Gene engineering and biotechnology of higher plants II

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Biotechnology of Drugs 2024

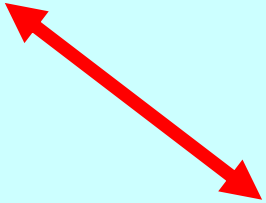
Content

1. Cloning of secondary metabolites
2. Gene knocks-...
3. Colour changing in flowers
4. Transgenic plants identification
5. Plantibodies
6. Examples of other recombinant plants

Molecular farming

- **Application of molecular biology methods to the synthesis of commercial products from plants**

Increased production of compounds that are “natural“ for plant



Production of completely new (for plant) compounds

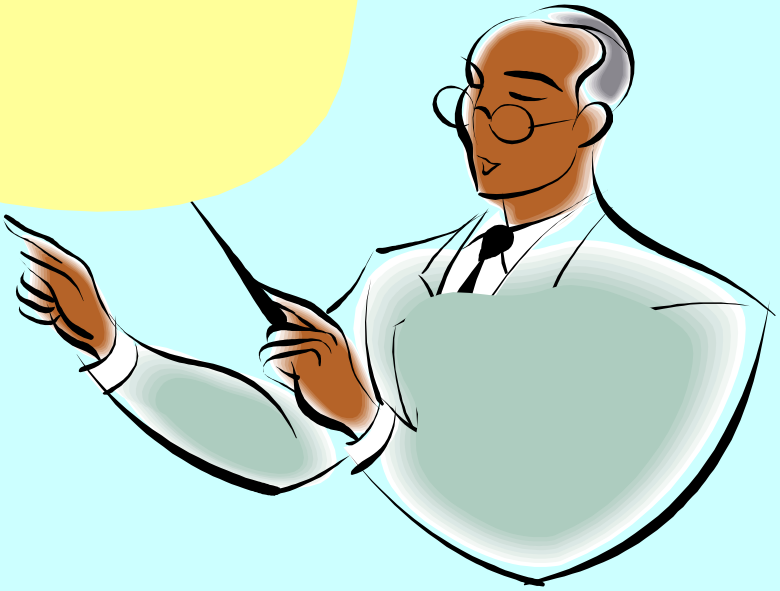
- **Proteins**
- **Carbohydrates and plant oils**
- **Secondary metabolites**

What can be manipulated in plants?

- **Structural proteins - e.g. Zein**
- **Phytohormones - genes for enzymes**
- **Secondary metabolites - genes for enzymes**
- **Expression intensity - regulatory genes**

Cloning of secondary metabolites

… cloning of genes for enzymes



Manipulation with secondary metabolites

Objective ?

- better nutritional properties of plants
- disease prevention

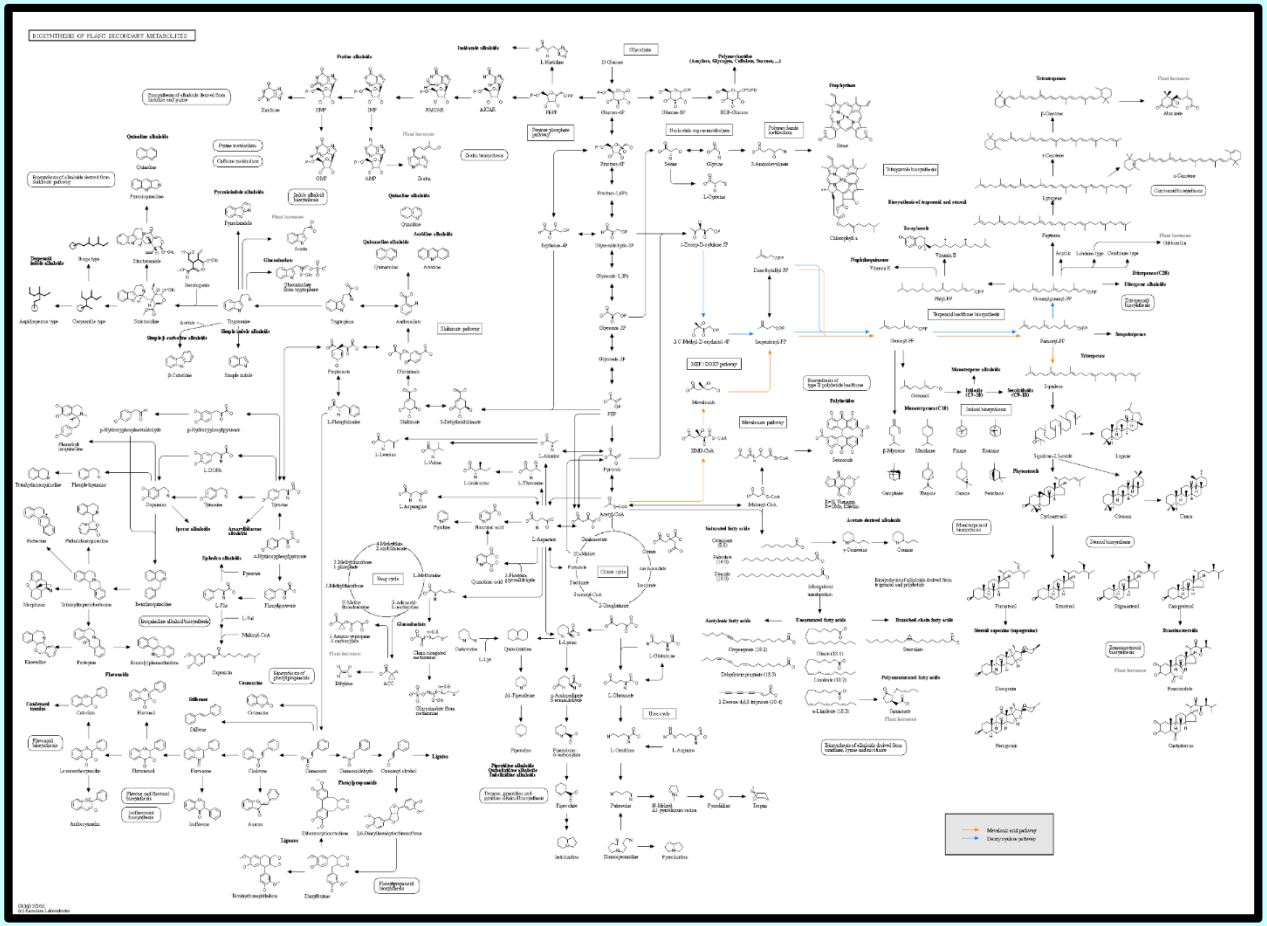
How ?

- enrich plants with functional components or modify them
- eliminate antinutritional and allergenic substances
- genetic modification of biosynthetic pathways

What all do we need to know ?

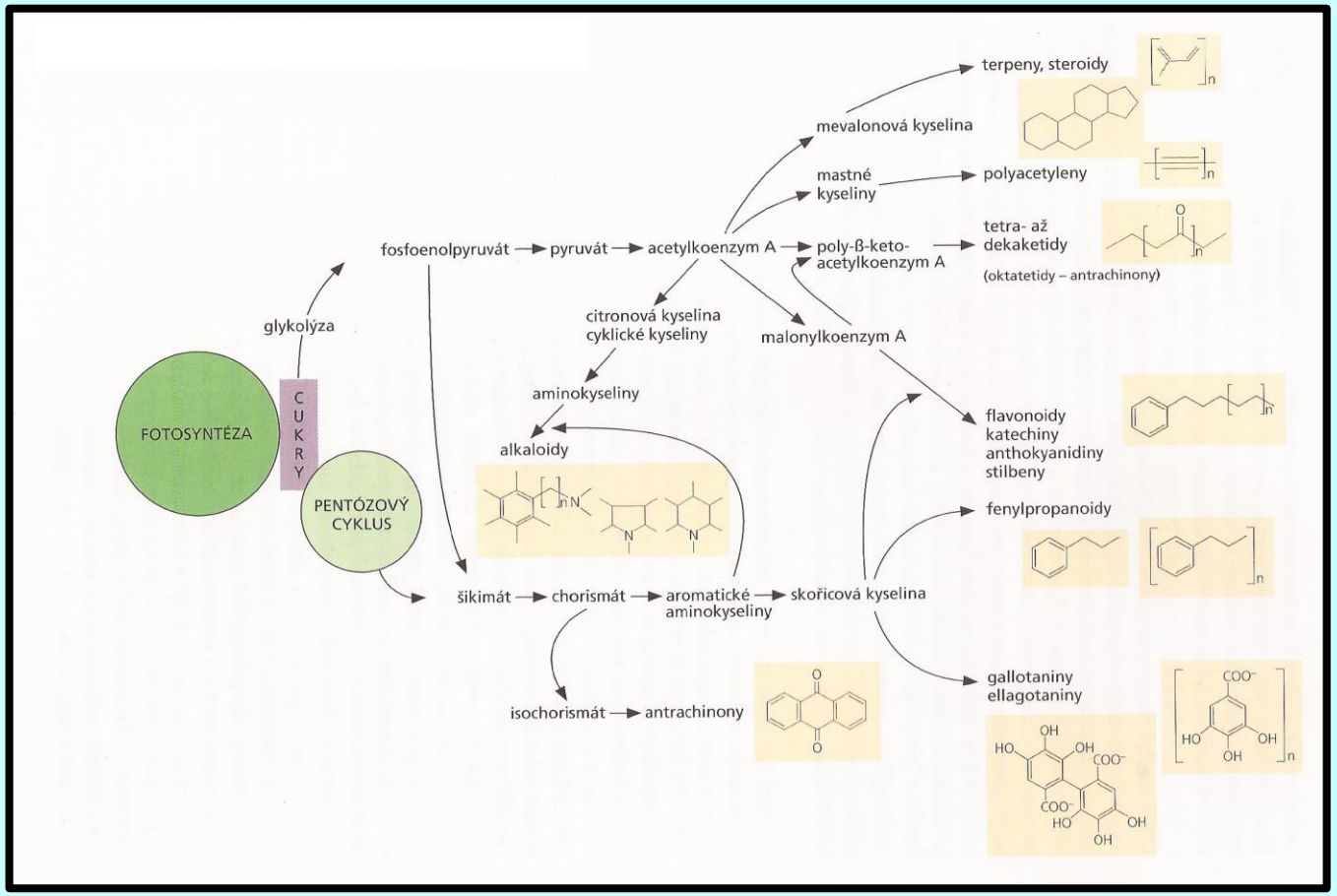
- biosynthetic pathways of the substance
- genes/proteins (enzymes) involved in biosynthesis
- the physiology of the plant used

