Biotechnology Applications of Sugar cane Genetic Transformation

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Sugarcane in Brazil

- Largest world Producer
- Availability of land with good soil fertility
- Good clime conditions
- Generate almost 1 million direct jobs and supports 70,000 independent farmers
- Sugar Production Plants well established
- Government resource incentive to ethanol production
- The use of Ethanol, greenhouse emissions were reduced by 43 million tons of CO2 (2004-2008), equivalent to plant 150 million trees

Source: UNICA
## Perspective of Expansion of Sugarcane Production in Brazil

<table>
<thead>
<tr>
<th></th>
<th>2007/08</th>
<th>2015/16</th>
<th>2020/21</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Production of Sugarcane (millions ton)</strong></td>
<td>469</td>
<td>829</td>
<td>1.038</td>
</tr>
<tr>
<td><strong>Cultivated Area (millions ha)</strong></td>
<td>7.8</td>
<td>11.4</td>
<td>13.9</td>
</tr>
<tr>
<td><strong>Sugar (million ton)</strong></td>
<td>31.0</td>
<td>41.3</td>
<td>45.0</td>
</tr>
<tr>
<td>Int. consumption and storage</td>
<td>12.4</td>
<td>11.4</td>
<td>12.1</td>
</tr>
<tr>
<td>Exportation</td>
<td>18.6</td>
<td>29.9</td>
<td>32.9</td>
</tr>
<tr>
<td><strong>Ethanol (billions liters)</strong></td>
<td>22.5</td>
<td>46.9</td>
<td>65.3</td>
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<tr>
<td>Int. consumption and storage</td>
<td>18.9</td>
<td>34.6</td>
<td>49.6</td>
</tr>
<tr>
<td>Exportation</td>
<td>3.6</td>
<td>12.3</td>
<td>15.7</td>
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<tr>
<td><strong>Bioeletricity (MW average)</strong></td>
<td>1.800</td>
<td>11.500</td>
<td>14.400</td>
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<tr>
<td>Participation in the electrical matrix</td>
<td>3%</td>
<td>15%</td>
<td>15%</td>
</tr>
</tbody>
</table>

**Source:** UNICA, nov 2008
87% of Brazilian sugarcane production

50% of gasoline consumption
Replaced by ethanol produced on
Nearly 1% of Brazilian arable land
(3 million ha)
Kingdom: Plantae
Phylum: Magnoliophyta
Class: Liliopsida
Order: Cyperales
Family: Poaceae
Genus: Saccharum
Species: S. officinarum, S. spontaneum, S. robustum, S. sinense, S. barberi, S. edule
CLASSICAL BREEDING

Results of genetic crossings:

• High level of sucrose
• Disease resistance cultivars
• improved ratooning ability

Limitations of the classical breeding:

• Complex polyploid-aneuploid genome
• Narrow genetic basis
• Poor fertility
• Long breeding program (12 - 15 years)
  (back-crossing to recover elite germoplasms with desired agronomic traits is time consuming)
Biotechnology offers excellent opportunities for sugarcane crop improvements
BIOTECHNOLOGY

Research Areas:

4.1. Genetic maps by molecular markers

4.2. Tissue and cell culture

4.3. Incorporation of desired genes – Transgenics
MOLECULAR MARKERS

Applications:

• Understanding commercial cultivar origins
• Identification of diversity and genetic variability
• Introgression and QTLs identification
• Diagnostics of disease resistance or tolerance
• Structural and functional genomics
Research Areas:

4.1. Genetic maps by molecular markers

4.2. Tissue and cell culture
TISSUE and CELL CULTURE

- Callus culture: Nickell, 1969
- Plant regeneration: Barba e Nickell, 1969
  Heinz e Mee, 1969

Success on plant regeneration:
- Micropropagation
- Somaclonal variation
- Basis for Genetic transformation
Explants: Immature Leaves
TISSUE and CELL CULTURE

Sugarcane Callus Induction

2,4-D

BAP
TISSUE and CELL CULTURE

Embryogenic Callus

RB72454
TISSUE and CELL CULTURE

Sugarcane Plant Regeneration from embryogenic callus
Shoot regeneration in MS medium with BA (0.1 mg/L), after callus induction on MS with 2,4-D (8.0 mg/L) in the dark.
Research Areas:

4.1. Genetic maps by molecular markers

4.2. Tissue and cell culture

4.3. Introduction of desired genes – Transgenics
Sugarcane Transformation

- Protoplasts with PEG: Chen et al, 1987
  - low efficiency and poor reproducibility

