10th Scientific Conference of the Bulgarian Focal Point of EFSA
"10 years of food science in service of consumers"
31 October – 2 November 2017, Sofia, Grand Hotel Sofia

New plant breeding techniques. Are there rational reasons to stop knowledge?

Krasimir Rusanov, Daniela Moyankova, Dimitar Djilianov,
Agrobioinstitute, Agricultural Academy

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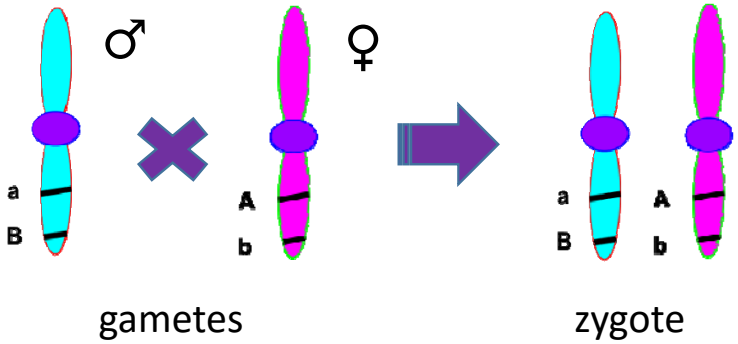
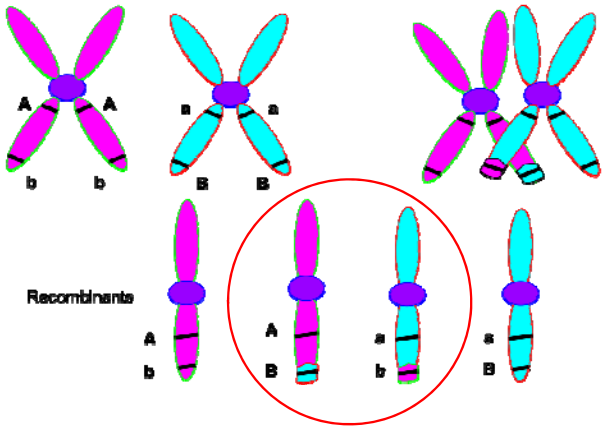
How does nature maintain and increase diversity?



gametes zygote



During meiosis allelic rearrangements occur along the homologous chromosome arms

Combining alleles

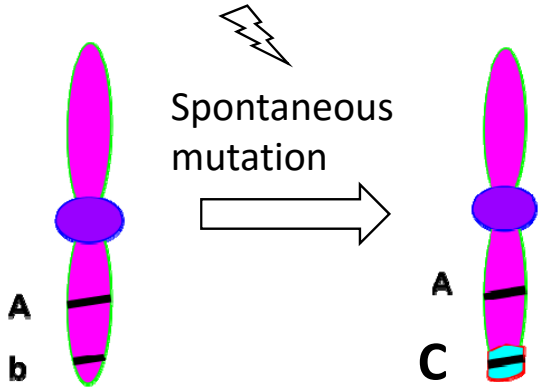
During cross pollination alleles from both parents are combined in a single organism

However, during cross-pollination, the variety of genes/alleles is mostly limited to those available among species that are capable of crossing

Horizontal gene transfer is relatively rare event in plants but there are evidences that it has happened many times during evolution

Recombination

Due to crossingover aleles A and B, which are located on two different homologous chromosome in somatic cells can be found on one chromosome (same DNA chain) in gametes

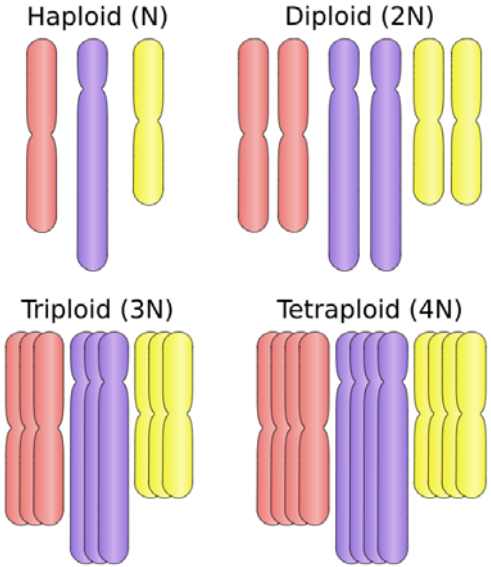


Spontaneous mutations

Spontaneous mutations occur frequently and sometimes they remain in the offspring. This leads to occurrence of new alleles which previously did not exist on the population or even the species level

Gene duplication

During evolution many genes were duplicated and formed gene families where gene function of a member of the family could become different from the original gene



