Eukaryotic Gene Expression: Basics & Benefits

P N RANGARAJAN

Lecture 36

Transgenic plants

Introducing genes into plants

Agrobacterium method (biological)

Biolistics using “Gene Gun“ (physical)

Somatic tissues of plants grown in culture can be transformed in the laboratory with the desired gene using one of the above methods and grown into mature plants with flowers.

Following gene transfer, the transgene is incorporated into the pollen and eggs and passed on to the next generation.

Agrobacterium-mediated gene transfer

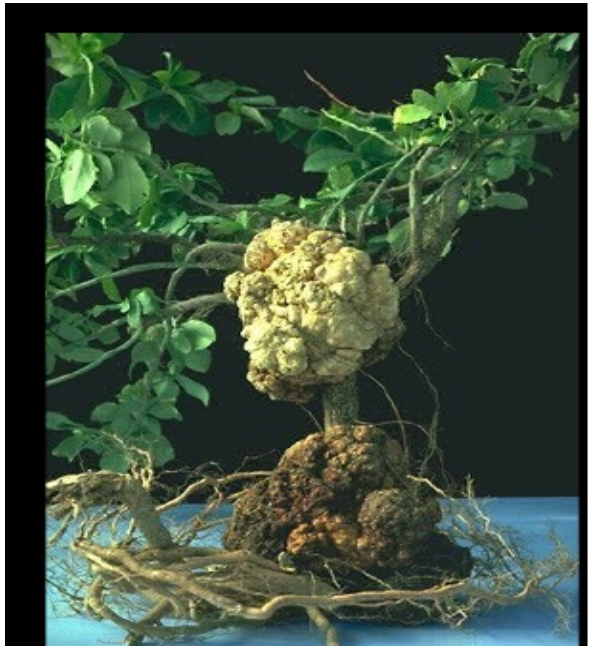
Just as animal viruses can be used as gene delivery vehicles for the introduction and expression of genes in animal cells, bacteria which infect plants can be exploited for delivering genes into plant cells.

Agrobacterium tumifaciens, is a plant pathogen found in soil.

It causes a disease known as the ‘crown gall disease’ in a variety of dicotyledonous plants, especially members of the rose family such as apple, pear, peach, cherry, almond, raspberry and roses.

Plants infected with this bacterium develop large tumour-like swellings (galls) that typically occur at the crown of the plant, just above soil level.

Following infection, the bacterium transfers part of its DNA to the plant, and this DNA integrates into the plant’s genome, causing the production of tumours and associated changes in plant metabolism.



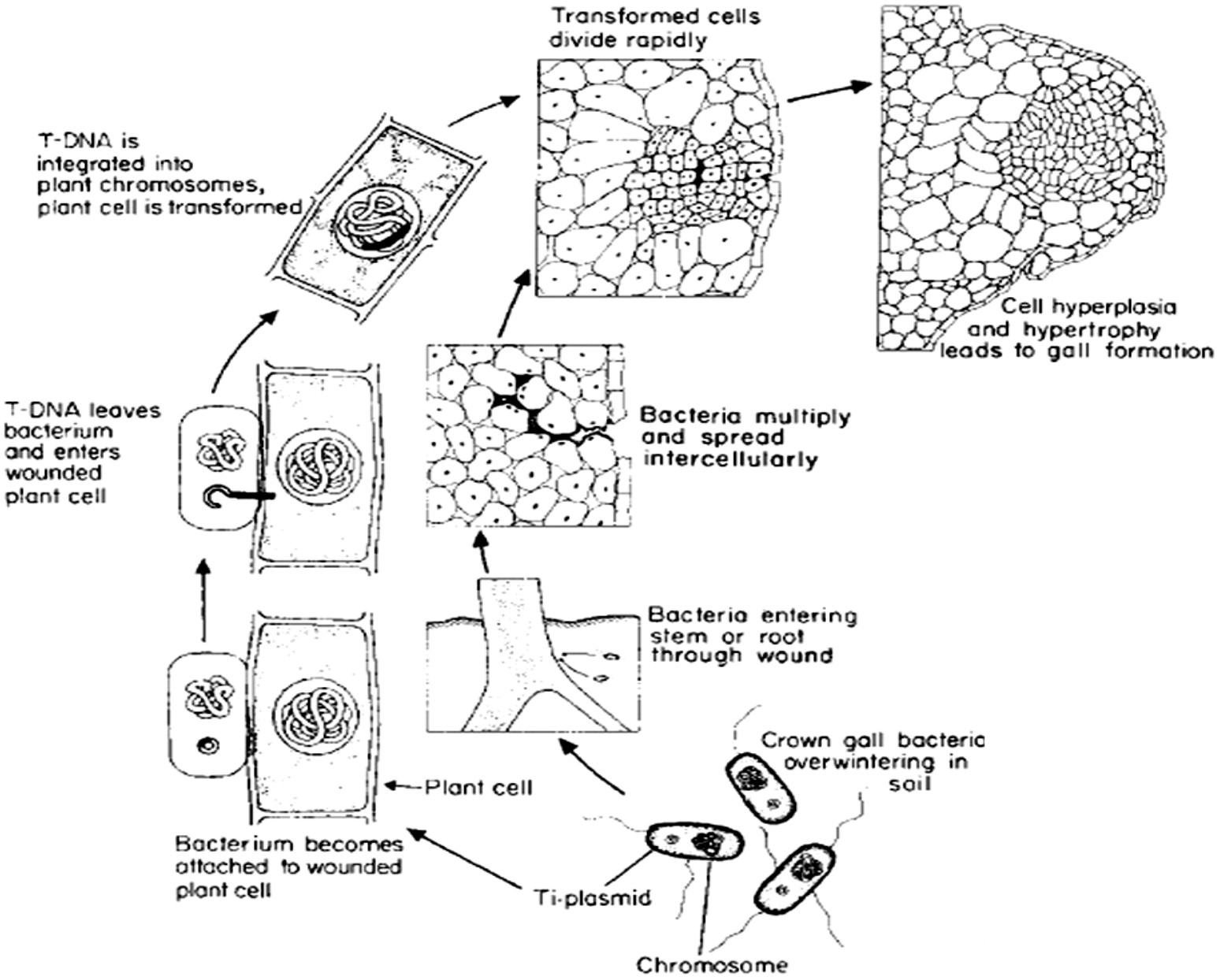
The process of infection

Agrobacterium tumefaciens infects plants through wounds. The motile bacterial cells are attracted to the wound site by the phenolic compounds such as acetosyringone secreted at the wound site through specific chemotactic receptors present in the bacterial membrane.

In addition to being involved in this chemotaxis, acetosyringone, at higher concentrations (about 10^{-5} - 10^{-4} M) activates the virulence genes (*Vir* genes) encoded by the bacterial plasmid known as the T*i* plasmid leading to the production of proteins (permeases) that are inserted in the bacterial cell membrane for uptake of compounds (opines) that are produced by the tumours.

Acetosynringone also causes the production of an endonuclease that excises part of the *Ti* plasmid termed the T-DNA (transferred DNA).

