Development in gene editing in Brazil and at EMBRAPA

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Brazilian Soybean Research Center – Embrapa Soja
NEW Biosafety Law - 11.105/05
Brazilian Biosafety Technical Commission - CTNBio

CNBS

National interest and socioeconomic factors (not an obligation)

CTNBio
Risk Assessment

Registration and Inspections agencies
- MAPA
  - Agriculture
- Anvisa
  - Human Health
- Ibama
  - Environmental

Maintenance of biosafety

National Biosafety Council
- National Biosafety Technical Commission
- Internal Biosafety Commissions
- Registration and Inspection Bodies
Do you know where biotech crops are grown?

More than 30 countries have planted biotech crops since 1995. See where they were grown in 2019.

USA
Is the largest producer of biotech crops in the world, planting 37.6% of global biotech crop area.

Spain
Is the leading country planting biotech maize in Europe.

SPAIN
More than 6 million farmers in India planted 11.9 million hectares of Bt cotton in 2019.

Malawi, Ethiopia, & Nigeria
Planted Bt cotton for the first time in 2019.

10 countries in Latin America planted biotech crops in 2019.

2.7 Billion hectares of biotech crops planted since 1996.


For more information on biotech crops, visit www.isaaa.org.
Today there are many GM products being produced and consumed...
Mosquito GM

- Insertion of genes to induce male sterility (Tetracycline)
- GM Males compete with Wild Males reducing total population.
- First results were presented to the scientific community recently in Juazeiro, Bahia, Brazil, where the experiment was conducted in open areas with high Mosquito infestations.

Liberated for Commercial use in Brazil: Abr/2014


Aedes do Bem™ é uma solução inovadora no controle biológico do mosquito transmissor da dengue, zika, chikungunya e febre amarela.

Chegou a hora de viver uma nova vida fora da caixa.

Assine agora por toda a temporada de mosquitos
COM PREÇO ESPECIAL ESQUENTA BLACK FRIDAY por apenas R$ 145/mês e receba seu Kit Caixas + Refis em até 8 dias com frete grátis!
GM Male Sterile Carterpillar (Lagarta do cartucho)  
*Spodoptera frugiperda*

Primeira LPMA para pesquisa autorizada em Maio 2019 pela CTNBio
First genetically engineered salmon sold in Canada

US firm AquaBounty Technologies says that its transgenic fish has hit the market after a 25-year wait.

Emily Waltz

04 August 2017

Liberado para consumo no BR em Maio/2021
OGM: Each country created its own rule

Phases and Costs to Development of a GM Crop

<table>
<thead>
<tr>
<th>Phases</th>
<th>Gene Discovery</th>
<th>Prove of Concept</th>
<th>Product Development (13.6)</th>
<th>Advanced Development (45.9)</th>
<th>Pre-Comercialization (17.2)</th>
<th>Launch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated Costs</td>
<td>~U$136 million</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Years</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

Today, basically, only four companies can place GM Crop Varieties in the Market:

- **Bayer (+Monsanto)**
- **BASF**
- **Corteva (Dow+DuPont+Pioneer)**
- **Syngenta (+ChemChina)**

Also, limited the use of Biotech in Agriculture to major crops (Soybean, Cotton, Corn, Eucalyptus, Sugarcane, etc...)

Estimated Costs: ~U$136 million

Estimated Costs of Deregulation Phase: ~U$75 million

It can take ~12-20 years from discovering a gene(s) and placing a GM Commercial Variety in the Market.
... but evolution on genetics keeps moving fast...
CRISPRs Technology brought a revolution in Genome Editing and is democratizing the use of biotechnology in agriculture
RESOLUÇÃO NORMATIVA Nº 16, DE 15 DE JANEIRO DE 2018

ANEXO I

Estabelece os requisitos técnicos para apresentação de consulta à CTNBio sobre as Técnicas Inovadoras de Melhoramento de Precisão

A COMISSÃO TÉCNICA NACIONAL DE BIOSSEGURANÇA - CTNBio, no uso de suas atribuições legais e regulamentares e em observância às disposições contidas nos incisos XV e XVI do art. 14 da Lei nº 11.105, de 24 de março de 2005;