Polyploidization

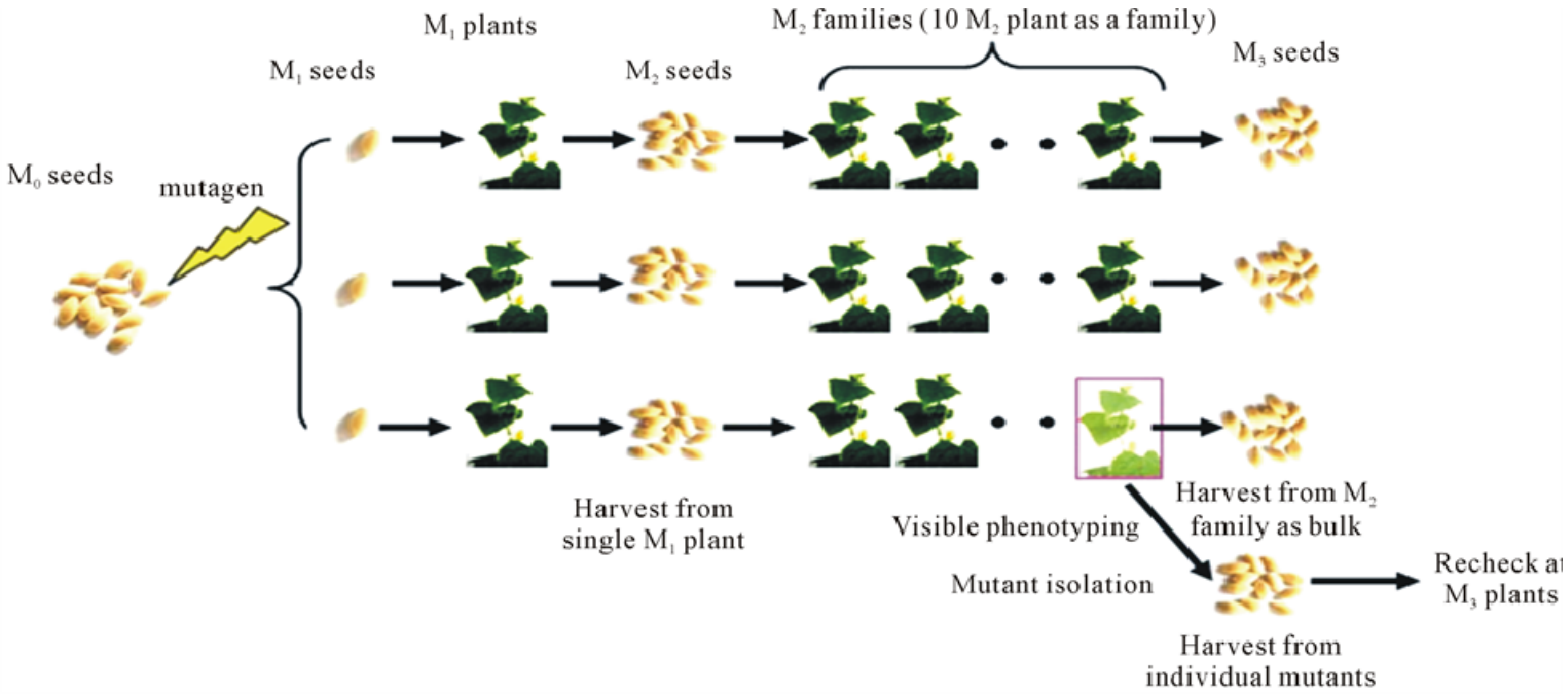
More than two copies per homologous chromosome. Multiple number of alleles per locus. Many plants are polyploids including wheat, tobacco, sugarcane, apple, cotton, rose etc.

What does man do to increase diversity?

All that nature does (cross pollination, selection) plus:

Breeding methods using radiation or chemical mutagenesis

- Radiation mutagenesis - gamma rays, X-rays
- Chemical mutagenesis - alkylating agents, ethylmethanesulfonate



Wang, L. , Zhang, B. , Li, J. , Yang, X. and Ren, Z. (2014) Ethyl Methanesulfonate (EMS)-Mediated Mutagenesis of Cucumber (*Cucumis sativus* L.). *Agricultural Sciences*, **5**, 716-721. doi: 10.4236/as.2014.58075.

- Authorized for use!
- Working blindly
- Multiple mutations with unknown localization in the genome are generated including SNPs, large deletions, chromosome aberrations, etc.
- Plants with positive qualitative or quantitative traits are selected
- Increasing the genetic diversity in species with low diversity
- A number of varieties have been created using mutagenesis. For example, the Bulgarian oil-bearing rose (*Rosa damascena* Mill.) var. Janina (radiation mutagenesis) and var. Elejna (chemical mutagenesis) (Assoc. Prof. Dr. Raycho Tsvetkov)

Genetic engineering in plants
How do we make GMO plants?

Many gene functions have already been known since the 1980's

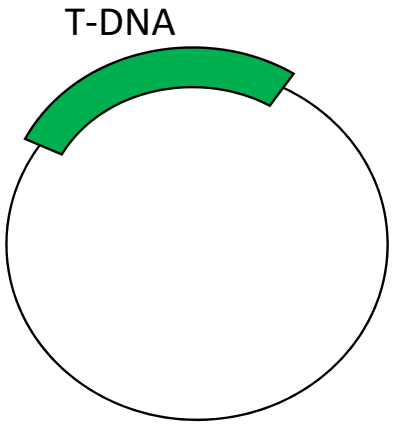
Can we transfer a "good" gene to help a plant be more profitable?

