Part VI
TRANSGENIC PLANTS: SECOND GENERATION

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Plant Biotechnology
Vietnam OpenCourseWare
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Second generation (1995-): ouput traits (enhanced nutrition, amino acid rich…)

6.1. Improve the nutritive value of plants

The second generation of transgenic plants use **metabolic engineering** to insert new pathways into plants or improve expression of enzymes in existing ones. The target includes:

- Increase protein content and quality
- Increase vitamins, antioxidants, iron and other minerals etc
- Remove antinutritional factors e.g. phytase inhibitors, lectins
- Modify saturated fat content
- Modify flower pigmentation of plants
- Increase/decrease phenolics and fibre

Improving protein content and quality

Since animals and humans are incapable of making 10 ‘essential’ amino acids, they must obtain them in their diet.

Nutritional value of seed storage proteins is often limited. Most crop plants are deficient in one or more amino acids. Eg., maize is low in Lys and Trp.

Amino acid balance in seeds has been limited.

Similar strategies have been used to improve protein content and composition in nonseed food crops.

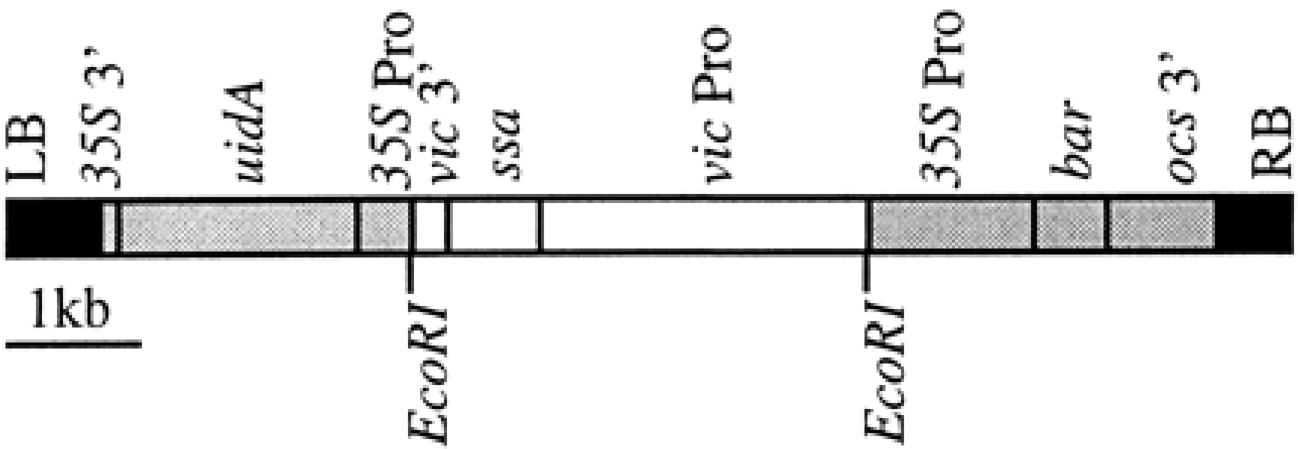
Enhanced methionine levels and increased nutritive value of seeds

A chimeric gene specifying seed-specific expression of a sulfur-rich, sunflower seed albumin was stably transformed into lupin (*Lupinus angustifolius L.)*. The transgenic seeds contained less sulfate and more total amino acid sulfur than the nontransgenic parent line. This was associated with a 94% increase in methionine content and a 12% reduction in cysteine content. These findings demonstrate the feasibility of using genetic engineering to improve the nutritive value of grain crops

Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (*Lupinus angustifolius* L.) expressing a sunflower seed albumin gene.
Molvig L, Tabe LM, Eggum BO, Moore AE, Craig S, Spencer D, Higgins TJ.Proc Natl Acad Sci U S A. 1997; 94:8393-8.

Image source: *www.api.ning.com*

albumin (141 amino acids): rich in methionine and cysteine



	WT	Transgenic
Met	**0.55**	**1.07**

Photo source: http://static.panoramio.com

Enhanced nutritive value and amino acid balance in seeds

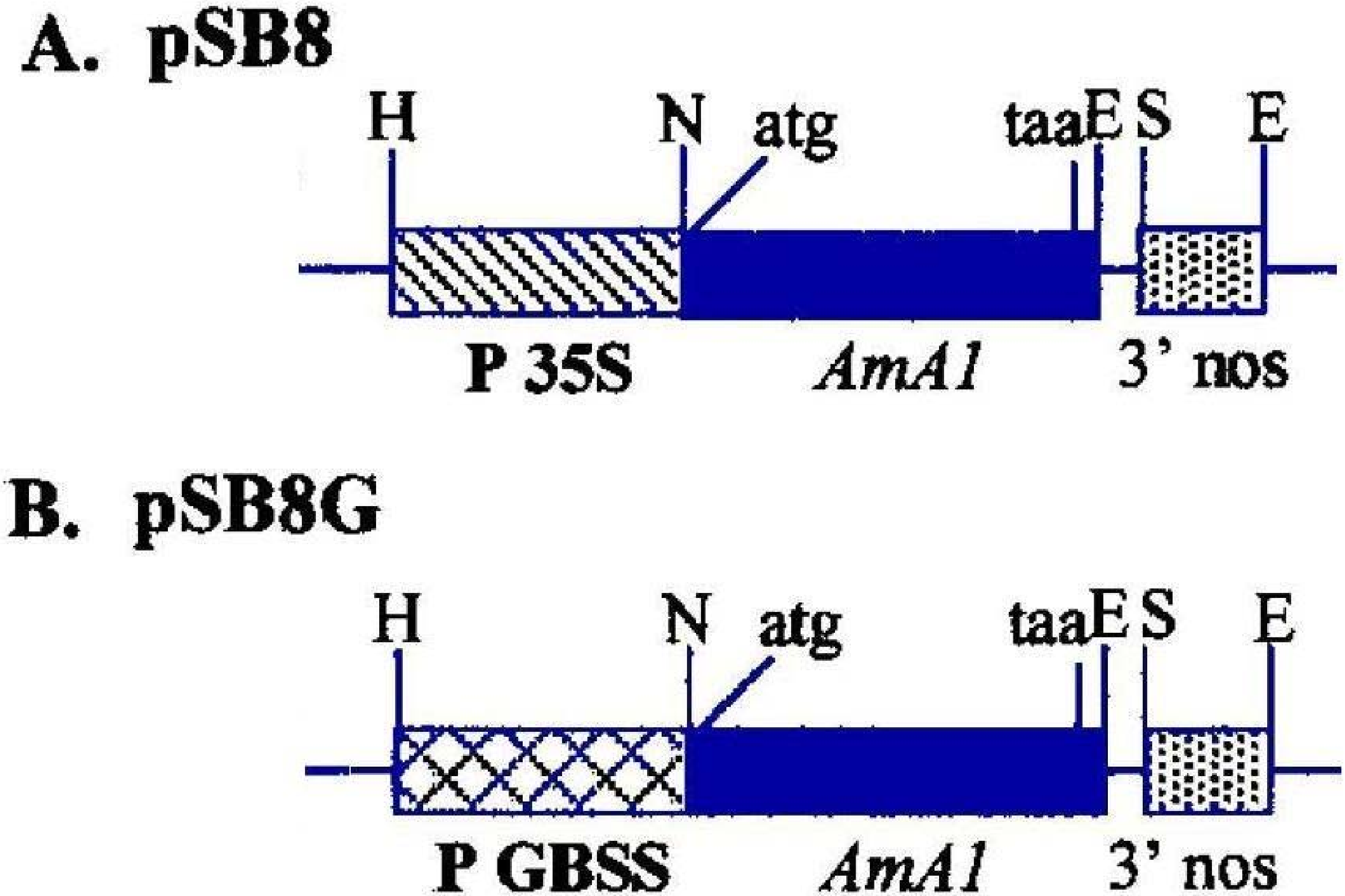
The AmA1 protein is nonallergenic in nature and is rich in all essential amino acids, and the composition corresponds well with the WHO standards for optimal human nutrition. In an attempt to improve the nutritional value of potato, the seed albumin gene *AmA1 from Amaranthus hypochondriacus was successfully introduced* and expressed in tuber-specific. Scientists have been able to use a seed albumin gene with a well-balanced amino acid composition as a donor protein to develop a transgenic crop plant.

‘Increased nutritive value of transgenic potato by expressing a nonallergenic seed albumin gene from Amaranthus hypochondriacus’

Chakraborty et al., PNAS 97, 3724-3729 (2000)

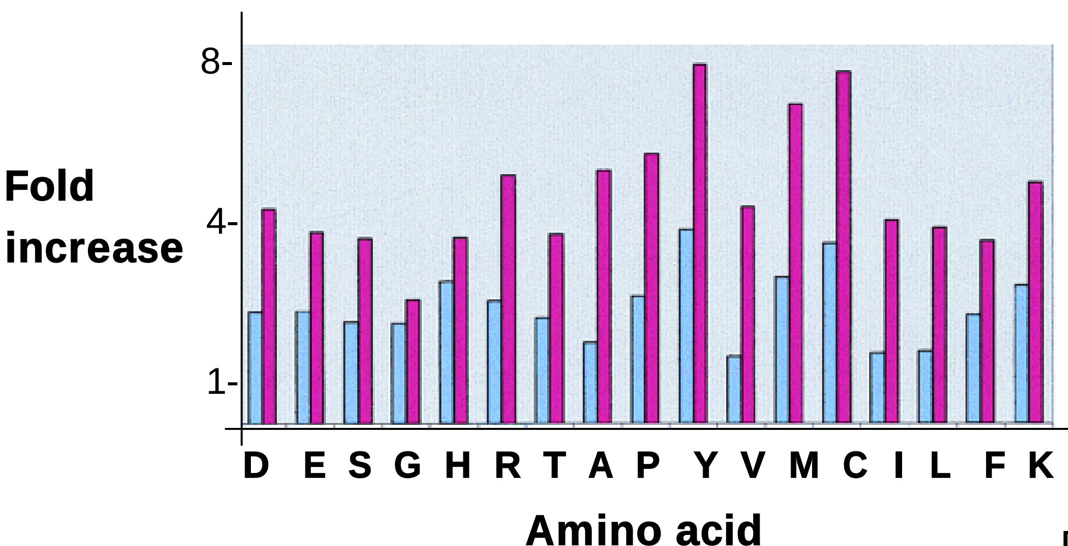
- Potato is the fourth most abundant global crop and used for food, animal feed and production of starch and alcohol.
- Limited in lysine, tyrosine, methionine and cysteine.
- Transformed potato with the seed albumin gene *AmA1 from Amaranthus hypochondriacus* . by using granulebound starch synthase (GBSS) and cauliflower mosaic virus (CaMV) 35S promoters, respectively.

