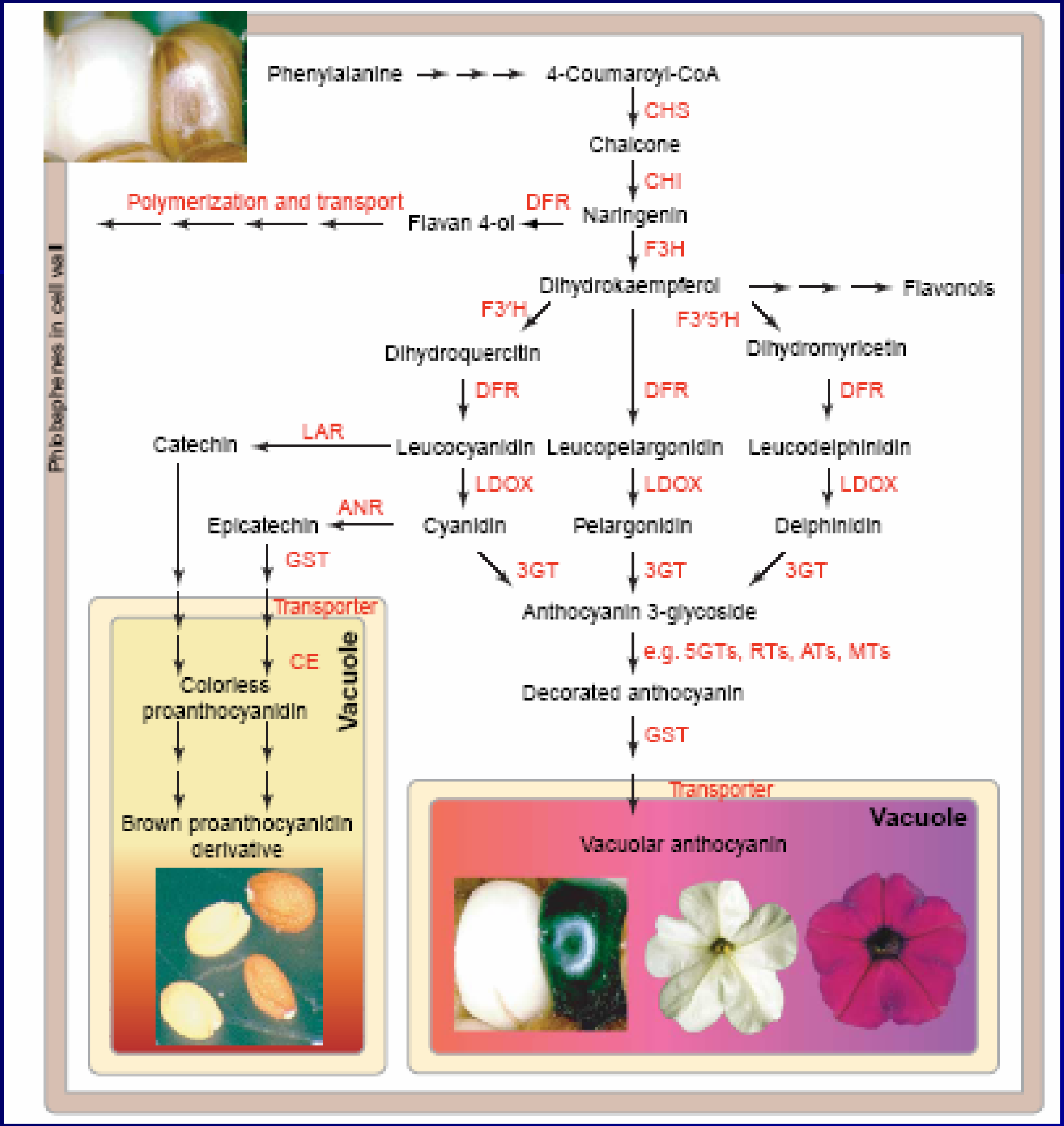
Regulation of Flavonoid Biosynthesis
(in seeds and other tissues)



Koes et al., 2005

Flavonoids in seeds

- Found in most seeds and grains
- Major ones are:
    a. Flavonols
    b. Anthocyanins
    c. Phlobaphenes
    d. Isoflavones
    e. Proanthocyanidins (PAs, also condensed tannins)

Flavonoids in seeds

Flavonols:

- contribute to seed pigmentation mainly as co-pigments with anthocyanins
- most frequent are glycosides derivatives (glucose and rhamnose at C3 and C7)

Anthocyanins:

- in seeds of both mono and dicots
- barley grains, bean seed coat, aleurone layer in maize

Flavonoids in seeds

Phlobaphenes (deoxyflavonoids):

- red polymers of flava-4-ol precursors found in maize and other monocots in the seed coat

Isoflavones:

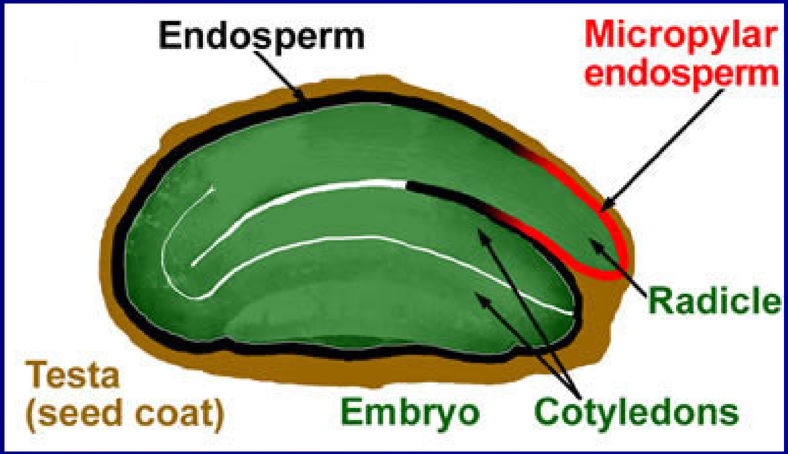
- colorless compounds, occur at the subfamily Leguminosae (soybean)
- major metabolites in the embryo and seed coat

Flavonoids in seeds

Proanthocyanidins (PAs, also condensed tannins):

Described in detail (in Arabidopsis)

Flavonols and PAs in Arabidopsis seeds



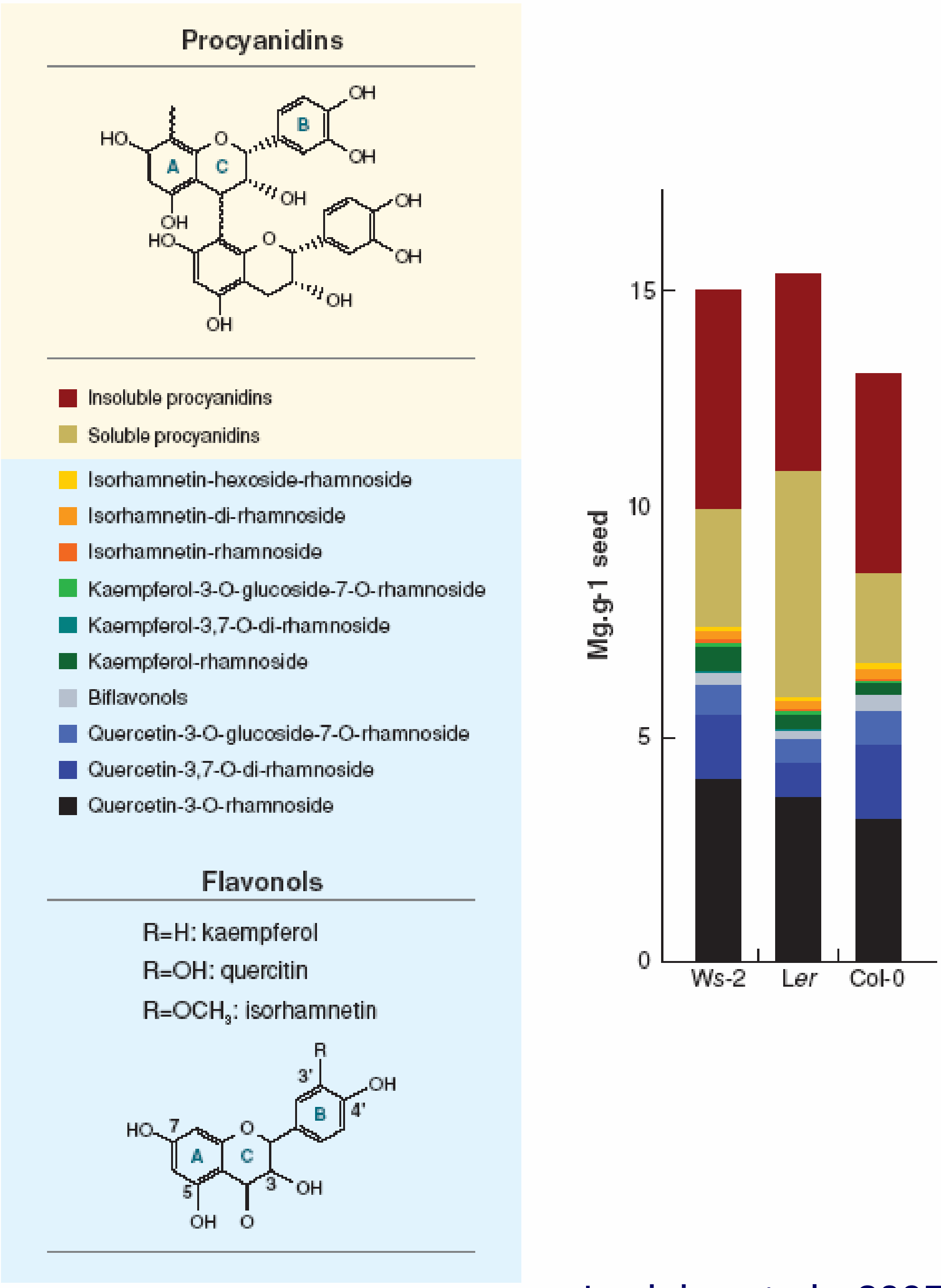
Flavonols and PAs in Arabidopsis seeds

- Arabidopsis seeds accumulate only flavonols and PAs (50/50)

- Flavonols present in testa (seed coat) and the embryo

- Quercetin (quercetin-3-O-rhamnoside) is the major flavonol

Flavonols and PAs in Arabidopsis seeds



Lepiniec et al., 2005

PAs in Arabidopsis seeds

- PAs accumulate in the seed coat

- Protect the embryo and endosperm

- Oxidation of PAs during seed desiccation- brown pigment to mature seeds

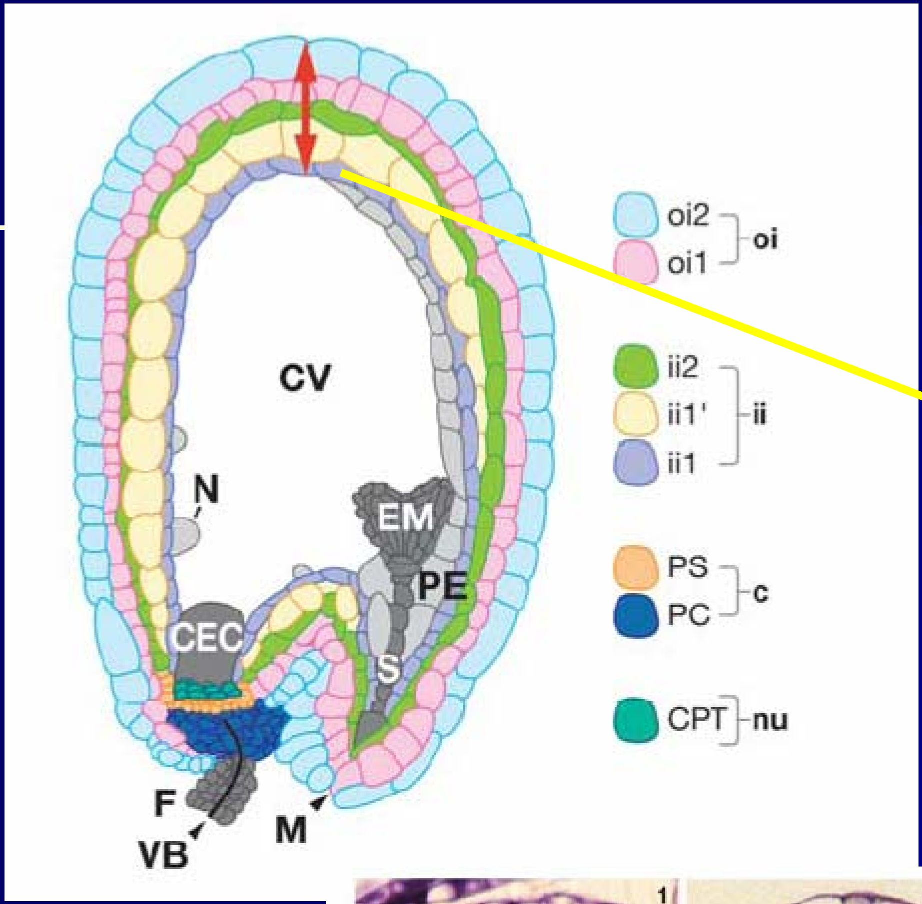
PAs in Arabidopsis seeds

- The seed coat results from differentiation of the integument and chalazal tissue after fertilization

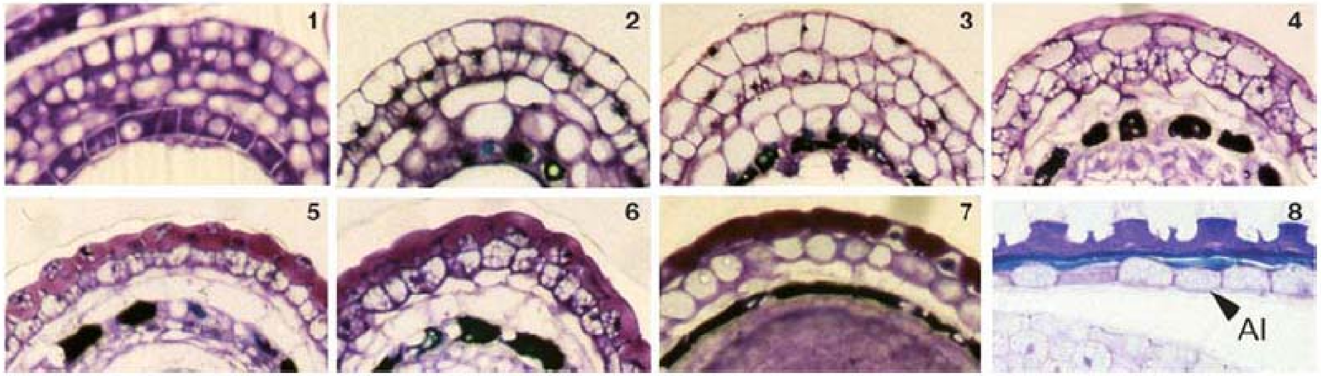
- The seeds have an outer and inner integument

- PAs are synthesized in the endothelium, the inner most cell layer of the inner integument

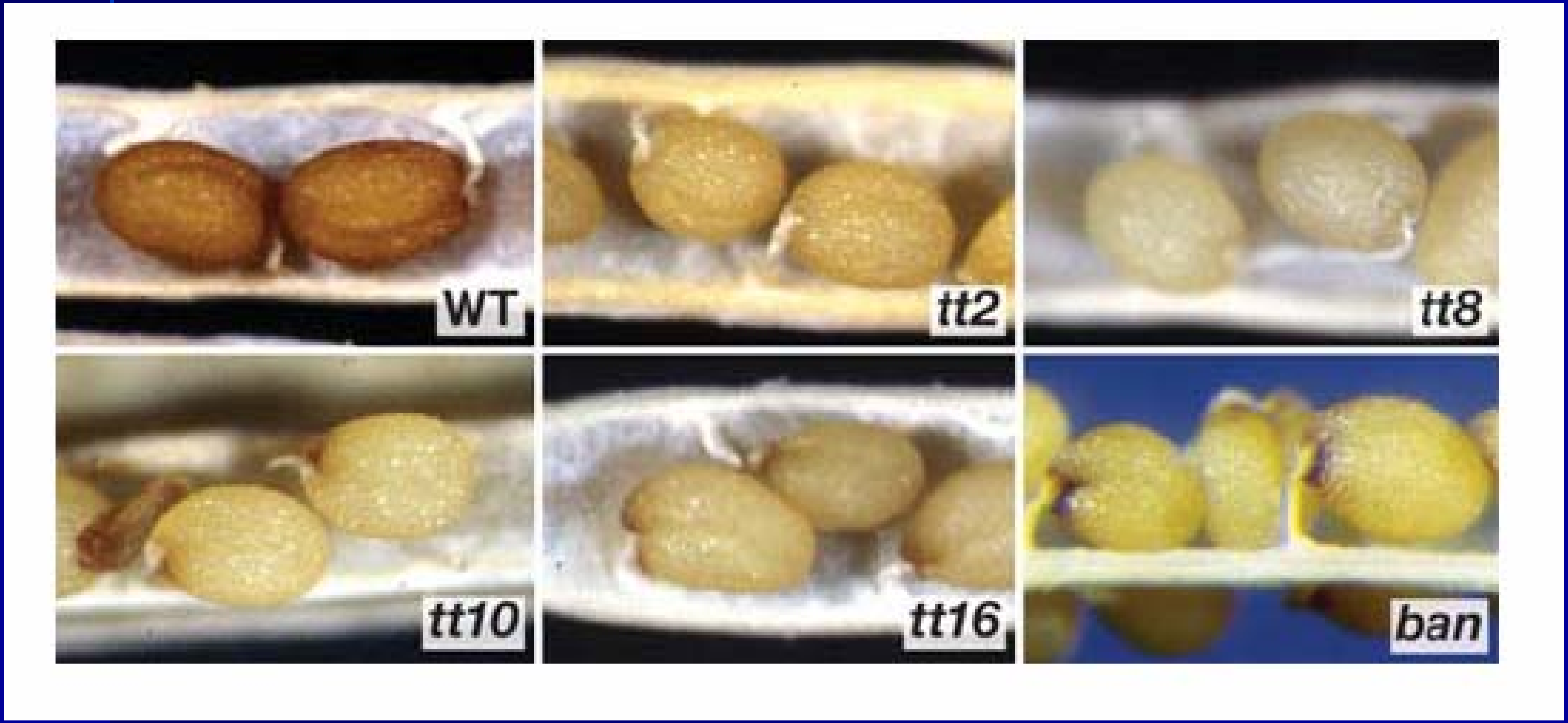
PAs in Arabidopsis seeds



Endothelium
(PAs synthesis)