CONSIDERANDO a necessidade de avaliar as Técnicas Inovadoras de Melhoramento de Precisão (TIMP), do inglês Precision Breeding Innovation (PBI) e que também englobam as denominadas Novas Tecnologias de Melhoramento, do inglês New Breeding Technologies -NBTs, à luz dos preceitos previstos na Lei nº 11.105, de 24 de março de 2005;

Considerando que a Lei nº 11.105, de 2005, define moléculas de ADN/ARN recombinante, engenharia genética e organismo geneticamente modificado - OGM nos incisos III, IV e V de seu art. 3º, respectivamente;

Considerando que as TIMP abrangem um conjunto de novas metodologias e abordagens que diferem da estratégia de engenharia genética por transgenia, por resultar na ausência de ADN/ARN
Global Regulatory Status

- Red: Specific genome editing rules – most will not be GMOs
- Green: Specific genome editing rules – are GMOs
- Blue: Continued discussions on genome editing rules
- Light blue: No specific rule but signed onto WTO statement suggesting most genome editing will not be GMOs
- Yellow: Current ban on all GMOs (assumed to include genome editing)

☐ No published rule on genome editing.
Site Directed Mutagenesis type:

SDN1
SDN2
SDN3

DNA cutting is done in regions (sequences) chosen with precision

Similar to mutations that occur in nature and are responsible for evolution on planet earth
Genome Edited Organisms in Brazil
GE Yeast for Alcohol Production from Sugarcane

First Genome-Edited Organism in Brazil for commercial use
Saccharomyces cerevisiae

Development Procedures
Yeast Germplasm Bank: 80 Strains tested for high alcohol and low glycerol production

Three S. cerevisiae strains chosen and crossed by classical breeding

Yeast Breeding

Excellomol

A Forth strain with very high alcohol production was identified. Mutation in 4 genes are responsible for this high efficiency.

Excellomol 4.0 Next
All four mutations were introduced by CRISPR/Cas9 into the Excellomol strain

Considered Non GM by CTNBio (2018)
Genome Edition on Diazotrophs organisms

Nitrogen Fixation Bacteria

Klebsiella varicola

Source: CTNBio, 2021
A new generation of genetically edited organisms has arrived and if early results are any indication, it has the potential to revolutionize the food manufacturing industry, as well as numerous industries.

On Monday, DuPont Pioneer announced the USDA will not subject a waxy corn hybrid created with CRISPR Cas9 gene-editing technology to regulations applied to traditional GMOs. The gene changes the functionality of starch. DuPont’s officials believe the corn can be planted in fields in five years. The company is also growing CRISPR edited wheat.

Usually Corn Starch has 75% Amylopectin and 25% Amylose

Also authorized to be sowed as conventional variety in the US, Canada and Argentina

Usually Corn Starch has 75% Amylopectin and 25% Amylose

α-amylose KnockOut

100% Amilopectina
0% Amilose

Considered Non GM by CTNBio (2018)
Considered Non GM by CTNBio (2019)

Genome Edited Tilapia

Gene edited tilapia secure GMO exemption

by The Fish Site
18 December 2018, at 2:06 p.m.

A line of tilapia that has been gene edited will not be classified as a genetically modified organisms (GMOs) in Argentina, according to a government advisory commission.

The line, known as FLT 01, has been developed by Intrexon and its subsidiary AquaBounty Technologies, the biotechnology company best known for its AquaAdvantage salmon strain. The tilapia were developed using gene editing.
Meat quality - Myostatin

Considered Non GM by CTNBio (2021)

Nelore bull semen with genome edited to Myostatin inactivation

Considered Non GM by CTNBio

Increase of ~20% in meat production
Hornless Animals

- **Polled allele** introduced by Homologous Recombination in fibroblast
  - Calves produced by somatic cell nuclear transfer (animal cloning)