Seed albumin gene AmA1 Construct



Transformed potato with seed albumin from *Amaranthus* which has good amino acid balance: Expression in tuber 5-10 fold higher with GBSS promoter than with 35S promoter.Total protein content also increased (35-45%)

Photo source: www.gardenseeker.com



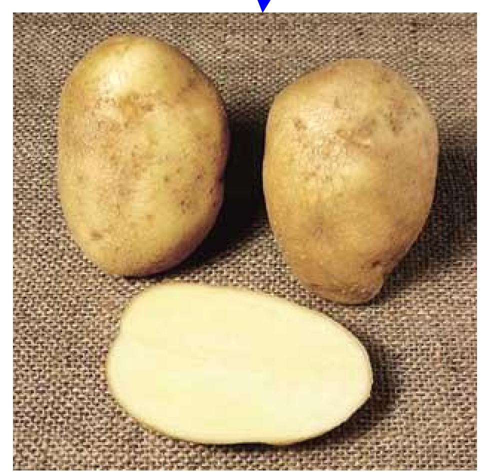


Photo source: www.gardenaction.co.uk

''Golden rice'', Engineering the provitamin A biosynthetic pathway into rice Endosperm

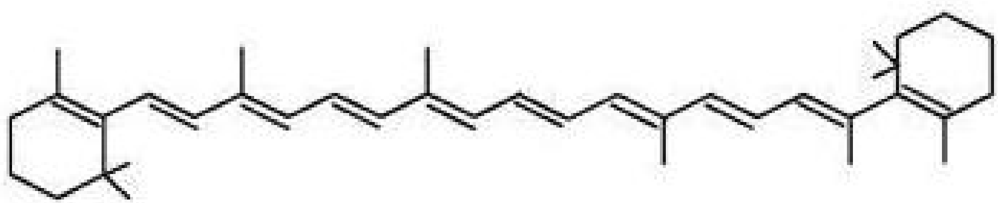
Vitamin A deficiency is prevalent in the developing world and is probably responsible for the deaths of two million children annually. In surviving children, vitamin A deficiency is a leading, but avoidable, cause of blindness (WHO report ,1995) Humans can synthesize vitamin A if provided with the precursor molecule β-carotene (also known as provitamin A), a pigment found inmany plants but not in cereal grains. Therefore, a strategy was devised to introduce the correct metabolic steps into rice endosperm to facilitate β-carotene synthesis.

Vitamin A deficiency (VAD) in rice

β-carotene is a pigment required for photosynthesis and is produced in all plant green tissues. Also produced in endosperm of yellow maize and sorghum.

Rice is a staple crop for much of the world, but rice grains do not produce vitamin A or its precursor, β-carotene. VAD is most serious in regions where rice is the staple food ; up to 70% children under 5 affected.

Have unsuccessfully searched for rice mutants with β-carotene in the grain.



β-carotene

Photos source:
www.vnexpress.net

Production of Golden Rice

- Ingo Potrykus and Peter Beyer with funding from The Rockefeller Foundation and EU. worked on 8 year long project to introduce genes for pro-vitamin A into rice.

- Initially showed that last precursor of carotenoid pathway made in rice endosperm isgeranylgeranyl pyrophosphate (GGPP).

- Enhance β-carotene synthesis via 4 enzymes:

• phytoene synthase, phytoene desaturase, β-carotene desaturase, lycopene cyclase

- These genes were isolated from Erwina (bacteria), daffodil (a monocot plant) and introduced into rice one by one

β-Carotene Pathway in Plants

IPP

↓

Geranylgeranyl diphosphate

↓ **Phytoene synthase**

Phytoene

↓ **Phytoene desaturase**

↓ **ξ -carotene desaturase**

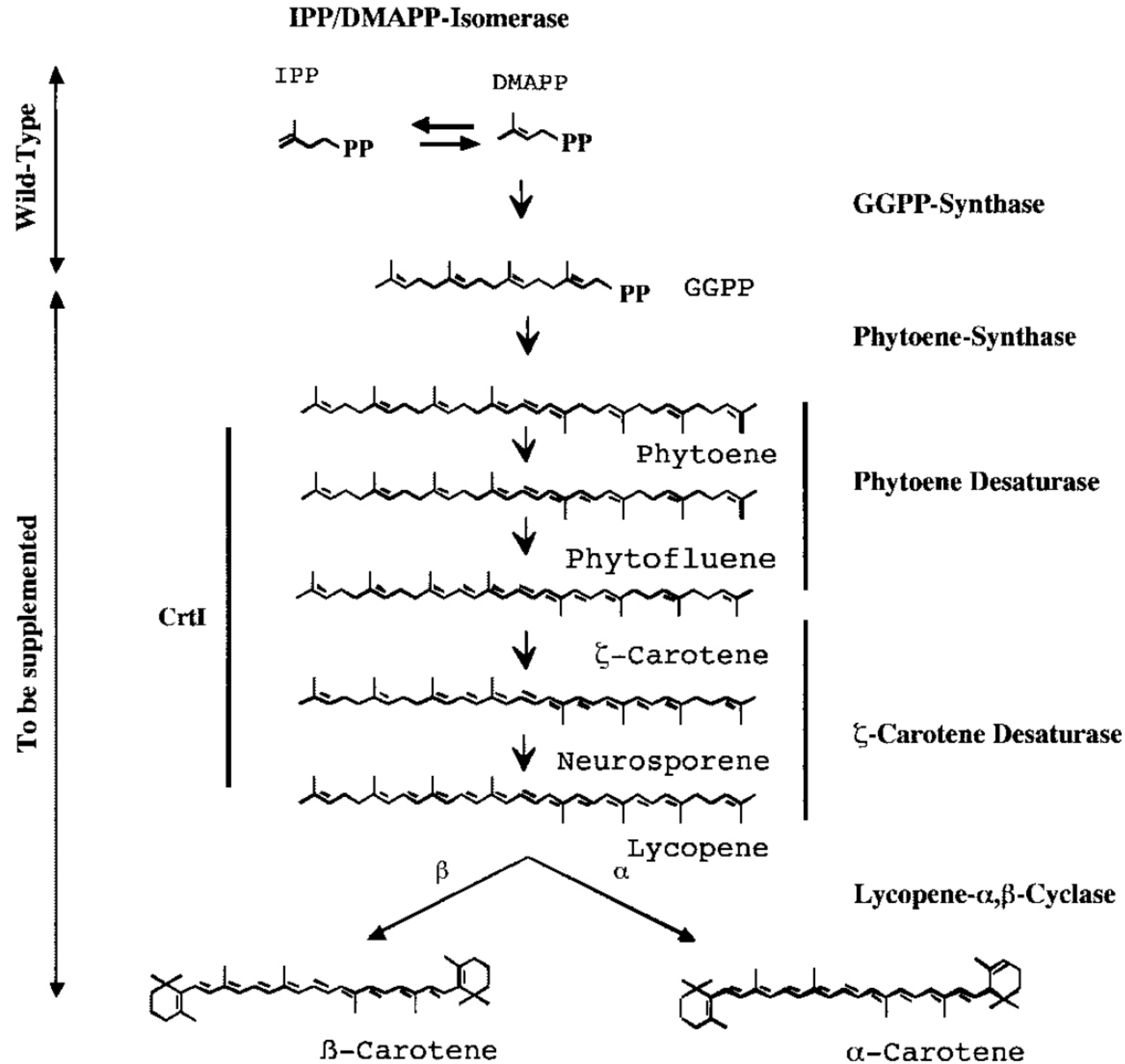
Lycopene

↓ **Lycopene-beta-cyclase**

β -carotene
(vitamin A precursor)

Problem:
Rice lacks
these enzymes

Normal
Vitamin A
"Deficient"
Rice


IPP/DMAPP-Isomerase
IPP
DMAPP
PP
PP
Wild-Type
GGPP-Synthase
PP
GGPP
Phytoene-Synthase
Phytoene
Phytoene Desaturase
Phytofluene
CrtI
To be supplemented
ζ-Carotene
ζ-Carotene Desaturase
Neurosporene
Lycopene
β
α
Lycopene-α,β-Cyclase
ß-Carotene
α-Carotene

The Golden Rice Solution

β-Carotene Pathway Genes Added

IPP

↓

Geranylgeranyl diphosphate

Daffodil gene ↓ Phytoene synthase

Phytoene

Single bacterial gene; performs both functions
↓ Phytoene desaturase
↓ ξ -carotene desaturase

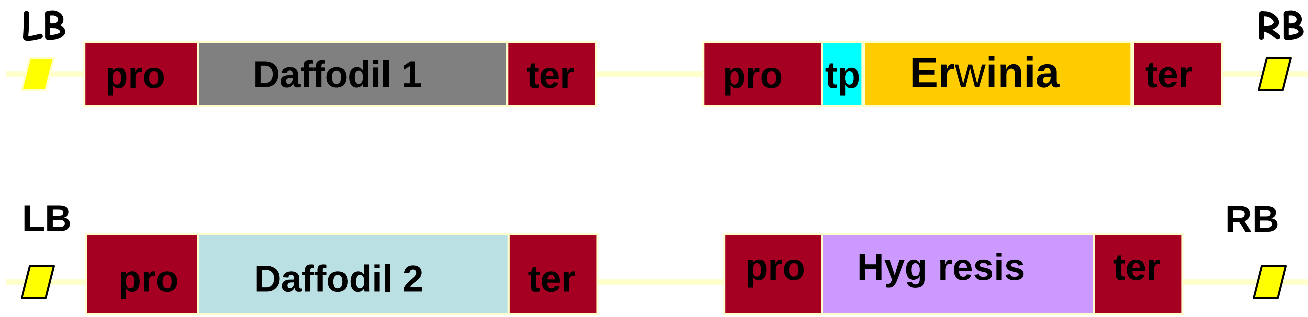
Lycopene

Daffodil gene ↓ Lycopene-beta-cyclase

β -carotene
(vitamin A precursor)

