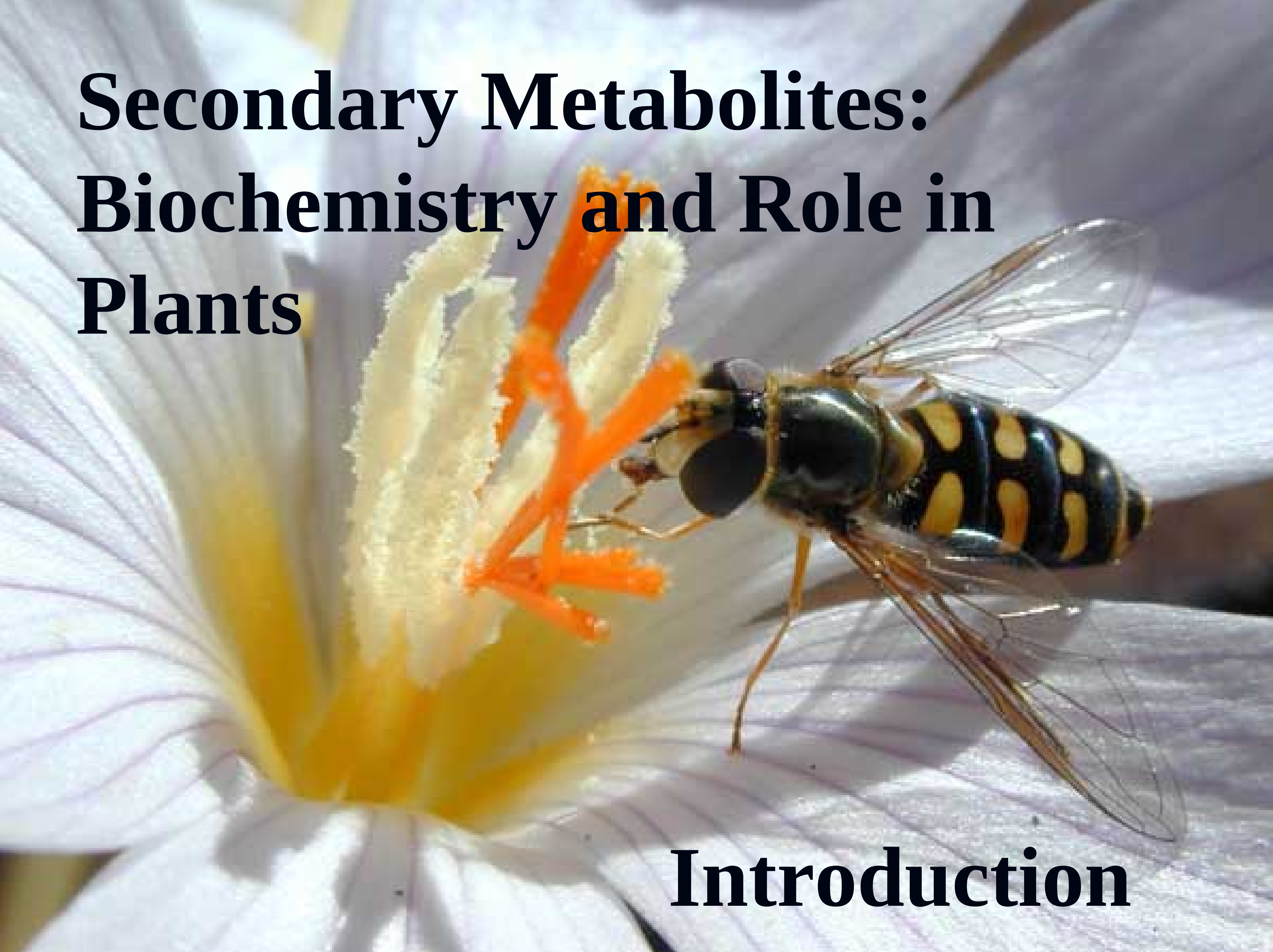
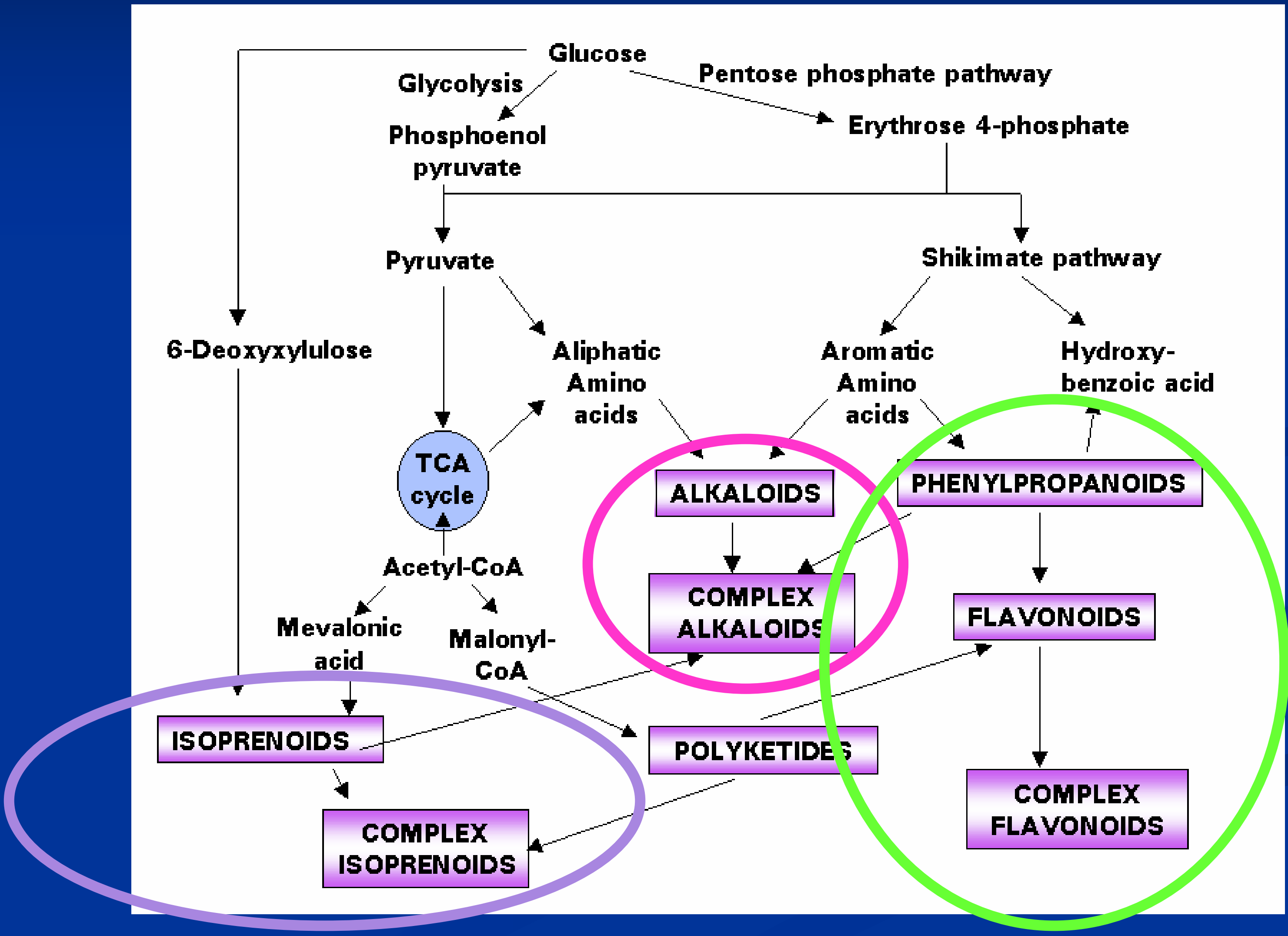


