UPOV

International Union for the Protection of New Varieties of Plants

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**Working Group on Biochemical and Molecular Techniques and DNA-Profiling in Particular**

**BMT/16/20**

**Sixteenth Session**
**La Rochelle, France, November 7 to 10, 2017**

**Original:** English
**Date:** November 6, 2017

# SDN-ASSISTED PLANT BREEDING AND POTENTIAL IMPACT ON DUS TESTING

*Document prepared by an expert from the European Union*

*Disclaimer: this document does not represent UPOV policies or guidance*

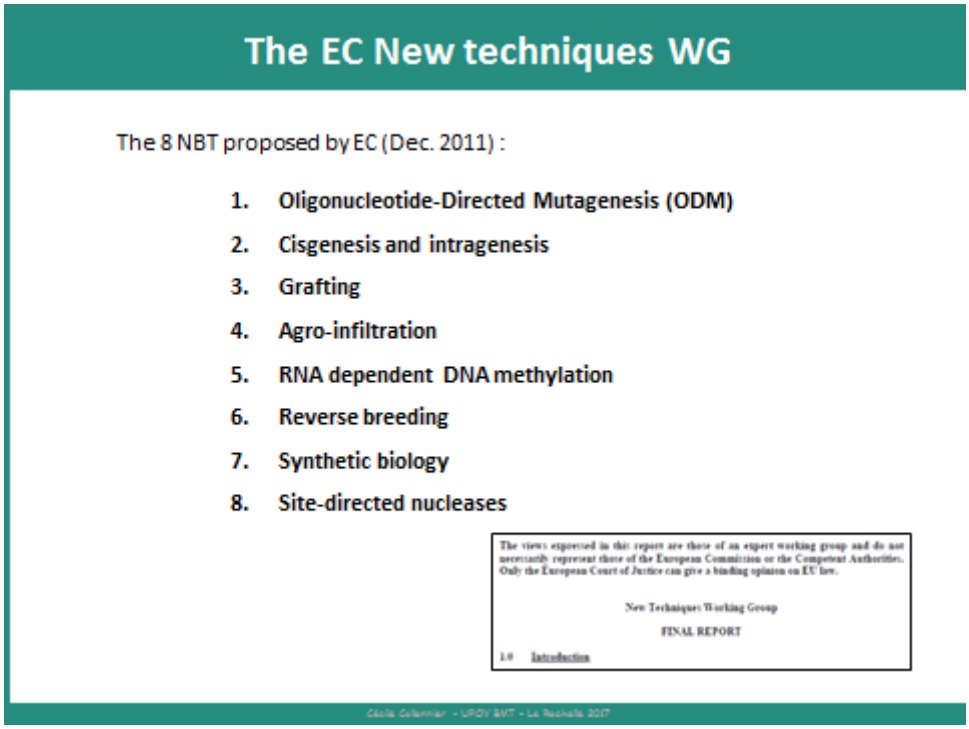
The Annex to this document contains a copy of a presentation on "SDN-assisted plant breeding and potential impact on DUS testing", prepared by an expert from the European Union, to be made at the sixteenth session of the Working Group on Biochemical and Molecular Techniques and DNA-Profiling in Particular (BMT).

[Annex follows]

