Part V
TRANSGENIC PLANTS: FIRST GENERATION

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Plant Biotechnology

Vietnam OpenCourseWare

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Classical Plant Breeding

Genetic modification following introduction of large amount of undefined DNA from a genetically similar source.

Plant Biotechnology

Genetic modification of a plant cell by introduction of defined DNA(s) from a genetically different source.

The goal of plant biotechnology is to produce plants with a variety of desirable traits in high yielding seed cultivars.

At present, transformation capability has been extended to more than 120 species in at least 35 families.

✓ *Success includes most major economic crops, vegetables, ornamental, medical, fruit, tree and pasture plants*

✓ *Benefits include resistance to viruses, insects, herbicides, post-harvest deterioration, and accumulation of useful modified storage products ...*

Progression of GM plants

First generation *(1994~) Input trait (herbicide tolerance, insect resistance, virus resistance, disease tolerance, delayed ripening…)*

Second generation *(1995~) Output trait (enhanced nutrition, amino acid rich….)*

Third generation *(1998~) Non-traditional (vaccine, antibody, pharmaceutical protein, phytoremediation, Phytosensors….)*

Next-generation*: Biofuels ….*

These are the so-called "first generation genetically modified (GM) plants", and include the Flavr SavrTM tomato that had been modified with a native gene inserted in reverse, giving it a delayed-ripening trait, first commercialized in 1994 Large-scale commercial cultivation of GM plants began in 1996 with a total global area of 1.7 million hectares in six countries. In 2008, almost all GM acreage was occupied by first generation plants, dominated by herbicide-tolerance , followed by insect resistance and a combination of these traits.

The last decade has largely been dedicated to the development of "second generation" GM plants with traits beneficial to consumers such as high nutritional value, amino acid rich*…. The second generation includes soybeans with high oleic acid- or laurate-content and rice with high vitamin A or iron content. Development of a "third generation" such as protein factories of vaccines and therapeutic medicines is in progress.*

First generation (1994-):
Input trait (herbicide tolerance, insect resistance, virus resistance, disease tolerance, delayed ripening)

5.1. Herbicide tolerance plants

Why develop herbicide tolerance plants?

Weeds compete with crops for water, nutrients, light and space resulting in reduced crop quality and yields.

Approximately 20% of global crop production lost through weed infestations per annum.

Traditionally, weeds are controlled either mechanically (e.g. hand or tractor cultivation) or culturally (crop-rotation).

These methods are, however, expensive and can cause damage to crops and soil compaction and erosion.

What are herbicides ?

Herbicides are chemicals that kill plants.

Herbicides can be divided into two broad classes:

- *narrow-spectrum (selective) - affects only certain species or families of weeds*
- *broad-spectrum (non-selective) - affects almost every type of plant they contact eg. glyphosate and glufosinate*

Herbicide Group	Mode of Action	Example
ALS inhibitors	Inhibition of acetolactate synthase ALS	Chlorsulfuron
Photosystem II inhibitors	Inhibition of photosynthesis at photosystem II	Atrazine
ACCase inhibitors	Inhibition of acetyl CoA carboxylase (ACCase)	Diclofop-methyl
Synthetic Auxins	Synthetic auxins (action like indoleacetic acid)	2,4-D
Bipyridiliums	Photosystem-I-electron diversion	Paraquat
Ureas and amides	Inhibition of photosynthesis at photosystem II	Chlorotoluron
Dinitroanilines and others	Microtubule assembly inhibition	Trifluralin
Thiocarbamates	Inhibition of lipid synthesis	Triallate
Triazoles, ureas	Inhibition of carotenoid biosynthesis	Amitrole
Glycines	Inhibition of EPSP synthase	Glyphosate
Carotenoid inhibitors	Inhibition of carotenoid biosynthesis	Flurtamone
Chloroacetamides and others	Inhibition of cell division	Butachlor
Nitriles and others	Inhibition of photosynthesis at photosystem II	Bromoxynil
PPO inhibitors	Inhibition of protoporphyrinogen oxidase (PPO)	Oxyfluorfen
Mitosis inhibitors	Inhibition of mitosis	Propham
Phosphinothricin	Inhibition of glutamine synthase	Glufosinate

Herbicide tolerance (HT) crops

- ✓ Herbicide tolerance can be engineered in crops that are not naturally tolerant against compounds that are broad spectrum (non-selective) in nature.
- ✓ Herbicide tolerance is an easily engineered trait since it usually involves a single gene and the biochemistry of tolerance to certain herbicides is well understood.
- ✓ Herbicide tolerance crops are, as a result, the most frequent application of genetic engineering to crop plants.

How to make a plant HT

There are four main ways of introducing herbicide tolerance in plants:

1) Inhibit the uptake of the herbicide.

2) Introduce a gene that over-expresses the herbicide-sensitive target protein.

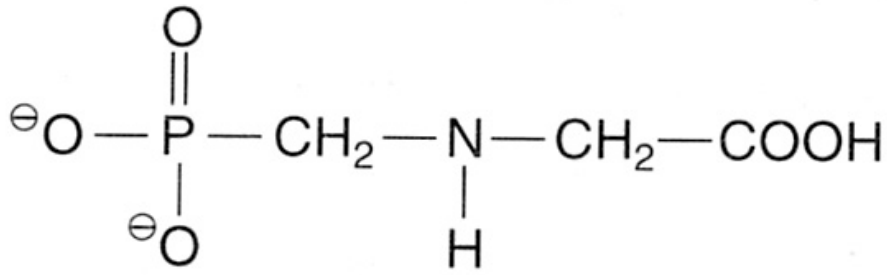
3) Introduce a gene that codes for an herbicide resistant form of the target protein.

4) Introduce a gene that codes for an enzyme that metabolically inactivates the herbicide.

Crops can be engineered to tolerate particular herbicides by introducing a single new gene.

Why Glyphosate and Glufosinate?

- **Broad-spectrum herbicide**
- **Bind to organic particles in soil**
  - Less carryover in soil and few restrictions for planting in subsequent years
  - Less contamination of ground water
- **Lower toxicity to animals** than many other herbicides



Glyphosate

Glyphosate tolerance plants

Mechanism action of glyphosate;

- *Glyphosate act by interfering with key enzymes (EPSPS) and in the chloroplast.*
- *Glyphosate specifically binds to and inactivates EPSPS, an enzyme which is involved in the biosynthesis of the aromatic amino acids tyrosine, phenylalanine and tryptophan. EPSPS is present in all plants, bacteria and fungi, but not in animals, which do not synthesize their own aromatic amino acids.*
- *Plants die because they lack the key amino acids.*

Glyphosate is a broad-spectrum herbicide (Active ingredient in RoundUp herbicide).

In order to increase its utility, Monsanto wanted to develop strains of commercial crops that were resistant to glyphosphate. It has not been possible to generate glyphosate tolerant plants by traditional mutagenesis and selection.

Methodology: Modify the enzyme to function in the presence of the herbicide (Roundup Ready®).

RoundUp Sensitive Plants

Shikimic acid + Phosphoenol pyruvate

+ Glyphosate

Plant EPSP synthase X

3-Enolpyruvyl shikimic acid-5-phosphate (EPSP) X

Without amino acids, plant dies

X 

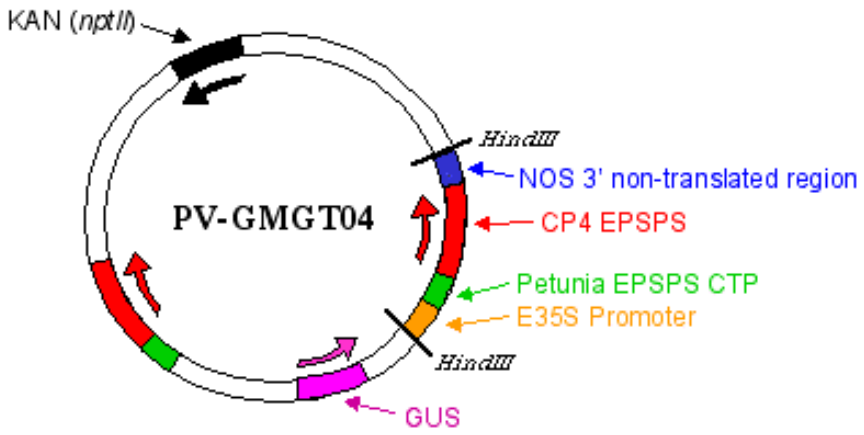
Aromatic amino acids X

A mutant EPSPS synthase gene (retaining catalytic activity but insensitive to glyphosate) has been isolated from *Agrobacterium* strain CP4.