Secondary Metabolites: Biochemistry and Role in Plants

Introduction

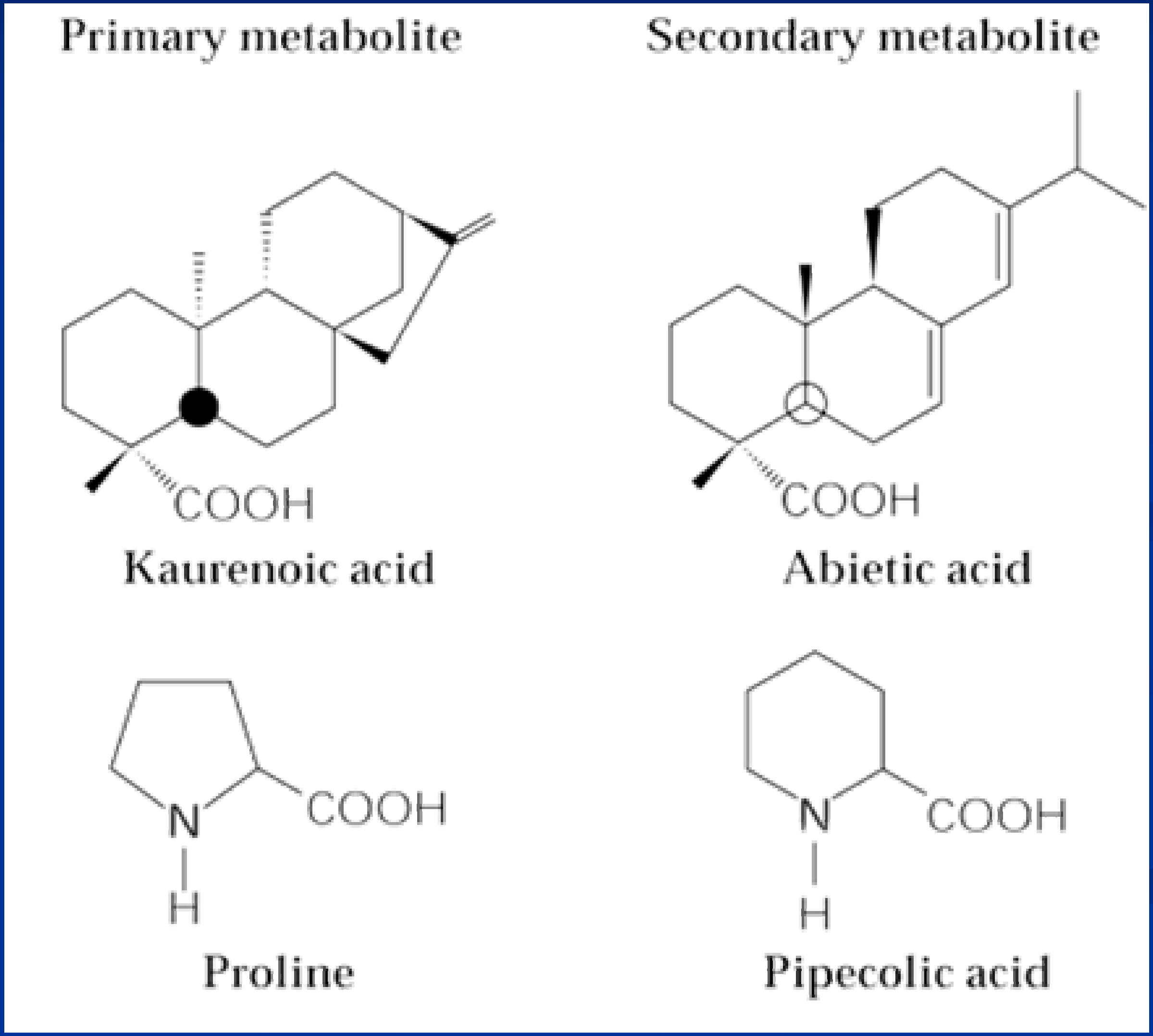
Secondary Metabolites are Derived from Primary Metabolites



Primary-Secondary metabolites boundary ??

GA biosynthesis

Essential amino acid



Resin component

Alkaloid

Main Groups of Secondary Metabolites in Plants

29,000 terpenes- derived from the C5 precursor isopentenyl diphosphate (IPP)

12,000 alkaloids- derived from amino acids

8,000 phenolics- shikimate pathway or malonate/acetate pathway

Main Secondary metabolites

Nitrogen containing:

- Alkaloids (12,000)
- Non protein amino acids (600)
- Amines (100)
- Cyanogenic glycosides (100)
- Glucosinolates (100)

Main Secondary metabolites

Without nitrogen:

- Terpenoids (29,000):

mono- 1000
sesquiterpene- 3000
diterpenes-1000
triterpenes, steroids, saponines- 4,000

- Phenolics (8,000):

Flavonoids- 2000
Polyacetylens-1000
Polyketides- 750
Phenylpropanoids- 500

Compartmentation of SMs biosynthesis

Mostly in the Cytosol: hydrophilic compounds

Chloroplasts: alkaloids (caffeine) and terpenoids (monoterpenes)

Mitochondria: some amines, alkaloids

Vesicles: alkaloids (protoberberines)

Endoplamic reticulum: hydroxylaton steps, lipophilic compounds

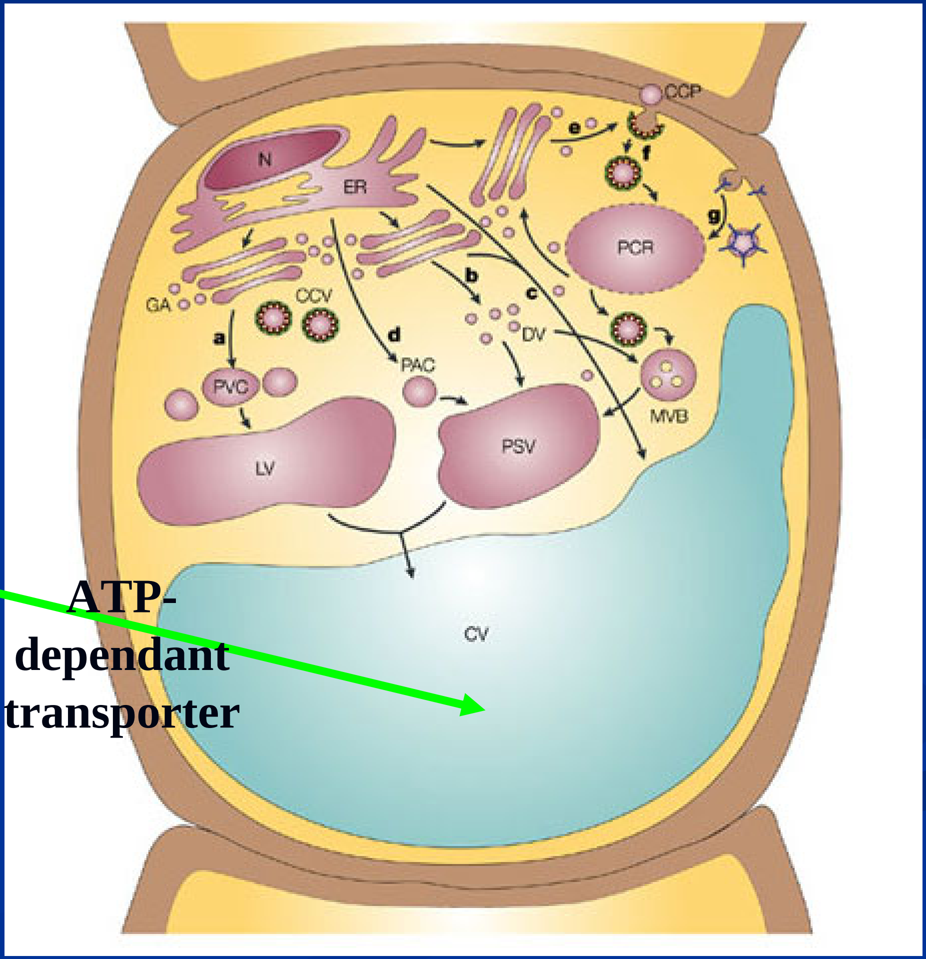
SMs sequestration

- Water soluble compounds are usually stored in the vacuole

- Lipophilic substances are sequestered in resin ducts, laticifers, glandular hairs, trichomes, in the cuticle, on the cuticle

SMs sequestration in to Vacuoles

Water soluble compounds- alkaloids, NPAAs, cyanogenic glucosides, glucosinolates, saponines, anthocyanines, flavonoids, cardenolides



SMs sequestration in to Vacuoles- Anthocyanin example

- Anthocyanines- blue-red flavonoid pigments

- They are stabilized in the vacuole

- Oxidized in the cytosol

- The sequestration is a detoxification process

SMs sequestration in to Vacuoles-
Anthocyanin example- Bz2 mutant

- When the *BRONZE2* gene is not active, anthocyanines accumulate in the cytosol and a tan bronze phenotype of tissue is obtained

- *BRONZE2* is a Glutathione-*S*-transferase

- Glutathionation of anthocyanines is a pre-requisite for the targeting to the vacuole through a GST-x-pump in the tonoplast membrane