Vitamin A Pathway is complete and functional

Two T-DNAs encoding 3 genes for pro-vitamin A (plus selectable marker gene) introduced together via *Agrobacterium* by co-transformation



Daffodil 1 = phytoene synthase
Daffodil 2 = lycopene ß-cyclase

} *With own native transit peptides and endosperm-specific promoter from rice glutelin (GT1 promoter)*

Erwinia = Erwinia double desaturase - *with added transit peptide, expressed from 35SCaMV promoter*

Grain of resulting transgenic rice has light golden-yellow colour

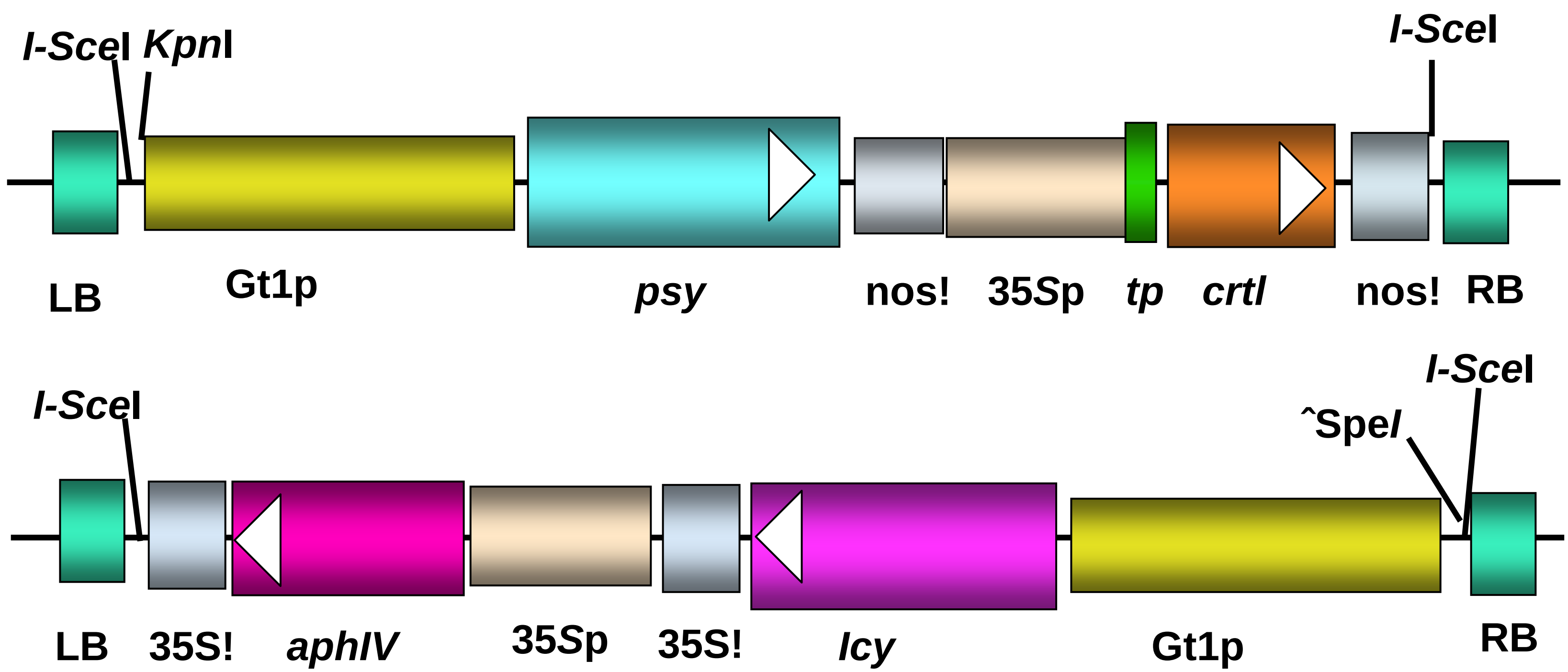
- best line had 85% of its carotenoids as ß-carotene

Photo source: *www.goldenrice.org*

Goldm Rice Gene Constructs

'Engineering provitamin A (ß -carotene) biosynthetic pathway into (carotenoid-free) rice endosperm'.

Ye et al., Science 287,303-305 (2000).



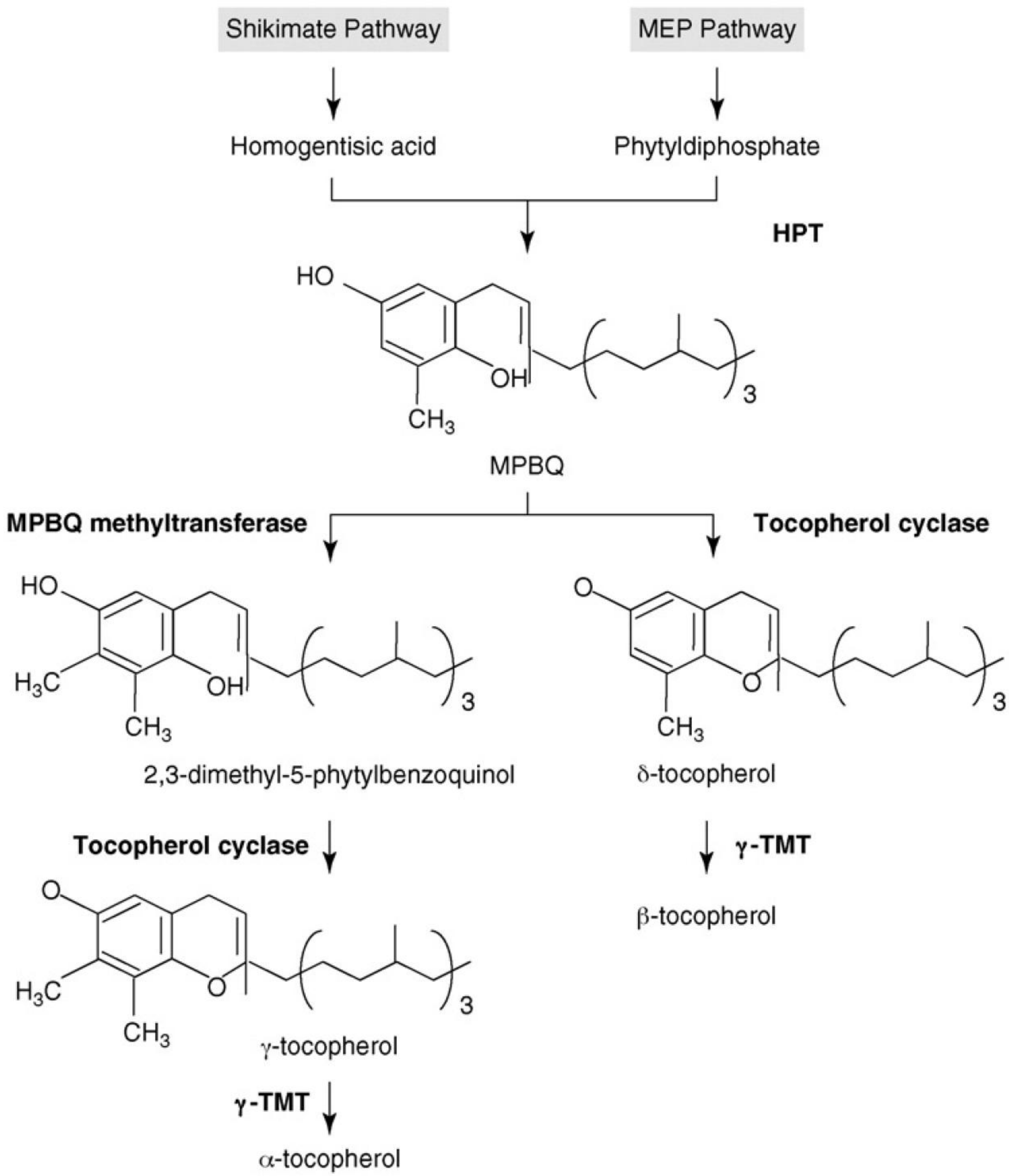
Now Golden rice version 2 (Pain et al, *Nature Biotechnology* 2005)
23 fold increase of caroten content Goldenrice version 1 (16 / 37 μg/g)

Photos source: *www.goldenrice.org*

303. 2006

Vitamin E and others

Whereas vitamin A is a single molecule, vitamin E is a group of eight hydrophobic compounds (known as vitamers), the most potent of which is a-tocopherol. Dietary vitamin E is obtained mainly from seeds, and its function in the body is to prevent the oxidation and polymerization of unsaturated fatty acids. Vitamin E deficiency leads to general wasting, kidney degeneration and infertility. In plants, tocopherol synthesis requires input from two metabolic pathways


Shikimate Pathway
MEP Pathway
Homogentisic acid
Phytyldiphosphate
HPT
HO
OH
CH_3
3
MPBQ
MPBQ methyltransferase
Tocopherol cyclase
HO
H_3C
OH
CH_3
3
2,3-dimethyl-5-phytylbenzoquinol
O
O
CH_3
3
δ-tocopherol
Tocopherol cyclase
γ-TMT
β-tocopherol
O
H_3C
O
CH_3
3
γ-tocopherol
γ-TMT
α-tocopherol

- ✓ A more recent study was based on the simultaneous expression of multiple genes in soybean; in this case, the Arabidopsis genes encoding 2-methyl-6-phytylbenzoquinol (MPBQ) methyltransferase and γ-tocopherol methyltransferase (γ-TMT) were used. Transgenic soybeans showed a significant elevation in the total amount of vitamin E activity (fivefold greater than that of wild-type plants), which was attributable mainly to an eightfold increase in the levels of α-tocopherol, from its normal 10% of total vitamin E to over 95%.
- ✓ Recently, plants have been engineered to accumulate several other vitamins, including folate and ascorbate (vitamin C).

Iron and phytate in rice

- Studies of the mineral content of crop plants have concentrated mainly on iron and zinc (which are most frequently deficient in human diets). The approach to mineral biofortification is to express recombinant proteins that enable minerals to be stored in a bioavailable form, for instance, overexpression of soybean ferritin in rice using an endosperm-specific promoter .
- Ferritin is an iron-storage protein. This produced rice grains with three and 4.4 times the wild-type iron levels.

