Biotechnology Applications of Sugar cane Genetic Transformation

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Sugarcane in Brazil

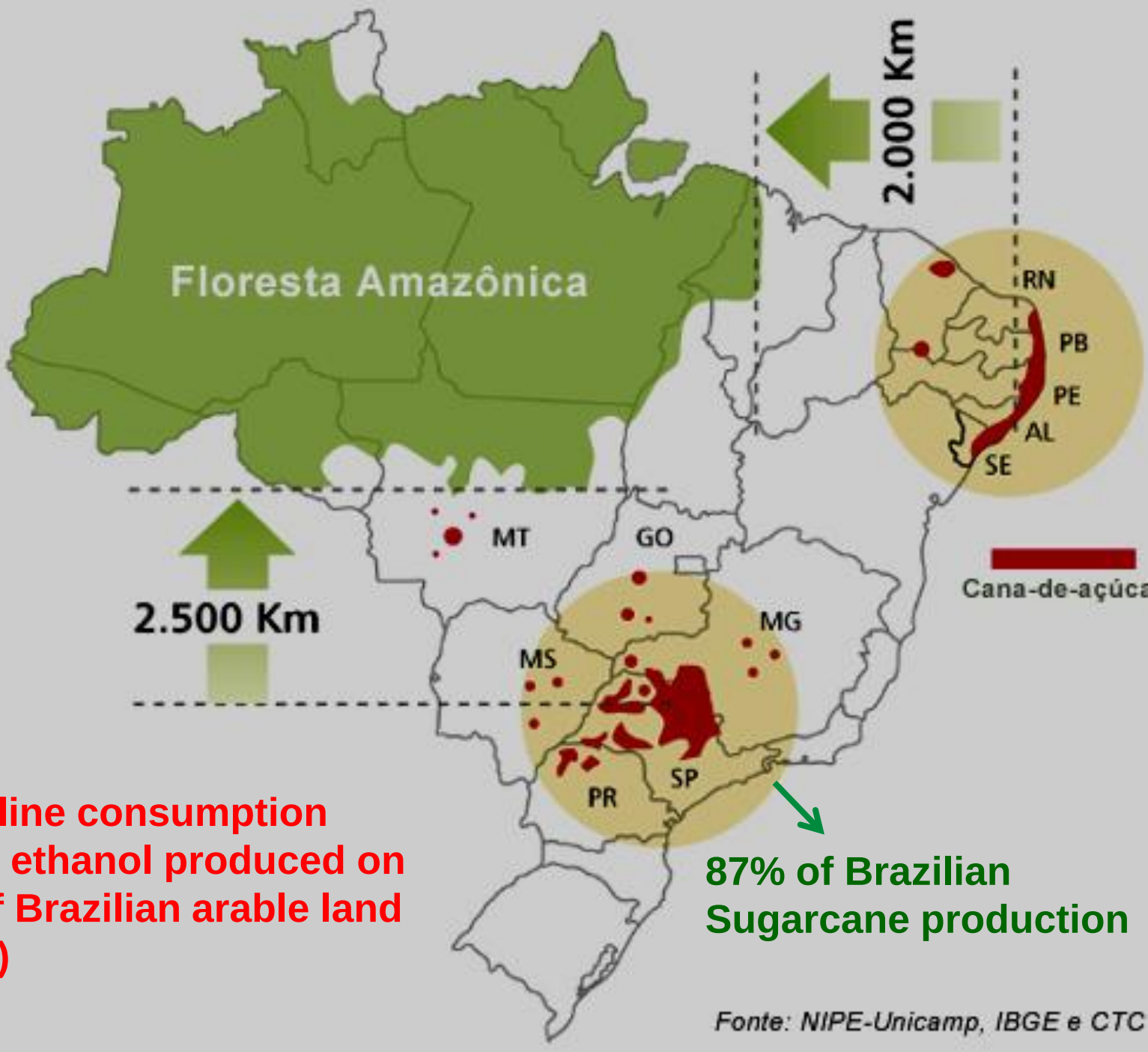
- Largest world Producer
- Availability of land with good soil fertility
- Good clime conditions
- Generate almost 1 million direct jobs and supports 70,000 independent farmers
- Sugar Production Plants well established
- Government resource incentive to ethanol production
- The use of Ethanol, greenhouse emissions were reduced by 43 million tons of CO_2 (2004-2008), equivalent to plant 150 million trees

Source: UNICA

Perspective of Expansion of Sugarcane Production in Brazil

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

Source: UNICA, nov 2008



50% of gasoline consumption
Replaced by ethanol produced on
Nearly 1% of Brazilian arable land
(3 million ha)

87% of Brazilian
Sugarcane production

PLANT TAXONOMY

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 germoplasm 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

BIOTECHNOLOGY

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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