Considered GM by CTNBio (2018)
<table>
<thead>
<tr>
<th>Requerente</th>
<th>Processo</th>
<th>Parecer</th>
<th>produto</th>
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<tbody>
<tr>
<td>Globalyeast JV CO Brasil S.A</td>
<td>01250.011076/2018-19</td>
<td>5905/2018</td>
<td>levedura para produção de bioetanol</td>
</tr>
<tr>
<td>Globalyeast JV CO Brasil S.A</td>
<td>01250.011076/2018-19</td>
<td>5904/2018</td>
<td>levedura para produção de bioetanol</td>
</tr>
<tr>
<td>Ourofino</td>
<td>01250.017539/2018-56</td>
<td>6236/2018</td>
<td>cepa vacinal de parvovírus canino</td>
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<tr>
<td>Agro Partners Consulting</td>
<td>01250.045811/2018-98</td>
<td>6125/2018</td>
<td>Bovino sem chifres (Parecer Cancelado)</td>
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<tr>
<td>Lallemand Brasil Ltda.</td>
<td>01250.014024/2018-15</td>
<td>6167/2018</td>
<td>linhagem M15980 de Saccharomyces cerevisiae para produção de etanol</td>
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<td>Du Pont do Brasil</td>
<td>01250.033737/2018-67</td>
<td>6208/2018</td>
<td>milho ceroso</td>
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<td>AquaBounty Technologies</td>
<td>01250.012915/2019-05</td>
<td>6527/2019</td>
<td>tilápia com um fenótipo de maior rendimento de filé</td>
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<tr>
<td>Forest</td>
<td>01200.700832/2016-10</td>
<td>6655/2019</td>
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<tr>
<td>Lallemand Brasil Ltda.</td>
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<td>Lallemand Brasil Ltda.</td>
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<td>Lallemand Brasil Ltda.</td>
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<td>Evolutta Consultoria Regulatória</td>
<td>01250.020936/2020-05</td>
<td>7098/2020</td>
<td>aditivo seco para ração e água destinado à criação de aves</td>
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<td>Evolutta Agro Biotecnologia Ltda</td>
<td>01245.003068/2020-92</td>
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<td>dsRNA para o silenciamento de genes de Spodoptera frugiperda e Helicoverpa armigera que atacam as lavouras cultivadas</td>
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<td>Pivot Bio</td>
<td>01250.010577/2020-01</td>
<td>7271/2020</td>
<td>Produto Kv137-3933 um inoculante para cultura do milho a base de Klebsiela varicola, visando a otimização do nitrogênio</td>
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<td>Pivot Bio</td>
<td>01250.010588/2020-82</td>
<td>7248/2020</td>
<td>Produto Kv137-1034 um inoculante para cultura do milho a base de Klebsiela varicola, visando a otimização do nitrogênio</td>
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<tr>
<td>YesSinergy Agroindustrial Ltda</td>
<td>01245.003594/2021-33</td>
<td>7519/2021</td>
<td>Produto produto Saccharomyces cerevisiae CEPA YS2101 - produção de etanol</td>
</tr>
<tr>
<td>Acellogen do Brasil Biotecnologia e Pesquisa Científica Ltda</td>
<td>01245.006161/2021-30</td>
<td>7520/2021</td>
<td>Sêmen de touro da raça Nelore (Samson) com aumento da massa muscular por edição gênica do gene da Miostatina</td>
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<tr>
<td>Evolutta Agro Biotecnologia Ltda</td>
<td>01245.003068/2020-92</td>
<td>7581/2021</td>
<td>dsRNA para o silenciamento de genes de Spodoptera frugiperda e Helicoverpa armigera</td>
</tr>
</tbody>
</table>
Research on Genome Edition in Brazil
Leading project on Genome Edition at EMBRAPA

**Knock-out (SDN1)**
- Soybean: Anti-nutritional Factors
- Sugarcane: Cell wall structure (2G Ethanol)
- Corn: Cell wall structure (2G Ethanol)
- Common Bean: Tegument Color

**HDR (SDN2)**
- Soybean: Drought
- Sugarcane: Drought
- Corn: Drought
- Common Bean: Drought
Embrapa Soybean and Embrapa Cenargen

CRISPRs Systems in Soybean

Soybean: Seed Quality, Anti-Nutricional factors and Drought Tolerance

Knock Out and HR at promoter elements

Candidate genes for Knock Out and HR:
Stay green1 (D1); Stay green2 (D2);
Pheophorbidase (PH2), DREB, AREB, DRIP, etc
SDN1 and SDN2 are for drought tolerance.