Biosynthesis of secondary metabolites



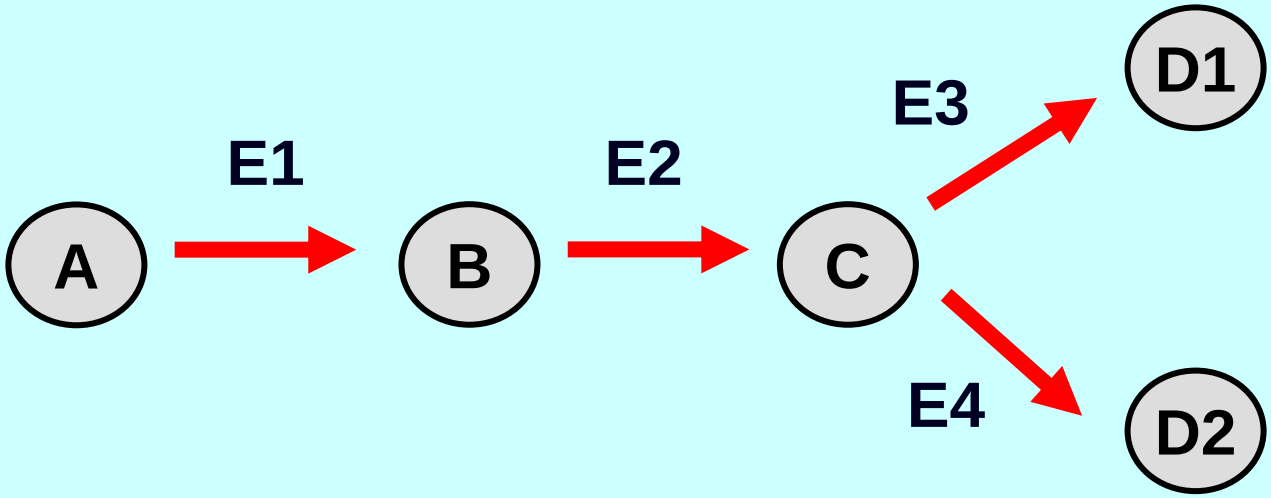
https://www.genome.jp/pathway/map01060

Biosynthesis of secondary metabolites – specific part

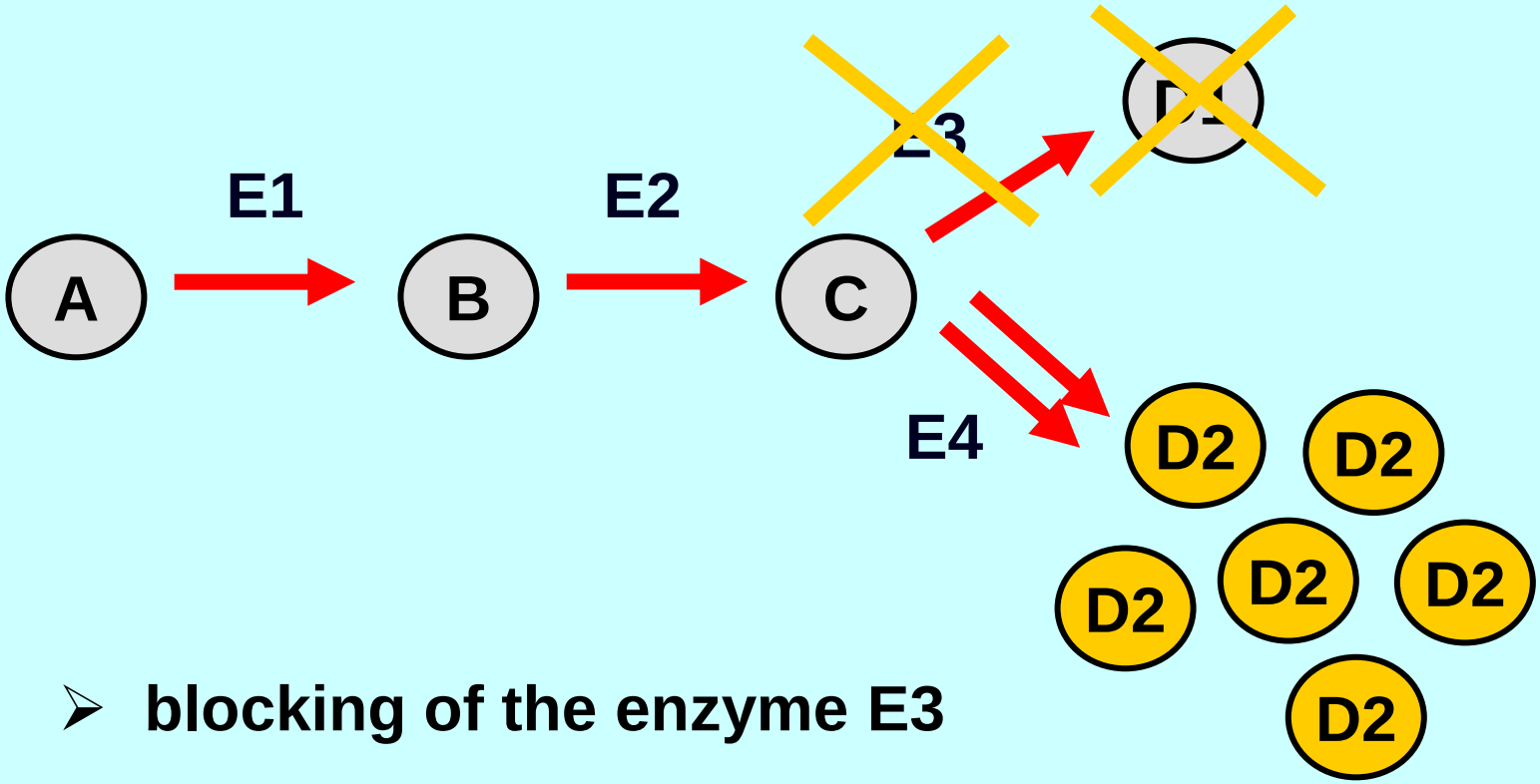


according to Jahodář: Farmakobotanika 2006

“Cloning“ of secondary metabolites



Increasing the production of metabolite D2



- blocking of the enzyme E3
- E3 = gene “knock-out“

Gene “knock-out“

Removing the gene function

- **Exchange of gene by its non-functional copy by recombination – insertion, deletion, substitution**
- **Insertion mutagenesis**
- **Non-sense codon formed by mutation**
- **Mutation, which changes the purpose of codon; function of enzyme is changed**
- **Blocking the function of gene by RNA interference**
- **Using CRISPR/Cas9 system**

Next “knock-...“

Knock-down

- Deactivation of gene function
- E.g. expression of a suppressor, use of a weaker promoter

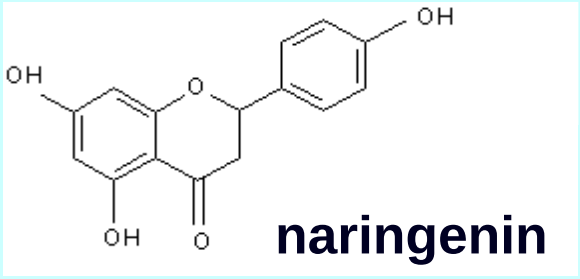
Knock-in

- Introducing a new gene with a new function

Knock-up

- Amplification of gene function
- E.g. expression of a strong transcription factor, use of a strong promoter or enhancer

Biosynthesis of isoflavonoids



naringenin

chalcone isomerase

CHALCONE

KNOCK-OUT

hydroxy-isoflavone synthase

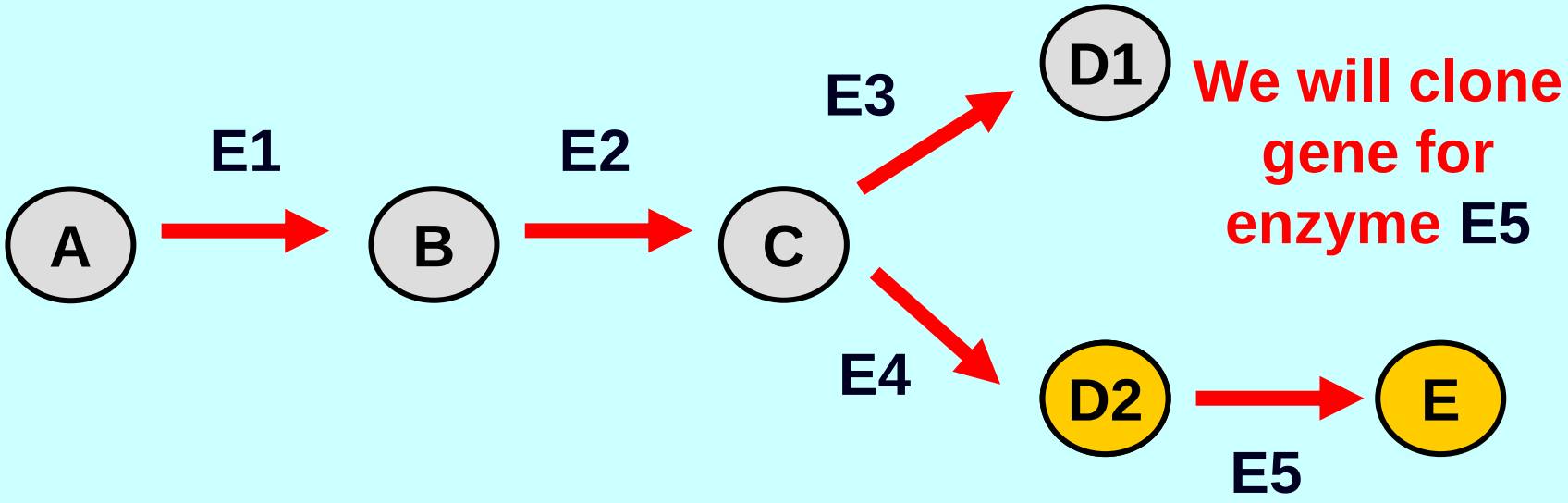
AURONES
FLAVONES
FLAVONOLS

isoflavones

Increased production

Formation of a new product

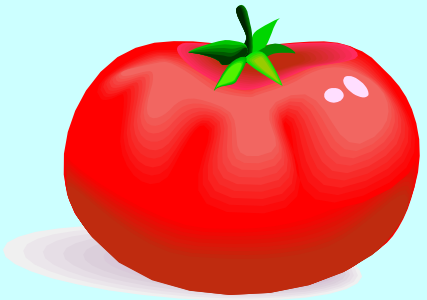
Gene “knock-in“



- **we want to add functional group to the metabolite D2**
- **we will create a new secondary metabolite E**

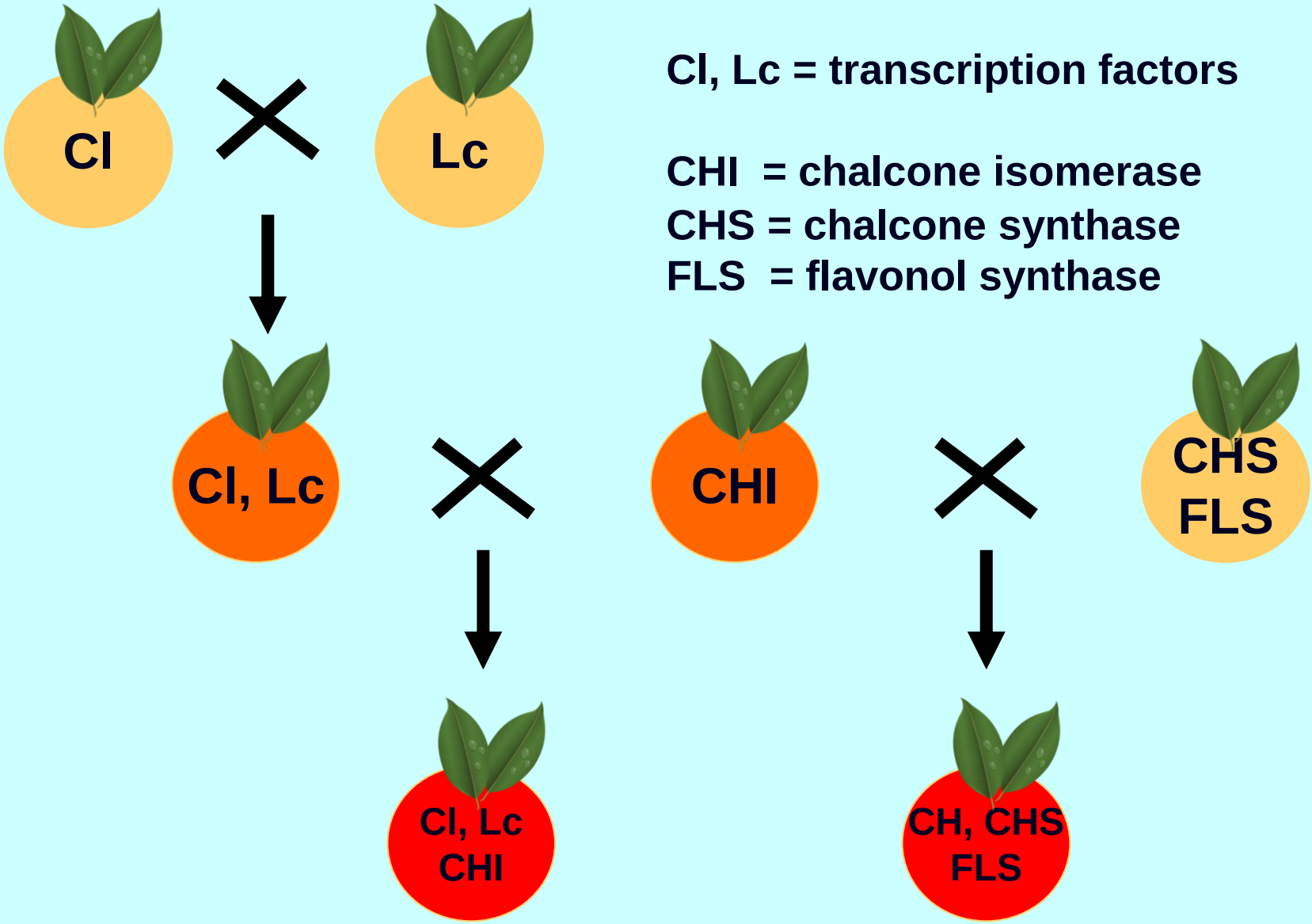
Accumulation of flavonols in tomatoes

In the flesh of tomato, the expression of genes for the synthesis of flavonoids (pal, chs, chi, f3h and fls) is very low



1. **Ectopic expression of suitable regulatory factors**
2. **Expression of 2-hydroxy-isoflavone synthase (HIS)**
3. **Expression of HIS with chalcone isomerase**
4. **Combined expression of HIS, genes or transcription factors responsible for the early phases of synthesis and inhibition of genes for competitive synthesis**