Altered seed pigmentation in Arabidopsis:
the transparent testa (tt) mutants

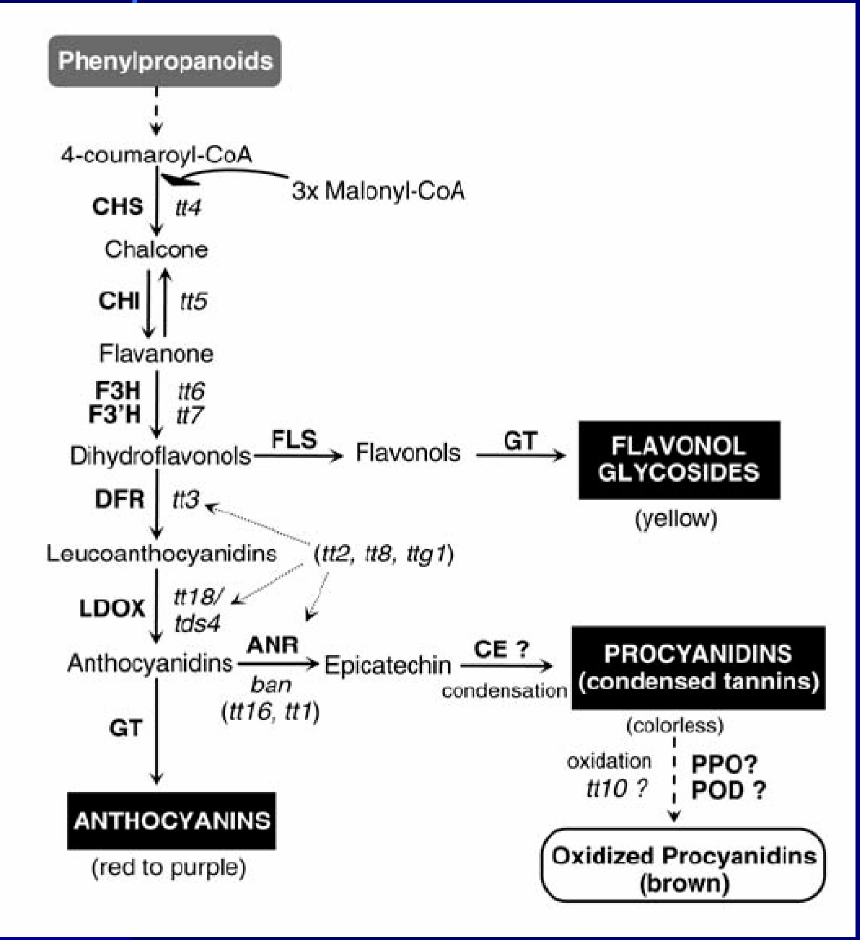


Altered seed pigmentation in Arabidopsis: the transparent testa (tt) mutants

Table 1 *Loci* involved in PA biosynthesis in *Arabidopsis* seed

Locus	Seed coat color[a]	Gene product	Branch[b]	References
Structural genes				
tt3	Yellow	Dihydroflavonol reductase (DFR)	P, A	(66, 128, 129)
tt4	Yellow	Chalcone synthase (CHS)	P, F, A	(33, 66, 129)
tt5	Yellow	Chalcone isomerase (CHI)	P, F, A	(66, 128, 129)
tt6	Pale brown spotted	Flavanone-3-hydroxylase (F3H)	P, F, A	(66, 102, 129, 154)
tt7	Pale brown spotted	Flavanone-3′-hydroxylase (F3′H)	P, F, A	(66, 67, 121, 129)
tt10	Dark yellow/brown C[c]	Polyphenol oxydase (PPO)	P, F	(66, 129; *e*)
tt12	Pale brown	MATE secondary transporter	P	(24)
tt15	Pale brown/brown CM	Glycosyltransferase (GT)	P	(34; *f*)
tt18/ tds4/ tt11	Yellow	Leucocyanidin dioxygenase (LDOX)[d]	P, A	(2, 93, 126; *g*)
tt19/tt14	Dark yellow[3]	Glutathione S-transferase (GST)	P, A	(24, 63; *g*)
ban	Pale gray/gray CM	Anthocyanidin reductase (ANR)	P	(25, 139, 156)
aha10	Pale brown	Autoinhibited H^+-ATPase isoform 10	P	(7)
Regulatory genes				
tt1	Yellow/brown CM	Transcription factor WIP-type Zn-Finger	P	(66, 111, 129)
tt2	Yellow	Transcription factor AtMYB123	P	(66, 93, 129)
tt8	Yellow	Transcription factor AtbHLH042	P, A	(66, 91, 129)
tt16 /abs	Yellow/brown CM	Transcription factor MADS AtAGL32	P	(59, 92)
ttg1	Yellow	Regulatory protein ("WD40" or "WDR")	P, A	(65, 129, 149)
ttg2	Yellow	Transcription factor AtWRKY44	P	(55)
Other loci				
tt9	Pale gray/dark CM	Unknown	?	(66, 129)
tt13	Pale brown	Unknown	?	(24)
tt17	Pale brown	Unknown	?	(11)
tds1,3, 5, 6	Pale brown	Unknown	P	(3)
tds2	Pale brown	Unknown	P, A	(3)

PA metabolism in Arabidopsis seeds: tt10- PAs oxidative polymerization (a polyphenol oxidase)



- PAs in Arabidopsis testa are the flavan-3-ol 2,3-cis-(-)-epicatechin oligomers

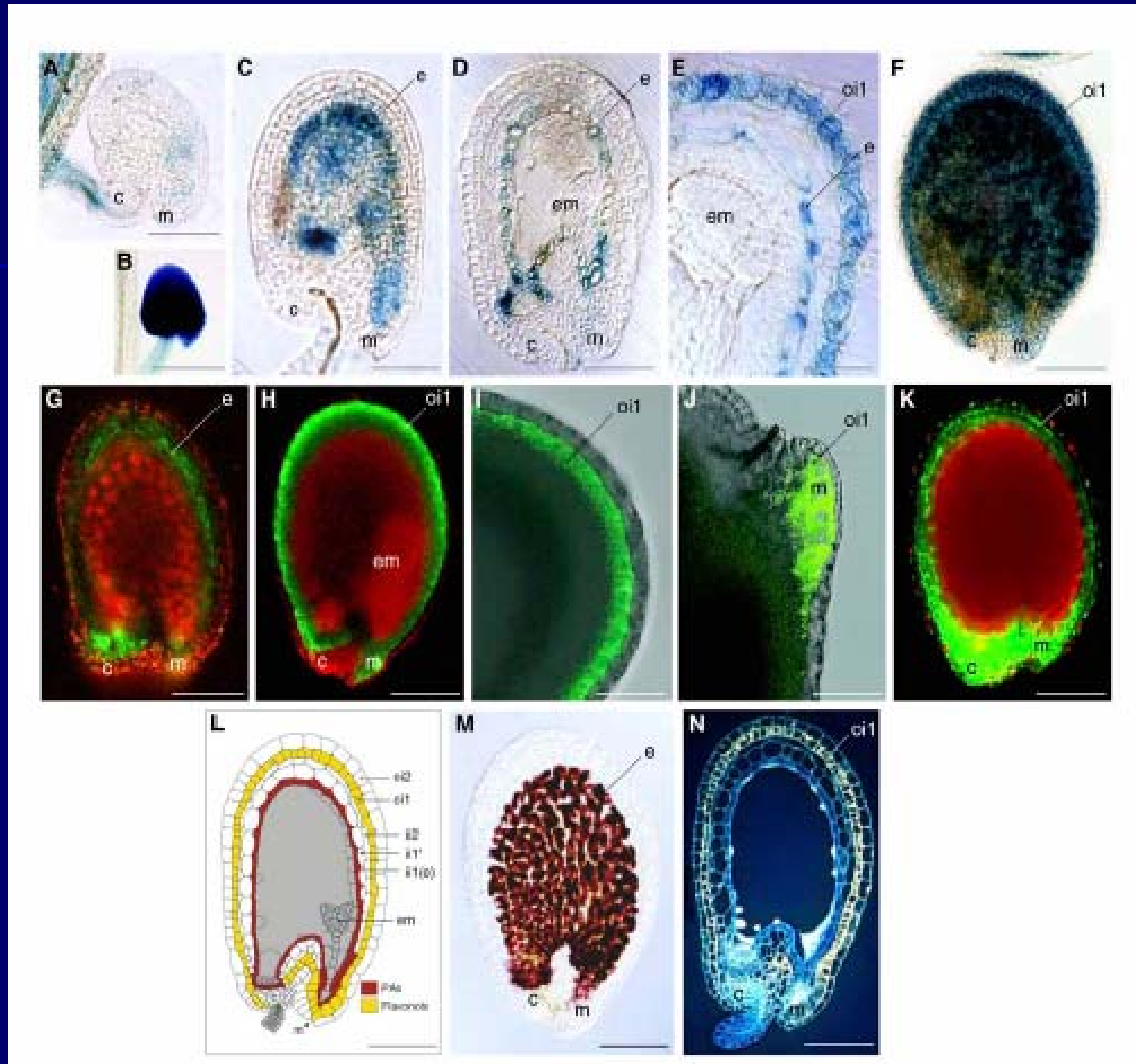
- PAs are colorless polymers localized to the vacuole

- During seed desiccation, vacuole burst will localize a polyphenol oxidase enzyme (TT10?) with PAs in the appoplast and this will result in browning

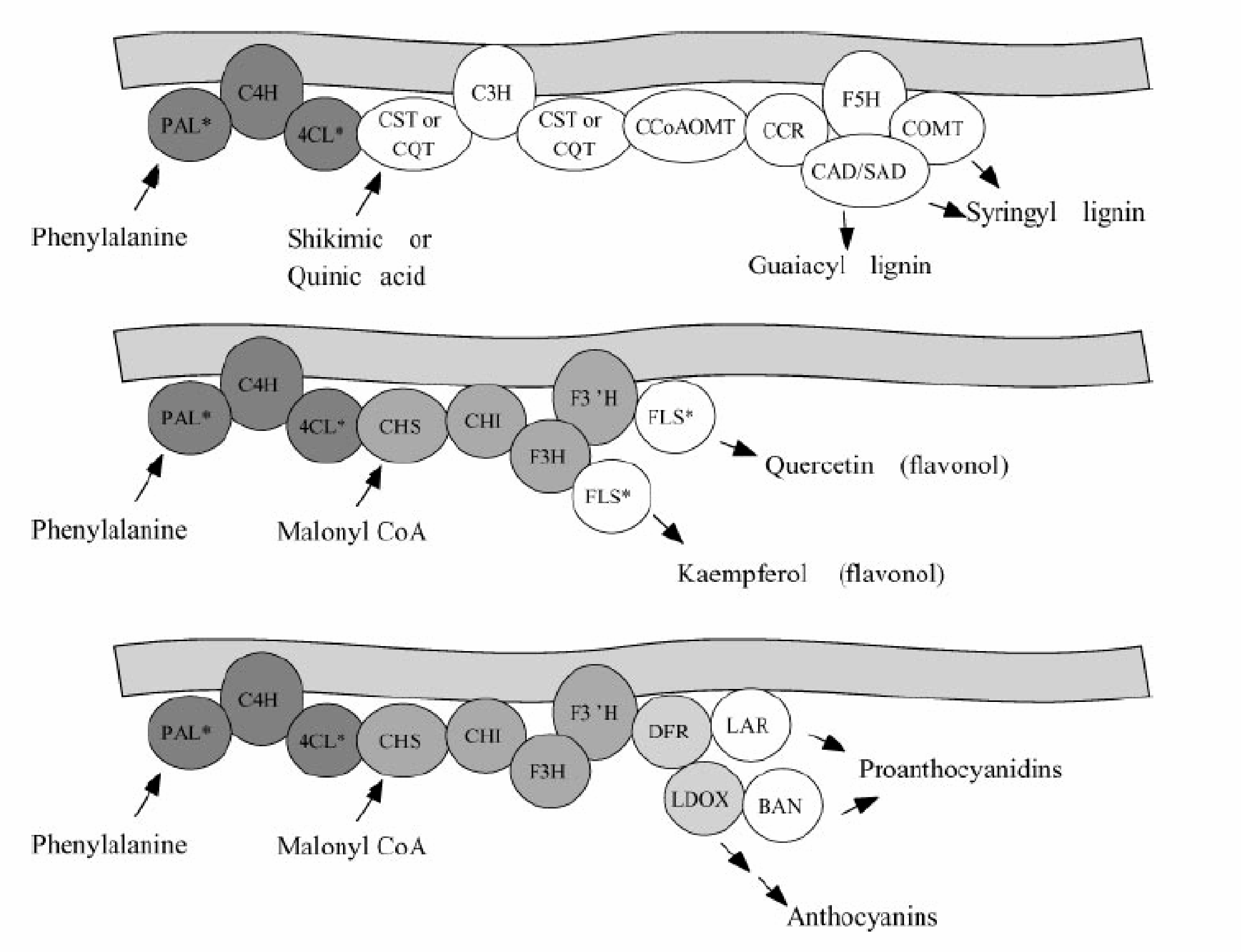
PA metabolism in Arabidopsis seeds: tt10- PAs oxidation

Gene expression localizes with PAs and flavonols synthesis

TT10 can oxidize epi-catechin and quercetin



Flavonoids in Arabidopsis seeds- Metabolon formation?

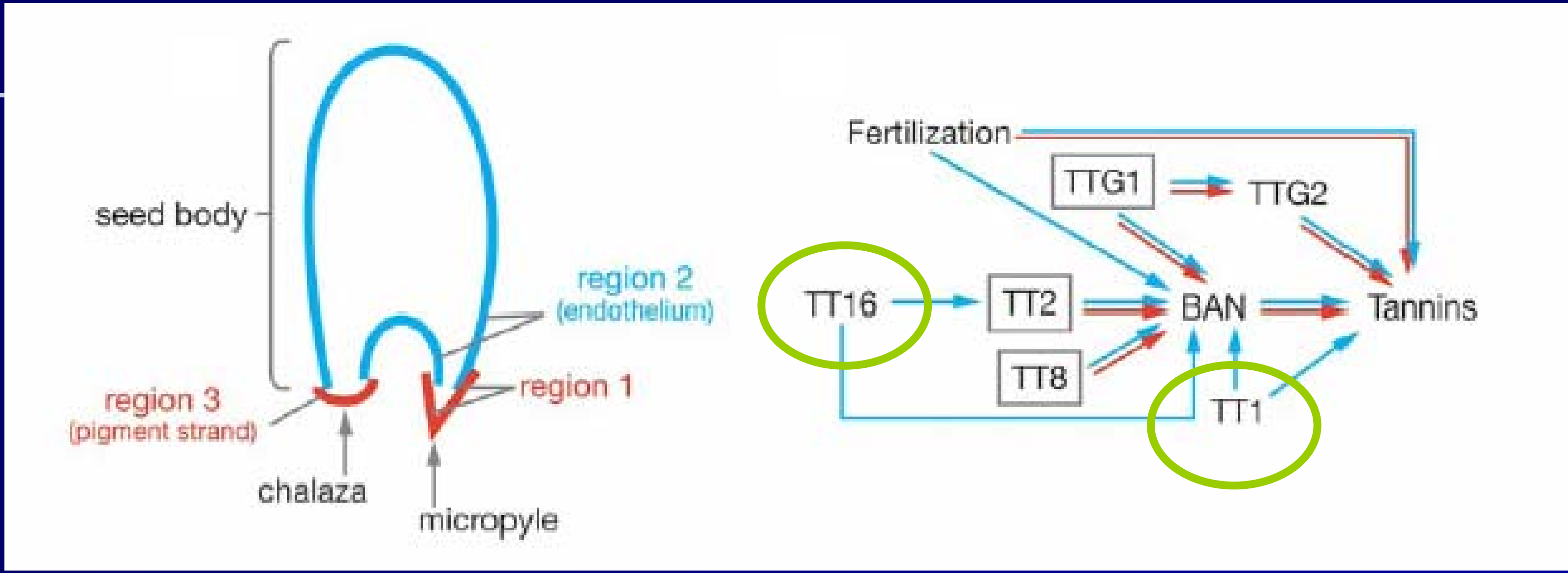


Winkel BS. 2004

Regulation of PAs biosynthesis in Arabidopsis seeds

Locus	Seed coat color[a]	Gene product	Branch[b]
Regulatory genes			
tt1	Yellow/brown CM	Transcription factor WIP-type Zn-Finger	P
tt2	Yellow	Transcription factor AtMYB123	P
tt8	Yellow	Transcription factor AtbHLH042	P, A
tt16 /abs	Yellow/brown CM	Transcription factor MADS AtAGL32	P
ttg1	Yellow	Regulatory protein ("WD40" or "WDR")	P, A
ttg2	Yellow	Transcription factor AtWRKY44	P

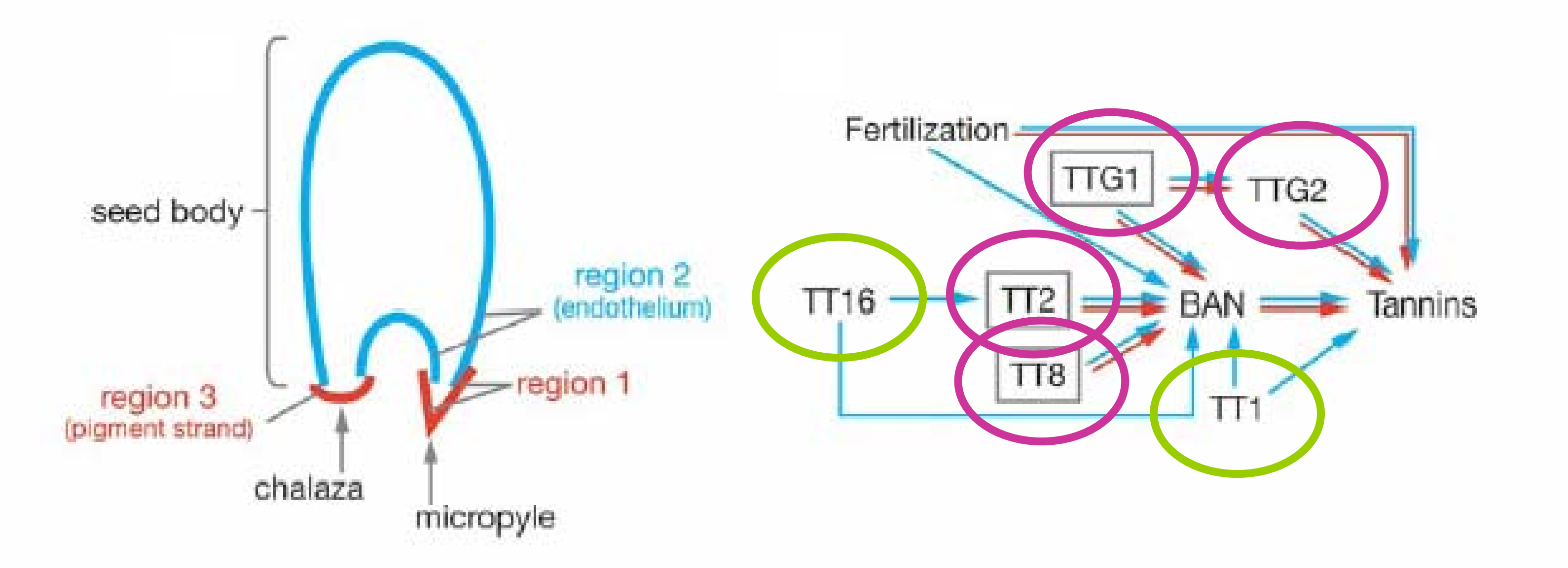
Regulation of PAs biosynthesis in Arabidopsis seeds



- TT1 (zinc-finger) and TT16 (MADS box), may control cellular differentiation leading to PA accumulation and not PA biosynthesis directly

- Needed for PA accumulation in the endothelium (region 2) but not in micropyle and chalaza area