SMs sequestration in Vacuoles- Anthocyanin example- bz2 & the an9 mutant

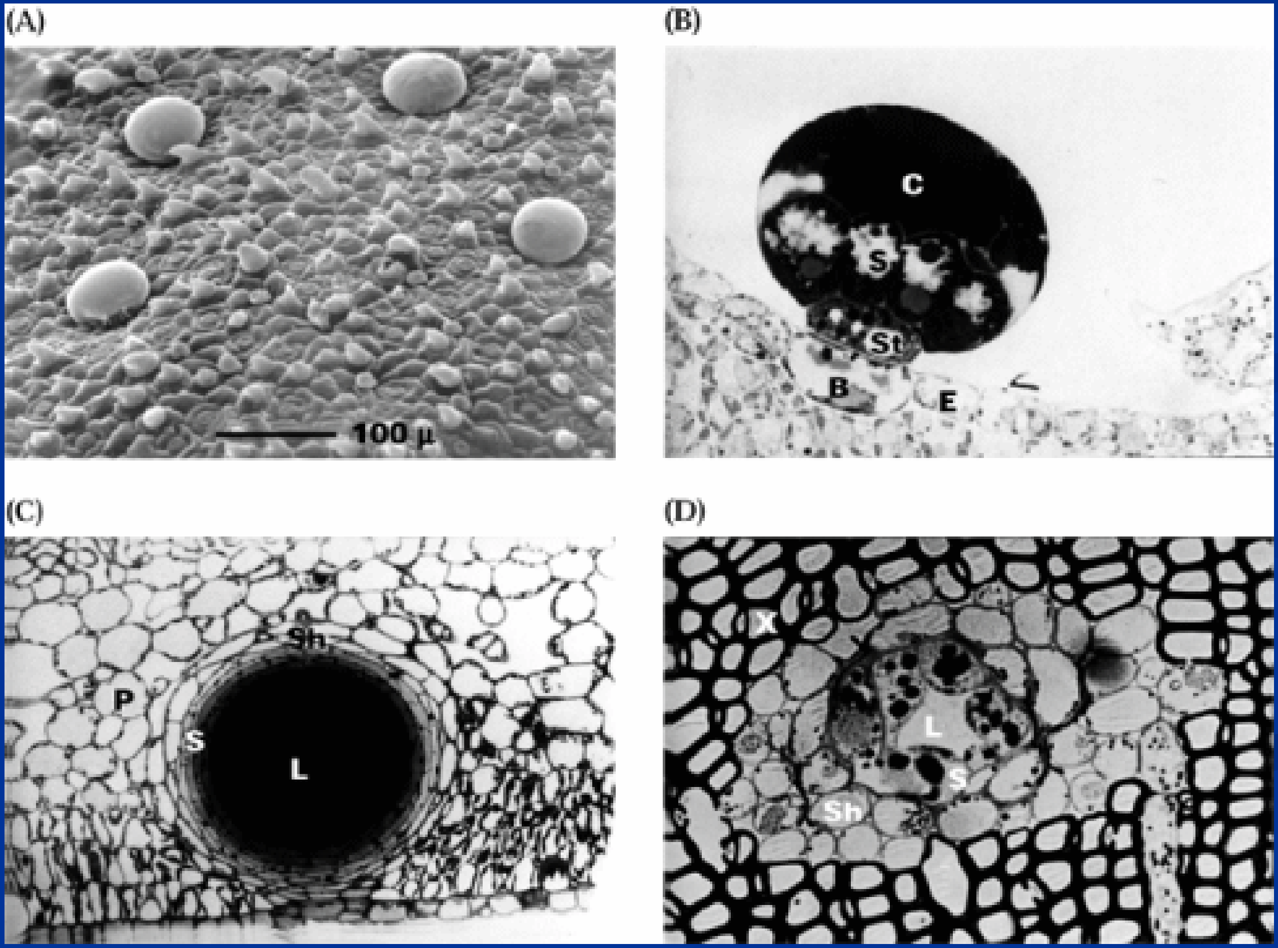
bz2

an9

SMs sequestration to a location with a solid barrier and not with a biomembrane (interfered by lipophilic SMs)

Thyme- glandular trichomes

Mint- glandular trichomes



Lemon leaf- secretory cavity

Pine- resin duct

Storage in LATICIFERS

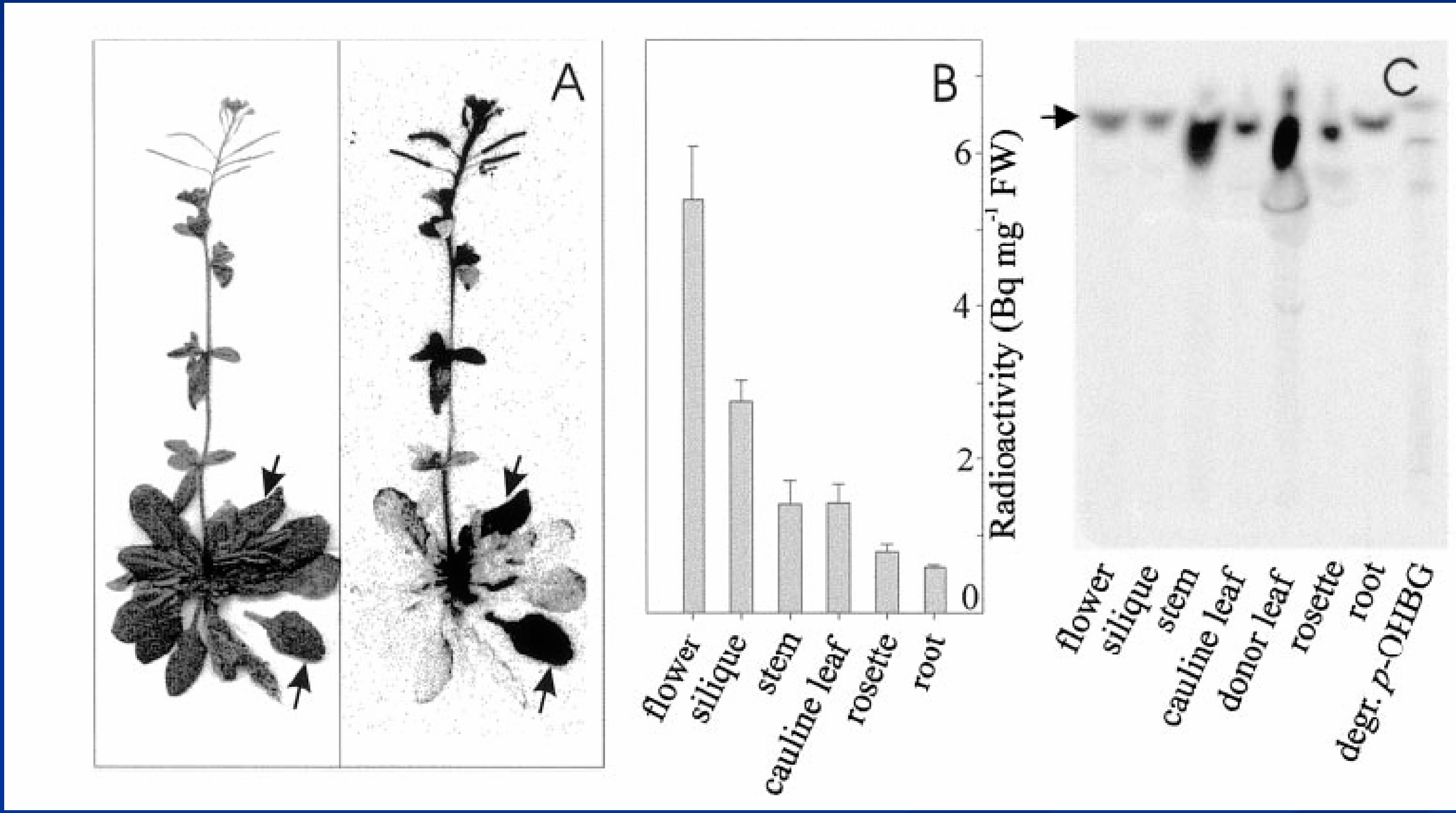
- Latex is a sap mixture of compounds stored in special structures called LATICIFERS

- Rubber was isolated from it in the past

- The composition is typically water, terpenes, sugars, enzymes, etc.