Accumulation of flavonols in tomatoes

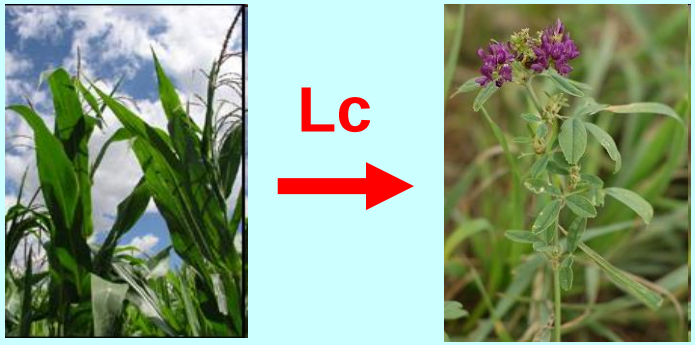


Cl, Lc = transcription factors

CHI = chalcone isomerase
CHS = chalcone synthase
FLS = flavonol synthase

Increasing the proanthocyanide levels

Transformation by transcription factor Lc from *Zea mays*



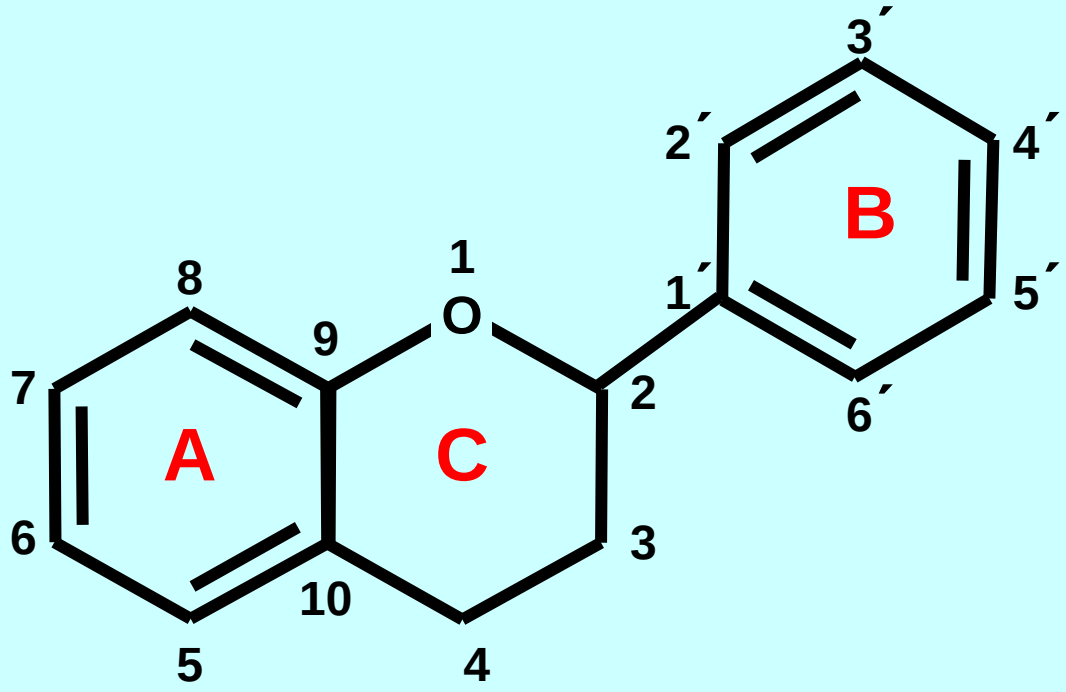
Lc transgenic alpha alpha (*Medicago sativa*)

- **Increased level of proanthocyanides influence the digestion of ruminants → pasture with better nutrient features**
- **It is still influenced by the environment – light stress, low temperatures…**