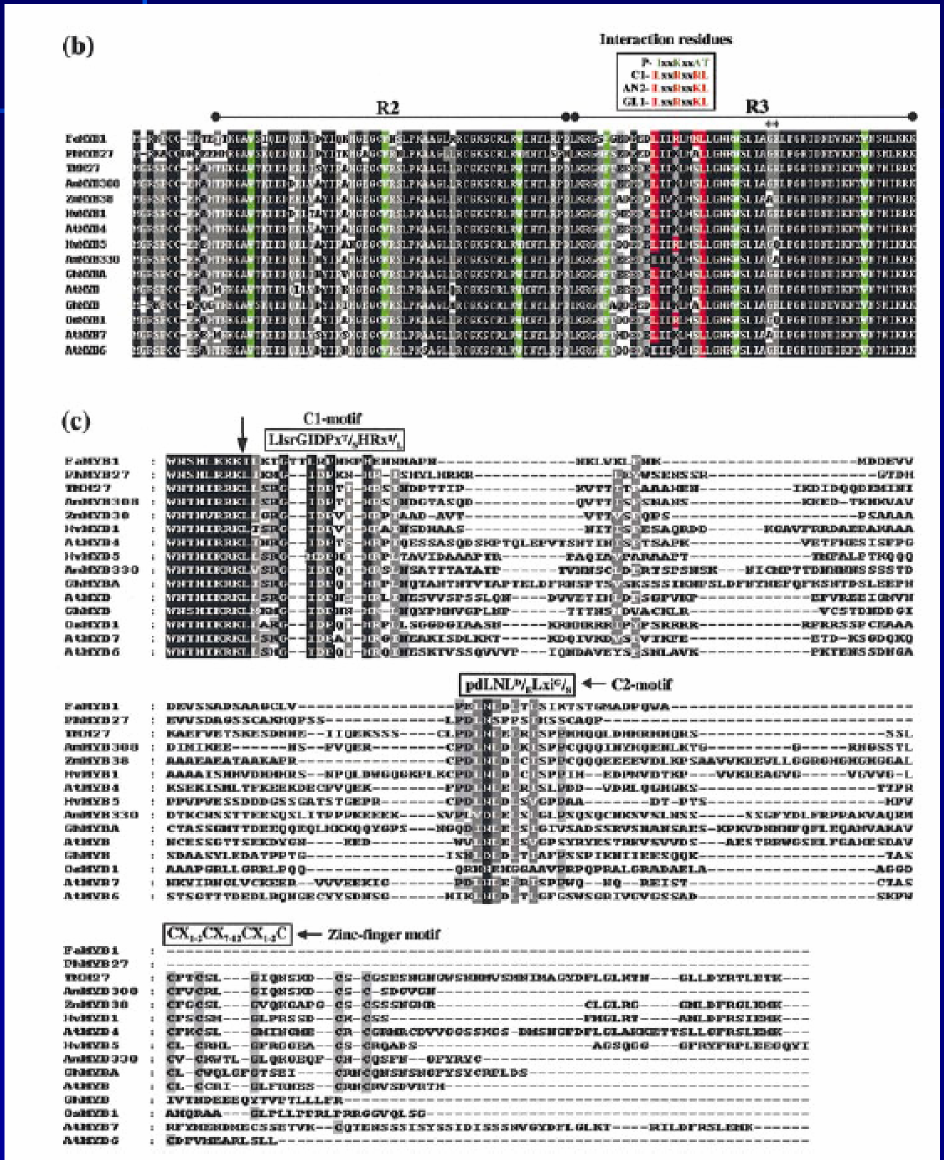
Regulation of PAs biosynthesis in Arabidopsis seeds

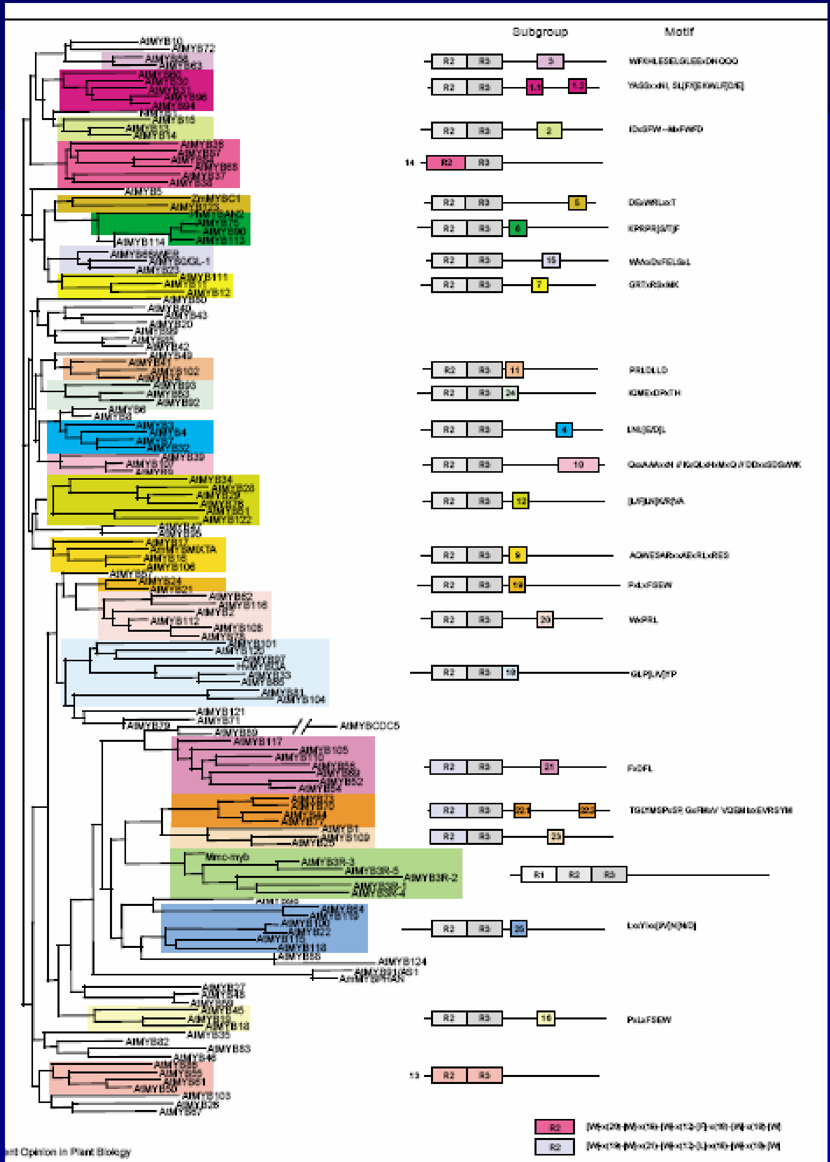


- TT2- R2-R3-MYB protein
- TT8- bHLH protein
- TTG1- WDR protein
- TTG2- WRKY transcription factor

Plant MYB Factors

Stracke et al., 2001





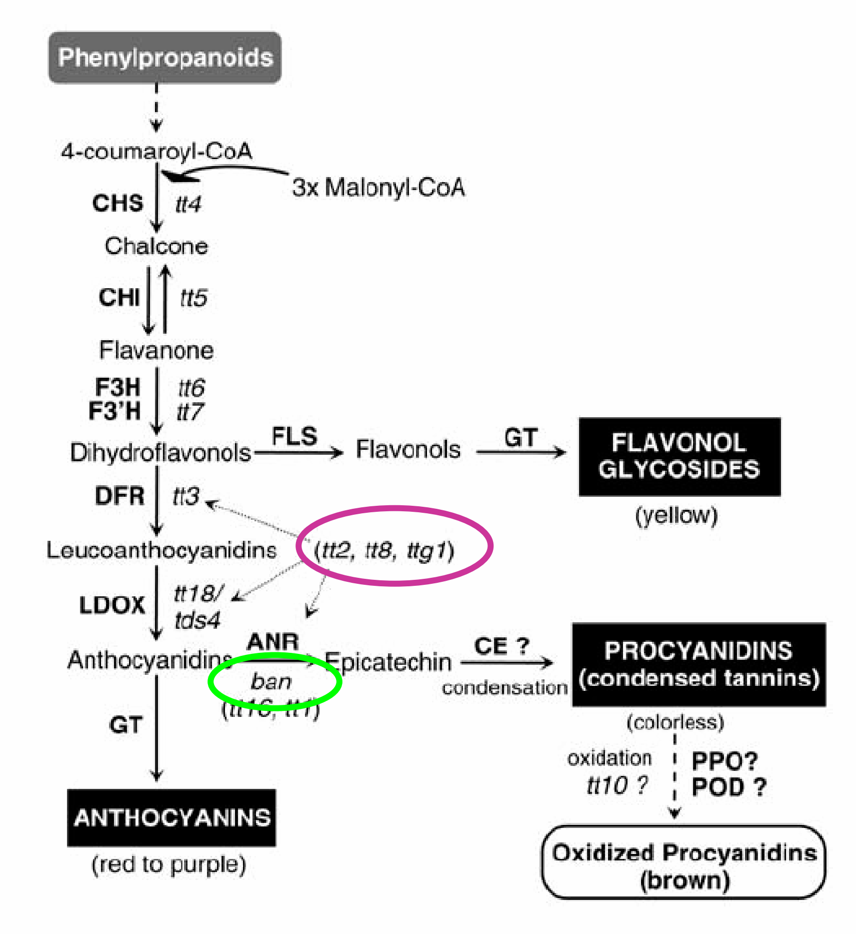
Plant Basic Helix-Lop-Helix Factors

- More than 160 members in Arabidopsis

- Contain a bHLH domain that is involved in binding DNA

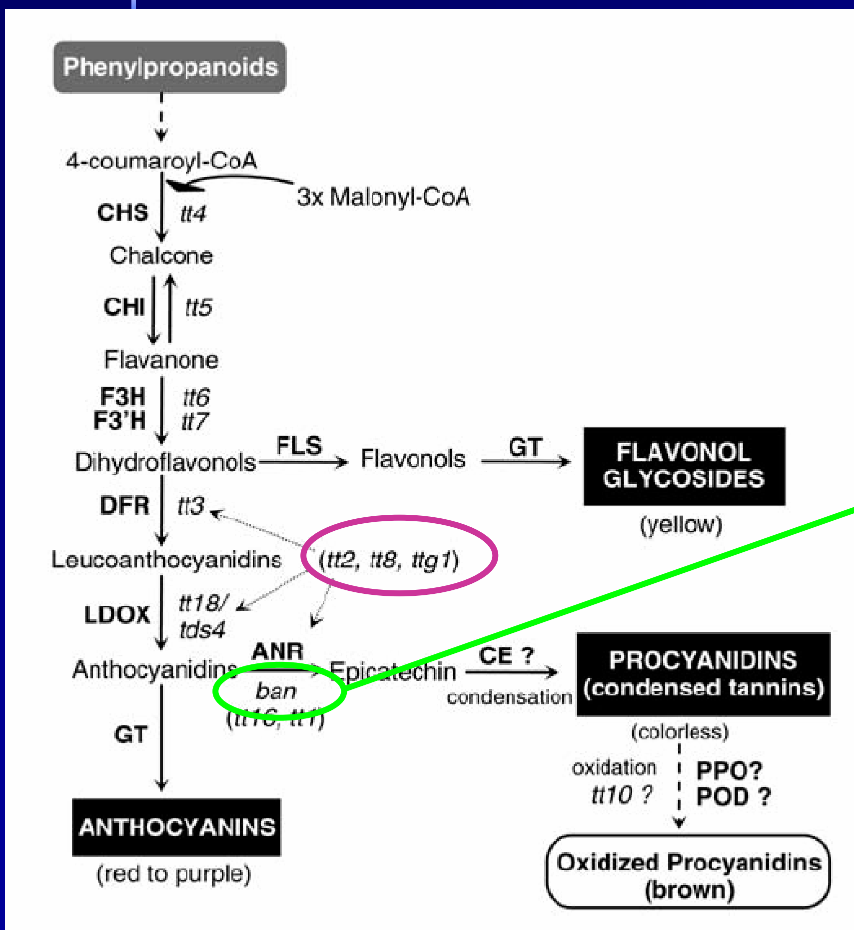
Regulation of PAs biosynthesis in Arabidopsis seeds

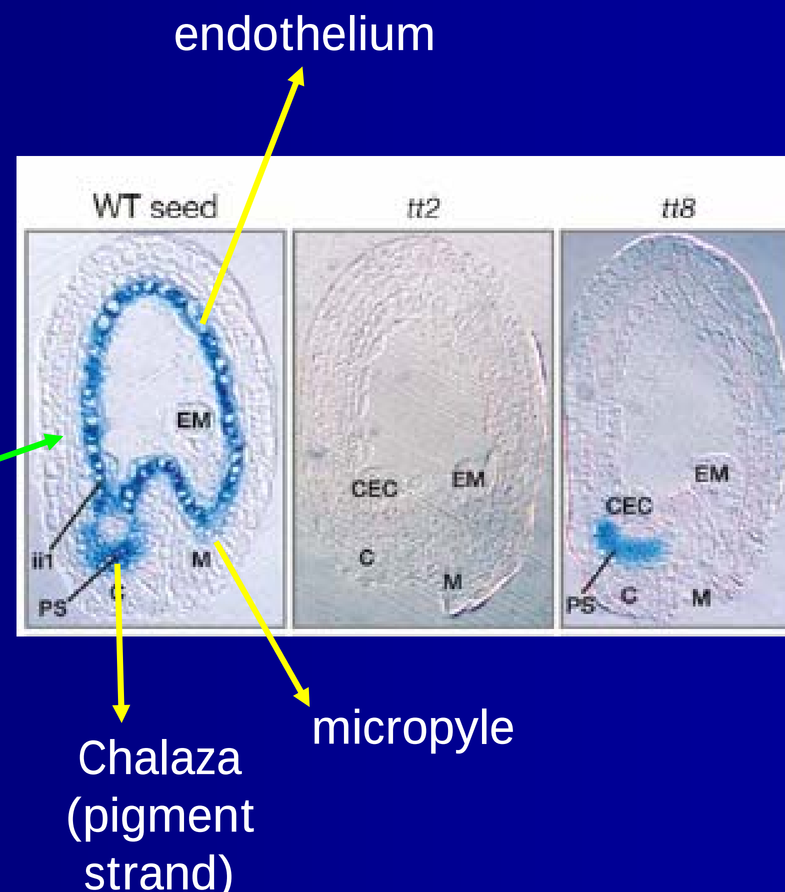
TT2, TT8 and TTG1 regulate BAN but also other flavonoid pathway genes



Regulation of PAs biosynthesis in Arabidopsis seeds

TT2, TT8 and TTG1 regulate BAN but also other flavonoid pathway genes





Regulation of PAs biosynthesis in Arabidopsis seeds
TT2, TT8 and TTG1 forming the MBW complex

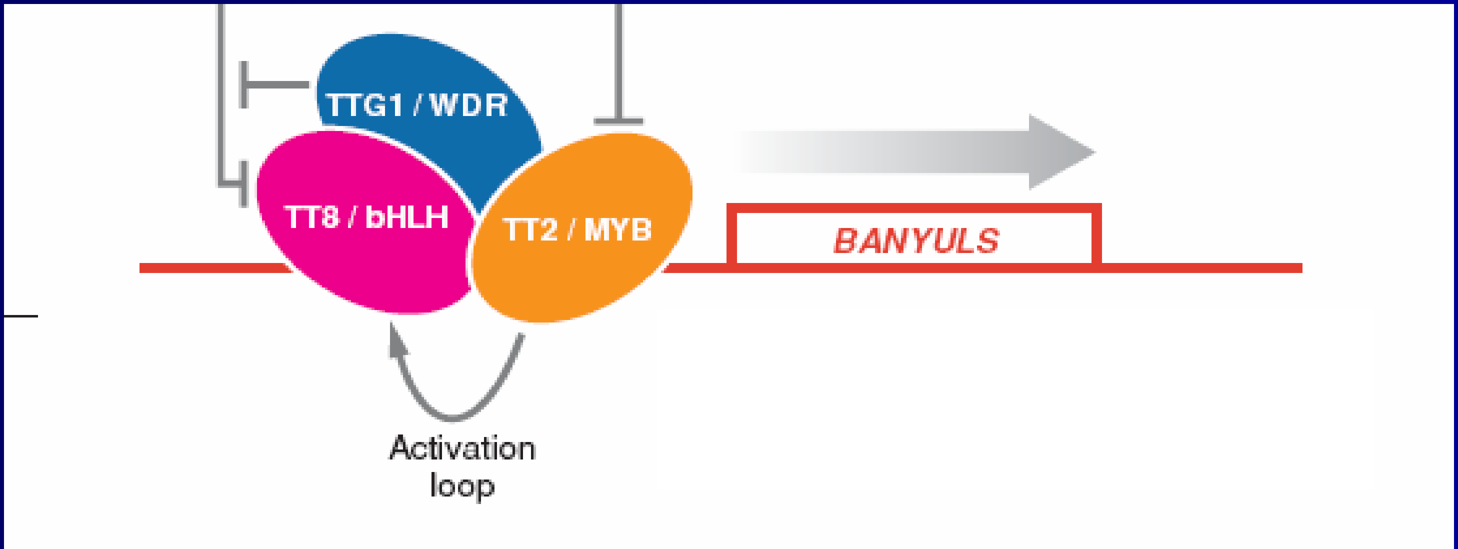
- TT2- R2-R3-**M**YB protein
- TT8- **b**HLH protein
- TTG1- **W**DR protein

Regulation of PAs biosynthesis in Arabidopsis seeds
TT2, TT8 and TTG1 forming the MBW complex

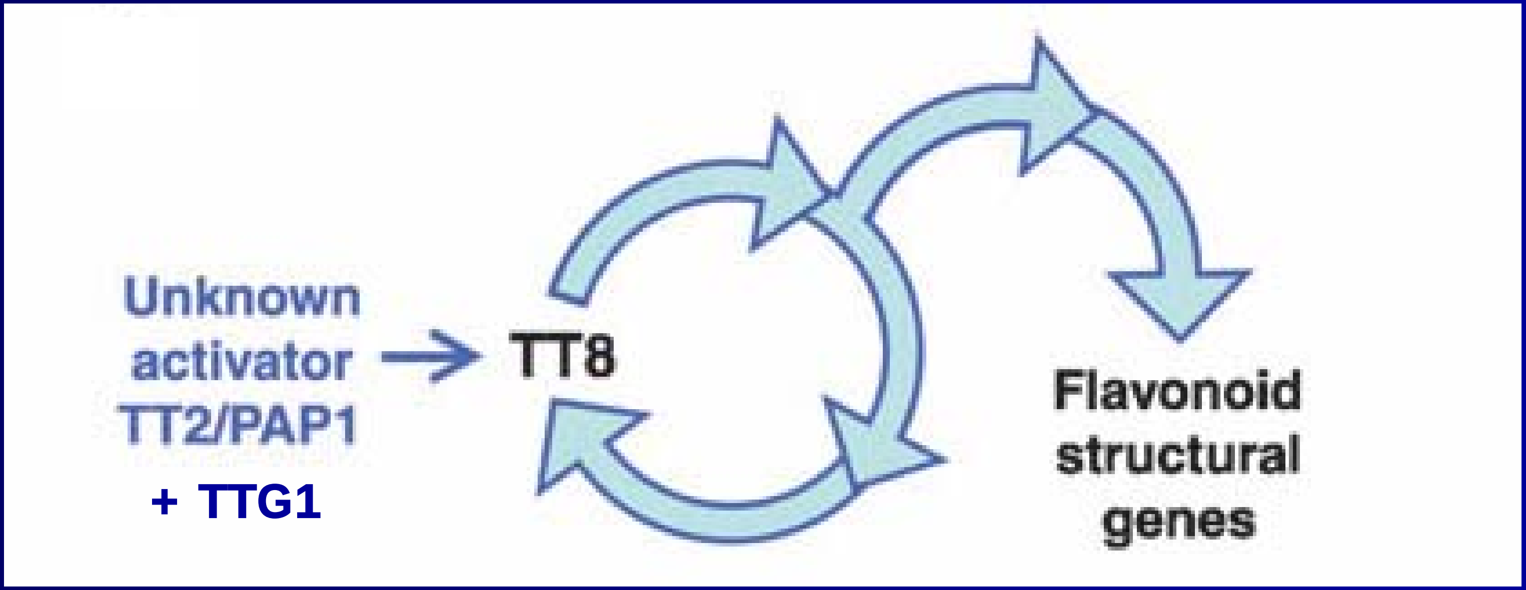
- R2-R3 MYBS can bind directly to DNA, either on their own or in the presence of bHLH proteins
- bHLH binding alone was never demonstrated
- Interaction MYB-bHLH was detected a few times (N-termini of bHLH interacts with R3 repeat of MYBS)
- WDR interact with MYB and bHLH

Regulation of PAs biosynthesis in Arabidopsis seeds
TT2, TT8 and TTG1 forming the MBW complex

- a ternary complex is able to bind the BAN promoter



Regulation of PAs biosynthesis in Arabidopsis seeds
What regulates TT8?