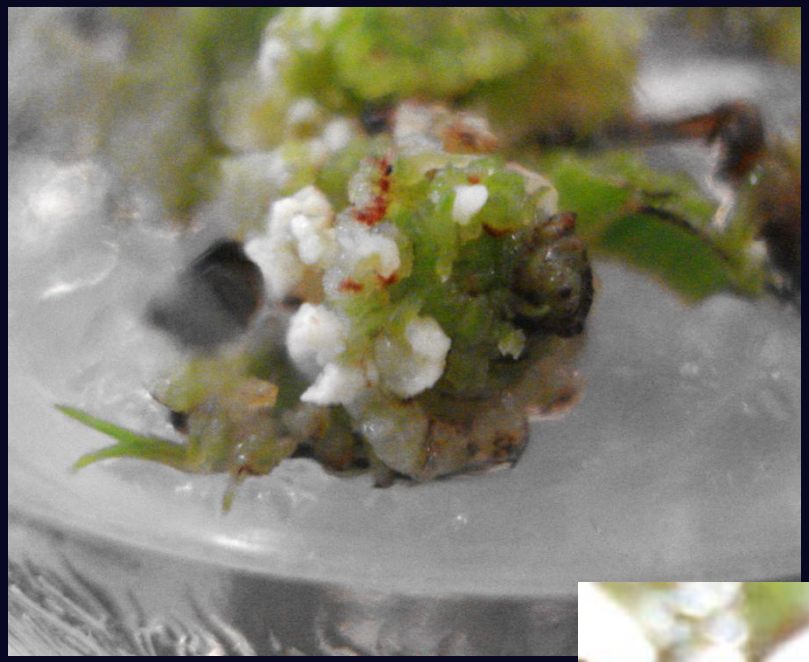
Sugarcane

Callus Induction

TISSUE and CELL CULTURE

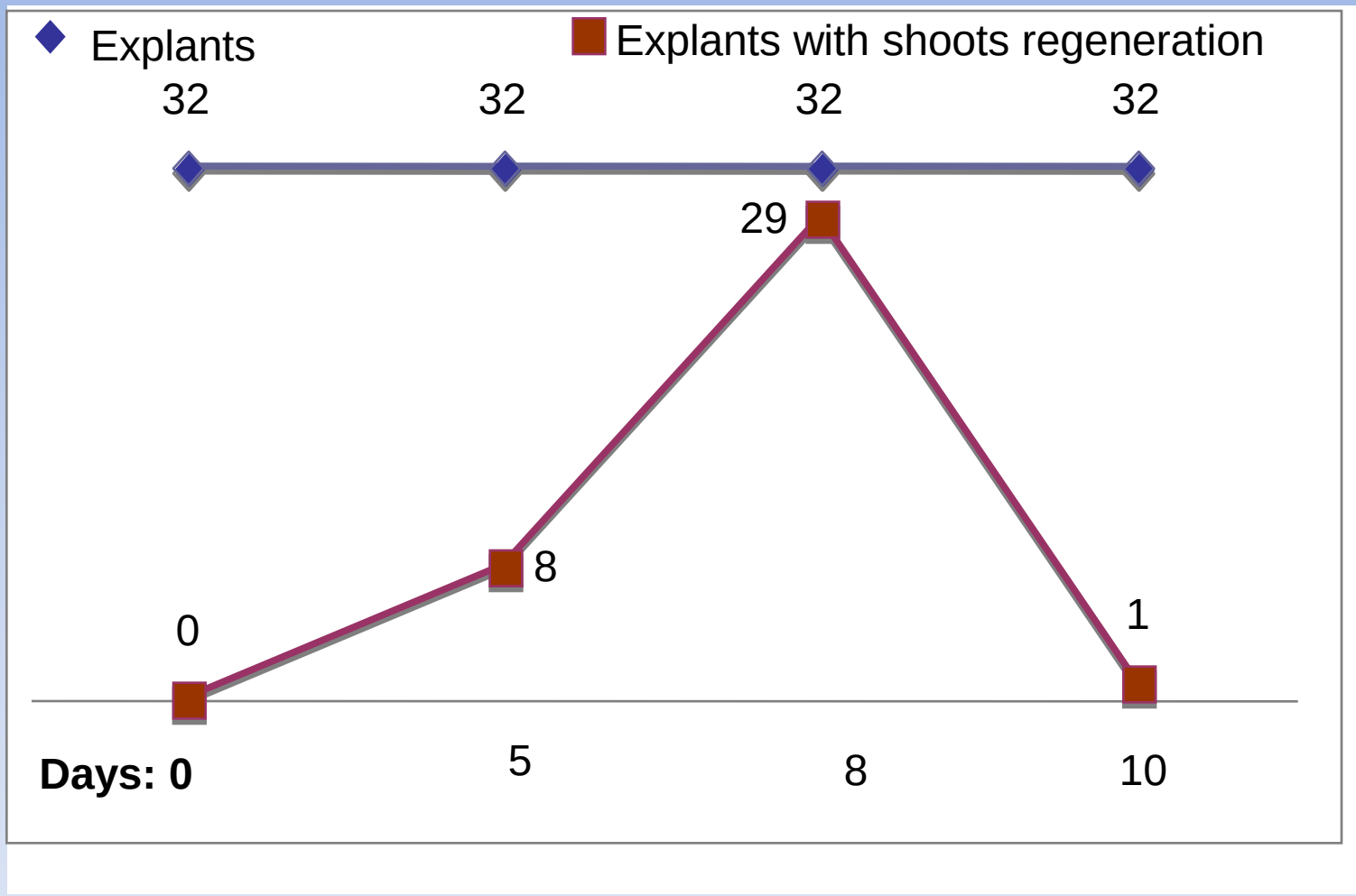
Embryogenic Callus

TISSUE and CELL CULTURE



Sugarcane Plant Regeneration from embryogenic callus

Shoots Regeneration



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

BIOTECHNOLOGY

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

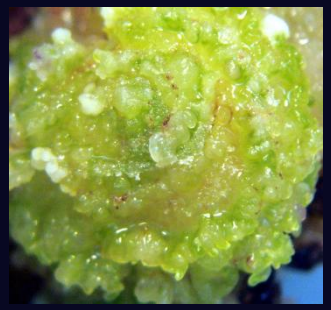
- **Electroporation: Rathus and Birch, 1992**
  - no plant regeneration

First Transformed Commercial Cultivar:

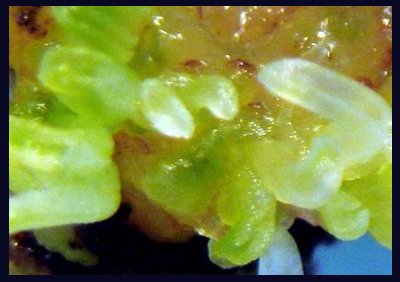
- **gene *npt-II*, in Australia: Bower e Birch, 1992 (microprojectile-mediated transformation)**

