Chloroplast Genomics and Genetic Engineering: Crop Improvement and Bioreactors

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Advantages of Chloroplast Transformation

- Multigene Engineering
- Maternal Inheritance
- Hyper-expression
- Gene Containment
- No Vector Sequences
- No Gene Silencing
- No Position Effect
- No Pleiotropic Effects

Foreign genes are integrated into the chloroplast genome

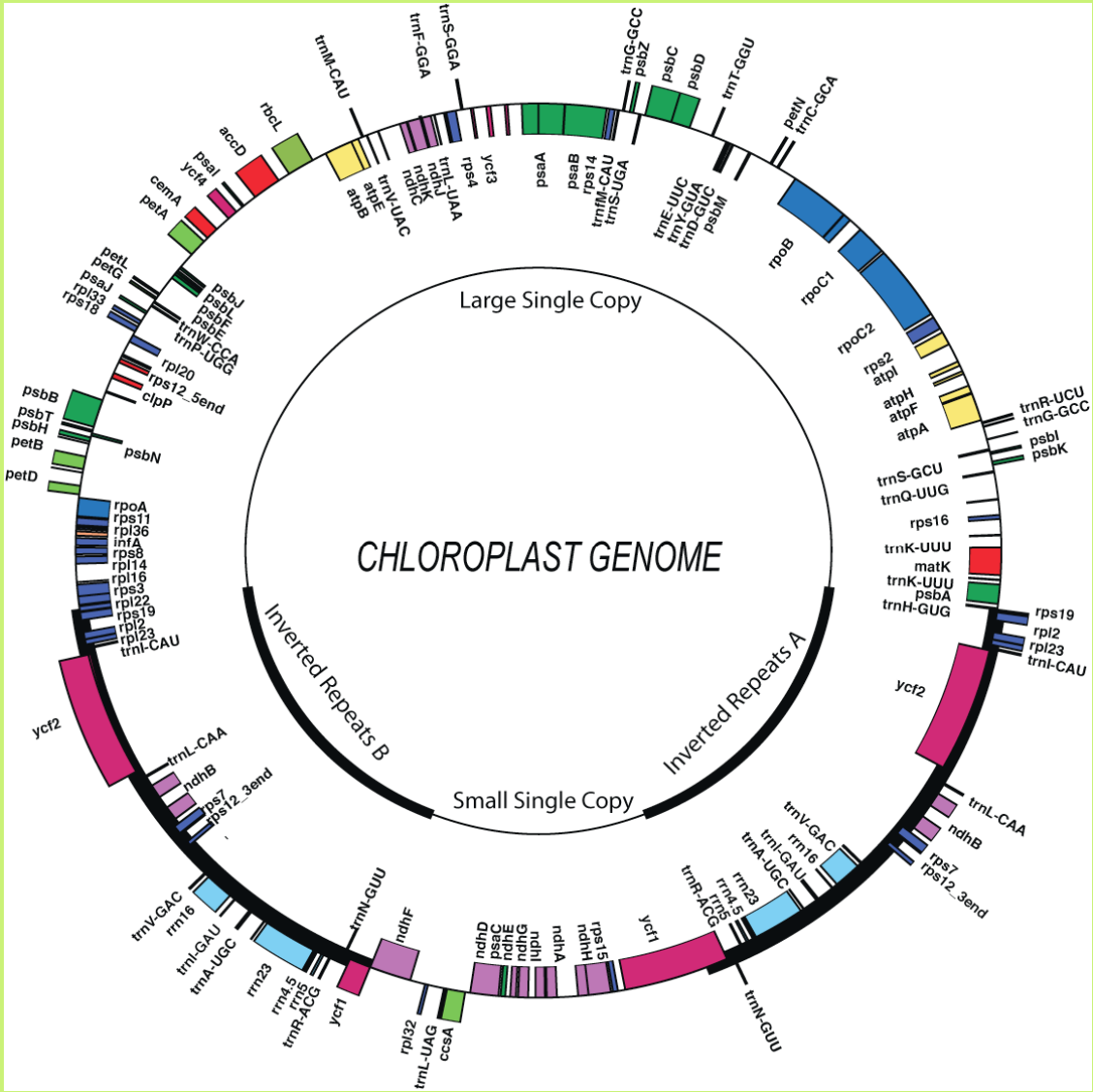
Up to 10,000 copies of foreign genes per plant cell

Up to 75% of foreign protein in the total leaf protein

Expression in leaves eliminates any reproductive structures

Efficient foreign gene Containment

Daniell lab sequenced >25 crop chloroplast Genomes; other labs have added more genomes



For optimal levels of gene expression in leaves
***trn*I-Prrn-ggagg-*aadA*-*Trps*16-P*psb*A-GOI-T*psb*A-*trn*A**

Selectable Marker

Regulatory Elements

3′ Untranslated region

trnI	Prrn	ggagg	*aadA*	*rps*16		Herbicide resistance	*trnA*
*rbc*L	P*psb*A	psbA	*nptII*	*rbc*L		Insect resistance	*acc*D
*rpl*32	Pr*bc*L	rbcL	*aphA6*	*psb*A		Drought tolerance	*trn*L
*pet*A		atpB	*badh*	*pet*D		Phytoremediation	*psb*J
3'rps7/12		Cry2a	*nptII aphA6*			Salt tolerance	*trn*V
*rrn16 trn*V		T7gene 10				Male sterility	*3'rps7/12*
*trnf*M						Biopharmaceuticals	*trn*G
*trn*N						Vaccine antigens	*trn*R
ycf3						Biomaterials	*trn*S
						Enzymes and amino acids	

Gene of interest for following traits

Homologous recombination
PEP RNA polymerase (light)
NEP RNA polymerase (dark)
5’ UTR Ribosome binding sites
3’ UTR transcript stability
No polyA tail
No transcript termination
Read through transcription
Translation of polycistrons