Transfer mutated EPSP synthase gene to make resistant (Roundup Ready) plants

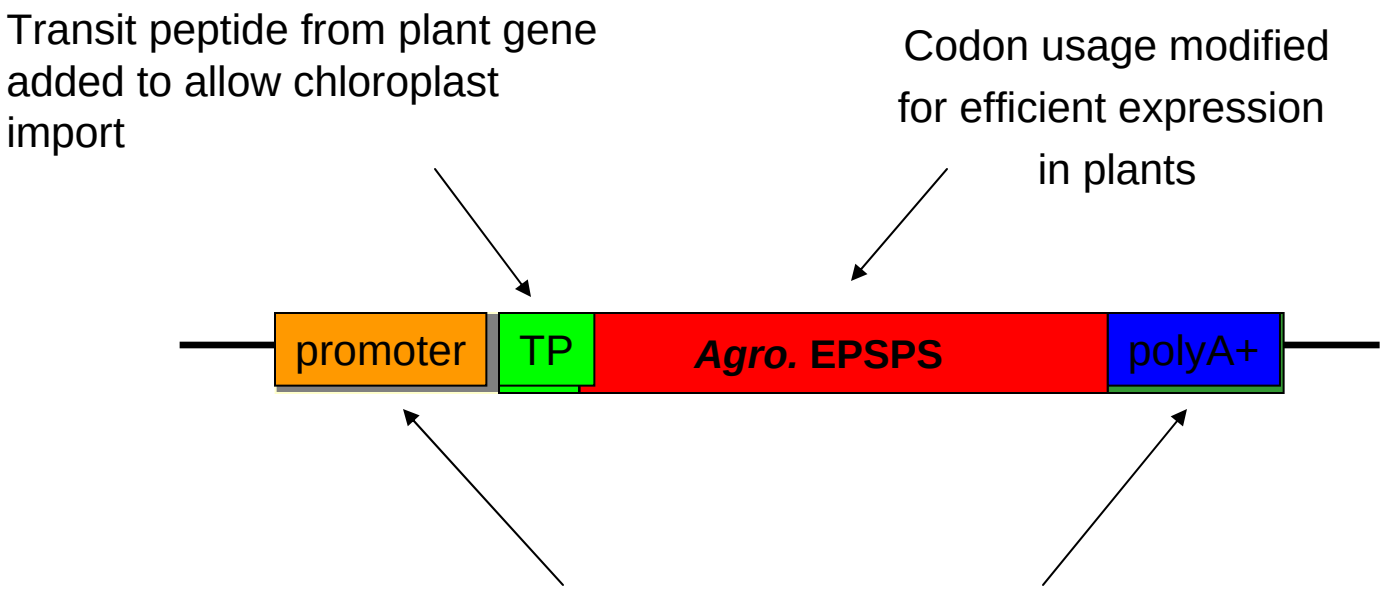
A resistant EPSP synthase gene allows crops to survive after spraying.

Vector Used for Transfection



E35S - cauliflower mosaic virus promoter
CTP - petunia chloroplast transit peptide
CP4 EPSPS -*Agrobacterium* strain CP4 EPSPS
NOS - nopaline synthase terminator / polyadenylation signal
GUS - β-glucoronidase gene
KAN - neomycin phosphotransferase

Glyphosate-tolerance soybean Construct



Regulatory sequences recognised by plant (either from plant gene or plant virus gene). In this case 35S CaMV promoter

RoundUp Resistant Plants

Shikimic acid + Phosphoenol pyruvate

+ Glyphosate

Bacterial

EPSP synthase

RoundUp has no effect;
enzyme is resistant to herbicide

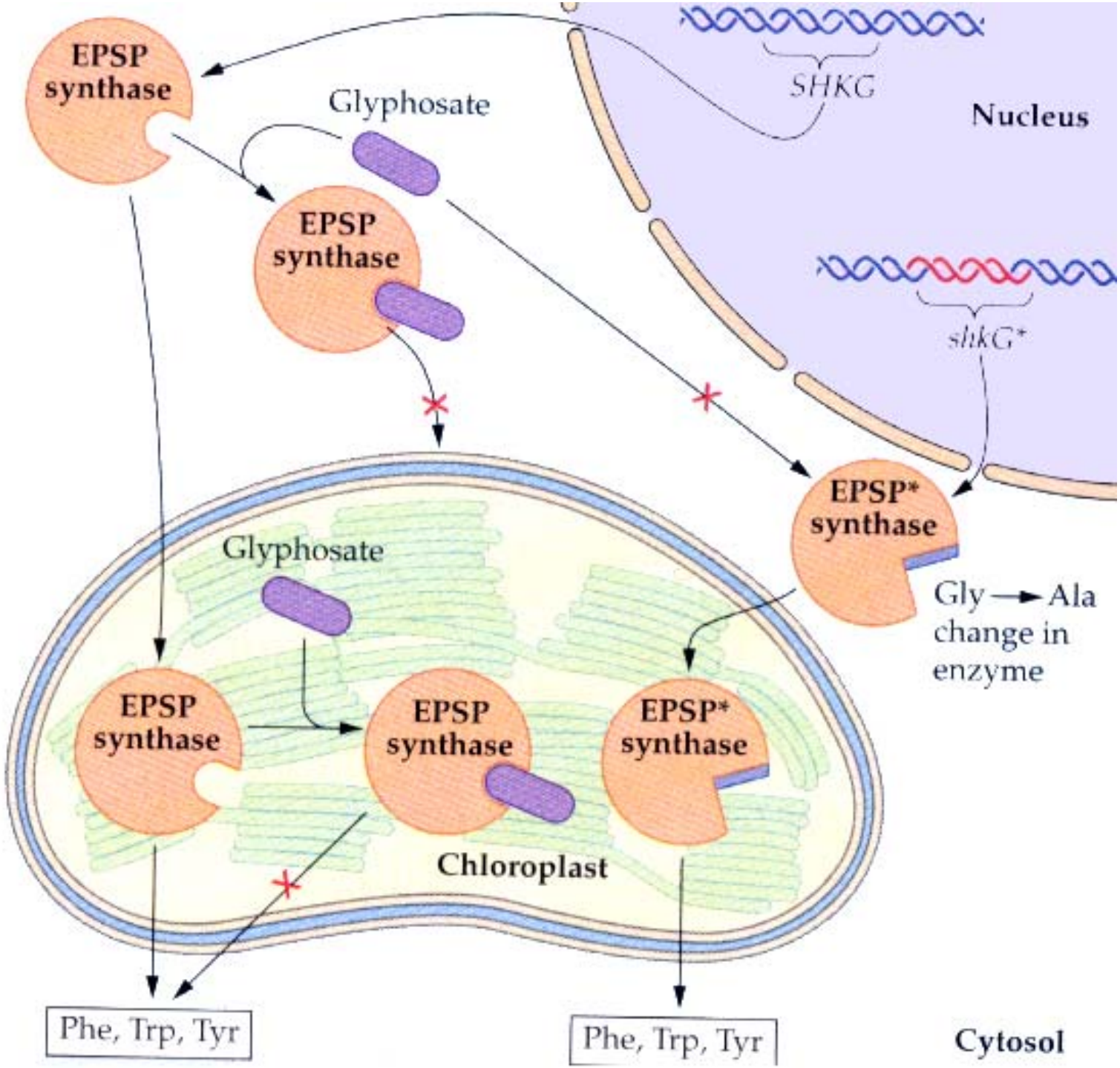
3-enolpyruvyl shikimic acid-5-phosphate

(EPSP)

With amino acids, plant lives

Aromatic

amino acids


EPSP synthase
Glyphosate
SHKG
Nucleus
EPSP synthase
shkG*
EPSP* synthase
Gly → Ala change in enzyme
Glyphosate
EPSP synthase
EPSP synthase
EPSP* synthase
Chloroplast
Phe, Trp, Tyr
Phe, Trp, Tyr
Cytosol

PV-GMGT04 used to transfect soybeans (microparticle acceleration method)

↓

Seeds collected from R_0 plants expressing GUS activity

↓

Seeds collected from R_1 plants displaying glyphosate resistance

↓

R_2 plants field trials

↓

Plant line 40-3-2 selected for possessing high EPSPS activity and low inhibition by glyphosate

Characterisation of plant line 40-3-2

- ✓ Fragment of the PV-GMGT04 plasmid including the entire CP4 EPSPS and CTP genes had become stably integrated into the soybean DNA.
- ✓ Strain 40-3-2 three times more resistant to glyphosate than the wild type species.
- ✓ Increase in yields resulting from the use of glyphosate treatment on the glyphosate-resistant crops claimed to be 7%.

Phosphinothricin herbicide

The herbicide phosphinothricin:

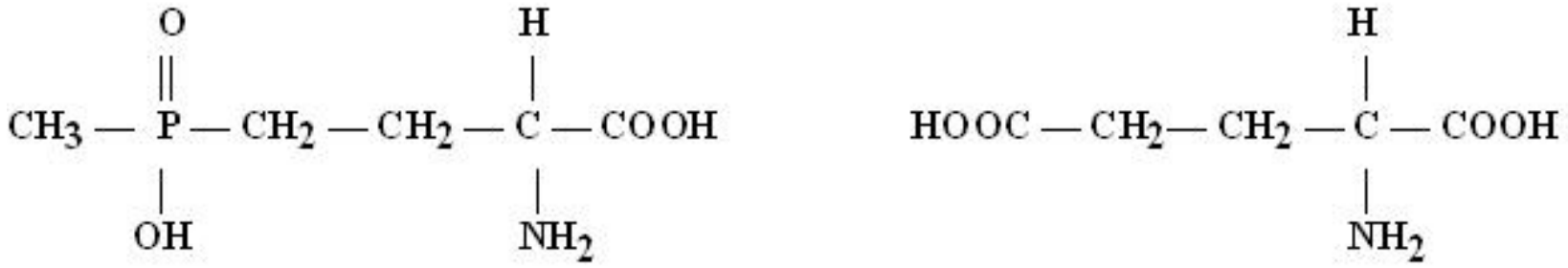
The L-isomer amino acid *of phosphinothricin (L-PPT) is considered a broad-spectrum herbicide because it is herbicidal to a wide range of plant species. L-PPT is the active ingredient of the herbicide glufosinate ammonium or in a number of commercial herbicide formulations (Bialaphos, Basta).*