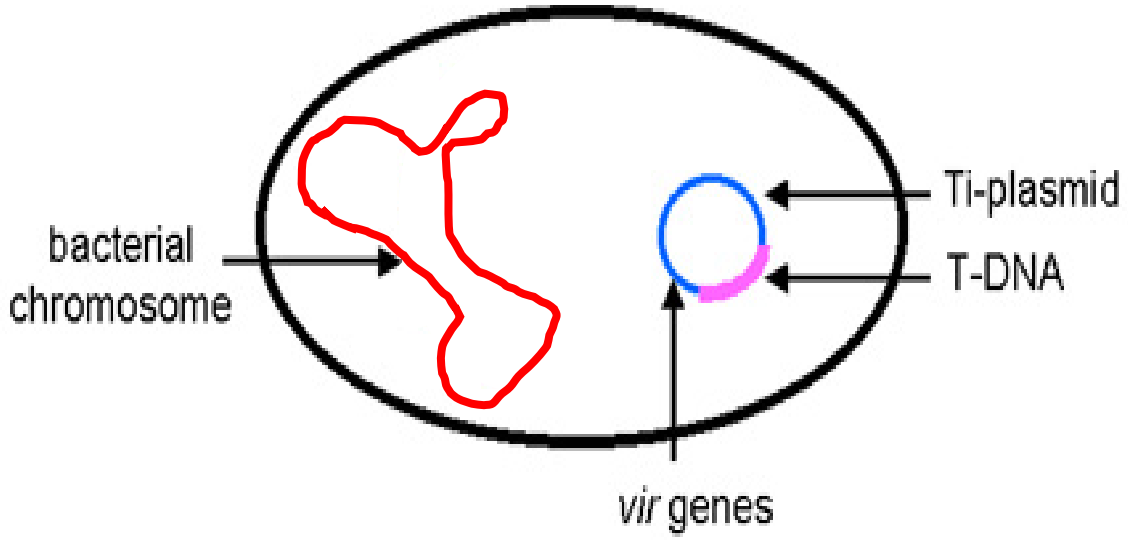
The process of infection



Tumour inducing (Ti) plasmid

A circular bacterial plasmid (~200 kb) known as the tumour inducing (Ti) plasmid is responsible for the production of the tumor as well as transformation. The Ti plasmid transfers and inserts a region of the plasmid, known as T-DNA, into the host chromosome. Genes known as the *vir* genes essential for T-DNA transfer are located outside the T-DNA on the Ti plasmid. The T-DNA region codes for the production of the tumor as well as synthesis of opines.

The Ti plasmid is lost when *Agrobacterium* is grown above 28°C. Such cured bacteria do not induce crown galls and thus become avirulent.



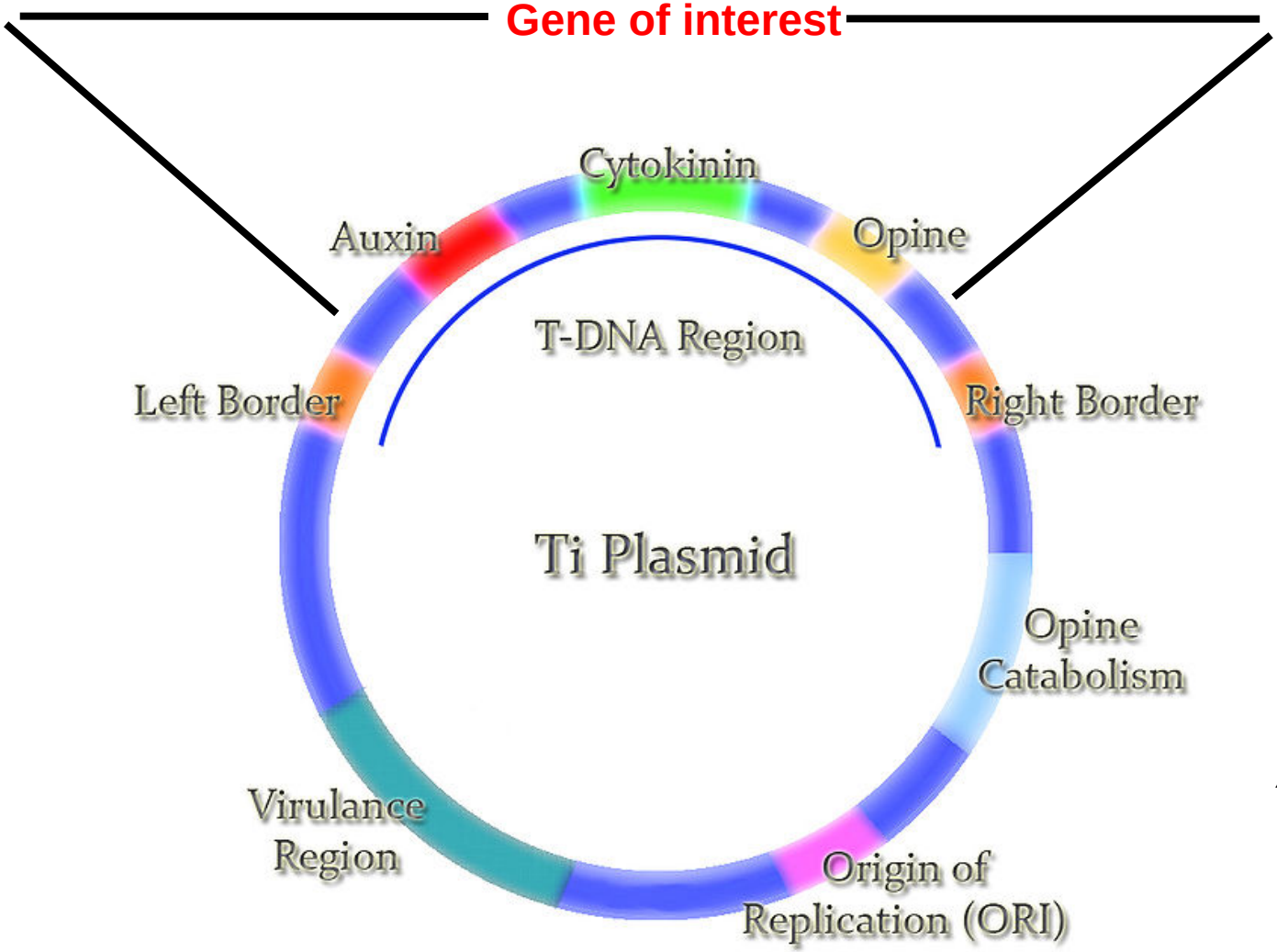
Ti plasmids are classified according to the opines produced

1.Nopaline plasmids: carry gene for synthesizing nopaline in the plant and for utilization (catabolism) in the bacteria. Tumors can differentiate into shooty masses (teratomas).

2.Octopine plasmids: carry genes to synthesize octopine in the plant and catabolism in the bacteria. Tumors do not differentiate, but remain as callus tissue.