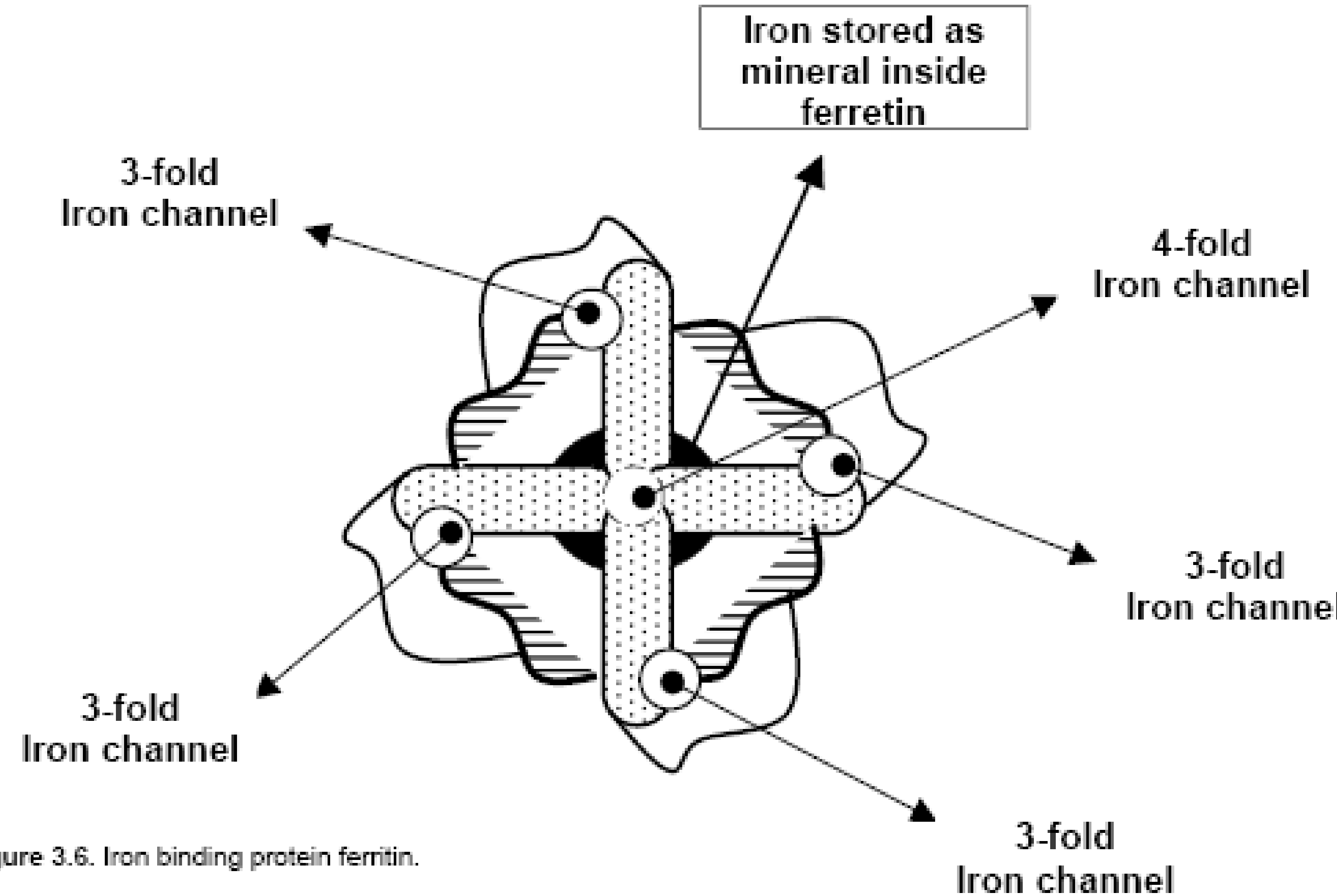
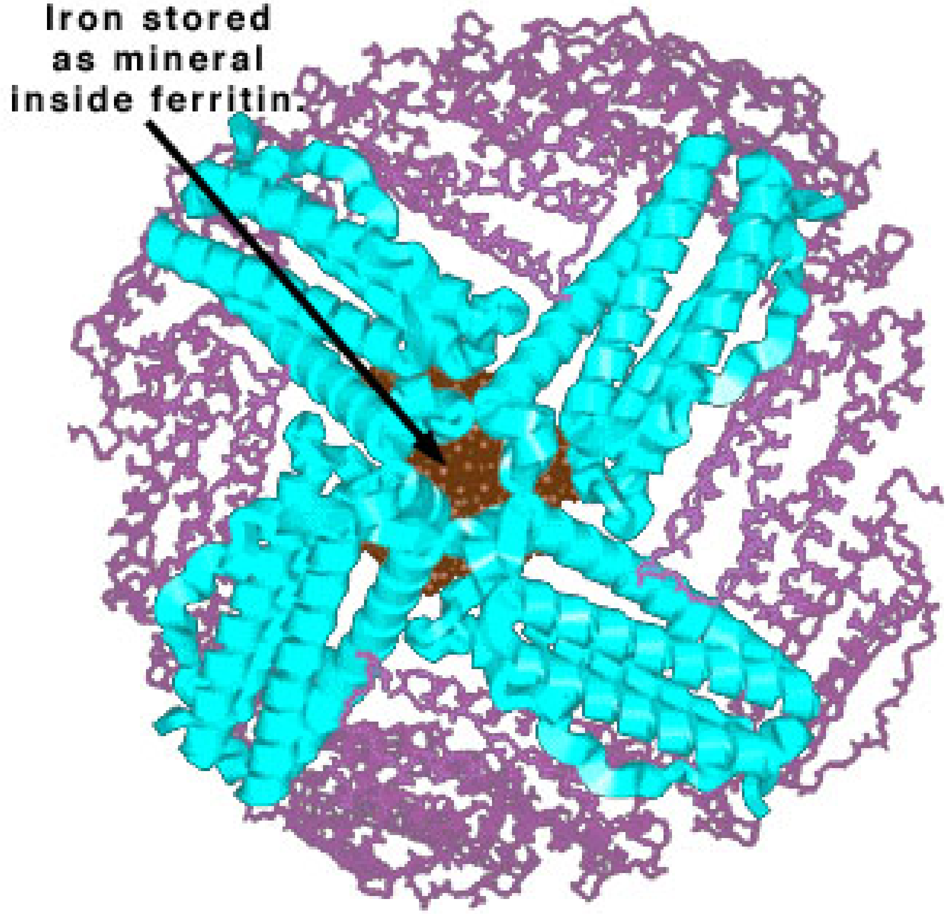
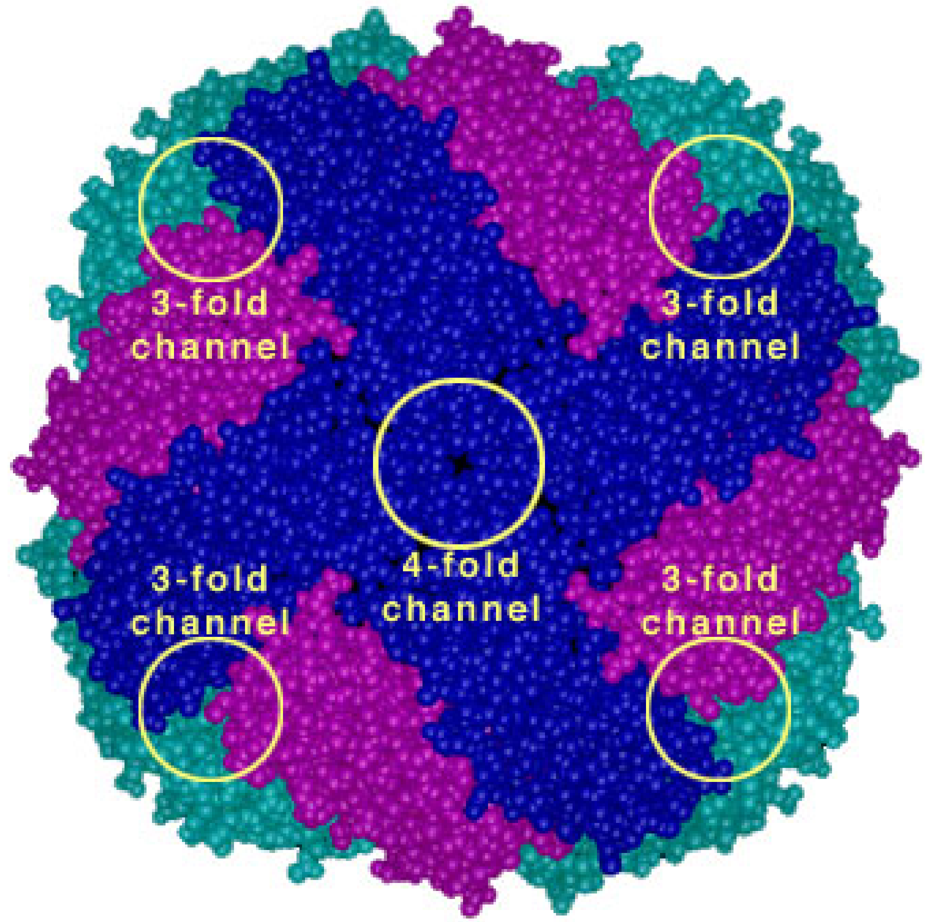


Figure 3.6. Iron binding protein ferritin.





Images source: *www.chemistry.wustl.edu*

Phytic acid (also known as phytate) is an antinutritional compound that chelates minerals and reduces their bioavailability in the gut. Therefore, a combined approach has been developed that involves the expression of both ferritin and phytase (a fungal enzyme that breaks down phytate); this has been achieved in rice and maize.The rice grains contained twice the wild-type amount of iron, and simulations of digestion and absorption using the maize kernels showed that the amount of bioavailable iron had also increased. The combined use of multiple strategies for iron fortification therefore provides the maximum levels of bioavailable iron.