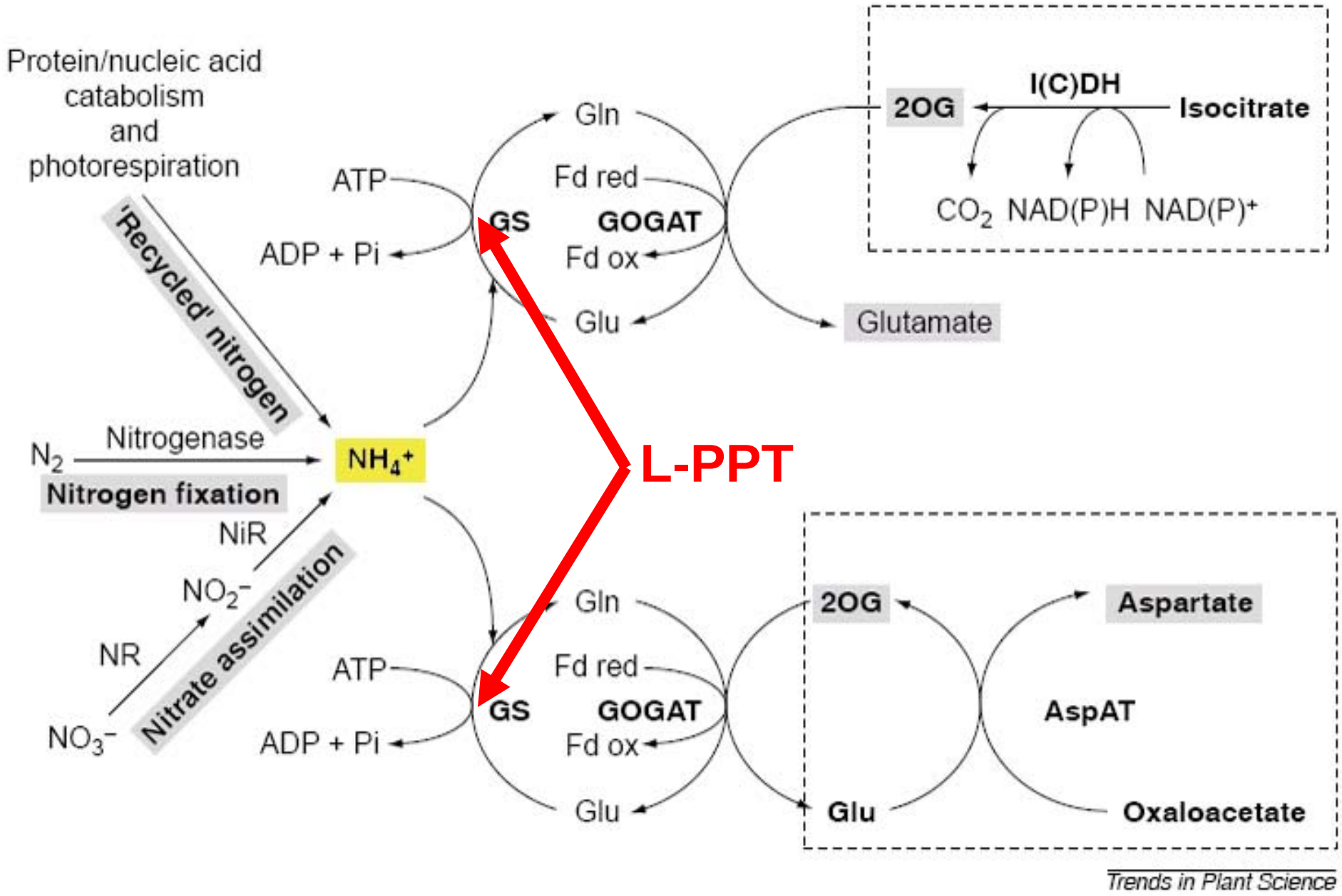
L-PPT inhibits glutamine synthetase (GS) of susceptible plants and results in the accumulation of lethal levels of ammonia.

L-PPT is a structural analogue of glutamate, the substrate of glutamine synthetase. L-PPT exerts its herbicidal effect through the inhibition of glutamine synthetase.

In the presence of ATP, L-PPT inhibits glutamine synthetase irreversibly. When L-PPT inhibits glutamine synthetase, phytotoxic levels of ammonia accumulate in the plant.

L-isomer of phosphinothricin (left) compared to glutamate (right)




Protein/nucleic acid catabolism and photorespiration
'Recycled' nitrogen
N_2
Nitrogenase
Nitrogen fixation
NH_4^+
NiR
NO_2^-
NR
NO_3^-
Nitrate assimilation
ATP
ADP + Pi
GS
Gln
Fd red
GOGAT
Fd ox
Glu
2OG
I(C)DH
Isocitrate
CO_2 NAD(P)H $NAD(P)^+$
Glutamate
L-PPT
ATP
ADP + Pi
GS
Gln
Fd red
GOGAT
Fd ox
Glu
2OG
Aspartate
AspAT
Glu
Oxaloacetate
Trends in Plant Science

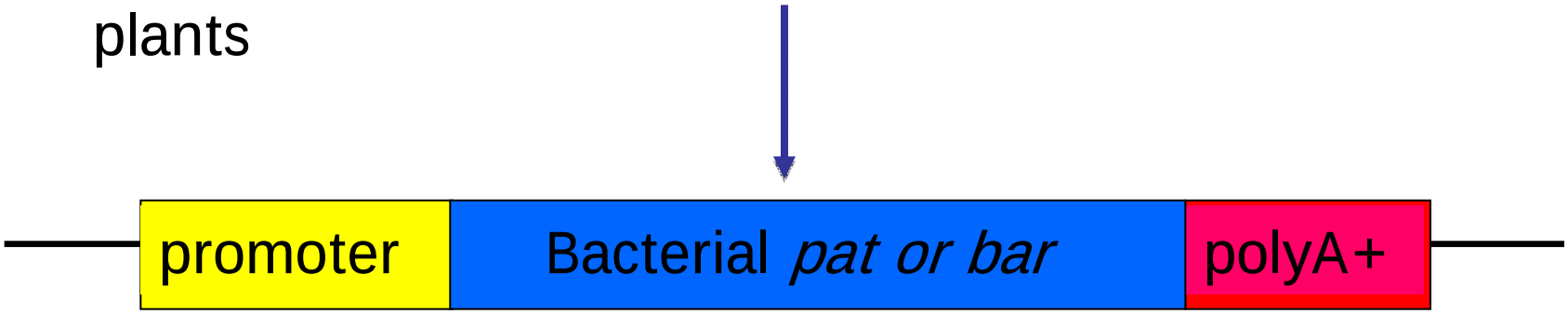
Phosphinothricin tolerant Plants

Transgenic strategies could be used to:

- ✓ Transform plants with a mutant GS insensitive to phosphinotricin.
- ✓ Transform a plant gene encode an enzyme that inactivates phosphinotricin.
- ✓ Detoxify L-phosphinotricin by acetylation, so GS not inhibited. Bacterial genes (either pat or bar) encoding phosphinothricin acetyl transferase have been transferred to plants.

Phosphinothricin-tolerance gene Construct

Codon usage modified for efficient expression in plants



Regulatory sequences recognised by plant (either from plant gene or plant virus gene). Frequently use 35S CaMV promoter

In some of the plants engineered with the pat or bar gene, the gene serves as a selectable marker gene. Such plants may not necessarily express agronomically useful levels of tolerance to L-PPT. Marker genes are routinely used in developing transgenic plants because they enable the researchers to select successful transformants in the laboratory. In addition, tolerance to L-PPT can be used as a selectable marker in the field.

L-PPT tolerant chicory, rape and maize are grown commercially.

Annals of Botany **88**: 597–603, 2001

Herbicide-tolerant Transgenic Pineapple (*Ananas comosus*) Produced by Microprojectile Bombardment