3. Agropine plasmids: carry genes for agropine synthesis and catabolism. Tumors do not differentiate and die out.

4.Ri plasmids: induce hairy root disease on some plants and crown gall on others; have agropine-type genes and may have segments from both nopaline and octopine plasmids



http://en.wikipedia.org/wiki/File:Ti_Plasmid.jpg

Strategy

Clone an ori for *E. coli* into the Ti plasmid

Clone gene of interest into Ti plasmid by replacing genes in the T-DNA (but keeping the left and right borders of the T-DNA intact).

Introduce the engineered Ti plasmid back into *Agrobacterium* and allow it to infect plant cells in culture.

Binary vectors

Disarmed Ti plasmid with gene of interest (no vir genes)

Helper vector with vir genes for infection

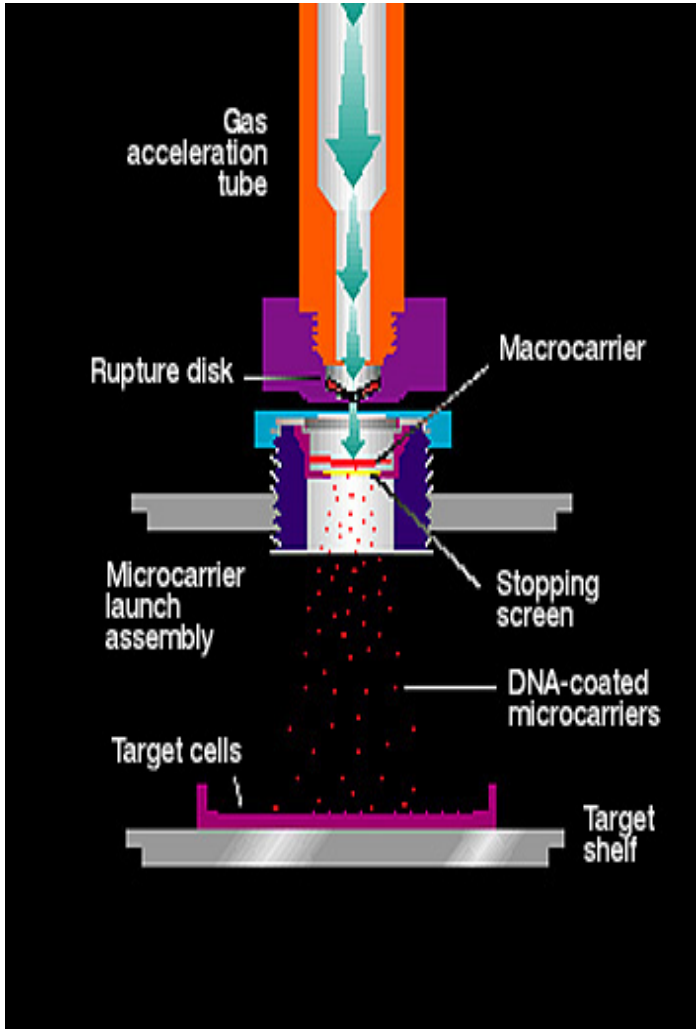
Procedure for creation a transgenic plant

1. Both plasmids are transfected into A.tumefaciens
2. Plant cell culture is infected with A.tumefaciens
3. Products of Vir genes excised gene of interest within T-DNA and transfer it to plant genome
4. Plant cells are selected on kanamycin
5. Presence of transgene confirmed by PCR

A binary plant vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid

A. Hoekema et al., *Nature* **303**, 179 - 180 (12 May 1983)

The particle bombardment/gene gun method is used for generating transgenic monocots



Agrobacterium mediated transfer was reported in monocot species including the most important food crops like rice (1994), maize (1996) & wheat (1997).

Efficient transformation of rice (Oryza *sativa L.)*mediated by *Agrobacterium and sequence analysis*of the boundaries of the T-DNA

Yukoh Hiei et al., *The Plant Journal (1994) 6(2), 271-282*

High efficiency transformation of maize (*Zea Mays* L.) mediated by *Agrobacterium tumefaciens*.

Ishida Y et al., Nature Biotechnol (1996)14: 745-750

Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*.

Cheng M et al., *Plant Physiol* (1997) 115:971-980.

Promoters used for expression in transgenic plants

Cauliflower mosaic virus 35S promoter

CaMV 35S is a strong promoter that is active in essentially all dicot plant tissues

US 6255560
Chimeric genes for transforming plant cells using viral promoters

(U.S. patent expired in 2005)

Selectable markers used in generation of transgenic plants

nptI, nptII

Two neomycin phosphotransferase genes are used in selection of transformed organisms: the neomycin phosphotransferase I (***npt*I***)* gene and the neomycin phosphotransferase II (***npt*II**) gene.

It was initially isolated from the transposon Tn5 present in *Escherichia coli* K12.

The gene codes for the aminoglycoside 3'-phosphotransferase (denoted NPTII) enzyme, which inactivates by phosphorylation a range of aminoglycoside antibiotics such as kanamycin, neomycin, geneticin (G418), and paromomycin.

Selectable markers used in generation of transgenic plants

hpt

The hygromycin phosphotransferase (***hpt***) gene was originally derived from *Escherichia coli*.

The gene codes for hygromycin phosphotransferase (HPT), which detoxifies the aminocyclitol antibiotic hygromycin

Selectable markers used in generation of transgenic plants

pmi

pmi encodes phosphomannose-isomerase that converts mannose-6-phosphate to fructose-6-phosphate, a useable carbohydrate

Zhengquan He et al., (2004) Plant Science166: 17-22
Phosphomannose-isomerase (*pmi*) gene as a selectable marker for rice transformation via *Agrobacterium.*

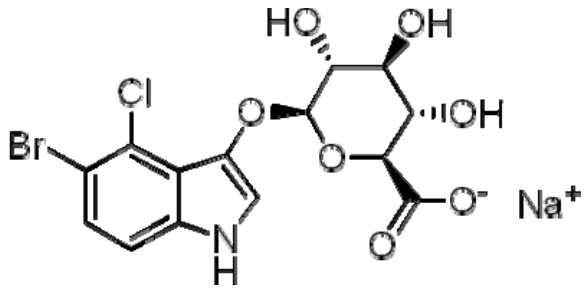
Reporter genes used in transgenic plants

The *gus*A gene encodes ß-glucuronidase (GUS)

GUS is a hydrolase that cleaves a wide variety of ß-glucuronides, is the most widely used reporter system for plants.

It is easy to quantify, highly sensitive and very specific.

Substrates for GUS are available for spectrometric, fluorometric and histochemical detection assays.



5-Bromo-4-chloro-3-indolyl β-D-glucuronide (X-glu)

	GUS		oxidative dimerization	
X-glu	→	colourless soluble intermediate	→	Blue precipitate of diX-indigo

Transgenic plants - benefits

Improved Nutritional Quality

Insect Resistance

Disease Resistance

Herbicide Resistance

Salt Tolerance

Delaying fruit ripening

Biopharmaceuticals & vaccines

Improved Nutritional Quality

Milling rice removes the husk and any beta-carotene it contained.