Steps involved in lettuce chloroplast transformation

1st round of selection

2nd round of selection

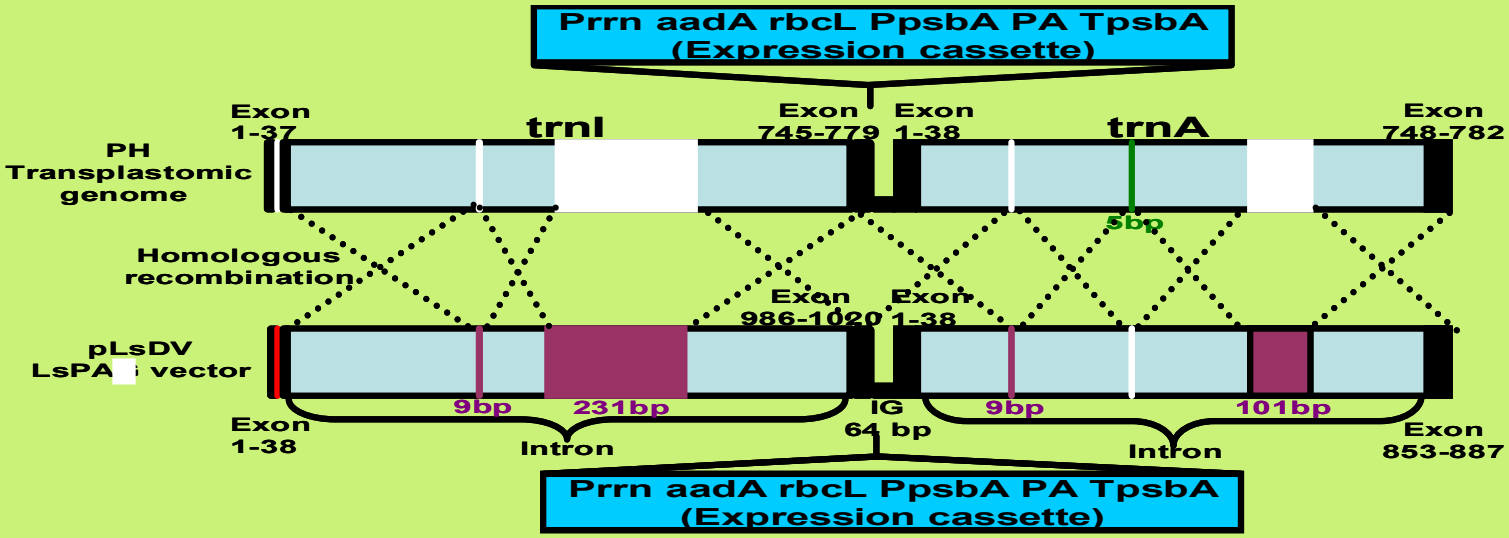
3rd round of selection and rooting

Acclimatization

transplantation

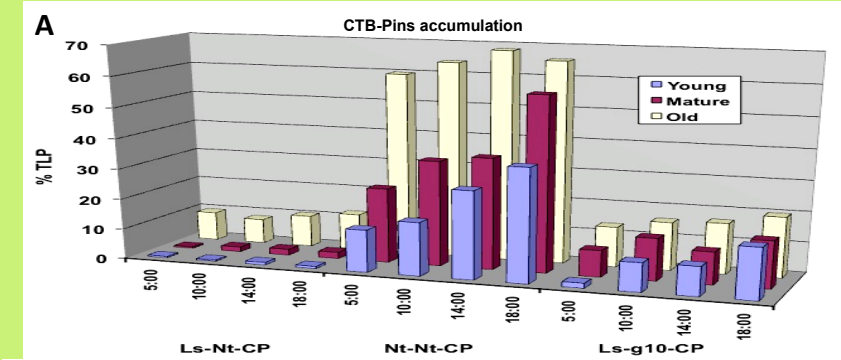
Mass propagation

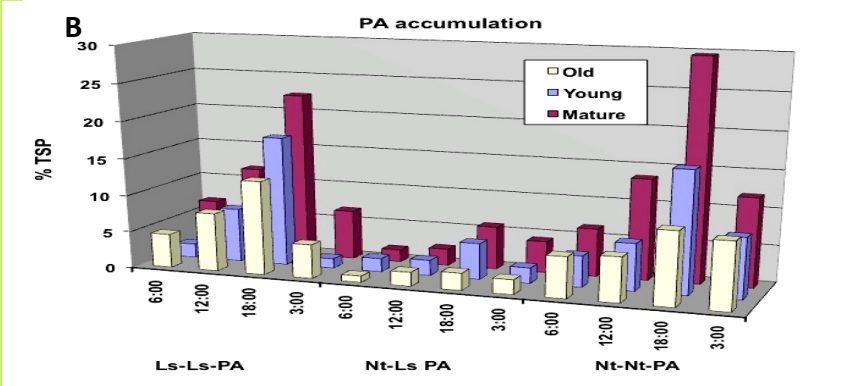
Flowering and seed setting

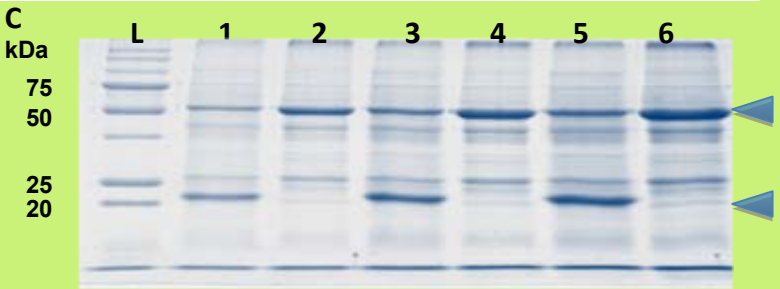


Schematic representation of homologous recombination between tobacco transplastomic genome and lettuce transformation vector. Total DNA was isolated from the *N. tabacum* Nt-Ls-PA transplastomic lines, sequenced using appropriate primers and aligned with *N. tabacum* and *L. sativa* sequences from the NCBI database. Unique *L. sativa* intron sequence of the transformation vector is indicated by purple bars. Unique *N. tabacum* intron sequence of the plastome is indicated by green bar. Blank indicates looped out sequence (s). Filled black bars indicate the exon region.

PLANT PHYSIOLOGY Ruhlman et al., April 2010





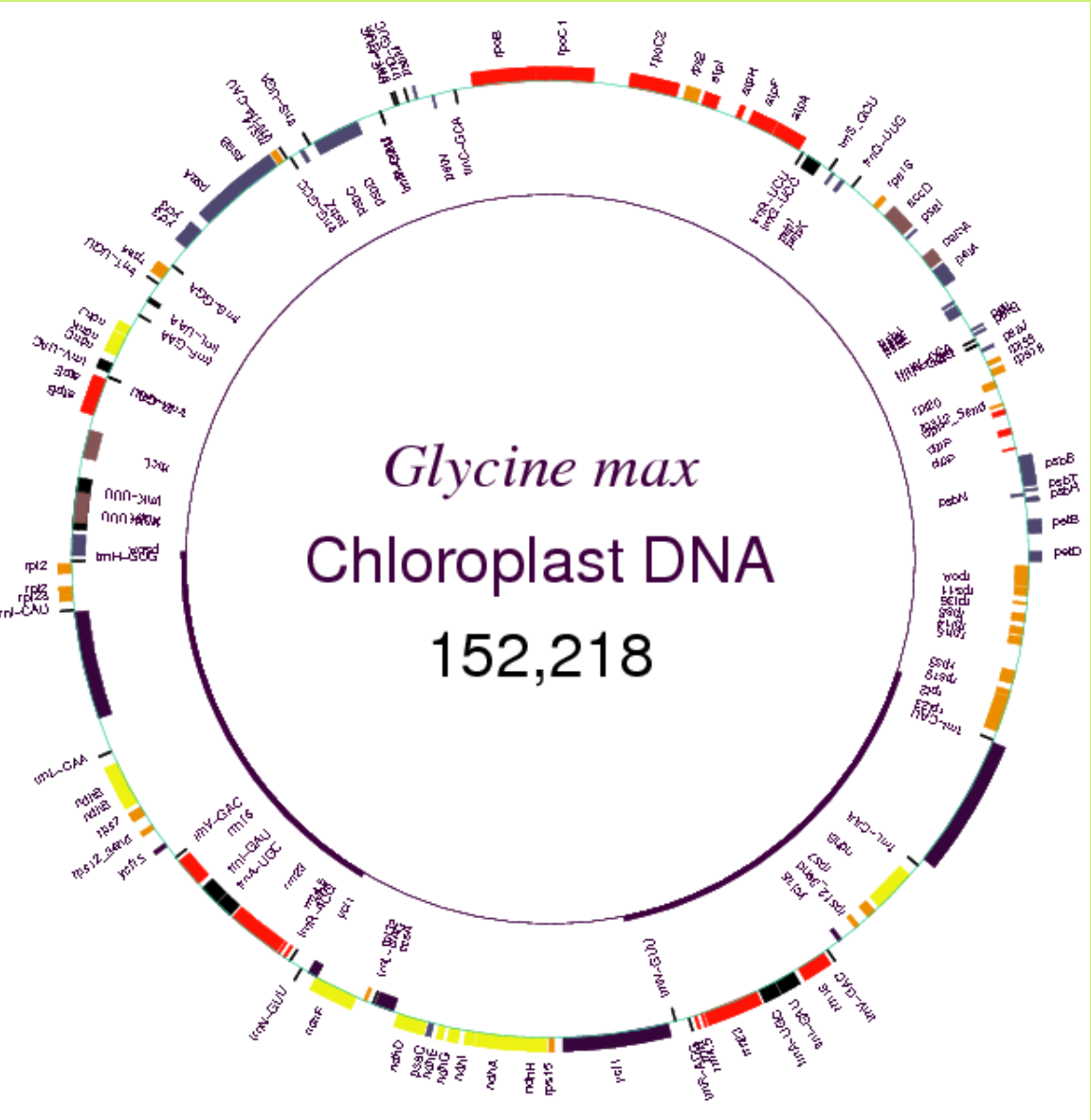


Chloroplast psbA promoter is regulated by light – compare expression at 6 AM & 6 PM

Compare expression of endogenous (LsLs or NtNt) with hetrologous (NtLs or LsNt) psbA regulatory elements

For endogenous NtNt Expression is so high that It is more than the most abundant protein RuBisCO

PLANT PHYSIOLOGY Ruhlman et al., April 2010



Between 1986-2004 Only 6 CROP cp genomes sequenced!!

In 2005/11, complete chloroplast genomes of soybean (PMB), potato & tomato (TAG), grape (BMC), barley, sorghum, Turfgrass (TAG), peach, chestnut, cocoa (MBE) were sequenced by BAC clones. Citrus (BMC), cotton (BMC), coffee (PBJ), carrot (BMC), cassava (TAG) chickpea (MPE), bamboo (TP), orchids (BMC) cp genomes were sequenced by RCA.

Chloroplast transformation by organogenesis

Cabbage	Spectinomycin; First selection – 50-200 µg mL^{-1}	Liu et al., 2007
Cauliflower	Spectinomycin; Selection - 50 µg mL^{-1}	Nugent et al., 2006
Lettuce	Spectinomycin; Selection - 50 µg mL^{-1} spectinomycin;.	Ruhlman et al., 2007
Oilseed rape	Spectinomycin; Selection - 10 µg mL^{-1} spectinomycin;	Cheng et al., 2010
Eggplant	Spectinomycin and Streptomycin; Selection - 200 µg mL^{-1} spectinomycin and 200 µg mL^{-1} streptomycin.	Singh et al., 2010
Poplar	Spectinomycin; Selection - 30 mg/l spectinomycin.	Okumura et al., 2006
Sugarbeet	Spectinomycin; Selection - 40, 300 and 400 µg mL^{-1} spectinomycin	De Marchis et al., 2009
Potato	Spectinomycin; Selection - 300 µg mL^{-1} spectinomycin, Rooting - 400 mg/l spectinomycin.	Nguyen et al., 2005
Tomato	Spectinomycin; Selection - 300 or 500 µg mL^{-1} spectinomycin	Ruf et al., 2001

Crop	Explant	Selection agent and conditions for first, second and third round of selection	Literature cited
Chloroplast transformation by embryogenesis			
Carrot	Fine cell suspension cultures derived from stem	Spectinomycin; First selection - 150 µg mL^{-1} of spectinomycin; Second selection on 350 µg mL^{-1} spectinomycin for a month; Multiplication using 500 µg mL^{-1} of spectinomycin;	Kumar et al., 2004
Cotton	Grayish-green friable callus from hypocotyls	Kanamycin; First selection - 50 µg mL^{-1} kanamycin; Second selection with 100 µg mL^{-1} kanamycin for 4-5 months. Transformed calli were converted into somatic embryos and plantlets.	Kumar et al., 2004
Rice	Calli derived from mature seeds	Streptomycin; First selection - 200 µg mL^{-1} of streptomycin; Rooting - 500 µg mL^{-1} of streptomycin.	Lee et al., 2006
Soybean	Embryogenic calli	Spectinomycin; First selection - 200 or 300 µg mL^{-1} spectinomycin Subsequent steps - 150 µg mL^{-1} of spectinomycin	Dufourmantel et al., 2004
Wheat	Immature Scutella	Kanamycin; First selection - 50 µg mL^{-1} kanamycin; Second selection with 100 µg mL^{-1} kanamycin for 4-5 months. Transformed calli were converted into somatic embryos and plantlets.	Cui et al, 2011

Expression and Characterization of Antimicrobial Peptides Retrocyclin-101 and Protegrin-1 in Chloroplasts to Control Viral and Bacterial Infections

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Plant Biotechnology Journal (2011) 9:100-115