Change of colour in flowers

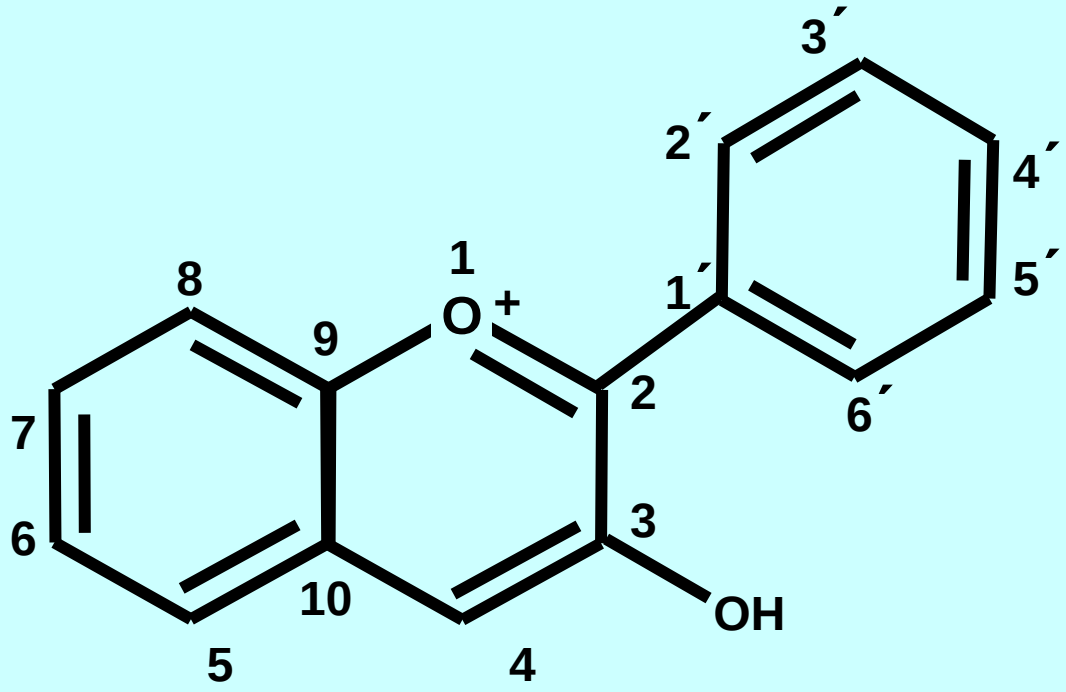
Flavonoids

General structure of flavonoids

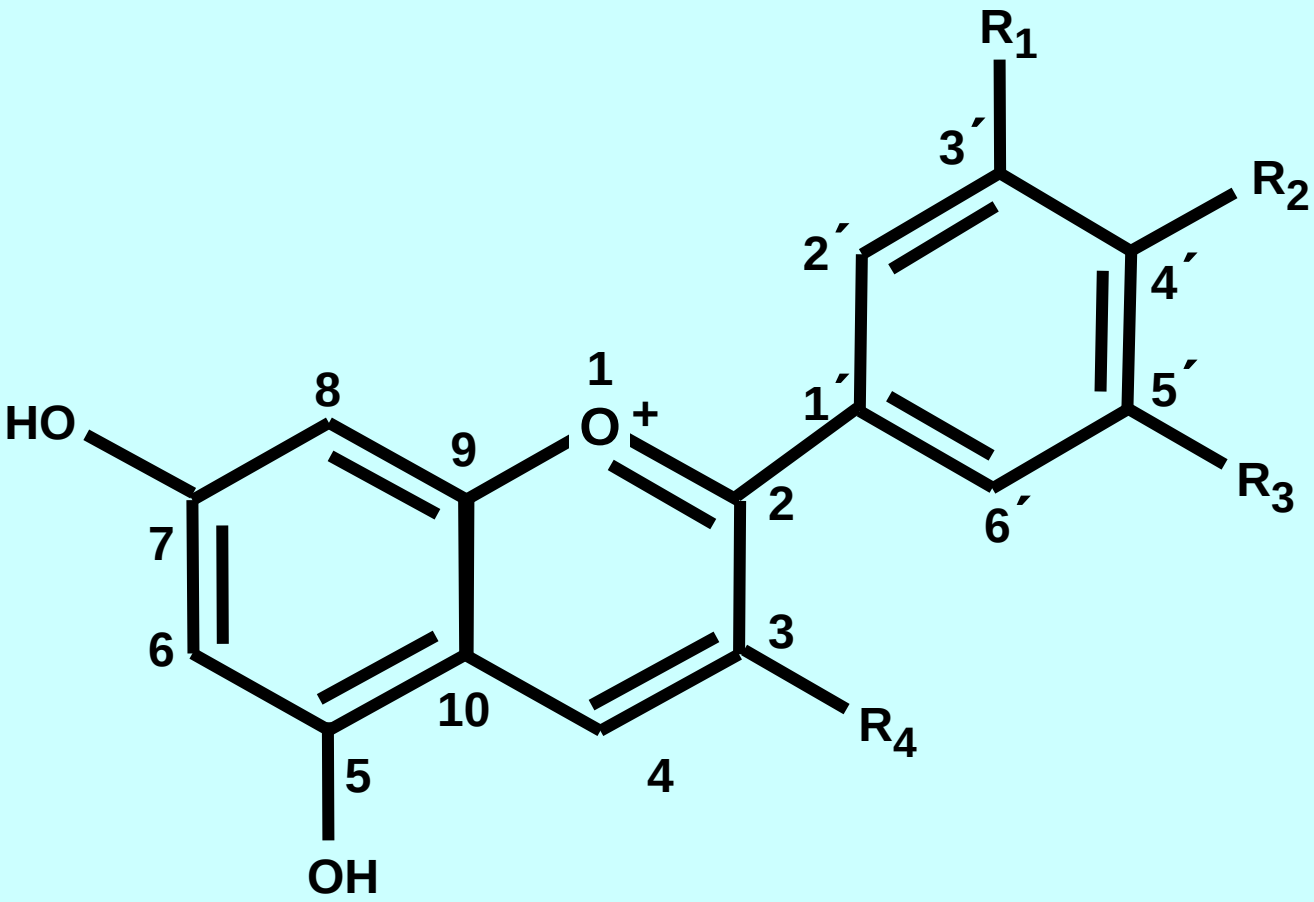


Anthocyans, Flavonols, Flavones, Catechins, Flavanols

Anthocyans

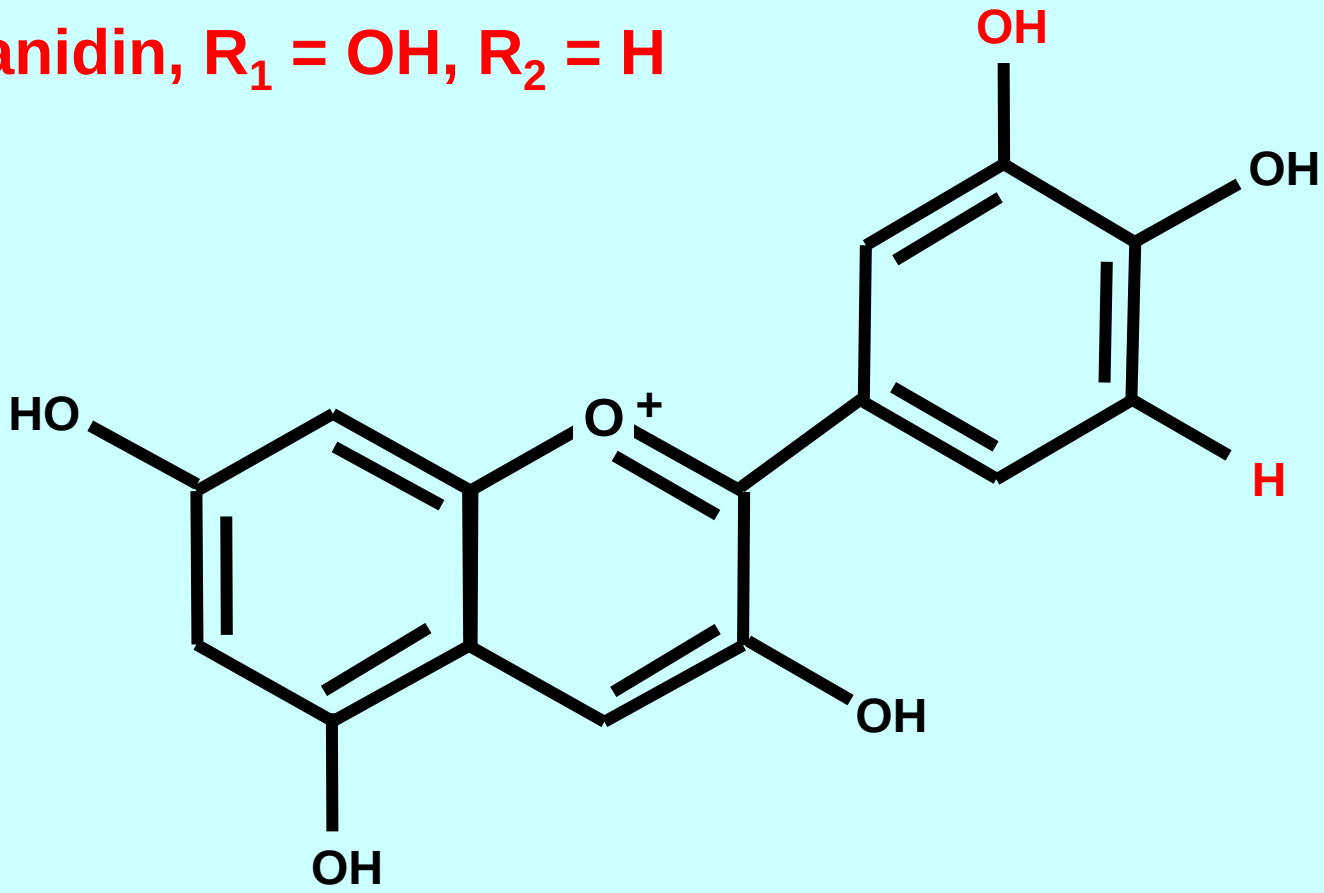


Anthocyans



Anthocyanidins

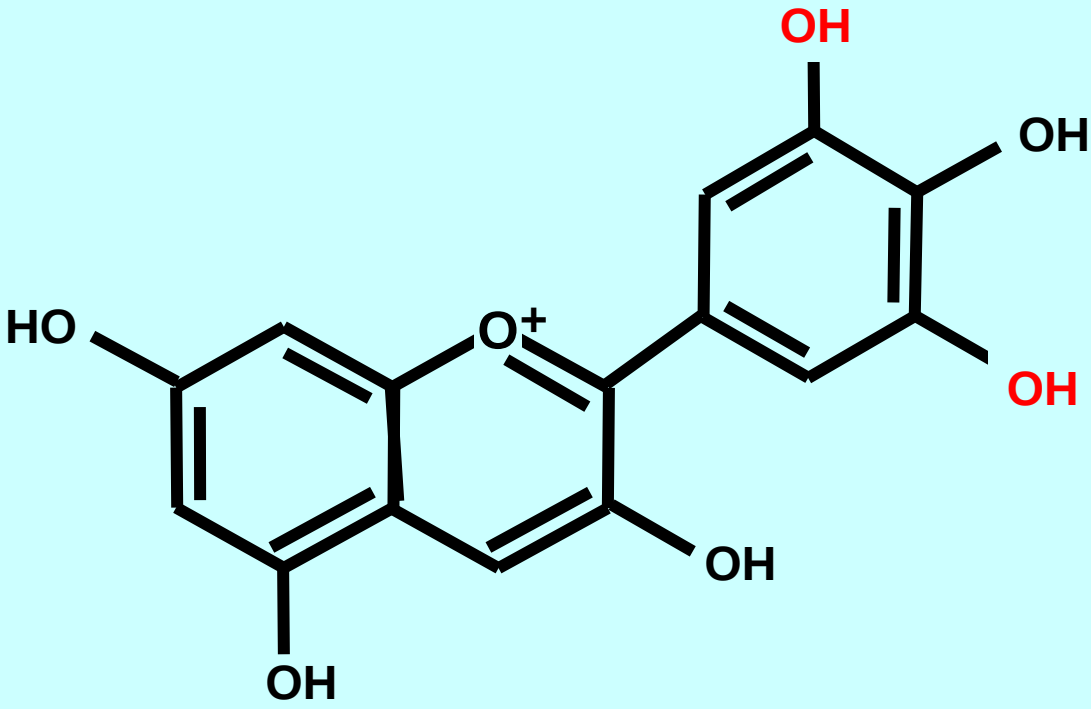
Cyanidin, R_1 = OH, R_2 = H



Cyanidin, Delphinidin, Malvidin, Peonidin, Petunidin

Change of colour in flowers

Hydroxylation of B ring of anthocyans



- **Gene for F3‘5‘H + specific cytochrome b5 → blue and violet gillyflowers, roses**

Blue lavender carnation in USA

- **The Florigene carnation varieties are genetically modified to alter the flower colour.**
- **They are intended for ornamental use only. Florigene has approval to market these flowers in the European Union**

https://www.florigene.com/product

BlueOcean chrysanthemums

- **Launched in fall of 2023.**
- **These spray type chrysanthemums are in a violet-blue color range going from a deep purple to a lavender-blue tone.**

Coral

Jade

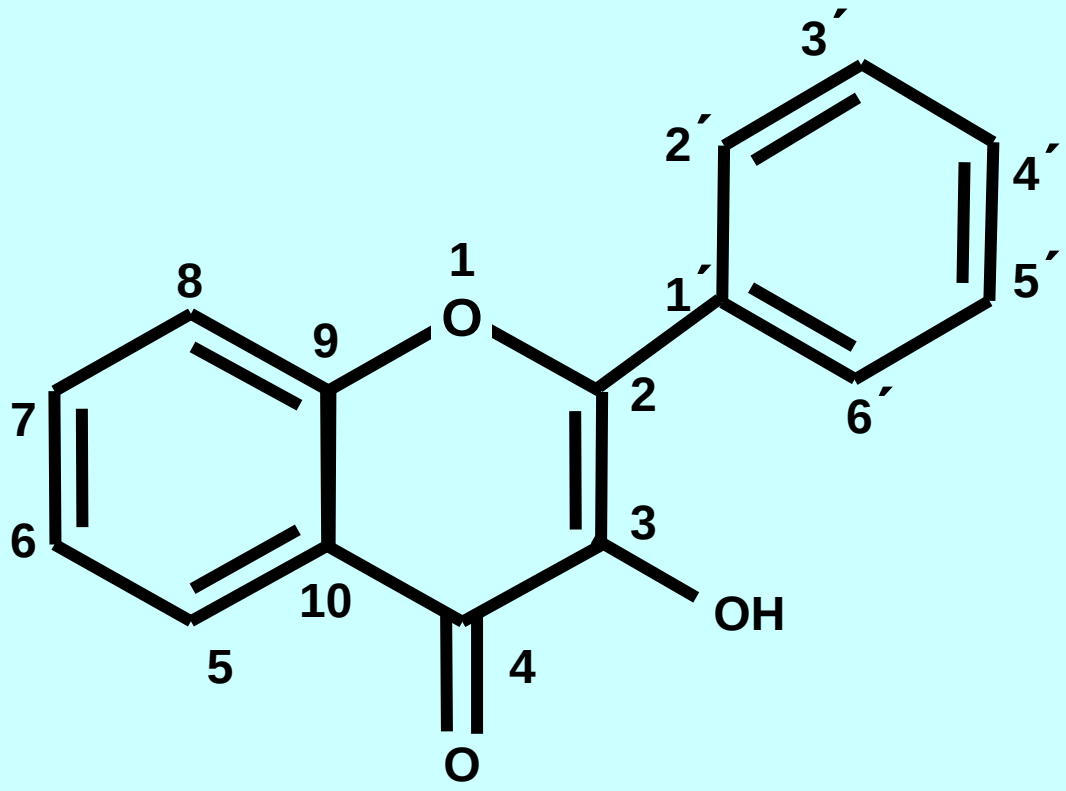
Opal

Purple saphire

Saphire

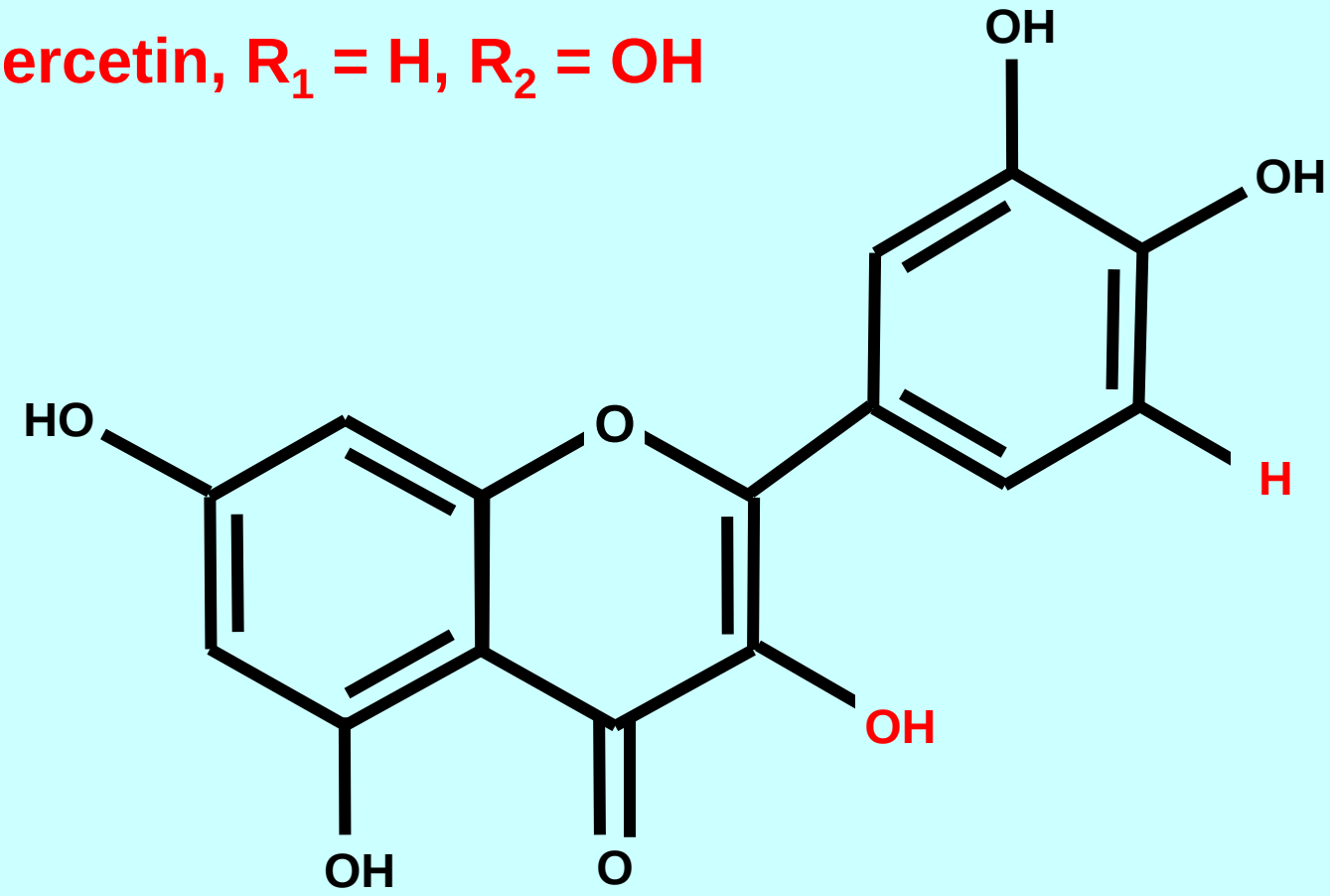
https://dinosaur-radish-hbwt.squarespace.com/bluocean

Flavonols



Flavonols

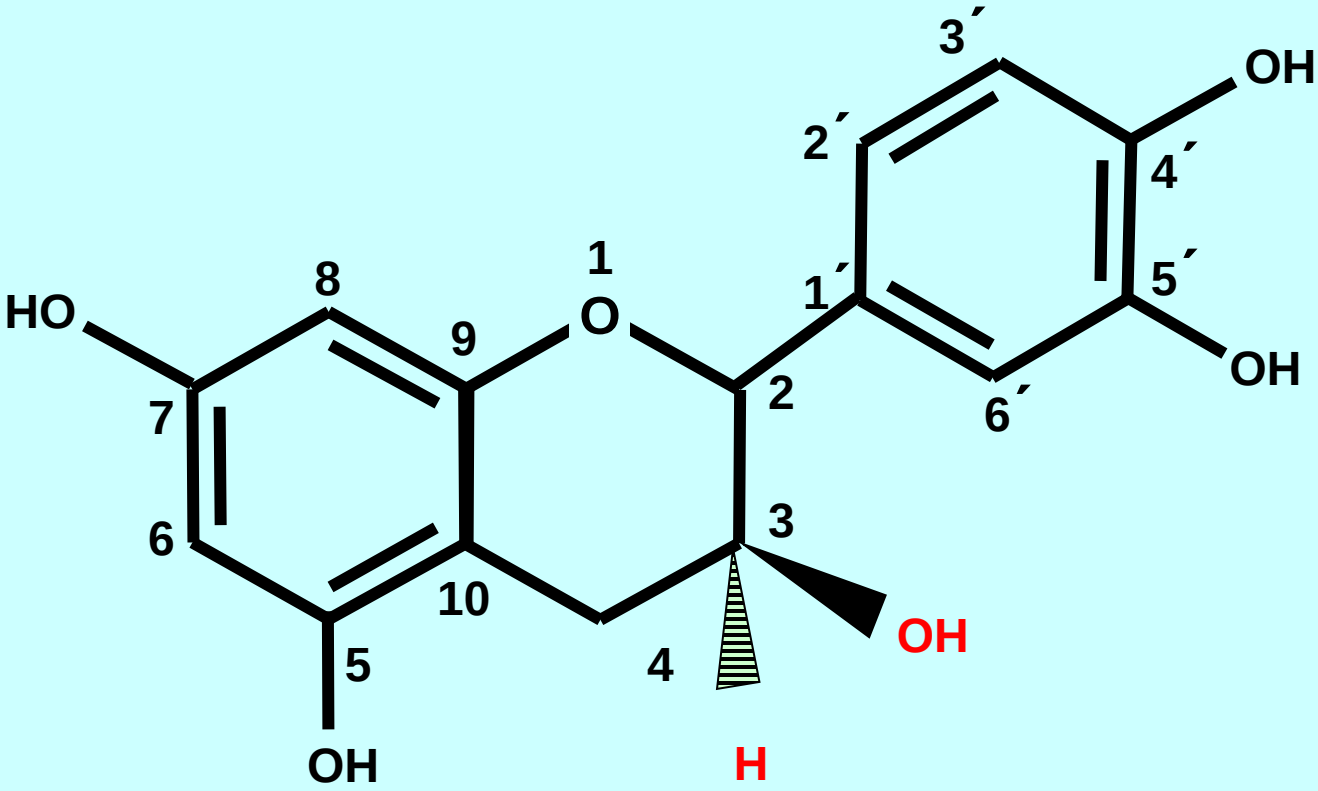
Quercetin, R_1 = H, R_2 = OH



Quercetin, Quercitrin, Myricetin, Rutin

Flavanols and catechins

Catechin, R_1 = OH, R_2 = H



Monomeric - Catechin, Epikatechin

Oligomeric - Procyanidin

Blue rose?