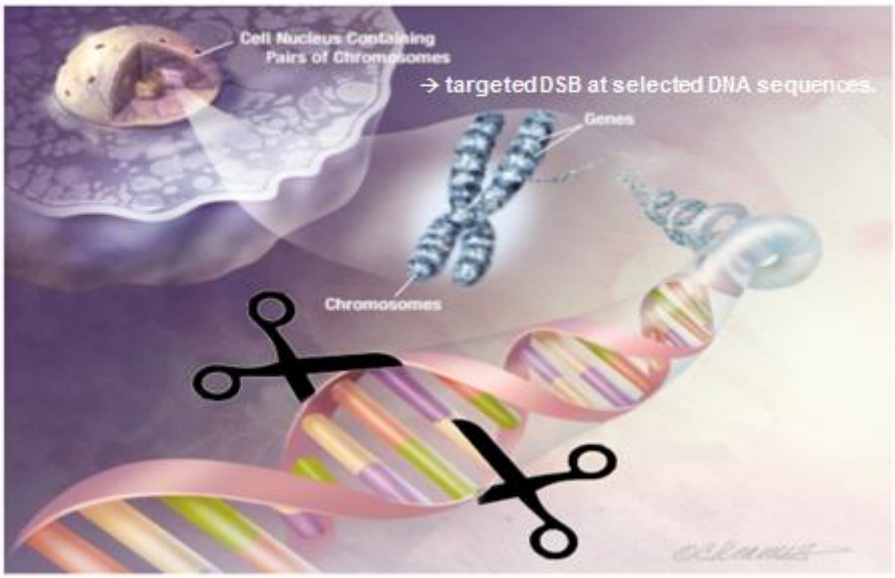
ANNEX

# SDN-ASSISTED PLANT BREEDING AND POTENTIAL IMPACT ON DUS TESTING

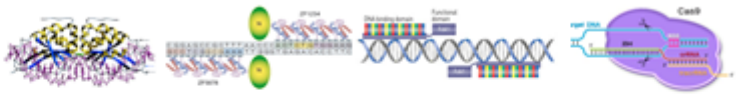
Presentation prepared by an expert from the European Union

## 8 – Site Directed Nucleases




## Site-Directed Nucleases

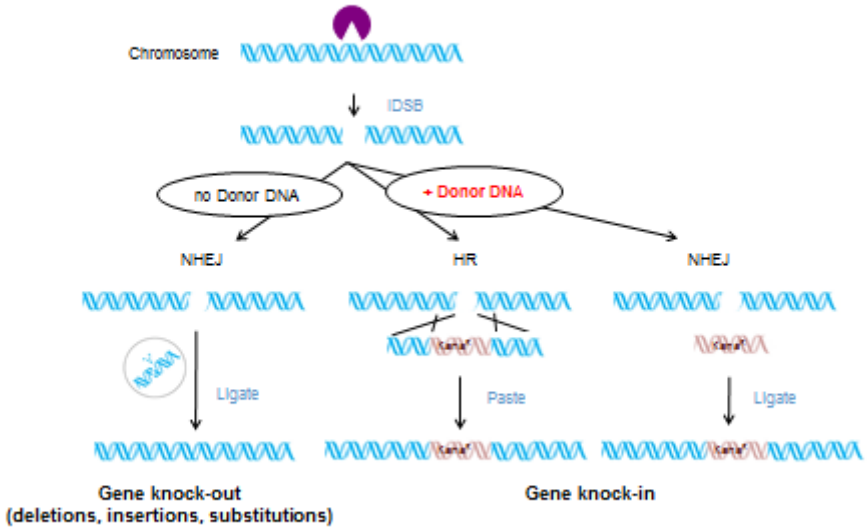


| | Meganuclease | ZFN | TALEN | CRISPR/Cas |
|---|---|---|---|---|
| Nb of proteins required | 1 | 2 | 2 | 1 + gRNA |
| Off-target | Very low (target = 18-24 bp) | Medium high (target = 2 x 12-18 bp) | Low (target = 2 x 10-30 bp) | Low (target = 19-23 bp) |
| Diversity of target sequences | One cutting site every 250 bp | One cutting site every 500 bp | One cutting site every 35 bp | One cutting site every 8 pb |
| Difficulty of production | Heavy (modification of the peptid chain and selection) | Uneasy (interaction effects on specificity) | Relatively easy (modular assembly) | Very easy (synthesis of one oligonucleotid) |
| Approximate price | 50 000 € | 5000 € | 1000 € | 100 € |

+ Hyper-specific MegaTALE (Boissel et al., 2013)

+ Hybrid nucleases (one subunit of ZFN + one subunit of TALEN) (Yan et al., 2013)