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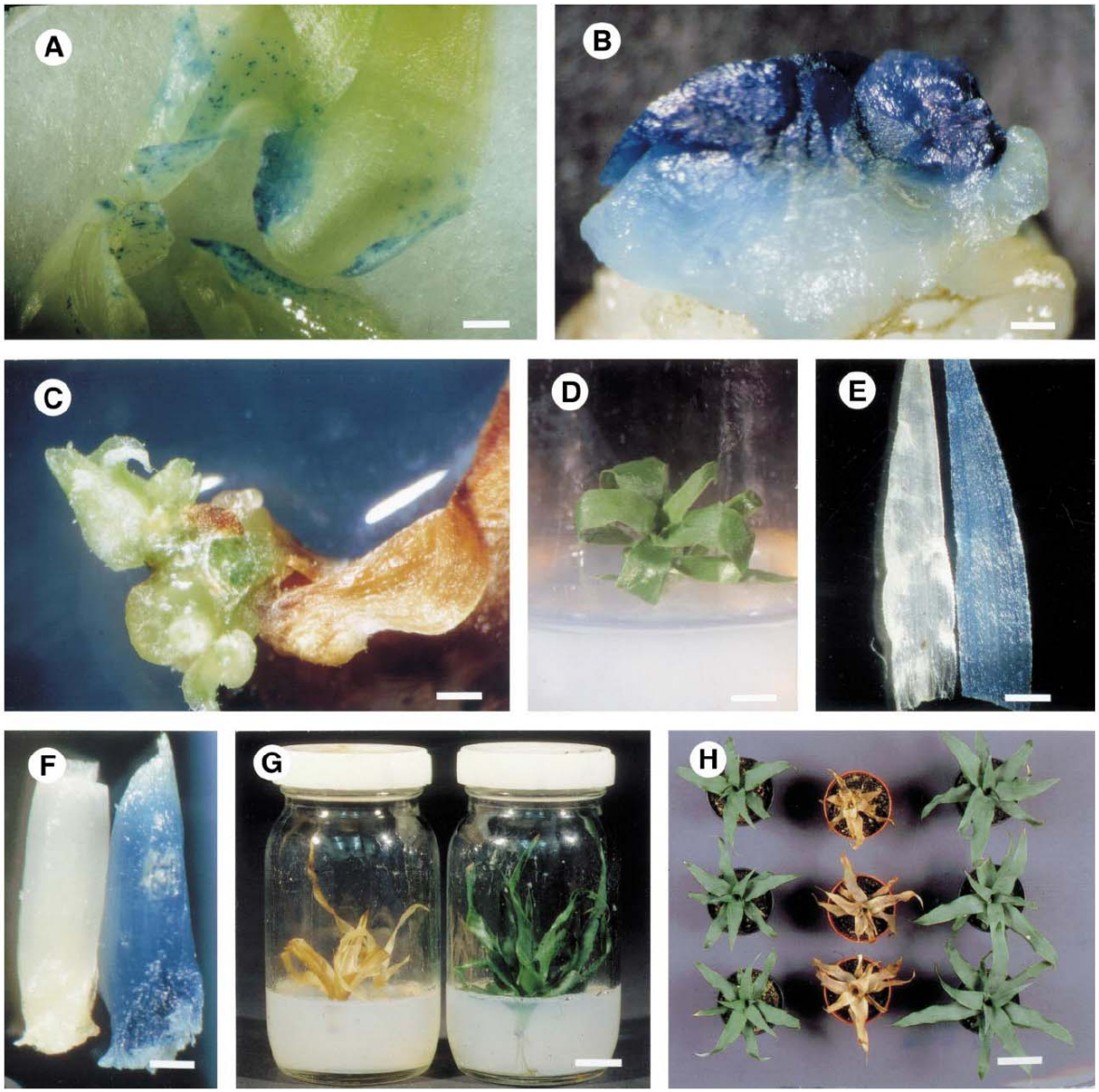
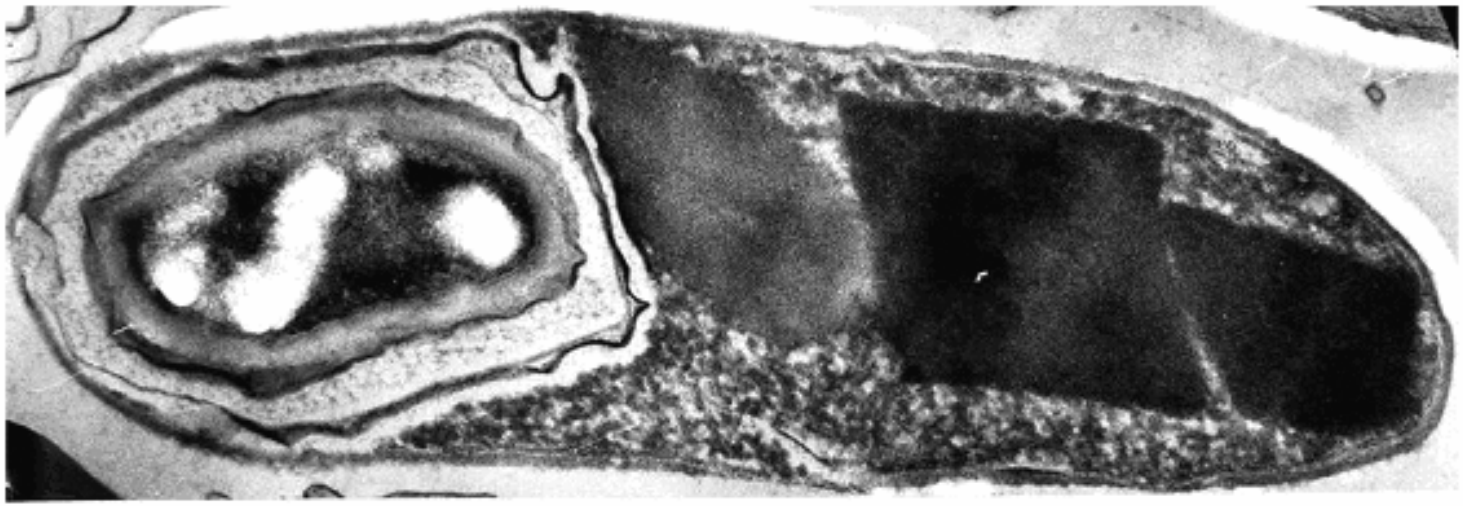


5.2. Insect resistant plants

Bacillus thuringiensis source of insecticidal toxins

The source of the insecticidal toxins produced in commercial transgenic plants is the soil bacterium *Bacillus thuringiensis* (Bt). One of the main insecticides Bt produces is a large protein that must be activated before it has any effect. The protein forms highly insoluble crystals under normal conditions, so it is safe to most animals. It is solubilised in the conditions found in the gut of lepidopteron larvae. For this reason, Bt is a highly specific insecticidal agent.

Bt strains show differing specificities of insecticidal activity toward pests, and constitute a large reservoir of genes encoding insecticidal proteins which are accumulated in the crystalline inclusion bodies produced by the bacterium on sporulation (Cry proteins, Cyt proteins) or expressed during bacterial growth (Vip proteins).



***Bacillus thuringiensis*; bacterial spore, mother cell and parasporal crystals**

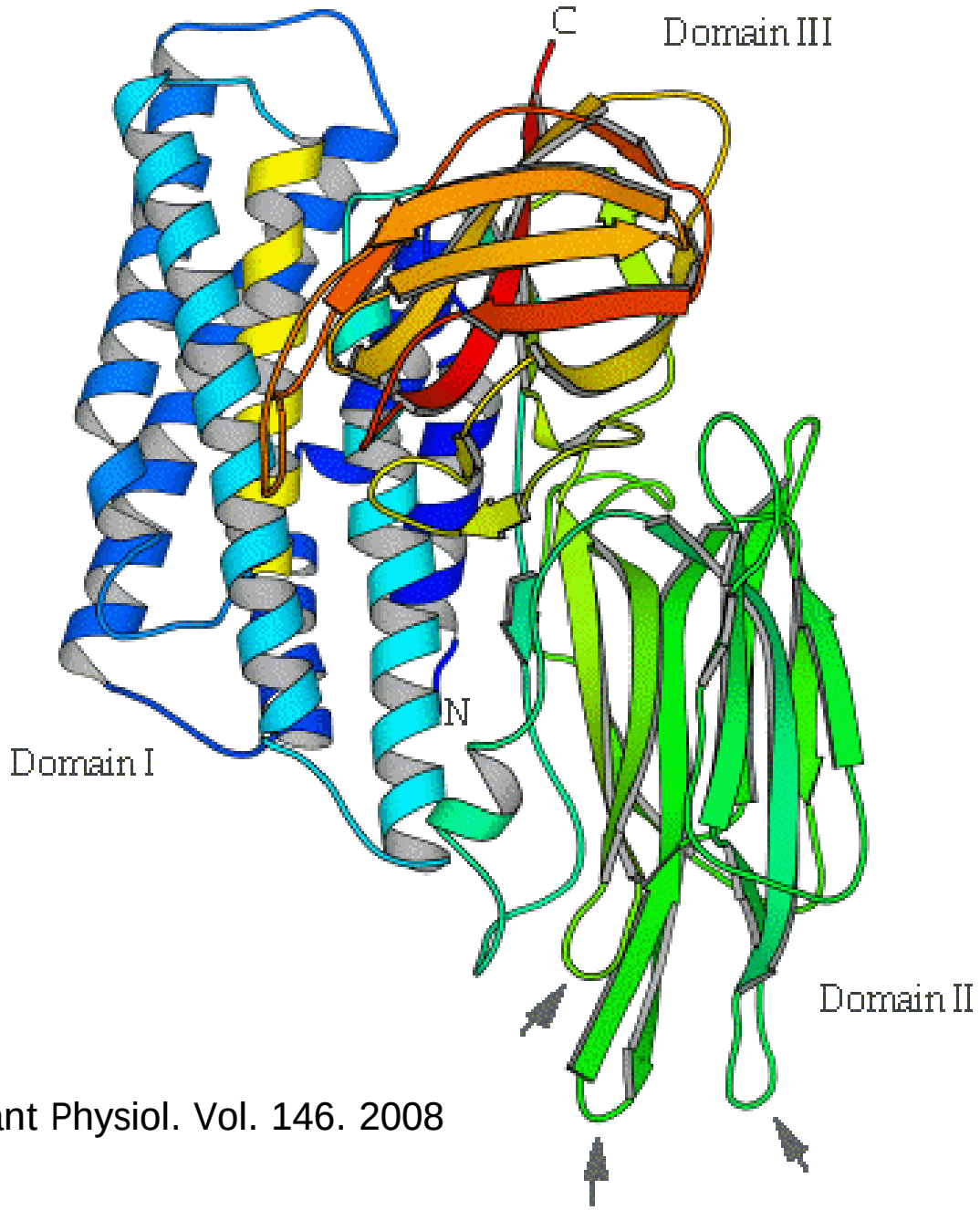
Photo source: *www.pathmicro.med.sc.edu*

Bt gene produces proteins that selectively kill certain groups of insects:

- *Toxicity in alkaline pH stomach of insect, must be ingested to kill*
- *Proteins bind to receptors intestines -> Insect stops eating.*
- *Used in granular or liquid form> 30 years as a pesticide*