- Often latex has a milky appearance

Long Distance Transport of SMs
In Xylem, Phloem or Apoplastic transport



Long-distance phloem transport of glucosinolates

Chen et al., 2001

Long-distance phloem transport of glucosinolates

- Intact Glucosinolates are transported

- Selection of a specific glucosinolate to be loaded into the phloem

- Presence of glucosinolates in the phloem provide means of defense against insects

- Export of glucosinolates from fully expanded leaves and senescent parts

- Export to sink tissues, seeds, flowers

Chen et al., 2001

Costs of Secondary metabolism (ATP / NADPH2 consumption)

Often needed in HIGH concentrations (1-3% of dry weight are regularly seen)

- Biosynthesis of precursors and secondary metabolites

- Transport and storage

- Formation of specialized storage compartments (e.g. trichomes)

- Synthesis of mRNA and proteins (transcription translation)

Function of Secondary Metabolites

Often arguments that SMs are waste products but this cannot explain:

- production of SMs in young tissues

- plants are autotrophs and waste products are typical and needed for heterotrophic animals that cannot degrade their food completely for energy production

- many SMs could be metabolized further (SMs that contain nitrogen stored in seeds and metabolized during germination)

- tight spatial and temporal regulation of SMs biosynthesis

- proven biological activity

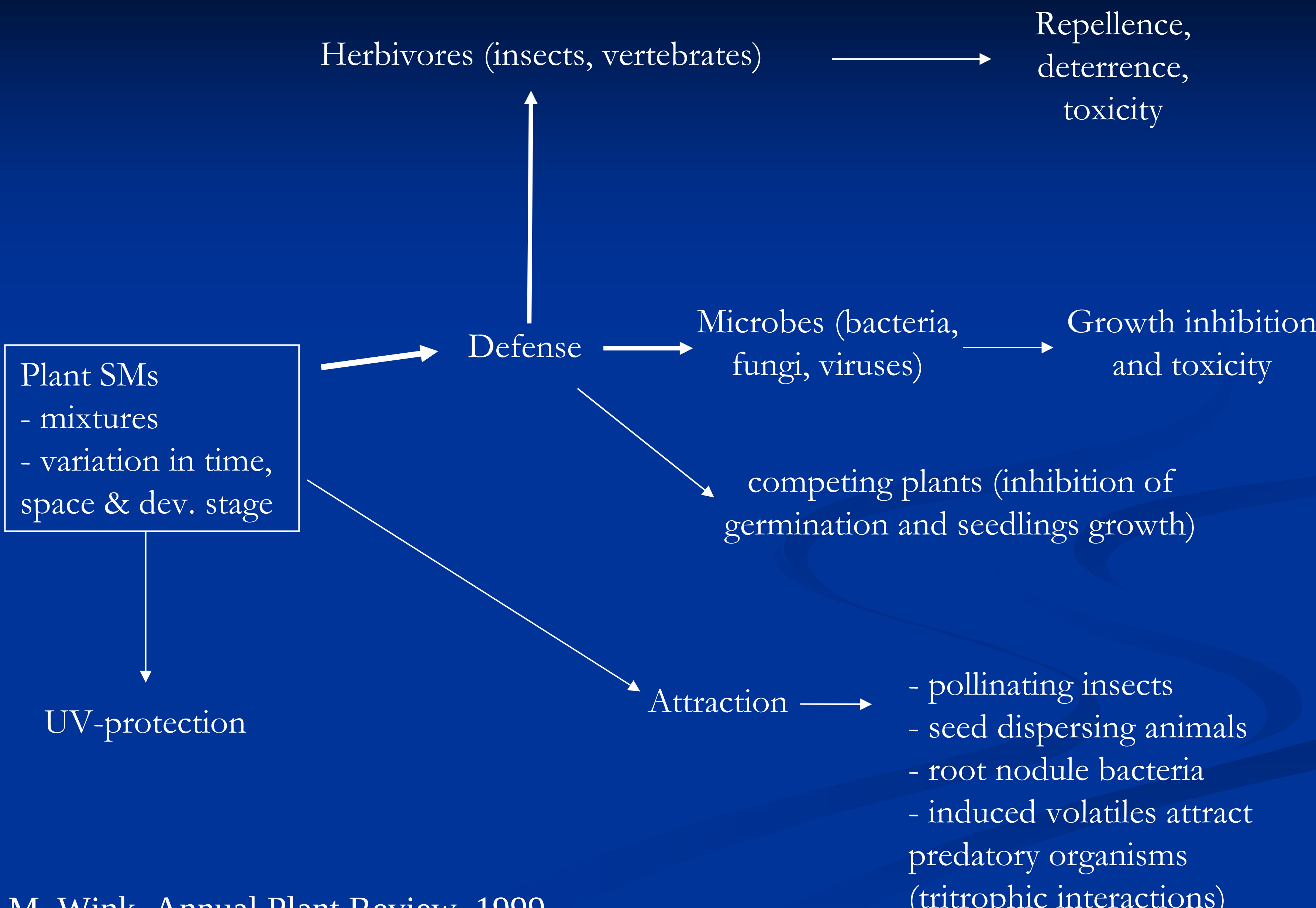
Function of Secondary Metabolites - DEFENSE - ATTRACTION - PROTECTION (uv)

- Most animals can move-run away and posses an immune system

- Plants are attacked by herbivores, microbes, (bacteria and fungi) and by other plants competing for light, space and nutrients

- Abiotic stresses such as radiation

Function of Secondary Metabolites:

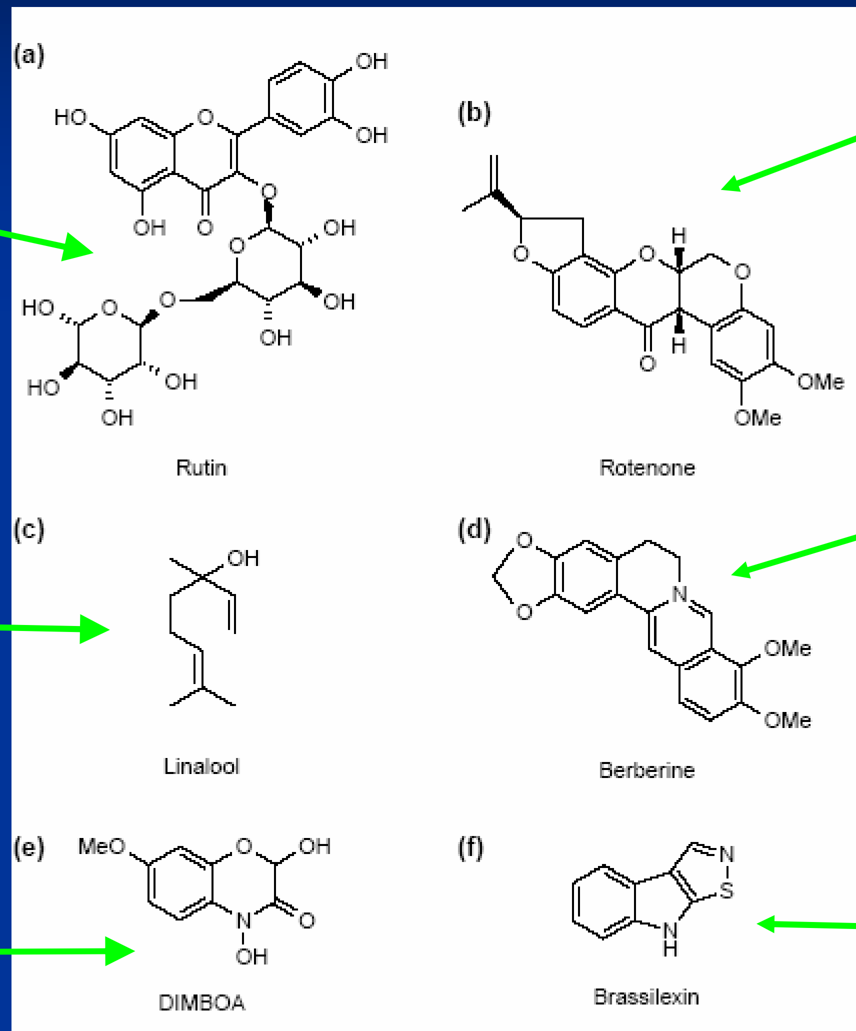


M. Wink, Annual Plant Review, 1999

Examples of plant SMs and their proposed function

Visual pollinator attractant

Insect feeding deterrent



Olfactory pollinator attractant

Defense toxin

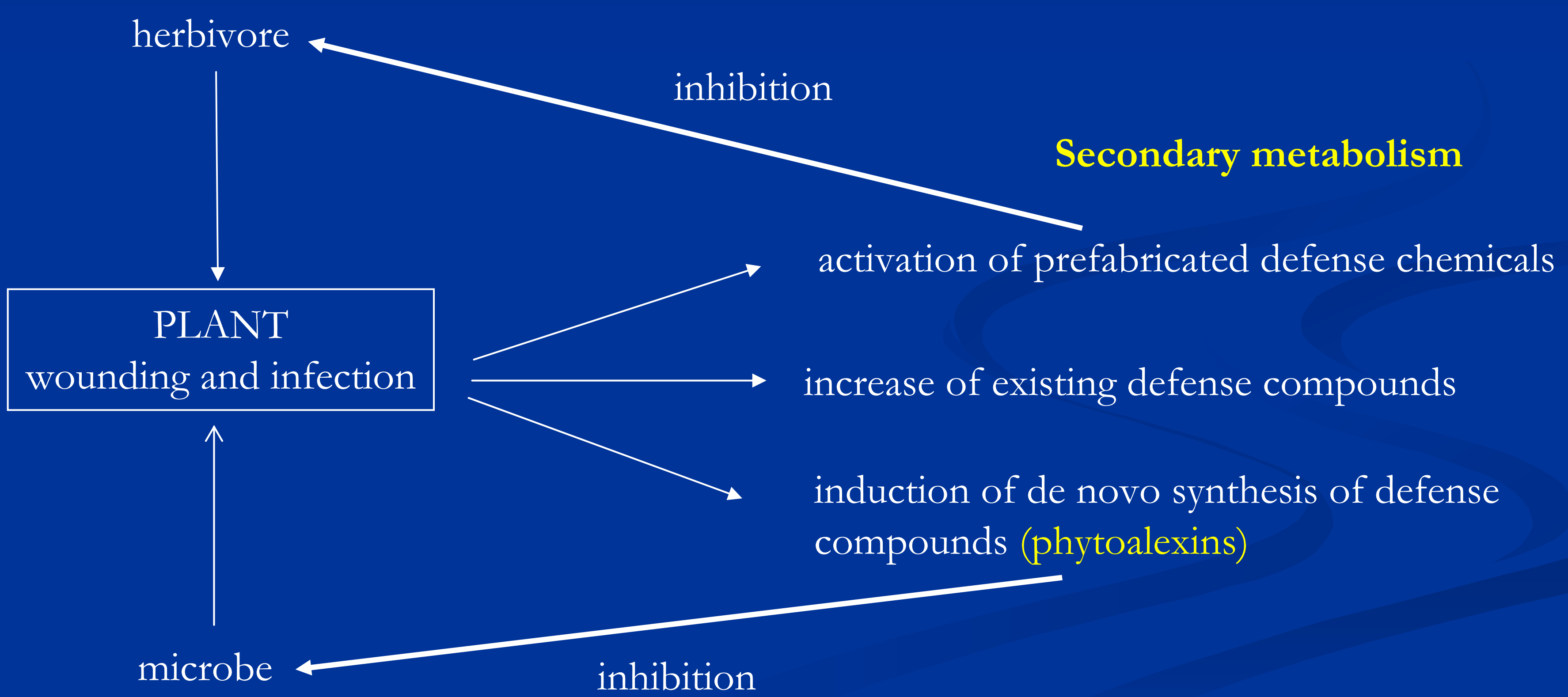
Defense toxin

Antifungal toxin

From Pichersky and Gang, 2000

Production of SMs for defense against herbivores and pathogens is not necessarily constitutive

- Wounding and infection trigger INDUCED accumulation of SMs



M. Wink, Annual Plant Review, 1999

Function of Secondary Metabolites

- Wounding can lead to release of a pre-fabricated compound from a compartment

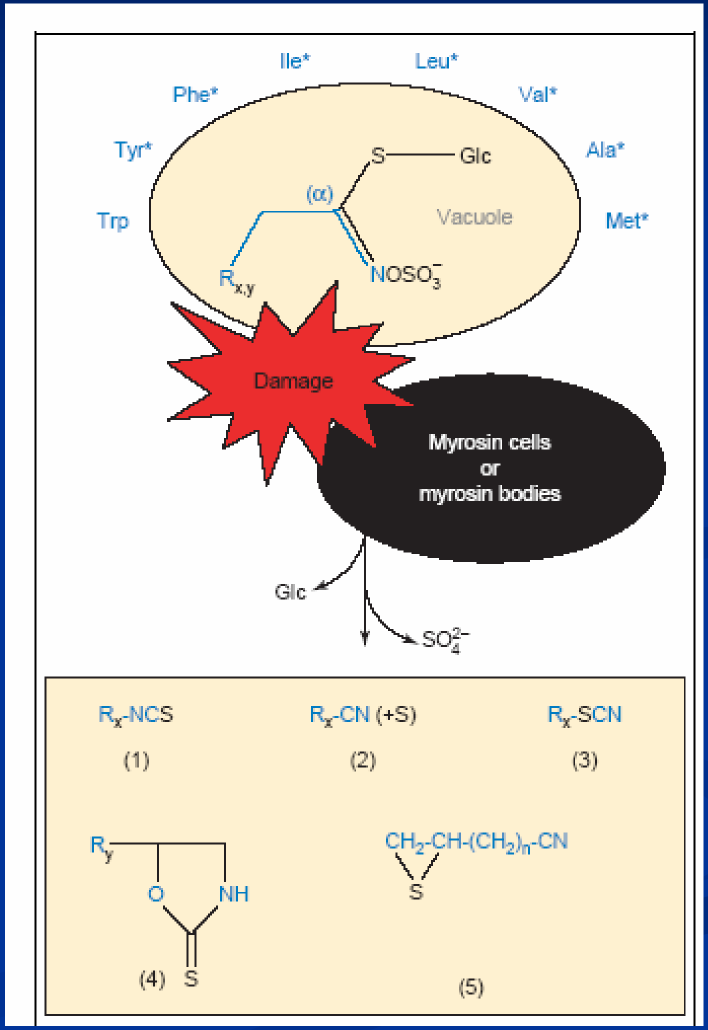
- The mix with an enzyme (often an hydrolaze) will result in production of an active form of the chemical

- Example: myrosinase-glucosinolates

The "mustard oil bomb"-- *A binary Glucosinolate-Myrosinase chemical defense system*

Glucosinolates breakdown products

1- isothiocyanates
2- nitriles and elemental sulfur
3- thiocyanates
4- oxazolidine--thiones
5- epithionitriles



Grubb and Abel, TIPS, 2006

Targets for SMs in animal systems

- Nervous system (perception, processing, signal transduction
- Development
- Muscles and motility
- Digestion
- Respiration
- Reproduction and fecundity

Co-evolution in plant SMs - natural enemy

- The SM defense system works in general but not always

- Some herbivores and microorganisms have evolved that have overcome the defense barrier (like viruses, bacteria or parasites that bypass the human immune system)

- These organisms developed different strategies of adaptations to the SMs

- They can either tolerate them or even use them for their diet

Adaptations of specialist herbivores & pathogens

Herbivores:

- Avoidance of toxic plants, except host plant

- Cutting laticfers and resin ducts filled with SMs

- Non-resorption or fast intestinal food passage

- Resorption followed by detoxification and elimination (urine and others)
    - Hydroxylation
    - Conjugation
    - Elimination

Adaptations of specialist herbivores & pathogens

Herbivores (continued)-

- Resorption and accumulation:
    - Specific compartments / cells / tissues for sequestration
    - Evolution of insensitivity

- Use of plant SMs in diet:
    - defense against predators
    - signal molecules (pheromones)
    - morphogen

Adaptations of specialist herbivores & pathogens

Microorganisms:

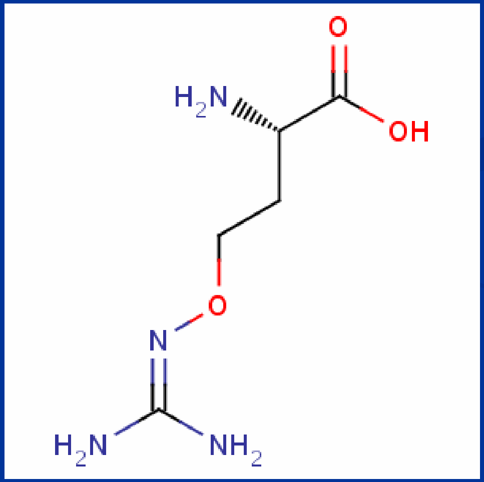
- Inactivation of SMs

- Evolution of insensitivity

Co-evolution in plant SMs - natural enemy

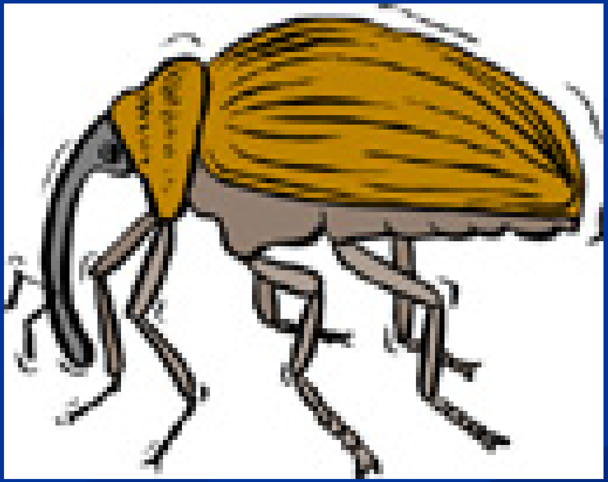
Plant defense	Plant taxon	Natural enemy	Counter resistance
Toxic amino acids	Various Leguminosae	Bruchid weevil	Modified tRNA synthase

Dioclea seed



L-canavanine

(similar to arginine, no protein amino acid)



Weevil

Co-evolution in plant SMs - natural enemy

- Canavanine is toxic due to its incorporation into proteins that rise to functionally aberrant polypeptides

- The tRNA- Arginine in insects uses also Canavanine

- The insect mutated its tRNA and will not incorporate canavanine instead of Arginine

Adaptations of specialist herbivores & pathogens

The process of co-evolution between plants and their natural enemies is believed to have generated much of the earth's biological diversity

This includes chemical diversity!!