- Electroporation: Rathus and Birch, 1992
  - no plant regeneration

First Transformed Commercial Cultivar:
- gene \textit{npt-II}, in Australia: Bower e Birch, 1992 (microprojectile-mediated transformation)
Explants: Immature Leaves

Callus Induction

Embryogenic Callus

Direct Embryogenesis

Transformation: Bombardment and A. tumefaciens

Plant Regeneration

Selective Medium

Greenhouse

André Barbosa
<table>
<thead>
<tr>
<th>Traits</th>
<th>Gene</th>
<th>Transformation method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reporter and selection systems</strong></td>
<td></td>
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<tr>
<td>Neomycin phosphotransferase</td>
<td>npt-II</td>
<td>Microprojectile</td>
<td>Bower and Birch, 1992</td>
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<tr>
<td>β-Glucuronidase</td>
<td>uidA</td>
<td>Microprojectile</td>
<td>Bower and Birch, 1992</td>
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<td>Hygromycin phosphotransferase</td>
<td>hpt</td>
<td>Agrobacterium</td>
<td>Arencibia et al., 1998</td>
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<td>Green fluorescent protein</td>
<td>gfp</td>
<td>Agrobacterium</td>
<td>Elliott et al., 1998</td>
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<tr>
<td>Phosphinothricin acetyl transferase</td>
<td>bar</td>
<td>Agrobacterium</td>
<td>Elliott et al., 1998</td>
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<td>Phosphinothricin acetyl transferase</td>
<td>bar</td>
<td>Agrobacterium</td>
<td>Manickavasagam et al., 2004</td>
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<td><strong>Herbicide resistance</strong></td>
<td></td>
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<tr>
<td>Bialaphos</td>
<td>bar</td>
<td>Microprojectile</td>
<td>Gallo-Meagher and Irvine, 1996</td>
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<td>bar</td>
<td>Agrobacterium</td>
<td>Enriquez-Obregon et al., 1998</td>
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<td>bar</td>
<td>Microprojectile</td>
<td>Falco et al., 2000</td>
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<td>Glufosinate ammonium</td>
<td>pat</td>
<td>Microprojectile</td>
<td>Leibbrandt and Snyman, 2003</td>
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<td><strong>Disease resistance</strong></td>
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<td></td>
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<tr>
<td>SCMV</td>
<td>SCMV-CP</td>
<td>Microprojectile</td>
<td>Joyce et al., 1998a, b</td>
</tr>
<tr>
<td>SrMV</td>
<td>SrMV-CP</td>
<td>Microprojectile</td>
<td>Ingelbrencht et al., 1999</td>
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<td>Sugarcane yellow leaf virus</td>
<td>SCYLV-CP</td>
<td>Microprojectile</td>
<td>Rangel et al., 2003</td>
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<tr>
<td>Sugarcane yellow leaf virus</td>
<td>SCYLV-CP</td>
<td>Microprojectile</td>
<td>Gilbert et al., 2009</td>
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<td>Fiji leaf gall</td>
<td>FDVS9 ORF 1</td>
<td>Microprojectile</td>
<td>McQualter et al., 2004a</td>
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<td>Sugarcane leaf scald</td>
<td>albD</td>
<td>Microprojectile</td>
<td>Zhang et al., 1999</td>
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<tr>
<td>Pest resistance</td>
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<tr>
<td>Sugarcane stem borer</td>
<td>cry1A</td>
<td>Electroporation</td>
<td>Arencibia et al., 1999</td>
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<td>Sucargane stem borer</td>
<td>cry1Ab</td>
<td>Microprojectile</td>
<td>Braga et al., 2003</td>
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<td>Sugarcane canegrub resistance</td>
<td>gna or pinII</td>
<td>Microprojectile</td>
<td>Nutt et al., 1999</td>
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<td>Mexican rice borer</td>
<td>gna</td>
<td>Microprojectile</td>
<td>Legaspi and Mirkov, 2000</td>
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<td>Sugarcane stem borer and Mexican rice borer</td>
<td>gna</td>
<td>Microprojectile</td>
<td>Setamou et al., 2002</td>
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<td>Metabolic engineering and alternative products</td>
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<td>Sucrose accumulation</td>
<td>Antisense soluble acid invertase</td>
<td>Microprojectile</td>
<td>Ma et al., 2000</td>
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<tr>
<td></td>
<td>Soluble acid invertase</td>
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<td>Fructo oligosaccharide</td>
<td>lsdA</td>
<td>Agrobacterium</td>
<td>Enriquez et al., 2000</td>
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<td>Polyphenol oxidase</td>
<td>ppo</td>
<td>Microprojectile</td>
<td>Vickers et al., 2005a</td>
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<tr>
<td>Polyhydroxybutyrate</td>
<td>phaA, phaB, and phaC</td>
<td>Microprojectile</td>
<td>Brumbley et al., 2003</td>
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<td>p-Hydroxybenzoic acid</td>
<td>hchl and cpl</td>
<td>Microprojectile</td>
<td>McQualter et al., 2004b</td>
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<tr>
<td>Tripsin inhibitors</td>
<td>Kunitz and Bower-Birk</td>
<td>Microprojectile</td>
<td>Falco and Silva-Filho, 2003</td>
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<td>Mannose</td>
<td>manA</td>
<td>Microprojectile</td>
<td>Jain et al., 2007</td>
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<tr>
<td>Store sugar level</td>
<td>SI</td>
<td>Microprojectile</td>
<td>Wu and Birch, 2007</td>
</tr>
</tbody>
</table>
Bax inhibitor-1: BI-1: PCD Regulatory inhibitor Protein