Genetic transformation

Agrobacterium tumefaciens

Natural genetic engineer

- Soil bacteria causing tumors in plants
- Capable of transferring a fragment of its DNA called T-DNA into the host plant genome
- T-DNA leads to tumor formation



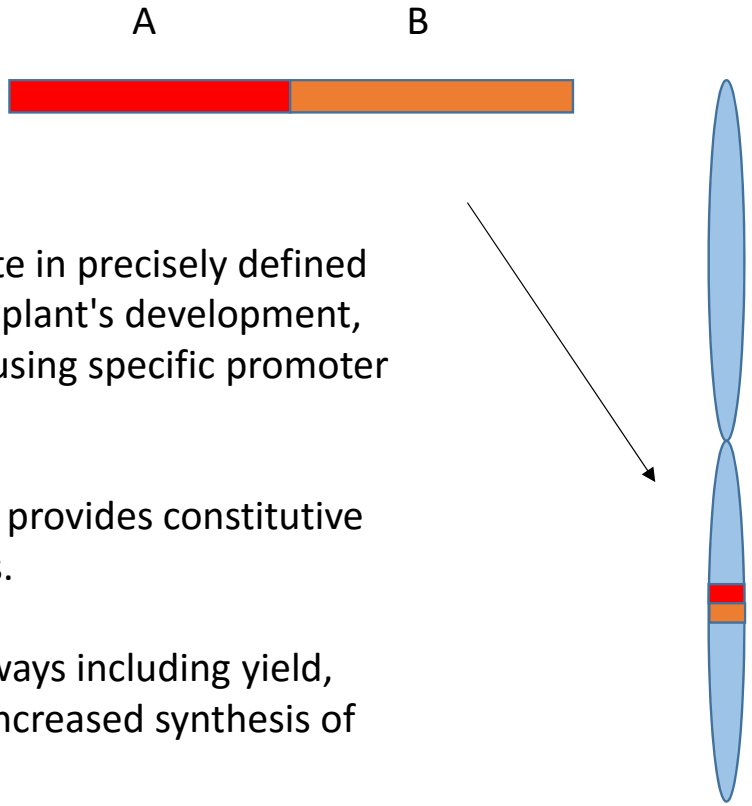
Tumor inducing plasmid

Chromosome of a host plant with integrated T-DNA

Tumor formation in roots

Schell, J; Van Montagu, M (1977). "The Ti-plasmid of Agrobacterium tumefaciens, a natural vector for the introduction of nif genes in plants?". *Basic life sciences*. **9**: 159–79

In reality, in addition to the transgene bearing the desired trait, another gene is also provided which allows selection of plants bearing the transgene
A - transgene
B – selectable marker (e.g., antibiotic or herbicide resistance gene)



Chromosome containing a transgene and a selectable marker

The transgene may be “tuned” to operate in precisely defined tissues and organs, at a specific stage of plant's development, or when exposed to external factors by using specific promoter regions.

The most commonly used 35S promoter provides constitutive expression of the transgene in all tissues.

Plants can be improved in a number of ways including yield, resistance to biotic and abiotic factors, increased synthesis of healthy metabolites etc.

Issues concerning the use of GMOs

Moral and ethical issues

- Transfer of genes between unrelated species and even between kingdoms that can not be combined naturally into one organism for long periods of time during evolution or even never. Do we have the moral right to do this?

Environmental issues

- Is it possible to control the spread of plant GMOs in nature as a result of free pollination?
- Development of organisms that have genetic advantages which can not be naturally acquired through crossing. Imbalance of populations resulting from the appearance of a transgenic species with significant advantages

A number of projects have been carried out to study the spread of pollen from GMO plants (eg. FP7 CoExtra) and methods have been proposed for the cultivation of GMO plants. Because of the integration of "foreign DNA" in the host, there are molecular methods which can identify the presence or absence of GMOs in plants and foods

Potential health problems

- So far, there are no convincing evidences that consumption of GMOs creates greater risk for human health
- Some people may have or develop allergic reaction to proteins or compounds that are present in GMO cultivars
- Therefore each food needs to be tested before release on the market

The boom of GMO plants after the development of technology in the 1980s

- Rapid commercialization of GMO plants by large companies in the US. In 2016 78% of soybean, 64% of the cotton and 33% of corn world production is GMO based
- Insufficient clarification of the technology used and too rapid commercialization by companies. Serious resistance from environmental organization

Current state of legislation on GMO cultivation in EU

- Each country in the EU decides whether to grow GMOs on its own territory
- Legislation in Bulgaria is very restrictive, as GMOs can not be cultivated in field conditions
- Work with GMOs in laboratory conditions is permitted following permission by the MOEW of BG

Molecular Breeding based on genomic studies

Classical breeding helped by DNA markers linked to loci controlling specific traits. Rapid development since the 1990s. Includes:

- Development of plant segregating populations (as in classical breeding)
- Development of genetic maps including DNA markers
- Phenotyping of the developed populations
- Discovery of QTLs and DNA markers linked with specific traits
- Breeding using the linked markers

Whenever sequences of compete genomes are available markers can be linked to physical loci in the genome. Genes can be functionally characterized, used for marker development and breeding

New Plant Breeding Techniques

Editing the genome of living organisms

Two parallel mechanisms to repair double-strand breaks in DNA

Non-Homologous End Joining (NHEJ)

- often associated with minor deletions or insertions at the repair site

Homology-Directed Repair (HDR)