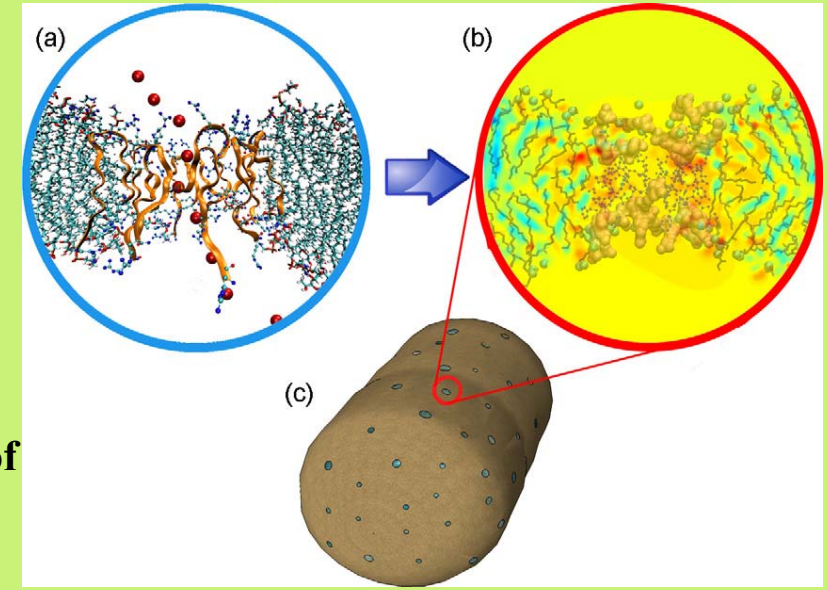
Current IF 4.9; next year IF >5.6

Retrocyclin 101

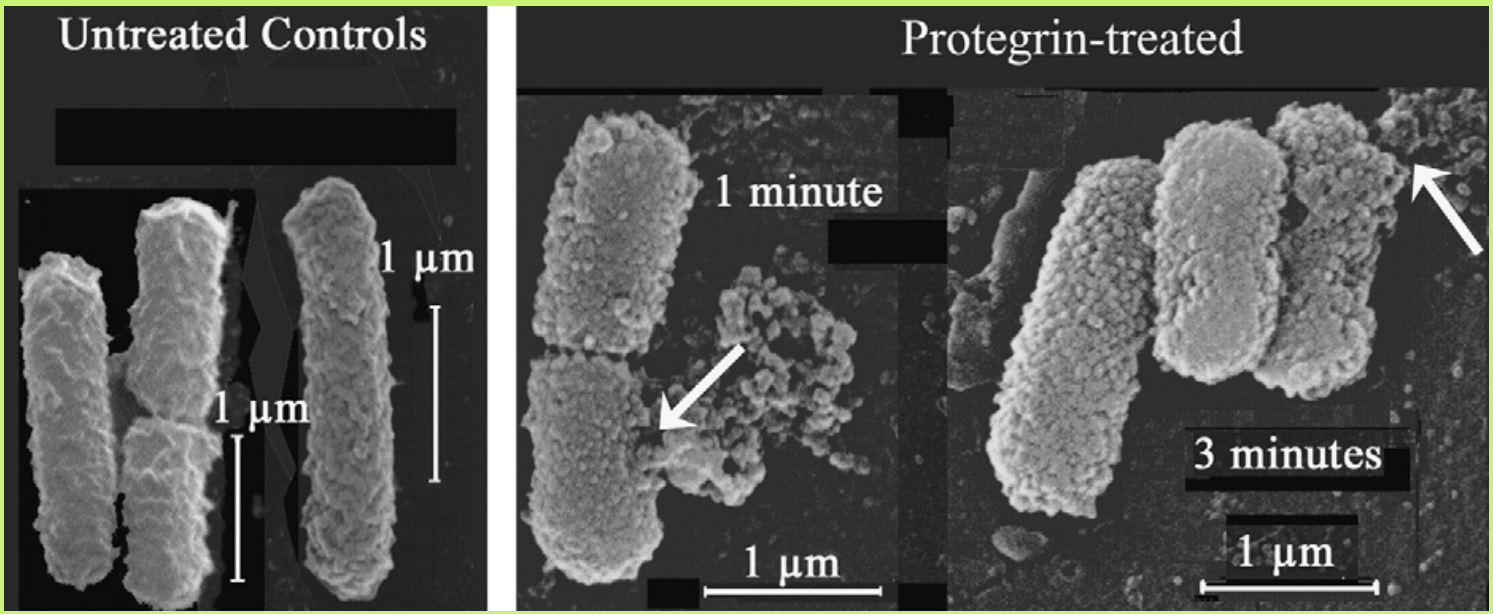
- Retrocyclin is a cyclic octadecapeptide, which is artificially synthesized based on a human pseudogene that is homologous to rhesus monkey circular minidefensins.

Retrocyclin contains six cysteines, and has largely β-sheet structure that is stabilized by three intramolecular disulfide bonds.

The S state and I state.

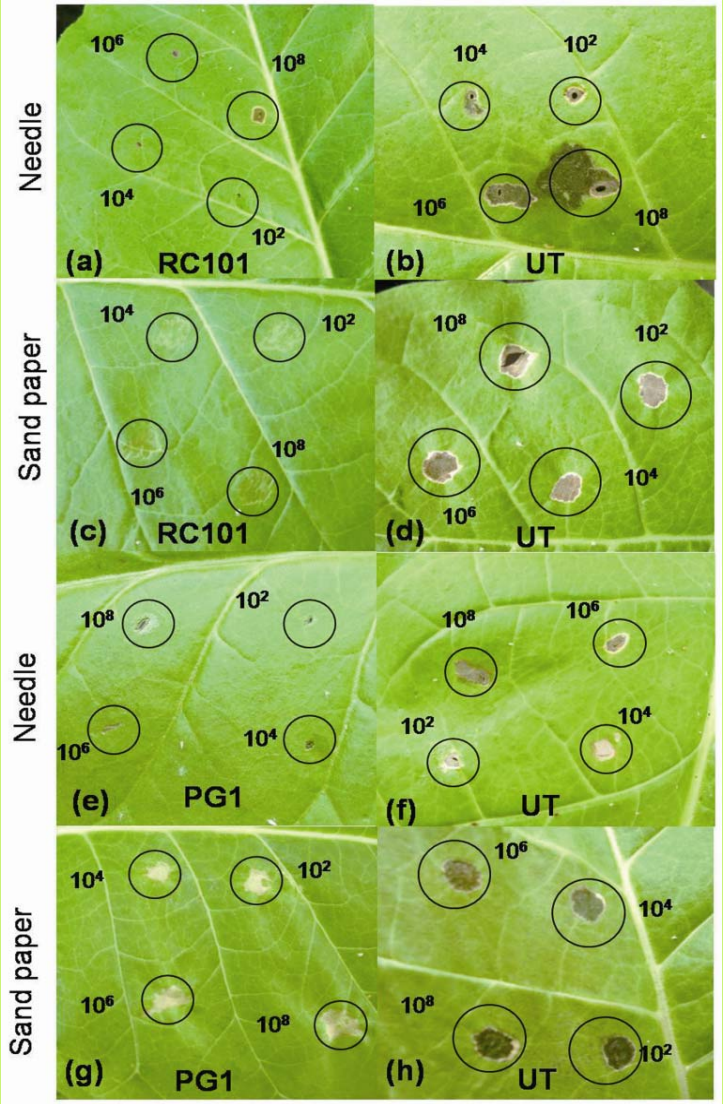


Transmission electron microscopy images of E. coli treated with 25 mg/mL of PG-1.



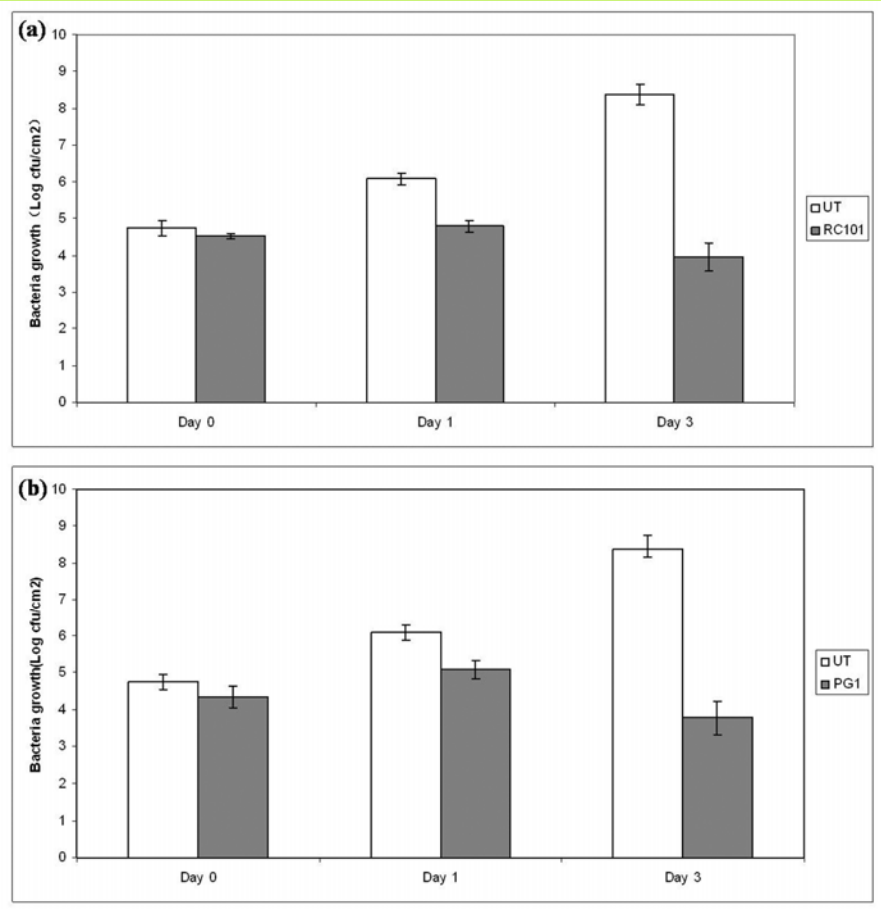
Bolintineanu, D., Hazrati, E., Davis, H. T., Lehrer, R. I. and Kaznessis, Y. N. (2010) Antimicrobial mechanism of pore-forming protegrin peptides: 100 pores to kill E. coli. Peptides **31**, 1-8.