Use of Rice to prevent and treat vitamin A *and* iron deficiencies

Iron deficiency is the most common nutritional disorder

- Iron-enriched transgenic rice:
- **1 gene increases Fe content (↑ ferritin)**
- **2 genes increase Fe absorption (↓ phytate, ↑ cysteine)**

DOUBLE TRANSGENIC RICE

β-carotene-enriched rice crossed with iron-enriched rice;

β-carotene enhances iron uptake

Free distribution to farmers in developing world

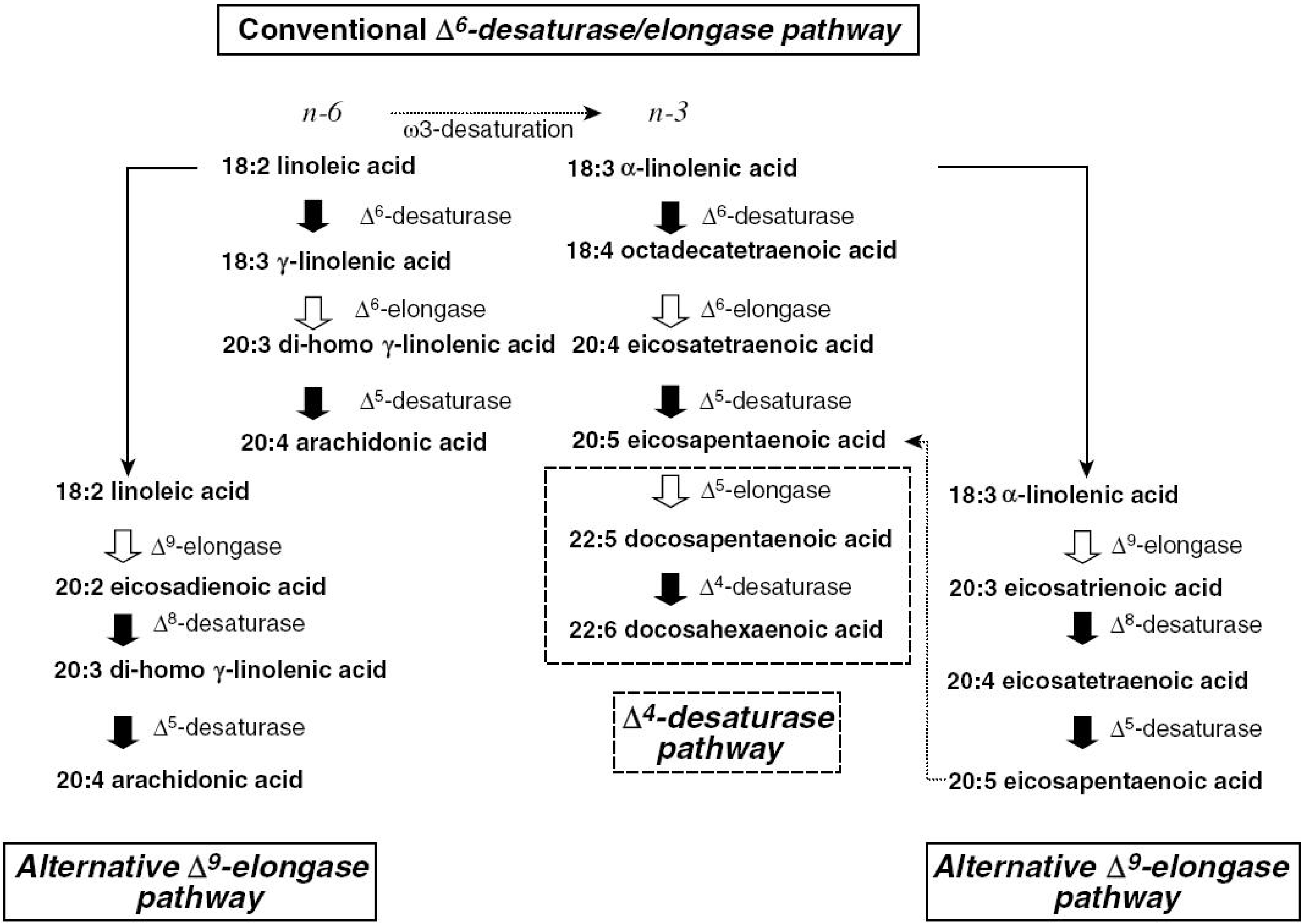
6.2. Modify Long-chain polyunsaturated fatty acids (LC-PUFAs) contents

Long-chain polyunsaturated fatty acids (LC-PUFAs) are important in human health and nutrition. The human diet must contain essential fatty acids or their precursors to synthesize LC-PUFAs. Mammals, including humans, have an absolute requirement for the dietary ingestion of the two essential fatty acids, linoleic acid (18:2, LA) and a-linolenic acid (18:3, ALA), because they lack the appropriate desaturases to convert monounsaturates into these two essential fatty acids. Since plants are rich in both linoleic acid and a-linolenic acid, normal diets usually provide sufficient essential fatty acids.

Unsaturated Plant Fatty Acids are essential in the Animal diet

Plant		Animal
18:0		18:0
↓		↓
18:1 (9c)		18:1 (9c)
↓		
18:2 (9c, 12c)	Diet →	18:2 (9c, 12c)
↓		↓
18:3 (9c, 12c, 15c)		18:3 (6c, 9c, 12c)
↓		↓

All higher plants have the ability to synthesize the main LA, ALA and some can also synthesize g-linolenic acid (GLA) and octadecatetraenoic acid (18,4, SDA). However, higher plants are unable to further elongate and desaturate these C18 fatty acids to produce LC-PUFAs) (long-chain polyunsaturated fatty acids). The synthesis of LC-PUFA in higher plants therefore requires the introduction of genes that encode all of the biosynthetic enzymes required to convert either LA into LC-PUFA such as 20:5 eicosapentaenoic acid and 20:6 docosahexaenoic acid.These genes can be obtained from a range of organisms such as fungi, bacteria that synthesize the LC-PUFA.


Conventional Δ6-desaturase/elongase pathway
n-6
ω3-desaturation
n-3
18:2 linoleic acid
Δ6-desaturase
18:3 γ-linolenic acid
Δ6-elongase
20:3 di-homo γ-linolenic acid
Δ5-desaturase
20:4 arachidonic acid
18:3 α-linolenic acid
Δ6-desaturase
18:4 octadecatetraenoic acid
Δ6-elongase
20:4 eicosatetraenoic acid
Δ5-desaturase
20:5 eicosapentaenoic acid
Δ5-elongase
22:5 docosapentaenoic acid
Δ4-desaturase
22:6 docosahexaenoic acid
Δ4-desaturase pathway
18:2 linoleic acid
Δ9-elongase
20:2 eicosadienoic acid
Δ8-desaturase
20:3 di-homo γ-linolenic acid
Δ5-desaturase
20:4 arachidonic acid
Alternative Δ9-elongase pathway
18:3 α-linolenic acid
Δ9-elongase
20:3 eicosatrienoic acid
Δ8-desaturase
20:4 eicosatetraenoic acid
Δ5-desaturase
20:5 eicosapentaenoic acid
Alternative Δ9-elongase pathway

Fatty acid desaturases of *Arabidopsis*

Name	Subcellular location	Fatty acid substrates	Site of double-bond insertion	Notes
FAD2	ER	$18{:}1^{\Delta 9}$	$\Delta 12$	Preferred substrate is phosphatidylcholine
FAD3	ER	$18{:}2^{\Delta 9,12}$	$\omega 3$	Preferred substrate is phosphatidylcholine
FAD4	Chloroplast	16:0	$\Delta 3$	Produces 16:1-*trans* at *sn*-2 of phosphatidylglycerol
FAD5	Chloroplast	16:0	$\Delta 7$	Desaturates 16:0 at *sn*-2 of monogalactosyldiacylglycerol
FAD6	Chloroplast	$16{:}1^{\Delta 7}$ $18{:}1^{\Delta 9}$	$\omega 6$	Acts on all chloroplast glycerolipids
FAD7	Chloroplast	$16{:}2^{\Delta 7,11}$ $18{:}2^{\Delta 9,12}$	$\omega 3$	Acts on all chloroplast glycerolipids
FAD8	Chloroplast	$16{:}2^{\Delta 7,11}$ $18{:}2^{\Delta 9,12}$	$\omega 3$	Isoenzyme of FAD7 induced by low temperature
FAB2	Chloroplast	18:0	$\Delta 9$	Stromal stearoyl-ACP desaturase

The Successful Synthesis of C20 PUFAs in Transgenic Plants

Expression (under the control of a constitutive promoter) of the Isochrysis C18 A9-elongating activity in transgenic Arabidopsis (*Arabidopsis thaliana*) resulted in the synthesis of the C20 elongation products 20:5 eicosapentaenoic acid and ETriA to significant levels (15% of total fatty acids) in all vegetative tissues. This demonstrated for the first time the efficient reconstitution of an LC-PUFA elongase in transgenic plants and confirmed the feasibility of engineering transgenic plants to accumulate C20 polyunsaturated fatty acids (Qi et al., 2004).