Explants:
Immature Leaves

Callus
Induction



Embryogenic
Callus



Direct
Embryogeneis

Transformation: Bombardment and *A. tumefaciens*

Plant Regeneration
Selective Medium

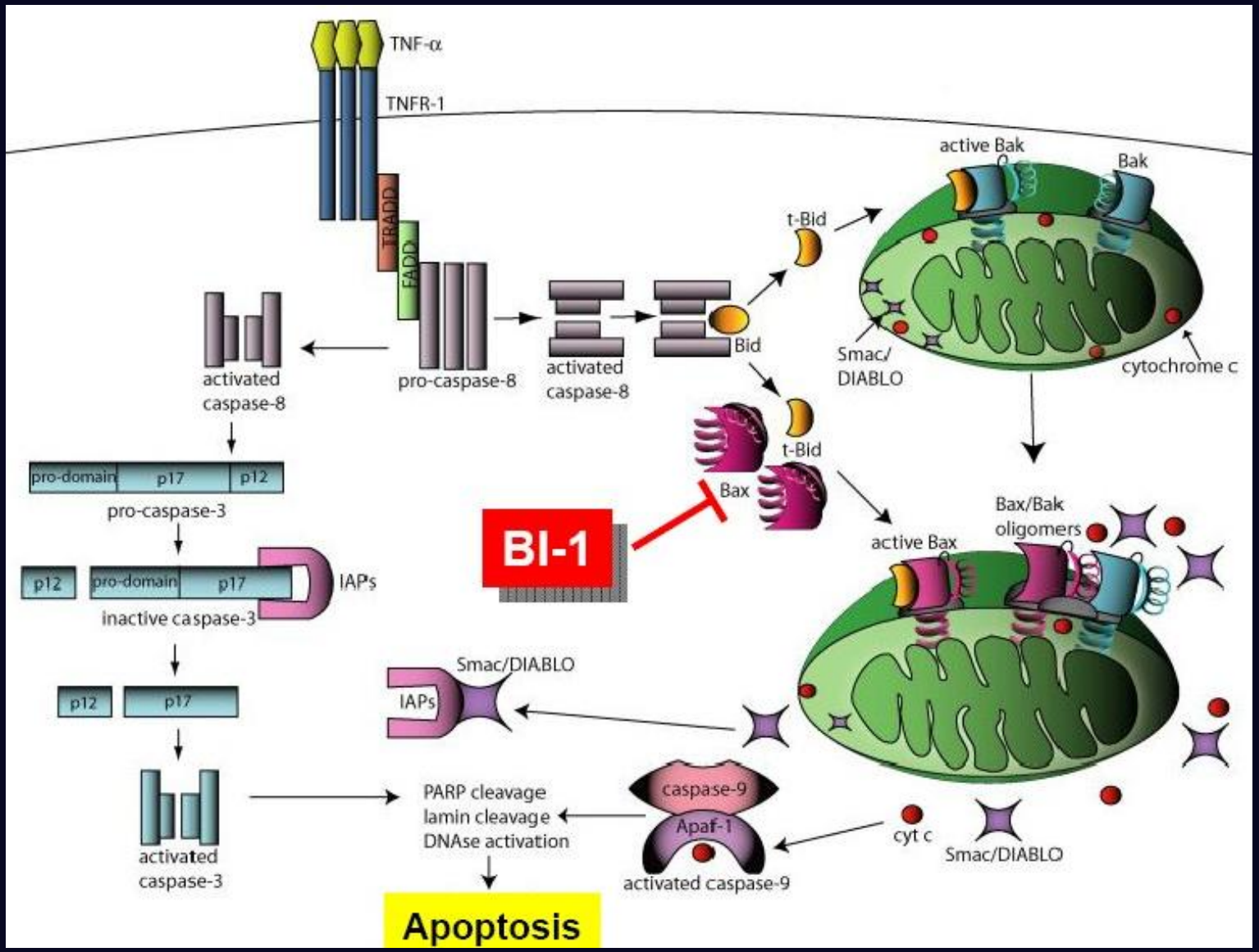
Greenhouse

André Barbosa

Traits	Gene	Transformation method	Reference
Reporter and selection systems			
Neomycin phosphotransferase	*npt-II*	Microprojectile	Bower and Birch, 1992
β-Glucuronidase	*uidA*	Microprojectile	Bower and Birch, 1992
		Electroporation	Arencibia et al., 1995
		Agrobacterium	Arencibia et al., 1998
Hygromycin phosphotransferase	*hpt*	*Agrobacterium*	Arencibia et al., 1998
Green fluorescent protein	*gfp*	*Agrobacterium*	Elliott et al., 1998
Phosphinothricin acetyl transferase	*bar*	*Agrobacterium*	Elliott et al., 1998
Phosphinothricin acetyl transferase	*bar*	*Agrobacterium*	Manickavasagam et al., 2004
Herbicide resistance			
Bialaphos	*bar*	Microprojectile	Gallo-Meagher and Irvine, 1996
Phosphinothricine	*bar*	*Agrobacterium*	Enriquez-Obregon et al., 1998
Phosphinothricine	*bar*	Microprojectile	Falco et al., 2000
Glufosinate ammonium	*pat*	Microprojectile	Leibbrandt and Snyman, 2003
Disease resistance			
SCMV	*SCMV-CP*	Microprojectile	Joyce et al., 1998a, b
SrMV	*SrMV-CP*	Microprojectile	Ingelbrencht et al., 1999
Sugarcane yellow leaf virus	*SCYLV-CP*	Microprojectile	Rangel et al., 2003
Sugarcane yellow leaf virus	*SCYLV-CP*	Microprojectile	Gilbert et al., 2009
Fiji leaf gall	*FDVS9 ORF 1*	Microprojectile	McQualter et al., 2004a
Sugarcane leaf scald	*albD*	Microprojectile	Zhang et al., 1999

Traits	Gene	Transformation method	Reference
Pest resistance			
Sugarcane stem borer	*cry1A*	Electroporation	Arencibia et al., 1999
Sucargane stem borer	*cry1Ab*	Microprojectile	Braga et al., 2003
Sugarcane canegrub resistance	*gna* or *pinII*	Microprojectile	Nutt et al., 1999
Mexican rice borer	*gna*	Microprojectile	Legaspi and Mirkov, 2000
Sugarcane stem borer and Mexican rice borer	*gna*	Microprojectile	Setamou et al., 2002
Metabolic engineering and alternative products			
Sucrose accumulation	Antisense soluble acid invertase Soluble acid invertase	Microprojectile	Ma et al., 2000
Fructo oligosaccharide	*lsdA*	*Agrobacterium*	Enriquez et al., 2000
Polyphenol oxidase	*ppo*	Microprojectile	Vickers et al., 2005a
Polyhydroxybytyrate	*phaA*, *phaB*, and *phaC*	Microprojectile	Brumbley et al., 2003
ρ-Hydroxybenzoic acid	*hchl* and *cpl*	Microprojectile	McQualter et al., 2004b
Tripsin inhibitors	*Kunitz* and *Bower-Birk*	Microprojectile	Falco and Silva-Filho, 2003
Mannose	*manA*	Microprojectile	Jain et al., 2007
Store sugar level	*SI*	Microprojectile	Wu and Birch, 2007

Bax inhibitor-1: BI-1: PCD Regulatory inhibitor Protein

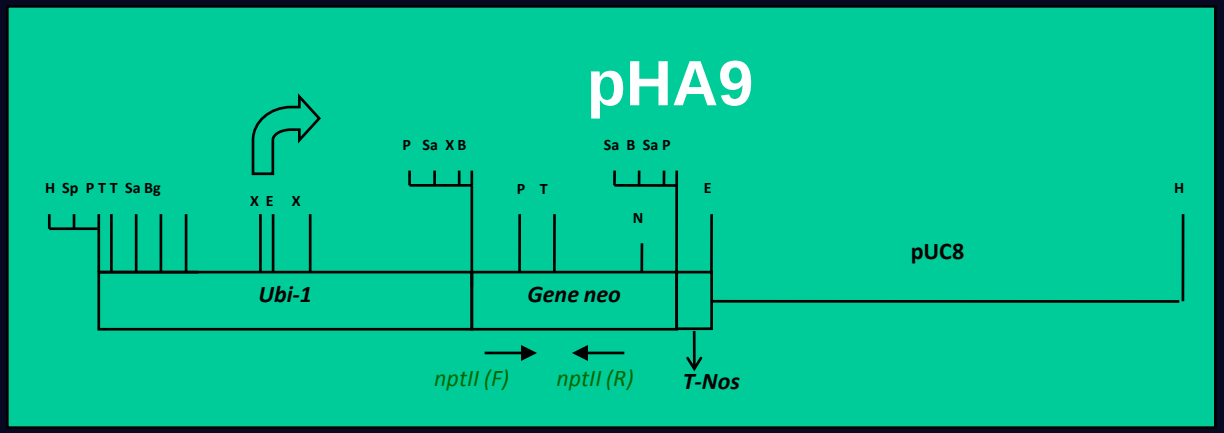


(source: homepage http://cabm.rutgers.edu/research.html

Co-transformation of variety RB835089 with plasmids: pHA9 (*Ubi-1 :: neo:: T-Nos*) and pDM8 (*CaMV35S:: AtBI-1-V5His6:: T-Nos*) pDM9 (*Ubi-1 :: AtBI-1-V5His6:: T-Nos*)

Experiments	N of bombarded plates	N of bombarded calli	N of shoots Resistants to Geneticin	Plants PCR (+) *neo*	Plants PCR (+) *neo/AtBI-1*	Co-transformation Efficiency (%)[a]
pHA9+pDM8	66	3,300	42	36	30	0.91
pHA9+pDM9	120	6,000	139	94	67	1.12

[a]Co-transformation efficiency (%): total of plants with positive PCR for *neo* and *AtBI-1* divided by number of bombarded calli.