SMs in Arabidopsis

Major classes of secondary metabolites found in *A. thaliana*.

Class	Approximate number of structures[a]	Suggested functions
Indole and indole-sulfur compounds	10	Defense against pathogens
Glucosinolates	35	Defense against pathogens and herbivores
Phenylpropanoids	20	UV protection. Defense against pathogens?
Benzenoids	25	Pollinator attractants? Defense against pathogens?
Flavonoids	15	UV protection. Auxin transport. Seed dormancy. Defense against pathogens?
Terpenes	50	Herbivore feeding deterrents? Resistance to oxidative stress? Defense against pathogens?
Fatty acid derivatives	15	Defense against pathogens? Volatile signals?

The secondary metabolism of *Arabidopsis thaliana*: growing like a weed

John C D'Auria and Jonathan Gershenzon

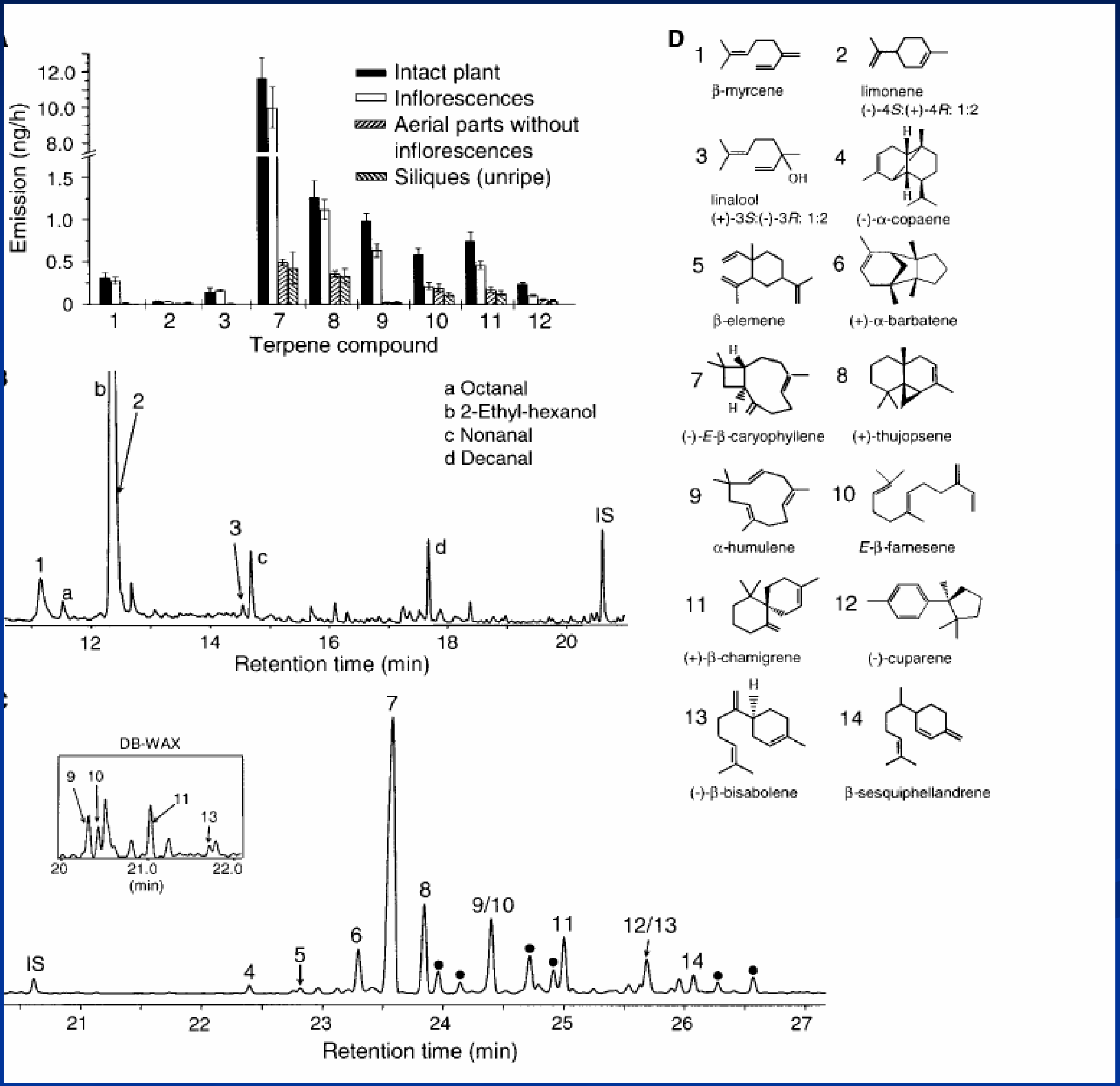
SMs in Arabidopsis

Representative gene families encoding enzymes participating in secondary metabolism in *A. thaliana*.

Gene family	Number of reported members existing in *A. thaliana*[a]	Secondary metabolites formed
Acyltransferases		
BAHD	64	Acylated anthocyanins, aliphatic and aromatic esters
Serine carboxy-peptidase like	53	Hydroxycinnamate esters
Methyltransferases		
SABATH	24	Aliphatic and aromatic methyl esters
Type I OMT	6	Flavonoid methyl ethers
Carboxy methyl esterases	20	Carboxylic acids
Cytochrome P450 monooxygenases	272	Hydroxylated phenylpropanoids, glucosinolates
Glutathione *S*-transferases	48	Glutathione conjugates
Aldehyde dehydrogenases	14	Aromatic and aliphatic acids
Terpene synthases	30	Mono-, sesqui-, and diterpenoids
Oxidosqualene cyclases	13	Triterpenoids
Glycosyl transferases	107	Glycosides (e.g. glucosinolates and anthocyanins)
Glycoside hydrolases family I	47	Aglucones (e.g. flavonols and phenylpropanoids)
Pathogenesis related lipase-like proteins	6	Fatty-acid-derived compounds
Acyl-activating enzymes/CoA ligases	63	CoA thioesters, amino acid conjugates