Many (60) different Cry proteins ->effective against different insects

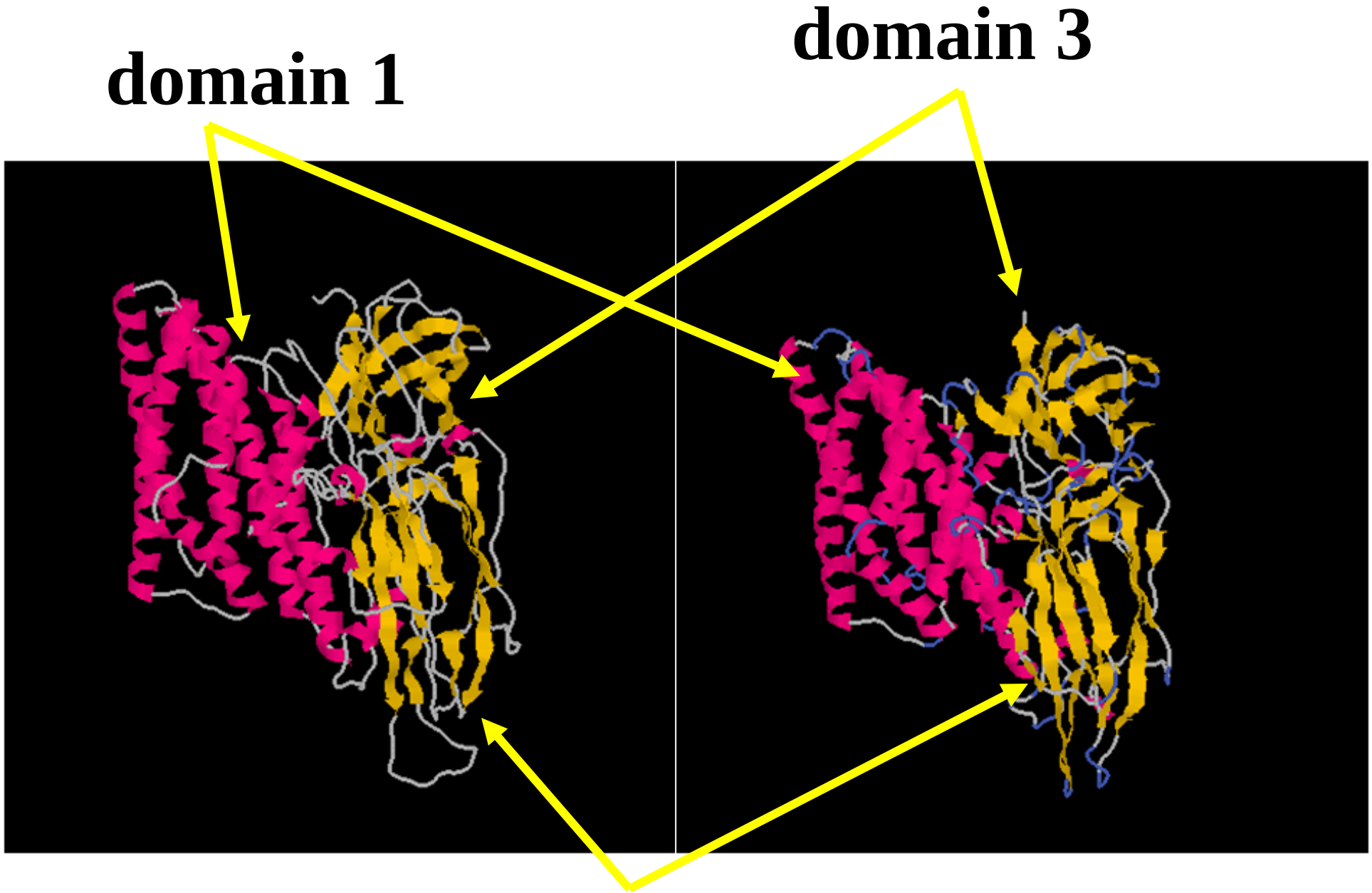
There are several Bt strains that can synthesize more than one crystal, formed by different cry toxins.



Source from Plant Physiol. Vol. 146. 2008

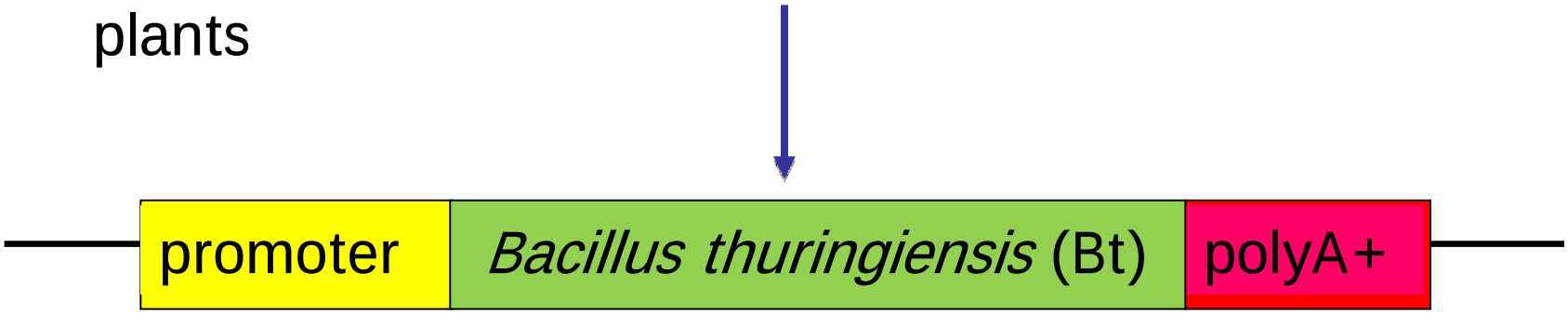
Classes of crystals proteins (> 120 allelles & 19 groups)

Class	Size (KD)	Insects affected
I	**130**	Lepidoptera (moth/butterfly)
II	**60-71**	lepidoptera & diptera
III	**73**	Coleoptera (beetle/weevil)
IV	**135,128,74,72**	Diptera (mosquito/flies)


domain 1
domain 3
CryIA
domain 2
CryIIIA

Bt-resistant genes Construct

Codon usage modified for efficient expression in plants



Regulatory sequences recognised by plant (either from plant gene or plant virus gene). Frequently use 35S CaMV promoter

The three domain Cry proteins have been extensively studied. Their mechanism of action involves a proteolytic activation step, which occurs in the insect gut after ingestion, followed by interaction of one or both of domains II and III with “receptors” on the surface of cells of the insect gut epithelium. This interaction leads to oligomerization of the protein, and domain I is then responsible for the formation of an open channel through the cell membrane. The resulting ionic leakage destroys the cell, leading to breakdown of the gut, bacterial proliferation, and insect death.

Mechanism of toxicity of Crystal proteins

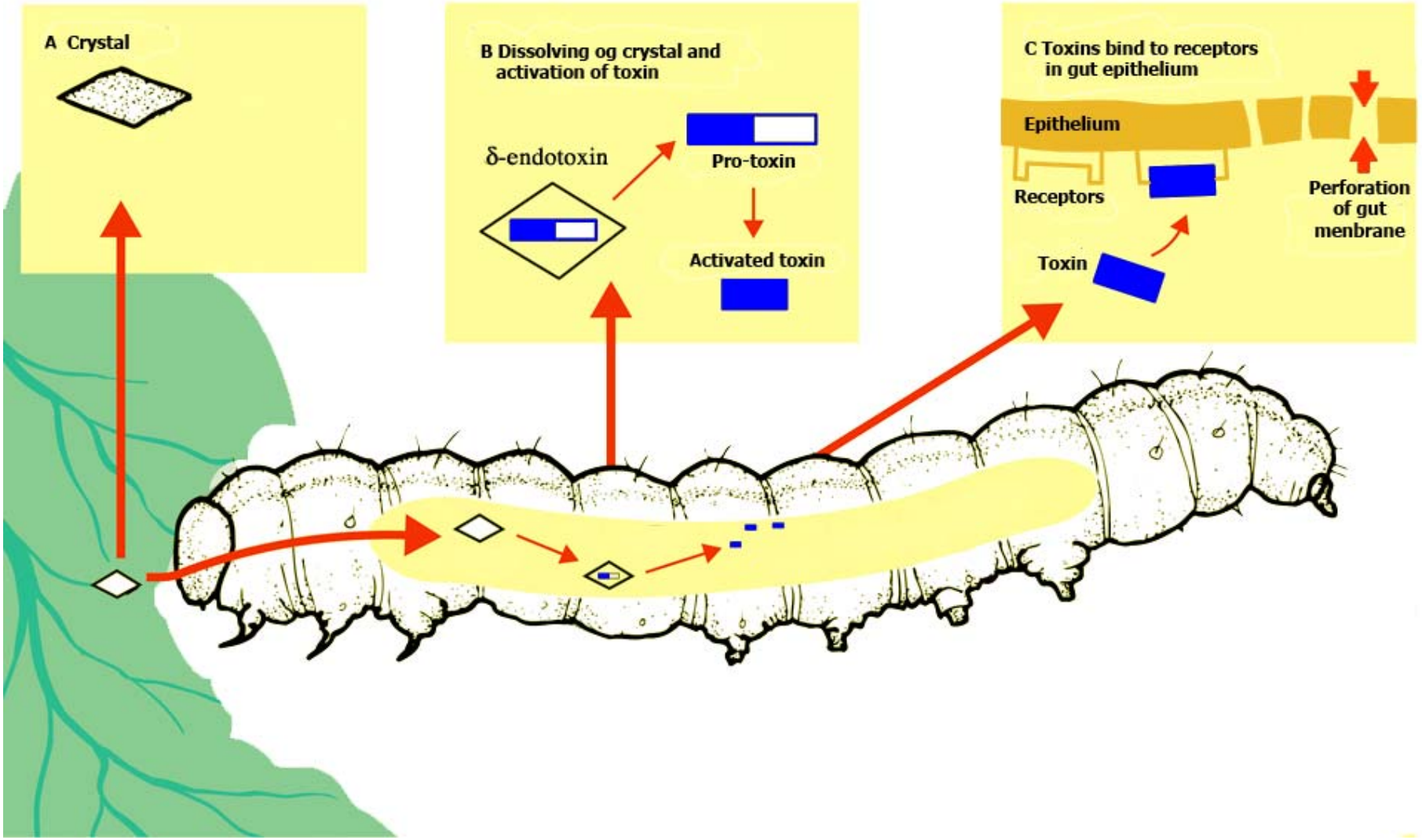
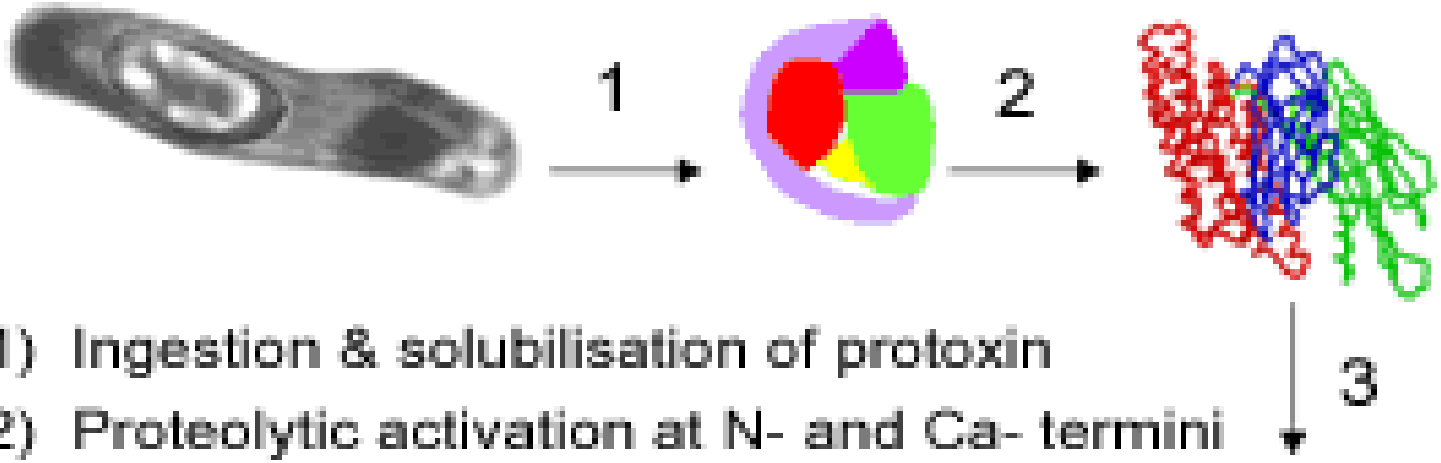