- TTG1, TT2 and PAP1 regulate TT8
- TT8 activates itself to synchronize its levels with those of its partners in the MBW complex

Baudry et al., 2006

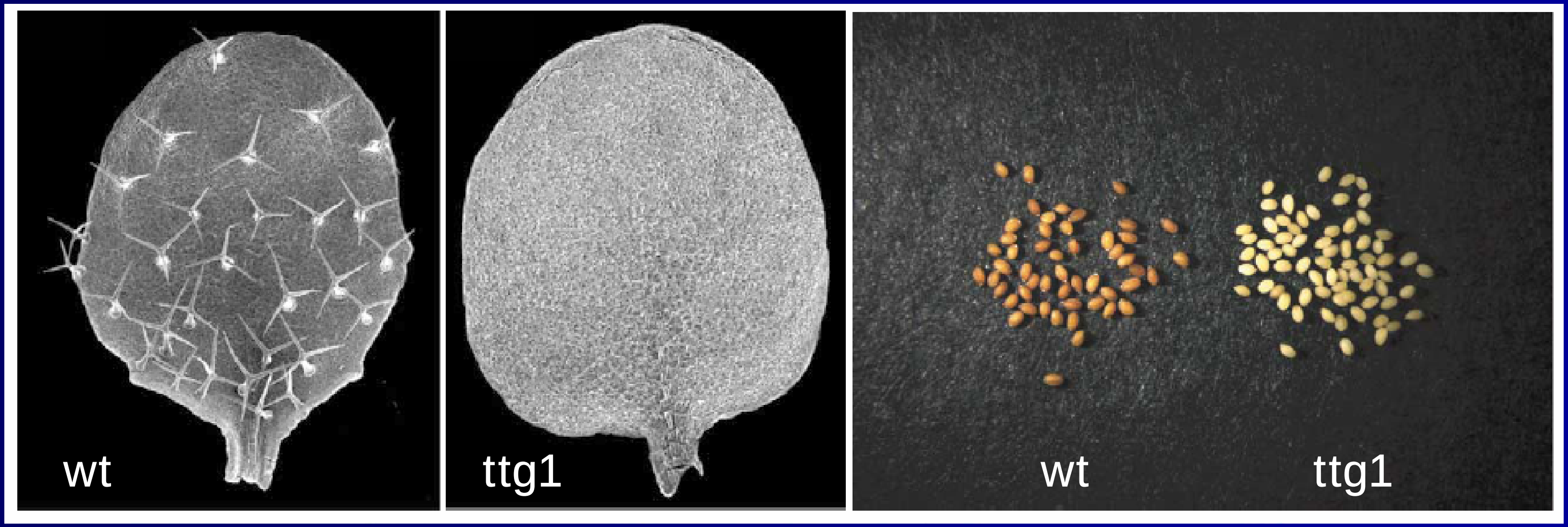
Epidermal cell fate, seed-coat development and flavonoid biosynthesis are linked in Arabidopsis

- The above relationship first became apparent with the discovery of the *transparent testa glabra1* (*ttg1*) mutants

- The *ttg* mutants typically:

    - produce few or no trichomes
    - have an aberrant pattern of root hair initiation
    - produce yellow seeds (reduction in PAs)

Epidermal cell fate, seed-coat development and flavonoid biosynthesis are linked in Arabidopsis



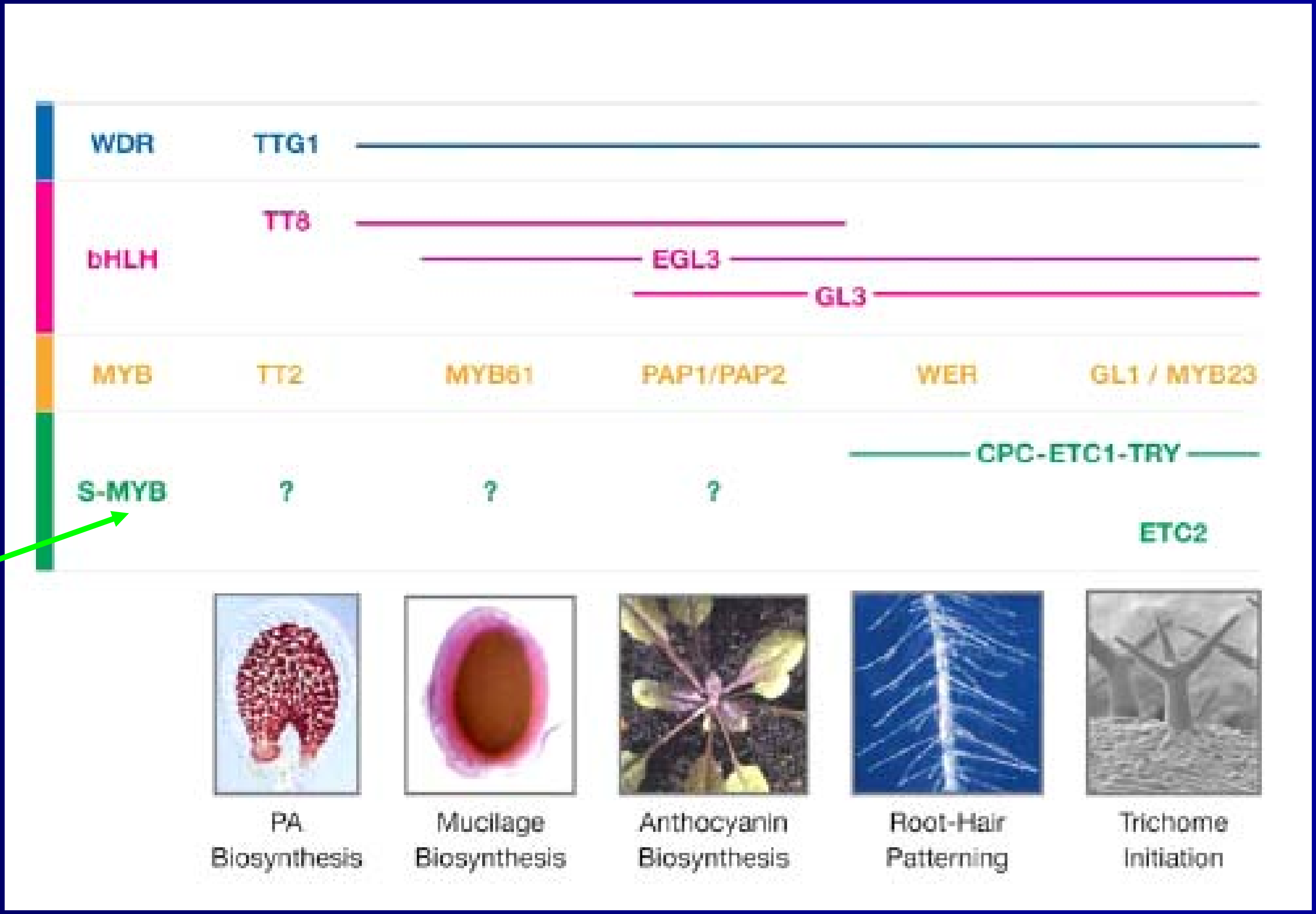
Broun, 2005

Epidermal cell fate, seed-coat development and flavonoid biosynthesis are linked in Arabidopsis

- The *ttg* mutants also:

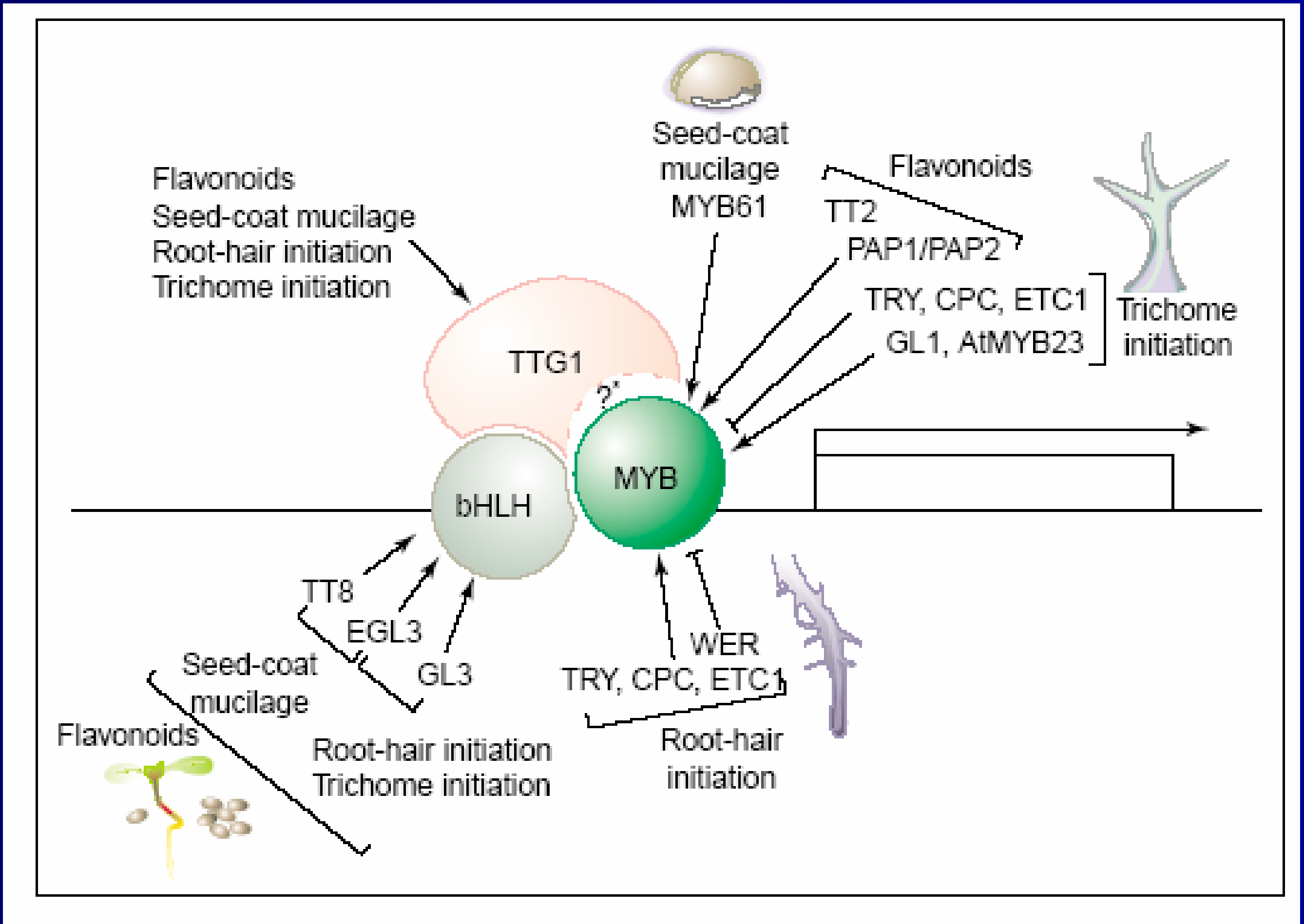
  - Mucilage production in the seed coat is impaired
  - Anthocyanins in leaves, stems do not accumulate or are reduced in levels

Epidermal cell fate, seed-coat development and flavonoid biosynthesis are linked in Arabidopsis

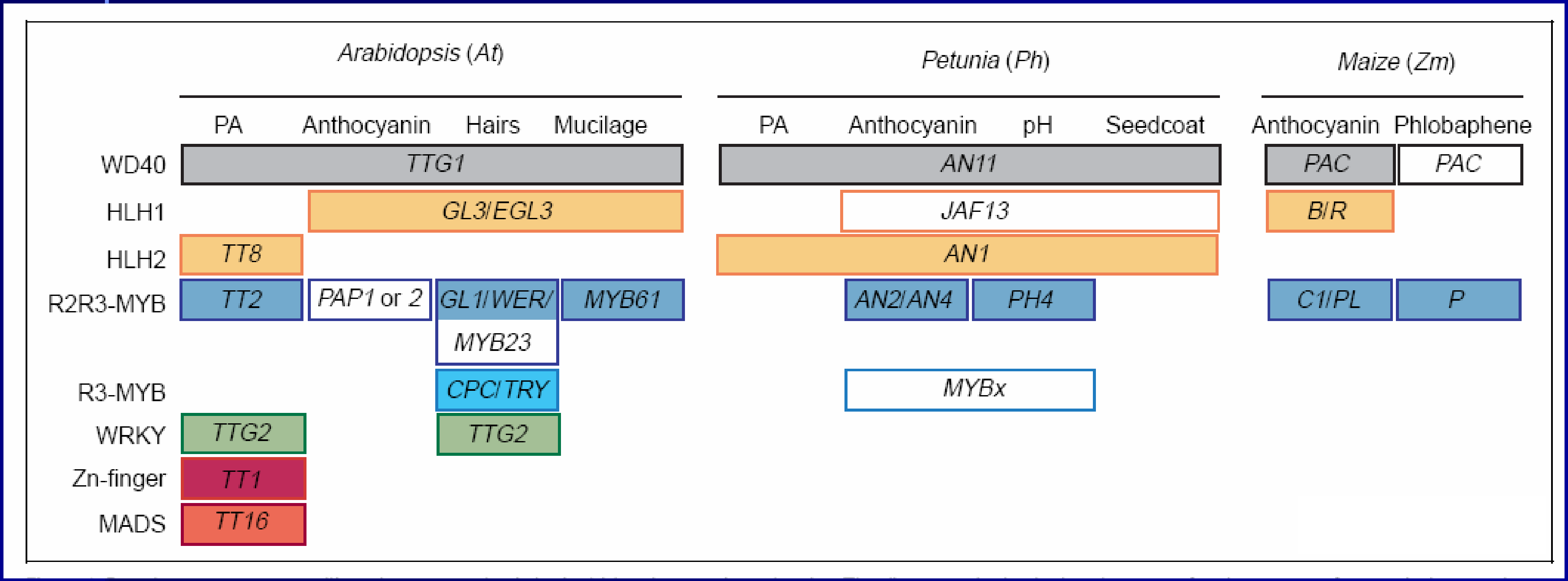


Single repeat MYBs (R3), lack an activation domain

Epidermal cell fate, seed-coat development and flavonoid biosynthesis are linked in Arabidopsis

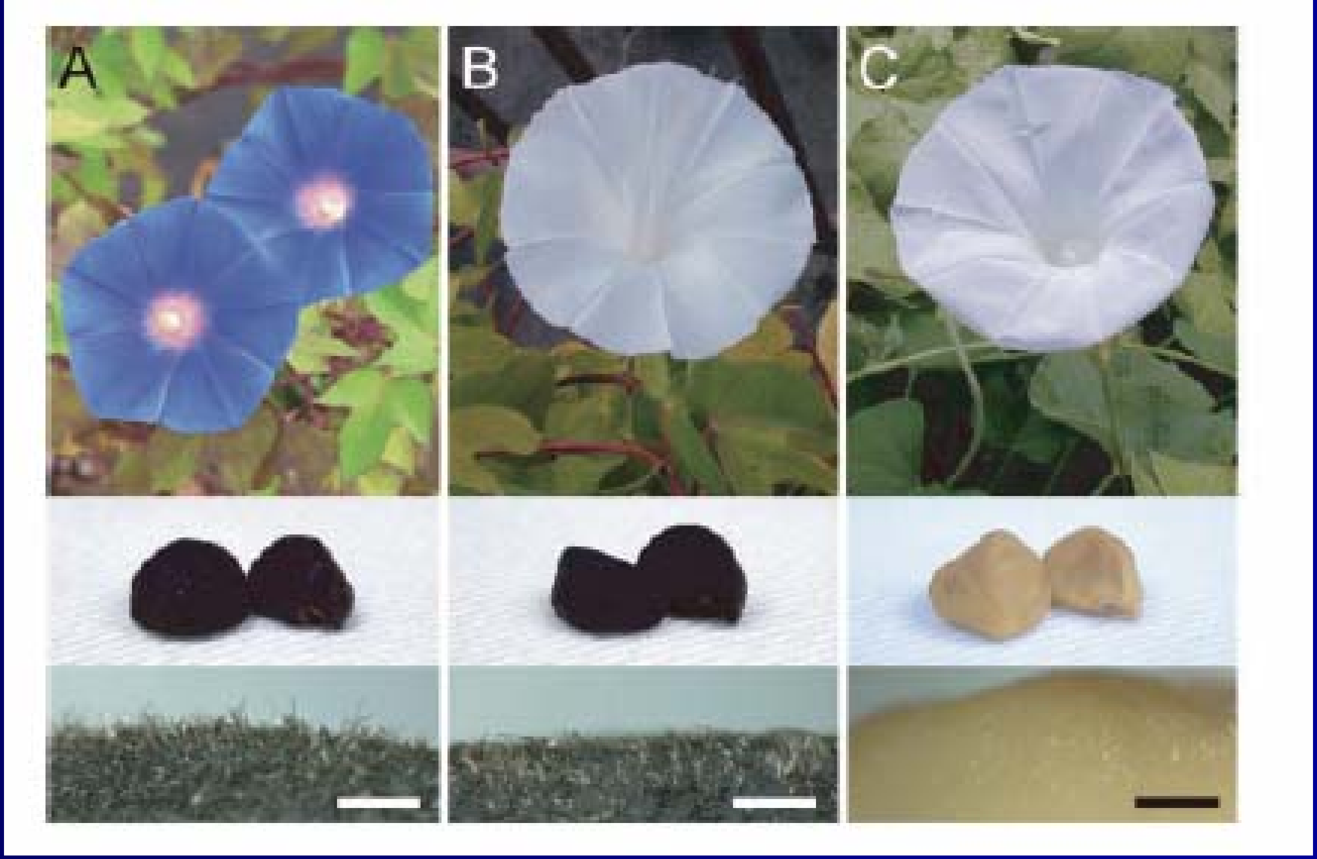


Regulatory genes controlling pigment synthesis in Arabidopsis, petunia and maize



What pathway and in what tissue will be largely determined by expression patterns of the regulators, their interactions and the *cis*-elements on the promoters of structural genes