6.3. New horticultural varieties

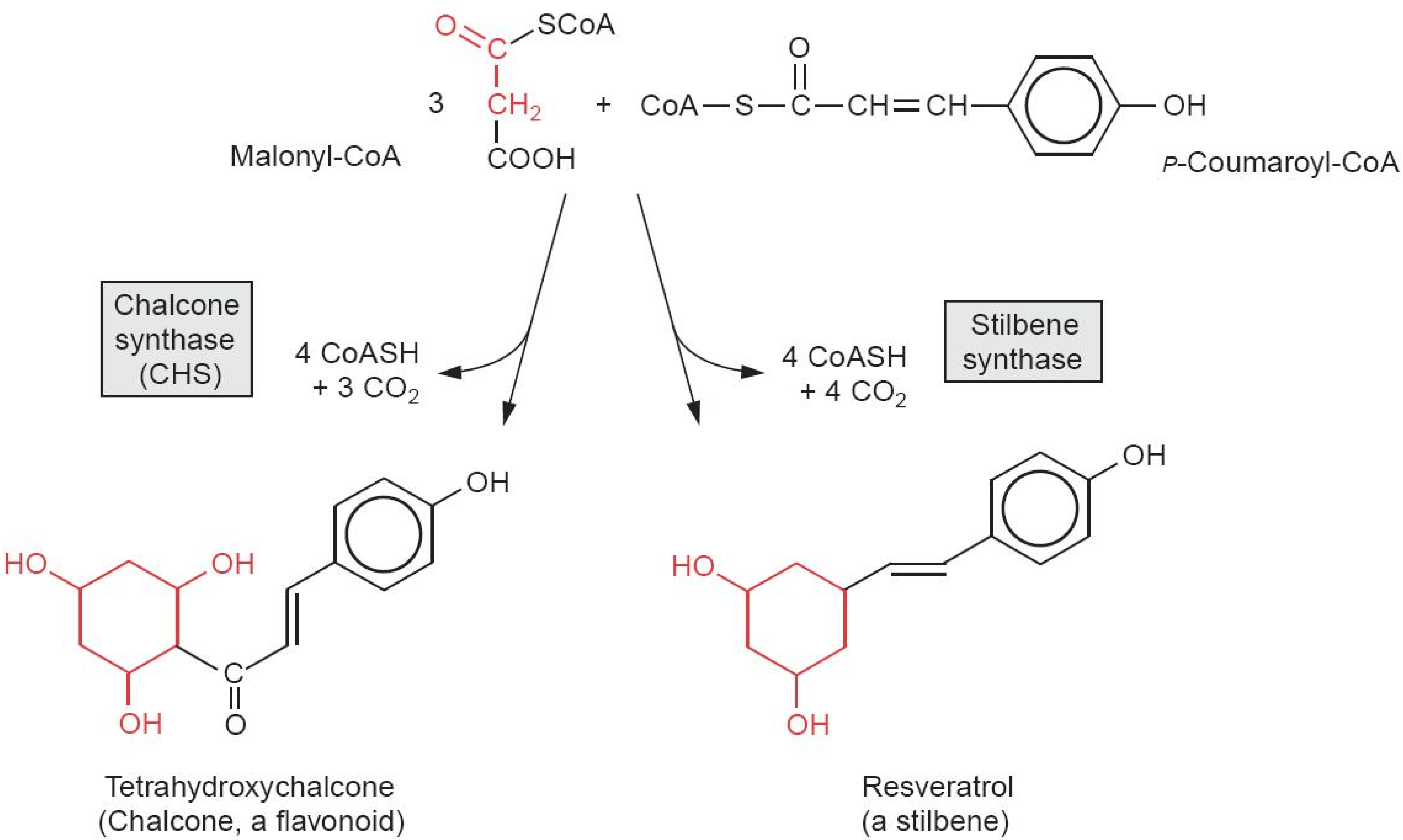
Understanding the biosynthetic pathways of flavonoid provide background to produce novel varieties.

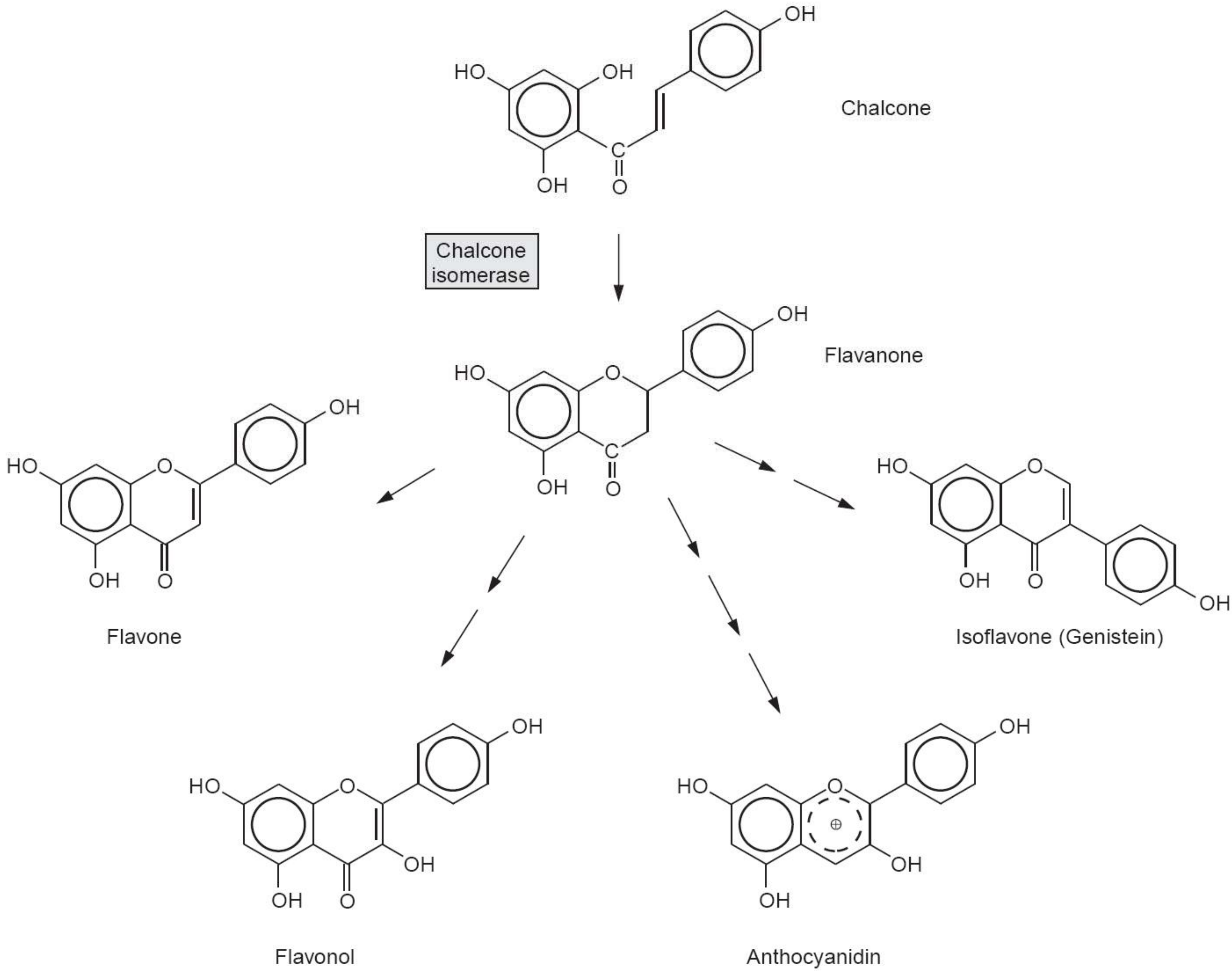
Flavonoid constructed from the products of two different pathways and catalyzed by CHALCONE SYNTHASE

Flavonoid combine to create flower pigmentation: serve as signals for pollinators

Seem to have major health benefits for humans.

Various classes of Flavonoids: Anthocyanins (Main constituents of plant flower color), Flavonols, Flavones, Isoflavones.


SCoA
O
C
3
CH2
+
CoA—S—C—CH=CH—
OH
Malonyl-CoA
COOH
P-Coumaroyl-CoA
Chalcone
synthase
(CHS)
4 CoASH
+ 3 CO2
4 CoASH
+ 4 CO2
Stilbene
synthase
OH
HO
OH
C
O
OH
HO
OH
Tetrahydroxychalcone
(Chalcone, a flavonoid)
Resveratrol
(a stilbene)



Chalcone
Chalcone isomerase
Flavanone
Flavone
Isoflavone (Genistein)
Flavonol
Anthocyanidin

Flavonoid Functions

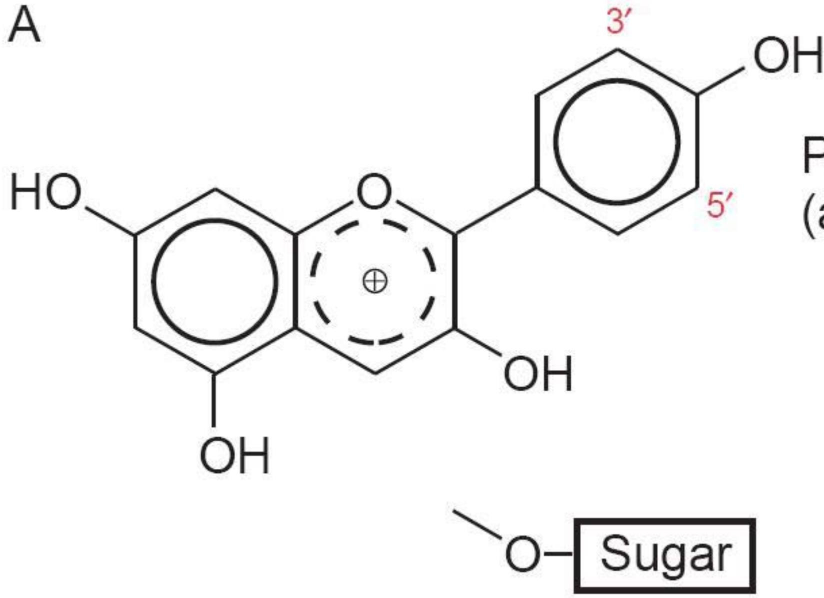
- ✓ The flavonoids include protectants against herbivores and many phytoalexins.
- ✓ The isoflavone medicarpin from lucerne (alfalfa) is a phytoalexin.
- ✓ Flavonoids also serve as signals for interactions of the plant with symbionts.
- ✓ Flavones and flavonols have an absorption maximum in the UV region. As protective pigments, they shield plants from the damaging effect of UV light.

- Many flavonoids act as antioxidants. As constituents of nutrients, they are assumed to be protectants against cardiovascular disease and cancer (e.g., green tea, soy sauce, and red wine).
- Recently, particular attention has been focused on certain isoflavones that are found primarily in legumes. It turns out that these forage plants contain isoflavones, which in animals (and in humans) have an effect similar to that of estrogens. For this reason, they have been named phytoestrogens. A strong estrogen effect has genistein. Investigations are in progress to use phytoestrogens for medical purposes.

Anthocyanin and flower colour

- Anthocyanins are flower pigments and protect plants against excessive light.
- Other widely distributed flower pigments are the yellow **chalcones,** light yellow **flavones, and red and blue anthocyanins.**
- Anthocyanins are glucosides of anthocyanidins in which the sugar component, consisting of one or more hexoses, is usually linked to the -OH group of the pyrylium ring.
- Anthocyanins are deposited in the vacuole.

