**LGN5809 - Genética Molecular**
Docente: Dra. Maria Carolina Quecine Verdi

# GENÉTICA REVERSA

Discentes
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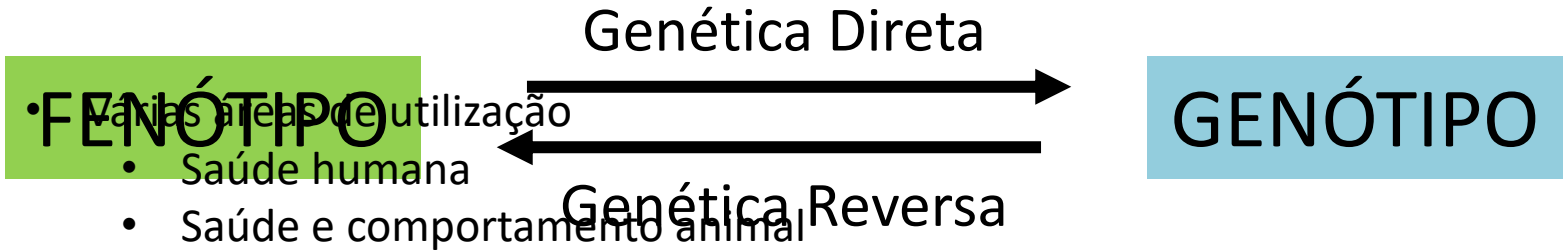
## Genética Direta

- Observações (fenótipo)
- Isolamento
- Identificar gene (mapeamento, sequenciamento)

X

## Genética Reversa

- Escolha de um gene
- Alteração de seu funcionamento (*knockdown*, *knockout*)
- Observações (fenótipo)




- Várias áreas de utilização
  - Saúde humana
  - Saúde e comportamento animal
  - Genética e melhoramento de plantas

- Importante papel complementar à genética direta
  - Compreender famílias de genes
  - Identificar mutações deletérias
  - Identificar genes de resistência

# Genética Reversa

Vantagens

- Velocidade
- Dispensa conhecimento do gene estudado
- Grande número de possibilidades de exploração

Desvantagens

- Em alguns casos inconclusiva / incoerente
- Especificidade de métodos (necessidade constante de atualização)

# Técnicas *Knockout* e *Knockdown*

**RECOMBINAÇÃO HOMÓLOGA**

**MUTAGÊNESE DE INSERÇÃO**

**RNAi**

# Recombinação homóloga

## *KNOCKOUT* DIRECIONADO

- Entrega de **DNA exógeno** por métodos **indiretos** (*Agrobacterium*) ou **diretos** (físicos e químicos);
- Substituição de um alelo selvagem funcional por um alelo inativo.

### Desvantagens

- Eficiência **variável** entre grupos;
- Em plantas, estudos desenvolvidos como prova de conceito.

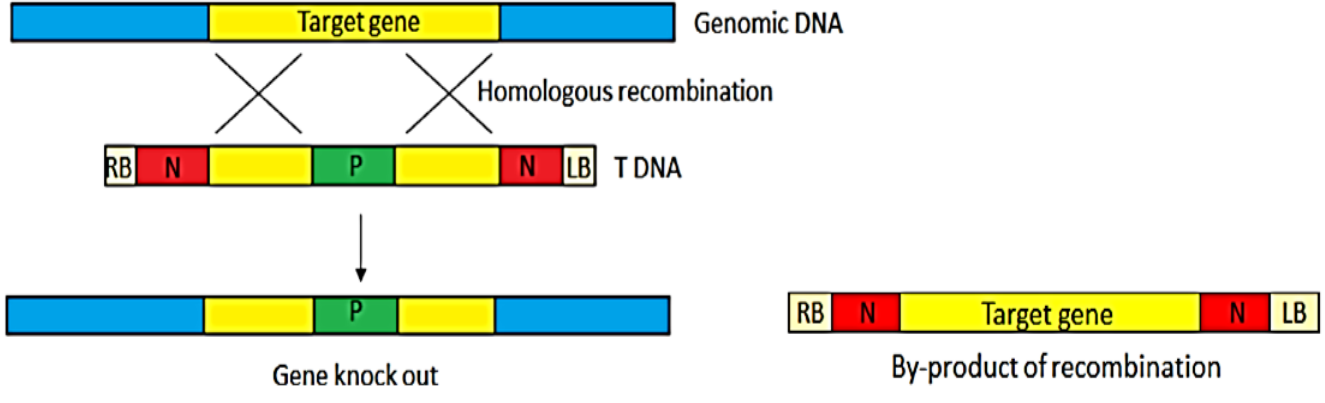



**Fig. 1** Illustration of homologous recombination (HR)-mediated gene targeting. Right border (RB), left border (LB), positive selection marker (P), negative selection marker (N). HR at the target site results incorporation of positive selection marker. Negative selection marker for the removal of ectopic recombinants

Fonte: AMRITHA; SHAH, 2021

# Mutagênese de inserção

## *KNOCKOUT* ALEATÓRIO

- Entrega de **DNA exógeno** por métodos **indiretos** (*Agrobacterium*) ou **diretos** (físicos e químicos);
- Integração **aleatória** no genoma para a geração de fenótipos alterados;
- Triagem para a **identificação** do **mutante** de interesse: PCR; sequenciamento.

**Desvantagens**

- Efeitos **variáveis** dependendo do local de inserção;
- Distribuição **tendenciosa** de **inserções** no genoma;
- **Incapacidade** de caracterizar **mutações letais**;
- **Inviabilidade** em **gerar** e **armazenar** população necessária.

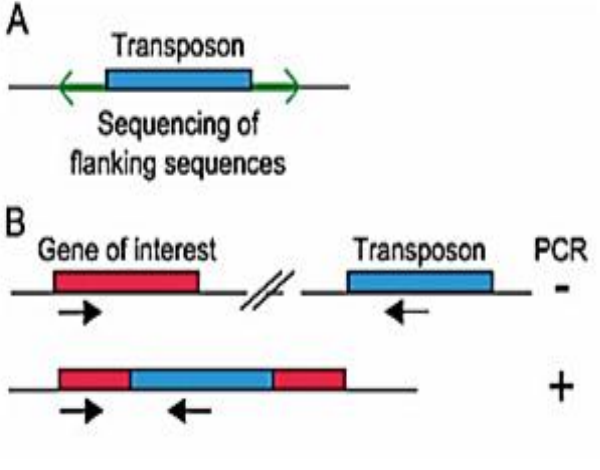



**Figure 1 | Screening for random mutations**
(**A**) Large collections of insertion mutants are generated. For each mutant, the regions flanking the transposon are sequenced, to identify the site of insertion. (**B**) After transposon insertional mutagenesis, screening is achieved by PCR using one primer that hybridizes in the transposon and another that hybridizes in the gene of interest. A PCR product will be obtained only if the transposon is inserted by chance in the gene of interest. To increase throughput, mutants are first tested in pools and then individually for mutants that belong to positive pools.

Fonte: Adaptado de HARDY et al., 2010

# RNAi

## *KNOCKDOWN* DIRECIONADO

- Entrega de **DNA exógeno** por métodos **indiretos** (*Agrobacterium*) ou **diretos** (físicos e químicos);
- **dsRNA**: expressão constitutiva ou transiente

- Elevada eficiência
- Rotineiramente utilizado para a determinação da função gênica

## DESVANTAGENS

- Inconsistência fenotípica
- Silenciamento de genes não-alvo

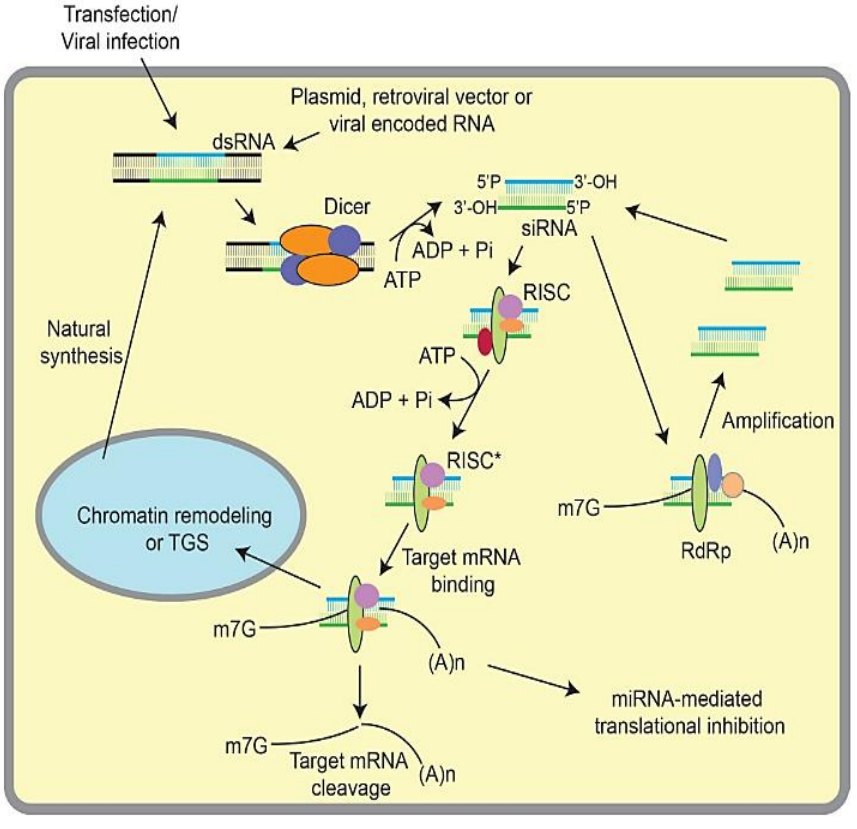



**Figure 1.** Mechanism of RNAi-mediated silencing. The model demonstrates double-stranded RNA (dsRNA) can generate either from exogenous natural sources, such as a viral infection, exogenous artificial sources such as transfection, or natural synthesis. The dsRNA is then processed by a multimeric Dicer enzyme to generate siRNA that can be further amplified by RNA-dependent RNA polymerase (RdRp). The siRNA subsequently interacts with an array of proteins to form RNA-induced silencing complex (RISC) that is activated in an ATP-dependent manner. The activated RISC (RISC*) can then induce chromatin remodeling or TGS, or induce target RNA cleavage, or cause miRNA-mediated translational inhibition.