## SDN-induced DSB

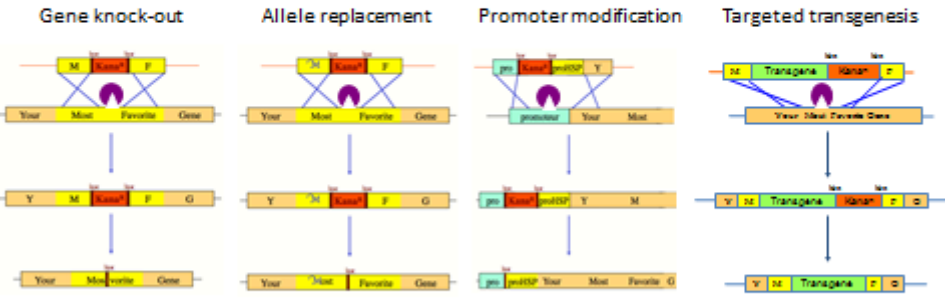





## Applications of SDNs-induced Gene Targeting

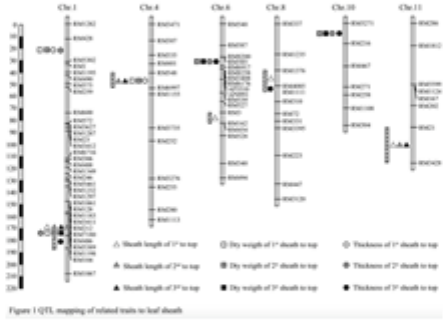
Precise modifications of the genome:

- Functional analysis of genes
- Biotechnological improvement of species



## Potential applications of SDNs for Plant Breeding

1) **Validation of candidate genes** by SDN-induced gene knock-out



2) **Knock-out (loss of function) or knock-in (allele replacement) of the validated genes.**

➢ Potentially all characters for which the genes involved are known can be modified by using SDNs.



## Potential applications of SDNs for Plant Breeding

- Fast assembling of alleles and genes (simplification of QTL pyramiding, ...)
  + interest for perennials, species propagated vegetatively, polyploid species...


Fruit trees

- Specific applications : elimination of unfavorable alleles, selection of recessive alleles, breaking of linkage drags
- Transfer of genes of interest from a wide range of species (exploitation of genetic resources)

- Possible insertion of alleles of interest in minor or orphan crops


sorghum


Cow pea


Pigeon pea

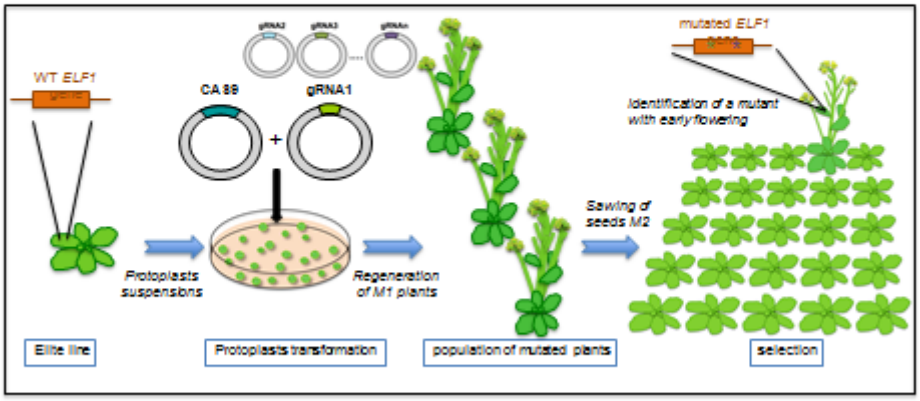

Cassava


Yam

- Design of traits adapted to very specific environments
- Opportunities to increase genetic diversity
  - ➔ Induction of targeted genetic variability (TIGV)
  - ➔ cis regulatory alleles
  - ➔ « rewilding » concept

## Targeted induced genetic variation (TIGV)

➢ Inducing targeted mutations in genes of interest in order to create and select new phenotypes.