A

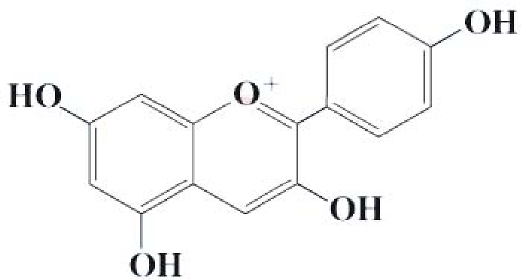


Pelargonidin
(anthocyanidin)

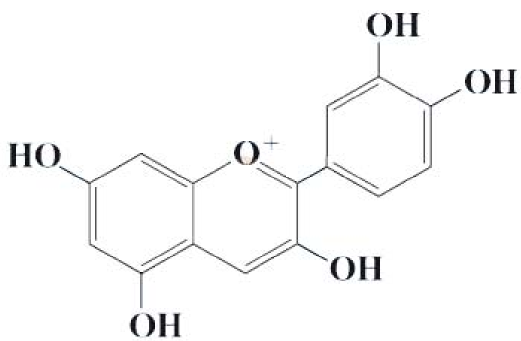
Pelargonin
(anthocyan)

B

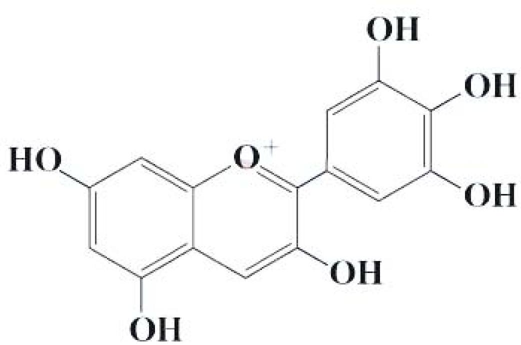
Anthocyanidin	Substituent	Color
Pelargonidin	—	Orange-red
Cyanidin	3′-OH	Red
Peonidin	3′-OCH_3	Pink
Delphinidin	3′-OH, 5′-OH	Bluish-purple
Petunidin	3′-OCH_3, 5′-OH	Purple
Malvidin	3′-OCH_3, 5′-OCH_3	Reddish-purple



pelargonidin



cyanidin



delphinidin

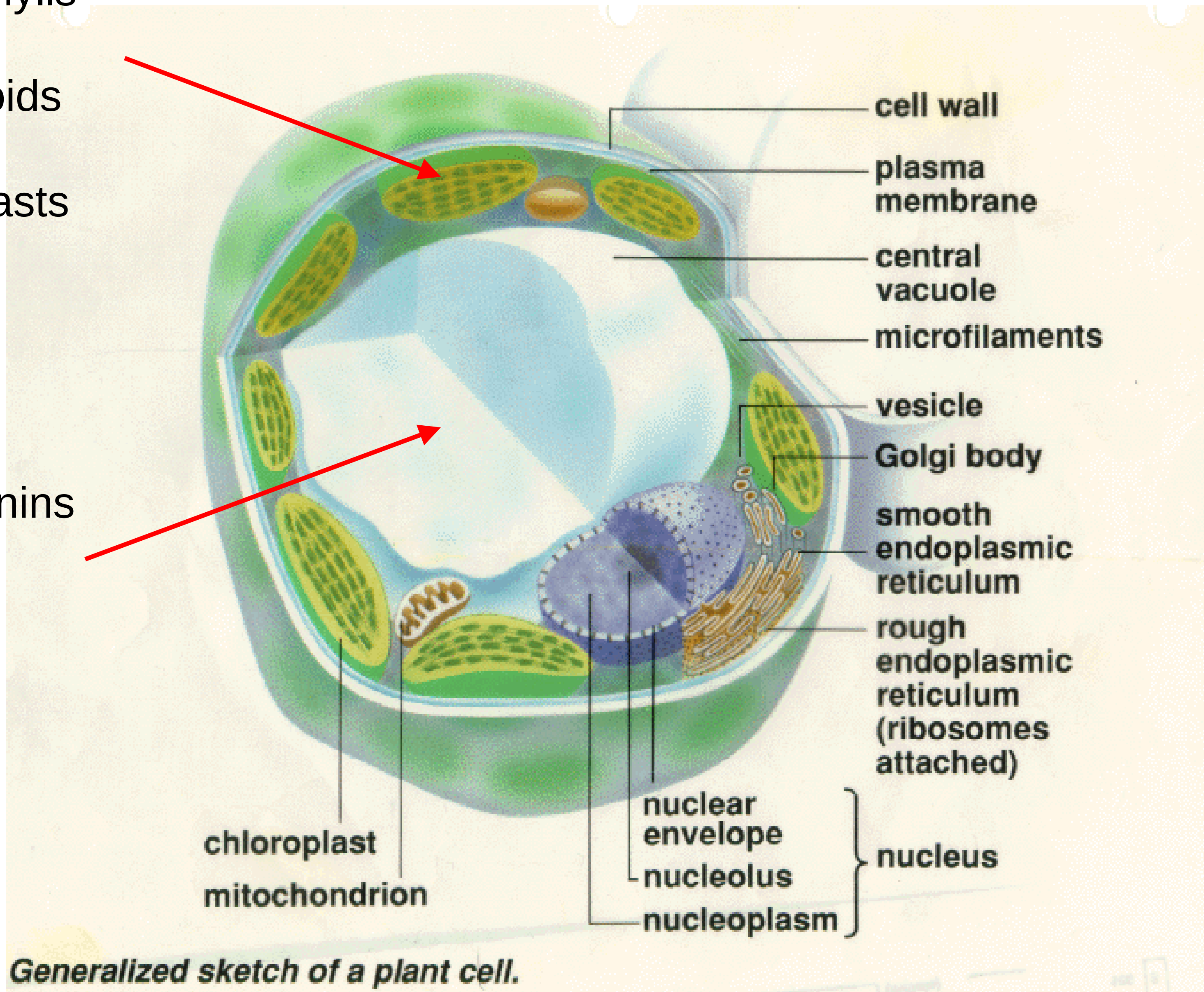
(a)