# Técnica *Knockdown* - Aplicação RNAi

frontiers in Plant Science

ORIGINAL RESEARCH
published: 29 September 2015
doi: 10.3389/fpls.2015.00808

## RNAi-based functional elucidation of *PtrPRP*, a gene encoding a hybrid proline rich protein, in cold tolerance of *Poncirus trifoliata*

*Ting Peng[1,2], Mao-Mao Jia[1] and Ji-Hong Liu[1]**

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Hybrid proline-rich proteins (HyPRPs) have been suggested to play important roles in various plant development and stress response. In this study, we report the cloning and functional analysis of *PtrPRP*, a HyPRP-encoding gene of *Poncirus trifoliata*. PtrPRP contains 176 amino acids, among which 21% are proline residues, and has an 8-cysteine motif (8 CM) domain at the C terminal, a signal peptide and a proline-rich region at the N terminal. *PtrPRP* is constitutively expressed in root, stem and leaf, with the highest expression levels in leaf. It was progressively induced by cold, but transiently upregulated by salt and ABA. Transgenic *P. trifoliata* plants with knock-down *PtrPRP* by RNA interference (RNAi) were generated to investigate the role of *PtrPRP* in cold tolerance. When challenged by low temperature, the *PtrPRP*-RNAi plants displayed more sensitive performance compared with wild type (WT), as shown by higher electrolyte leakage and malondialdehyde content. In addition, the RNAi lines accumulated more reactive oxygen species (ROS) and lower levels of proline relative to WT. These results suggested that PtrPRP might be positively involved in cold tolerance by maintaining membrane integrity and ROS homeostasis.

**Keywords: cold stress, *Poncirus trifoliata*, hybrid proline-rich protein, RNA interference, ROS**

OPEN ACCESS

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*liujihong@mail.hzau.edu.cn*

## CONTEXTUALIZAÇÃO

- Gene **PtrPRP**
- Altamente responsivo ao frio
- HyPRP

## OBJETIVO

- Realizar a caracterização e a análise funcional do gene PtrPRP

# Técnica *Knockdown* - Aplicação RNAi

## PRINCIPAIS RESULTADOS

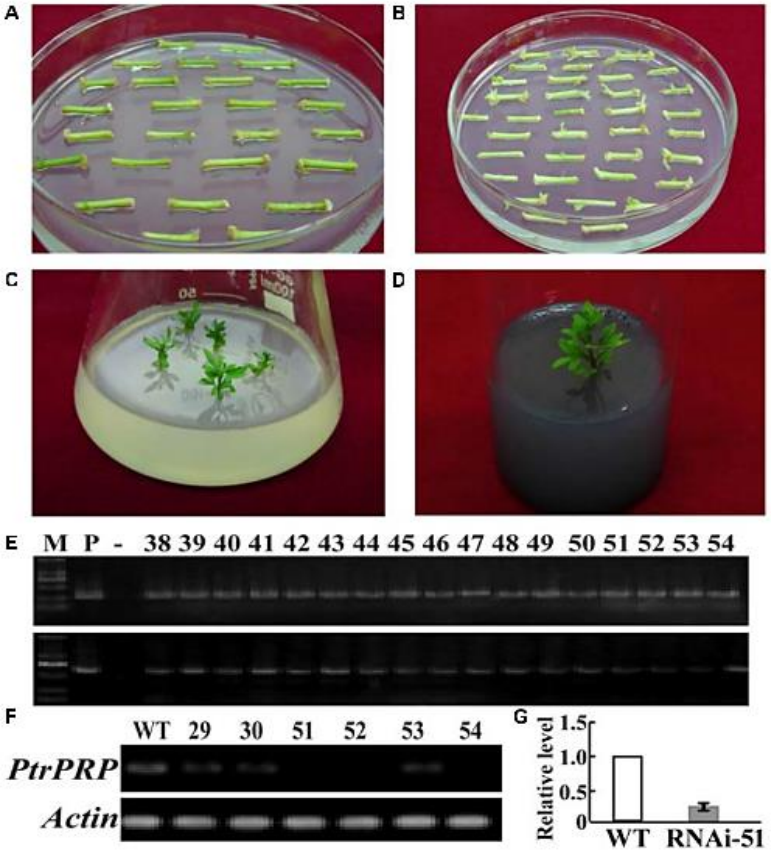


**FIGURE 6 | Transformation, regeneration, and characterization of trifoliate orange transgenic plants. (A,B)** Culture of the stem segments on selection medium for 30 d **(A)** and 60 d **(B)**, respectively. **(C)** Regeneration of kanamycin -resistant shoots on the selection medium. **(D)** A rooting plant on the root-inducing medium. **(E)** Genomic PCR of the kanamycin-resistant plants using designed primers specific to PtrPRP (upper) and NPTII (bottom), respectively. **(F)** Expression analysis of *PtrPRP* in six positive transgenic plants, as revealed by RT-PCR. Actin gene was used as an internal control. **(G)** Analysis of *PtrPRP* expression level in RNAi-51 using qRT-PCR.

# Técnica *Knockdown* - Aplicação RNAi

## PRINCIPAIS RESULTADOS

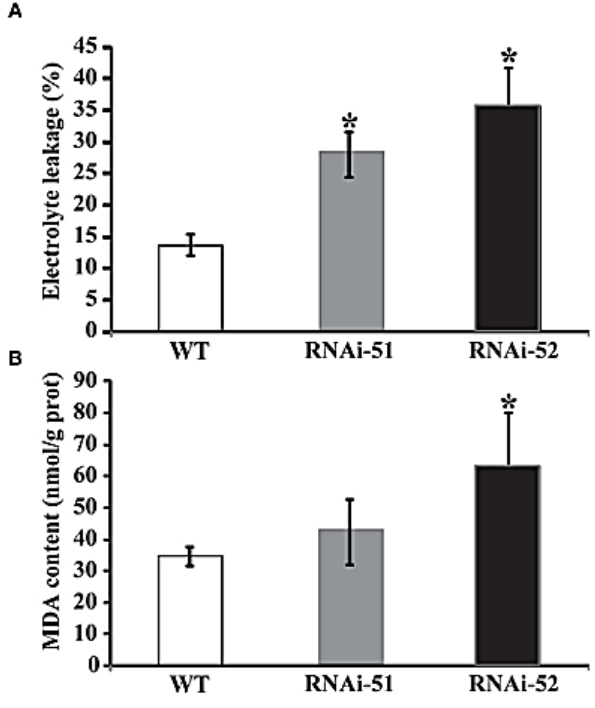


**FIGURE 7 | Analysis of electrolyte leakage (EL) (A) and malondialdehyde (MDA) (B) in wild type (WT) and two RNAi lines after cold treatment.** Asterisks indicate that the values of corresponding transgenic lines are statistically significantly different from that of WT (*$P < 0.05$).

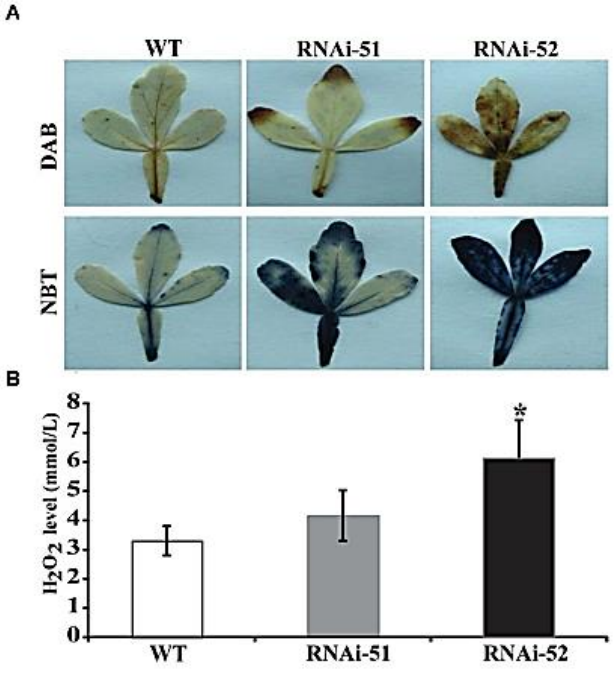


**FIGURE 8 | Histochemical staining and quantitative measurement of ROS. (A)** Histochemical staining with DAB (upper) and NBT (lower) in WT and two RNAi lines after cold treatment. **(B)** Quantitative measurement of $H_2O_2$ in WT and the RNAi lines. Asterisks indicate that the values of corresponding transgenic lines are statistically significantly different from that of WT (*$P < 0.05$).

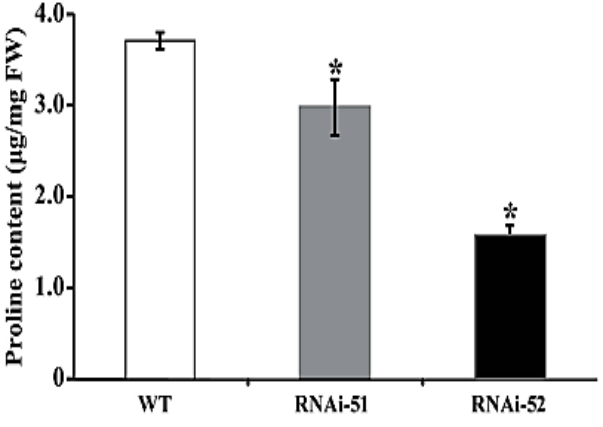


**FIGURE 9 | Analysis of proline levels in WT and two RNAi lines after cold treatment.** Asterisks indicate that the values of corresponding transgenic lines are statistically significantly different from that of WT (*$P < 0.05$).