RC101 and PG1 retained their antimicrobial activity when expressed in chloroplasts



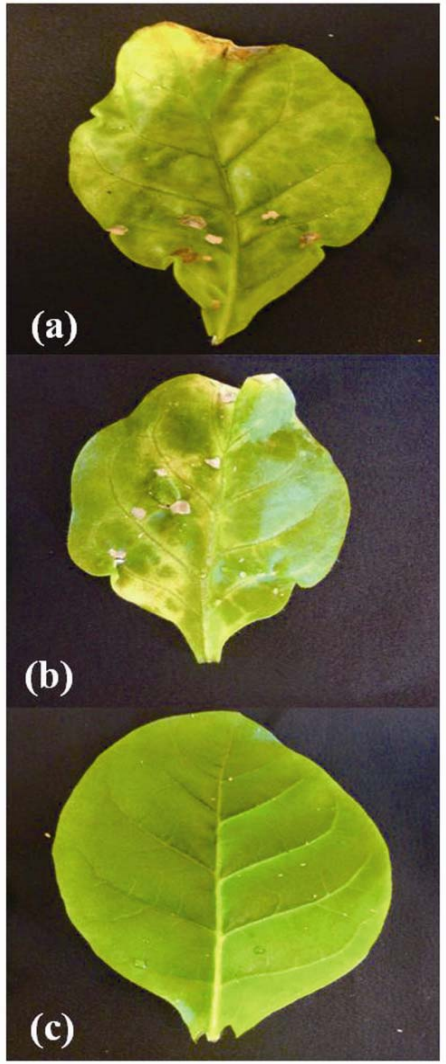
- **Figure 8.** In planta antimicrobial bioassays to evaluate functionality of RC101 and PG1 expressed in tobacco chloroplasts. Twenty microliter of the 10^8, 10^6, 10^4 and 10^2 cells from an overnight culture of *E. carotovora* were injected into leaves of RC101 (a), PG-1 transplastomic (e) and untransformed (UT) tobacco (b, f) plants using a syringe with a precision glide needle. In the parallel study, Five- to 7-mm areas of T1 transformants RC101(c), PG-1(g) and non-transformed tobacco leaves (d, h) were scraped with fine-grain sandpaper. Twenty microliter of the same dilutions of *Erwinia* was inoculated to each prepared area. Photos were taken 5 days after inoculation.

RC101 and PG1 retained their antimicrobial activity when expressed in chloroplasts



- **Figure 9.** Bacterial population in the PG1, RC101 and untransformed (UT) tobacco plants inoculated with *E. carotovora*.
- (A) Bacterial population in RC101 and untransformed plants.
- (B) Bacterial population in PG1 and untransformed plants.
- Bacterial suspensions (1.0×10^5 cfu/ml) of *E. carotovora* were infiltrated into the leaf of transplastomic and untransformed tobacco, respectively. The bacterial population in the inoculated plant was detected on 0, 1 and 3 days after inoculation. All values represent means of 6 replications with standard deviations shown as error bars.

RC101 and PG1 retained their antimicrobial activity when expressed in chloroplasts



- **Figure 10.** Response of untransformed and RC101/ PG1 transplastomic plants to TMV.
- (**a**) TMV inoculated leaf from untransformed plant;
- (**b**) TMV inoculated leaf from transplastomic PG1 plant.
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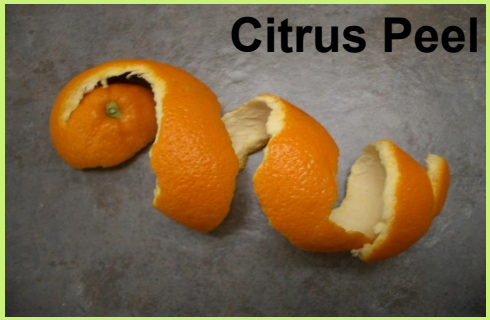
- (**c**) TMV inoculated leaf from transplastomic RC101 plant. Pictures were taken on 20 days after inoculation.

Ethanol From Agricultural Products



Tobacco producing: Required cellusases, hemicellulases, pectinases, etc.

Pretreatment

Processed Material

There is no need to purify enzyme

Fermentable Sugars

Fermentation

Distillation

Ethanol

Cost of biomass degrading enzymes

Cellulase-$186 (2000U)

Glucosidase-$282 (200U)

Mannanase-$177(500U)

Xylanase-$180 (2000U)

Pectate lyase-$177 (600U)

Chloroplast-derived Enzyme Cocktails Hydrolyse Lignocellulosic Biomass and Release Fermentable Sugars

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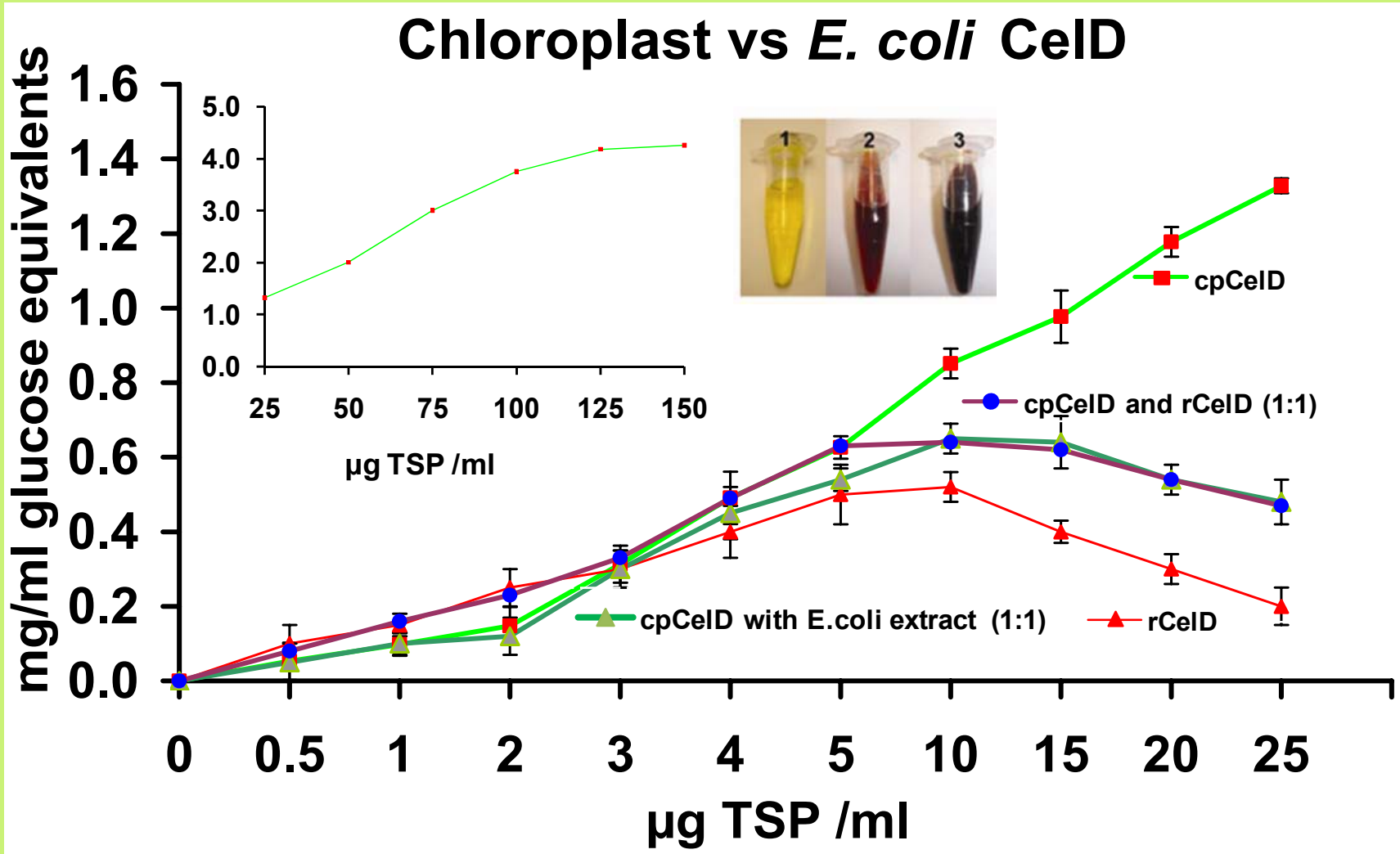
Plant Biotechnology Journal, March 2010
8: 332-350

First Enzyme cocktails produced in plants

Enzyme activity of cell wall degrading enzymes expressed in *E. coli* and chloroplast

Enzyme	pH	ºC	Substrate	Enzyme activity (units/mg) in crude total soluble protein		Activity of transplastomic over *E.coli* (fold)
				E.coli	Transplastomic	
CelD	5.2	60 ºC	CMC (2%)	349 ±36	493 (±21)	1.41
EG1	5.2	50 ºC	CMC (2%)	28 ±7	339 (±12)	12.10
CelO	5.2	60 ºC	β-D-glucan (1%)	18 ±2	442 (±19)	24.55
Bgl1	5.2	50 ºC	p-nitrophenyl-β-D-glucopyranoside (4mM)	2 ±0.02	14 (±2)	7.0
Xyn2	5.2	50 ºC	Oat spelt xylan (1%)	89 ±3	421 ±9	4.73
PelB	6 & 8	40 ºC	Polygactouronic acid (0.25%)	2.17 ±0.2	2.42 ±0.1	1.12
PelD	6 & 8	40 ºC	Polygactouronic acid (0.25%)	2.09 ±0.3	2.31 ±0.4	1.10
PelA	6 & 8	40 ºC	Polygactouronic acid (0.25%)	2.50 ±0.5	2.81 ±0.9	1.12
Cutinase	8.0	30 ºC	p-nitrophenyl butyrate (0.03%)	24 ±4	15 ±4	<0.625
SwoI	Swelling of cotton fiber was observed with *E. coli* and chloroplast-derived crude extract as described earlier (Saloheimo *et al.*, 2002).					
Axe1	Color change with *E. coli* enzyme extract was observed using 1 mM α-naphthyl acetate as described earlier (Poutanen and Sundberg, 1998)					

Enzyme assays were done in triplicates and standard errors were calculated. Untransformed tobacco leaves and *E.coli* did not show detectable level of hydrolysis of any of the above substrates