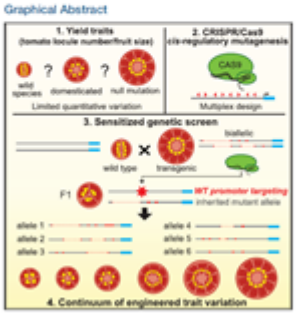





## CRISPR gene editing to advance traits discovery : creation of a new variability

**CRISPR/Cas9 mutagenesis of promoters to generate novel cis-regulatory alleles to achieve a range of variation for quantitative traits**

**Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing**

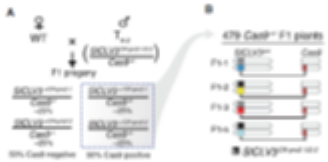



Authors
Daniel Rodriguez-Leal, Zachary H. Lemmon, Jarrett Man, Madelaine E. Bartlett, Zachary B. Lippman

Correspondence
lippman@cshl.edu

In Brief
In this article, we discuss how to use the CRISPR/Cas9 genome editing approach to dissect the biology of quantitative trait loci.

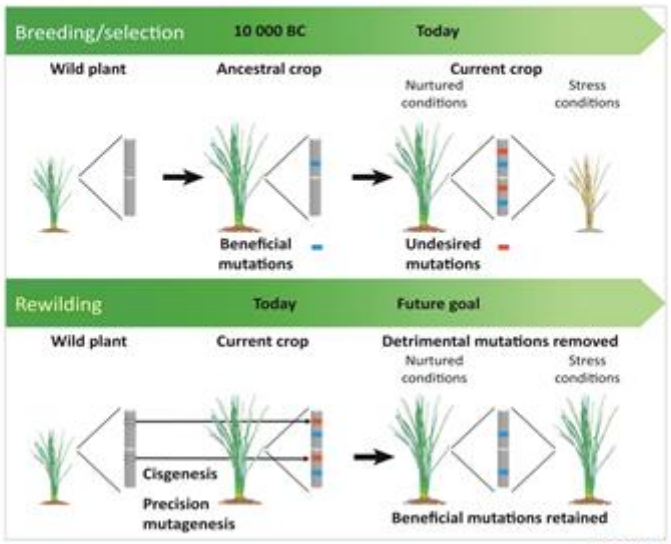
Cell



a simple genetic scheme which exploits trans-generational heritability of Cas9 activity

## Rewilding concept

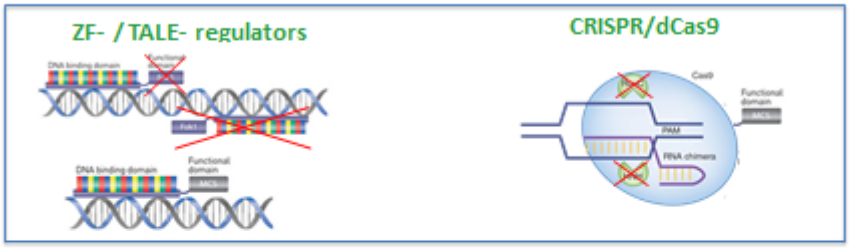
➢ Domestication of wild plants by inactivating the already identified domestication genes.

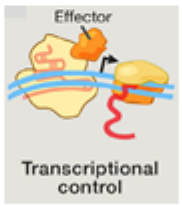



(Palmgren et al., 2014)



## Site-Directed Effectors





- activation of gene expression
- repression of gene expression
- activation of noncoding genes to enhance repression

Fusion to epigenetic enzymes (DNA methyltransferase or histone deacetylase) to install or remove specific epigenetic marks and induce gene silencing.