## CONCLUSÃO

O ***knockdown*** de **PtrPRP** conferiu **maior sensibilidade ao frio** nas linhagens de **RNAi sob resfriamento**, manifestada por **dano de membrana mais severo**, **maior acúmulo de EROS** e **redução do conteúdo de prolina** sob resfriamento. Portanto, o **PtrPRP** pode contribuir para a **tolerância ao frio** via modulação da **prolina**, um importante osmólito e **eliminador de EROS**.

## Targeting Induced Local Lesions IN Genome (TILLING)

- Anos 2000 - Início do crescimento dos bancos de dados - Popularização da genética reversa
- Técnica desenvolvida na tentativa de criar uma estratégia que seja automatizada, funcione de forma geral para todo o genoma e produza ampla quantidade de alelos.
- *Arabidopsis thaliana* - única planta com um banco de dados genético quase completo

Scientific Correspondence

# Targeting Induced Local Lesions IN Genomes (TILLING) for Plant Functional Genomics

**Claire M. McCallum, Luca Comai, Elizabeth A. Greene, and Steven Henikoff***

Basic Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109–1024 (C.M.M., E.A.G., S.H.); Molecular and Cellular Biology Program (C.M.M.) and Department of Botany (L.C.), University of Washington, Seattle, Washington 98195; and Howard Hughes Medical Institute Research Laboratories, Seattle, Washington 98109 (S.H.)

One of the most important breakthroughs in the history of genetics was the discovery that mutations can be induced (Muller, 1930; Stadler, 1932). The high frequency with which ionizing radiation and certain chemicals can cause genes to mutate made it possible to perform genetic studies that were not feasible when only spontaneous mutations were available. As a result, much of our understanding of genetics of higher organisms is based upon studies utilizing induced mutations for analyzing gene function. Alkylating agents, which yield predominantly point mutations, have been especially valuable, since the resulting altered and truncated protein products help to precisely map gene and protein function. Because of the high mutational density and the great utility of point mutations, traditional chemical mutagenesis methods have continued to be popular in phenotypic screens despite the development of other mutagenic tools such as transposon mobilization (Bingham et al., 1981).

With the recent expansion of sequence databanks, locus-to-phenotype reverse genetic strategies have become an increasingly popular alternative to phenotypic screens for functional analysis. Sequence information alone may be sufficient to consider a gene to be of interest, because sequence comparison tools that detect protein sequence similarity to previously studied genes often allow a related function to be inferred. Hypotheses concerning gene function that are generated in this way must be confirmed empirically. Experimental determination of gene function is desirable in other situations as well, for example, when a genetic interval has been associated with a phenotype of interest. In such cases, the functions of genes in an interval can be inferred by using reverse genetic methods. Routine reverse genetics (Scherer and Davis, 1979) has been an important factor in the popularity of baker's yeast over the past two decades, and the RNAi technique (Fire et al., 1998) now provides *Caenorhabditis elegans* investigators with a routine knockout method that has enjoyed huge popularity over the past year (Sharp, 1999). In most other eukaryotes, however, the situation remains unsatisfactory.

In plants, the two most common methods for producing reduction-of-function mutations are antisense RNA suppression (Schuch, 1991; de Lange et al., 1995; Hamilton et al., 1995; Finnegan et al., 1996) and insertional mutagenesis (Altmann et al., 1995; Smith et al., 1996; Azpiroz-Leehan and Feldmann, 1997; Long and Coupland, 1998; Martienssen, 1998; Pereira and Aarts, 1998; van Houwelingen et al., 1998; Speulman et al., 1999). However, antisense RNA suppression requires considerable effort for any given target gene before knowing whether it will work, and insertional mutagenesis occurs at a low frequency per genome. There is current interest in RNAi-related suppression (Waterhouse et al., 1998). However, its efficacy is not yet clear; for example, epigenetic phenotypes can be variegated and unpredictable (Que and Jorgensen, 1998). Because these techniques rely either on *Agrobacterium* T-DNA vectors for transmission or on an endogenous tagging system, their usefulness as general reverse genetics methods is limited to very few plant species. Moreover, these techniques produce a very limited range of allele types. Therefore, as the amount of sequence data grows for Arabidopsis and other organisms, it is important to develop genome-scale reverse genetic strategies that are automated, broadly applicable, and capable of creating the wide range of mutant alleles that is needed for functional analysis.

We have introduced a new reverse genetic strategy that combines the high density of point mutations provided by traditional chemical mutagenesis with rapid mutational screening to discover induced lesions (McCallum et al., 2000). TILLING (Targeting Induced Local Lesions IN Genomes) combines chemical mutagenesis (Koornneef et al., 1982) with a sensitive mutation detection instrument. In a pilot experiment, DNA from a collection of EMS-mutagenized Arabidopsis plants was pooled, subjected to PCR amplification, and screened for mutations using denaturing HPLC (DHPLC). DHPLC detects mismatches in heteroduplexes created by melting and annealing of heteroallelic DNA. Among the lesions detected were base transitions causing missense and nonsense changes that can be used for phenotypic analyses.

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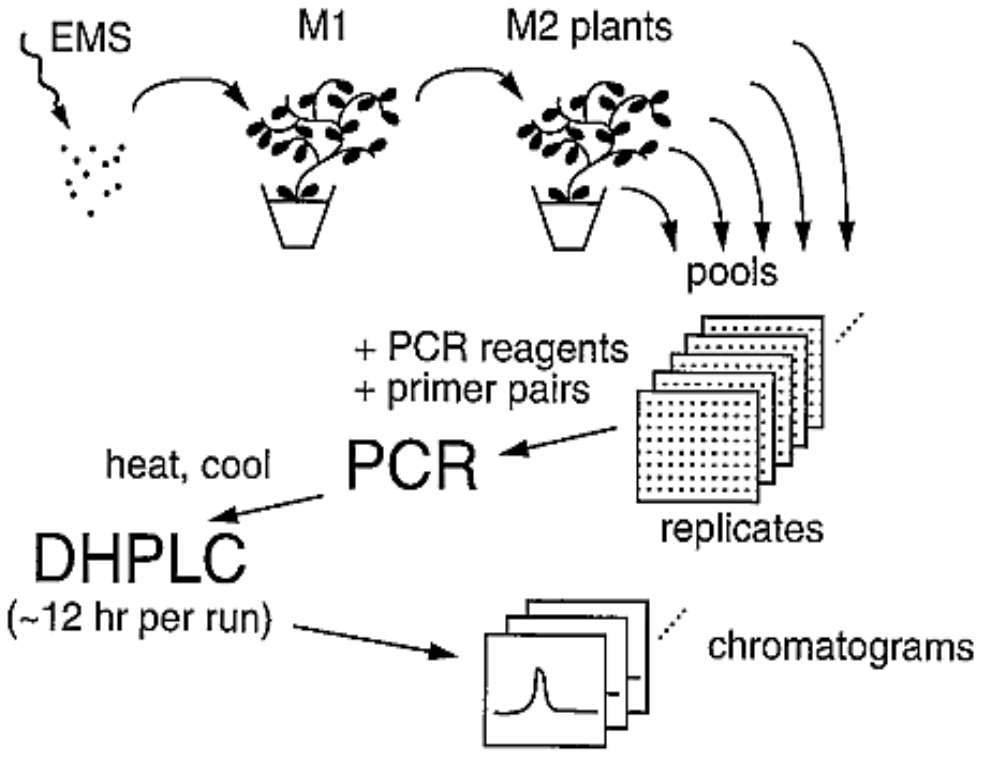
# O que é?

- Método que utiliza a técnica de **mutagênese**, unida a estratégias de busca de mutações para encontrar as mutações induzidas em um gene de interesse.

- **EMS** - Metanossulfonato de etila, mutagênico, tipicamente causa substituições de CG para AT

- **DHPLC** - Cromatografia líquida de alta performance desnaturante.



# Tipos de TILLING

- **TILLING Clássico** - Conforme originalmente descrito no artigo
- **TILLING enzimático** - Utiliza Nucleases para clivar o DNA na posição do mismatch (Cel I) seguido de eletroforese em gel
- **Eco-TILLING** - Busca mutações naturais em uma população, substituindo a etapa de mutagênese pela coleta de material biológico
- **TILLING por sequenciamento** - Utiliza métodos de sequenciamento de nova geração (NGS) para detectar as mutações

*cells*

MDPI

*Article*

# TILLING-by-Sequencing+ Reveals the Role of Novel Fatty Acid Desaturases (GmFAD2-2s) in Increasing Soybean Seed Oleic Acid Content

Naoufal Lakhssassi [1], Valéria Stefania Lopes-Caitar [2,†], Dounya Knizia [1,†], Mallory A. Cullen [1,†], Oussama Badad [1], Abdelhalim El Baze [1], Zhou Zhou [1], Mohamed G. Embaby [3], Jonas Meksem [4], Aicha Lakhssassi [5], Pengyin Chen [6], Amer AbuGhazaleh [3], Tri D. Vuong [6], Henry T. Nguyen [6], Tarek Hewezi [2] and Khalid Meksem [1,*]

## INTRODUÇÃO E OBJETIVOS

- **Altos** teores **de ácido oleico aumentam** o **tempo de vida** do **produto** e necessitam de **menos hidrogenação**, tornando-os mais **saudáveis** para o consumo