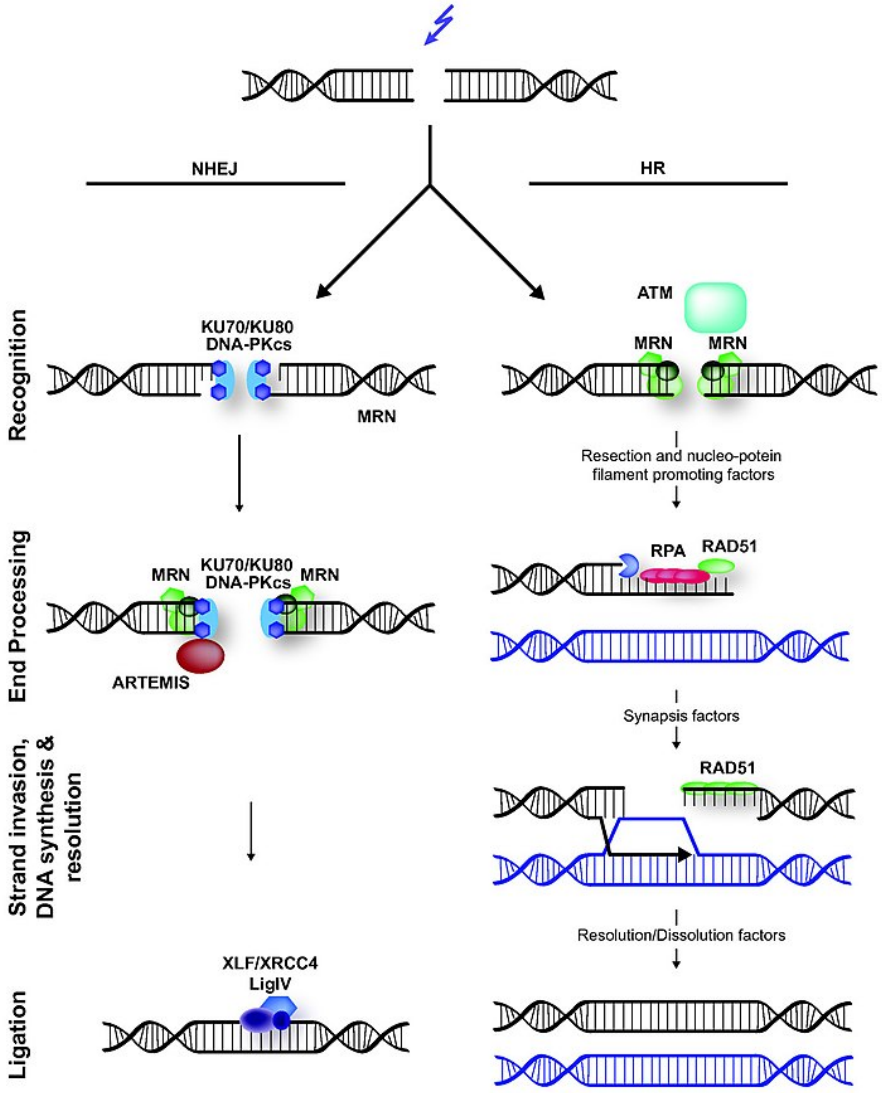
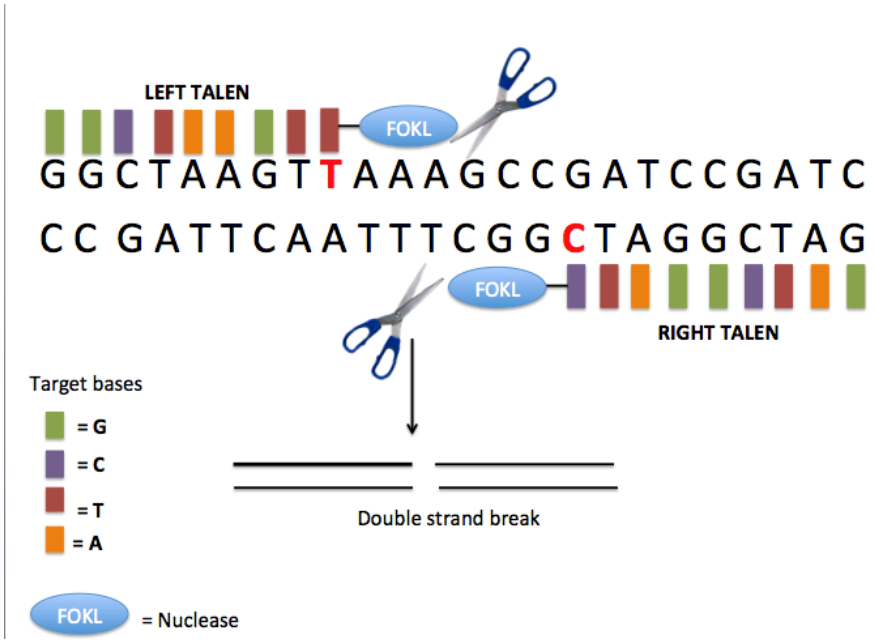


Fig adapted from Hannes Lans, Jurgen A. Marteijn & Wim Vermeulen (2012). "ATP-dependent chromatin remodeling in the DNA-damage response". *Epigenetics & chromatin* **5**: 4. DOI:10.1186/1756-8935-5-4

If we can create double-strand breaks at specific locations in the genome of organisms we will have the opportunity to:

- Induce mutations in desired genes in order to inactivate them
- Repair mutated genes to restore their reading frame
- Change the promoter regions of genes in order to alter their expression
- Replace one allele with another (from the same organism or another)
- Integrate new genes and create GMOs
- .....