## Potential applications of SDNs for Plant Breeding

**A great diversity of potential traits :**

- **Disease resistance** (eg. target: translation initiation factors)
- **Herbicide tolerance** (eg. target: acetolactate synthase gene)
- **Drought tolerance** (eg. Target : promoters of genes activated by water stress)
- **Low anti-nutritional compounds** (eg. erucic acid in Brassicas, target: fatty acid elongases)
  **and allergens** (eg. target: conglutin genes in peanut, gluten in wheat)
- **Improved nutritional value** (eg. via elevated carotenoids, target: zeaxanthin epoxidase)
- **Modified starches and fats for food and non-food uses**
  (eg. target: starch synthases, branching enzymes and fatty acid desaturases)
- **Longer shelf life/reduced wastage** (eg. targets: aminocyclo-propane (ACC) oxidase and polygalacturonase)
- **Reduced enzymatic** (eg. target: polyphenol oxidases)
  **and nonenzymatic browning** (eg. target: invertase genes for high quality and low acrylamide potato )
- **Improved yield via modified RuBisCO genes** (eg. increasing catalytic activity, decreasing oxygenation activity)
- **Improved biomass conversion for biofuels** (eg. lower lignin)
- **Control of meiosis** : Increase crossing overs, diplogametes, ...
- ...



## Potential applications of SDNs for Plant Breeding

**Qualitative and quantitative traits can be achieved.**

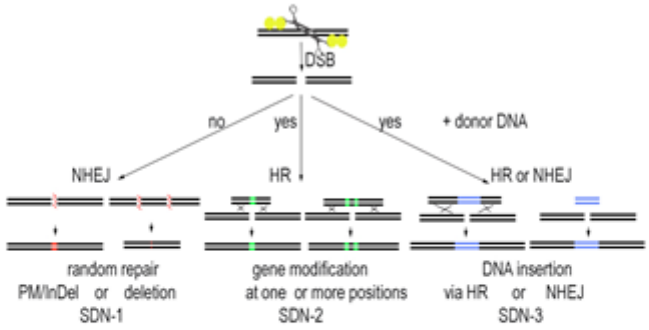
**Easier for monogenic or oligogenic traits (quality, resistance to biotic stresses...)**

**But more complex traits can also be improved (yield, resistance to abiotic stresses, metabolites...)**

**So far, easier by gene knock-out than by gene knock-in...**

**New products addressing from niche markets to large field crop markets**

## 3 types of events produced by using SDNs on plants




SDN-1 (random repair) : DSB result in site-specific random point mutations (PM) and short insertions/deletions (indel) or in excision. (no donor DNA)

SDN-2 (gene modification) : homologous donor DNA is used to induce specific nucleotide sequence changes by HR (= allele replacement)

SDN-3 (DNA insertion) : DNA is integrated at a specific site of the plant genome (landing pads) using either NHEJ (cassette without homology) or HR (cassette with homology).

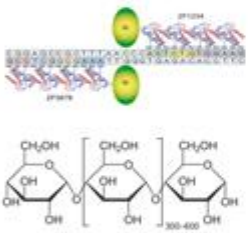


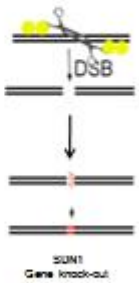
## SDN1 / ZFNs

Potato (2n = 4x)

Knock out the starch branching enzyme II (SBEII) gene to increase amylose content.

@WUR



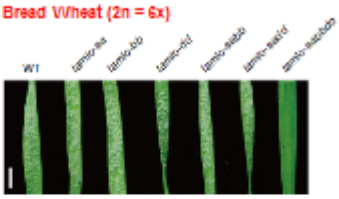



EXZACT™ Precision Technology (Dow AgroSciences)
http://www.exzactprecisiontechnology.com/index.htm

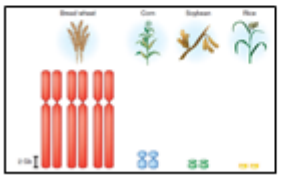
## SDN1 / TALEN

Particule gun

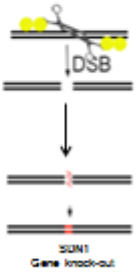

Bread Wheat (2n = 6x)




➤ knocking out all six alleles encoding the MILDEW-RESISTANCE LOCUS (MLO) protein
→ mutant line with strong resistance to powdery mildew.



Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew nature biotechnology (2014)

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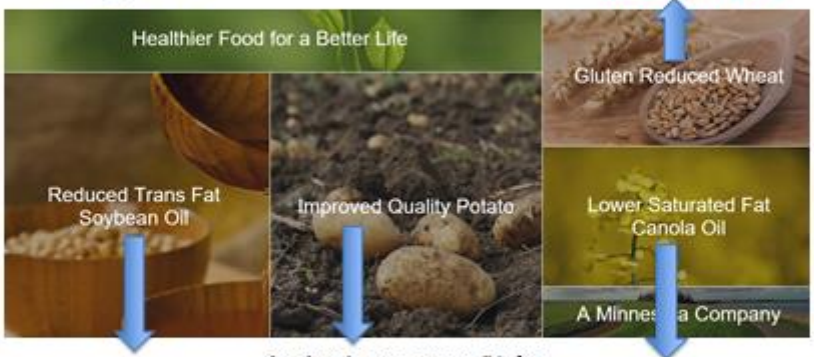
## SDN1 / TALEN

calyxt

+ High fiber wheat
→ still at an early stage.

Removal of the components of gluten responsible for the immune reaction
→ still at an early stage.




high oleic acid and low linolelc acid = ~20% decrease in saturated fatty acids compared to standard soybean oil
→ 30 tons produced in Argentine in 2016.

inactivated enzyme responsible for the degradation of sugars in tubers
→ reduction of sweetening of cold-stored potatoes and of creation of acrylamide during frying.
→ 2016 = 2nd year of field trials.

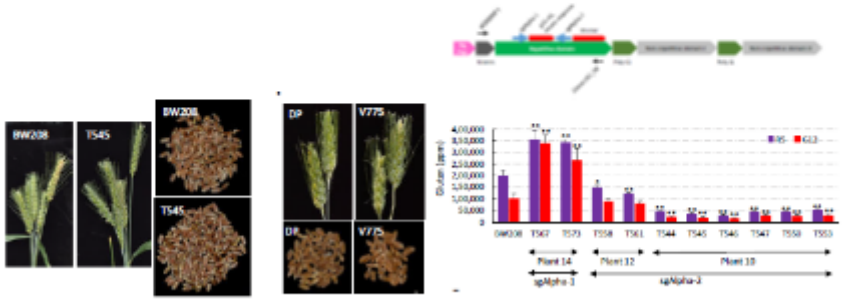
3.5% saturated fat (instead of 7%) by deactivating one enzyme responsible for the synthesis of saturated fatty acids
→ On going.

## SDN1 / CRISPR-Cas

### Low-gluten, non-transgenic wheat engineered with CRISPR/Cas9

Susana Sánchez-León1, 4, Javier Gil-Humanes2, 4, Carmen V. Ozuna1, María J. Giménez1, Carolina Sousa3, Daniel F. Voytas2, Francisco Barro1,*

two sgRNAs targeting a conserved region in the 45 gliadin genes of the WT
21 mutant lines, all showing strong reduction in gliadins.
Up to 35 different genes mutated → immunoreactivity reduced by 85%.

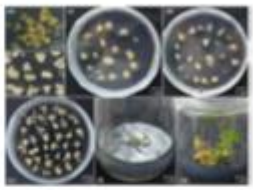




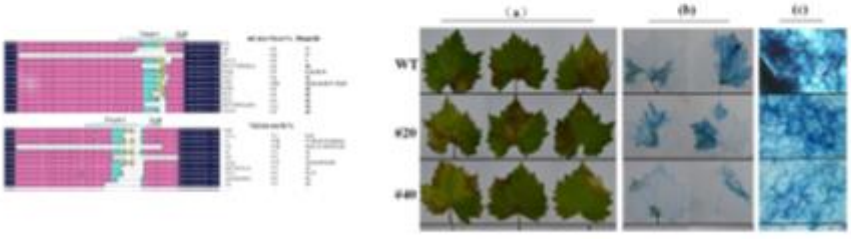

## SDN1 / CRISPR-Cas

**CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation**

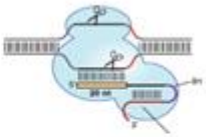
Xianhang Wang[1,†], Mingyang Tu[1,†], Dejun Wang[1,2], Jianwei Liu[1,2], Yuyuan Li[1,2], Zhi Li[1,2], Yuejin Wang[1,2], Xiping Wang[1,2*]



knock out of transcription factor *VvWRKY52* in grape increased the resistance to *Botrytis cinerea*.