- Mutações nos genes **GmFAD2-1A** e **GmFAD2-1B** são bem estudadas e demonstraram **alto teor de ácidos oleicos**, mas **prejudicam a germinação** da planta em climas **frios** (conversão de ácido oleico para ácido linoleico)

- Genes da família **GmFAD2-2** (5 ao todo), são relacionados mas mal caracterizados. Recentemente CRISPR mostrou que mutações em dois genes dessa família também causam acumulação de ácido oleico

- **Objetivo**: Desenvolver e caracterizar linhas de mutantes **EMS** para os genes da subfamília **GmFAD2-2**

    - Não transgênico
    - Potencial para o melhoramento

## MATERIAL E MÉTODOS

- **Desenvolvimento de uma linha mutagênica**
  - EMS 0,6% foi utilizado no cultivar Forrest (resistência a vários patógenos)
  - 4032 famílias na terceira geração

- **Obtenção das sequências e localizações dos genes**
  - UNIPROT, NCBI, Soybase, Phytozome

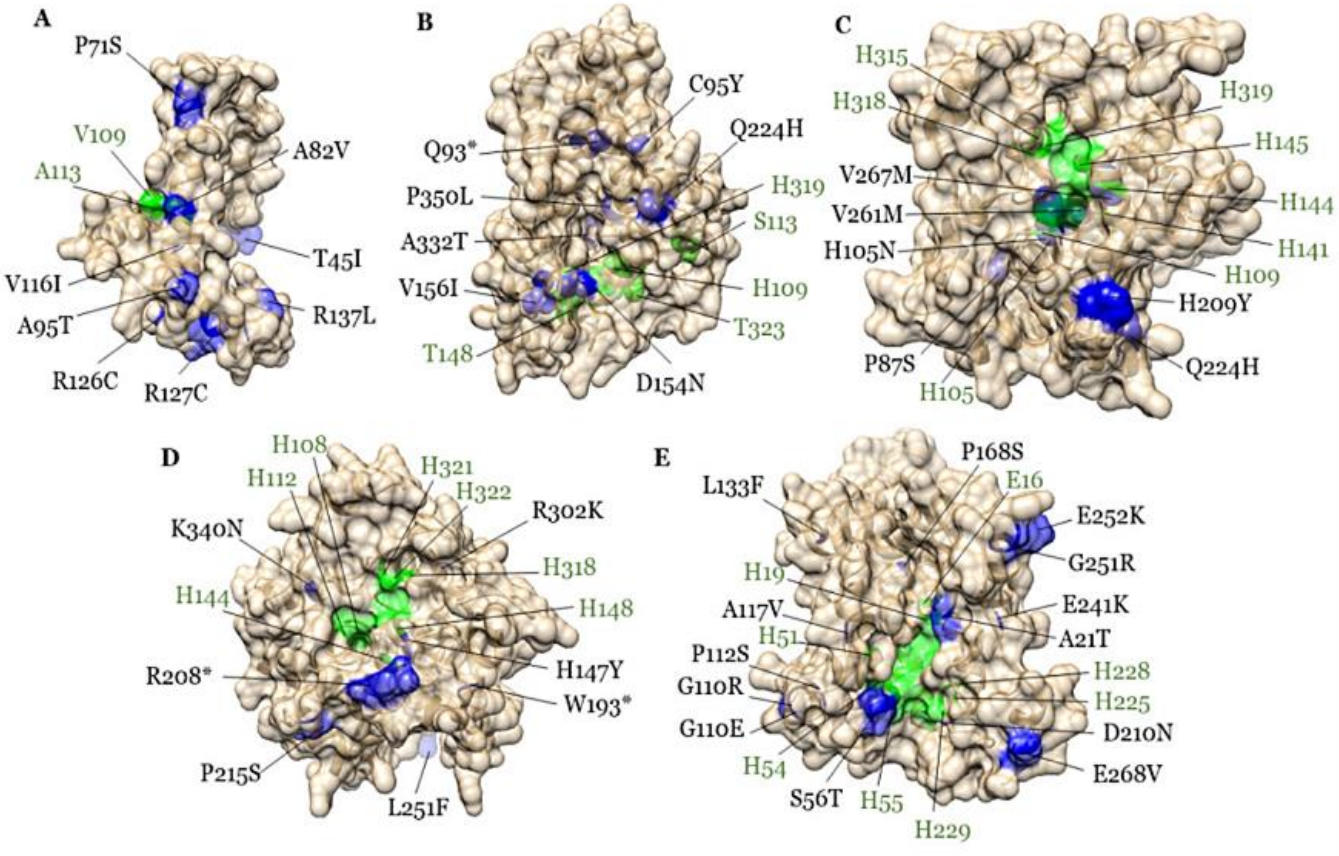
- **Pooling do DNA, design das sondas e TILLING-por-sequenciamento**
  - DNA foi organizado em grupos em 42 placas de 92 poços, amplificada por PCR e sequenciada por Illumina (NGS)
  - Controle de qualidade em todas as etapas

- **Densidade de mutações foi mensurada e em seguida, após a obtenção das sementes de cada linhagem mutante, o teor de ácido oleico foi medido**

- **EXTRA: Análise de proteínas, análises filogenéticas, níveis de expressão (rnaseq), clonagem e transformação em células de cebola e análises estatísticas**

## RESULTADOS

- 441 SNPs e 16 InDels com 74% típicas de **EMS**
- Mutações **missense** obtidas:
  - **12 GmFAD2-2A**
  - **8 GmFAD2-2B**
  - **10 GmFAD2-2C**
  - **9 GmFAD2-2D**
  - **19 GmFAD2-2E**

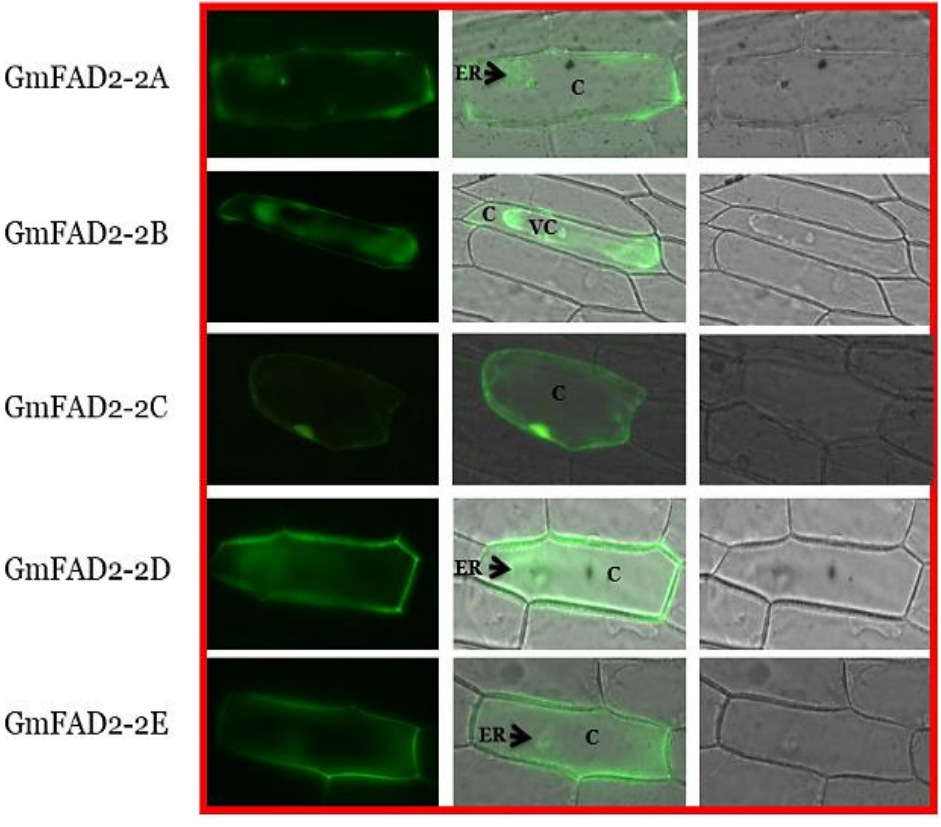


## RESULTADOS

- Todos os mutantes isolados **missense** e **nonsense** das proteínas da subfamília **GmFAD2-2** apresentaram **aumento** no teor de **ácido oleico** nas sementes!

  - **GmFAD2-2A - até 31,9%**
  - **GmFAD2-2B - até 28,1%**
  - **GmFAD2-2C - até 29,6%**
  - **GmFAD2-2D - até 32,7%**
  - **GmFAD2-2E - até 35,7%**

- Trabalho revela enorme papel da família **GmFAD2-2** via metabólica envolvendo **ácido oleico**