Gene for F3‘5‘H + specific cytochrome b5 → change of red colour to dark violet

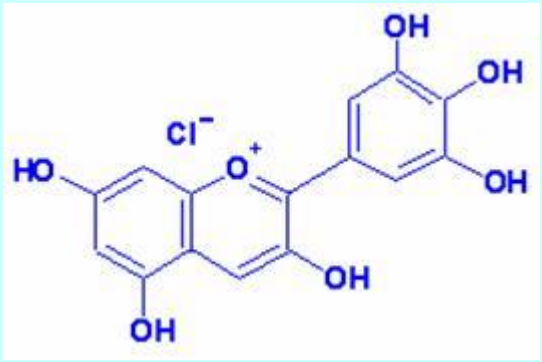
?

Nobody has succeeded, because of acidic content of rose vacuoles

The Florigene Company has been trying to do it for more than 20 years

Despite this, Blue Rose Applause started to blossom!

In 2004, Japanese brewery company Suntory



Gene for delfinidin

Price for the development 28 million USD, profit from such roses about 10 billion USD

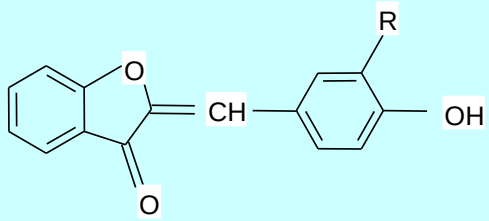
Aurones – yellow colour of flowers

1) Simultaneous expression of two genes

   - auresidin synthase

   - chalcone 4‘-O-glucosyl-transferase

2) Inhibition of anthocyanide synthesis



White flowers

- **Inhibition of genes for chalcone synthase → absence of flavonoids → white, colourless flowers**

- **But the absence of flavonoids is connected with the increased sensitivity of plants to phytopathogens**

Orange Petunia „African Sunset“

- **Gene knock-in by gene from corn**
- **Production of pelargonidin**

- **Modified in 1987 by Peter Mayer form MaxPlanck Institute in Köln (Germany)**

Secretly growing GMOs in Europe: *In March 2017, when Finnish professor Teemu Teeri, who studies agricultural crops, saw orange petunias at Helsinki train station, he linked them to an article Meyer had just published in Nature and subjected the orange petunia leaves to DNA analysis. Soon he was clear, it was a GMO.*

Firefly petunia

- Company Light Bio from Sun Vallye in Idaho (USA)

- Firefly petunias glow at night thanks to another organism, the glowing poisonous Asian fungus Neonothapanus nambi

- Only 29 USD per piece
- Available from April 2024!

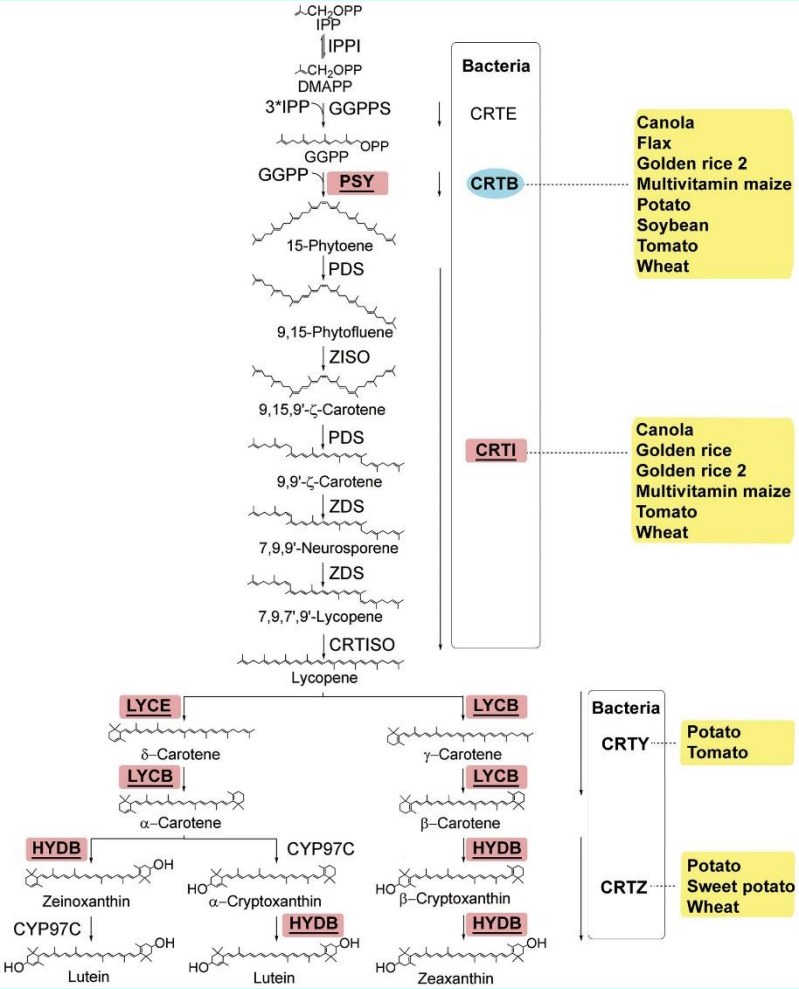
https://light.bio

Enhancing vitamin production

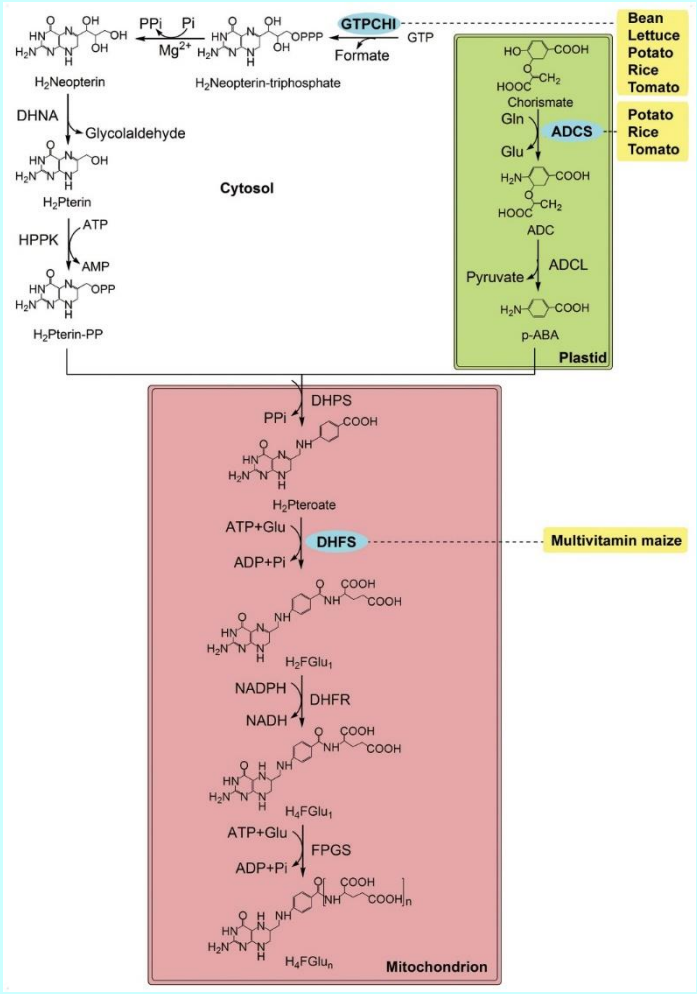
- **Biofortification - enhancing micronutrient production in plants**
- **GM plants with higher vitamin content = higher availability in poorer areas**
- **In most cases this involves inserting more efficient enzymes of a given synthetic pathway from other organisms**
- **What has been created?**
  - **Provitamin A (e.g. golden rice 2 - up to 37 µg carotenoids/g dry fruit)**
  - **Folate (vit. B9)**
  - **B vitamins (1, 2, 3, 5, 6, 7, 12)**
  - **Vitamin C**
  - **Vitamin E**

Enhancing vitamin production

Provitamin A



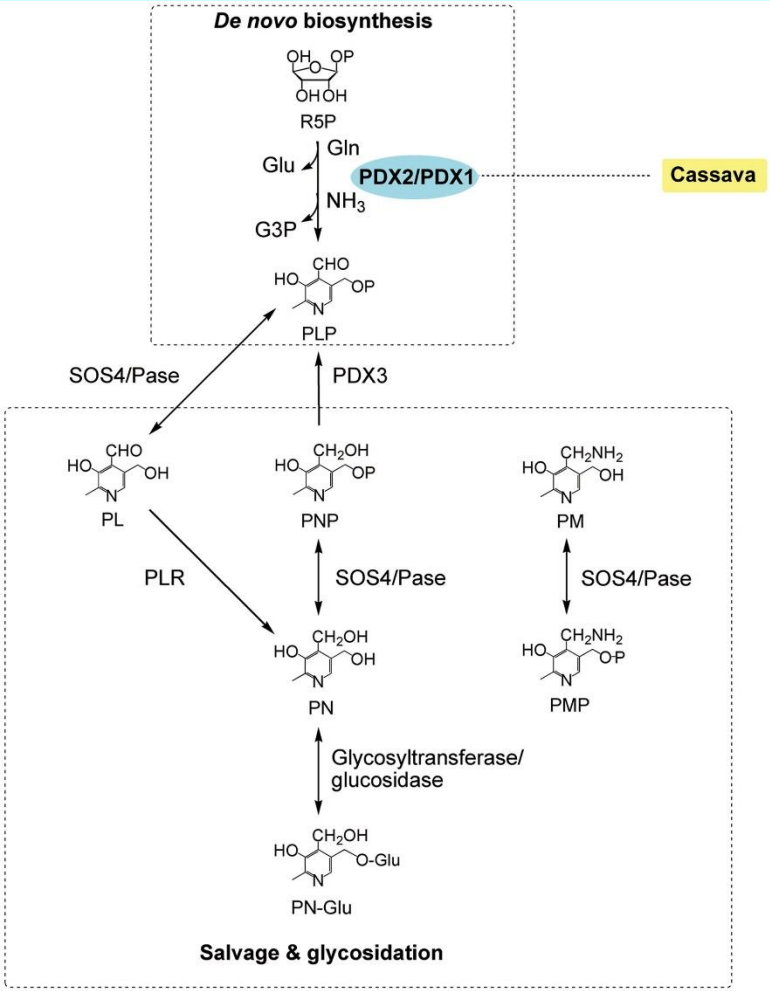
Folic acid



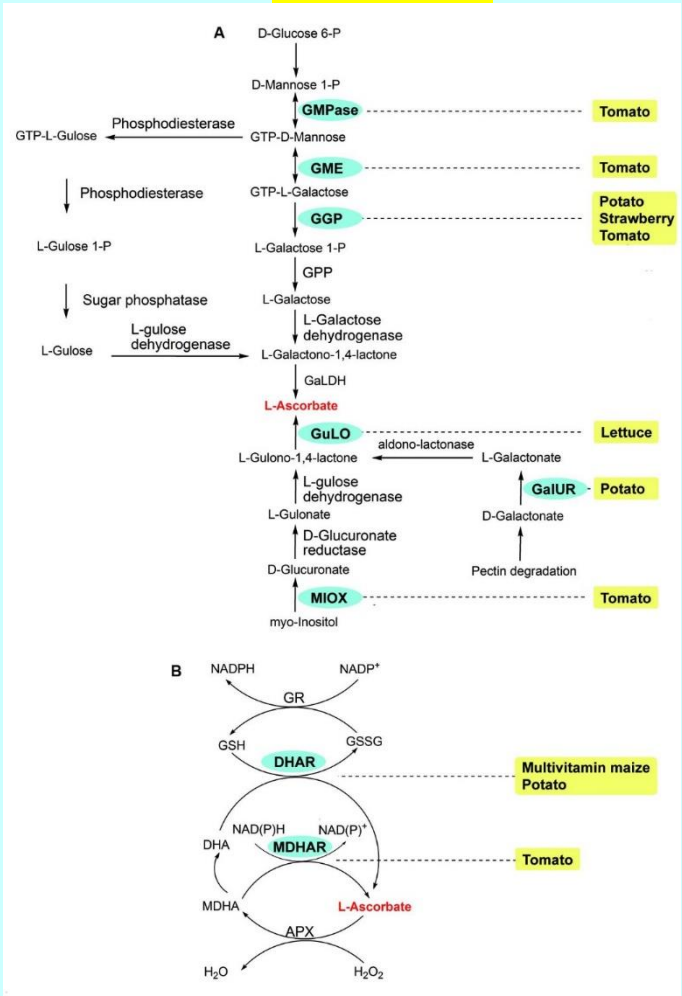
Front. Plant Sci., 06 June 2017 | https://doi.org/10.3389/fpls.2017.00937