## SDN1 / CRISPR-Cas

Tomato

Mutations in 3 regions of the RIN gene (transcription factor regulating fruit ripening)

→ incomplete-ripening fruits in homozygous mutants
(longer shell-life, easier processing).

CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening (2015)

Biochemical and Biophysical Research Communications



## SDN1 / CRISPR-Cas


Dr Yinong YANG

CRISPR/Cas9-edited white button mushroom (Agaricus bisporus)

Transient transformation of protoplasts (no transgene)
Small deletions (1-14 bp) in the polyphenol oxydase gene
→ anti-browning phenotype

USDA considers it as non regulated (April 13, 2016).

## SDN1 / CRISPR-Cas

(KO of 1 gene (wx) to block the production of amylose)

DUPONT PIONEER

100% amylopectin corn
→ Feed, industry.

(conventional waxy cvs = 3,5 % less yield)

**waxy corn hybrids**

USDA considers it as **non regulated** (April 18, 2016).

## SDN2 / CRISPR-Cas

Accepted Date : 15-Jul-2016
Article type : Research Article

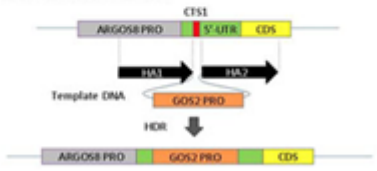
Plant Biotechnology Journal
Open Access

**ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions**

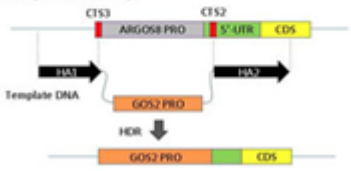
*Jinrui Shi*, Huirong Gao, Hongyu Wang, H. Renee Lafitte, Rayeann L. Archibald, Meizhu Yang, Salim M. Hakimi, Hua Mo, and Jeffrey E. Habben*

DUPONT PIONEER

**GOS2 promoter insertion**



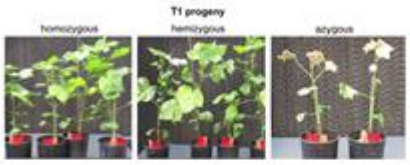

**GOS2 promoter swap**



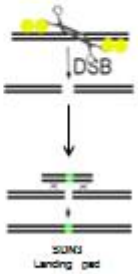
## SDN3 / Meganuclease

Particule gun

Cotton




- gene stacking using I-CreI
- precise insertion of two herbicide tolerance genes (*hppd*, *epsps*)
  next to pre-existing Bt and bar loci in 2% of the embryogenic callus lines
- all trait genes were inherited as a single genetic unit




Plant Biotechnology Journal (2013) 11, pp. 933–941 doi: 10.1111/pbi.12085

**Targeted molecular trait stacking in cotton through targeted double-strand break induction**

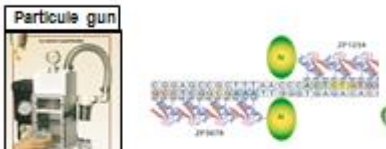
Kathleen D'Halluin[1,*], Chantal Vanderstraeten[1], Jolien Van Hulle[1], Joanna Rosolowska[1], Ilse Van Den Brande[1], Anouk Pennewaert[1], Kristel D'Hont[1], Martine Bossut[1], Derek Jantz[2], Rene Ruiter[1] and Jean Broadhvest[1]

[1]Bayer CropScience N.V., Gent, Belgium
[2]Metabolix BioSciences, Durham, NC, USA

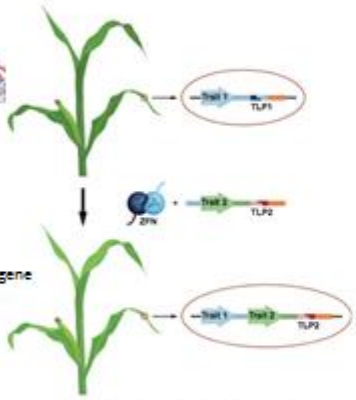


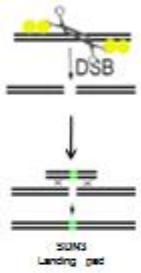
## SDN3 / ZFN

Particule gun



Maize




- stacking :
  5% of transgenic events integrated the transgene precisely at the landing pad.