Beta-carotene is a precursor to vitamin A, and consuming milled rice leads to so vitamin A deficiency, especially in the countries of Southeast Asia.

The synthesis of beta-carotene requires a number of enzyme-catalyzed steps.

In January 2000, a group of European researchers reported that they had succeeded in introducing **three transgenes** into rice that enabled these plants to manufacture beta-carotene in their endosperm.

Carotenoid	-----	1.6 mg/g	37 mg/g

Expression of two genes from snapdragon that induce the production of anthocyanins in tomatoes, generates purple tomatoes

Anthocyanins offer protection against certain cancers, cardiovascular disease and age-related degenerative diseases. There is evidence that anthocyanins also have anti-inflammatory activity, promote visual acuity and hinder obesity and diabetes.

'Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors'
Nature Biotechnology doi: 10.1038/nbt.1506

Plant Biotechnology Journal (2007) **5**, pp. 000–000 doi: 10.1111/j.1467-7652.2007.00283.x

Genetically stable expression of functional miraculin, a new type of alternative sweetener, in transgenic tomato plants

Hyeon-Jin Sun, Hiroshi Kataoka, Megumu Yano and Hiroshi Ezura*

Graduate School of Life and Environmental Sciences, Gene Research Center, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8572, Japan

Miraculin - taste-modifying protein – miracle fruit, the red berries of *Richadella dulcifica* - shrub native to West Africa

Active principle - protein miraculin

Sour foods - lemons, limes & grapefruit, taste sweet when tasted together with this protein

Tearless Onion

Dr Colin Eady of Crop & Food Research in New Zealand

As onions are sliced, cells are broken, alliinases - break down aa sulphoxides - generate sulphenic acids - unstable - rearrange into a volatile gas - diffuses by air - reaches the eye - reacts with the water to form a diluted solution of sulphuric acid –
Tear glands produce tears to dilute and flush out the irritant

By shutting down the lachrymatory factor synthase gene
using RNAi technology, the conversion of valuable
sulphur compounds to the tearing agent was inhibited

http://www.sciencedaily.com/releases/2008/02/080202115345.htm

"rainbow cauliflowers"

PRODUCED BY TRADITIONAL BREEDING – NONTRANSGENIC

The orange cauliflower has higher than normal levels of beta carotene, that encourages healthy skin.

The purple colour comes from anthocyanin, which may help prevent heart disease by slowing blood clotting.

Syngenta

http://www.phxbodymindandsoul.com/home/909nourishcoloredcauliflower

Insect Resistance

Bacillus thuringiensis is a bacterium that is pathogenic for a number of insect pests. Its lethal effect is mediated by a protein toxin (Bt toxin) it produces.

When the toxin gene was introduced into economically important crop plants, they develop resistance for major insect pests obviating the need for the use of insecticides.

Disease Resistance

Genes that provide resistance against plant viruses have been successfully introduced into such crop plants as tobacco, tomatoes, and potatoes.

Herbicide Resistance

Genes for resistance to certain herbicides have been introduced into crop plants so that they can thrive even when exposed to herbicides that kill weeds.

>99% of all transgenic crops are either herbicide or insect resistant

<1% have other traits

Salt Tolerance

A large fraction of the world's irrigated land cannot be used to grow most important crops due to increases salinity in the soil.

Researchers have created transgenic tomatoes that grew well in saline soils.

The transgene introduced was a sodium/proton antiport pump that sequestered excess sodium in the vacuole of leaf cells.

Transgenic plants expressing Antisense RNA

The Flavr Savr tomato

Most tomatoes that have to be shipped to market are harvested before they are ripe. Otherwise, ethylene synthesized by the tomato causes them to ripen and spoil before they reach the customer.

Transgenic tomatoes have been constructed that express an antisense RNA complementary to the mRNA for an enzyme involved in ethylene production. These tomatoes make only 10% of the normal amount of the enzyme, thus delaying ethylene production.

Production of therapeutic proteins

Recombinant proteins from transgenic plants

Eva Franken, Ute Teuschel and Rüdiger Hain

http://www.ufv.br/DBV/PGFVG/BVE684/htms/pdfs_revisao/trangenicos_transformacao/recomprotplant.pdf

Production of vaccines

Expression of hepatitis B surface antigen in transgenic plants.
Mason HS, Lam DM, Arntzen CJ.
Proc Natl Acad Sci U S A. 1992 Dec 15;89(24):11745-9.

Tobacco plants were genetically transformed with the gene encoding hepatitis B surface antigen (HBsAg) linked to a nominally constitutive promoter were generated.

Recombinant HBsAg purified from transgenic plants had properties similar to human serum-derived HBsAg.

We conclude that transgenic plants hold promise as low-cost vaccine production systems.

Biotechnology (N Y). 1995 Dec;13(13):1484-7.
Expression of the rabies virus glycoprotein in transgenic tomatoes.
McGarvey PB, Hammond J, Dienelt MM, Hooper DC, Fu ZF, Dietzschold B, Koprowski H, Michaels FH.

Gene linked to CaMV35S promoter

Introduced to tomato plants by *Agrobacterium*- mediated transformation

Expression of recombinant glycoprotein in leaves and fruits

Protein localized in golgi bodies, vesicles and plasmalemma

Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice.

Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ.
Proc Natl Acad Sci U S A. 1996 May 28;93(11):5335-40.

- Causative agent for acute epidemic gastroenteritis
- NVCP was fused to CaMV35S or patatin promoter
- Transformation by *Agrobacterium*
- Expression level:

  0.37% TSP in potato tubers

  0.23% TSP in tobacco leaves

Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato

CAROL O. TACKET[1], HUGH S. MASON[2], GENEVIEVE LOSONSKY[1], JOHN D. CLEMENTS[3], MYRON M. LEVINE[1] & CHARLES J. ARNTZEN[2]

NATURE MEDICINE • VOLUME 4 • NUMBER 5 • MAY 1998

Antigenic proteins produced in transgenic plants retained immunogenic properties when purified

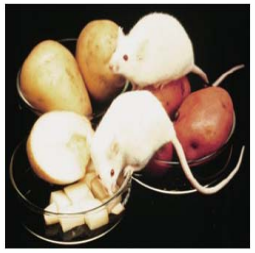
When injected into mice the antigen caused production of protein-specific antibodies.

Moreover, in some experiments, if the plant tissues were simply fed to mice, a mucosal immune response occurred.

This study was conducted as a proof of principle to determine if humans would also develop a serum and/or mucosal immune response to an antigen delivered in an uncooked foodstuff.