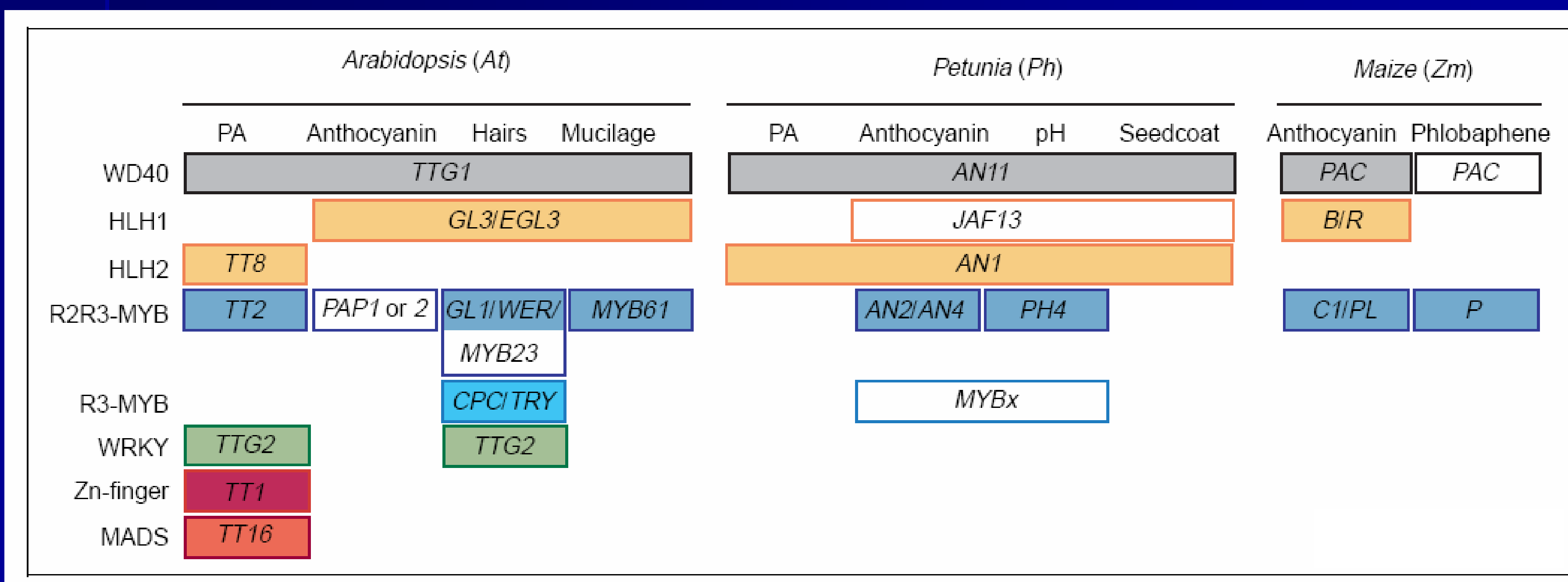
Activity of a similar complex in Morning Glory



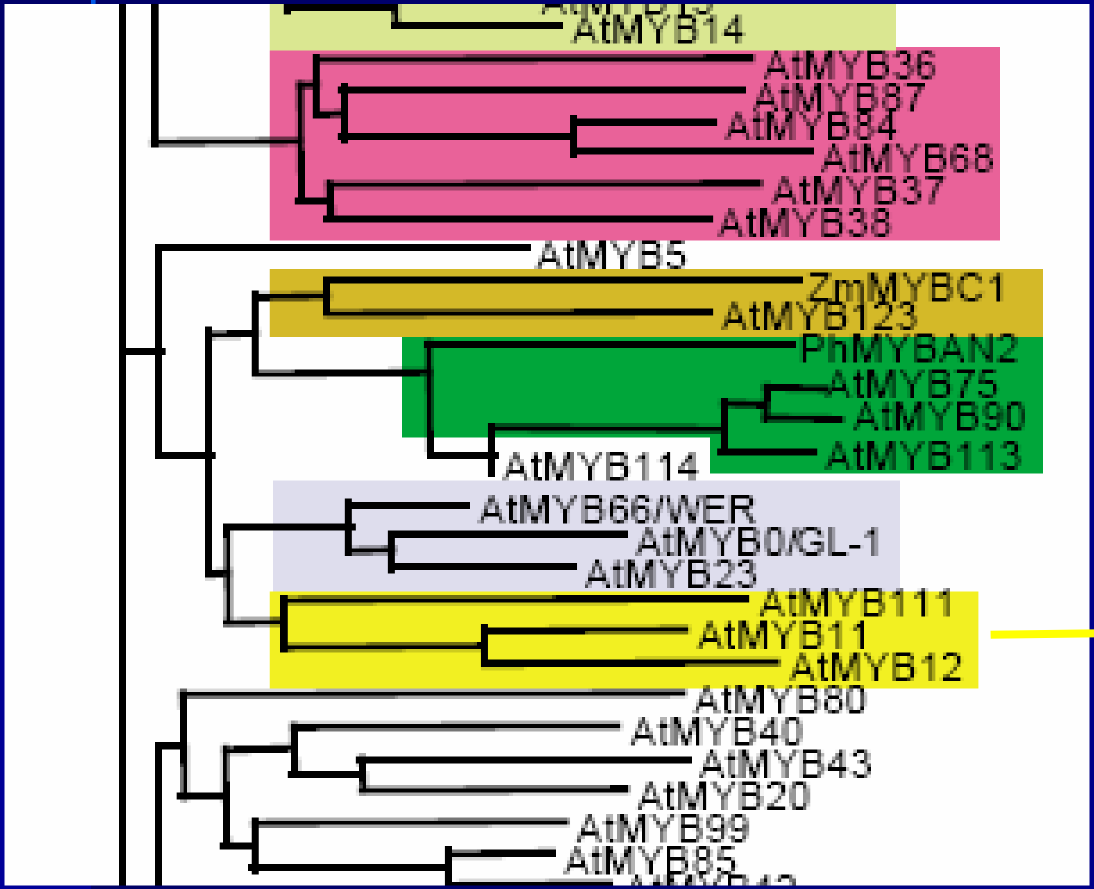
Morita et al., 2006

PH4 of Petunia Is an R2R3 MYB Protein That Activates Vacuolar Acidification through Interactions with Basic-Helix-Loop-Helix Transcription Factors of the Anthocyanin Pathway

Francesca Quattrocchio,[1] Walter Verweij,[1] Arthur Kroon, Cornelis Spelt, Joseph Mol, and Ronald Koes[2]



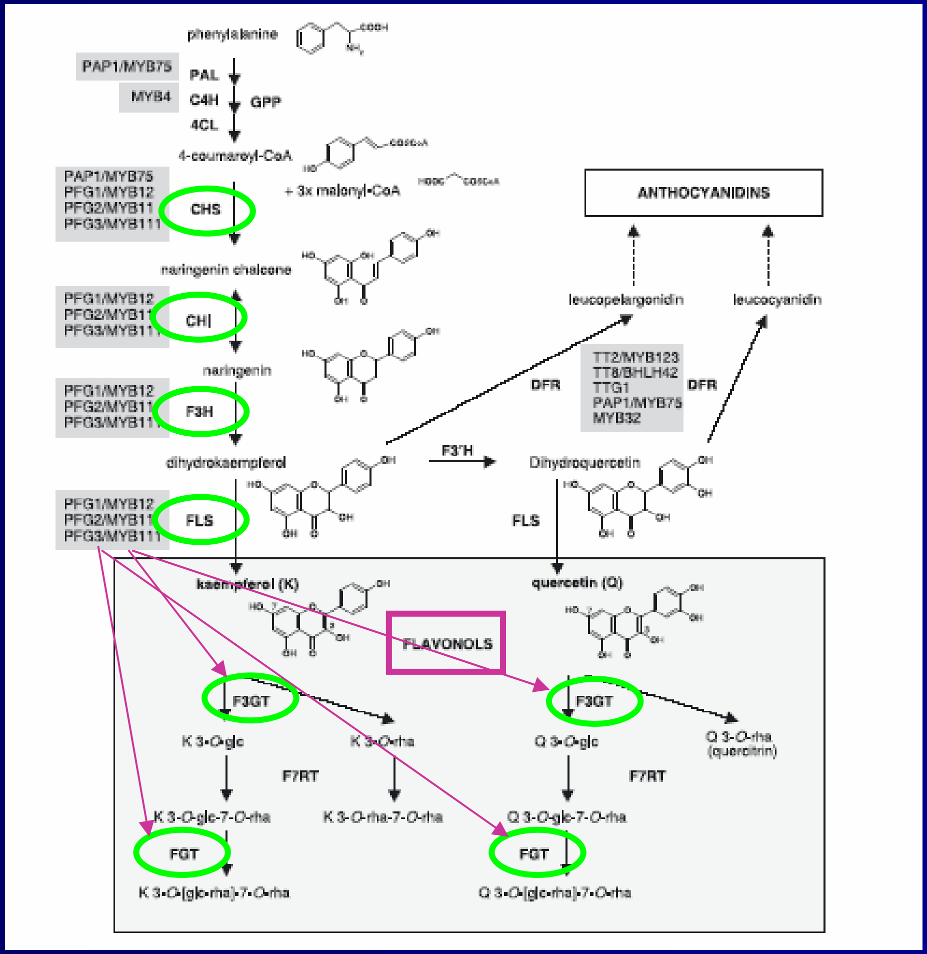
Regulation of flavonol metabolism and not anthocyanins by MYB sub-group 7



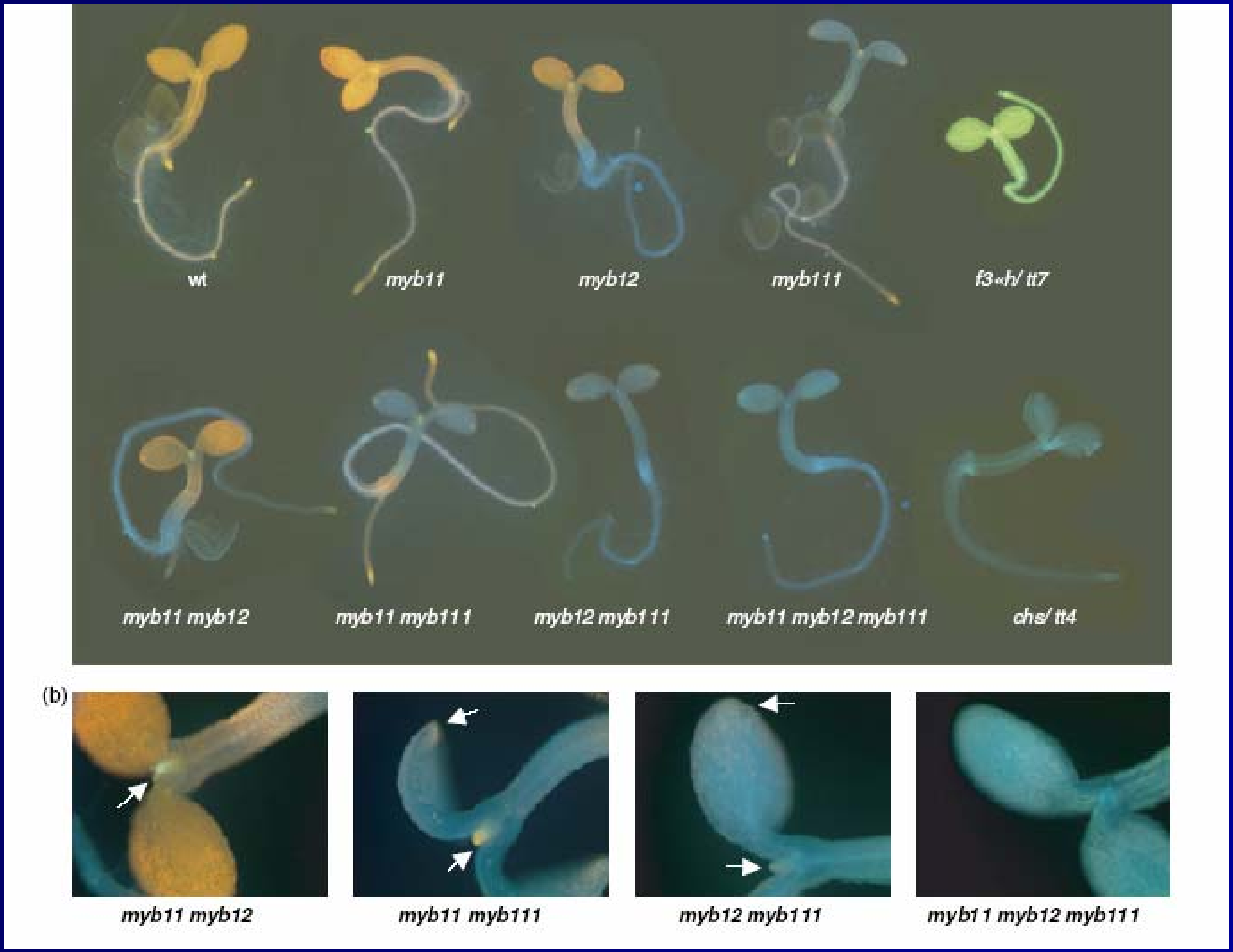
Sub-group 7

Stracke et al., 2005, 2007

Regulation of flavonol metabolism and not anthocyanins by MYB sub-group 7



Regulation of flavonol metabolism and not anthocyanins by MYB sub-group 7



Regulation of flavonol metabolism and not anthocyanins by MYB sub-group 7

- In the flavonoid pathway a single target gene is controlled by multiple transcription factors
- The factors belong to the same transcription factor family and share the same DNA binding domain
- Different expression patterns (spatial, temporal and stimulus responsive transcript accumulation patterns) could explain the regulation of production of a certain phenylpropanoid

Regulation of floral pigmentation intensity & patterning

- Many color patterns provide guides that direct pollinators towards the reproductive organs and the source of nectar within flowers

- In snapdragon - yellow is an Aurone and magenta is Anthocyanin

Schwinn et al., 2006

Regulation of floral pigmentation intensity & patterning

- Some natural isolates of snapdragon have different patterning, with increased pigmentation in regions of the epidermis overlaying the vascular strands of the petal

- Mutation in regulatory genes do not abolish pigmentation (as in structural genes), but change the pattern of pigmentation in the flowers

Mutations in snapdragon regulatory genes

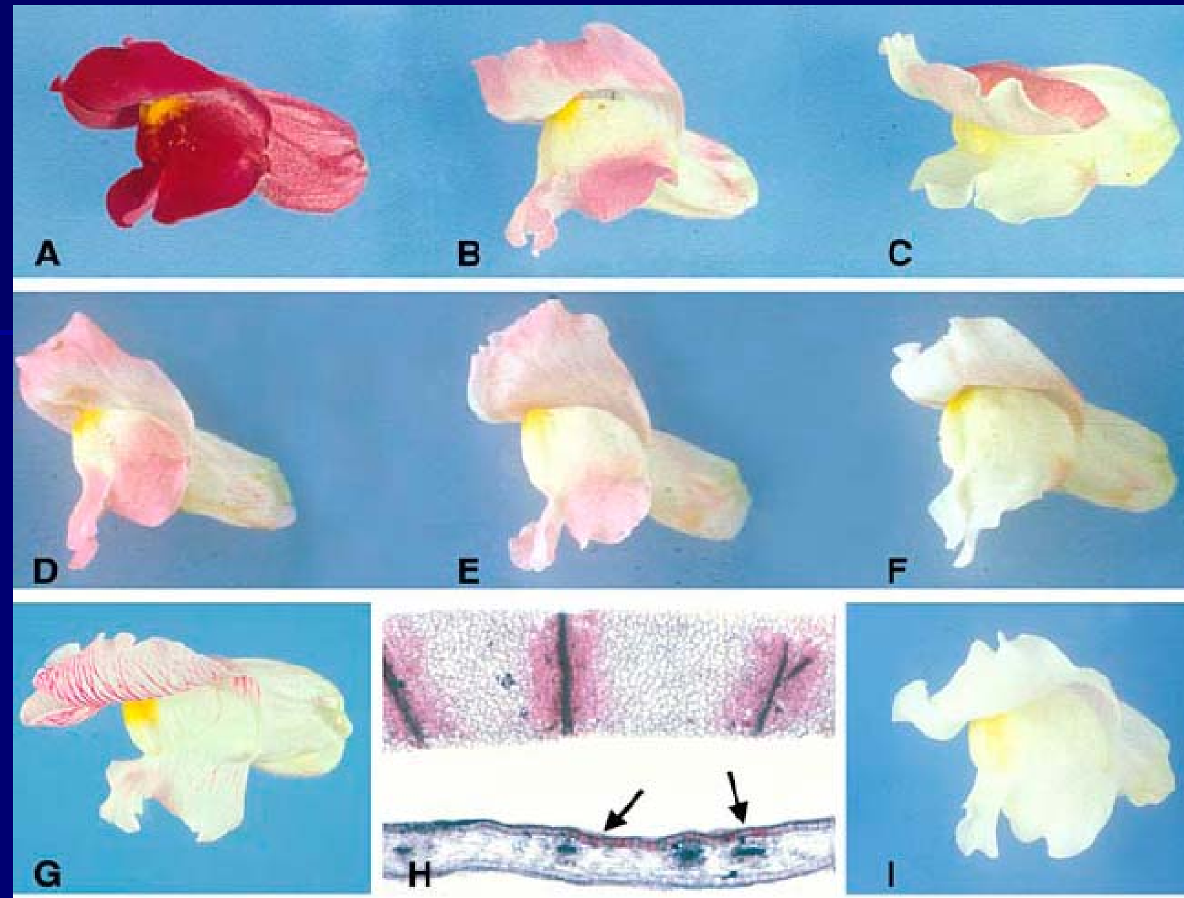
Delila - pigmentation in the corolla lobes (bHLH)

Mutabilis - pigmentation in the corolla lobes

Rosea - pattern and intensity of pigmentation in both lobes and tubes

*Venosa (*dominant*)* - pigmentation of the epidermis overlaying the veins in both lobes and tubes

rosea and venosa mutants



- 2 mutant alleles for rosea (colorata and dorsea)
- weak anthocyanin production

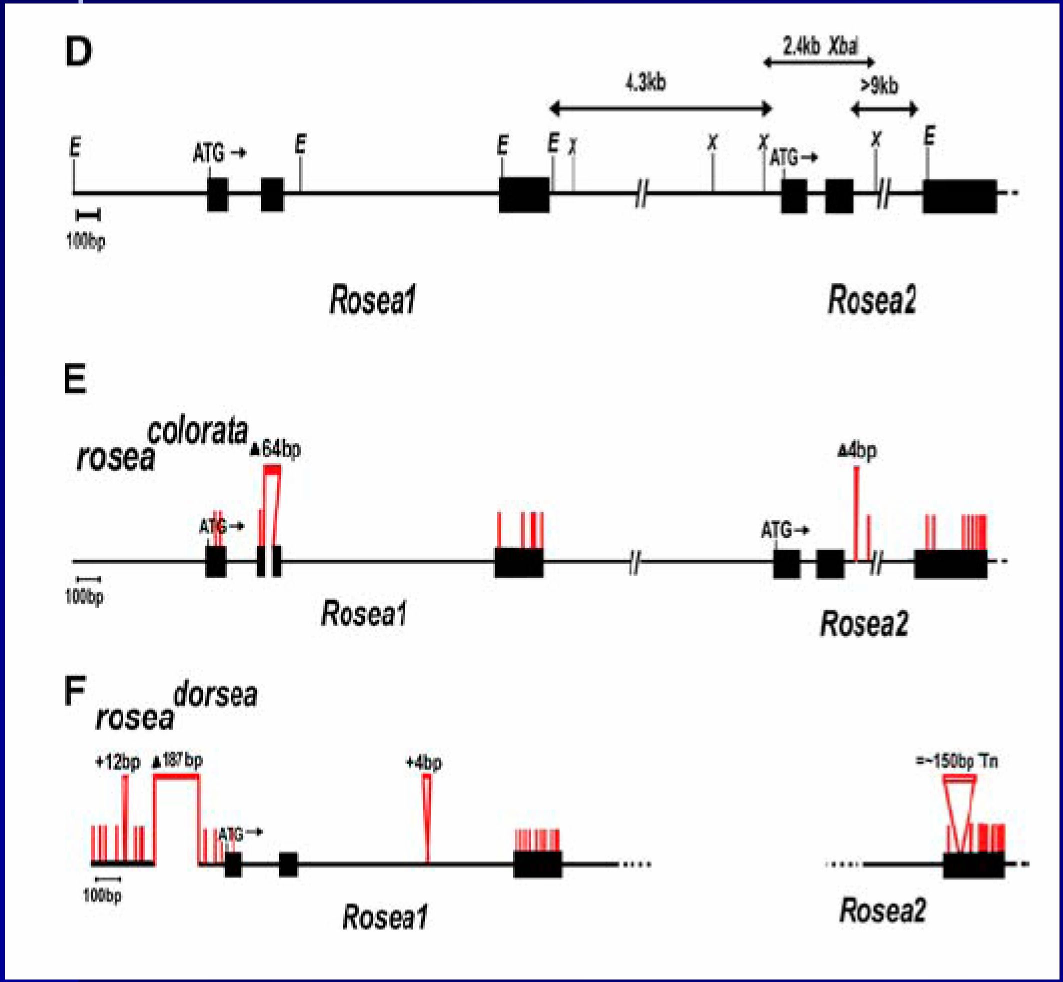
(A) Wild type.
(B) *ros*^{col} (grown outside).
(C) *ros*^{dor} (grown outside).
(D) to **(F)** Phenotypes of individuals in the F2 population of *ros*^{col} × *ros*^{dor}.
(D) *ros*^{col} homozygote.
(E) *ros*^{col} *ros*^{dor} heterozygote.
(F) *ros*^{dor} homozygote.
(G) *ros*^{dor} *Ve*$^{+}$.
(H) Surface view and transverse section of a dorsal petal from a *ros*^{dor} *Ve*$^{+}$ in
overlying the vascular tissue.
(I) *ros*^{dor} grown in the greenhouse.