Image source: *www.virtualsciencefair.org*

Proposed mode of action of Cry toxins



1) Ingestion & solubilisation of protoxin
2) Proteolytic activation at N- and Ca- termini
3) Interaction with cell surface binding protein
4) Conformational change exposing α4-5 helical hairpin
5) Oligermisation & insertion in membrane to form pore

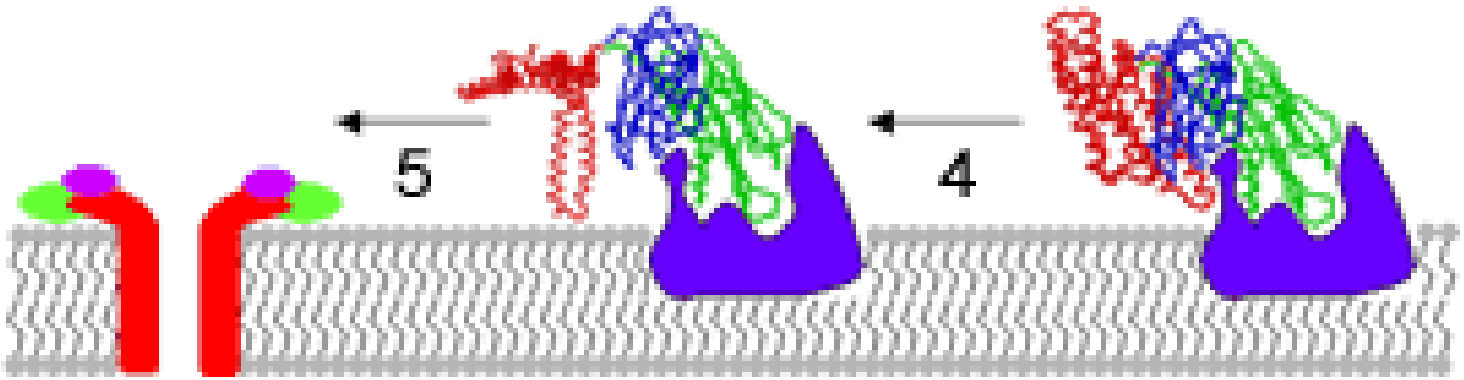
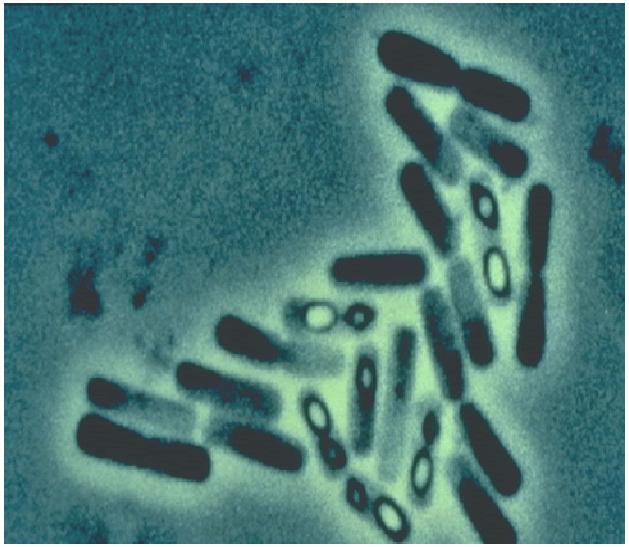


Image source : *www3.imperial.ac.uk*

Insect resistant plants

Bt gene → Bt toxin expression → pest control



Bacillus thuringensis

Transformed Plant

Lepidopteran Control

Photos source: *www.eoearth.org; www.biotechnology.gov.au; www.umbi.umd.edu*

Insect-resistant crops have been one of the major successes of applying plant genetic engineering technology to agriculture. Cotton (Gossypium hirsutum) resistant to lepidopteron larvae (caterpillars) and maize (Zea mays) resistant to both lepidopteran and coleopteran larvae (rootworms) have become widely used in global agriculture and have led to reductions in pesticide usage and lower production costs.

INGARD cotton in Australia

Successful in field operations

Photos source: www.acgra.net.

5.3. Virus resistance

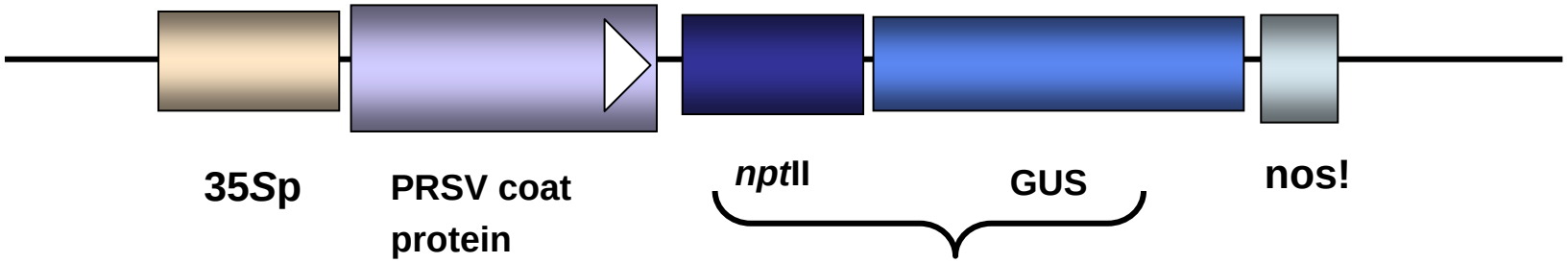
Transgenic Virus Resistant Papaya

Papaya ringspot virus (PRSV) was discovered in Hawaii in the 1940s and virtually eliminated large papaya production on Oahu island in the 1950s, causing the papaya industry to relocate to Puna district on Hawaii island in the early 1960s.

The papaya industry thrived in Puna because of ideal rainfall conditions, availability of land, and most importantly, because Puna was free of PRSV.

- *A gene from the pathogen was used to fight against the pathogen itself.*
- *Work progressed rapidly, primarily because the team (Gonsalves, Slightom, Manshardt, and Fitch) had a focused approach and had the right blend of expertise.*
- *Dr. John Sanford at Cornell University, Geneva, New York who had recently invented the gene gun, assisted in making a transgenic papaya.*
- *A papaya transformation system was developed whereby young embryos from papaya seeds of the commercial Hawaiian solo cultivar 'Sunset' were transformed with the coat protein gene of a PRSV isolate from Hawaii, and a promising transgenic papaya line (55-1) that showed resistance to PRSV from Hawaii was identified in 1991.*

Virus resistant Papaya Construct



Transgenic papaya inoculated with PRSV from Hawaii (left) and nontransgenic papaya inoculated with PRSV from Hawaii (right). Note the resistance of transgenic papaya.

Source: *www.apsnet.org*

Although the exact mechanism by which the viral protection occurs is unknown, most evidence suggests that expression of viral CP by a plant interferes with one of the first steps in viral replication, uncoating (removal of CP) from the incoming virus (Register & Nelson 1992).

Other modes of action of cross-protection have also been suggested.

Transgenic line 55-1 of Sunset was inbred to homozygosity for the single copy coat protein gene and named SunUp.

The Rainbow cultivar was developed to create a virus-resistant, transgenic yellow-fleshed papaya to replace virus-susceptible Kapoho.

Rainbow is an F1 hybrid from SunUp and nontransgenic Kapoho.

In 2001, 40 million pounds were produced from 1,675 bearing acres with Kapoho and Rainbow accounting for 39 and 41% of the acreage, respectively.

Photos source: *www.apsnet.org*

Varieties of transgenic papaya, squash, and potato have been produced that display resistance to infection and subsequent disease caused by plant viruses.

In the case of potatoes, the only one of these crops used as livestock feed, resistance to potato virus Y (PVY) and potato leafroll virus (PLRV) has been introduced by inserting DNA sequences corresponding to the virus coat protein (CP) or the viral replicase, respectively.

5.4. Improve the post-harvest biology of fruits and flowers

Biochemistry of Fruit Ripening and Softening

Fruit ripening and softening is a highly controlled developmental process. Firmness of fruit is a function of the properties of the cell wall (Cellulose fibers, Pectins, Hemicellulose and Proteins). Various enzymes that degrade specific components of the cell wall are synthesized during fruit ripening (i.e. Cellulase -breaks down cellulose; Polygalacturonase (PG) and pectin methyltransferase (PME) -break down cross-linking pectin molecules). To delay fruit ripening, slow down the ethylene response of the ripening pathway. This approach or technique could be used on any **climacteric** fruit.