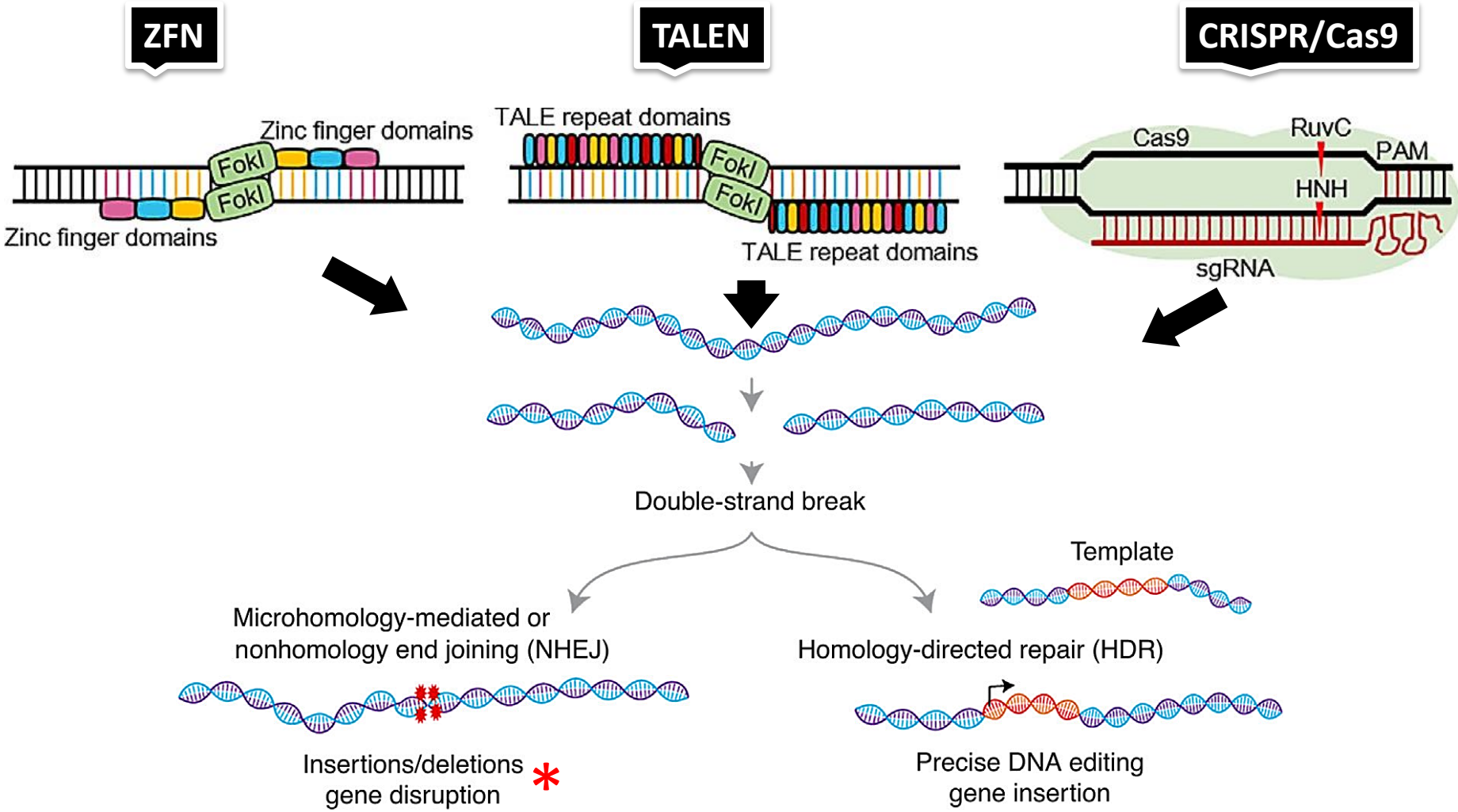
## DISCUSSÃO E CONCLUSÃO

- **Estudos anteriores** reportaram que a **síntese de ácidos graxos** não ocorre somente no **retículo endoplasmático**, mas também por uma via alternativa no **citoplasma**, onde vários das proteínas **GmFAD2-2** estão **presentes**
  - Alto potencial para o melhoramento da soja

- **Mutações** em locais de alta importância nas proteínas provavelmente **prejudicaram** suas atividades, levando a **inibição** na síntese de **ácido linoleico** e o acúmulo de **ácido oleico**
  - Processo **inverso** também pode ocorrer, com a **escassez** de ácido linoleico estimulando a **síntese** de ácido oleico

- O estudo mostra que genes previamente mal estudados da família **GmFAD2-2** podem ser uma poderosa ferramenta para o melhoramento da soja

# Técnicas *Knockout* - Edição de Genoma




Adaptado de:
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doi.org/10.1016/B978-0-12-817876-8.00009-7

# Zinc finger nuclease (ZFN) - 1996

*Proc. Natl. Acad. Sci. USA*
Vol. 93, pp. 1156–1160, February 1996
Biochemistry

## Hybrid restriction enzymes: Zinc finger fusions to *Fok* I cleavage domain

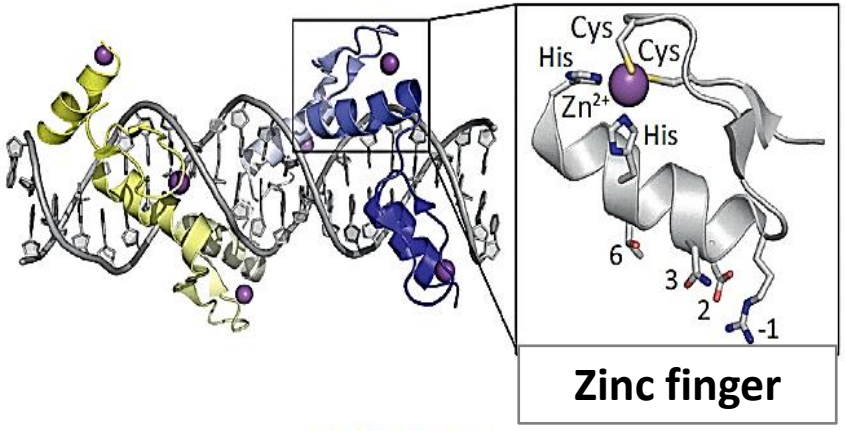
(*Flavobacterium okeanokoites*/chimeric restriction endonuclease/protein engineering/recognition and cleavage domains)

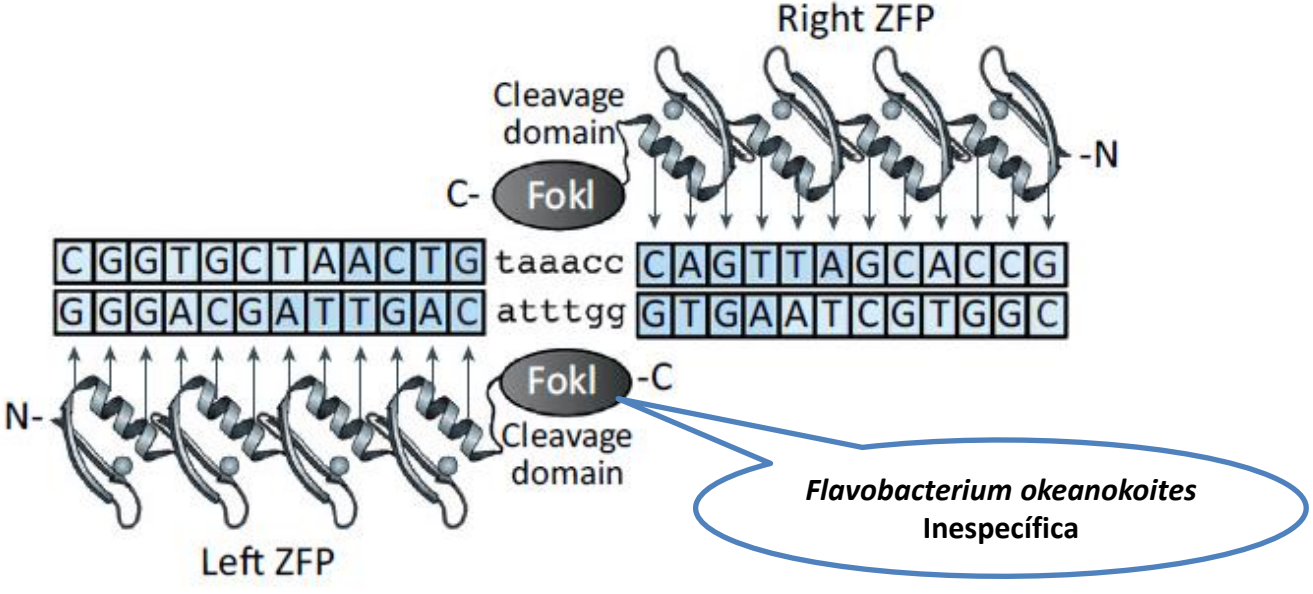
YANG-GYUN KIM, JOOYEUN CHA, AND SRINIVASAN CHANDRASEGARAN*

Department of Environmental Health Sciences, The Johns Hopkins University, School of Hygiene and Public Health, 615 North Wolfe Street, Baltimore, MD 21205-2179

*Communicated by Thomas Kelly, October 3, 1995 (received for review April 13, 1995)*

**ABSTRACT** **A long-term goal in the field of restriction-modification enzymes has been to generate restriction endonucleases with novel sequence specificities by mutating or engineering existing enzymes. This will avoid the increasingly arduous task of extensive screening of bacteria and other microorganisms for new enzymes. Here, we report the deliberate creation of novel site-specific endonucleases by linking two different zinc finger proteins to the cleavage domain of *Fok* I endonuclease. Both fusion proteins are active and under optimal conditions cleave DNA in a sequence-specific manner. Thus, the modular structure of *Fok* I endonuclease and the zinc finger motifs makes it possible to create "artificial" nucleases that will cut DNA near a predetermined site. This opens the way to generate many new enzymes with tailor-made sequence specificities desirable for various applications.**




doi.org/10.1073%2Fpnas.93.3.1156
dx.doi.org/10.1016/j.tibtech.2013.04.004

# Transcription Activator-Like Effector Nucleases (TALENs) - 2010


DOI: 10.1534/genetics.110.120717

Note

## Targeting DNA Double-Strand Breaks with TAL Effector Nucleases

Michelle Christian,* Tomas Cermak,* Erin L. Doyle,† Clarice Schmidt,† Feng Zhang,* Aaron Hummel,† Adam J. Bogdanove† and Daniel F. Voytas*,[1]