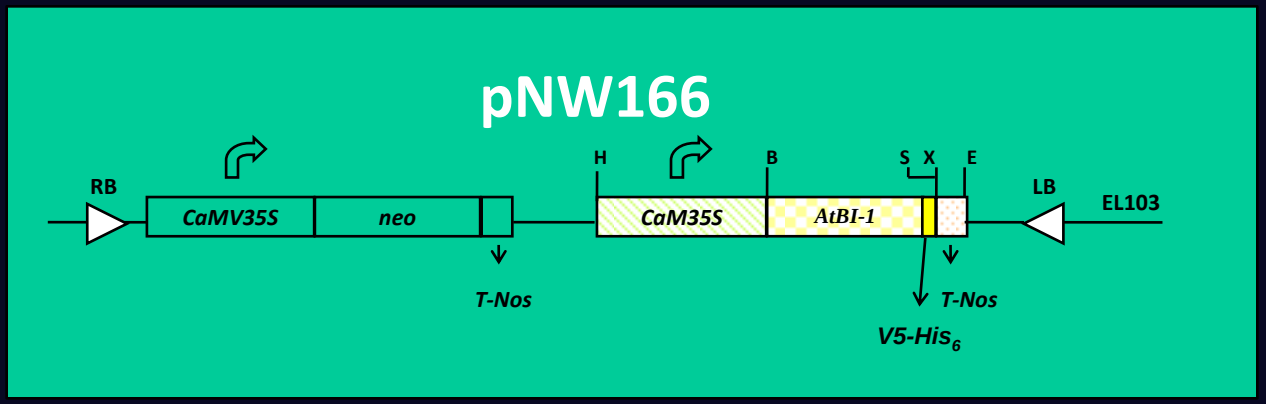


Christensen AH, Quail PH (1996) Transgenic Res 5:213–218

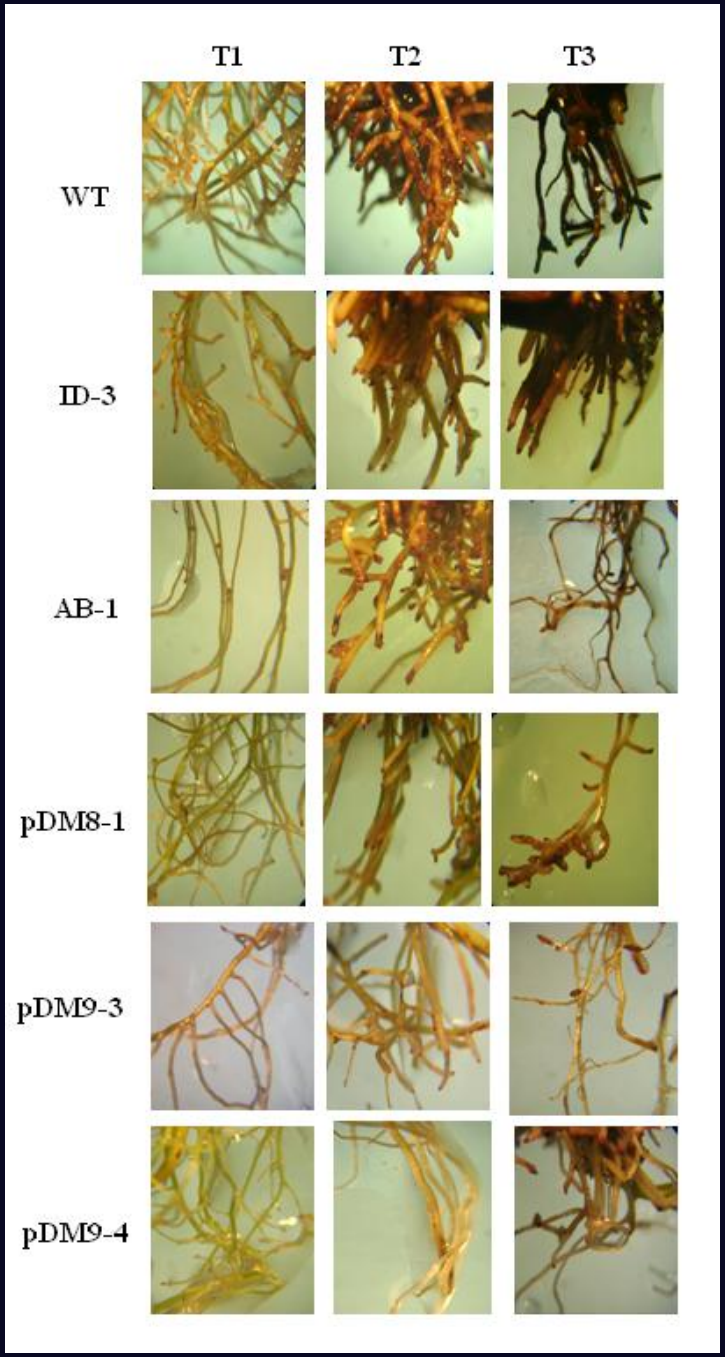
Genetic transformation of variety RB835089 mediated by *A. tumefaciens* EHA105 with pNW166

Experiment	N° of Plates	N° of inoculated calli	N° of shoots Resistants to Geneticin	Plants PCR (+) *neo*	Plants PCR (+) *neo/AtBI-1*	Transformation Efficiency (%)[a]
Agrobacterium	25	1,250	56	52	52	4.16
Agrolistic	25	1,250	86	78	78	6.24

[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*.



Watanabe & Lam, 2008

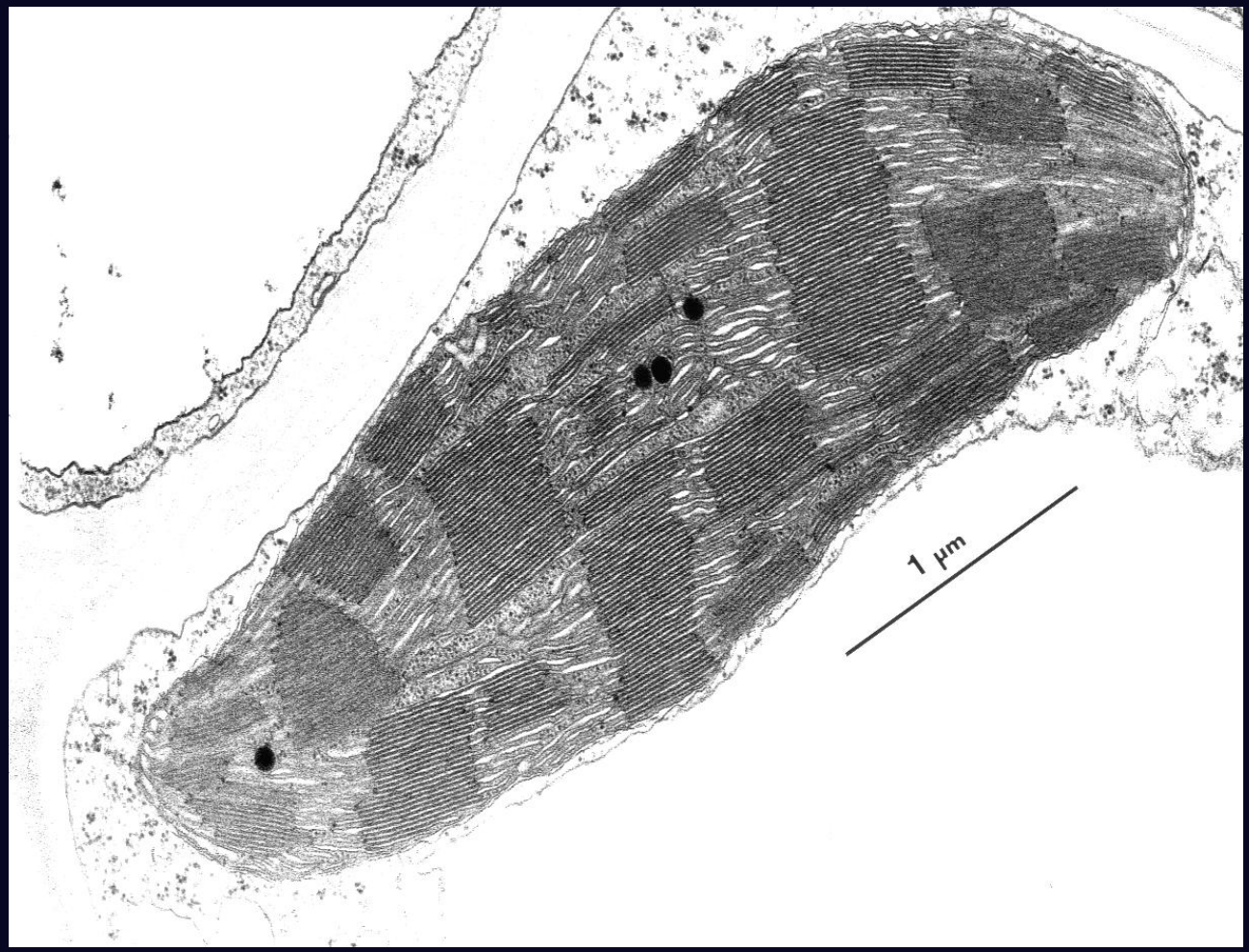


Phenotype of the root system of WT plants and transgenic plants incubated in liquid MS medium with:

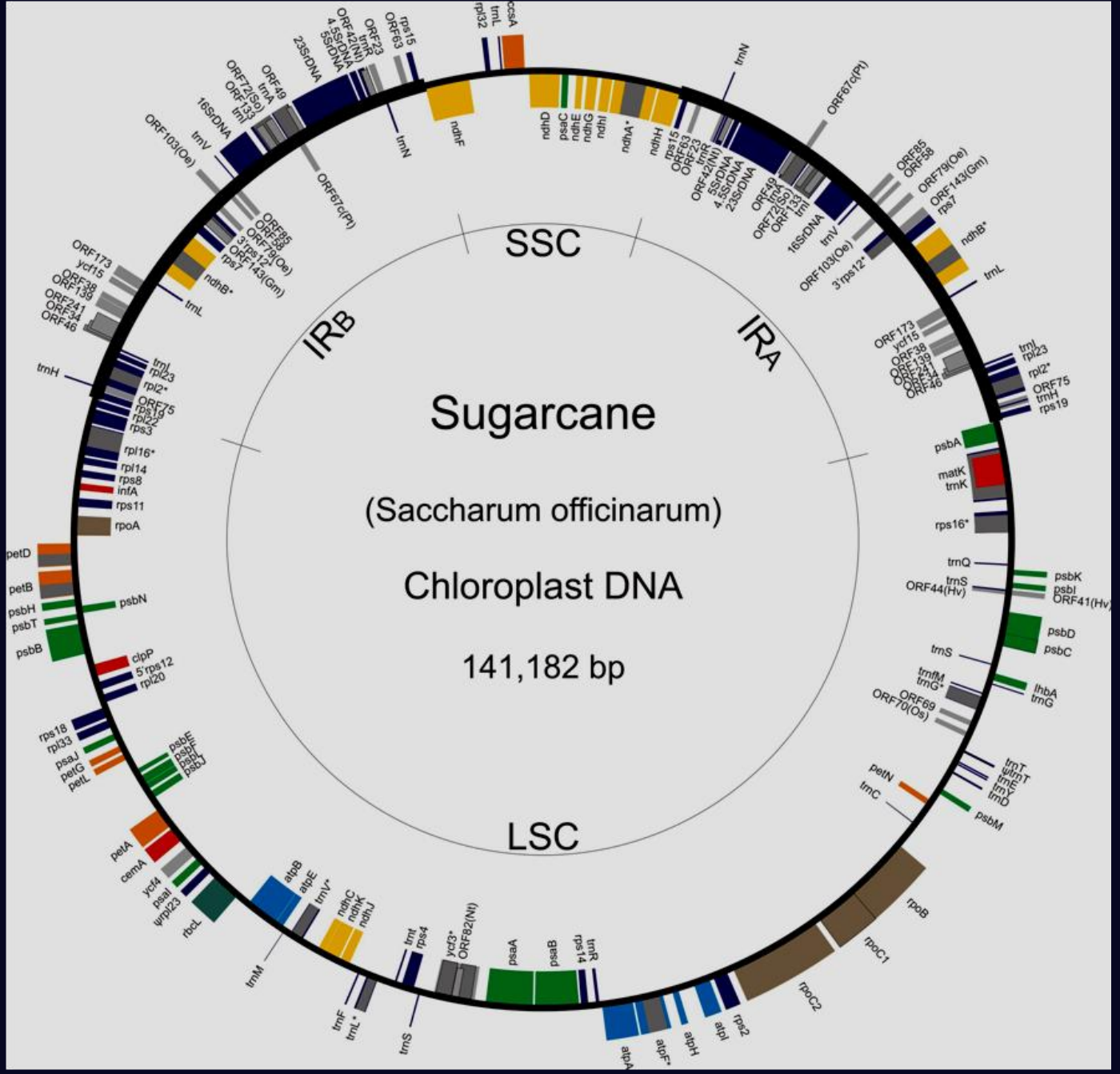
T1: 0.0 Tunicumacyn
T2: 0.5 mg.L^{-1} Tunicumacyn
T3 : 1.0 mg.L^{-1} Tunicumacyn