Enhancing vitamin production

Vitamin B6



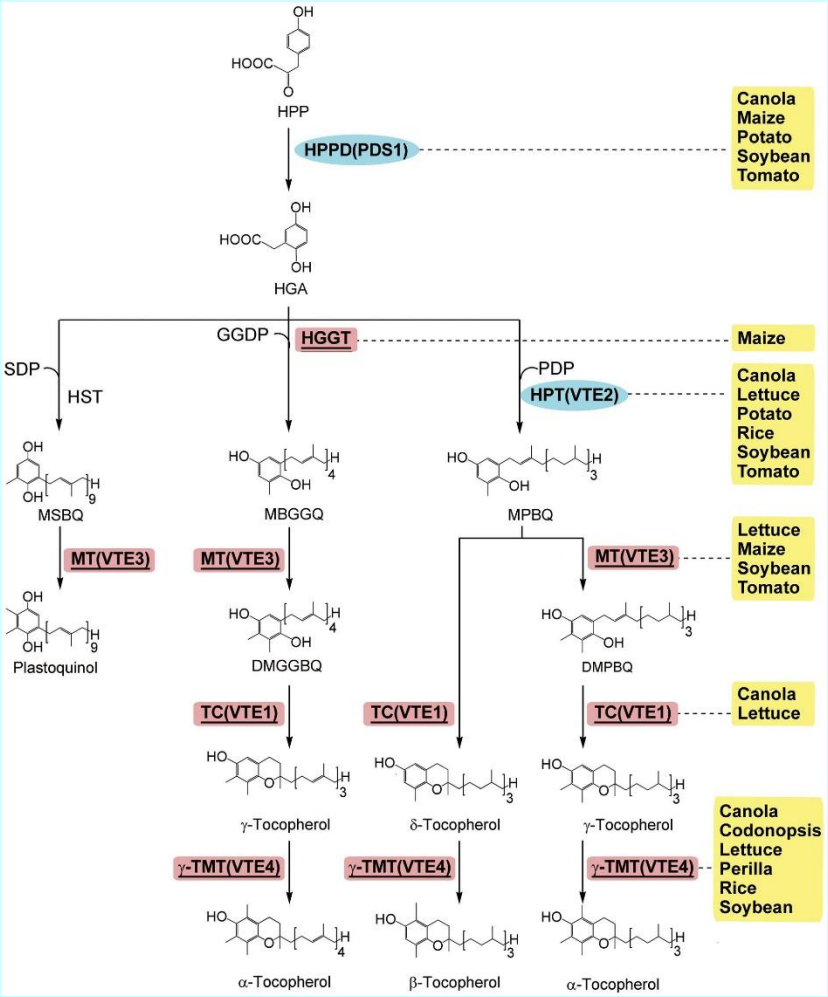
Vitamin C



Front. Plant Sci., 06 June 2017 | https://doi.org/10.3389/fpls.2017.00937

Enhancing vitamin production

Vitamin E



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Manipulation of other important plant metabolites

- **Change in fatty acid composition (canola, soy, sunflower,...)**
  - **18:0 → 18:1 delta9 desaturase**
  - **18:1 → 18:0 antisense construct for delta9 stearate desaturase**
- **Optimization of the amino acid spectrum in cereals and legumes, methionine enrichment**
  - **Zein from maize (28% methionine)**
  - **sunflower albumin (16 % met + 8 % cysteine)**
- **Change in starch composition of potatoes**
  - **starch with increased amylose content - SBE (starch branching enzyme) enzymes ensure the branching of linear amylose chains to form amylopectin**

Transgenic plants identification

A. Molecular detection of transgene

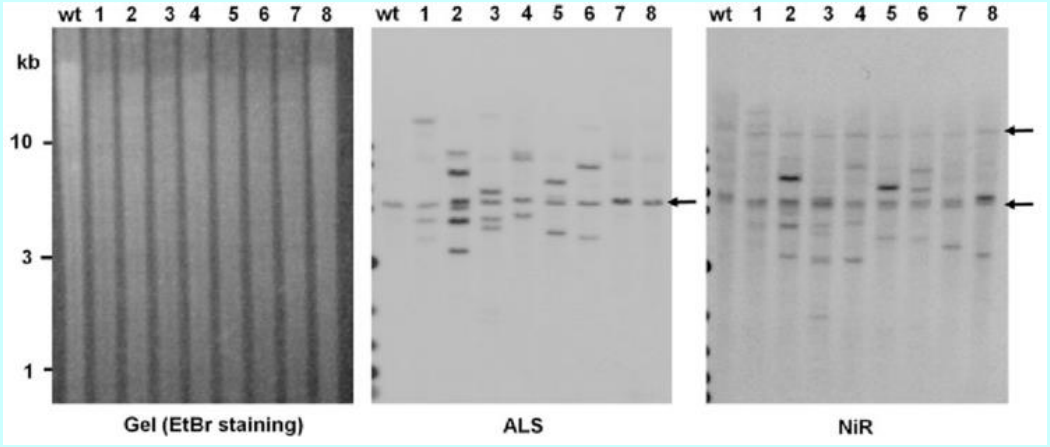
B. Transgene expression

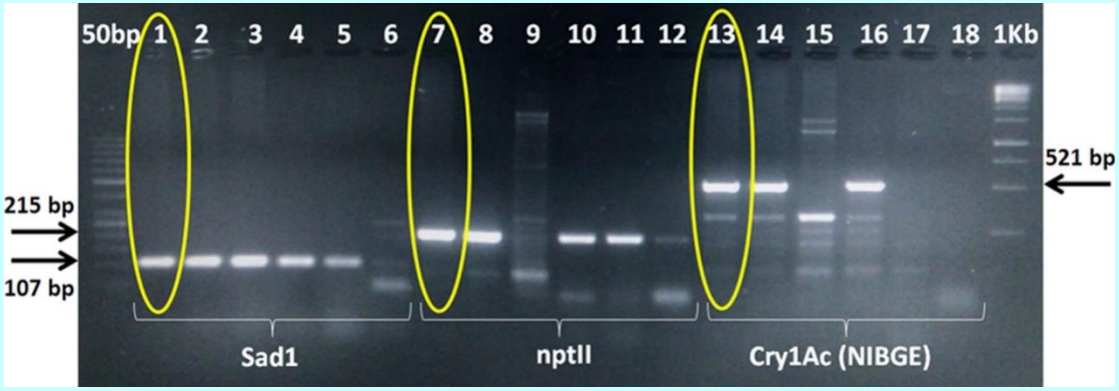
C. Transgene heredity

Molecular detection of transgene

1) DNA blot - Southern blot analysis with a probe prepared from a transgene

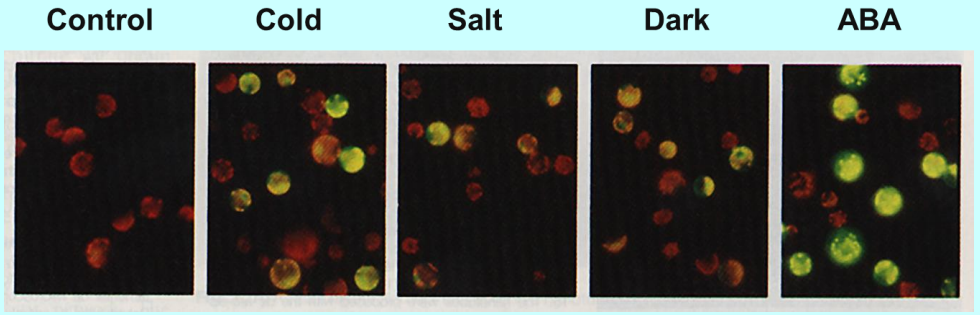
2) Polymerase chain reaction - transgene specific sequences - e.g. recombinant potatoes in practice

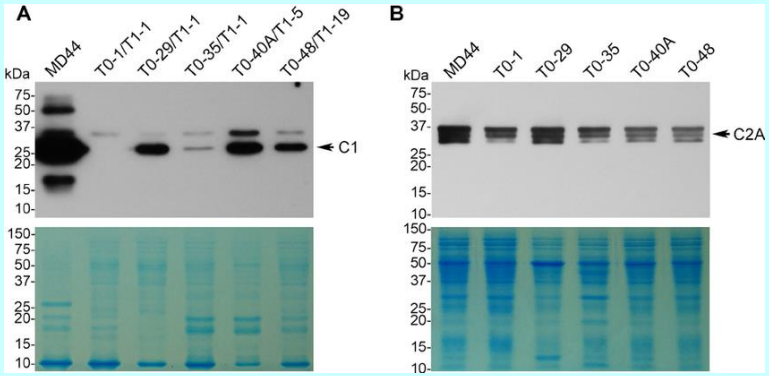


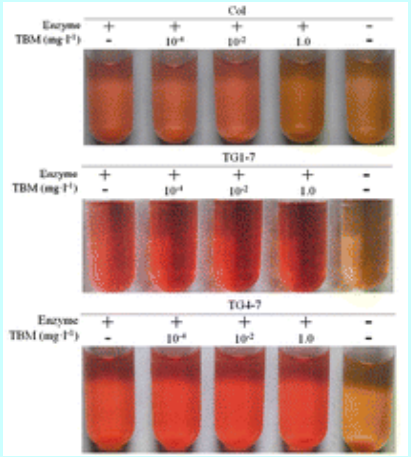


Transgene expression

1) Determination of reporter gene activity
2) Determination of the amount of transcribed RNA or protein level by northern or western blot
3) Enzyme activity assay

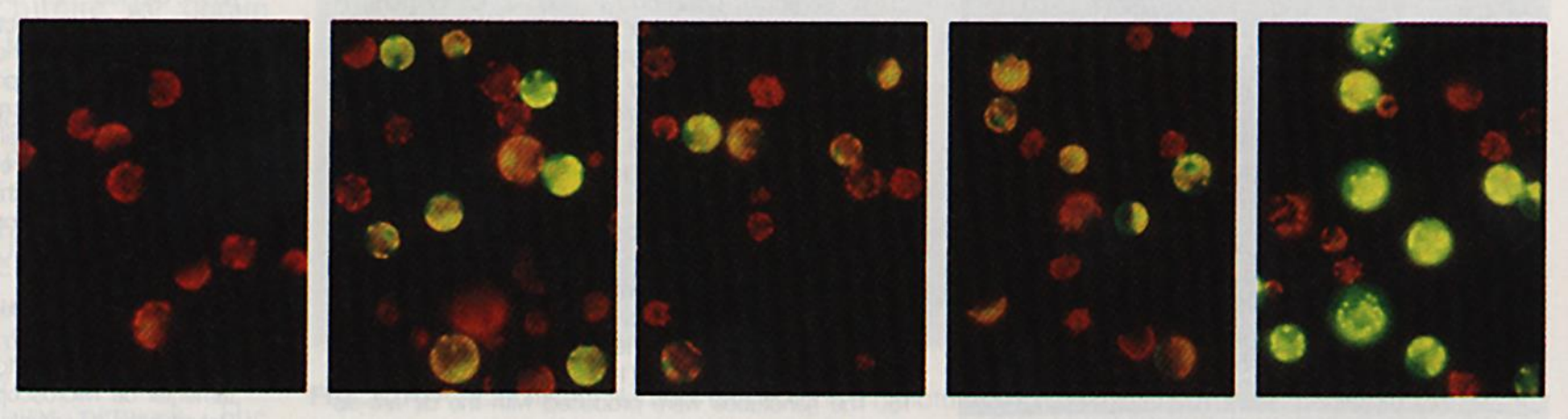






Stress-regulated expression of chimeric gene based on GFP

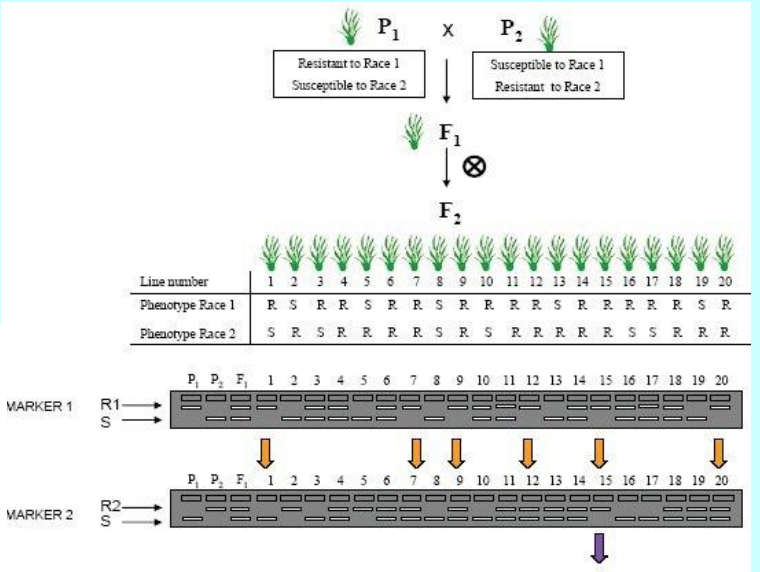
Control Cold Salt Dark ABA



Transgene heredity

The functional insertion at a single locus is phenotypically equivalent to the dominant allele, so it is heritable according to Mendelian rules