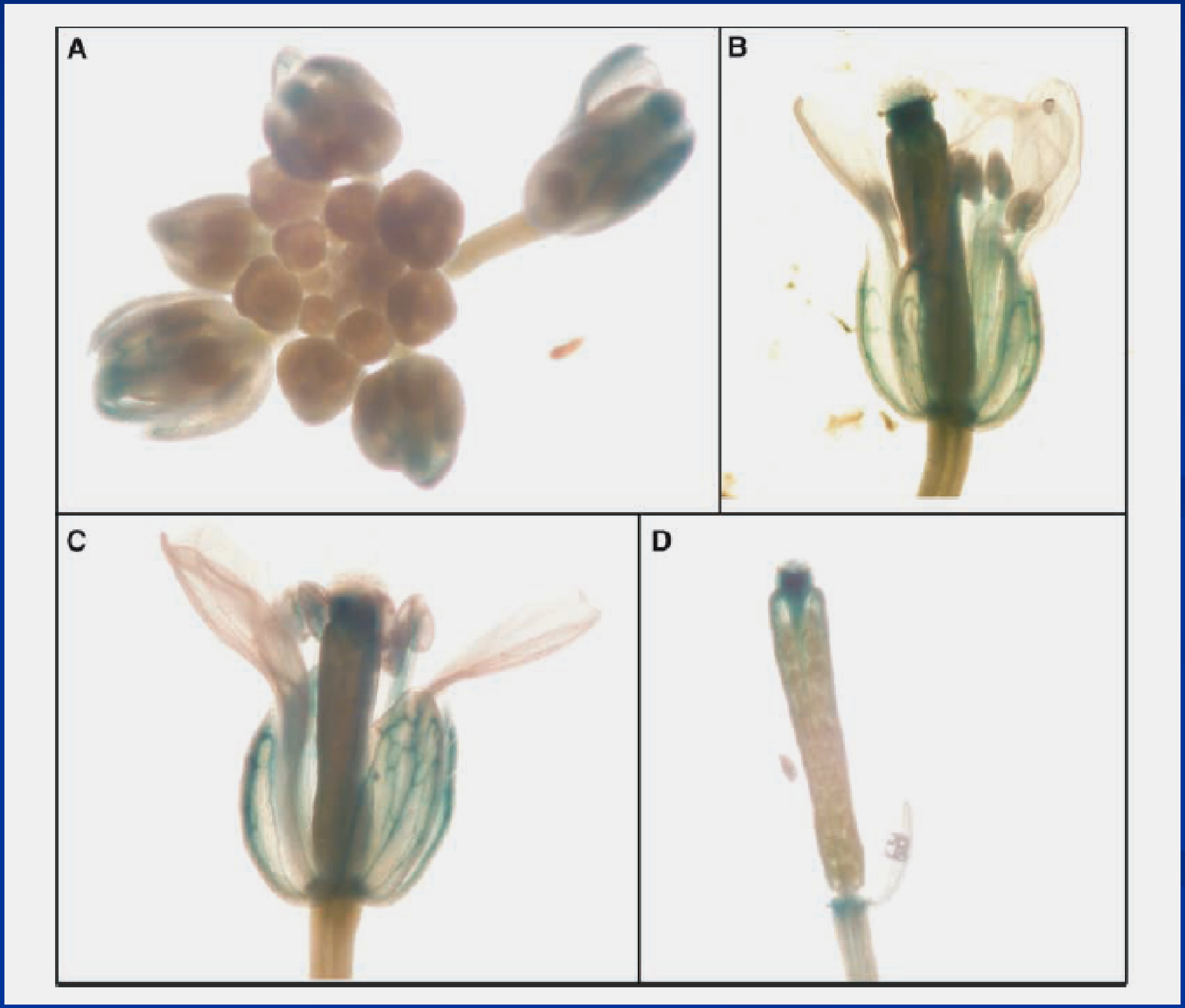
[a] Includes pseudogenes. Abbreviation: OMT, *O*-methyltransferase.

Terpenes (mono and sesquiterpenes in Arabidopsis



Chen et al., 2003

Expression pattern of a Terpene Synthase in Arabidopsis



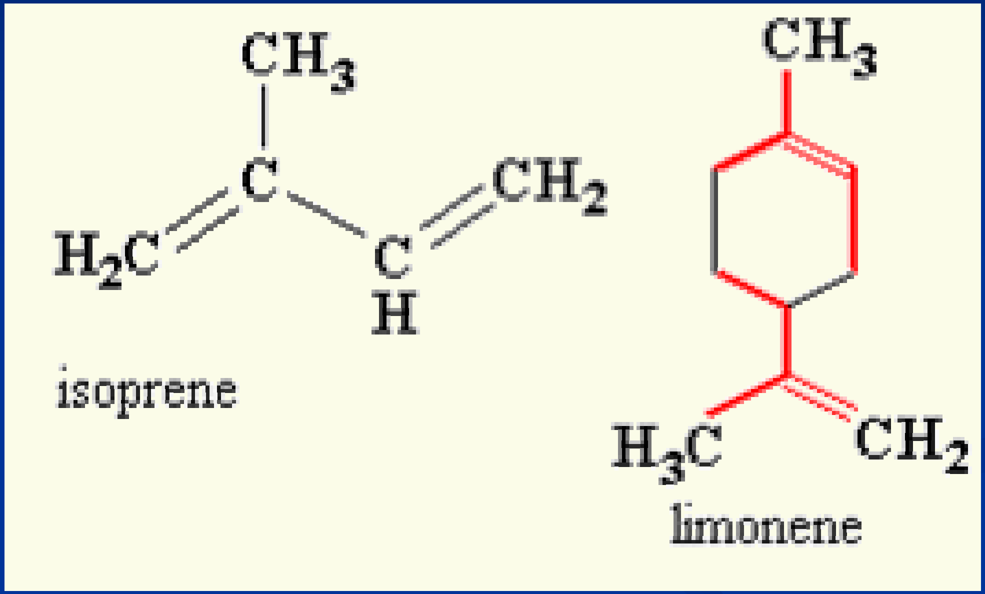
The Terpenoids or Isoprenoids

The name Terpenoid & Isoprenoid

- The name terpenoid derives from the fact that first members of the class were isolated from TURPENTINE [the distillate from tree (e.g.. pine) resins]

- Isoprenoid, since ISOPRENE is the basic unit of C5 building them (C_5H_8)

The biogenetic isoprene rule



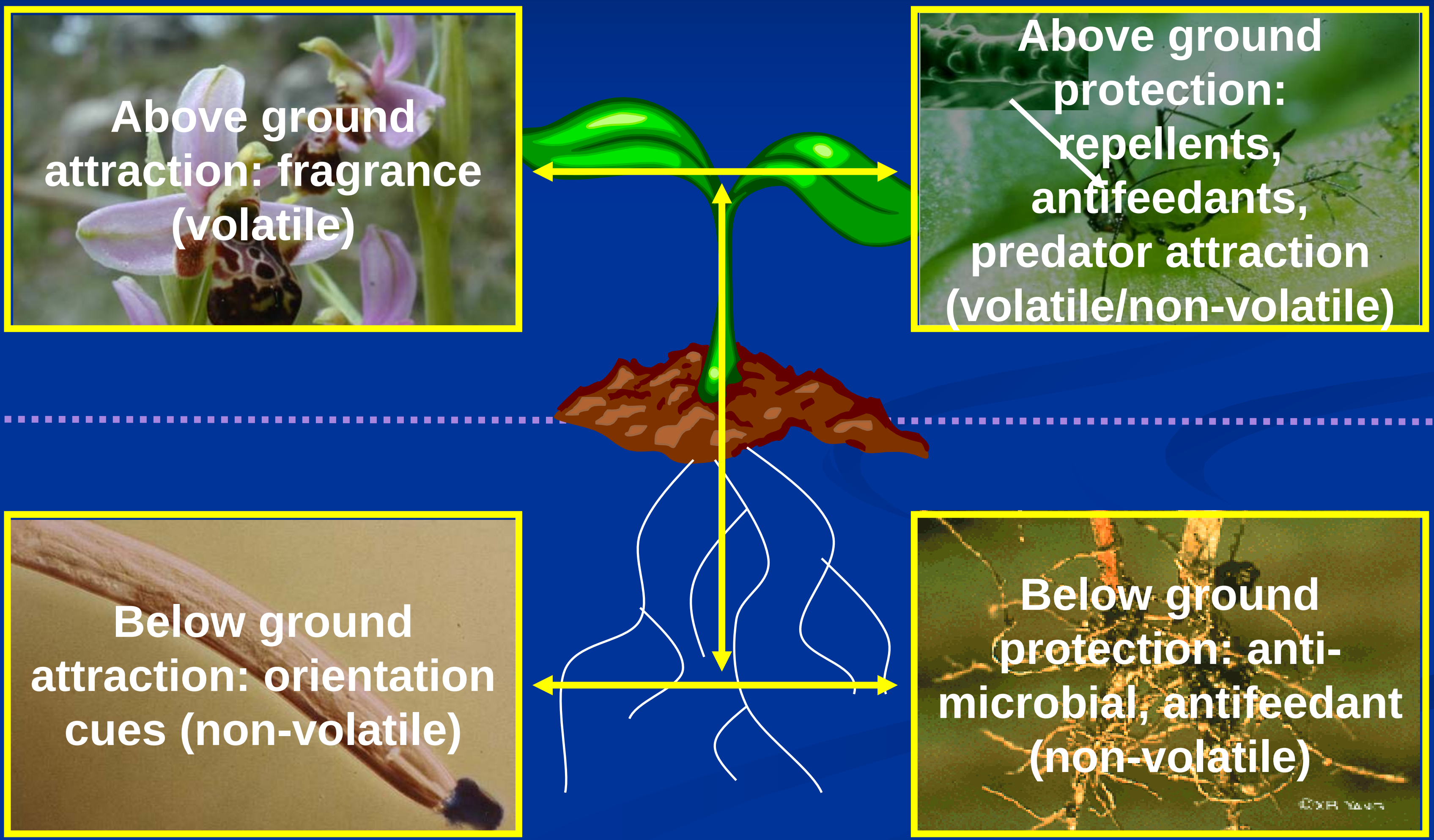
Leopold Ruzika (1930s; Nobel Prize chemistry 1910): A compound is an "isoprenoid" if it is derived biologically from an "isoprenoid" with or without rearrangements