viewed on the microscope in the 10th day after incubation

Plastid Genetic Transformation

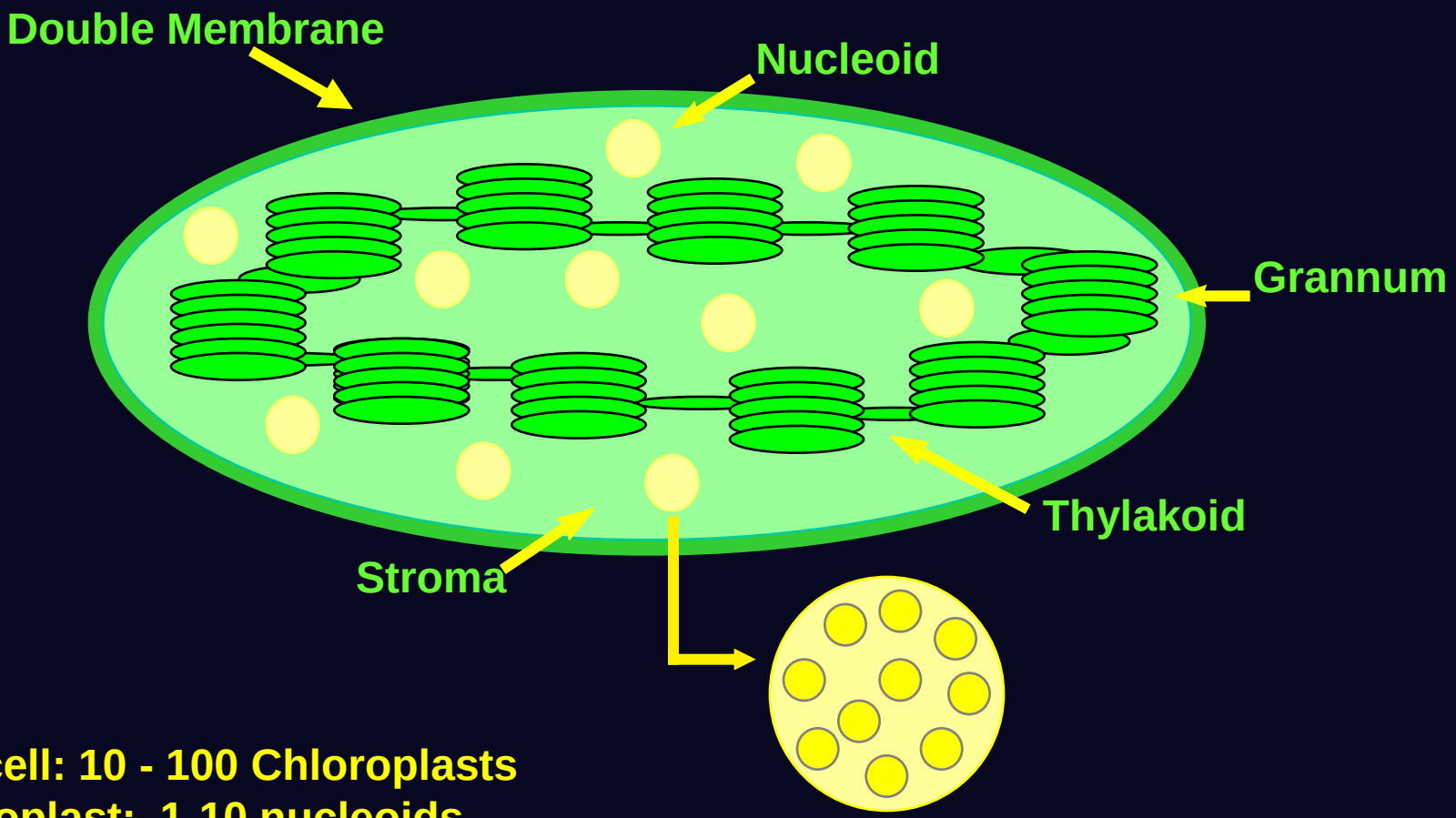


Saccharum officinarum



Calsa-Junior et al., Current genetics, 2004

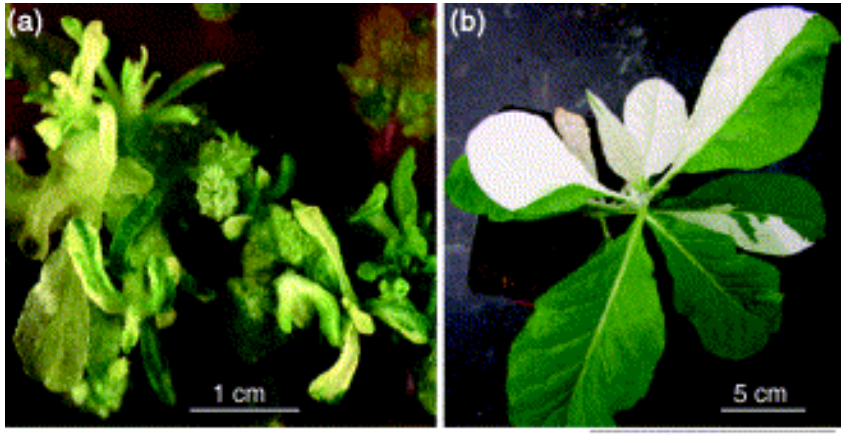
Chloroplast Organization



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

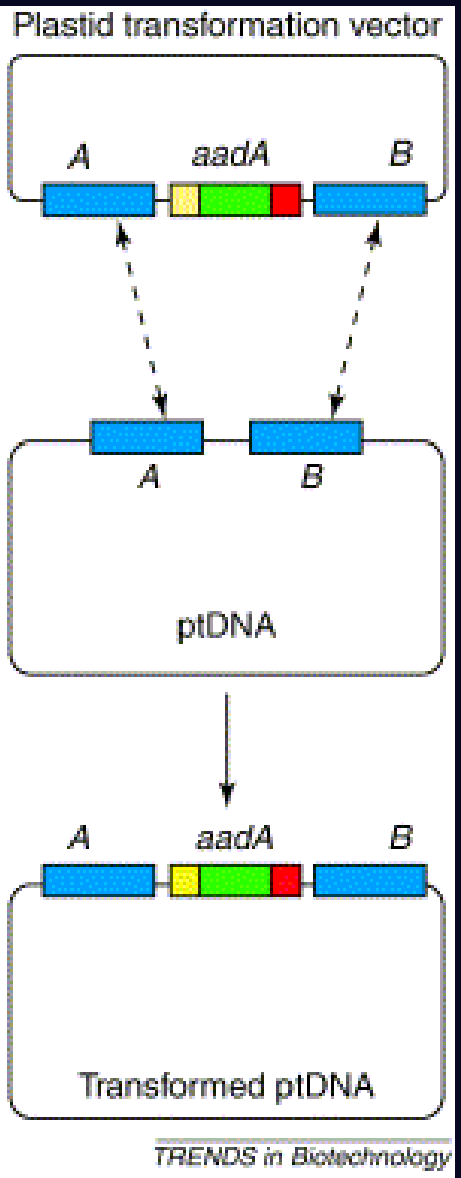
Chloroplast Transformation

Insertion of transgene by Homologous Recombination



TRENDS in Biotechnology

It is necessary to obtain homoplasmic plants

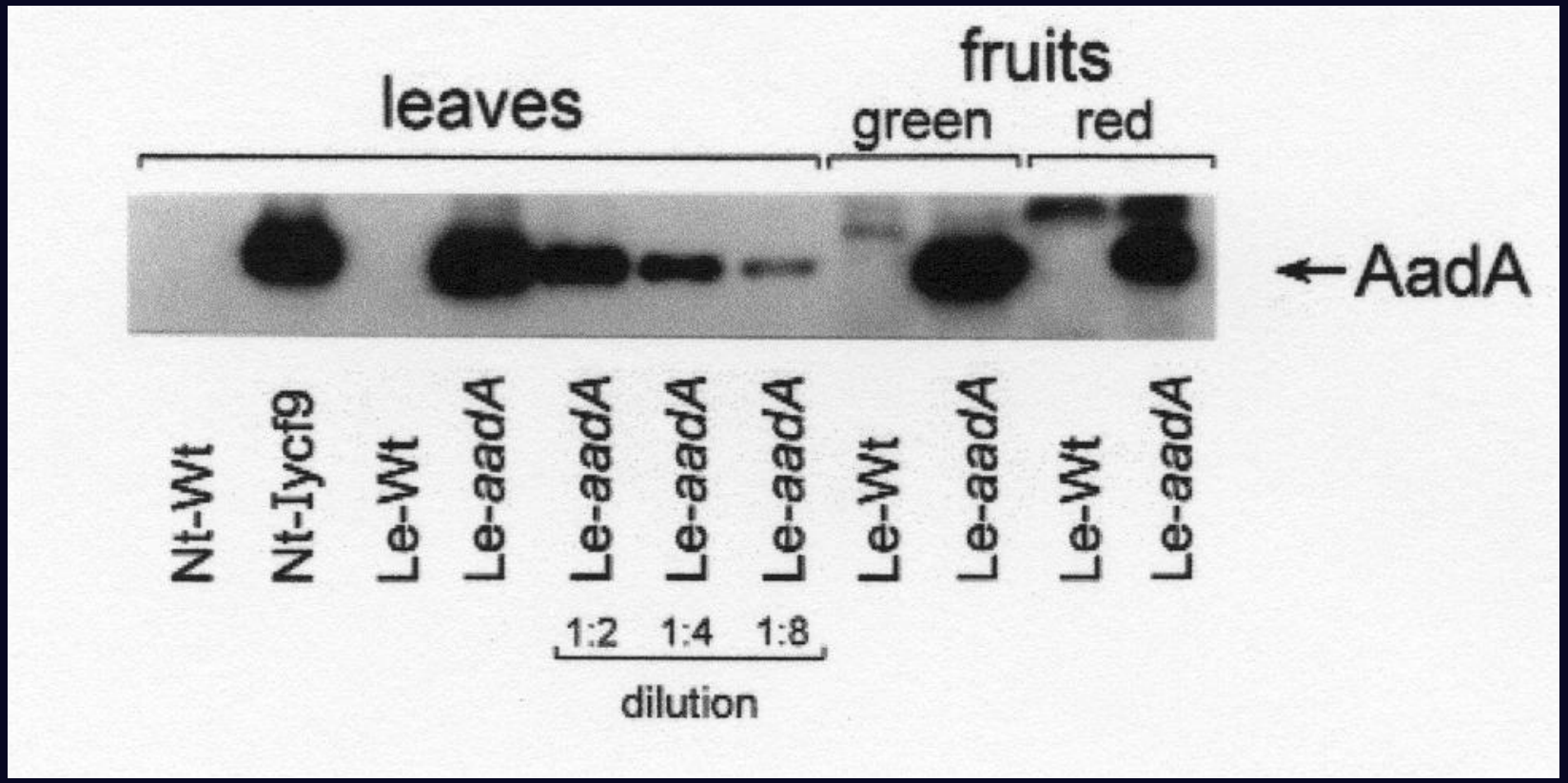


TRENDS in Biotechnology

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

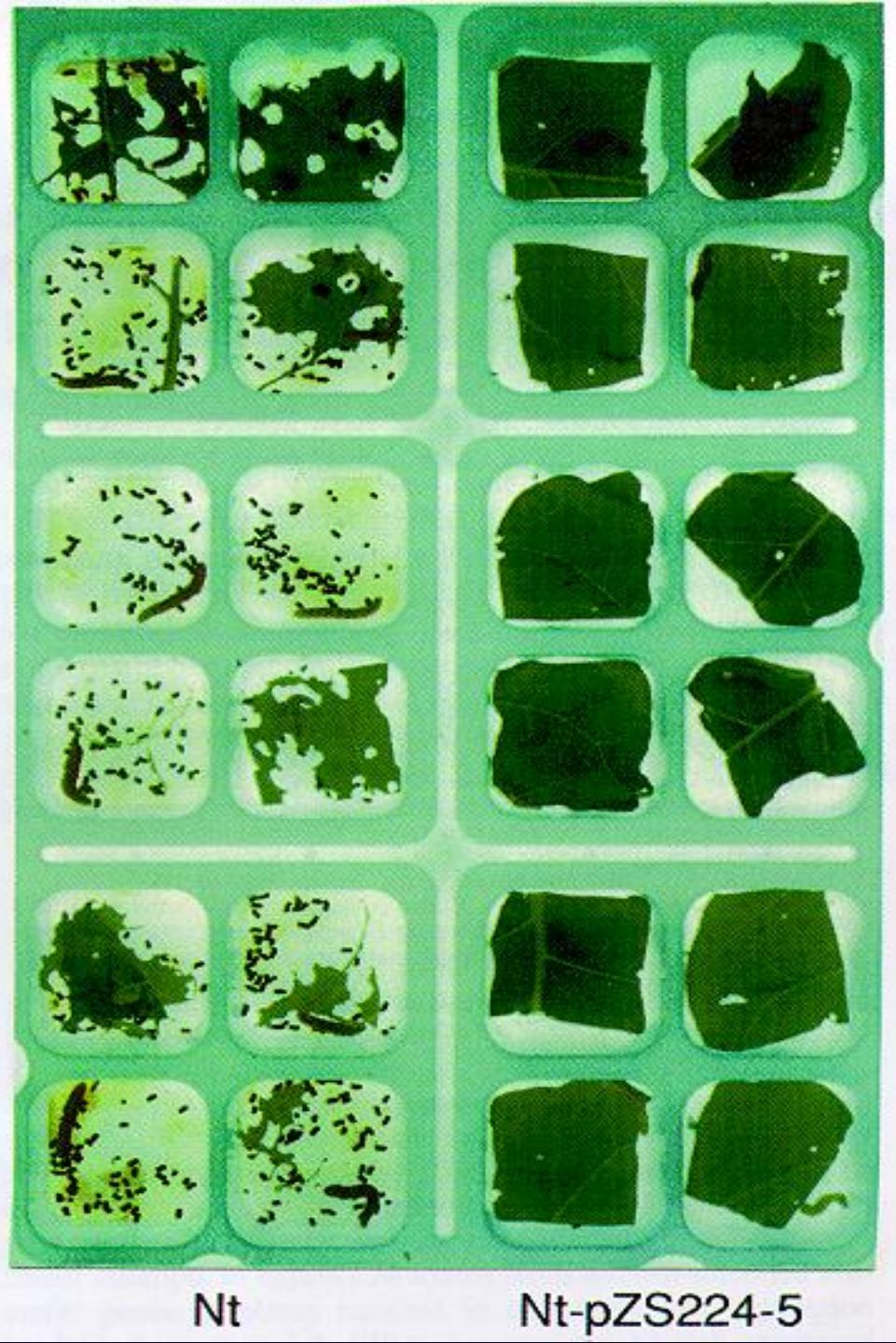


Ruf *et al*., 2001. Nature Biotech.