I. **Restriction fragments - DNA polymorphisms of the transgene**
II. **Phenotype visible in offspring**



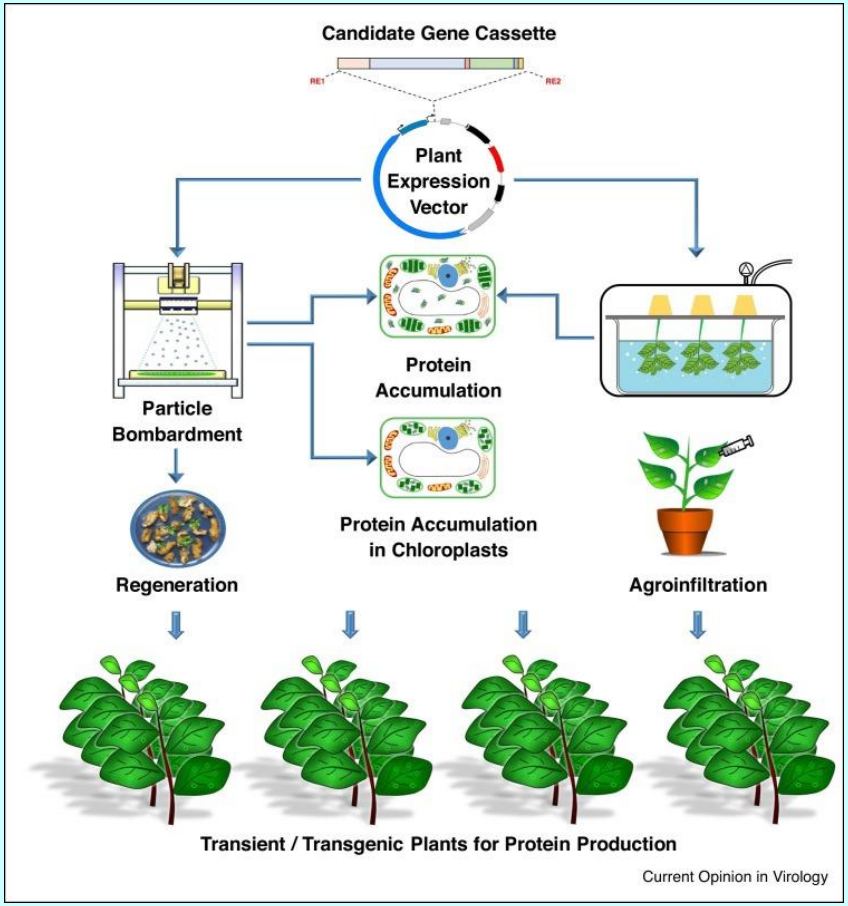
Not all transformations are stable

Stable transfection

- Regeneration of plants from callus
- Transgene transfer to the next generation

Transient transfection

- Agroinfiltration
- Transgene does not spread to the next generation



DOI: 10.1016/j.coviro.2017.07.019

Proteins usable in medicine

- **antibodies**
- **vaccines**
- **other important proteins**

Production of plantibodies

The basic strategy of plant antibodies (plantibodies) consists in

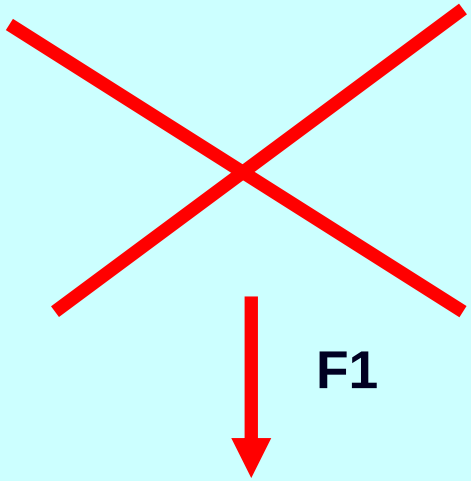
- **Construction of producers of single subunits in different plant lines**
- **Such lines are then hybridized**
- **And producers of complete immunoglobulins are selected**

An example of plantibody I

- **The first example in 1989 – mouse immunoglobulin IgG1**

1st line

Gene for heavy chain γ



F1

2nd line

Gene for light chain κ

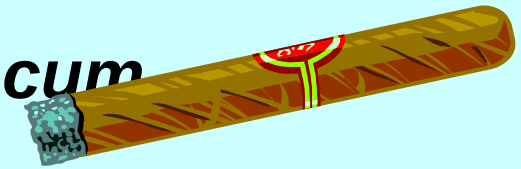
Both chains in the amount of up to 1.3% of total protein in leaves

An example of plantibody II

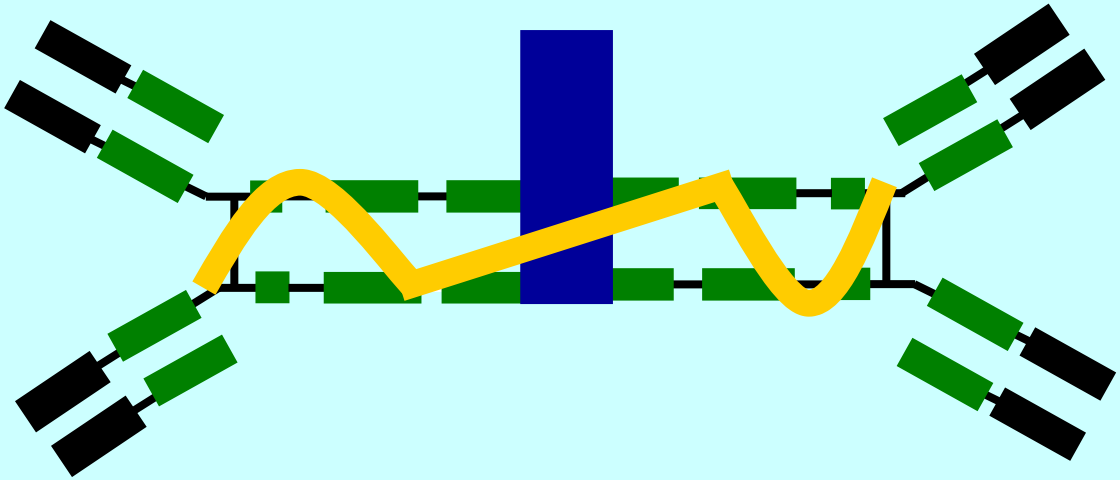
- **Immunoglobulins had a native signal sequence**
- **It directed the polypeptide into ER, which was necessary for the effective folding and stability of the protein**
- **Conditions in ER enabled the formation of disulphide bonds**
- **Chaperones participated in the folding process**
- **The amount of immunoglobulin in F1 generation was higher than the number of individual chains in parental lines**

Utilisation of plantibodies

- **Secretory sIgA from *Nicotiana tabacum***

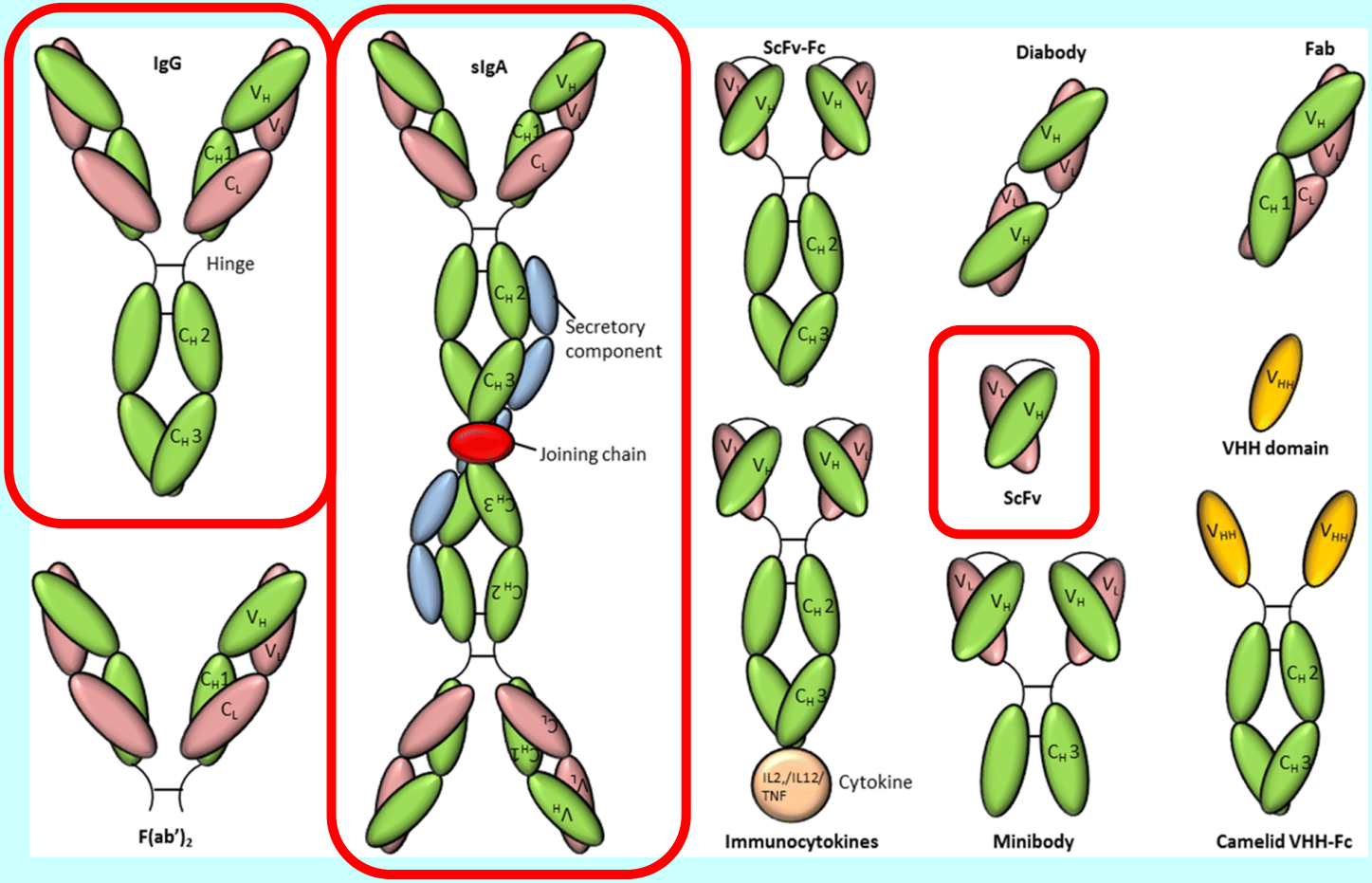


- **Protection against inflammation of teeth caused by *Streptococcus mutans***
- **It recognizes adhesin I/II, by which *S. mutans* bonds to the cell surface – prevention of colonisation**



4 starting lines, 3 hydridisation

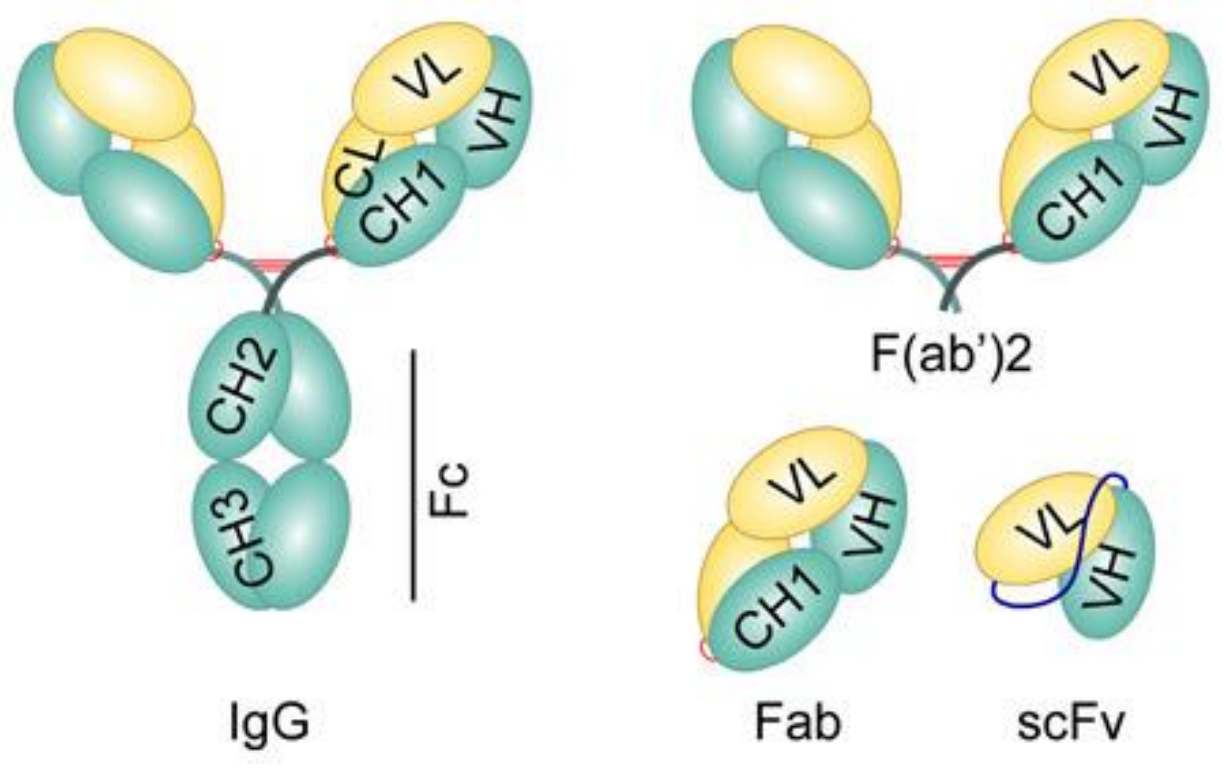
Types of produced antibodies



https://doi.org/10.1007/s10529-019-02651-z

scFv proteins

- **V_L and V_H parts of immunoglobulin connected by a suitable linker**



scFv proteins

- **They are prepared by using a vector based on tobamovirus TMV – tobacco mosaic virus**
- **Expression of scFv is directed by viral promoter**
- **Coding sequence of antibody is localised upstream to leader sequence for α-amylase from rice – directing the protein to extracellular spaces in a plant**