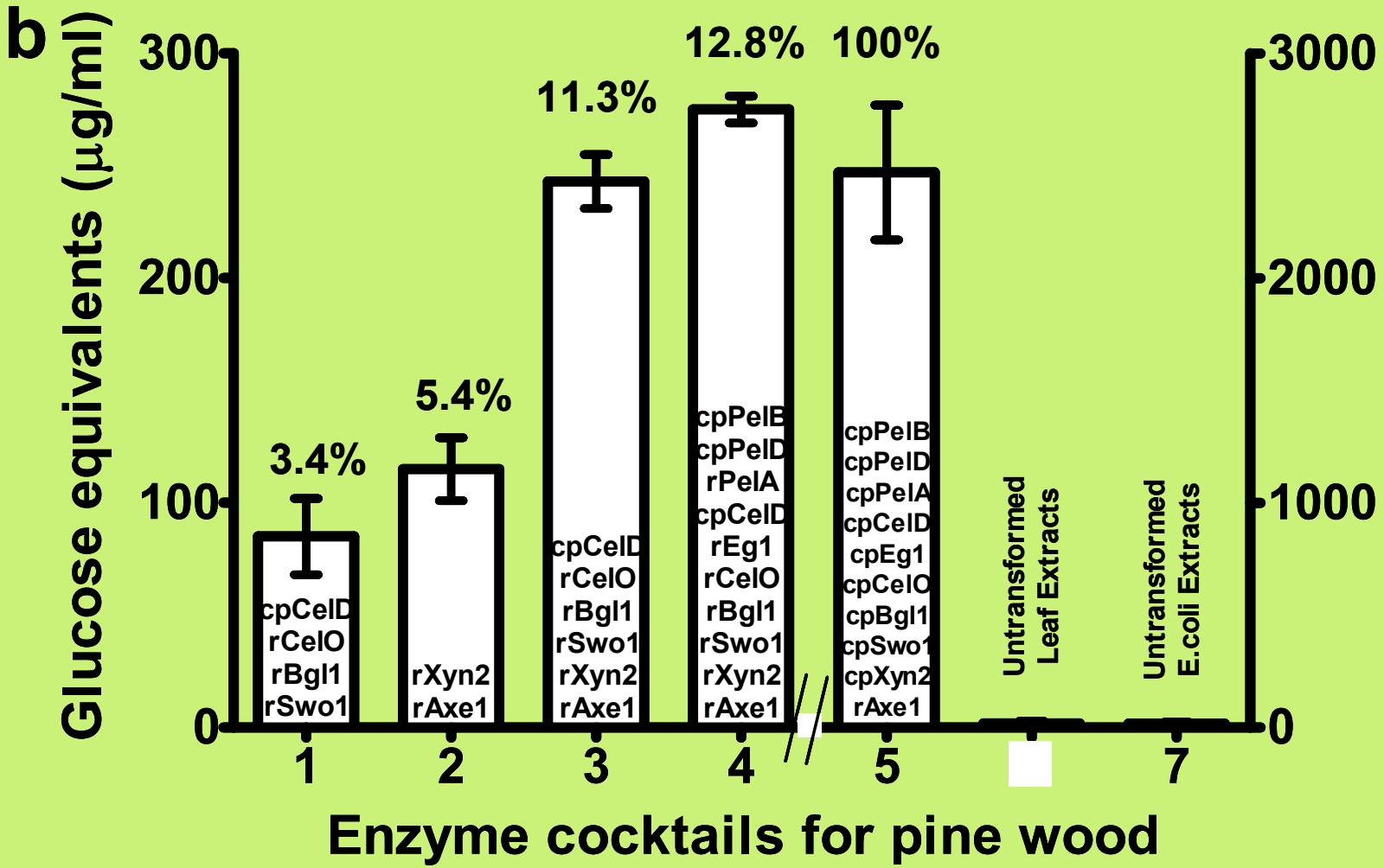
Enzyme kinetics of cpCelD and rCelD using carboxymethyl cellulose (2%) substrate. The reaction mixture contained increasing concentration of cpCelD and rCelD TSP (µg/ml). Enzyme hydrolysis was carried out for 30 minutes at 60ºC. Figure inset shows enzyme kinetics saturation point for cpCelD TSP amount (µg/ml) towards CMC (2%). Eppendorf tubes with reaction mixture shown in inset represents, 1 untransformed plant, 2 and 3 rCelD and cpCelD 10µg TSP.

Enzyme yield in transplastomic tobacco plants

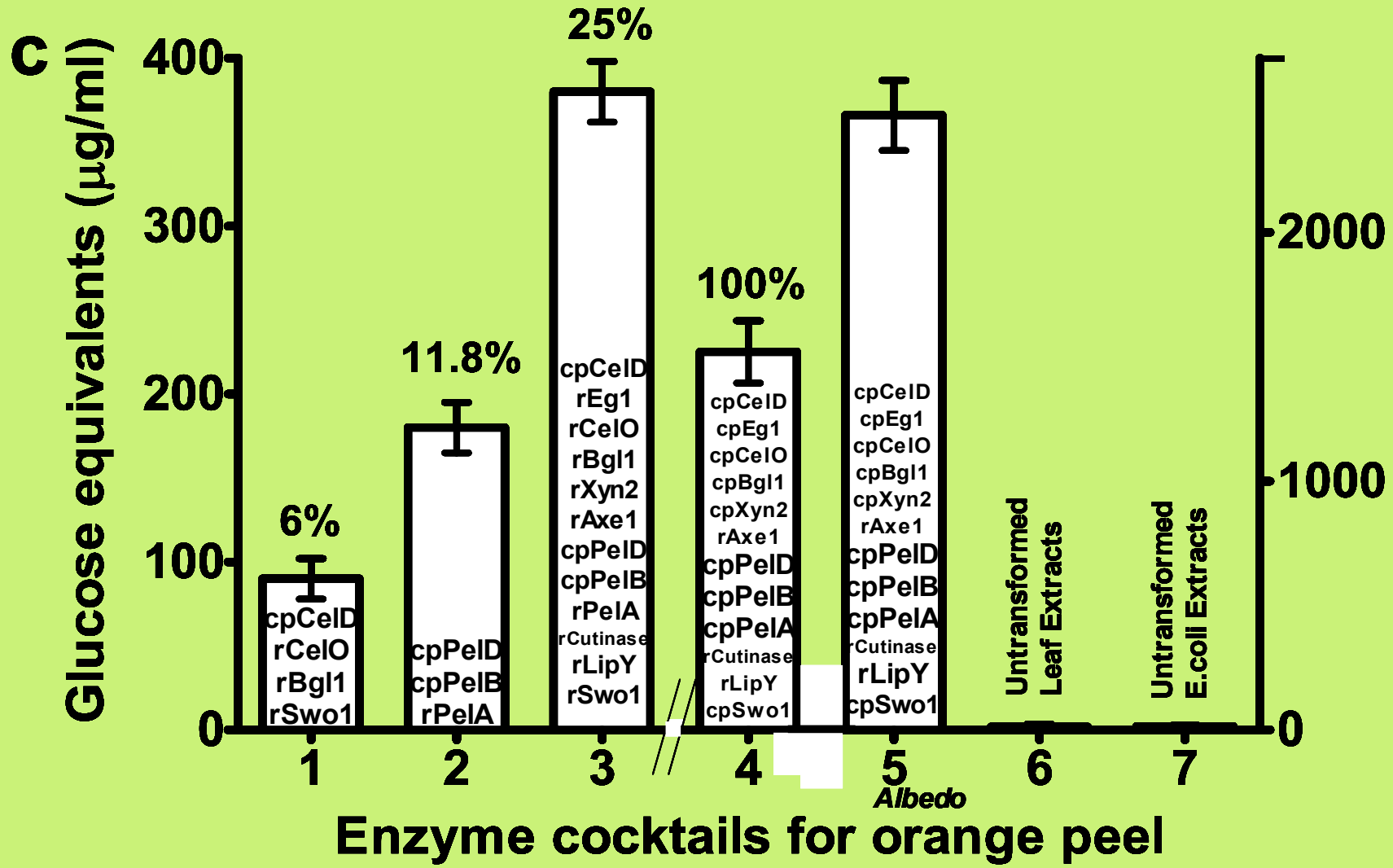
Enzyme	Leaf age	No of leaves/ plant	Avg. Wt (g)/leaf	Units/ g in fresh leaf	Units		Whole plant yield	Units(millions)/ acre/ cutting	Units(millions)/ acre/year
					Per leaf	Per age group			
PelB	Young	3.5	2.5	20.82	52.05	182.18	8.89 %	16.39	49.17
	Mature	8.2	8.0	25.56	204.48	1,676.74	81.84%		
	Old	4.5	5.6	7.54	42.22	190.00	9.27%		
PelD	Young	3.5	2.5	26.65	66.63	233.19	8.70%	21.43	64.3
	Mature	8.2	8.0	32.44	259.52	2,128.06	79.43%		
	Old	4.5	5.6	12.62	70.67	318.02	11.87%		
CelD	Young	3.5	2.5	3,000	7,500	26,250	5.86%	3,583.50	10751
	Mature	8.2	8.0	4,930	39,440	323,408	72.20%		
	Old	4.5	5.6	3,900	21,840	98,280	21.94%		

One unit of PelB and PelD enzyme is defined as the amount of enzyme which forms 1 µmol of unsaturated soluble oligogalacturonates per min with a molar extinction coefficient of 4,600 μmol^{-1} cm^{-1} in a 2.5 ml reaction containing 2.5 mg/ml polygalacturonic acid.

One unit of CelD enzyme is defined as the amount of enzyme that released 1 µmole glucose equivalents per minute in a 1ml reaction containing 2%CMC.



Enzyme cocktail activity on hydrolysis of pine wood sample (200 mg/5 ml reaction).



Enzyme cocktail activity on hydrolysis of Valencia orange peel and albedo portion (200 mg/5 ml reaction).