Agronomical traits introduced in Plastid Genome

Trait	Transgene	Promoter	5′/3′ UTRs	Homologous recombination site
Insect resistance	*Cry*1A (c)	Prrn	*rbc*L/T*rps*16	*trn*V/*rps*12/7
Herbicide resistance	*Aro*A	Prrn	ggagg/T*psb*A	*rbc*L/*acc*D
Insect resistance	*Cry*2A*a*2	Prrn	ggagg (native)/T*psb*A	*rbc*L/*acc*D
Herbicide resistance	*bar*	Prrn	*rbc*L/psbA	*rbc*L/*acc*D
Insect resistance	*Cry*2A*a*2 operon	Prrn	native 5′ UTRs/T*psb*A	*trn*I/*trn*A
Disease resistance	*MSI*-99	Prrn	ggagg/T*psb*A	*trn*I/*trn*A
Drought tolerance	*tps*	Prrn	ggagg/T*psb*A	*trn*I/*trn*A
Phytoremediation	*mer*A[a]/*mer*B[b]	Prrn	ggagg[a,b]/T*psb*A	*trn*I/*trn*A
Salt tolerance	*badh*	Prrn-F	ggagg/*rps*16	*trn*I/*trn*A
Cytoplasmic male sterility	*phaA*	Prrn	P*psbA*/T*psb*A	*trn*I/*trn*A