(Valério et al., 2016; Fuganti-Pagliarini et al., 2017)

It will be submitted soon to CTNBio under RN16.
Sugarcane BAHDO1 – Increase in glucose conversion

- More than 70% of the biomass cellulose was converted to glucose;
- No change in percentage of cellulose, hemicellulose and lignin;
- ART content of the biomasses Flex was equal to or greater than WT

Increased glucose in 48 hours of enzymatic hydrolysis

![Graph showing increased glucose conversion](image)

De Souza et al., 2019. *Biotechnology for Biofuels*, v. 12, n. 111, p. 1-14

Fonte: Hugo Molinari, 2021
Embrapa Maize and Sorghun

BAHD genes – New gene for sucrose improvement in Corn and Sugarcane

- Metabolic profile (HPLC-HRMS): greatest difference observed for the BAHD5 events;
- Desreplication: sucrose main discriminant metabolite in the BAHD5 events;
- We observed an increase in the sucrose content in leaf (Ev. 1.1 = 52.94%; Ev. 3.1 = 85.19%) and culm (Ev. 1.1 = 95.80%; Ev. 2.1 = 96.22%; Ev. 3.1 = 94.05%) in comparison with NT plants.
- No significant differences in biomass and structural carbohydrate content.
COMMON BEAN CRISPR TARGETS:
- Post Harvesting Darkening (PHD)
- Drought stress

EMBRAPA Rice and Beans Center

CRISPR in Common Beans

Dr. Josias Correa de Faria

Dr. Rosana Vianello
Increasing thermotolerance in European dairy (Holstein) and beef (Angus) cattle
• SNPs found in criollo breeds in Central and South American cattle are associated to high tolerance to heat
✓ Contribute to improve reproductive and productive performance of Angus and Holstein in the tropics

Increasing milk yield in Indian (Zebu) dairy cattle
• SNPs found in Holstein and Jersey breeds are associated to milk and protein production
✓ Contribute to improve productive performance of zebu cows (Gir)
Improving drought tolerance in maize via targeting of candidate genes with CRISPR

Team: José Hernandes Lopes, Viviane C.H. da Silva, Ricardo Dante, Isabel Gerhardt, Juliana Yassitepe, Paulo Arruda

1) **Overall problem:** Drought stress is an important concern for maize production, often resulting in large yield losses during extreme events. Discovery of new genes efficacious at improving drought tolerance via biotechnology approaches is challenging due to the trait genetic complexity and IP protection. We thus have focused discovery efforts at thus far uncharacterized genes associated with large-effect stress tolerance genes and pathways.

2) **Objective:** Development of non-GM (SND1) maize lines with improved drought tolerance via knock-out of thus far largely uncharacterized genes.

3) **Results:**

<table>
<thead>
<tr>
<th>Candidate gene</th>
<th>Modulation under drought</th>
<th>Score</th>
<th>Differential expression under drought</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Treatment</td>
</tr>
<tr>
<td>ZmUNK1</td>
<td>Up/Down</td>
<td>20</td>
<td>35.7%</td>
</tr>
<tr>
<td>ZmUNK3</td>
<td>Up/Down</td>
<td>29</td>
<td>57.1%</td>
</tr>
<tr>
<td>ZmUNK5a</td>
<td>Up</td>
<td>16</td>
<td>28.6%</td>
</tr>
<tr>
<td>ZmUNK8</td>
<td>Up</td>
<td>42</td>
<td>100.0%</td>
</tr>
<tr>
<td>ZmUNK10a</td>
<td>Up/Down</td>
<td>34</td>
<td>71.4%</td>
</tr>
<tr>
<td>ZmUNK11</td>
<td>Up</td>
<td>16</td>
<td>21.4%</td>
</tr>
</tbody>
</table>

1) Identification of genes of unknown function co-expressed with drought-response drivers;  
2) Selection of six candidate genes for knock-out by expression profiling under drought conditions;  
3) Two sets of sgRNAs were designed to knock-out (SDN1) each selected gene; Such sgRNAs were cloned into expression vectors containing the CRISPR machinery.  
4) In progress: transformation (Agrobacterium), tissue culture and selection of the edited plants via Sanger Sequencing.
Regulamentação no Mundo para uso da Biotecnologia no AGRO