Strategies to improve post-harvest shelf life

There are three main ways to improve post-harvest shelf life:

1)By blocking the expression of genes (such as PG and PME) that are induced in response to ethylene (i.e. Flavr Savr tomato).

2) By blocking ethylene synthesis.

3) By blocking the reception to ethylene.

Expression of a dominant mutant ethylene receptor

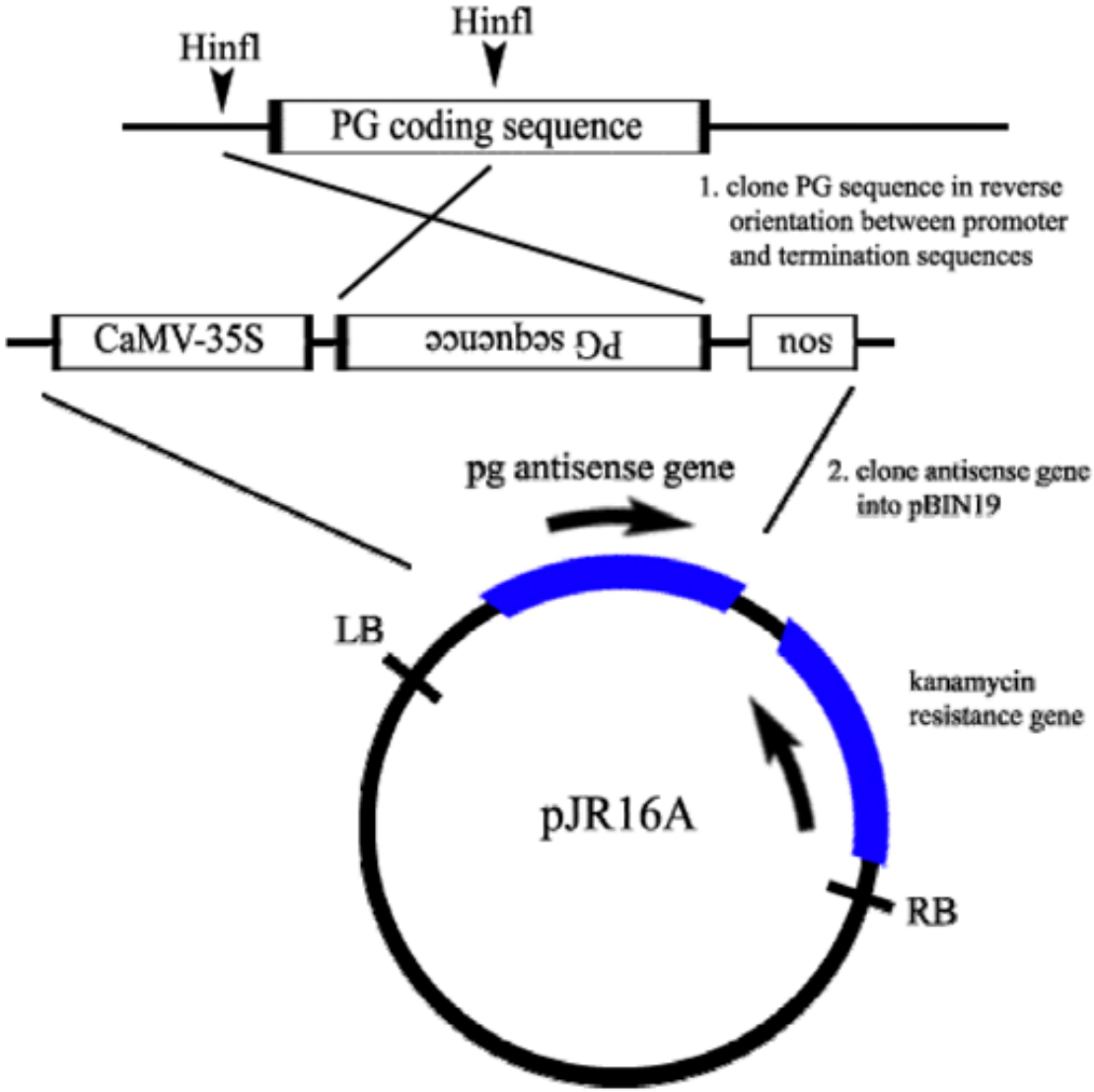
Using Antisen technique to Reduce PG Expression in Tomatoes

1. Clone the PG gene from tomato and construct a chimeric gene to express antisense RNA for PG in the fruit.

   A promoter that is highly active in fruits - The ORF of PG, flipped so that antisense RNA will be transcribed - A transcription terminator

2. Transform to produce transgenic fruit with this antisense RNA gene.
3. Evaluate these transgenic plants.

PG antisene gene Construct



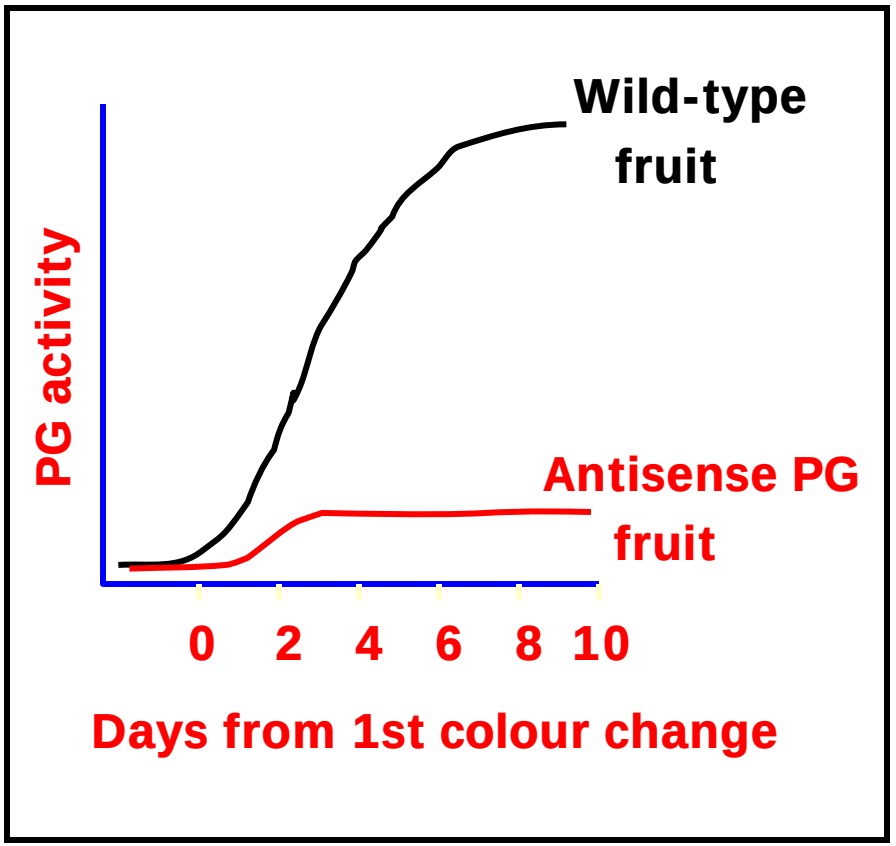
Altering Fruit Ripening with Antisense RNA

Polygalacturonase (PG) is an enzyme that breaks down pectin in ripening fruit walls.

Plants with an antisense PG transgene produce less PG.

Walls soften more slowly.

Many genes manipulated in the same way to answer basic questions: what is the role of hormones in ripening; what do particular enzymes do in fruit walls?



Using Antisense technique to Reduce PME Expression in Tomatoes

PME is involved in metabolism of pectins in the cell wall. Pectin in mature green fruits are long polymers. During fruit ripening PME breaks down the long polymers to short polymers.

The same antisense RNA technique was used to reduce the PME expression in fruits.

Longer polymers increased the viscosity of juice from transgenic low-PME fruits than the control samples.

Antisense PG and PME Transgenic Fruits

Antisense PG technique used by Calgene to produce FLAVR SAVR tomatoes failed to produce desired post-harvest quality.

Antisense PME technique was good for viscosity of juice and was used to make good quality tomato paste faster but did not improve shelf life.

In both cases, the reason may be that nothing was done to reduce ethylene production.

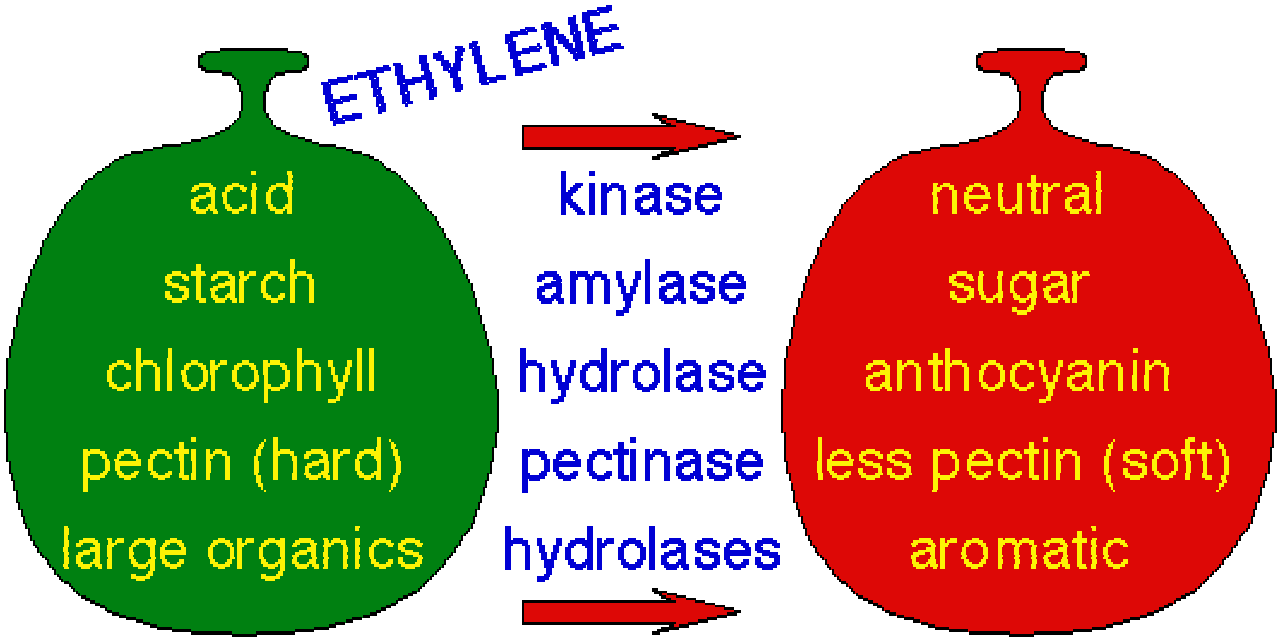
Using Antisen technique to Modify Ethylene Responses in Transgenic Plants

- ✓Ethylene, a natural hormone produced by some fruits as they ripen, promotes additional ripening of produce exposed to it.
- ✓Damaged or diseased apples produce high levels of ethylene and stimulate the other apples to ripen too quickly.
- ✓As the fruits ripen, they become more susceptible to diseases.