Utilisation of scFv proteins

- **To cure of lymphomas of B cells – individually specific antigens are produced**
- **Very quick production of specific antibodies in 6-8 weeks**
- **Tested on 16 patients in 2003**

Summary of plantibodies

Application	Plant	Antibody	Sig. sequence
Teeth inflam.	Tobacco	sIgA	mouse IgG
Teeth inflam.	Tobacco	IgG	mouse IgG
Tumour of colon	Tobacco	IgG	mouse IgG
Herpes simplex	Soya	IgG	extensin from tobacco
Lymphoma	Tobacco	scFv	α-amylase from rice
Embryonic carcinoma	Crops	scFv	mouse IgG

Vaccines produced by plants

- **Effort to produce eatable vaccines**
- **Cheap prevention of intestinal diseases in the developing countries**
- **Utilisation of available sources**
- **Initially tobacco, at present tomatoes, bananas and cereals**
- **Problems with dosage and movement through digestive tract (proteins are degraded in stomach)**

Examples of plant vaccines

Causative agent	Rec protein	Plant	Amount
Vibrio cholerae	subunits of CtoxA and B	Tobacco	0.001% SLP
Hepatitis B	surface protein	Potatoes	0.3% TSP
Rabies virus	glycoprotein	Tomatoes	1% TSP
Foot and mouth disease	viral epitop VP1	Alfha-alpha/ Arabidopsis	N/A
Pig coronavirus	viral glycoprotein	Tobacco/corn	0.2% TSP
Dog parvovirus	peptide from capsid VP2	Arabidopsis	3% SLP

TSP = total soluble protein, SLP = soluble lipoprotein

Other products of GM plants

- Glucocerebrosidase
- Human serum albumin
- Hormones
- Cytokines
- Structural proteins

Glucocerebrosidase

- Gaucher´s disease – hereditary disease in which cerebrosides accumulate in lysozymes because glucocerebrosidase is defective
- The disease causes oedema of spleen and liver, and serious damage of bones; may be extremely exhausting and painful
- Cerebrosides are purified from human placenta (1 year treatment of one patient requires about 10-12 tons of placentas)
- Production in cultures of mammal cells is much cheaper
- It is expected that the production utilising plants will become more and more cheaper

DOI:https://doi.org/10.1016/j.c

The first usable plant product

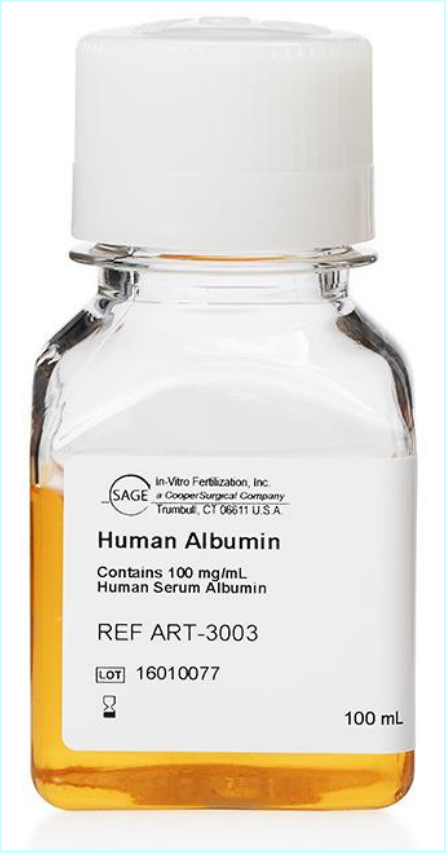
May 2012

- **Business name: Elelyso**
- **Enzyme: taliglucerase alfa**
- **Produced by: GM carrot culture**
- **Manufacturer: Protalix BioTherapeutics, Israel Pfizer, USA**

FDA approved for the treatment of Gaucher disease

Human serum albumin

- Curing of burns, cirrhosis, etc.
- Expression in tobacco and potatoes under the control of promoter 35S
- Two forms with different signal sequences
  - human, not properly folded
  - extracellular protein PR-S from tobacco, properly folded



Other examples of biopharmaceuticals I

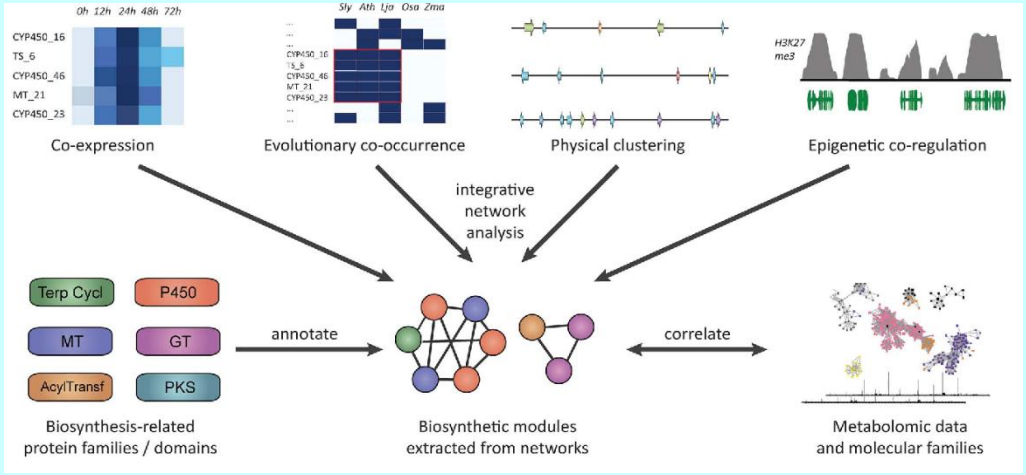
Rec. protein	Origin	Plant	Application
protein C	human	tobacco	anticoagulant
hirudin	leech	canola	anticoagulant
somatotrophine	human	tobacco	growth hormone
β-interferon	human	rice/turnip/tobacco	hepatitis B and C
haemoglobins	human	tobacco	blood recovery
collagen	human	tobacco	collagen

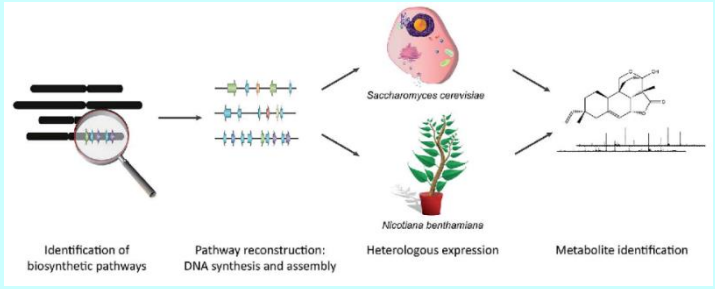
Other examples of biopharmaceuticals II

Rec. protein	Origin	Plant	Application
α1-antitrypsin	human	rice	cystic fibrosis
aprotinin (trypsin inhibitor)	human	corn	transplantation
lactoferrin	human	potatoes	antimicrobial effects
ACE	human	tobacco/ potatoes	hypertension
enkefalin	human	*Arabidopsis*	opiate
trichosanthin-α	*Trichosanthes kirilowii*	tobacco	HIV therapy, tumours

And it goes the other way, too ...

- **Reconstruction of a plant biosynthetic pathway in another organism, e.g. Saccharomyces cerevisiae**
- **Often complex approach - many genome modifications required**
- **Very often combination of genes/proteins from different organisms**
- **Beginning of synthetic biology?**





DOI: 10.1039/c6np00035e

Year 2015 - biotechnological prehistory

Heroin production in yeast

University of Carolina in Berkeley, 05/2015

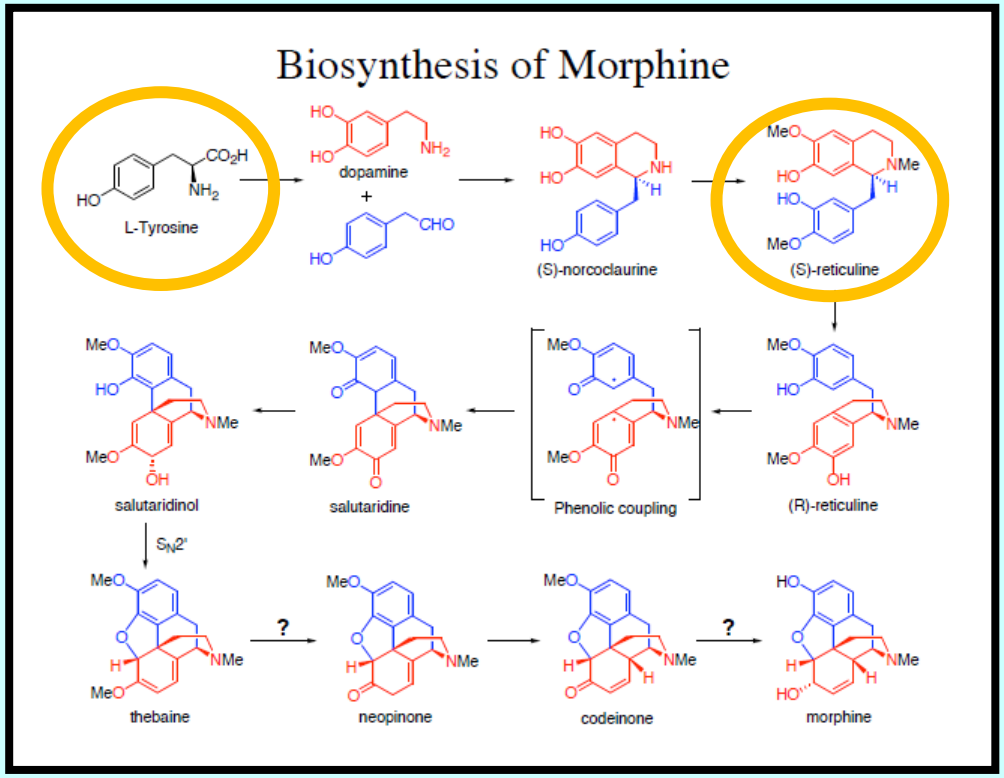
Alkaloid production required 16 steps

That's when scientists went from L-tyrosine to (S)-reticulin

http://www.rsc.org/chemistryworld/2014/08/yeast-turned-morphine-opioid-biotechnology-factories
http://www.scientificamerican.com/article/opiate-making-yeast-could-lead-to-home-brewed-heroin/
http://www.nature.com/nchembio/journal/vaop/ncurrent/full/nchembio.1816.html

Heroin production in yeast 2015

The situation at the time



Status in 2020

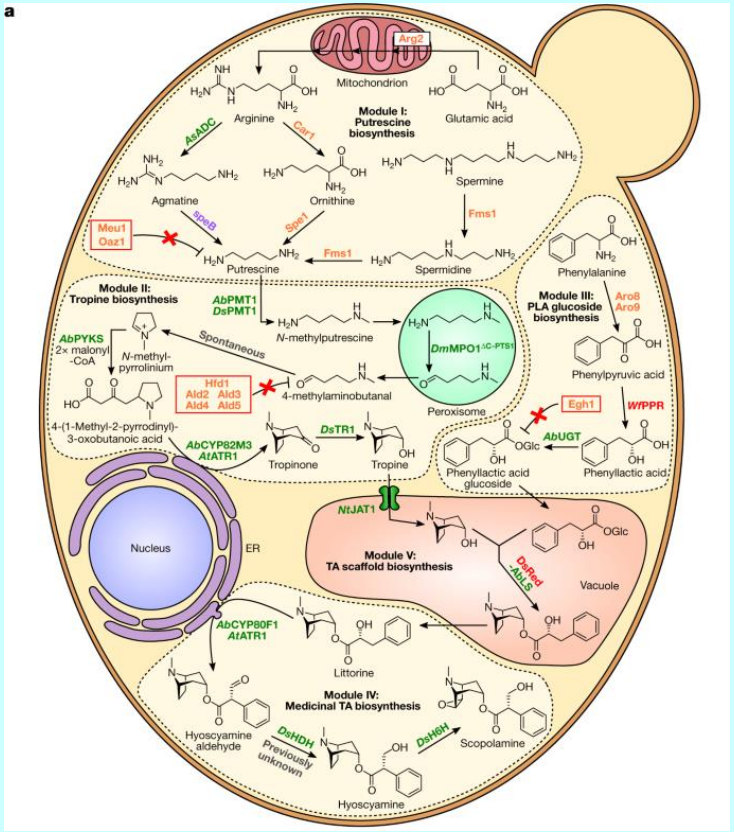
Saccharomyces cerevisiae

34 chromosomal modifications

- **26 gene knock-ins or knock-ups**
- **8 knock-out genes**

Result

tropane alkaloids hyoscyamine and scopolamine



https://doi.org/10.1038/s41586-020-2650-9

Summary

1. Cloning of secondary metabolites
2. Gene knocks-...
3. Colour changing in flowers
4. Transgenic plants identification
5. Plantibodies
6. Examples of other recombinant plants