NHEJ HDR

TALENs (Transcription activator-like effector nucleases)

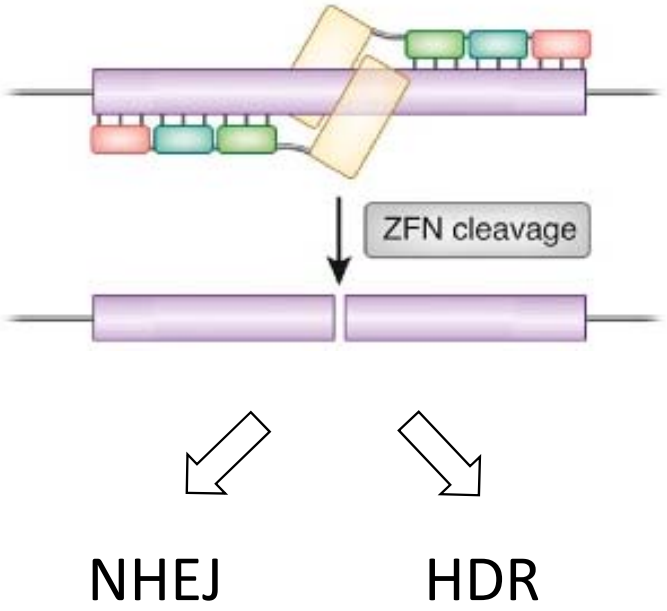
Artificially created restriction enzymes designed to cut at precisely defined nucleotide sequence
Consisting of **TAL (Transcription Activator-like)** domain and DNA-Cutting monain (**Nuclease Domain**)

TAL are proteins secreted by bacteria of the genus Xanthomonas when infecting a plant which bind to promoter regions and activate the expression of genes helping the bacterium to infect the plant

TALs contain a highly conservative domain of 33-34 amino acids except the 12th and 13th amino acids that vary and whose variation results in the recognition of a **specific nucleotide**

The nuclease domain is a FOKL nonspecific restriction enzyme from bacteria (*Flavobacterium okeanokoites*). Consists of two domains which become active when brought close to each other

Zinc finger nucleases (ZFNs)



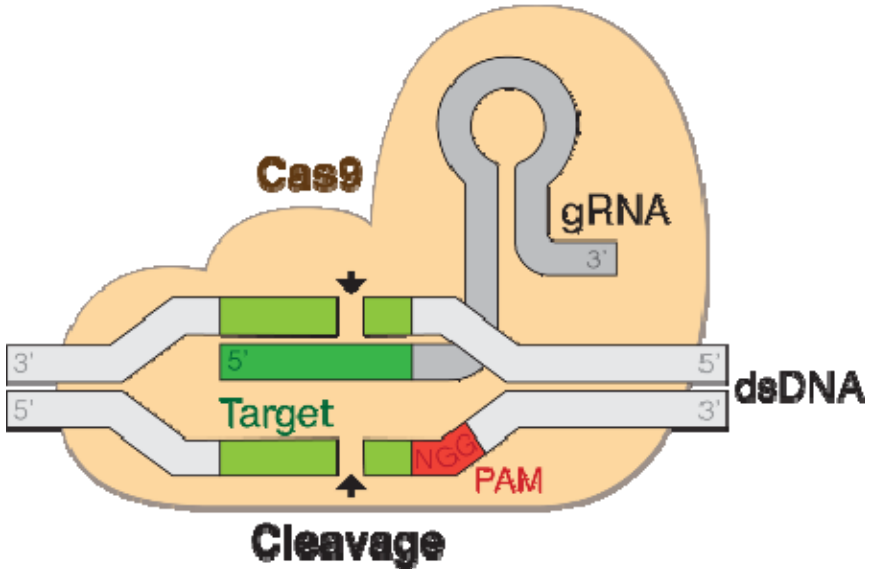
Artificially created restriction enzymes consisting **of a zinc finger DNA binding domain** and a **DNA-cleaving domain**

The zinc finger domain can be designed to recognize a specific DNA sequence

The zinc finger domain consists of individual zinc finger proteins which recognizes a specific 3 nucleotide sequence

Therefore combining different zinc finger proteins will create a zinc finger domain recognizing a specific DNA sequence

The DNA cleavage domain is a FOKL nonspecific restriction enzyme from bacteria (*Flavobacterium okeanokoites*). Consists of two domains which become active when brought close to each other



NHEJ HDR

CRISPR/Cas9*

CRISPR / Cas is a prokaryotic immune system

CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats

CRISPRs are regions of DNA in the genome of the bacterium containing short palindromic repeats + spacing areas containing DNA sequences of viruses that the microorganisms store to protect against future attacks from the same viruses

Consists of guide RNA (gRNA) and Cas9 (**C**RISPR **a**ssociated protein **9**) nuclease

Cas9 binds to the guide RNA (gRNA) and cuts the DNA at the position where homology exists

