of genetically modified crops

Jennifer Kuzma & Adam Kokotovich

RESEARCH PAPER New Biotechnology • Volume 31, Number 1 • Jan

ELSEVIER

Review

Engineering nucleases for gene targeting: safety and regulatory considerations

Katia Pauwels[1], Nancy Podevin[2], Didier Breyer[1], Dana Carroll[3] and Philippe Herman[1]

Currently no international consensus on the regulatory approach to NBT products

Acknowledgements

- DuPont Pioneer

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Tom Greene
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Sergei Svitashev
Alex Lyznik
Amit Das

- Precision BioSciences

Derek Jantz
Jeff Smith

DUPONT PIONEER