(source: homepage http://cabm.rutgers.edu/research.html)
Co-transformation of variety RB835089 with plasmids: pHA9 (\textit{Ubi-1 :: neo:: T-Nos}) and pDM8 (\textit{CaMV35S:: AtBI-1-V5His6:: T-Nos}) pDM9 (\textit{Ubi-1 :: AtBI-1-V5His6:: T-Nos})

<table>
<thead>
<tr>
<th>Experiments</th>
<th>N of bombarded plates</th>
<th>N of bombarded calli</th>
<th>N of shoots Resistant to Geneticin</th>
<th>Plants PCR (+)\textit{neo}</th>
<th>Plants PCR (+)\textit{neo}/\textit{AtBI-1}</th>
<th>Co-transformation Efficiency (%)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHA9+pDM8</td>
<td>66</td>
<td>3,300</td>
<td>42</td>
<td>36</td>
<td>30</td>
<td>0.91</td>
</tr>
<tr>
<td>pHA9+pDM9</td>
<td>120</td>
<td>6,000</td>
<td>139</td>
<td>94</td>
<td>67</td>
<td>1.12</td>
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</tbody>
</table>

\textsuperscript{a}Co-transformation efficiency (%): total of plants with positive PCR for \textit{neo} and \textit{AtBI-1} divided by number of bombarded calli.

\begin{center}
\includegraphics[width=0.8\textwidth]{pHA9_diagram.png}
\end{center}

Genetic transformation of variety RB835089 mediated by *A. tumefaciens* EHA105 with pNW166

<table>
<thead>
<tr>
<th>Experiment</th>
<th>N° of Plates</th>
<th>N° of inoculated calli</th>
<th>N° of shoots Resistant to Geneticin</th>
<th>Plants PCR (+) neo</th>
<th>Plants PCR (+) neo/AtBI-1</th>
<th>Transformation Efficiency (%)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrobacterium</td>
<td>25</td>
<td>1,250</td>
<td>56</td>
<td>52</td>
<td>52</td>
<td>4.16</td>
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<tr>
<td>Agrolistic</td>
<td>25</td>
<td>1,250</td>
<td>86</td>
<td>78</td>
<td>78</td>
<td>6.24</td>
</tr>
</tbody>
</table>

\(^a\) Transformation efficiency (%): total of plants with positive PCR for neo and AtBI-1 divided by number of calli inoculated into suspension of *A. tumefaciens*.

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![Diagram of pNW166](image)

Watanabe & Lam, 2008
Phenotype of the root system of WT plants and transgenic plants incubated in liquid MS medium with:

T1: 0.0 Tunicumacyn
T2: 0.5 mg.L\(^{-1}\) Tunicumacyn
T3: 1.0 mg.L\(^{-1}\) Tunicumacyn

viewed on the microscope in the 10\(^{th}\) day after incubation
Plastid Genetic Transformation

Saccharum officinarum
Sugarcane
(Saccharum officinarum)
Chloroplast DNA
141,182 bp