Advantages over Talens and Zinc finger nucleases in terms of ease of implementation

Jinek M., Chylinski K., Fonfara I., Hauer M., Doudna J. A., Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity 2012, Science, 337 (6096), 816-21

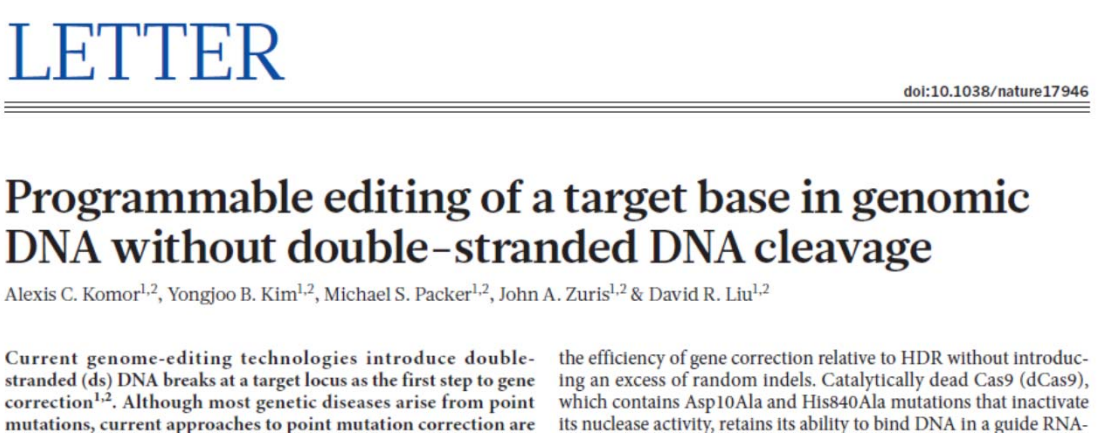
Editing the genome without double strand breakage

Komor et al. 2016* introduced programmable editing

Includes addition of cytidine deaminase enzyme to the CRISPR/Cas9 machinery (Cas9 nuclease activity inactivated)

Based on chemical modification cytidine to uridine in target regions followed by C→T (or G→A) substitution after replication

*Komor A. C., Kim Y. B., Packer M. S., Zuris J. A., Liu D. R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage 2016, Nature, 533 (7603), 420-4, Komor, Alexis C. Nature. 2016 May 19;533(7603):420-4



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 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-

No need to search for natural QTLs – just create them with CRISPR/Cas9

Rodrı´guez-Leal et al., Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing, Cell (2017), http://dx.doi.org/10.1016/j.cell.2017.08.030

Two loci (*fas* and *lc*) playing major role during tomato domestication related to fruit size. Both of this loci comprise mutations in cis-regulatory elements (promoter and repressor regions)

numerous promoter variants - allele development – QTL controlling tomato fruit size (increased number of carpels), inflorescence branching, and plant architecture

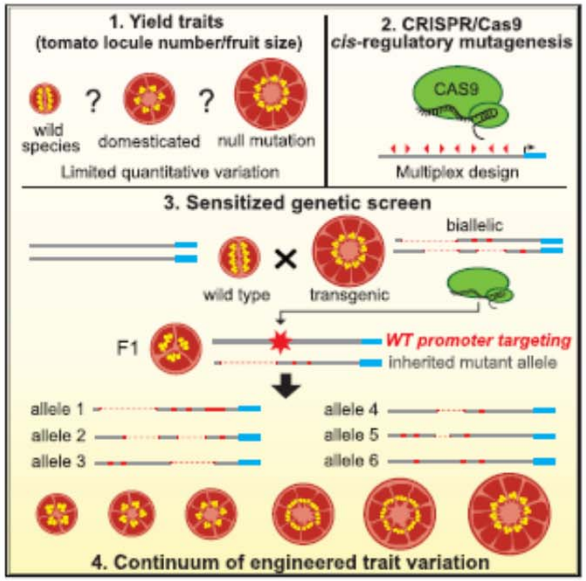
Recreate plant domestication using CRISPR-Cas9

Resource

Cell

Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing

Graphical Abstract



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

Highlights

- CRISPR/Cas9 targeting of a *cis*-regulatory motif recreated a domestication QTL
- CRISPR/Cas9 drove mutagenesis of promoters to create a continuum of variation
- Phenotypic effects were not predictable from allele type or transcriptional change
- Selected promoter alleles in developmental genes could improve yield traits

Overcoming male sterility in rice indica-japonica hybrids using CRISP/Cas9

Hybrids between indica and japonica rice are usually sterile thus hindering the use of heterosis effect in breeding

The Sa locus consists of two adjacent genes SaF and SaM which interact with each other to cause pollen abortion in hybrids carrying the japonica allele

Silencing of SaF or SaM using RNAi restores fertility

Xie et al. 2017 developed SaF and SaM knock-out mutants of using CRISPR/Cas9 to obtain the same results as with RNAi

JIPB Journal of Integrative Plant Biology

Suppression or knockout of *SaF*/*SaM* overcomes the *Sa*-mediated hybrid male sterility in rice[FA]