Concept

- Avoid costly down stream processing
- "Manufacture" in greenhouses

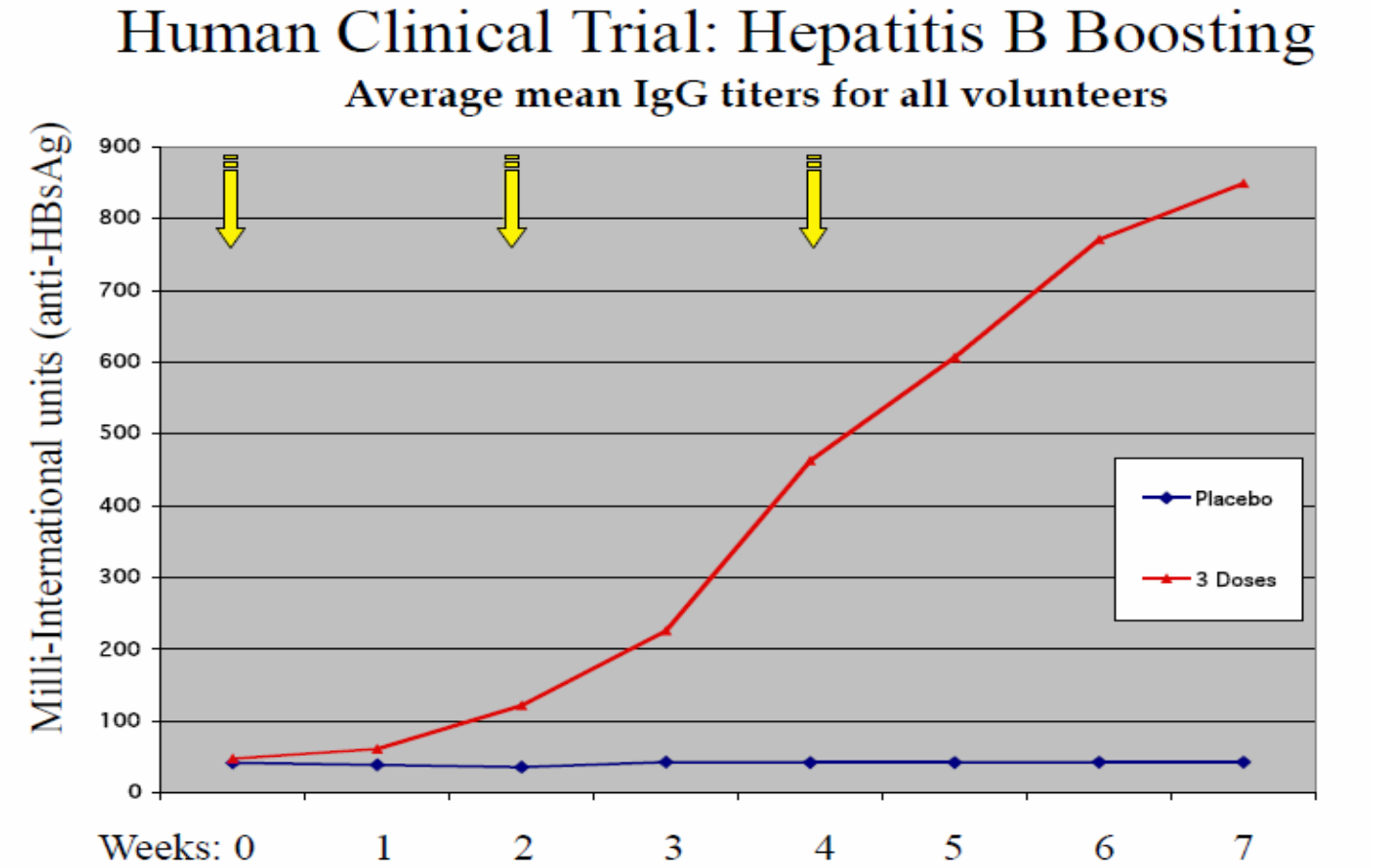


Regulatory:

- Pre-clinical studies with mice
- Vaccine is only a "food additive"

HBsAg Clinical trial in 1998

Thanavala, Y., et al. 2005
Immunogenicity in humans of an edible vaccine for HBV.
PNAS 102:3378-3382.



Edible vaccines not ready for main course

Nature Medicine **10**, 881 (2004)

Plant-based vaccines face big scientific and regulatory hurdles

Since 1992, when biologist Charles Arntzen proposed genetically modifying bananas to serve as cheap vaccines against infectious diseases, research on plant-based pharmaceuticals has grown rapidly. In the US, several acres of crops, most of them still experimental, are planted each year.

But even Arntzen now says his original idea of distributing vaccine-bearing fruit was naive, because regulatory agencies will not approve vaccines with variable dosing.

Vaccine manufacturers have little reason to replace existing production lines, as most vaccines are economically unattractive. The medical community is also focused on high-tech approaches, making farm-grown vaccines a tough sell.

If vaccines are intimately presented together with food, the gut's immune system faces a conundrum."The gut is designed not to react to antigens in food, but must produce a useful response against the vaccine. Instead of being immunized, patients could even end up being 'tolerized,' meaning an immune response against future invaders would be weakened, not intensified.

Dr. Charles Arntzen inspects tomatoes grown in the greenhouse facility at ASU.

Freeze dried potato media

Dr. Arntzen and David Julovich look at freeze dried tomatoes.

http://www.biodesign.asu.edu/people/charles-arntzen

Upstream and Downstream Manufacturing of Vaccines in Tobacco

Charles J. Arntzen
charles.arntzen@asu.edu

THE biodesign INSTITUTE Center for Infectious Diseases and Vaccinology
ARIZONA STATE UNIVERSITY

http://www.biotech.ucdavis.edu/MCB294/pdfs/MCB%20294%20-%20Charles%20Arntzen,%202010.pdf

Transgenic Plant-Based Oral Vaccines (2010)
Lugade AA et al., Immunological Investigations, 39:468-482

Controversies on transgenic / genetically modified plants

The introduction of transgenic plants into agriculture has been vigorously opposed by some. There are a number of issues that worry the opponents.

One of them is the potential risk of transgenes in commercial crops endangering native or nontarget species.

Examples:

A gene for herbicide resistance in, e.g. corn, escaping into a weed species could make control of the weed far more difficult.

The gene for Bt toxin expressed in pollen might endanger pollinators like honeybees.

However, a large number of field studies on transgenic cotton and maize indicate that the reduction in the number of certain non-target insects is much smaller than that observed in fields treated with insecticides.

Another worry is the inadvertent mixing of transgenic crops with nontransgenic food crops. Although this has occurred periodically, there is very little evidence to indicate that this poses a threat to human health.