Calsa-Junior et al., Current genetics, 2004
Chloroplast Organization

- **Double Membrane**
- **Nucleoid**
- **Grannum**
- **Thylakoid**
- **Stroma**

**Leaf cell:** 10 - 100 Chloroplasts
**Chloroplast:** 1-10 nucleoids
**Nucleoid:** 10 ptDNA

**Nucleoid (DNA + proteins)**
Chloroplast Transformation

Insertion of transgene by Homologous Recombination

It is necessary to obtain homoplasmic plants
Advantages of Plastid Transformation

- integration of transgene at specific local, in intergenic region;
- maternal inheritance;
- high protein accumulation in plastids;
- not occur genes silencing;
- it is possible to insert multiple genes in an unique transformation event;
- there are methods to eliminate the antibiotic resistance marker gene.
Accumulation of protein expressed in leaves and fruits of transplastomic tomato

# Agronomical traits introduced in Plastid Genome

<table>
<thead>
<tr>
<th>Trait</th>
<th>Transgene</th>
<th>Promoter</th>
<th>5’/3’ UTRs</th>
<th>Homologous recombination site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insect resistance</td>
<td>Cry1A (c)</td>
<td>Prrn</td>
<td>rbcL/Trps16</td>
<td>trnV/rps12/7</td>
</tr>
<tr>
<td>Herbicide resistance</td>
<td>AroA</td>
<td>Prrn</td>
<td>gagg/TpsbA</td>
<td>rbcL/accD</td>
</tr>
<tr>
<td>Insect resistance</td>
<td>Cry2Aa2</td>
<td>Prrn</td>
<td>gagg (native)/TpsbA</td>
<td>rbcL/accD</td>
</tr>
<tr>
<td>Herbicide resistance</td>
<td>bar</td>
<td>Prrn</td>
<td>rbcL/psbA</td>
<td>rbcL/accD</td>
</tr>
<tr>
<td>Insect resistance</td>
<td>Cry2Aa2 operon</td>
<td>Prrn</td>
<td>native 5’ UTRs/TpsbA</td>
<td>trnL/trnA</td>
</tr>
<tr>
<td>Disease resistance</td>
<td>MSI-99</td>
<td>Prrn</td>
<td>gagg/TpsbA</td>
<td>trnL/trnA</td>
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<tr>
<td>Drought tolerance</td>
<td>tps</td>
<td>Prrn</td>
<td>gagg/TpsbA</td>
<td>trnL/trnA</td>
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<tr>
<td>Phytoremediation</td>
<td>merA&lt;sup&gt;a&lt;/sup&gt;/merB&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Prrn</td>
<td>gagg&lt;sup&gt;a,b&lt;/sup&gt;/TpsbA</td>
<td>trnL/trnA</td>
</tr>
<tr>
<td>Salt tolerance</td>
<td>badh</td>
<td>Prrn-F</td>
<td>gagg/rps16</td>
<td>trnL/trnA</td>
</tr>
<tr>
<td>Cytoplasmic male sterility</td>
<td>phaA</td>
<td>Prrn</td>
<td>PpsbA/TpsbA</td>
<td>trnL/trnA</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> related to genes with their respective regulatory sequence
High level of Bt protein expression

(Insertion of *cry1A* gene)

Nuclear: 2 - 3%

Chloroplast: 5-20%

MacBride et al., Bio/Techn. 1995
Kota et al., PNAS, 1999
De Cosa et al., Nat Biotech, 2001
Expression of Betaine aldehyde-dehydrogenase confers saline tolerance in carrot

(embryogenic culture)

Kumar et al., 2004
Expression of fragment-C of tetanus toxin in chloroplast genome

High expression is prejudicial to plant development - Nt-pJST10, Nt-pJST11 show low protein expression
High level Bacterial Cellulase Accumulation In Chloroplast Tobacco mediated by Downstream Box fusion

*Thermobifida fusca* cl6A gene
Endoglucanase

Gray et al., 2008
Sugarcane as Biofactory

Important Characteristics:

- fast growth
- efficient pathway for carbon fixation
- production of high amount of biomass
- storage system well developed (stem)
Challenges

- Improve efficiency of genetic transformation;
- Isolation of suitable genes from Eukaryotic or Prokaryotic sources;
- Control the expression of the transgene;
- Identification of suitable gene promoter elements to direct strong tissue/organ-and cell-specific expression;
- Improve stability and storage of the transgene product in the stem;
- Development of the plastid transformation technology for sugarcane.
Acknowledgements:

Collaborators:

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Dr. Eric Lam
Dr. Pal Maliga
Dr. Michael Lawton

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