Yongyao Xie[1,2,3†], Baixiao Niu[3†,††], Yunming Long[3†††], Gousi Li[1,2,3], Jintao Tang[1,2,3], Yaling Zhang[1,2,3], Ding Ren[3††††], Yao-Guang Liu[1,3,4] and Letian Chen[1,2,3,4*]

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doi: 10.1111/jipb.12564

Abstract Hybrids between the *indica* and *japonica* subspecies of rice (*Oryza sativa*) are usually sterile, which hinders utilization of heterosis in the inter-subspecific hybrid breeding. The complex locus *Sa* comprises two adjacently located genes, *SaF* and *SaM*, which interact to cause abortion of pollen grains carrying the *japonica* allele in *japonica-indica* hybrids. Here we showed that silencing of *SaF* or *SaM* by RNA interference restored male fertility in *indica-japonica* hybrids with heterozygous *Sa*. We further agricultural traits, but did break down the reproductive barrier in the hybrids. We found that some rice lines have natural neutral allele *Sa-n*, which was compatible with the typical *japonica* or *indica Sa* alleles in hybrids. Our results demonstrate that *SaF* and *SaM* are required for hybrid male sterility, but are not essential for pollen development. This study provides effective approaches for the generation of hybrid-compatible lines by knocking out the *Sa* locus or using the natural *Sa-n* allele to overcome hybrid male

Research Article

Multisite genome editing in polyploids

Need to edit multiple sites in polyploid genomes

Wang et al. 2017 targeted a reporter gene DsRed2 previously inserted in the genome and an endogenous gene GhCLA1 of allotetraploid Mexican cotton

Coding regions of both genes were targeted using six sgRNAs used to target DsRed2 and 4 to target GhCLA1.

Two sgRNAs combined in one vector for transformation. 3 vectors for transformation and editing of DsRed2 and 2 vectors for GhCLA1 editing.

The efficiency of editing at each target site was 66-100%.

No off-targeting observed for the endogenous gene

Plant Biotechnology Journal

Plant Biotechnology Journal (2017), pp. 1–14 doi: 10.1111/pbi.12755

High efficient multisites genome editing in allotetraploid cotton (*Gossypium hirsutum*) using CRISPR/Cas9 system

Pengcheng Wang[1,†], Jun Zhang[1,†], Lin Sun[1], Yizan Ma[1], Jiao Xu[1], Sijia Liang[1], Jinwu Deng[1], Jiafu Tan[1], Qinghua Zhang[1], Lili Tu[1], Henry Daniell[2], Shuangxia Jin[1,*] and Xianlong Zhang[1,*]

[1]*National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, Hubei, China*
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[†]These authors contributed equally to this work.

Summary

Gossypium hirsutum is an allotetraploid with a complex genome. Most genes have multiple copies that belong to At and Dt subgenomes. Sequence similarity is also very high between gene homologues. To efficiently achieve site/gene-specific mutation is quite needed. Due to its high efficiency and robustness, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system has exerted broad site-specific genome editing from prokaryotes to eukaryotes. In this study, we utilized a CRISPR/Cas9 system to generate two sgRNAs in a single vector to conduct multiple sites genome editing in allotetraploid cotton. An exogenously transformed

Targeted gene replacement using CRISPR/Cas9 in Arabidopsis

Zhao et al. 2016 report targeted gene replacement using CRISPR/Cas9 in Arabidopsis

(construct 1) dual-sgRNA/Cas9 vector - deletion of a miRNA gene regions (MIR169α and MIR827α)

(construct 2) Donor vector containing GFP reporter gene and homology sequences for HDR

Co-transformation with both vectors

0.8% (4 of 500) of the T0 plants contained the replaced region

www.nature.com/scientificreports

SCIENTIFIC REPORTS

OPEN

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An alternative strategy for targeted gene replacement in plants using a dual-sgRNA/Cas9 design

Yongping Zhao[1,*], Congsheng Zhang[1,2,*], Wenwen Liu[1], Wei Gao[1], Changlin Liu[1], Gaoyuan Song[1], Wen-Xue Li[1], Long Mao[1], Beijiu Chen[2], Yunbi Xu[1], Xinhai Li[1] & Chuanxiao Xie[1]

Precision DNA/gene replacement is a promising genome-editing tool that is highly desirable for molecular engineering and breeding by design. Although the CRISPR/Cas9 system works well as a tool for gene knockout in plants, gene replacement has rarely been reported. Towards this end, we first designed a combinatory dual-sgRNA/Cas9 vector (construct #1) that successfully deleted miRNA gene regions (*MIR169a* and *MIR827a*). The deletions were confirmed by PCR and subsequent sequencing, yielding deletion efficiencies of 20% and 24% on *MIR169a* and *MIR827a* loci, respectively. We designed a second structure (construct #2) that contains sites homologous to *Arabidopsis TERMINAL FLOWER 1* (*TFL1*) for homology-directed repair (HDR) with regions corresponding to the two sgRNAs on the modified construct #1. The two constructs were co-transformed into *Arabidopsis* plants to provide both targeted deletion and donor repair for targeted gene replacement by HDR. Four of 500 stably transformed T0 transgenic plants (0.8%) contained replaced fragments. The presence of the expected recombination sites was further confirmed by sequencing. Therefore, we successfully established a gene deletion/replacement system in stably transformed plants that can potentially be utilized to introduce genes of interest for targeted crop improvement.