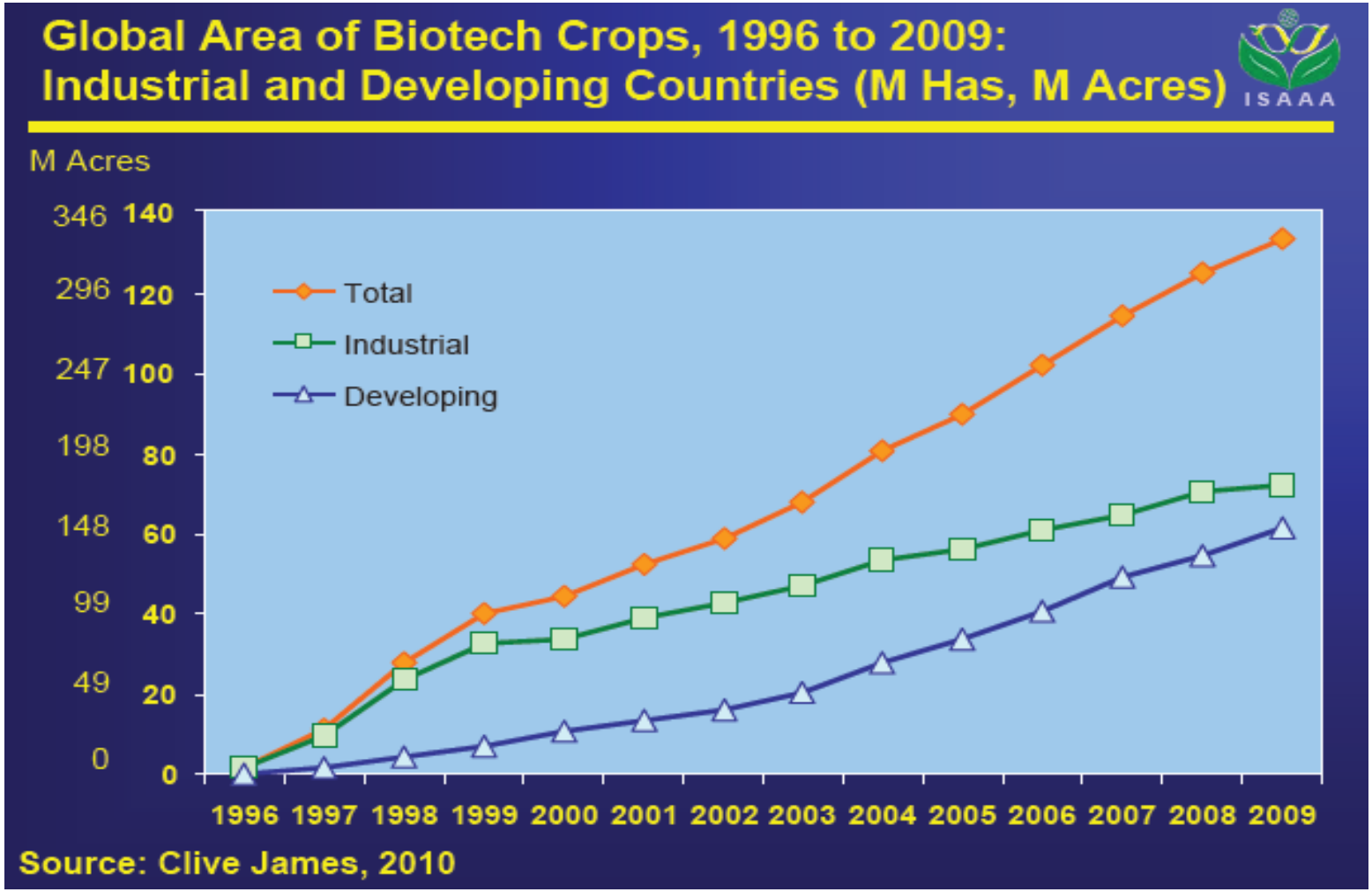
Despite these controversies, farmers around the globe are using transgenic crops.

In the United States >80% of the corn, soybeans, and cotton grown are genetically modified (GM) to develop resistance to the herbicide glyphosate ("Roundup Ready®") or resistance to insect attack (by expressing the Bt toxin).

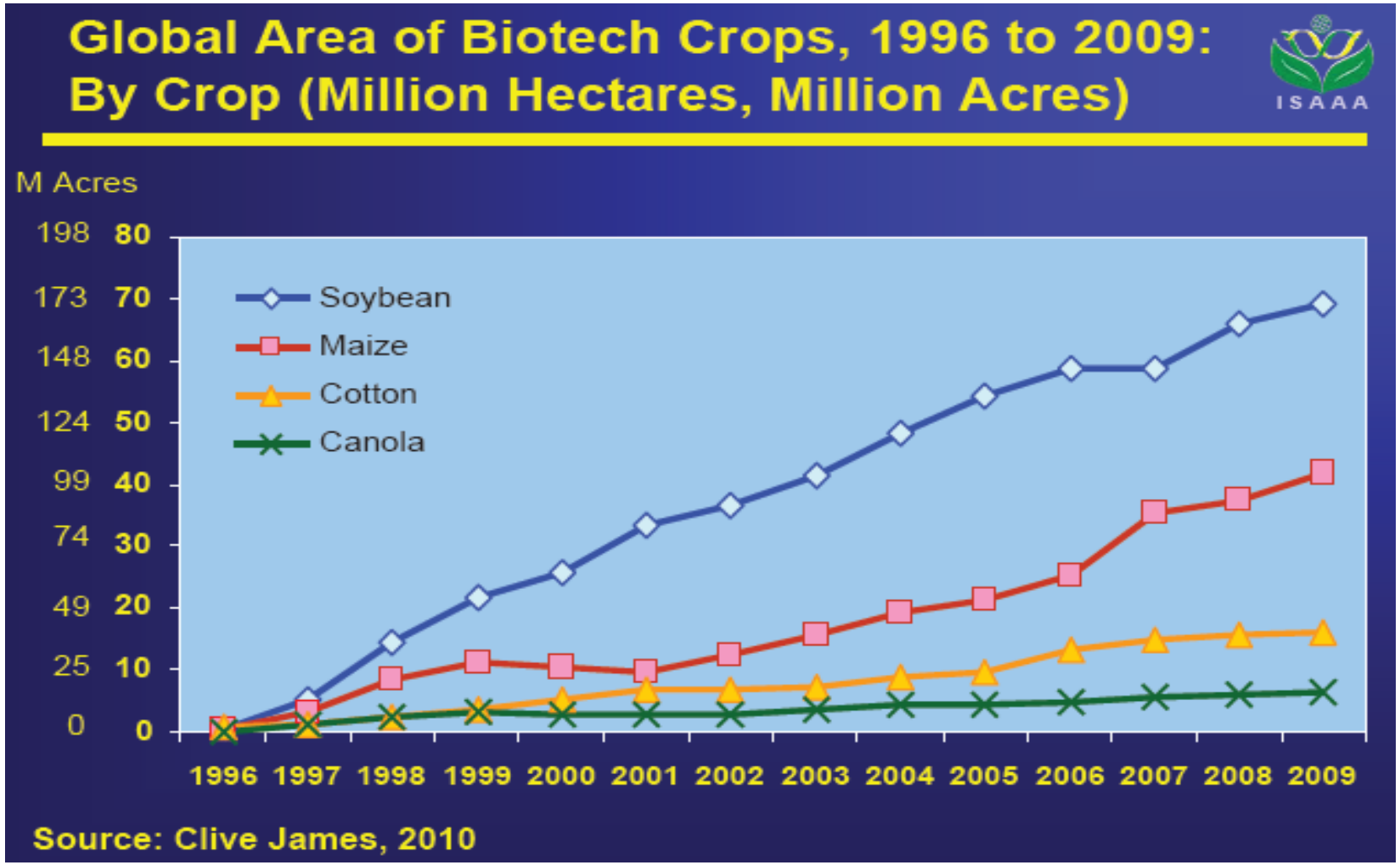
Approved Transgenic plants

Soybean
Corn
Cotton
Oil Seed rape
Sugarbeet
Squash
Tomato
Tobacco
Carnations
Potato
Flax
Papaya
Chicory
Rice
Melon