- *Venosa:* could be seen on the *ros* backgrounds only (*Ros*$^{+}$ epistatic to *Ve*$^{+}$)
- pigment over the veins

Schwinn et al., 2006

The Rosea Locus (Ros1 & Ros2 are linked genes)

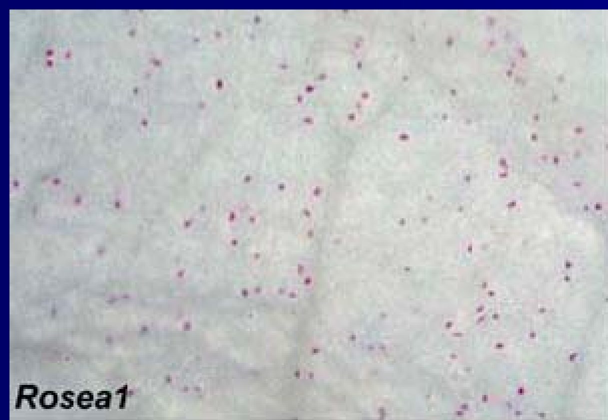
Three different MYB genes isolated



Loss of function in *Ros1*

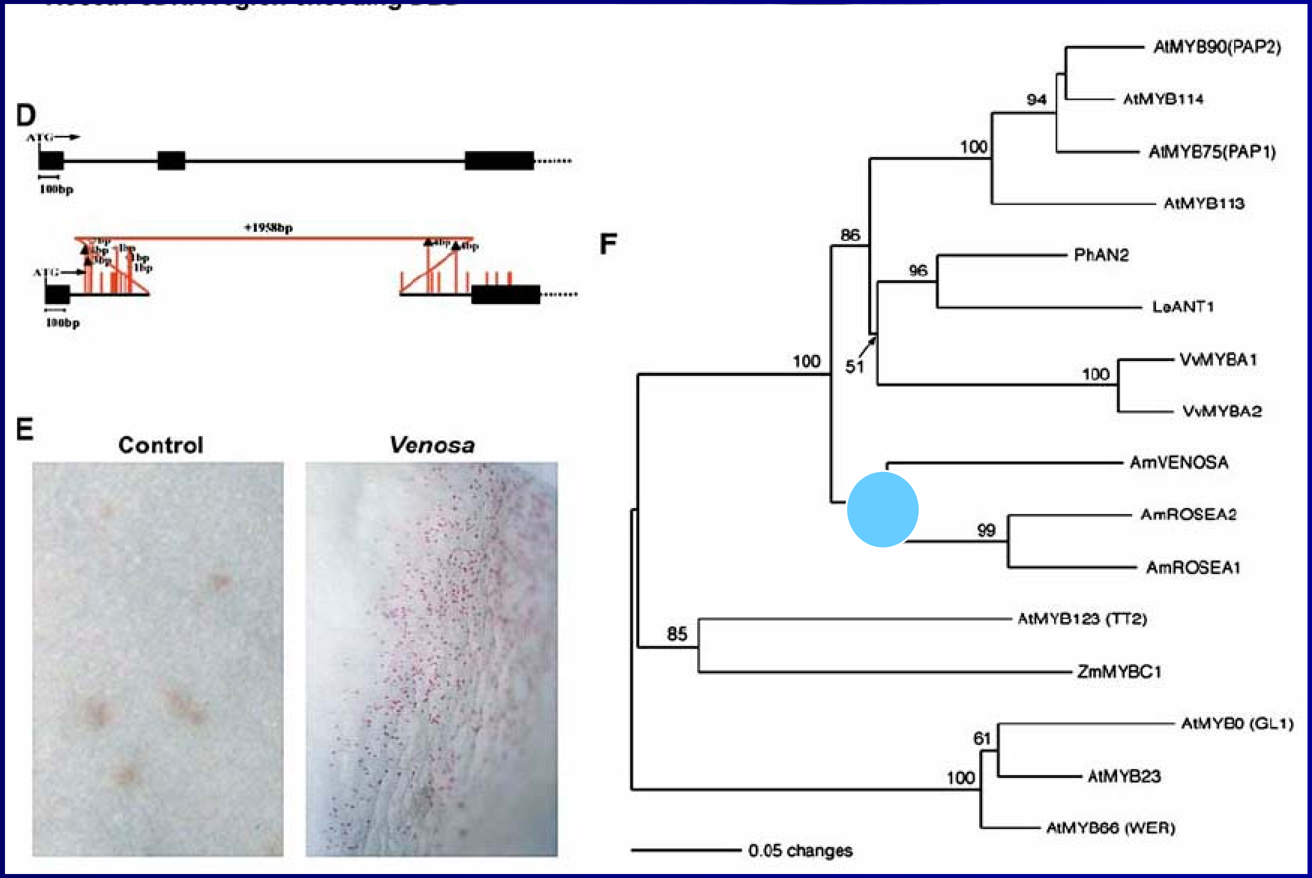
Reduced expression of *Ros1* and loss of function *Ros2*

The Rosea genes activate anthocyanin biosynthesis in a ros^{dor} *background*



Schwinn et al., 2006

The Venosa gene



Ros petal

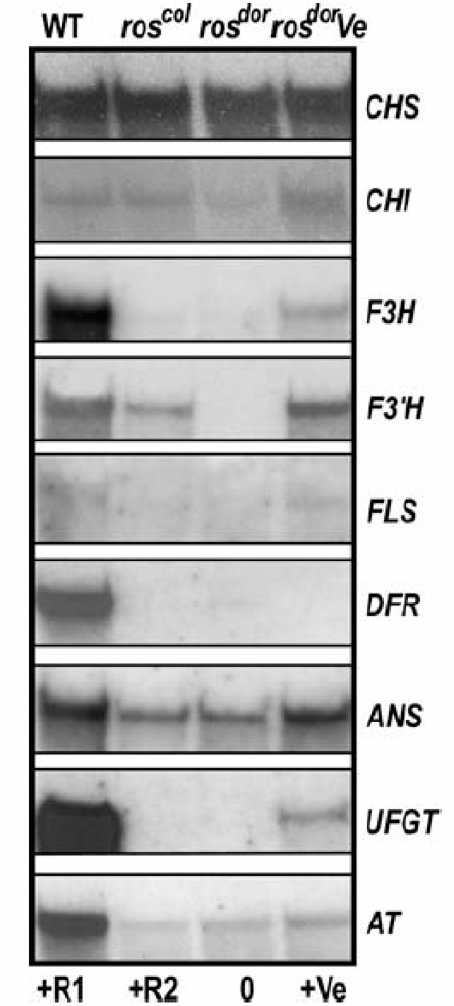
Schwinn et al., 2006

Ros1, Ros2 and Ve have similar but not identical targets

- *Ros1* increases LBGs expression *(F3H, DFR, ANS & UFGT)*

- *Ros2* regulates the expression of *F3'H*

- *Ve presence increase CHI, F3H, F3'H, FLS, ANS, UFGT and AT*



Schwinn et al., 2006

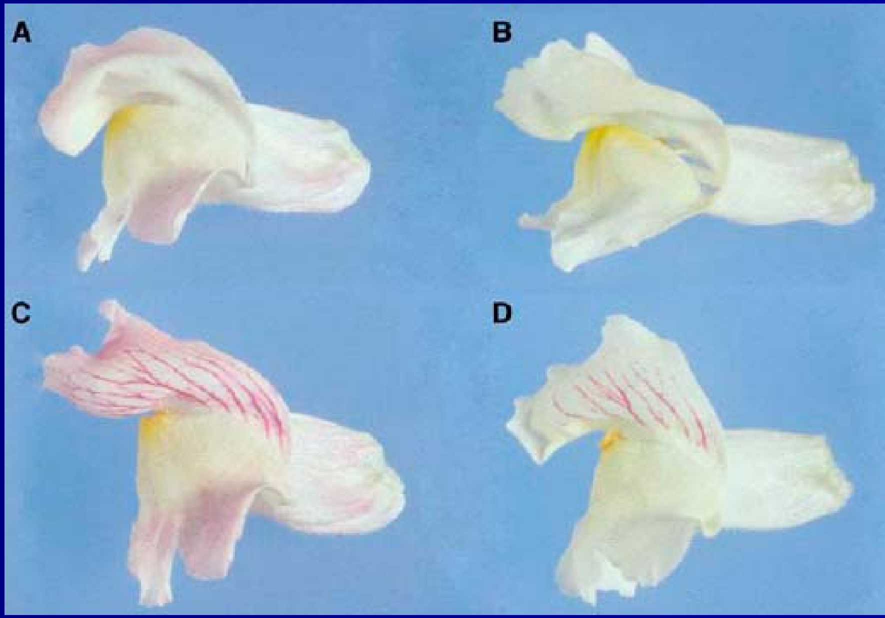
Interaction of Ros1, Ros2 and Ve (MYB) with Delila (Del) and Mutabilis (Mut) bHLH factors

A: ros^{col} (only Ros2 expressed, Ros1 missing)

B: ros^{col} del (Ros2 interacts with Del but not with Mut that needs to be activated by the missing Ros1)

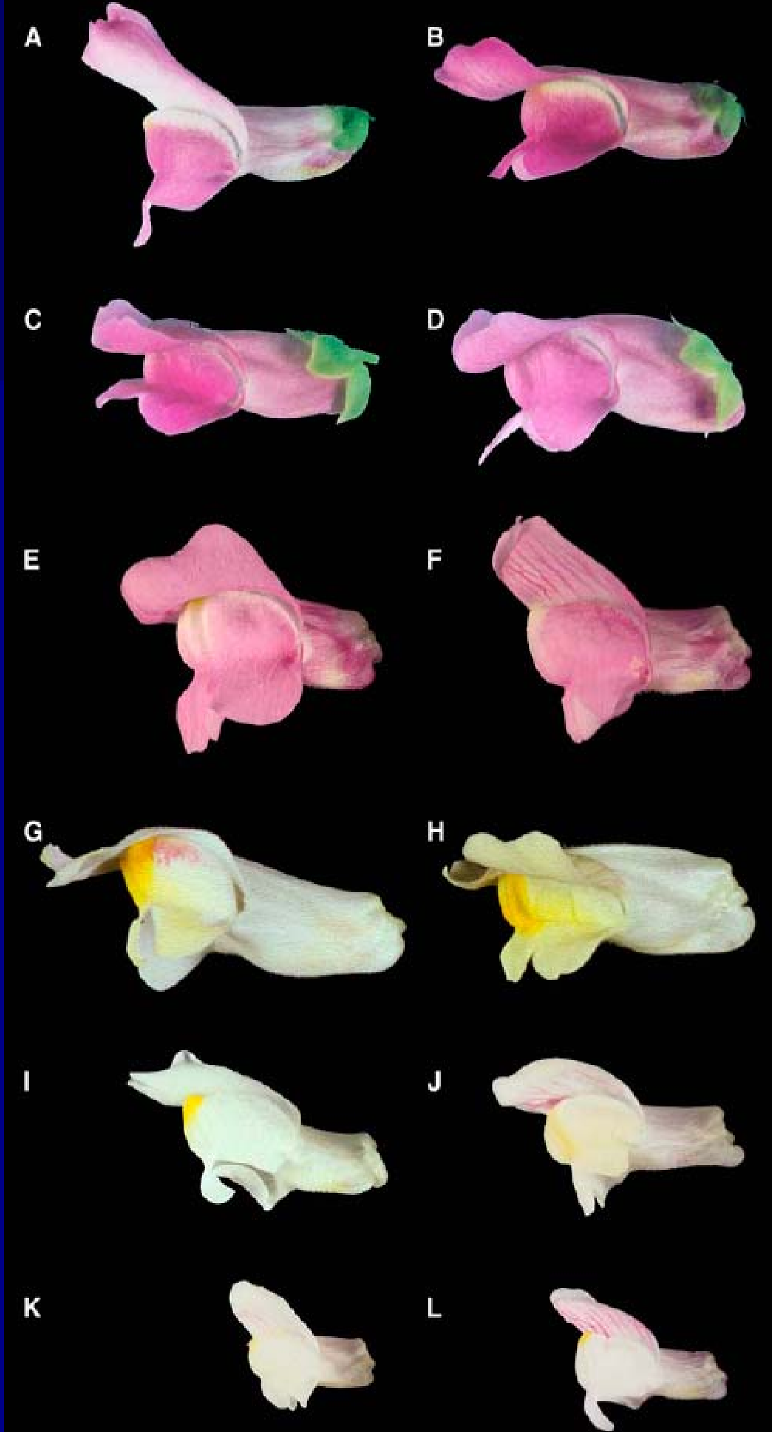
C: ros^{col} Ve+

D: ros^{col} Ve + del (Ros2 interacts with Del in the lobes and Ve can interact with Mut)



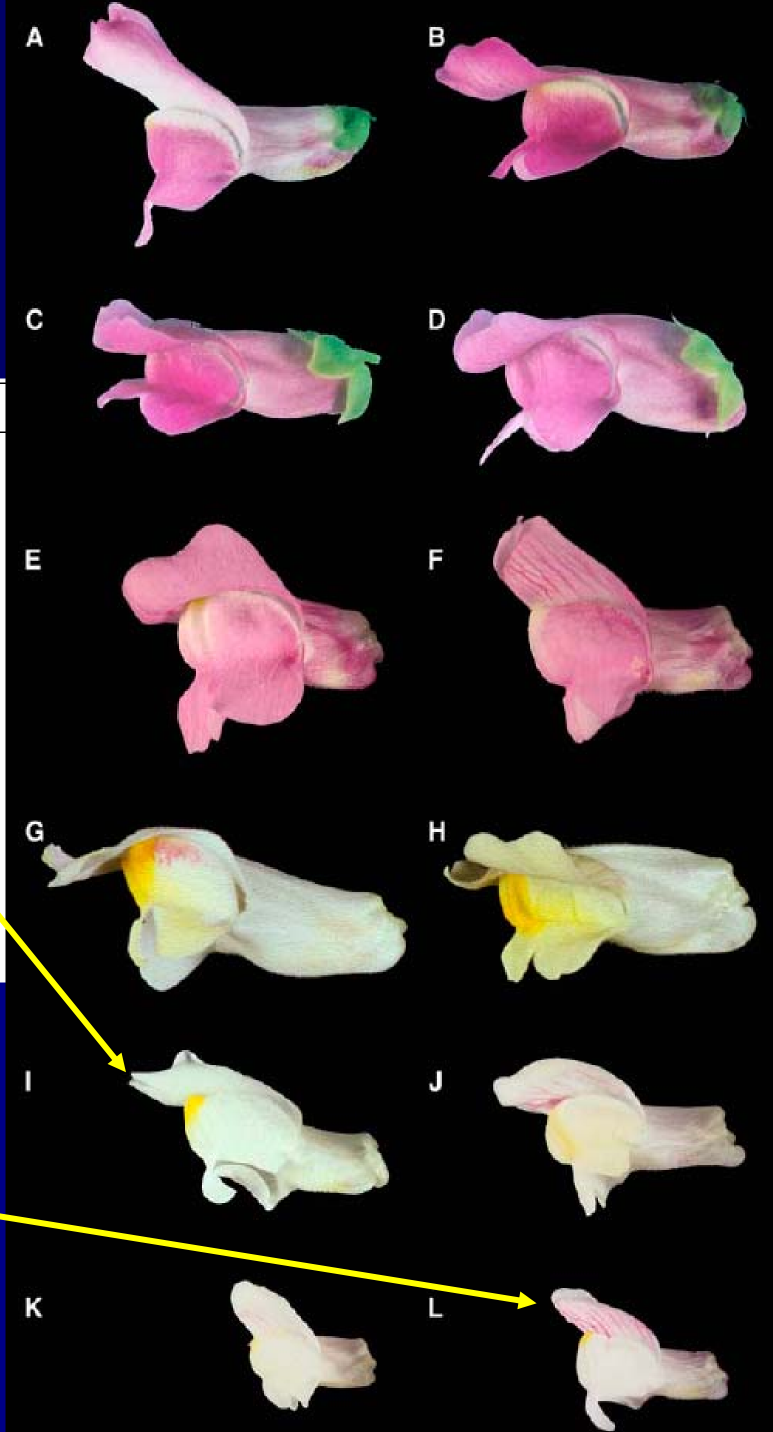
Schwinn et al., 2006

Variation in anthocyanin patterns in flowers of different snapdragon species results principally from variation in activity of MYB- related genes



Schwinn et al., 2006

Species	Floral Pigmentation Phenotype	Genotype
A. majus	Medium-intensity magenta pigmentation, weak venal pattern (Figures 8A and 8B)	*Ros*$^{+}$/*Ros*$^{+}$;*Ve/ve*
	Medium-intensity magenta pigmentation, weak venal pattern (Figures 8C and 8D)	*Ros*$^{+}$/*Ros*$^{+}$;*Ve/ve*
A. latifolium	No background magenta pigmentation; venal pattern restricted to central part of dorsal lobes; yellow lobes (Figure 8G)	*Ros*^{El}/*Ros*^{El};*Ve/Ve;sulf/sulf*
	No background magenta pigmentation; venal pattern on dorsal lobes; yellow lobes (Figure 8H)	*Ros*^{El}/*Ros*^{El};*Ve/Ve;sulf/sulf*
A. graniticum	Very pale magenta pigmentation/no pigmentation (Figure 8I)	*ros/ros ve/ve*
A. molle	No background magenta pigmentation; strong venal pattern on dorsal lobes; yellowish lobes (Figure 8K)	*ros/ros;Ve/Ve;sulf/sulf*
A. mollissimum	No background magenta pigmentation; strong venal pattern on dorsal lobes (Figure 8L)	*ros/ros;Ve/Ve*
A. meonanthemum	No background magenta pigmentation; strong venal pattern on dorsal lobes; yellow lobes (Figure 8J)	*Ros*^{El}/*Ros*^{El};*Ve/Ve;sulf/sulf*



Regulation of floral pigmentation intensity & patterning

- A small 3 members family in snapdragon controls the pattern and intensity of floral pigmentation

- The 3 genes are *not* functionally equivalent (target genes and expression pattern different)

- Seem to arise by gene duplication and sub-functialization

Regulation of floral pigmentation intensity & patterning

- Variation in anthocyanin production is attributed to MYB genes in flowers of petunia and snapdragon, berry skin color in grape and potato tuber