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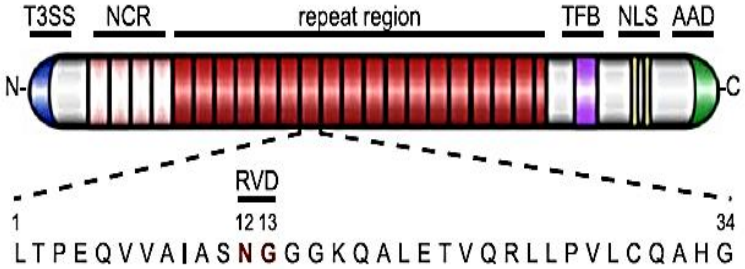
Manuscript received July 8, 2010
Accepted for publication July 20, 2010

ABSTRACT

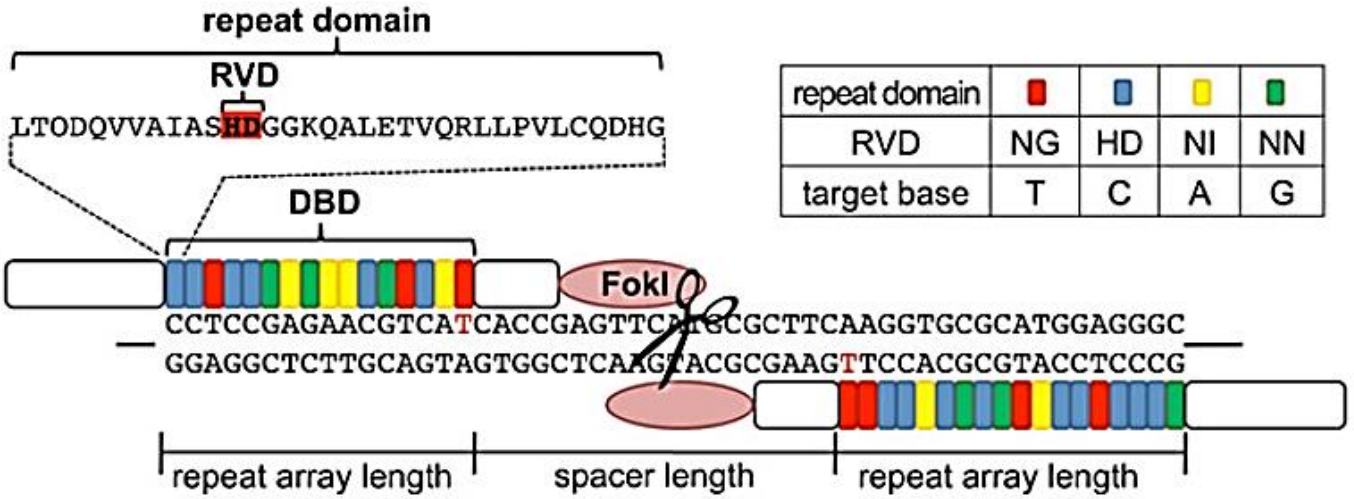
Engineered nucleases that cleave specific DNA sequences *in vivo* are valuable reagents for targeted mutagenesis. Here we report a new class of sequence-specific nucleases created by fusing transcription activator-like effectors (TALEs) to the catalytic domain of the *FokI* endonuclease. Both native and custom TALE-nuclease fusions direct DNA double-strand breaks to specific, targeted sites.

## Transcription activator-like effector (TALE)




| RVD | specificity |
|---|---|
| NG | T |
| NI | A |
| NN | G / A |
| HD | C |
| NS | A / G / C / T |

Proteínas efetoras de *Xanthomonas* spp.




| repeat domain | | | | |
|---|---|---|---|---|
| RVD | NG | HD | NI | NN |
| target base | T | C | A | G |

doi:10.1534/genetics.110.120717
doi.org/10.1016/j.ggedit.2021.100007
doi:10.1242/bio.20149738

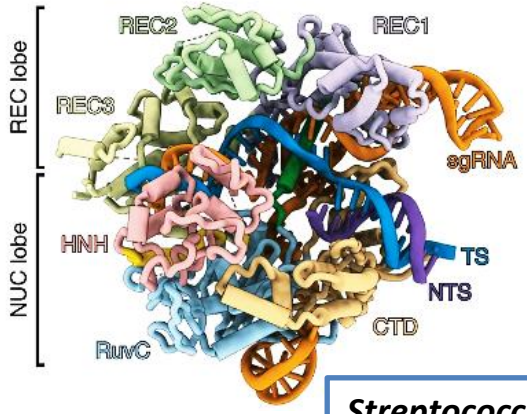
# Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) - 2012

RESEARCH ARTICLE

## A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

Martin Jinek,[1,2]* Krzysztof Chylinski,[3,4]* Ines Fonfara,[4] Michael Hauer,[2]† Jennifer A. Doudna,[1,2,5,6]‡ Emmanuelle Charpentier[4]‡

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. We show here that in a subset of these systems, the mature crRNA that is base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA structure that directs the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA. At sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the noncomplementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.



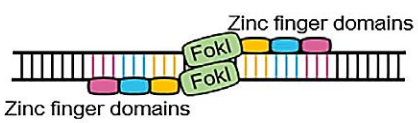

***Streptococcus pyogenes***

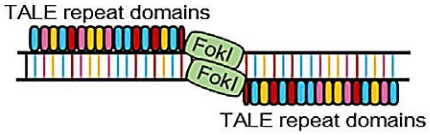


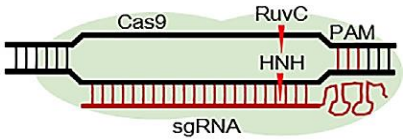

de VASCONCELOS, M. J. V., and José Edson Fontes FIGUEIREDO. "Tecnologia CRISPR-Cas para edição genômica." (2015).

doi: 10.1126/science.1225829
doi.org/10.1038/s41594-019-0258-2

# Técnicas *Knockout* - Edição de Genoma




| | ZFN | TALEN | CRISPR/Cas9 |
|---|---|---|---|
| **Sítio de rec.** | Zinc-finger | Regiões RVD (TALE) | crRNA / sgRNA |
| **Dimerização** | ✓ | ✓ | X |
| **Tamanho Seq. Alvo** | 9 a 18 pb/Monômero | 14 a 20 pb/Monômero | ~23 pb |
| **Ligação no DNA** | Ricos em G | 5’ T | NGG (PAM) |
| **Especificidade** | Média (Missmatches) | Alta | Média (Missmatches) |
| **Citotoxicidade** | Média - Alta | < ZFN | Baixa |
| **Efeito off-target** | Médio | Baixo | Médio |
| **Construção** | Difícil (Proteína) | Difícil (Proteína) | Fácil (sgRNA) |
| **Multiplexação** | X | X | ✓ |
| **Custo** | ↑ | ↑ | ↓ |

# *Rj4*, a Gene Controlling Nodulation Specificity in Soybeans, Encodes a Thaumatin-Like Protein But Not the One Previously Reported[1]

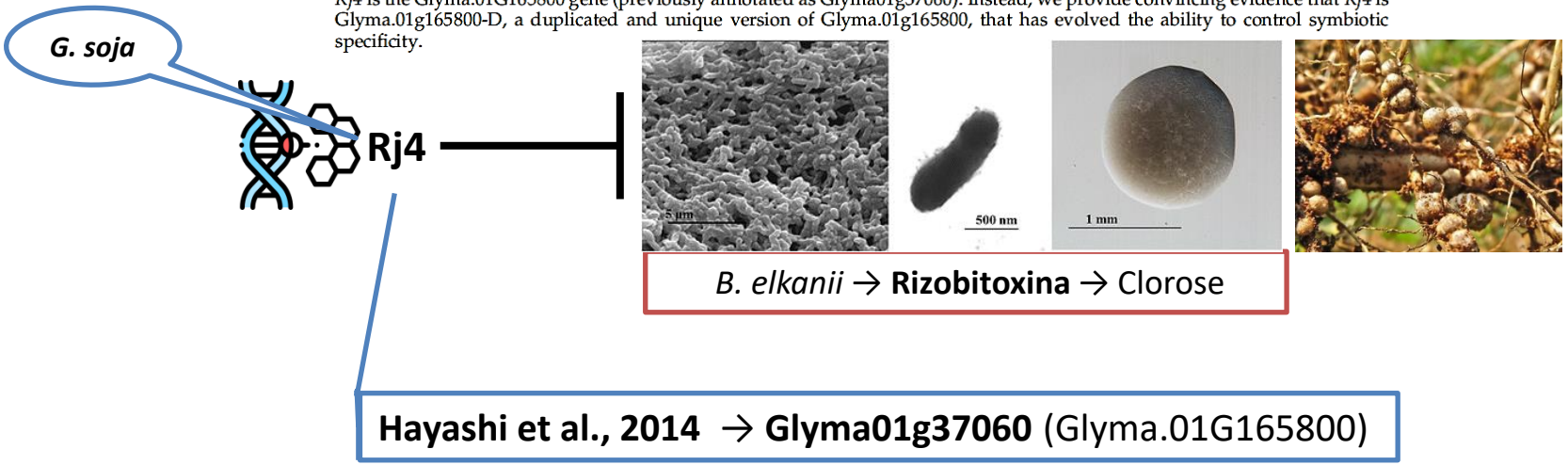
*Plant Physiology*®

**Fang Tang, Shengming Yang, Jinge Liu, and Hongyan Zhu***

Department of Plant and Soil Sciences, University of Kentucky, Lexington, Kentucky 40546–0312