**Trait stacking via targeted genome editing**

William M. Ainley[1,*], Lakshmi Sastry-Dent[1,*], Mary E. Welter[1], Michael G. Murray[1], Bryan Zeitler[2], Rainer Amora[2], David R. Corbin[1], Rebecca R. Miles[1], Nicole L. Arnold[1], Tonya L. Strange[1], Matthew A. Simpson[1], Zehui Cao[1], Carley Carroll[1], Katherine S. Pawelczak[1], Ryan Blue[1], Kim Weis[1], Lynn M. Rowland[1], Douglas Perkins[1], Pon Samuel[1], Cristie M. Dewey[1], Liu Shen[1], Shreedharan Sriram[1], Steven L. Evans[1], Edward J. Reba[2], Lei Zhang[2], Phillip D. Gregory[2], Fyodor D. Urnov[2], Steven R. Webb[1] and Joseph F. Petolino[1,*]

[1]Dow AgroSciences LLC, Indianapolis, IN, USA
[2]Sangamo BioSciences, Inc., Richmond, CA, USA

Plant Biotechnology Journal (2013) 11, pp. 1126–1134

## SDN-mediated varieties and DUS

### Description of SDN-mediated candidate varieties

For each PVP application CPVO Technical Questionnaire gives:

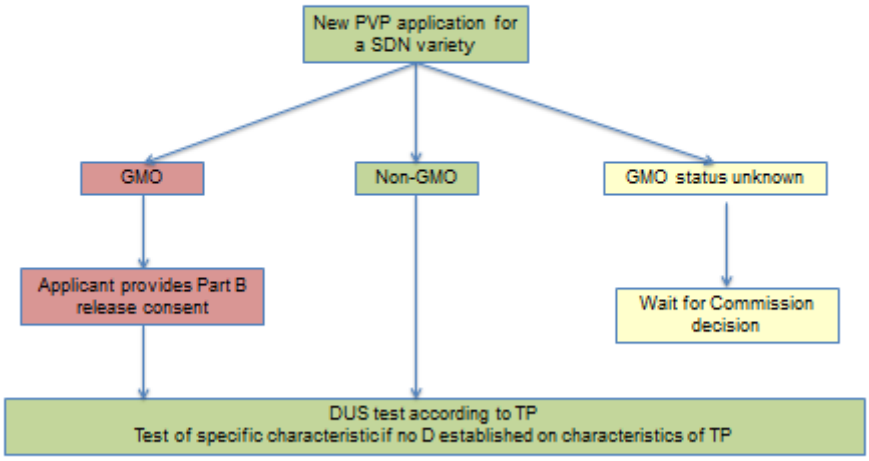
- Information on breeding scheme and multiplication
- Description of specific characteristics
- Declaration of GMO status according to Directive EC/2001/18

→ Necessary loyalty
since no detection possible without declaration for SDN1 and SDN2
(impossible to distinguish between targeted and natural mutations)



## SDN-mediated varieties and DUS

### DUS Procedure for SDN-mediated candidate varieties



## SDN-mediated varieties and DUS

- SDNs are breeding tools to introduce traits into varieties:
  - Either these traits contribute to characteristics evaluated in the TP,
  - Or they are specific characteristics that need to be treated as additional characteristics (fulfilling UPOV requirements).

    → Distinctness is established using the same minimum distances as usually.

A priori, the use of SDN for plant breeding should not change fundamentally the process of DUS testing, but could increase the diversity of traits to evaluate.



CPVO · OCVV
Community Plant Variety Office
Office Communautaire des Variétés Végétales

Thank you !

[End of Annex and of document]