Acreage of transgenic crops has gone from nothing in 1995
to around 345 million acres in 2009



http://www.isaaa.org



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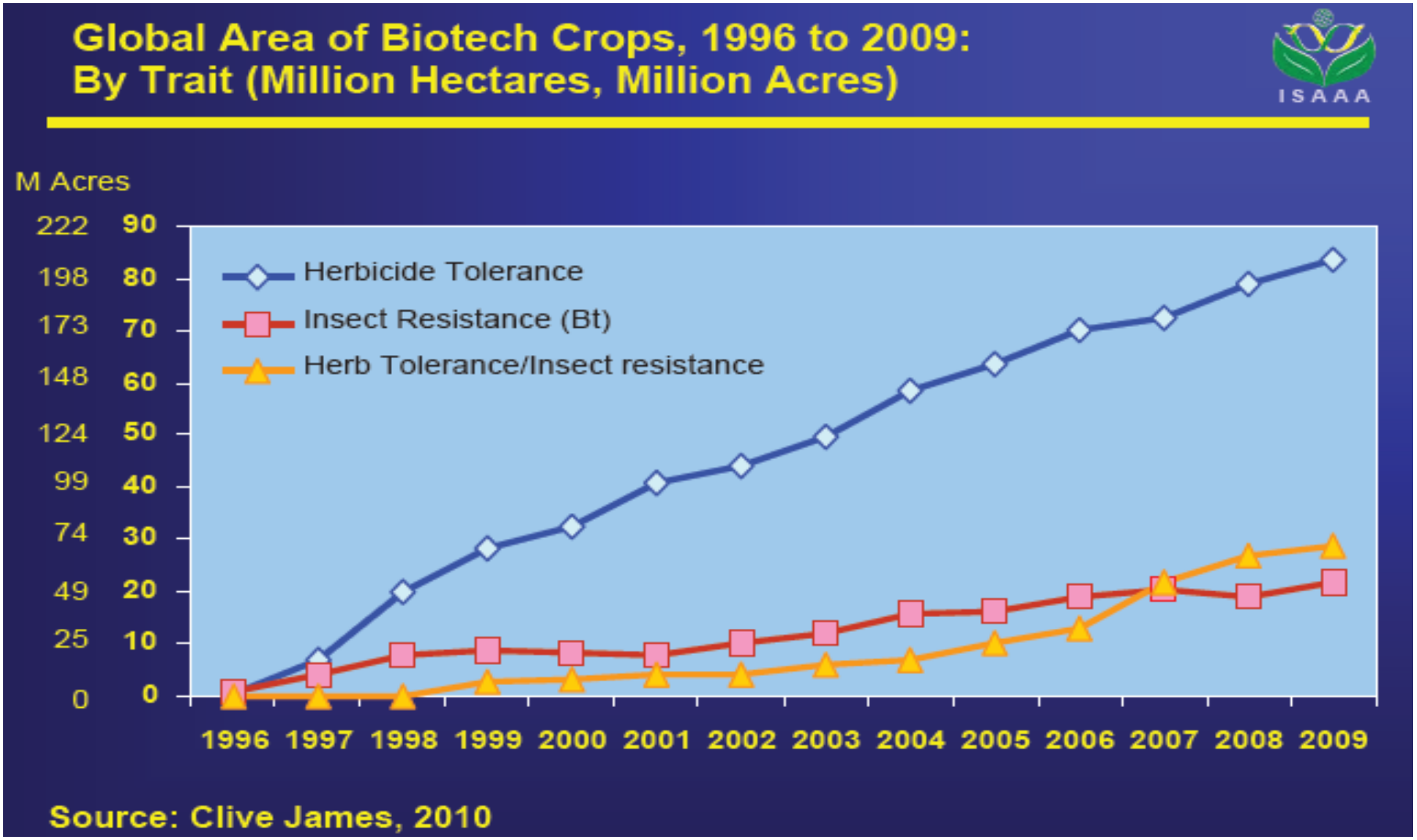