a,b related to genes with their respective regulatory sequence



High level of Bt protein expression

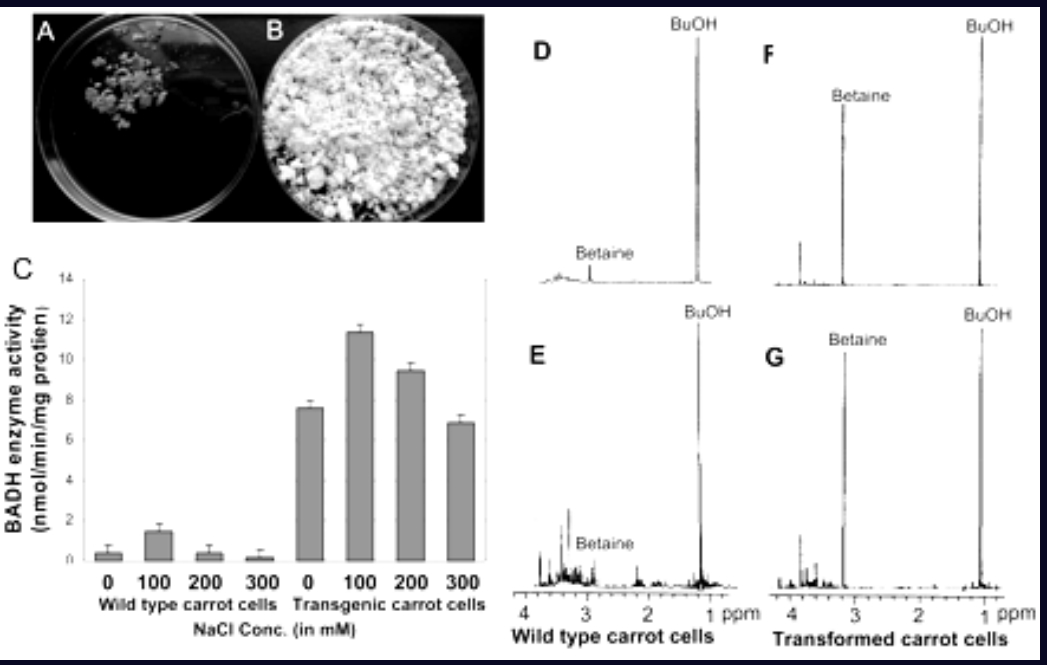
(Insertion of *cry1A gene*)

Nuclear: 2 - 3%

Chloroplast: 5-20%

MacBride et al., Bio/Techn. 1995
Kota et al., PNAS, 1999
De Cosa et al., Nat Biotech, 2001

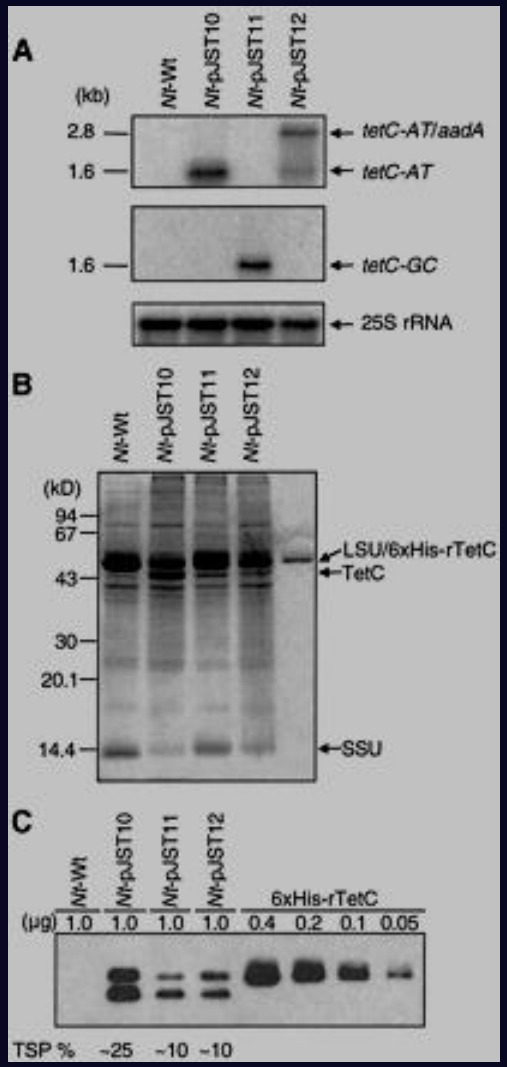
Expression of Betaine aldehyde-dehydrogenase confers saline tolerance in carrot



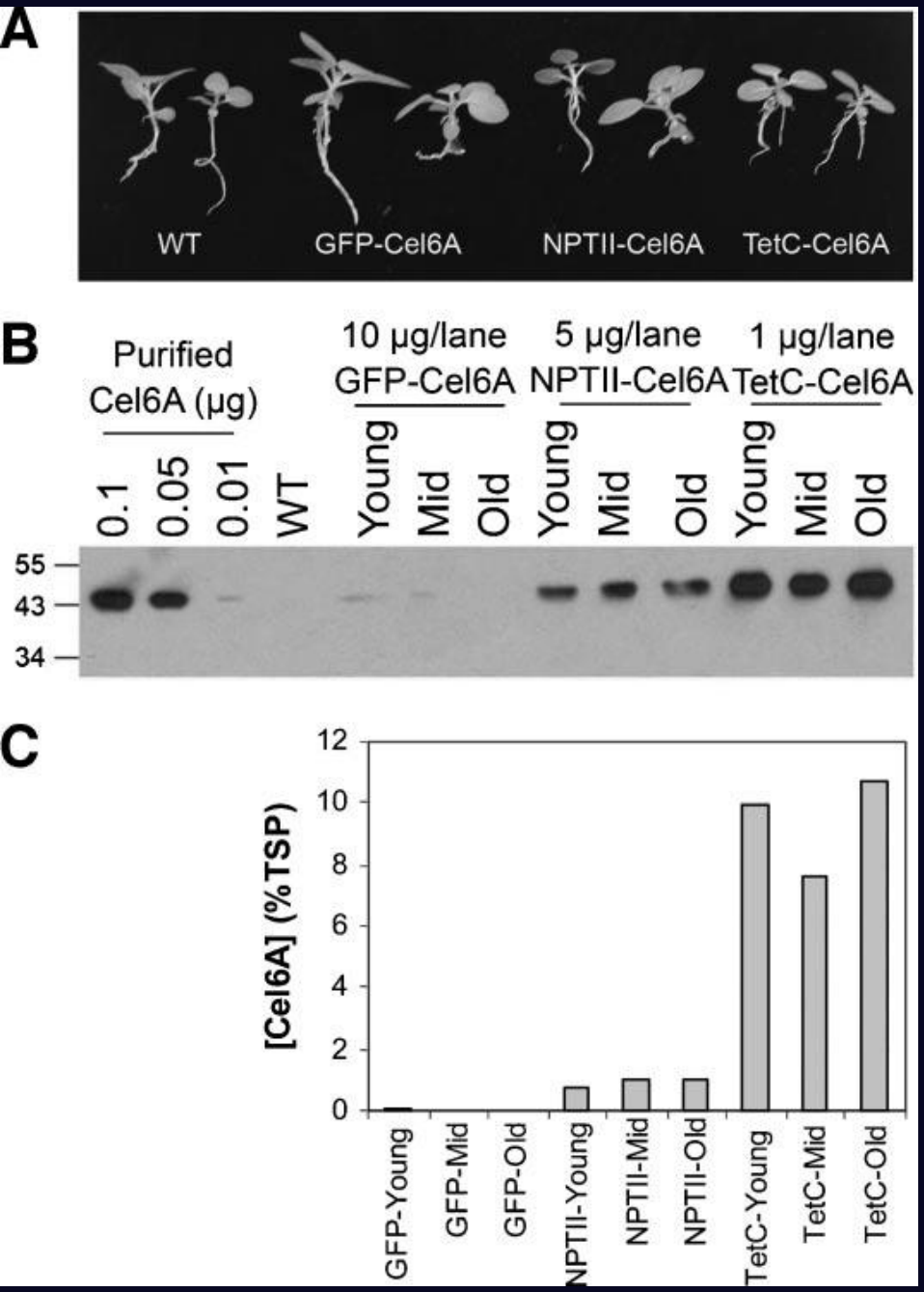
(embryogenic culture)

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

New Perspectives

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.

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Thank you!

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