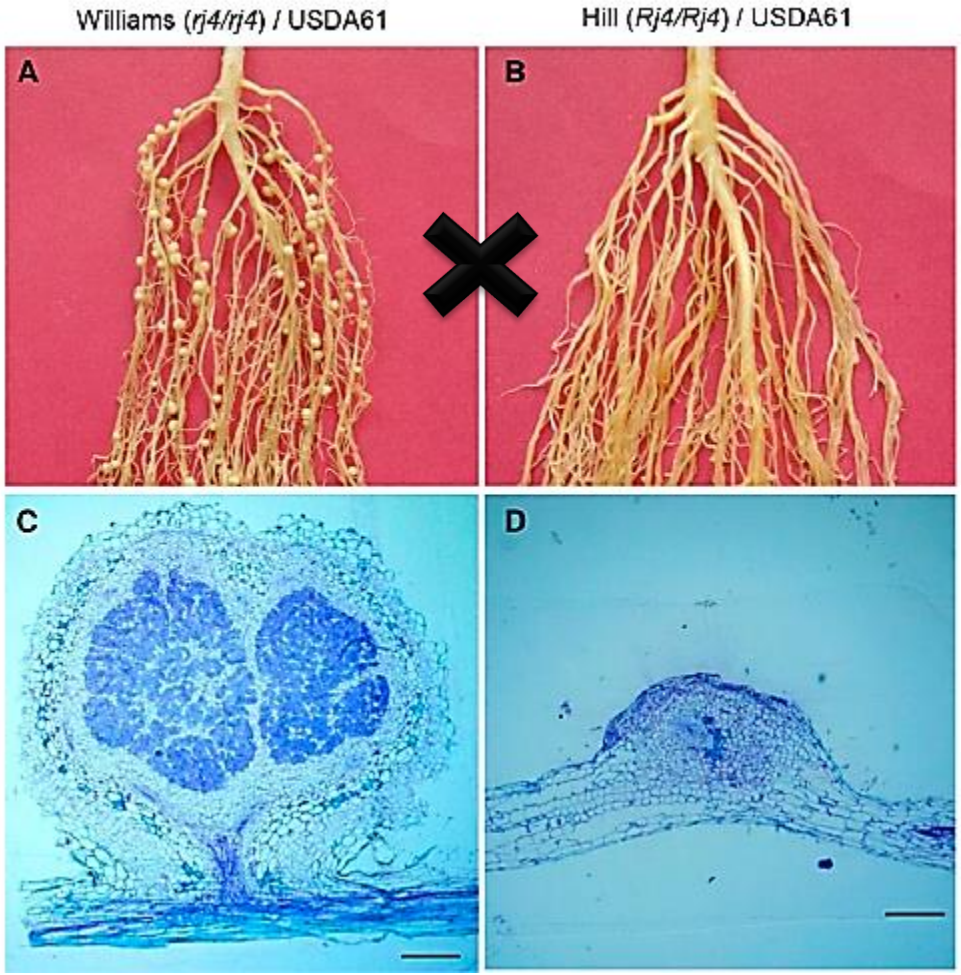
**ORCID ID: 0000-0002-8703-6289 (H.Z.).**

*Rj4* is a dominant gene in soybeans (*Glycine max*) that restricts nodulation by many strains of *Bradyrhizobium elkanii*. The soybean-*B. elkanii* symbiosis has a low nitrogen-fixation efficiency, but *B. elkanii* strains are highly competitive for nodulation; thus, cultivars harboring an *Rj4* allele are considered favorable. Cloning the *Rj4* gene is the first step in understanding the molecular basis of *Rj4*-mediated nodulation restriction and facilitates the development of molecular tools for genetic improvement of nitrogen fixation in soybeans. We finely mapped the *Rj4* locus within a small genomic region on soybean chromosome 1, and validated one of the candidate genes as *Rj4* using both complementation tests and CRISPR/Cas9-based gene knockout experiments. We demonstrated that *Rj4* encodes a thaumatin-like protein, for which a corresponding allele is not present in the surveyed *rj4* genotypes, including the reference genome Williams 82. Our conclusion disagrees with the previous report that *Rj4* is the Glyma.01G165800 gene (previously annotated as Glyma01g37060). Instead, we provide convincing evidence that *Rj4* is Glyma.01g165800-D, a duplicated and unique version of Glyma.01g165800, that has evolved the ability to control symbiotic specificity.




DOI: 10.1104/pp.15.01661

# Técnicas de Edição de Genoma – Aplicação CRISPR/Cas9




População F2 (1 semana)

↓

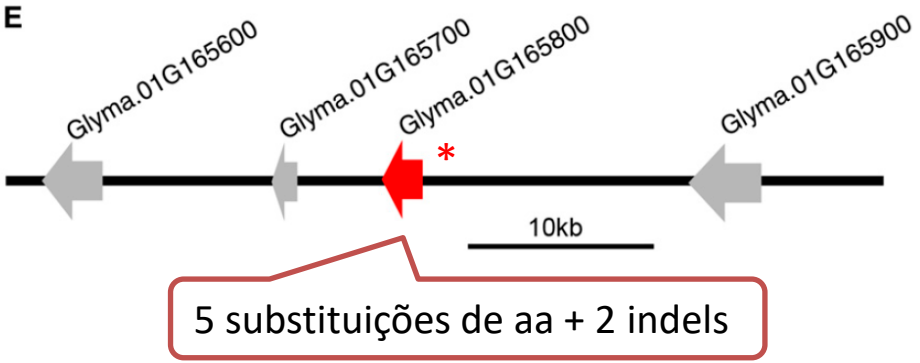
*Bradyrhizobium elkanii* USDA61

↓

Clonagem posicional - lócus **Rj4**
(Genoma de referência **Willians 82**)

↓

Sequência genômica 45 kb (Cr. 1)




* Possível gene candidato → Validação

DOI: 10.1104/pp.15.01661

# Técnicas de Edição de Genoma – Aplicação CRISPR/Cas9

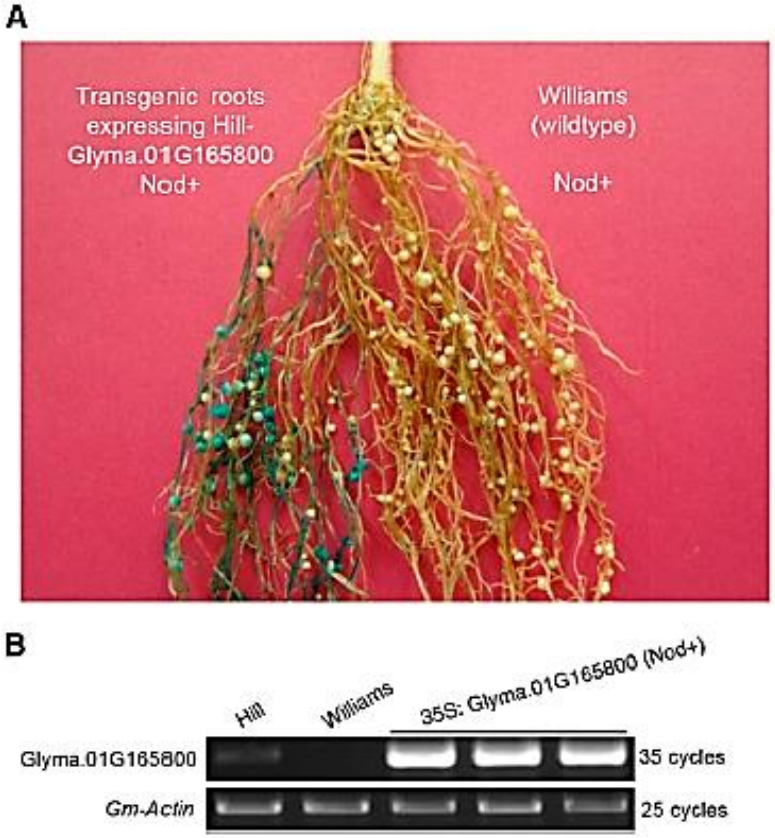



**Figure 2.** Complementation test using the Hill allele of Glyma.01G165800. A, Introduction of the Hill allele of Glyma.01G165800 into Williams (*rj4/rj4*) failed to block nodulation on the transgenic hairy roots (blue) by USDA61. B, Examples of transgenic hairy roots expressing Glyma.01G165800 of Hill in Williams. The transgenic hairy roots were first identified by GUS staining of a small portion of the root segments, followed by isolation of RNA from the transgenic hairy roots.

**Hayashi et al., 2014**

↓

**Glyma01g37060** (Glyma.01G165800)

↓

Rj4

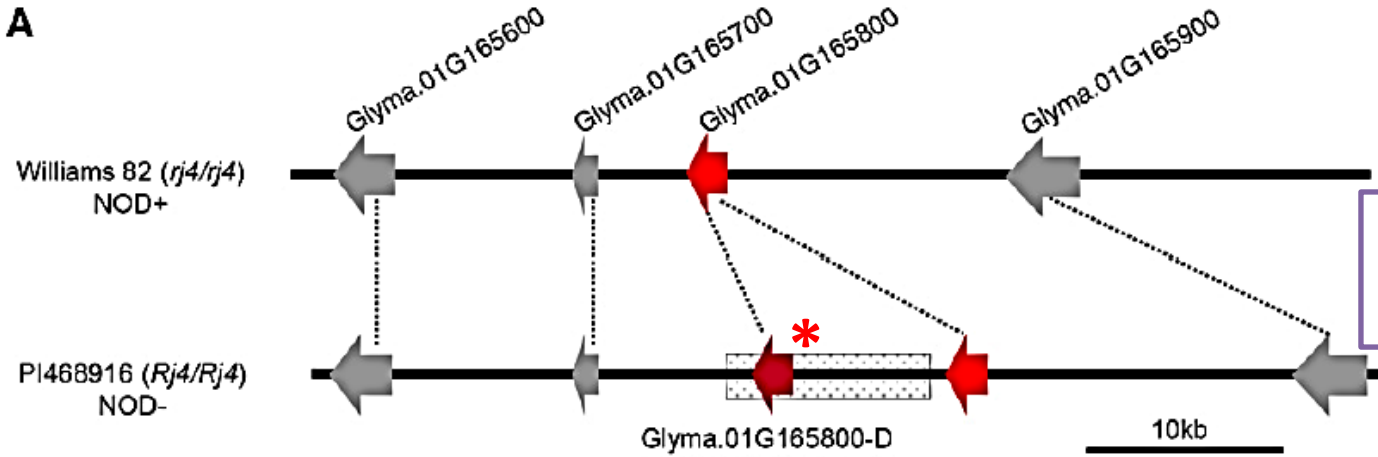
**(?)**

DOI: 10.1104/pp.15.01661

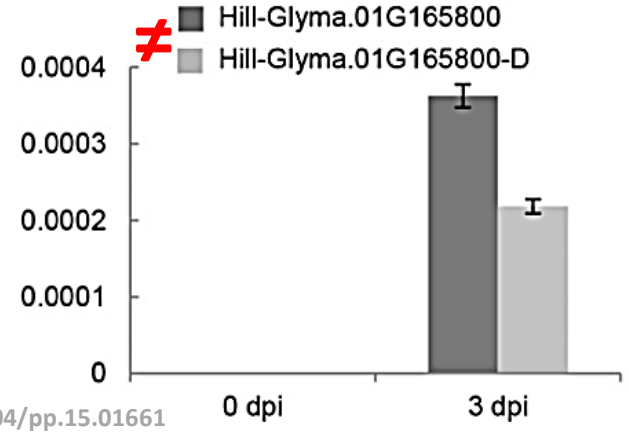
# Técnicas de Edição de Genoma – Aplicação CRISPR/Cas9