Photo source: *www.gardenseeker.com*

Photo source: www.mooseyscountrygarden.com

Chlorophylls and carotenoids are in chloroplasts

Anthocyanins are in the vacuole

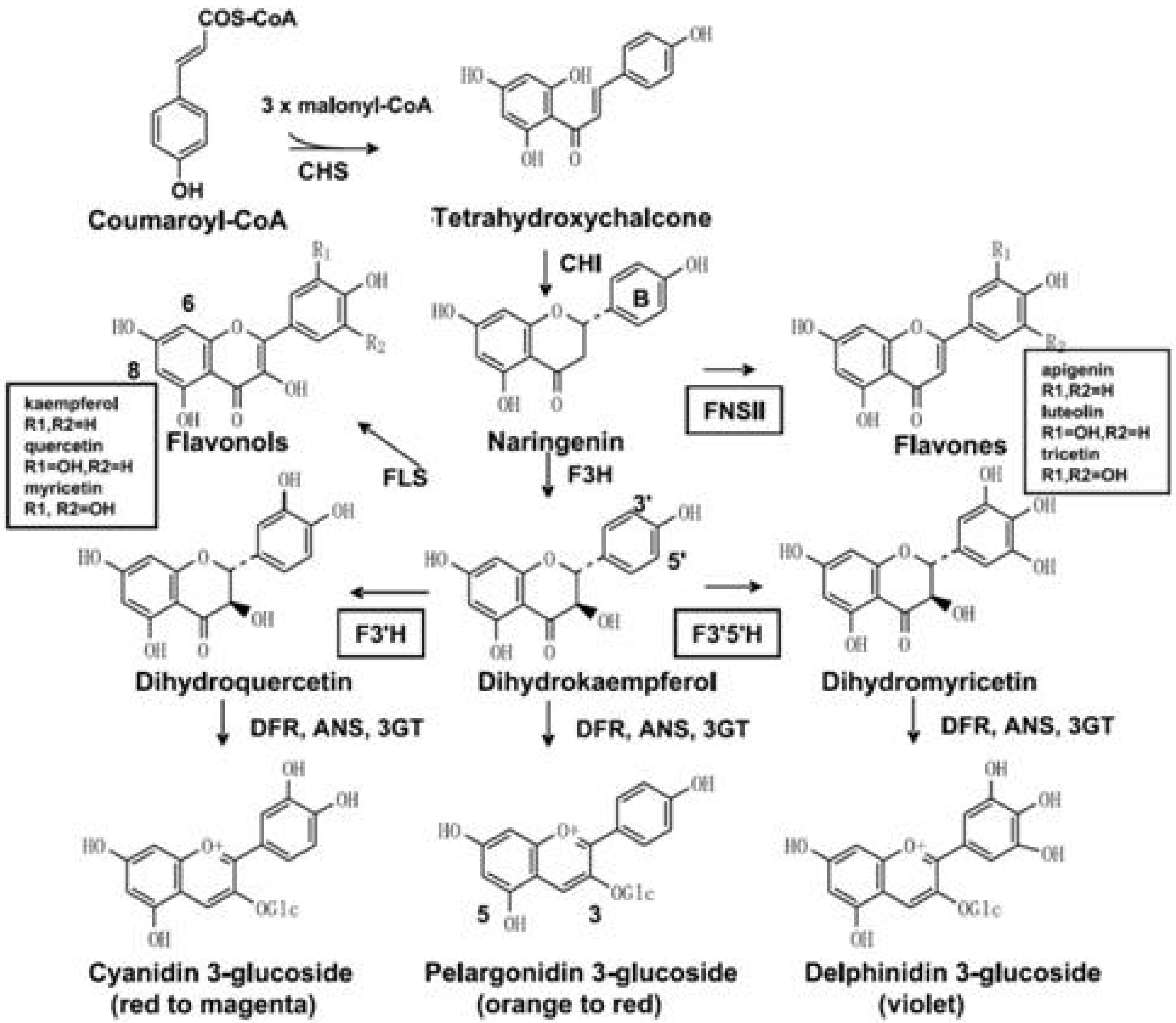


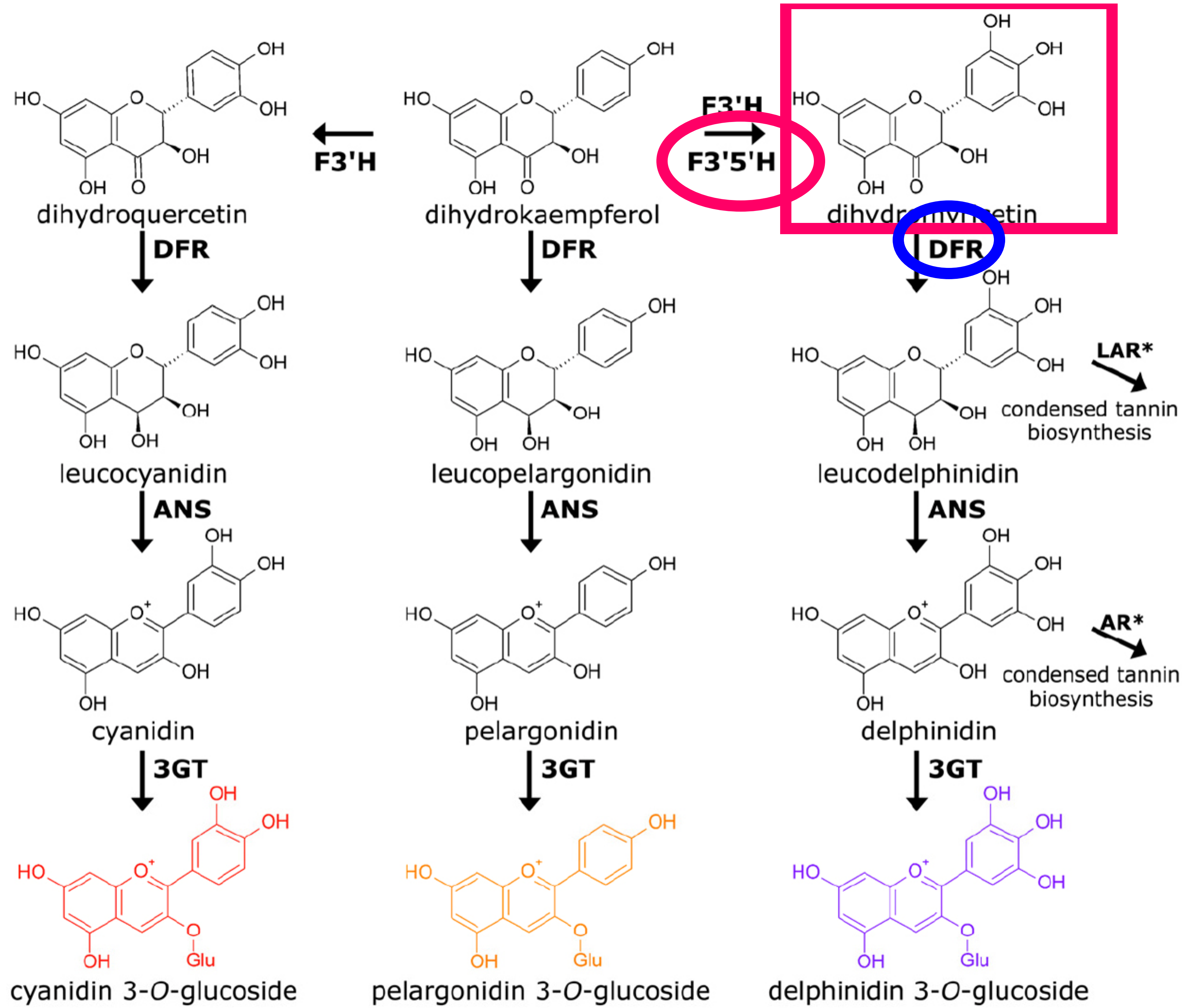
Generalized sketch of a plant cell.

Image source: *www.progressivegardens.com*

Plants accumulate specific flavonoids and thus every species often exhibits a limited flower colour range. Flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) catalyze the hydroxylation of the B-ring of flavonoids and are necessary to biosynthesize cyanidin-(red to magenta) and delphinidin-(violet to blue) based anthocyanins, respectively. Pelargonidin-based anthocyanins (orange to red) are synthesized in their absence. Some species such as roses, carnations and chrysanthemums do not have violet/blue flower colour due to deficiency of F3′5′H. Successful expression of heterologous F3′5′H genes in roses and carnations results in delphinidin production, causing a novel blue/violet flower colour.

The flavonoids biosynthetic pathway leading to the production of the first coloured anthocyanins




dihydroquercetin
F3'H
dihydrokaempferol
F3'H
F3'5'H
dihydromyricetin
DFR
DFR
DFR
leucocyanidin
leucopelargonidin
leucodelphinidin
LAR*
condensed tannin biosynthesis
ANS
ANS
ANS
cyanidin
pelargonidin
delphinidin
AR*
condensed tannin biosynthesis
3GT
3GT
3GT
cyanidin 3-O-glucoside
pelargonidin 3-O-glucoside
delphinidin 3-O-glucoside

Florigene Ltd. and Suntory Ltd. have successfully developed a range of transgenic violet carnations by introduction of a F3′5′H gene together with a petunia DFR gene into a DFR-deficient white carnation (Fukui et al.2003).

In April of 2005, they announced the production of a blue rose by introducing three transformation constructs simultaneously into roses: a pansy F3′5′H gene to produce delphinidin, an anti-rose DFR gene that silences the endogenous DFR to produce a white background, and an iris DFR gene to make specific modifications of delphinidin. Unfortunately, the details of the transgenic roses have not been revealed.

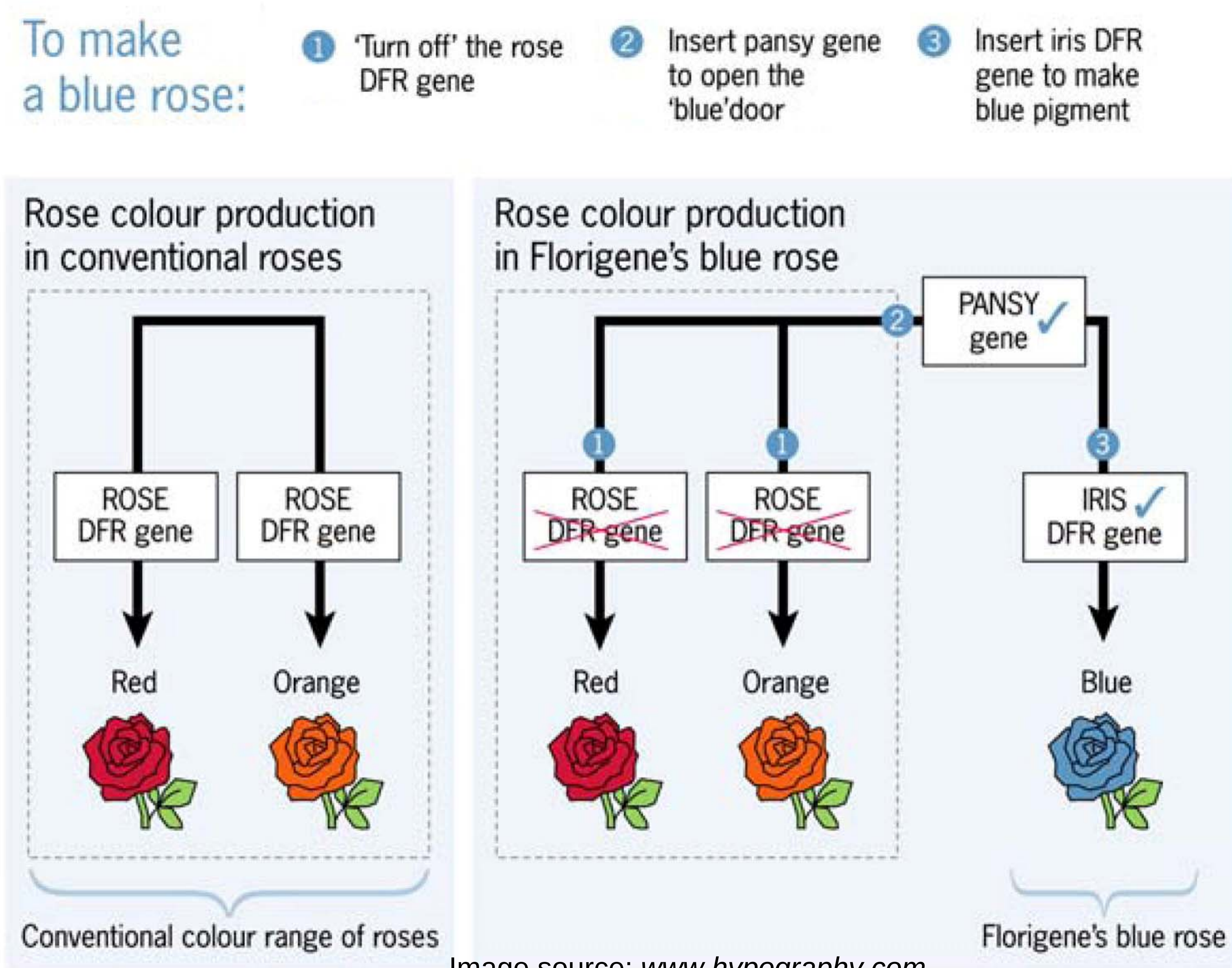


Image source: *www.hypography.com*

Source from Floriculture, Ornamental and Plant Biotechnology. Global Science Books, UK .Vol I (33) 2006

Fig. 4 Flower biotechnology has achieved success in the 150-year-long quest for the "holy grail" of blue roses. Blue roses (left) and purple carnations (right) produced through genetic engineering of flower pigments by Florigene Ltd. and Suntory Ltd. Photographs reproduced with permission from Dr. T. Tanaka of Suntory Ltd.

- ✓ Golden Rice is transformed with genes from maize and bacteria to acquire one trait, high b-carotene content (Golden rice version 2).
- ✓ Transgenic tomatoes with three kinds of promoters have a high content of b-carotene (up to 2-fold), lycopene (up to 10-fold) and flavonoids (chlorogenic acid).
- ✓ Flavonoids are a group of well-studied plant secondary metabolites that serve as flower and fruit pigments and as precursors of plant defense compounds.
- ✓ Flavonoids also play an important role for human health by their antioxidant activity and may be involved in protection against cancers, cardiovascular disease and age-related diseases.