- The above suggests that the same route was followed independently on a number of occasions

- Altering regulatory proteins rather than structural genes is more rapid

ANTHOCYANIN2 (AN2), a MYB factor detrmaining flower color variation and pollinator attraction

- Specific sets of floral traits are often associated with a particular group of pollinators (adaptation)

- Adaptation:
  - a single pollinator- some orchids
  - one class of pollinators (long-tongued nocturnal hawk moth
  - multiple pollinator types

Adaptation of flower by:
flower morphology, scent, nectar production, color

Hoballah et al., 2007

ANTHOCYANIN2 (AN2), a MYB factor determining flower color variation and pollinator attraction

- The genus petunia is pollinated with either hawk moth or bees

- Petunia axillaris- white flowers with long and narrow corolla tubes, emits a large amount of volatiles at night and contains a large amount of nectar.
Pollinated by hawk moth

- Petunia integrifolia- violet-reddish flower color, short corolla tube containing low amounts of nectar and emitting small amounts of volatiles
- Pollinated by bees

Hoballah et al., 2007

ANTHOCYANIN2 (AN2), a MYB factor determining flower color variation and pollinator attraction

Petunia axillaris

Petunia integrifolia

ANTHOCYANIN2 (AN2), a MYB factor determining flower color variation and pollinator attraction

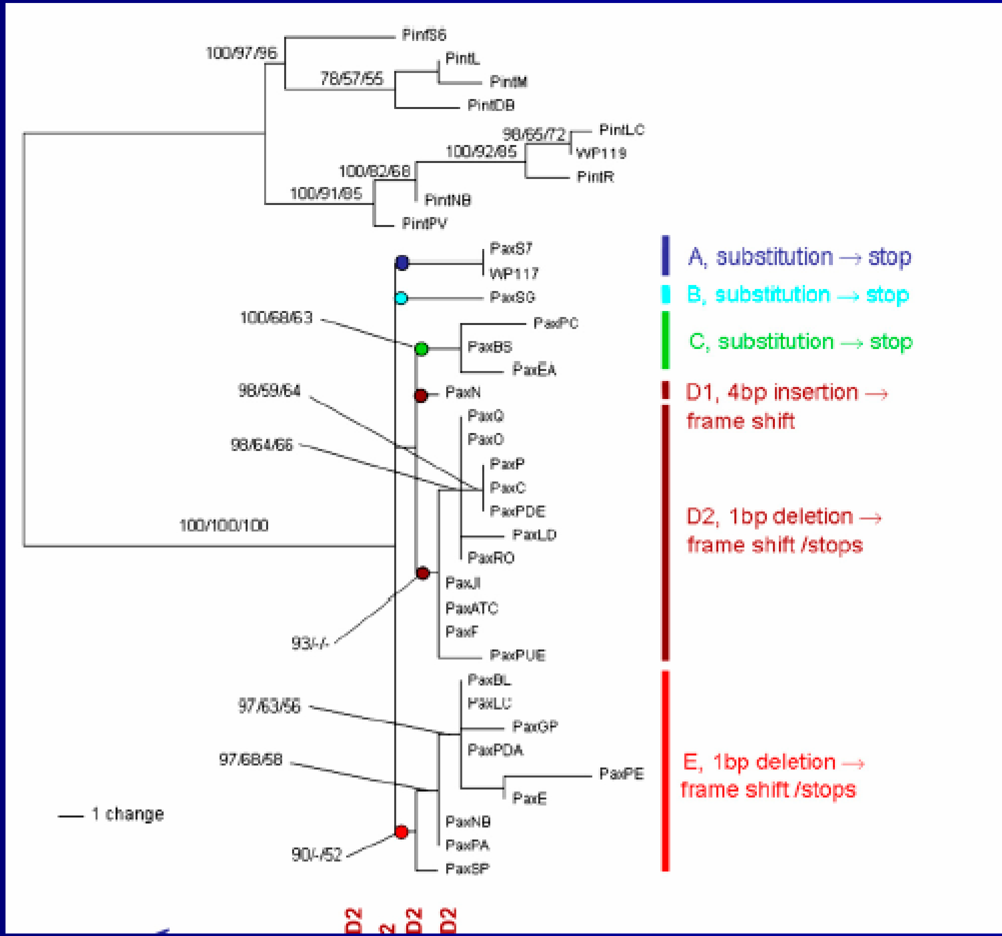
Table 1. Taxa, Abbreviations, Collection Localities, and Geographic Data of *Petunia* Accessions Used for This Study

Taxa	Abbreviation	Collection	Locality	GPS Coordinates, Sea Level
Petunia integrifolia inflata	(PintS6)	Vrije Universiteit, Amsterdam		
Petunia integrifolia integrifolia	(PintL)	Botanical Garden, Dresden		
Petunia integrifolia violacea	(PintM)	Botanical Garden, Rostock		
Petunia axillaris parodii	(PaxS7)	Vrije Universiteit, Amsterdam		
Petunia axillaris axillaris	(PaxN)	Botanical Garden, Rostock		
Petunia axillaris axillaris	(PaxQ)	Botanical Garden, Leipzig Northern Argentina		
Petunia axillaris axillaris	(PaxP)	Botanical Garden, Bern		
Petunia axillaris axillaris	(PaxO)	Botanical Garden, Dresden Argentina, provence Buenos Aires		
Petunia integrifolia integrifolia	(PintLC)	Uruguay (2004)	Las Canas	33°09'97.9''S 58°21'40.1''W, 16 m
Petunia integrifolia integrifolia	(PintNB)	Uruguay (2002)	Nuevo Berlin	32°59'01.0''S 58°03'80.7''W, 14 m
Petunia integrifolia integrifolia	(PintPV)	Uruguay (2004)	Puerto Viejo	32°38'14.4'S 58°08'83.7''W, 0 m
Petunia integrifolia integrifolia	(PintR)	Uruguay (2005)	Rivera	31°00'10.1''S 55°37'13.1''W, 224 m
Petunia axillaris axillaris	(PaxC)	Uruguay (2004)	Carmelo	33°56'18.4''S 58°22'13.3''W, 19 m
Petunia axillaris axillaris	(PaxPC)	Uruguay (2002)	Punta Colorada	34°54'15.1''S 55°15'67.4''W, 3 m
Petunia axillaris axillaris	(PaxJI)	Uruguay (2004)	José Ignacio	34°46'35.8''S 54°40'87.2''W, 9m
Petunia axillaris axillaris	(PaxBS)	Uruguay (2004)	Balnearis Solis	34°47'37.1''S 55°23'49.3''W
Petunia axillaris axillaris	(PaxBL)	Uruguay (2002)	Barra Santa Lucia	34°46'59.0''S 56°21'88.7''W, 0 m
Petunia axillaris axillaris	(PaxPE)	Uruguay (2002)	Punta Espinillo	34°50'48.6'' S 56°24'25.8'' W, 10 m
Petunia axillaris axillaris	(PaxPDE)	Uruguay (2002)	Punta dell'Este	34°57'97.5''S 54°57'26.5''W, 16 m
Petunia axillaris axillaris	(PaxB)	Uruguay (2004)	La Barra Km 172	34°52'18.9''S 54°45'02.8''W, 11 m
Petunia axillaris parodii	(PaxGP)	Uruguay (2002)	Gruta del Palacio	33°16'81.5''S 57°08'54.1''W, 94 m
Petunia axillaris axillaris	(PaxSG)	Uruguay (2002)	San Gregorio	33°54'95.4''S 56°45'40.1''W, 98 m
Petunia axillaris axillaris	(PaxF)	Uruguay (2004)	Flores	
Petunia axillaris parodii	(PaxNB)	Uruguay (2002)	Nuevo Berlin	32°59'01.0''S 58°03'80.7''W, 14 m
Petunia axillaris axillaris	(PaxLD)	Uruguay (2002)	Laguna del Diario	34°54'53.3''S 55°00'51.8''W, 12 m
Petunia axillaris parodii	(PaxLC)	Uruguay (2002)	Las Canas	33°09'97.9''S 58°21'40.1''W, 16 m
Petunia axillaris axillaris	(PaxLG)	Uruguay (2005)	Laguna del Garzon	34°48'10.5''S 54°34'56.6''W, 7 m
Petunia axillaris axillaris	(PaxE)	Uruguay (2005)	Ruta 12 Eucalyptus	34°33'95.3''S 55°05'21.5''W, 178 m
Petunia axillaris axillaris	(PaxPUE)	Uruguay (2005)	Pueblo Eden	34°37'59.4''S 55°03'26.7''W, 71 m
Petunia axillaris axillaris	(PaxRO)	Uruguay (2005)	Ruta 9 to Rocha	34°44'16.8''S 54°37'37.9''W, 60 m
Petunia axillaris axillaris	(PaxPA)	Uruguay (2005)	Playa Agraciada	33°48'64.7''S 58°25'58.8''W, 11 m
Petunia axillaris axillaris	(PaxPDA)	Uruguay (2005)	Pan de Azucar	34°46'73.7''S 55°11'07.5''W, 65 m
Petunia axillaris axillaris	(PaxATC)	Uruguay (2005)	Arroyo Terpes Chico	
Petunia axillaris axillaris	(PaxSP)	Uruguay (2005)	Salto del Penitente	34°22'33.2''S 55°03'18.7''W, 2 m
Petunia axillaris axillaris	(PaxEA)	Uruguay (2005)	Estancia Arteaga	
Recombinant inbred line	(WP 117)	Stuurman et al. (2004)		
Recombinant inbred line	(WP 119)	Stuurman et al. (2004)		

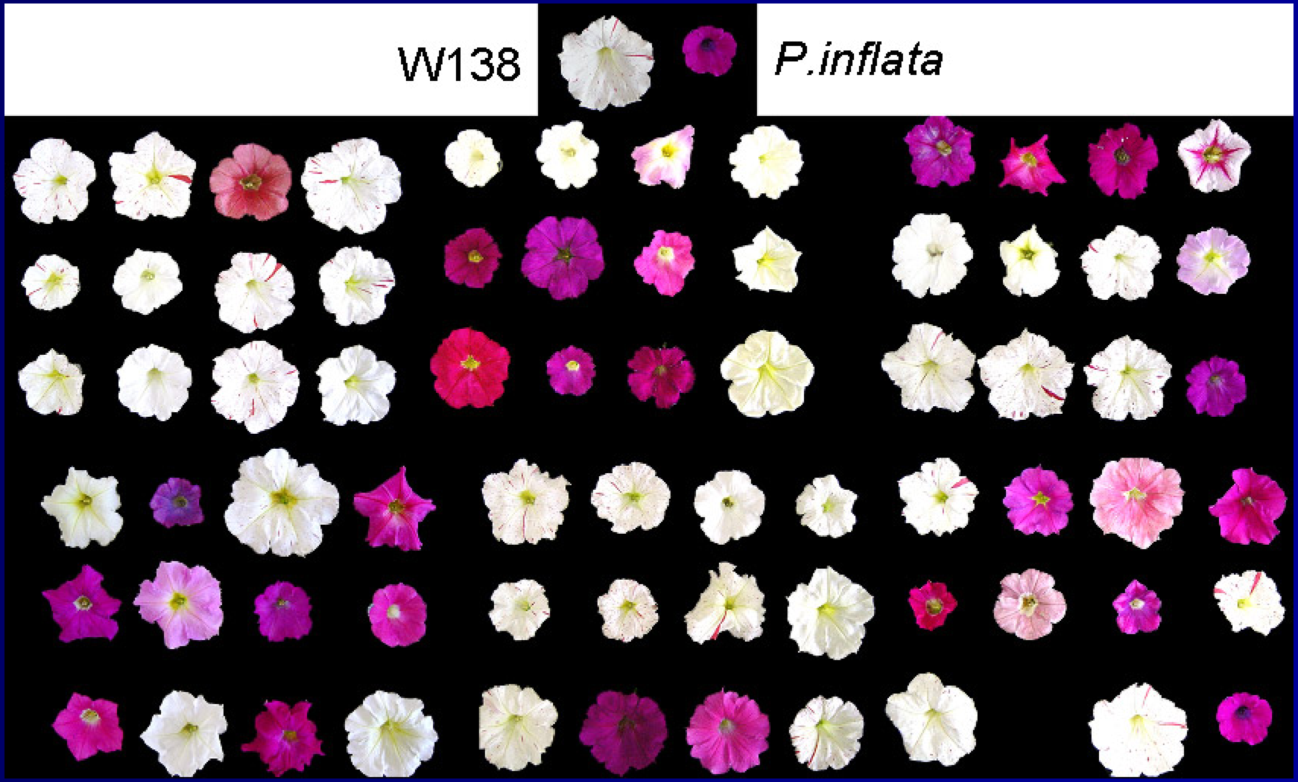
A cDNA sequence of *P. integrifolia* (PintDB) was obtained from GenBank (AF146704). Seed material is maintained at the University of Bern and is available upon request.

ANTHOCYANIN2 (AN2), a MYB factor determining flower color variation and pollinator attraction

- P. Axillaris carries mutations in the AN2 gene
- Loss of color arose multiple times
-



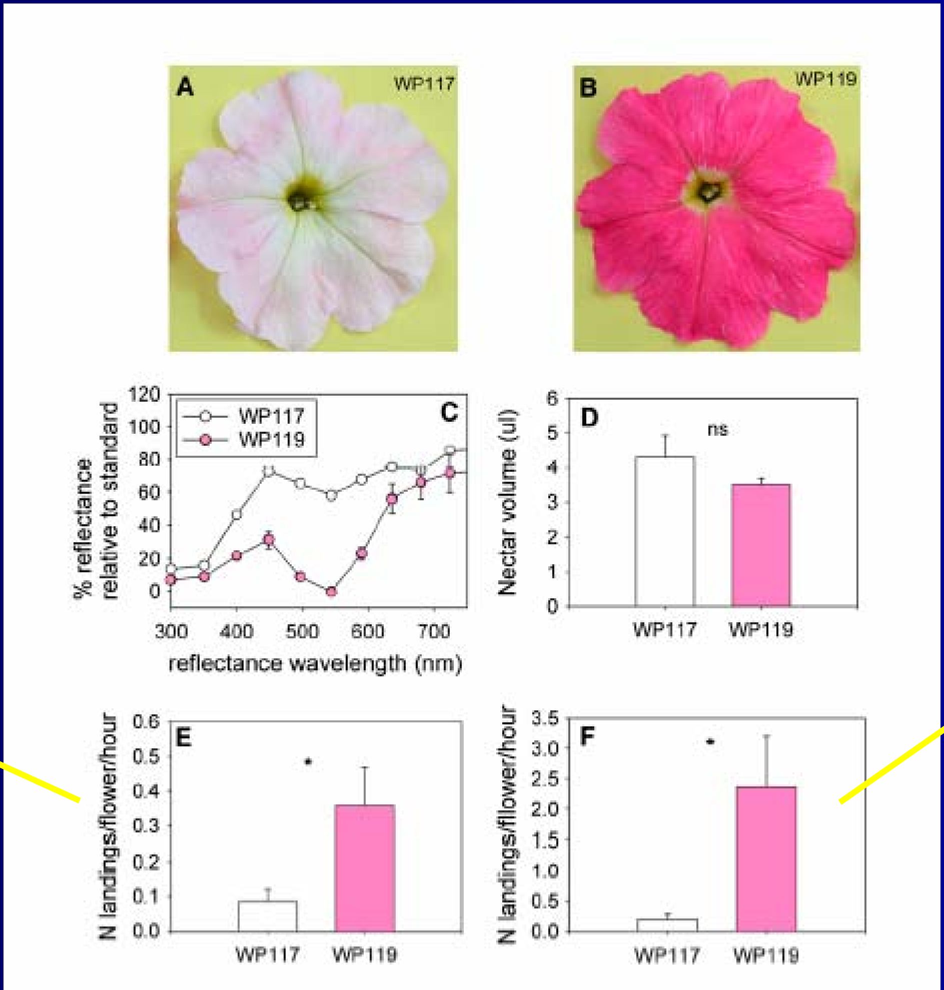
ANTHOCYANIN2 (AN2), a MYB factor determining flower color variation and pollinator attraction



Hoballah et al., 2007

Field tests for pollination

Diurnal butterflies



Diurnal hymenopteran (bees, wasps, ants)