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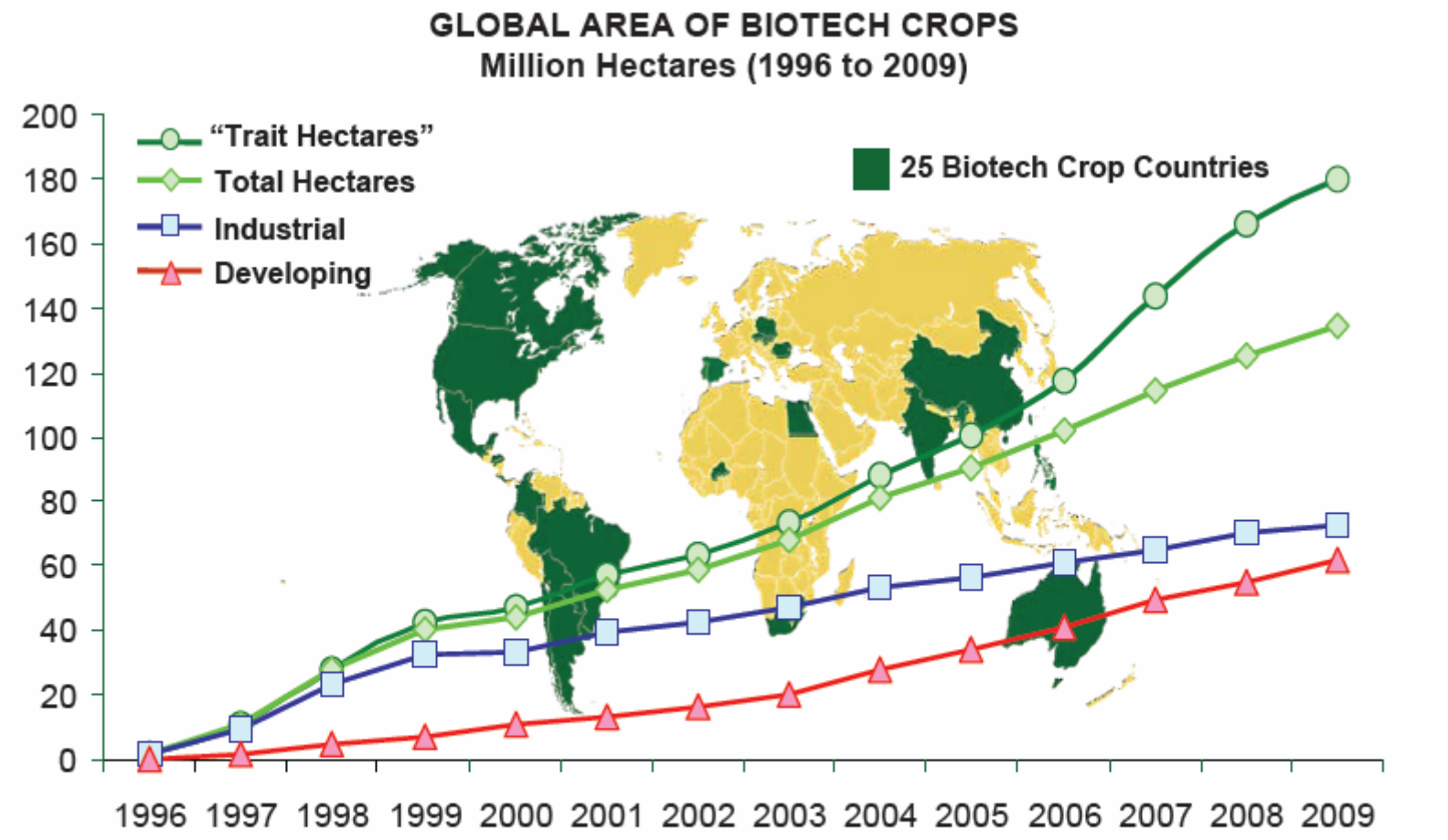
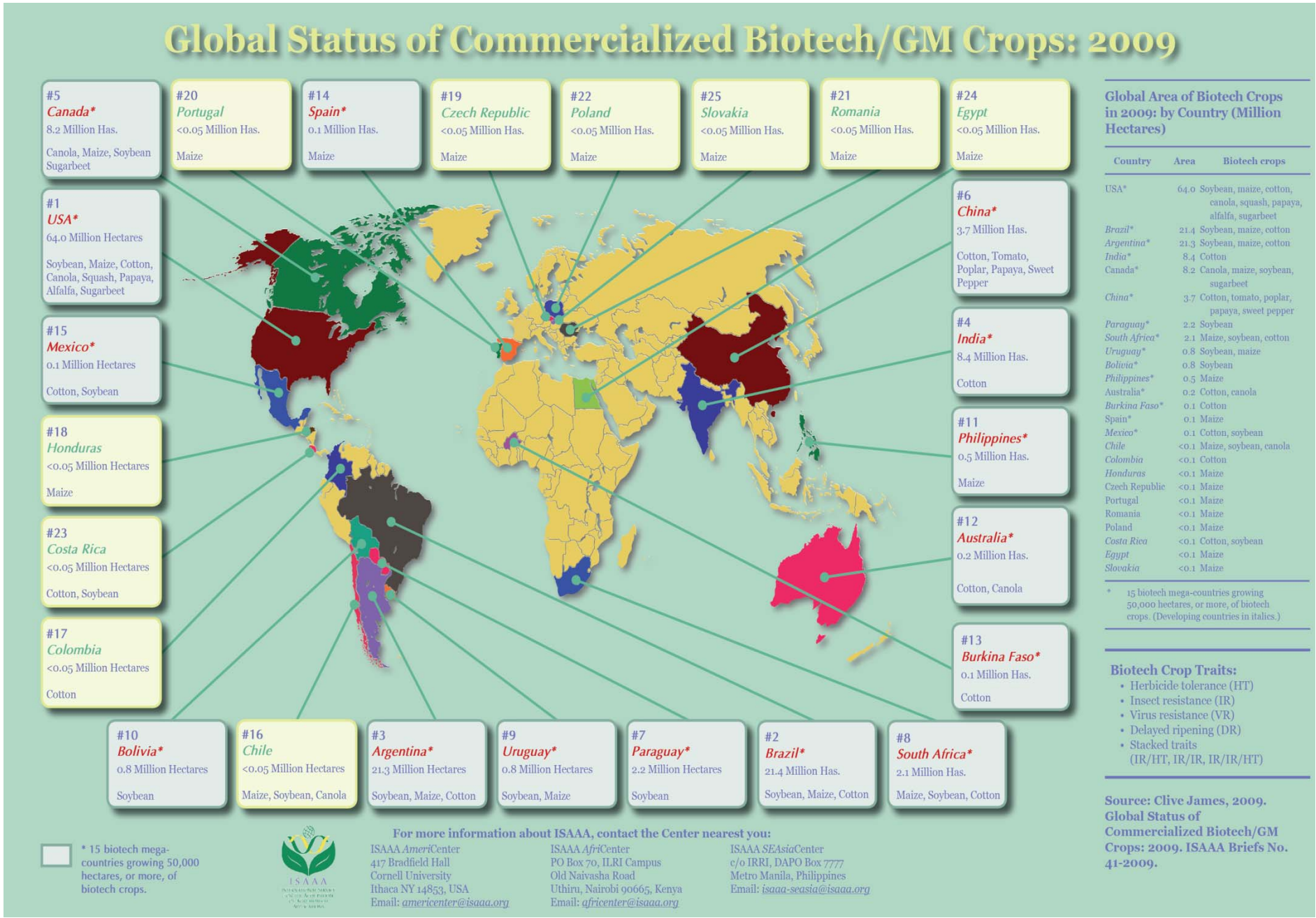


A record 14 million farmers, in 25 countries, planted 134 million hectares (330 million acres) in 2009, a sustained increase of 7% or 9 million hectares (22 million acres) over 2008.

Source: Clive James, 2009.

http://www.isaaa.org



http://www.isaaa.org/resources/publications/briefs/41/pptslides/Global_Status_Map-2009.pdf

“Terminator" Genes

Transgenes introduced into crop plants to make them produce sterile seeds thereby forcing the farmer to buy fresh seeds for the following season rather than saving seeds from the current crop are known as “terminator” genes.

The process involves introducing three transgenes into the plant:

A gene encoding a **toxin** which is lethal to developing seeds but not to mature seeds or the plant. This gene is normally inactive because of a stretch of DNA inserted between it and its promoter.

A gene encoding a recombinase that can excise the spacer in the toxin gene, thus allowing it to be expressed.

A repressor gene whose protein product binds to the promoter of the recombinase thus keeping it inactive.

Before selling, the seeds are soaked) in a solution of tetracycline so that synthesis of the repressor is blocked.

The recombinase gene is activated which removes the spacer so that the toxin gene is turned on.

Since the gene product is toxic only to developing seeds but not the growing plant, the crop can be grown normally but the seeds produced by it are sterile.

Cookbook For Eukaryotic Protein Expression: Yeast, Insect, and Plant Expression Systems

The Scientist 1998, **12**(22)**:**20

http://www.the-scientist.com/article/display/18282/#ixzz0zrHGti1D

csmith@the-scientist.com

Global Status of Commercialized Biotech/GM Crops

http://www.isaaa.org

Some of the leading agri biotech companies

Monsanto

BASF

Dow AgroSciences

Bayer CropScience

DuPont

Syngenta