Release of Hormones From Conjugates by β-glucosidase: A Novel Mechanism to Double Biomass and Confer Protection Against Insects

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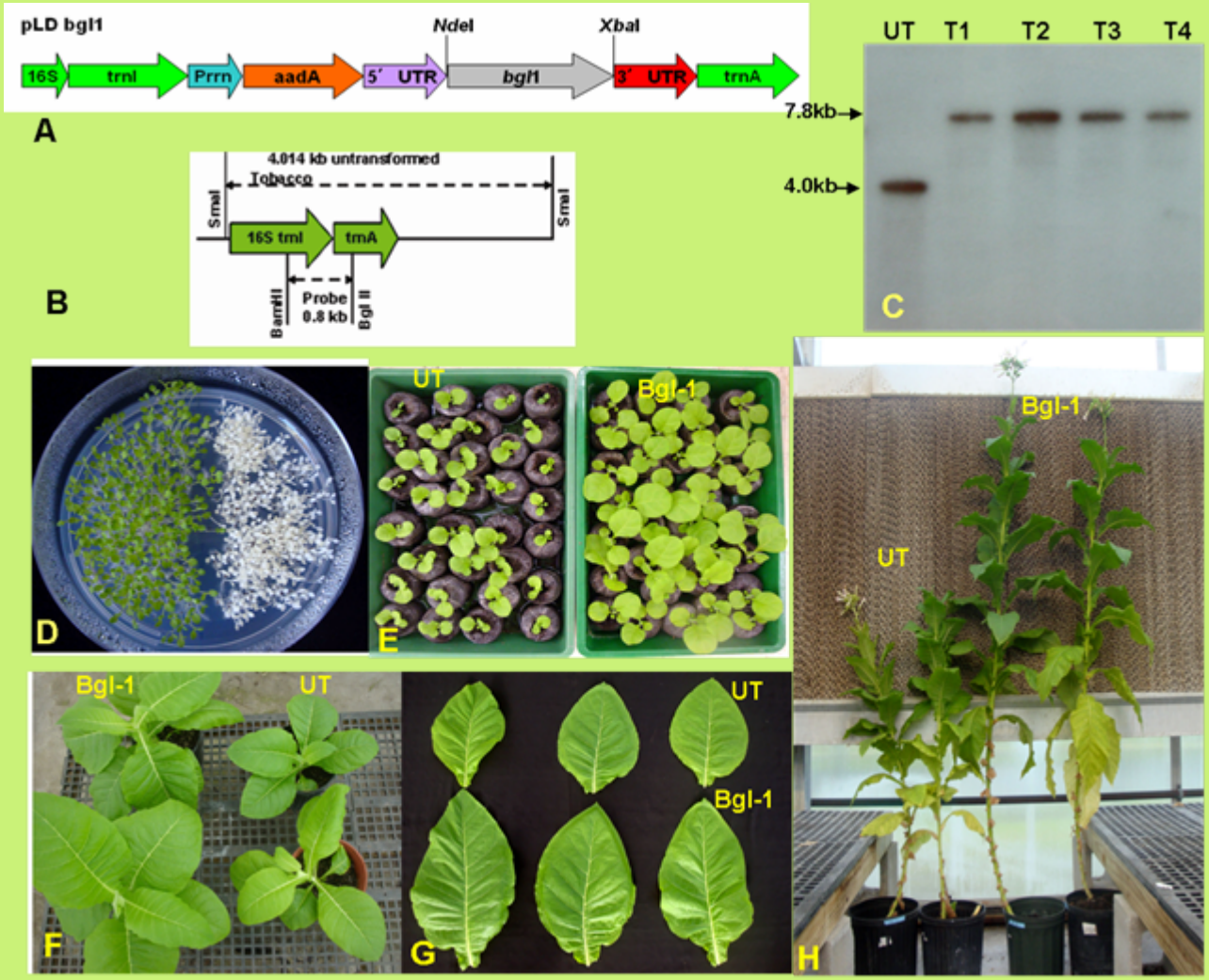


Figure 1. Evaluation of homoplasmy, maternal inheritance and β-glucosidase activity and phenotype of transplastomic (Bgl-1) and untransformed (UT) plants. (A) Schematic representation of the chloroplast transformation vectors. *bgl*1, β-glucosidase coding sequence; Prrn, rRNA operon promoter; *aad*A, aminoglycoside 3′-adenylytransferase gene; 5′ UTR, promoter and 5′ untranslated region of the *psb*A gene; 3′ UTR, 3′ untranslated region of the *psb*A gene. (B) Schematic representation of the chloroplast 16S *trn*I/*trn*A spacer region into which transgenes were inserted. (C) DNA gel blot hybridized with the flanking sequence probe showing homoplasmy of T1 to T4 independent transplastomic lines. (D) Untransformed and transplastomic seeds germinated on half-MSO medium containing spectinomycin (500 mg/L) showing absence of Mendelian segregation (E) Three weeks after seed germination. UT (left), Bgl-1(right). (F) Two-month old transplastomic (left) and UT (right) plants (G) Leaves of transplastomic (below) and UT (upper) (H) Mature (4-month old) transplastomic (right) and UT (left) plants

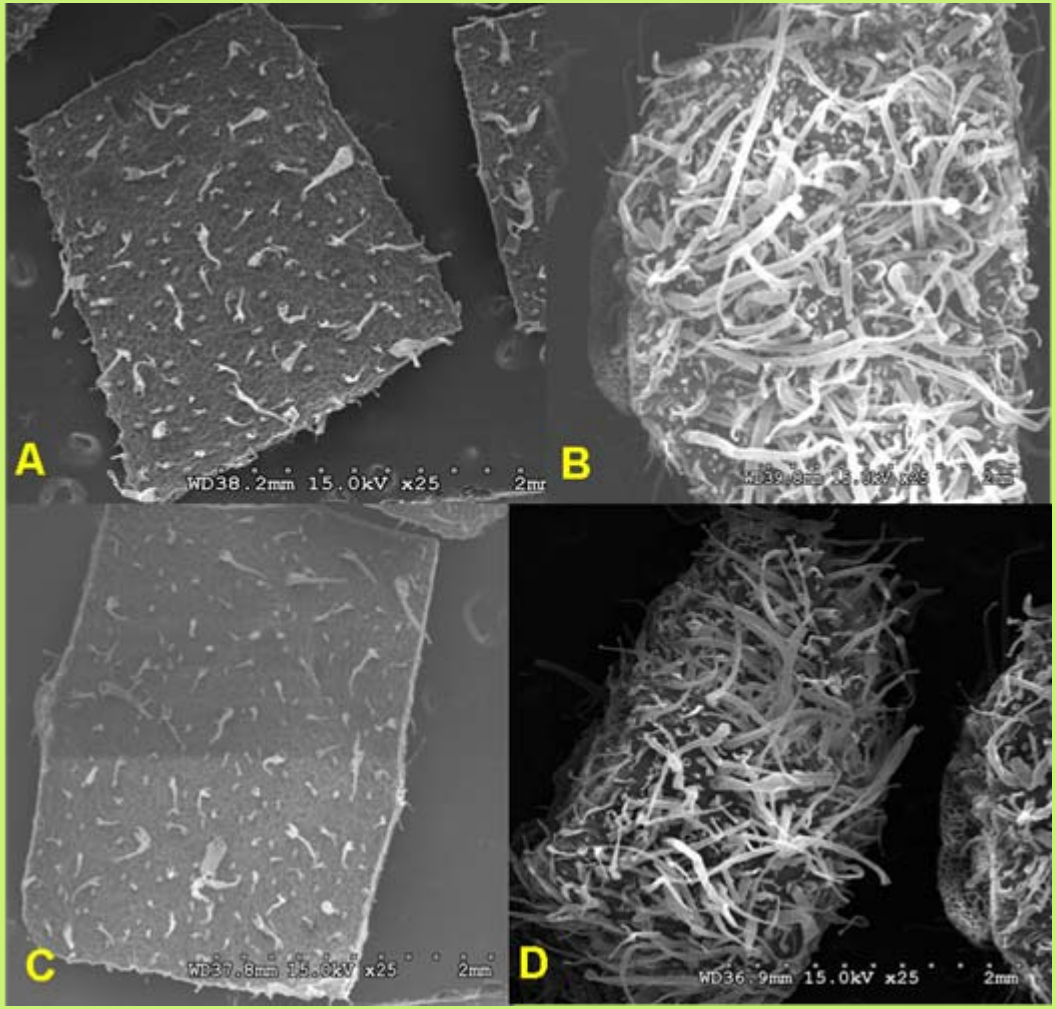


Figure 2. Evaluation of leaf surface by scanning electron microscopy. (A) Trichomes on leaf upper surface of (untransformed) UT; (B) Trichomes on leaf upper surface of Bgl-1 plants; (C) Trichomes on leaf lower surface of UT; (D) Trichomes on leaf lower surface of Bgl-1 plants