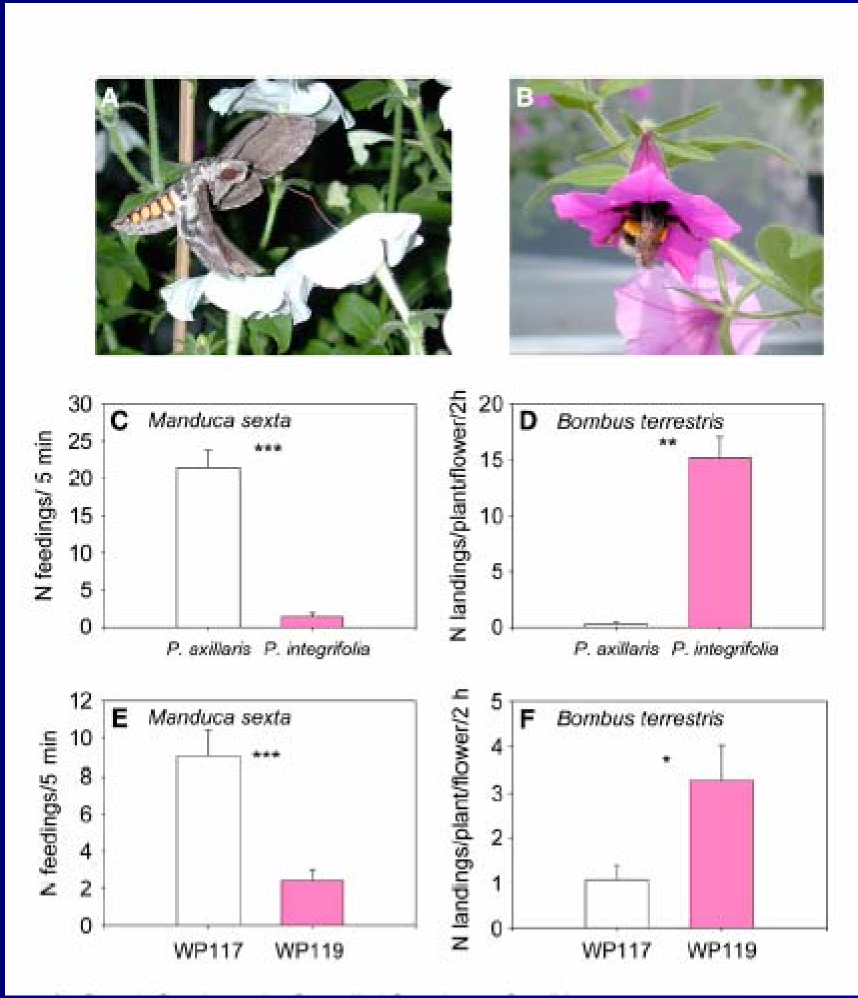
Hawk moth could not be assessed

Hoballah et al., 2007

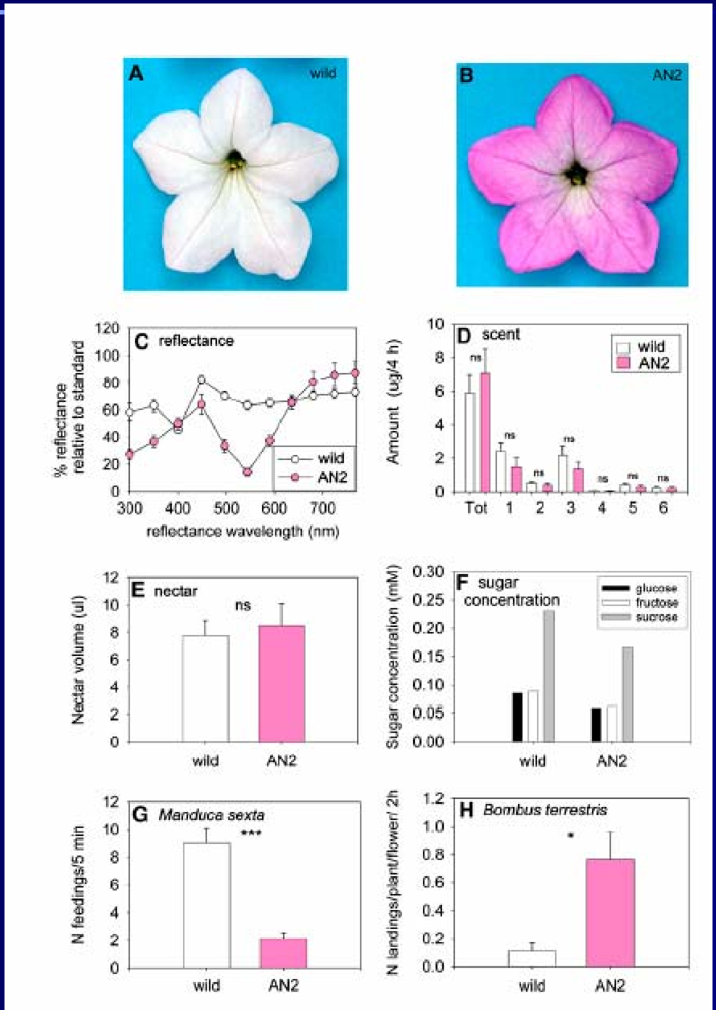
Green house tests for pollination

Wild species

Introgression lines



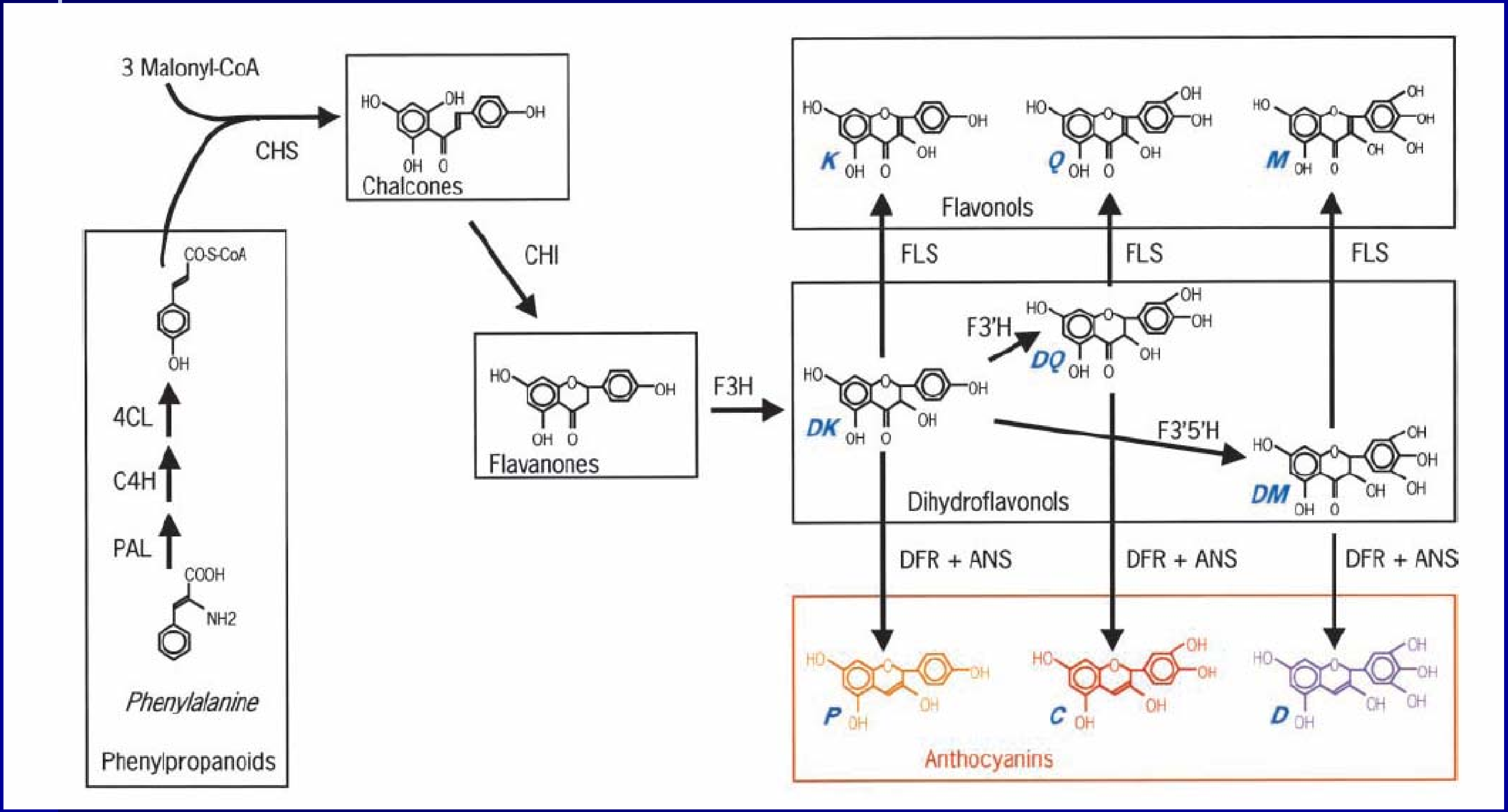
Green house tests for pollination in transgenic plants overexpressing AN2 in the white axilllaris petunia



2 on Pollinator Preference.

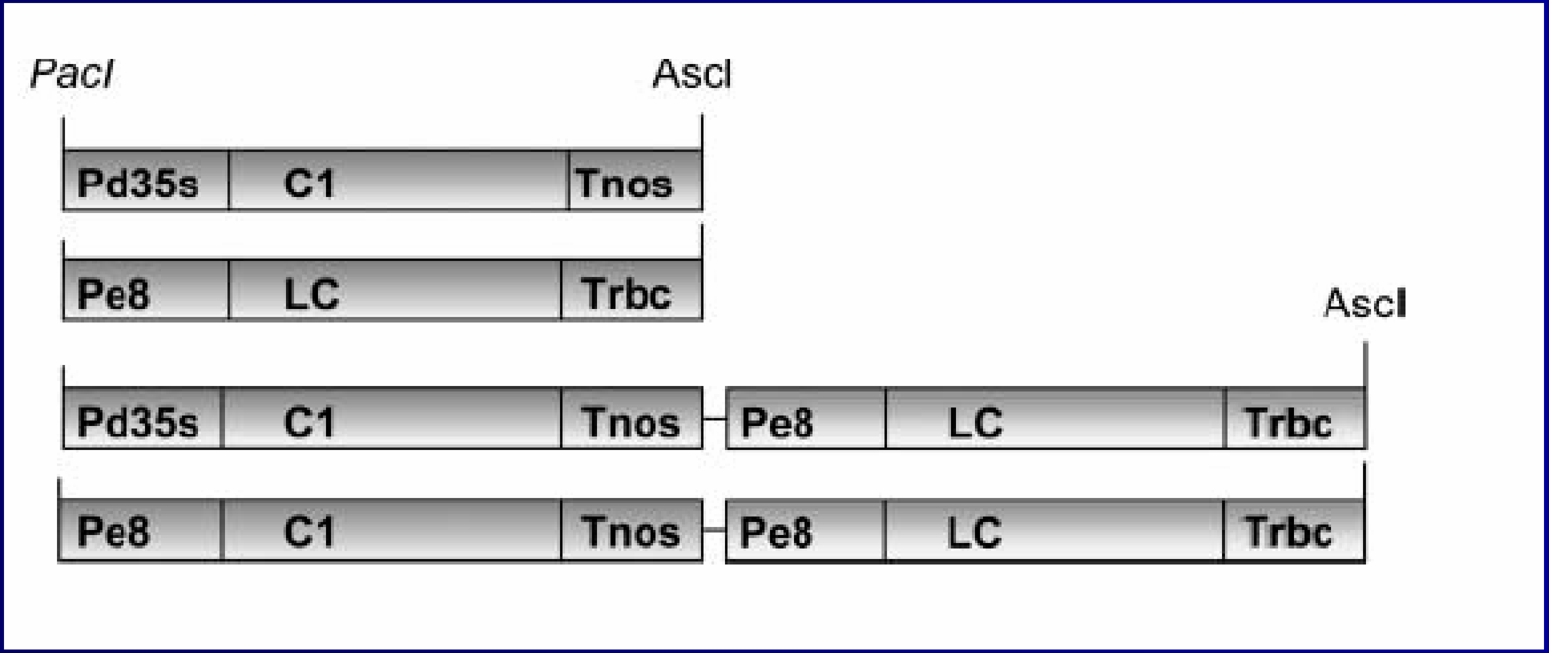
- Single gene-mediated shift in pollinator attraction in petunia flower
-AN2 is a major determinant of pollinator attraction
- Adaptation of plants to a new pollinator might be through single genes with large effects

Engineering Flavonoids with regulatory genes



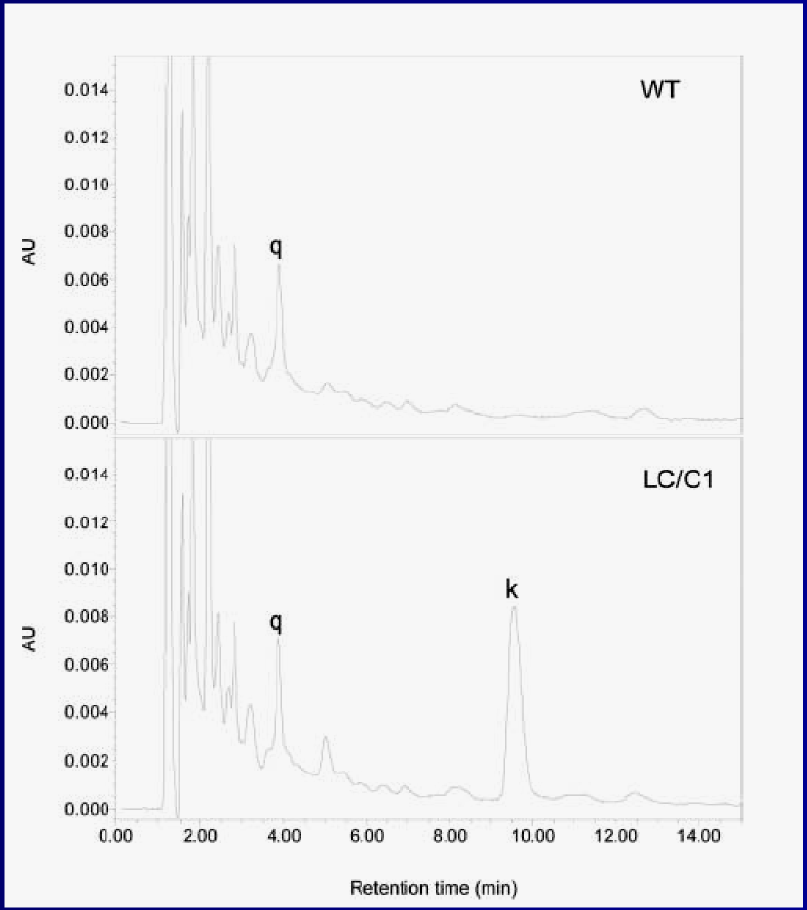
Bovy et al., 2002

Engineering Flavonoids with regulatory genes



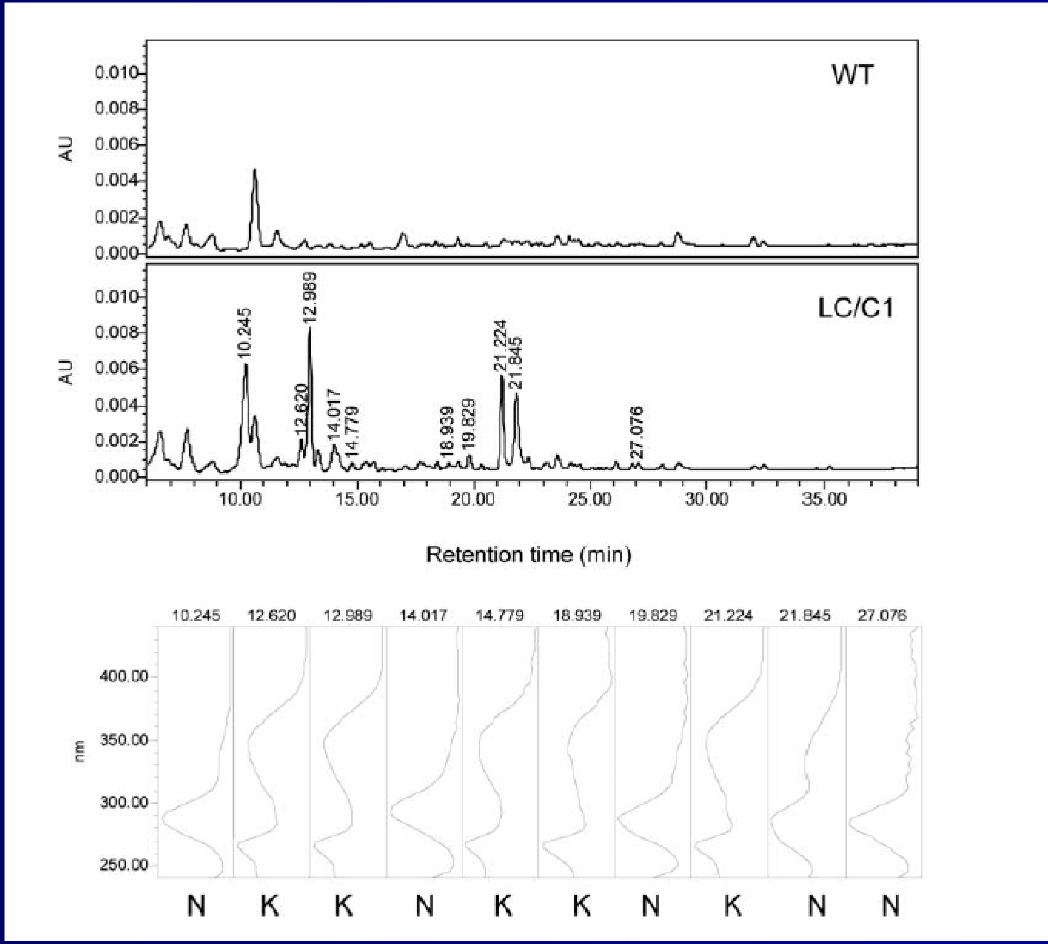
Bovy et al., 2002

Engineering Flavonoids with regulatory genes



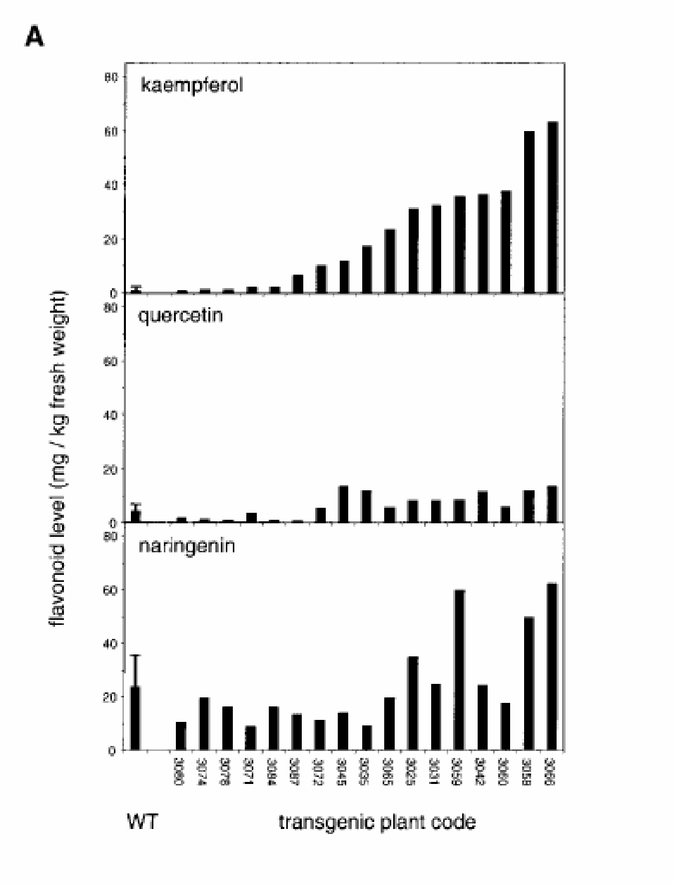
Bovy et al., 2002

Engineering Flavonoids with regulatory genes



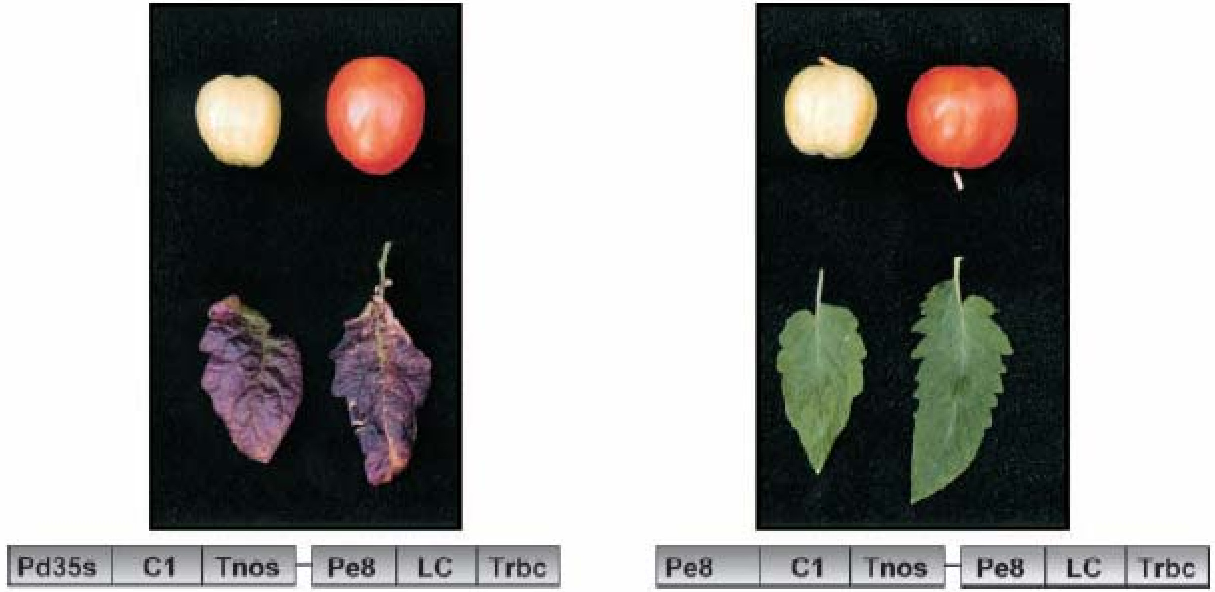
Bovy et al., 2002

Engineering Flavonoids with regulatory genes



Bovy et al., 2002

Engineering Flavonoids with regulatory genes



Bovy et al., 2002

Engineering Flavonoids with regulatory genes

- Using the regulatory genes LC/C1 from maize for overexpression in tomato

- A 60 fold increase in tomato flesh of the flavonol Kaempferol

- Flesh of tomato normally has very little flavonoids

- Both genes needed