- ✓ Some climatic fruits as: avocados, papayas, passion fruit, peaches, persimmons, plantains, prunes, tomatoes, etc. are highly sensitive to ethylene
- ✓ Ethylene "producers" should not be stored with fruits, vegetables, or flowers that are sensitive to it.
- ✓ The result could be loss of quality, reduced shelf life, and specific symptoms of injury.
- ✓ Most climacteric fruit picked green, shipped to market and treated with ethylene before sale at wholesale level.

The autocatalytic synthesis of the gaseous hormone ethylene regulates the expression of specific genes involved in tomato ripening.

In climacteric fruits like apple the exponential increase in ethylene production coincides with a rise in respiration and correlates with the development of FFC (Fruit Flavor Composition).

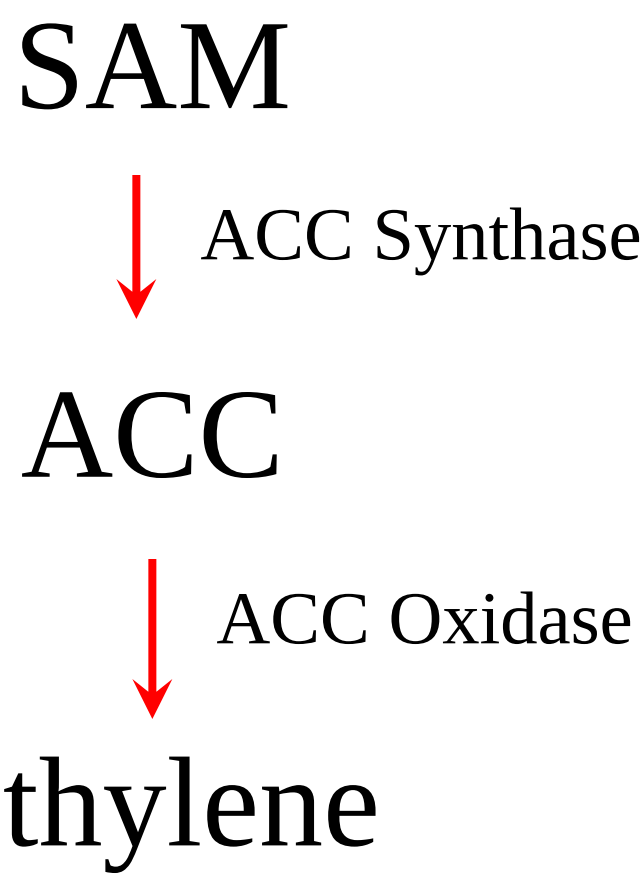


Biosynthesis of Ethylene

Biosynthesis of ethylene occurs in two enzymatic steps:

S-adenosyl methionine is converted to ACC (1-aminocyclopropane-1-carboxylic acid) by ACC synthase (ACS).

ACC is converted by ACC oxidase to ethylene.



Biotechnology to Modify Ethylene Responses in Transgenic Plants

Ethylene – changes in gene expression – initiation of ripening or senescence

Ethylene – binds to receptor – signal transduction pathway – changes in gene expression

Transgenic plants with ethylene response blocked:

- Fruit will not ripen, even if exposed to ethylene (i.e. Apple).
- Flowers will not abscise (i.e. Carnation).

Ethylene Suppressed Transgenic Plants Approach

- ✓ Transgenic apple plants were produced in which the genes coding for key enzymes of ethylene biosynthesis were silenced. (i.e. ACC synthase)
- ✓ Ethylene suppressed fruits were firmer and displayed increased shelf-life.
- ✓ Sugar and acid accumulation was not different from controls.
- ✓ Same approach with down regulated ACC synthase Carnation flowers.

Alternative Ansätze

60 Tage

Antisense
ACC-Synthase
78 Tage

Antisense
ACC-Synthase
78 Tage
(+ Ethylen)

Ethylen-Synthese blockieren

Methionin

↓

S-Adenosyl-Methionin

"Co-Suppression"
"Antisense"

↓ ACC-Synthase

1-Amino-Cyclopropan-1-Carboxylsäure (ACC)

↓

Ethylen

DNA Plant Technology Corp. (USA) - "Endless Summer" (1995)

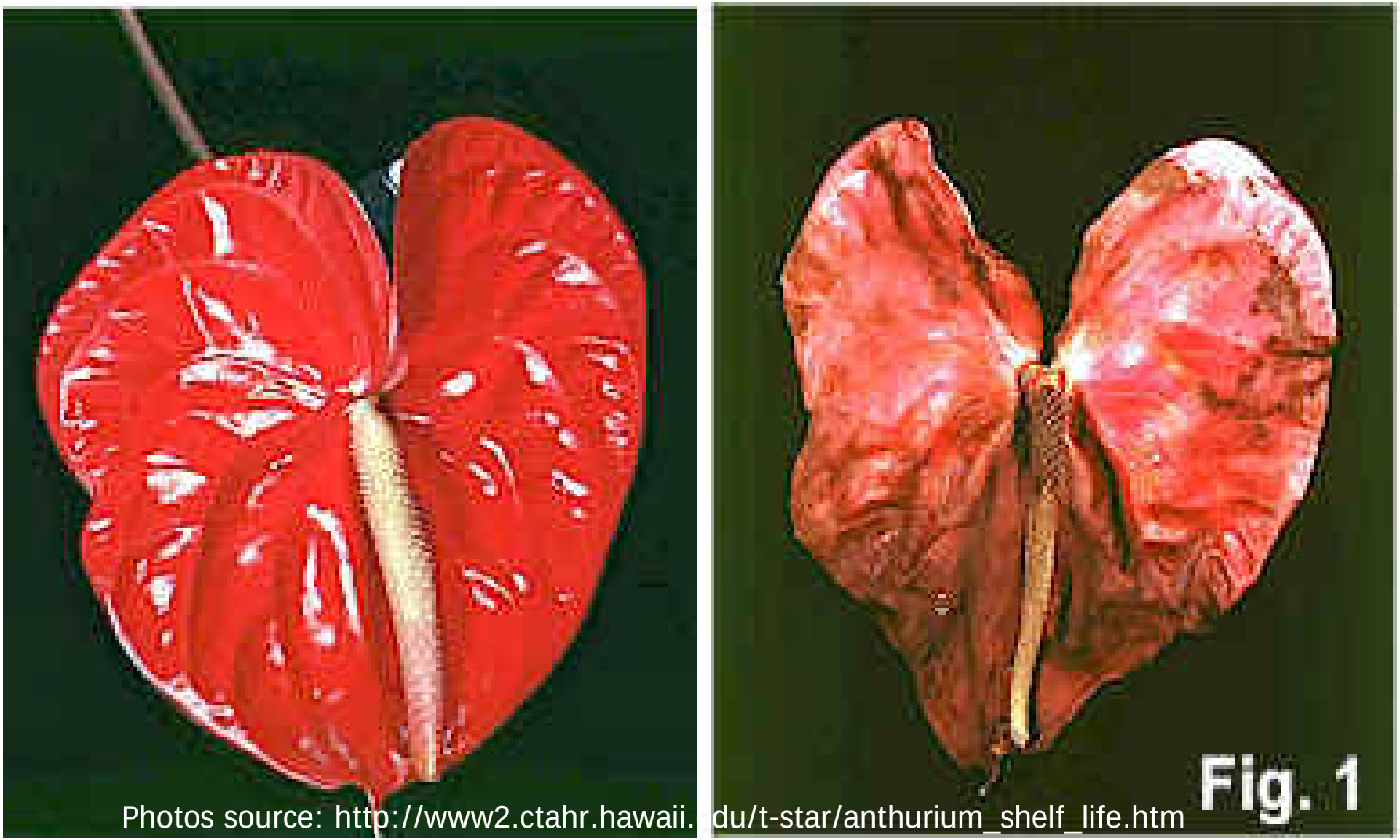
Long-life carnation with down regulated petal ACC synthase (Florigene Ltd.). The native carnations (left) senesced after 2 weeks of harvest while the transgenic carnations (right) have comparable vase-life to STS treated ones (center).

Source from Plant Cell, Tissue and Organ Culture 80: 1–24, 2005

Improving Post Harvest Shelf Life of Flowers

- ✓ Anthuriums are a major cut flower produced in Hawaii.
- ✓ Annual sales $ 5-7 million
- ✓ The natural plant hormone cytokinin plays an important anti-aging role in plants.
- ✓ Researchers have identified the gene that regulates cytokinin production in plants.
- ✓ Now they are trying to over-express those regulatory genes to increase cytokinin levels in *Anthurium* plants.

Increasing *Anthurium* Flower Production and Shelf Life



Photos source: http://www2.ctahr.hawaii.edu/t-star/anthurium_shelf_life.htm

Fig. 1