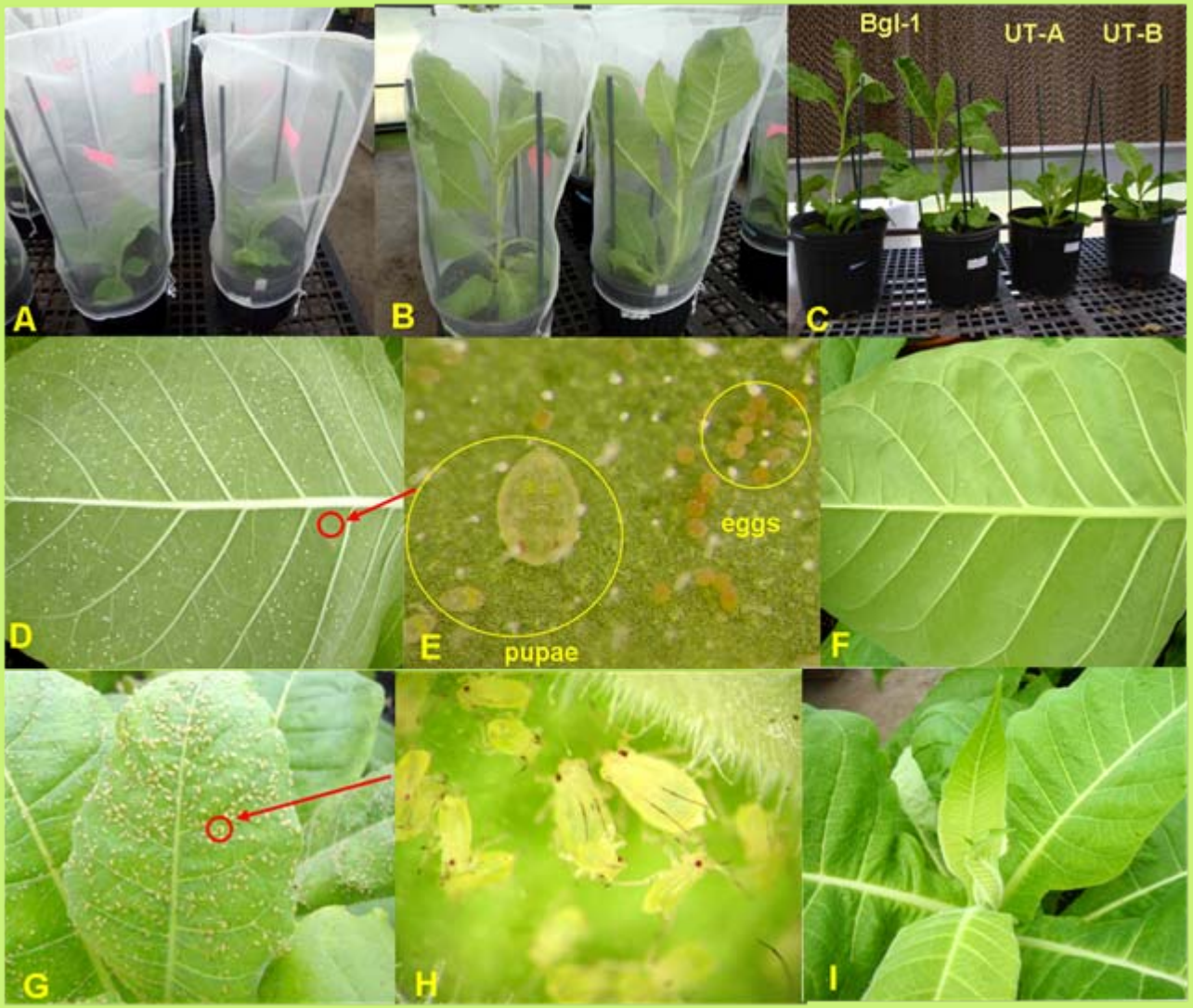
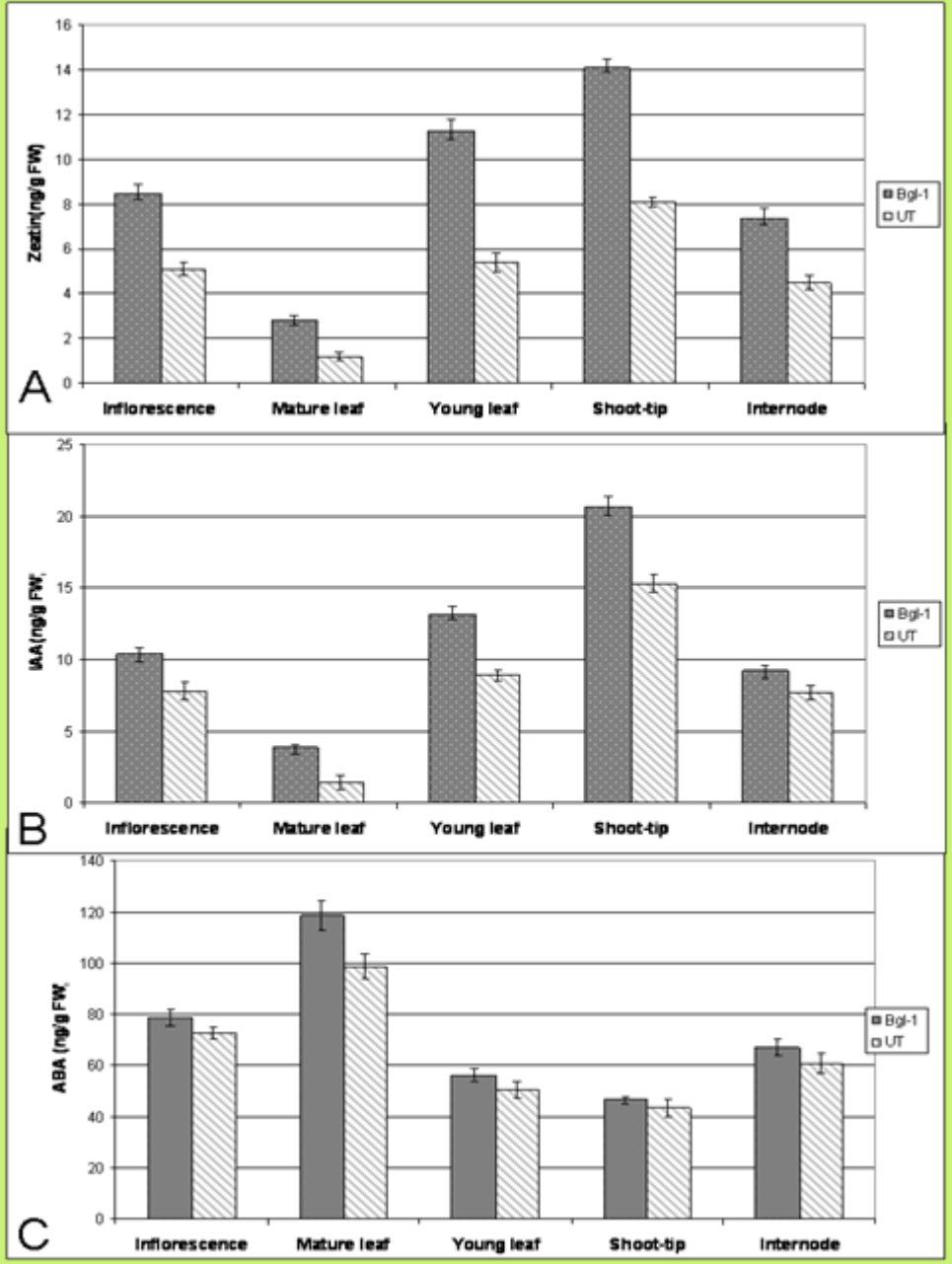


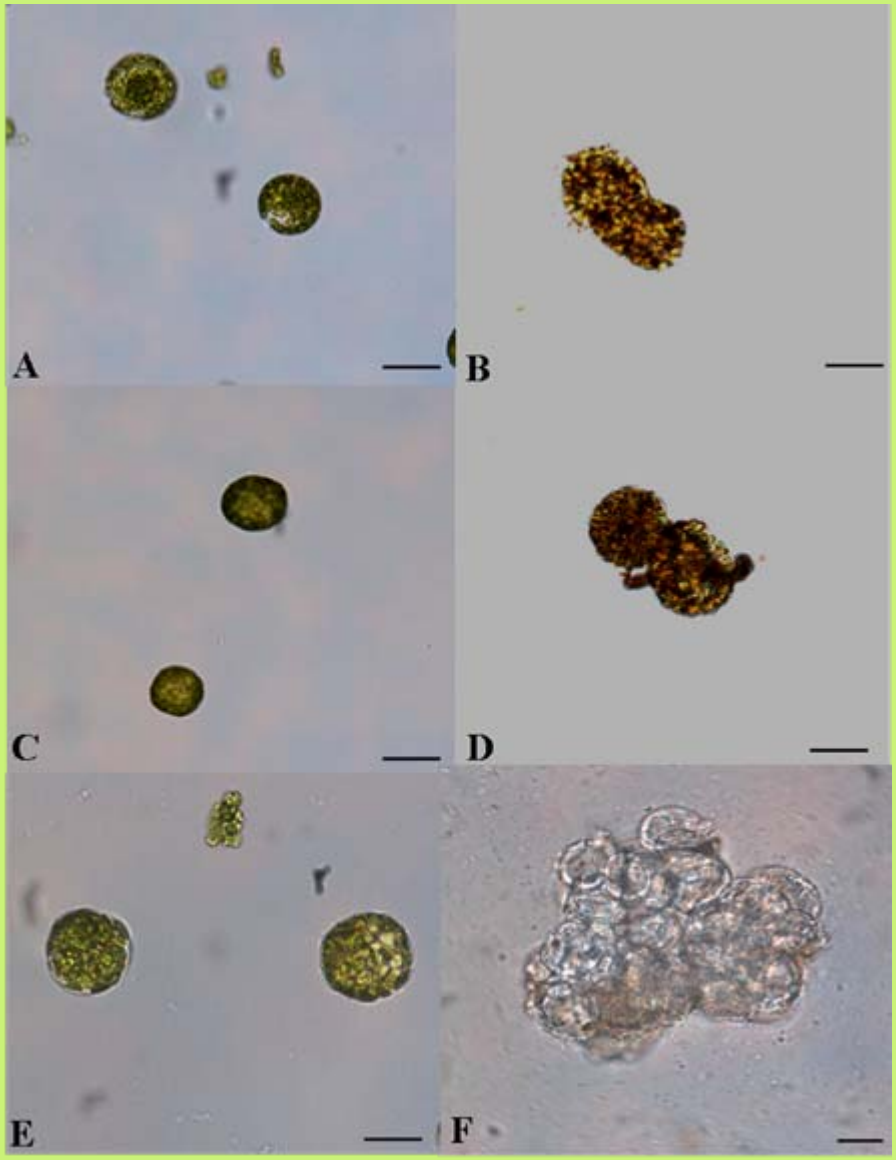
Figure 6. Aphid and whitefly bioassays on Bgl-1 and Untransformed (UT) plants. (A) Mesh-bag cage placed on each pot (40-day-old, 6-7 leaf stage) on day 0 for insect bioassays; (B) Plants 25 days after insect bioassays; (C) Release of plants from cage 25 days after insect bioassays; (D) and (E) UT plant heavily colonized with mature and immature whiteflies. Figure E is the enlarged view of figure D. (F) Bgl-1 tranplastomic plants with negligible colonization of whiteflies; (G) and (H) UT plant heavily colonized with mature and immature aphids. Figure H is the enlarged view of Figure G; (I) Bgl-1 tranplastomic plants with negligible aphids

Release of GA from conjugates

	GA-levels (ng/g DW) of Untransformed(UT) plants				
	Mature leaf	Inflorescence	Shoot-tip	Internodes	Young leaf
GA_4	0.3	0.2	0.3	0.5	1.8
GA_{34}	0.0	0.0	0.0	0.0	0.0
GA_{53}	0.2	2.7	0.9	8.6	0.1
GA_{44}	0.0	0.0	0.5	1.3	0.0
GA_{19}	6.3	9.9	14.4	26.8	10.5
GA_{20}	3.5	0.7	3.8	1.5	13.4
GA_1	3.7	1.1	2.4	0.9	10.2
GA_8	1.4	0.7	2.6	0.9	0.8
$GA_{4/34}$	0.3	0.2	0.3	0.5	1.8
$GA_{53/44/19/20}$	10.1	13.3	19.6	38.3	23.9
GA_1/GA_8	5.1	1.8	5.0	1.8	11.0
	Average GA-levels (ng/g DW) ±SD				
$GA_{4/34}$	0.4±0.1	0.4±0.1	0.4 ±0.1	1.0±0.4	2.4 ±0.6
$GA_{53/44/19/20}$	8.5±1.6	15.9±2.6	21.4±1.7	28.4±10.0	32.6±8.7
GA_1/GA_8	4.8±0.2	1.9±0.1	4.0±1.0	2.1±0.3	10.8±0.1
	GA-levels (ng/g DW) of transplamtomic Bgl-1 line				
	Mature leaf	Inflorescence	Shoot-tip	Internodes	Young leaf
GA_4	0.4	0.5	0.5	0.1	6.9
GA_{34}	0.0	0.0	0.0	0.0	0.3
GA_{53}	0.2	2.4	0,8	1.4	0.4
GA_{44}	0.1	0.4	0.6	1.3	0.3
GA_{19}	10.4	10.2	21.5	13.8	24.3
GA_{20}	9.7	0.6	8.2	7.2	42.3
GA_1	7.5	0.4	1.5	0.6	15.1
GA_8	2.7	0.2	0.9	0.8	0.7
$GA_{4/34}$	0.5	0.5	0.5	0.1	7.2
$GA_{53/44/19/20}$	20.4	13.6	31.1	23.7	67.3
GA_1/GA_8	10.2	0.5	2.4	1.4	15.8
	Average GA-levels (ng/gDW) ±SD				
$GA_{4/34}$	0.7±0.2	0.8±0.3	0.7±0.2	0.2±0.1	6.8±0.4
$GA_{53/44/19/20}$	18.5±1.9	15.2±1.6	30.7±0.4	39.5±15.8	61.2±6.1
GA_1/GA_8	9.6±0.6	1.0±0.4	2.3±0.1	1.1±0.3	16.3±0.5