NPBT can be considered as a much more precise, based on knowledge and responsible technique compared to the already accepted and widely used mutagenesis in breeding

Design plants following development of “artificial” QTLs

The first steps of NPBT application pass through genetic transformation for delivery of the genome editing machinery. However, the integrated in the genome transgene (genome editing machinery) is omitted in later generations to leave only the edited sequence which happens elsewhere in the genome.

NPBT can be used to integrate foreign DNA through HDR thus creating GMO as we know it

Questions arising

Should we be able to trace genome events developed through NPBT and when? How do we trace mutations which may already exist in nature or repaired genes?

Should we leave a molecular marker for every gene editing that we create using NPBT? If yes, then what do we do with classical mutagenesis we have used so far?

Should we revise definitions for GMO, breeding, mutagenesis? NPBTs smear the borders.

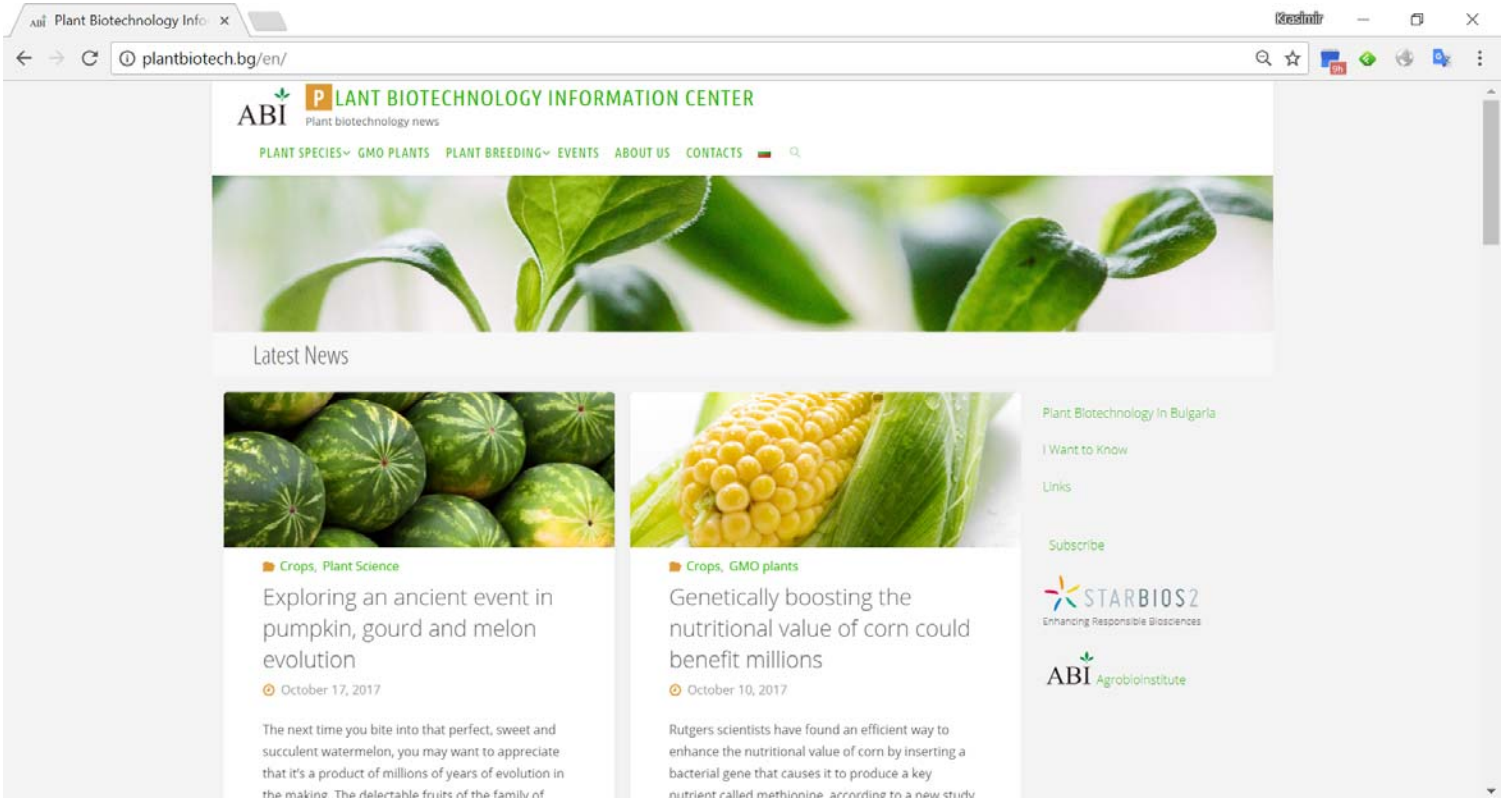
There is a need for clear legislation on when NPBT can be considered as GMO and under what legislation they should fall in

Plant Biotechnology Information Centre

http://plantbiotech.bg

Latest news on Plant biotechnology development worldwide including breeding, GMO plants and NPBT

Supported by Horizon 2020 Project STARBIOS2 and its team from the Agrobioinstitute, Sofia



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Thank you!

Our team:

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Agrobioinstitute, Agricultural Academy

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