**BAC**

| PI # | Species | Origin | Phenotype | *Rj4* |
|---|---|---|---|---|
| PI 468916 | *G.soja* | China | Nod- | + |




* **Inserções**
+
**Dulicata** Glyma.01G165800




**PCR:**
**Rj4** → Glyma.01G165800 + Glyma.01G165800-D
**rj4** → Glyma.01G165800

DOI: 10.1104/pp.15.01661

# Técnicas de Edição de Genoma – Aplicação CRISPR/Cas9

## Apenas Glyma.01G165800-D ou ambos genes restringem a nodulação por *B. elkanii*?

```
Hill-Glyma.01G165800     MGNSTKMASIVTMASLFFFQFLSGSCSTRLTITNKCSYTVWPAILSATGSSPLSTSGFVL
Hill-Glyma.01G165800-D   MASSTKKAFIITTACLFFFQFLYGSYSTRLTIINKCSYTVWPAILSVTGSSPLSTSGFVL

Hill-Glyma.01G165800     QPGDFKIVPVPPAWSGRLWGRTLCSLDITSTKFSCVTGDCGSTTIECVGGNAAPPVTLVK
Hill-Glyma.01G165800-D   QPGHFKIVPVPPAWSGRLWGRTLCSLDITSTKFSCVTGDCDSTTIECVGGNAAPPVTLVK

Hill-Glyma.01G165800     FTLNGTGGLDFYEVSLVDGFNLPVRVKPRGGRNCRATGCVMDLNLSCPTELKVIRDGDAV
Hill-Glyma.01G165800-D   FTLNGTGGLDFYEVSLVDGFNLPVRVKPRGGRNCRATGCVMDLNLSCPTELKVIRDGDAV

Hill-Glyma.01G165800     ACKSVCQAEPCLSSQFFKTACPGAHVHAYTCSSHDYTITFCPPPTPSSSYQEISRGSGGG
Hill-Glyma.01G165800-D   ACKSVCQAEPCLSSQFFKTACPGAHVHAYTCSSHDYTITFCPPPTPSSSYQEISRGSGGG

Hill-Glyma.01G165800     QPGSSPINVVVAVVVALASVCGLFIACKITIRLSNGDCVFGIGAGTRTGTIQDVTV
Hill-Glyma.01G165800-D   QPGSSPINVVVAVVVALASVCGLFIACKITIRLSNGDCVFGIGAGTRTGTIQDVTV
```

Glyma.01G165800 e Glyma.01G165800-D ≠*

↓

Vetores Genes individuais

↓

Ferramenta de **genética reversa** (CRISPR/Cas9)

↓

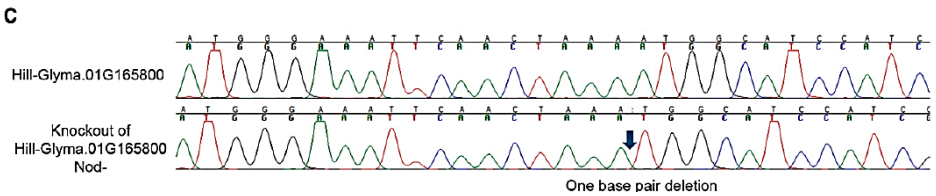
**KNOCKOUT** na cv. Hill

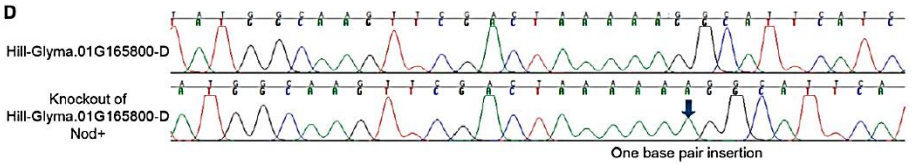
DOI: 10.1104/pp.15.01661

# Técnicas de Edição de Genoma – Aplicação CRISPR/Cas9

## Apenas Glyma.01G165800-D ou ambos genes restringem a nodulação por *B. elkanii*?

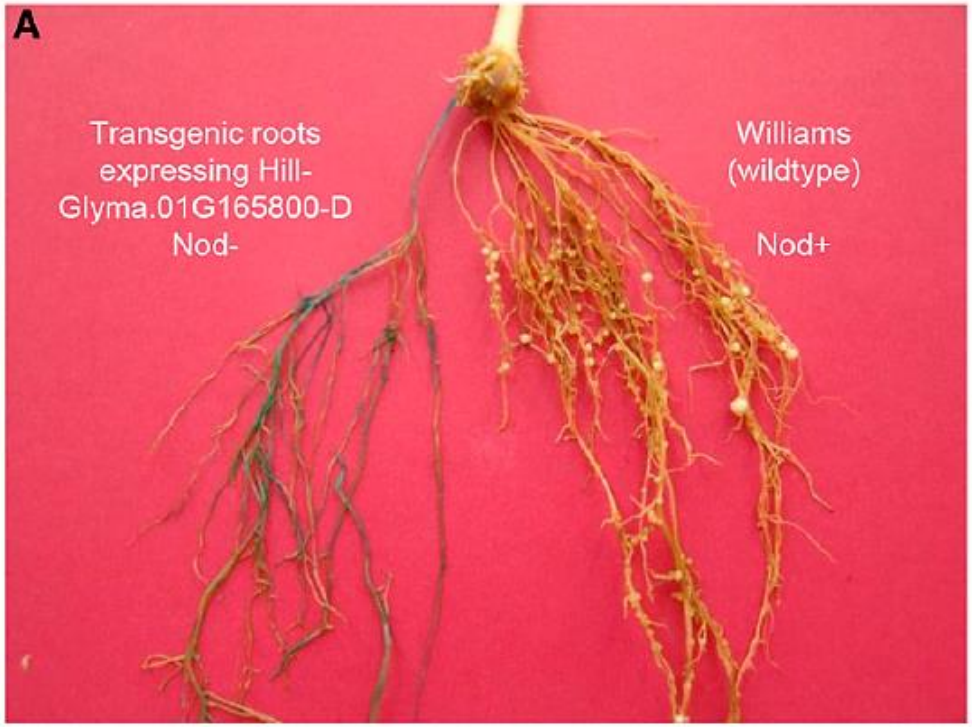


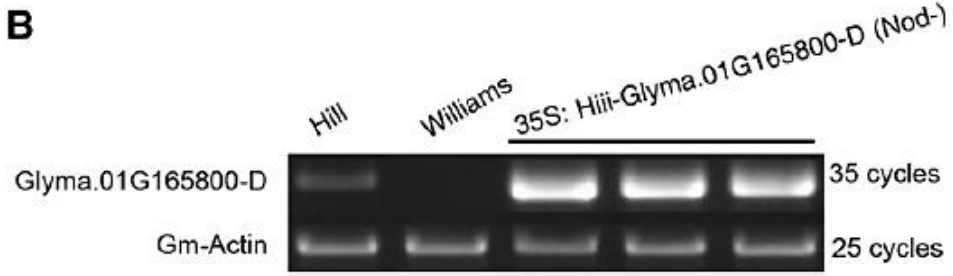



DOI: 10.1104/pp.15.01661

## Confirmação e Conclusão





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# Perspectivas: genética reversa

- Genética Reversa + Genética Direta
- Genética reversa exploratória
- Desenvolvimento e aprimoramento de técnicas

# Considerações Finais

Open Access Article

## Establishment of a Virus-Induced Gene-Silencing (VIGS) System in Tea Plant and Its Use in the Functional Analysis of *CsTCS1*

by Guodong Li [1], Yan Li [1], Xinzhuan Yao [1,2] and Litang Lu [1,2,*]

[1] The Key Laboratory of Plant Resources Conservation and Germplasm Innovation in Mountainous Region (Ministry of Education), College of Life Sciences, Guizhou University, Guiyang 550025, China

[2] College of Tea Sciences, Institute of Plant Health & Medicine, Guizhou University, Guiyang 550025, China

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*Int. J. Mol. Sci.* **2023**, *24*(1), 392; **https://doi.org/10.3390/ijms24010392**

Li, Guodong, Yan Li, Xinzhuan Yao, and Litang Lu. 2023. "Establishment of a Virus-Induced Gene-Silencing (VIGS) System in Tea Plant and Its Use in the Functional Analysis of *CsTCS1*" *International Journal of Molecular Sciences* 24, no. 1: 392. https://doi.org/10.3390/ijms24010392