Endogenous ABA, IAA and trans-Zeatin concentration in Bgl-1 and untransformed plants. (A) Trans- Zeatin concentration of Bgl-1 and Untransformed control; (B) IAA concentration of Bgl-1 and Untransformed control; (C) ABA concentration of Bgl-1 and Untransformed control. ABA, IAA and Trans- Zeatin concentrations were calculated as ng per g FW (Fresh weight). Each measurement was replicated 3–4 times using different pooled samples and the phytodetect competitive ELISA kit.



Protoplasts and protoplast-derived cells and cell colonies. (A) Protoplasts from untransformed leaf could not divide in the medium without hormones(Bar = 60μm); (B) First cell division of Bgl-1 sample without hormones; (C) Protoplasts from Untransformed leaf could not divide in the medium with Zeatin-O- glucoside; (D) First cell division of Bgl-1 sample in the medium with Zeatin-O-glucoside; (E) Protoplasts from Untransformed leaf could not form calli in the medium without hormones; (F) Protoplast-derived calli of Bgl-1 sample in the medium without hormones

Pinellia ternata agglutinin hyper-expression in chloroplasts confers broad spectrum resistance against aphid, whitefly, l*epidopteran* insects, bacterial, fungal and viral pathogens

Shuanxia Jin, Xianlong Zhang and Henry Daniell

Department of Molecular Biology and Microbiology, College of Medicine
University of Central Florida, Orlando, Florida

Chinese edible medicinal herb (crow dipper) produce antimicrobial peptides that control insects, bacterial, fungal and viral pathogens

Naturally grown in the wild and distributed throughout Asia

Single protein confers protection against multiple biotic stress

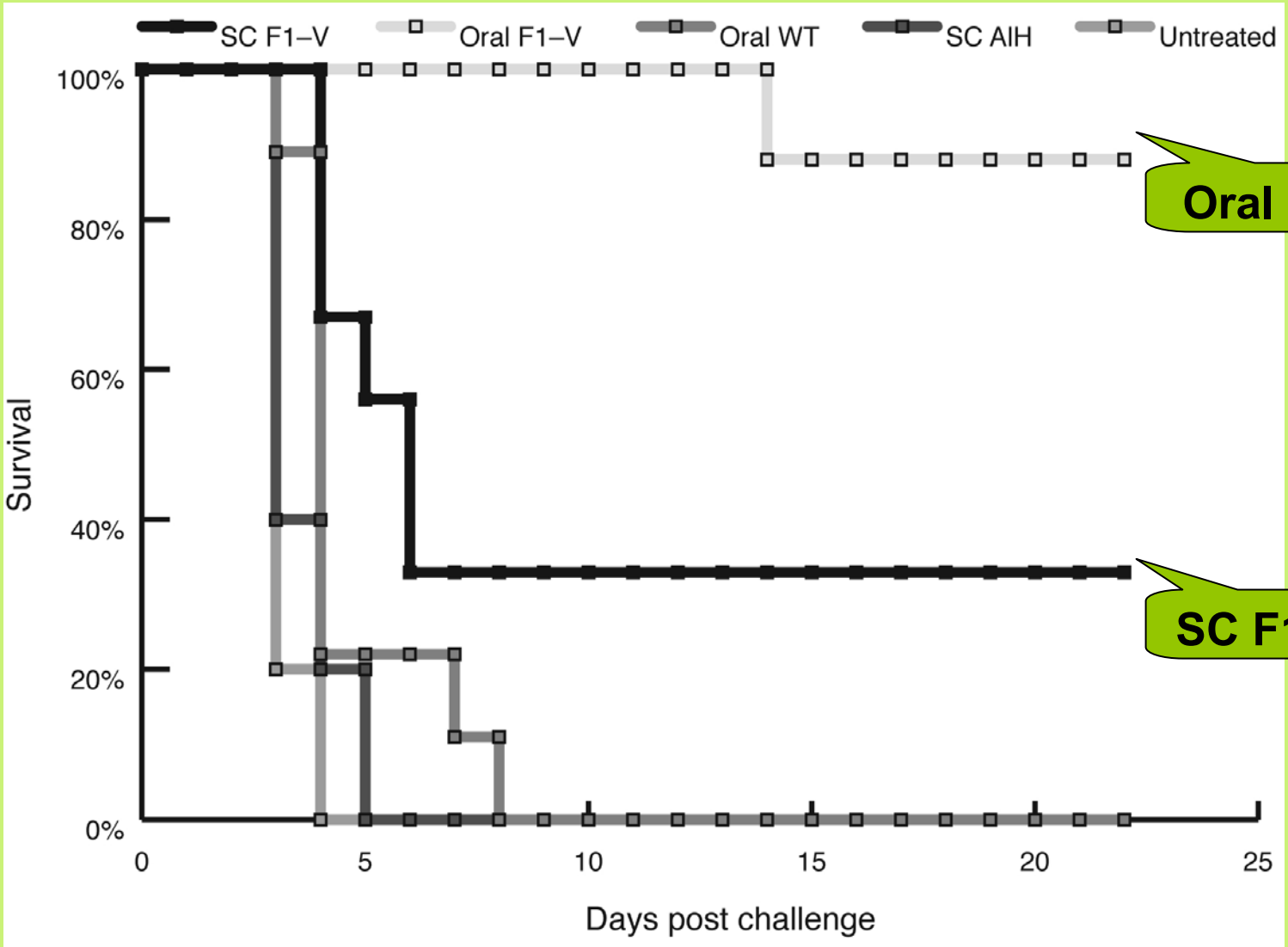
Oral Plague Vaccine is more effective than Injectable Vaccine

Philip A. Arlen, Mike Singleton, Jeffrey Adamovicz, Yi Ding and Henry Daniell

**Department of Molecular Biology and Microbiology,
College of Medicine
University of Central Florida,
Orlando, Florida
USAMRIID**

**Infection & Immunity, August 2008
76: 3640-3650**

Mice receiving oral boosts of chloroplast-derived F1–V survived better than those receiving injections



Dual Oral Vaccine for Cholera and Malaria

A. Davoodi-Semiromi, D. Singh, N. Samson, D. Verma, M. Shreiber, H. Daniell

University of Central Florida, College of Medicine

Plant Biotechnology Journal, February 2010, 8: 223-242

Oral administration protects against development of insulitis (Diabetes) in non-obese diabetic mice

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Plant Biotechnology Journal 5: 511-525

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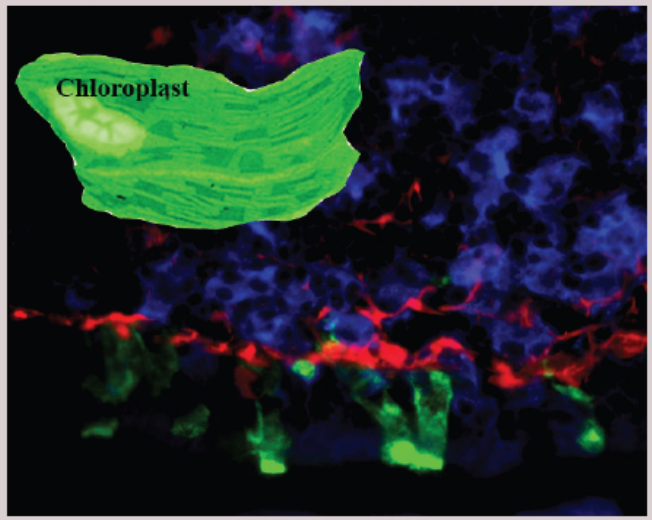
In This Issue

Chloroplast delivery may prevent allergic response

Chloroplasts are plant organelles that sustain life on earth by producing food and oxygen, and could be used as bioreactors to orally deliver therapeutic proteins, according to a study. Dheeraj Verma et al. (pp. 7101–7106) describe a method that conceals the coagulation protein Factor IX from the immune system, which prevents the development of an allergic response in mice. By introducing the gene for Factor IX into the chloroplast genome of plants, the authors induced the plants to produce high levels of the protein, which was engineered with a delivery molecule to aid its entry into gut cells once ingested. Because the immune system in some hemophiliac patients can develop an allergy against therapeutic treatments, the authors sought to present functional Factor IX to the immune system in a way that develops tolerance. The researchers found that mice that ingested the plant-produced protein after being given an intravenous administration of Factor IX treatment were far less likely than controls to develop inhibitory antibodies, which block Factor IX from working. The authors suggest that priming human immune systems in the same way could extend the reach of Factor IX and help prevent allergic reactions in hemophiliac patients unable to tolerate the treatment. — T.H.D.



Factor IX (red) expressed in chloroplasts (inset) to the gut immune system after oral delivery.

Bayer pharma funded F VIII made in chloroplasts for oral delivery

Gates Foundation, Bayer, JDRF, NIH Awards for Translational Research

For advancing inventions from Daniell lab to the Clinic:

- Gates Foundation to develop polio vaccine
- JDRF to develop Type 1 Diabetes vaccine
- Bayer Hemophilia Global award to block inhibitors against blood clotting factor VIII
- Two NIH R01 grants ($5.4 million) to develop hemophilia vaccines (F VIII, F IX)


Thanks to all lab
Colleagues and
Funding agencies
Thank you