Legislação mais assertiva mantendo a Biossegurança

Fases e Custos para desenvolver uma Planta GM

Custo Total Estimado: ~U$136 milhões

Pode levar de 12 a 20 anos desde a descoberta de um gene até a colocação de uma variedade comercial no mercado
Legislação Harmonizada entre vários países tem permitido surgimento de mais “players”

### Regulated Technologies: Consolidation

<table>
<thead>
<tr>
<th>Company</th>
<th>Market Share</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bayer</strong> (incl. Monsanto)</td>
<td>19.7</td>
</tr>
<tr>
<td>Syngenta (incl. Chemchina agricultural business)</td>
<td>14.1</td>
</tr>
<tr>
<td>Corteva*</td>
<td>12.7</td>
</tr>
<tr>
<td>BASF (incl. Bayer business)</td>
<td>7.9</td>
</tr>
<tr>
<td>FMC</td>
<td>3.4</td>
</tr>
</tbody>
</table>

**World market leaders in agrochemicals by pro forma sales 2017 in € bln**

*Handelsblatt // ‘Dow Dupont merged agricultural operation’*

### Non-Regulated Tech.: Diversification

- Caribou Bioscience
- Eden Research
- Inari
- Biothalyx
- Green Biologics
- Zymergen
- CiBO
- Indigo
- Invaio
- Pacific Bioscience
- BioAmber
- Arcadia
- EcoVative Design
- Pairwise
- Provivi
- Iden
- BioAtlantis
- Phyllotec
- Lemnatec
- Edison Agroscience
- AgriHouse
- Benson Hill
- Evogene
- Performance Plant
- GreenVenus
- Tropic Bioscience
- Agrosphere
- Agricell
- AgriScience
- Grassroots Biotech
- Eurogentec
- Mendel
- Oxitec
- ....

[https://www.ventureradar.com/keyword/Agricultural%20Biotechnology?](https://www.ventureradar.com/keyword/Agricultural%20Biotechnology?)
“Open source” new nucleases

Genome editing in plants with MAD7 nuclease

Qiupeng Lin a,b,1, Zixu Zhu a,b,1, Guanwen Liu c,d,1, Chao Sun a,b, Dexing Lin a,b,
Chenxiaoxue a,b, Shengnan Li c,d, Dandan Zhang c,d,1, Caixia Gao a,b, Yanpeng Wang a,b, *,
Jin-Long Qiu c,d, *

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A R T I C L E   I N F O

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Keywords:
MAD7 nuclease
CRISPR-Cas12a
Plant genome editing
Royalty-free
Commercial use

A B S T R A C T

MAD7 is an engineered nuclease of the Class 2 type V-A CRISPR-Cas (Cas12a/Cpf1) family with a low level of homology to canonical Cas12a nucleases. It has been publicly released as a royalty-free nuclease for both academic and commercial use. Here, we demonstrate that the CRISPR-MAD7 system can be used for genome editing and recognizes T-rich PAM sequences (YTN) in plants. Its editing efficiency in rice and wheat is comparable to that of the widely used CRISPR-LbCas12a system. We developed two variants, MAD7-RR and MAD7-RVR, that increase the target range of MAD7, as well as an M-ATD (a MAD7-APOBEC fusion-induced deletion) system that creates predictable deletions from 5′-adenylated Cs to the MAD7-cleavage site. Moreover, we show that MAD7 can be used for multiplex gene editing and that it is effective in generating indels when combined with other CRISPR RNA orthologs. Using the CRISPR-MAD7 system, we have obtained regenerated mutant rice and wheat plants with up to 65.6% efficiency.

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Challenges for the detection of genetically modified food or feed originating from genome editing

With respect to the challenges for GMO detection laboratories as framed in the scenario above the following can be concluded:

Most of the mutations induced by genome editing technologies cannot be unequivocally distinguished from natural mutations as they may also occur naturally. Plant genomes have inherently a considerable sequence variability which is at present not only insufficiently documented for any crop, but the genomes keep also changing over time.

Moreover, mutations obtained by new mutagenesis techniques can currently not be differentiated from those induced by conventional mutagenesis techniques, which have been incorporated in traditional breeding programs and are often not thoroughly documented.
Obrigado
Thank You

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