The Terpenoids of Plant origin

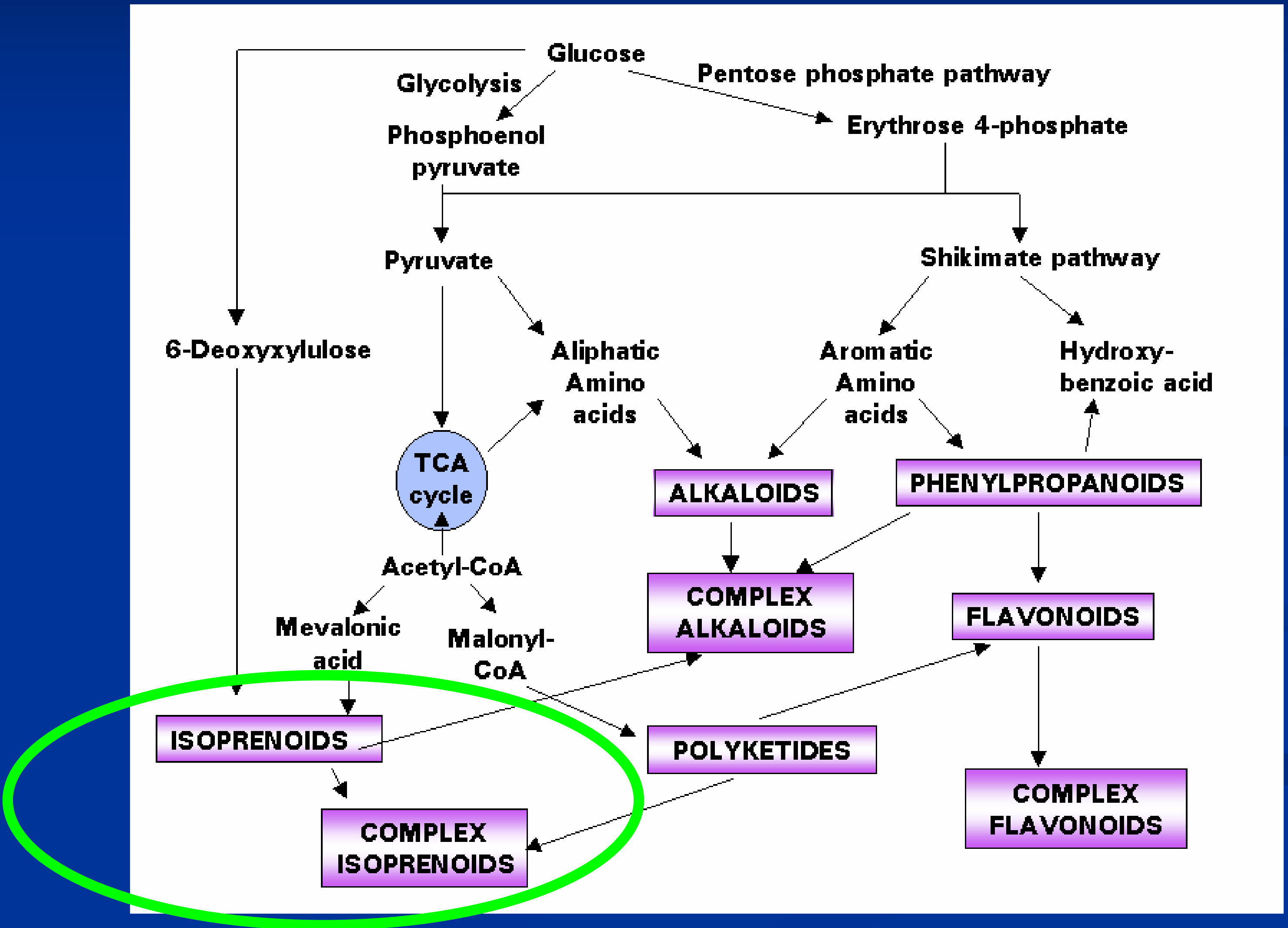
Biological Role (volatile and non volatile):

- Flavour, fragrance, scent
- Antibiotics
- Hormones
- Membrane lipids
- Insect attractants
- Insect antifeedants
- Mediate the electron transport processes (in respiration and photosynthesis)

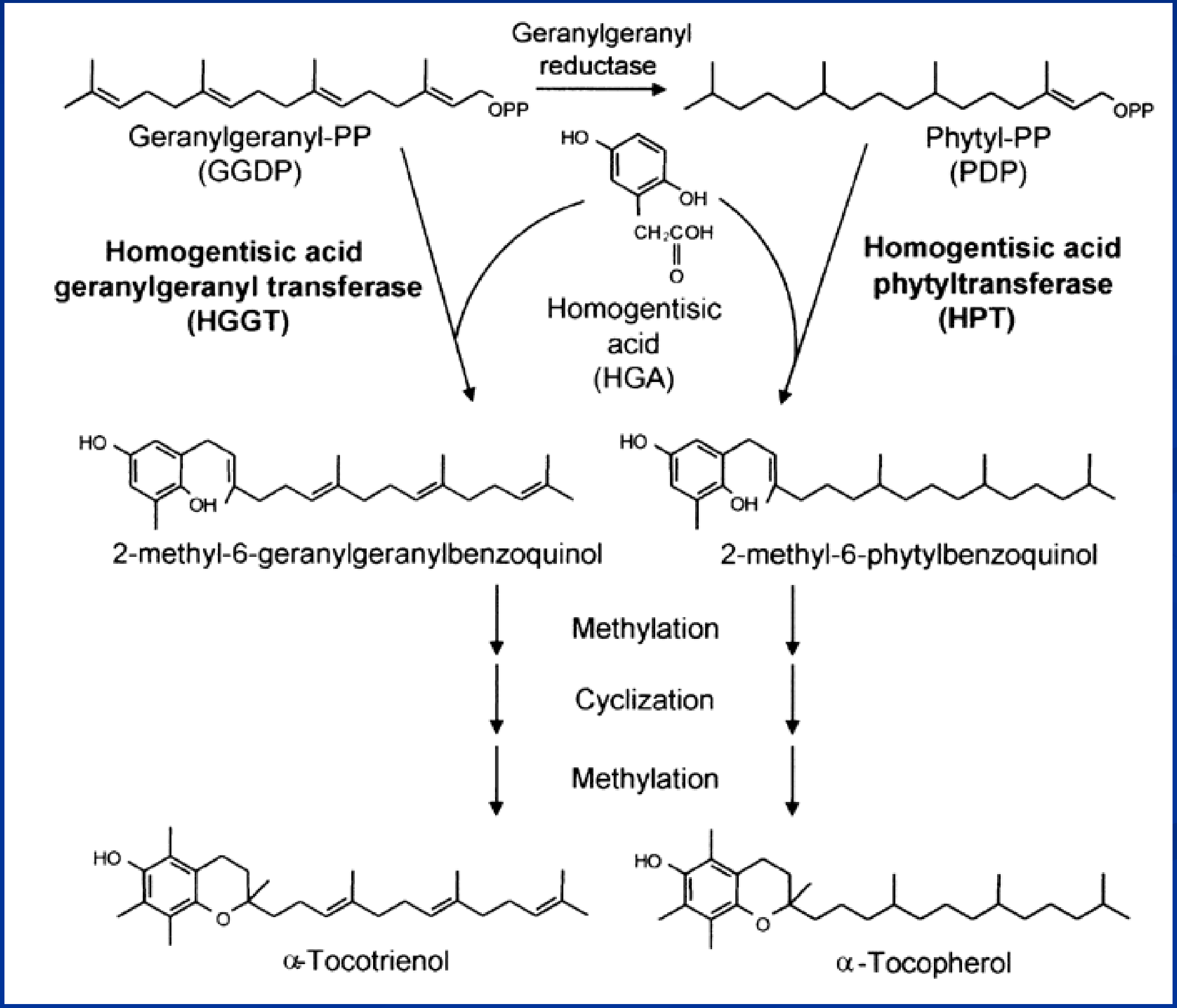
Terpenoids and Communication



Precursors of Terpenoids



Mixed Origins of Terpenoids Precursors (Meroterpenes)



Terpenoids - Important Molecules !

C_5 - hemiterpenes - e.g. isoprene

C_{10} - monoterpenes - e.g. limonene

C_{15} - sesquiterpene - e.g. abscisic acid (ABA)

C_{20} - diterpene - e.g. gibberellin

C_{30} - triterpne - e.g. brassinosteroids

C_{40} - tetraterpenes - e.g. carotenoids

> carbons - polyterpenes- e.g. ubiquinones, rubber

mixed biosynthetic origins - meroterpenes - e.g. cytokinines, vitamin E

Monoterpenes (C_{10})

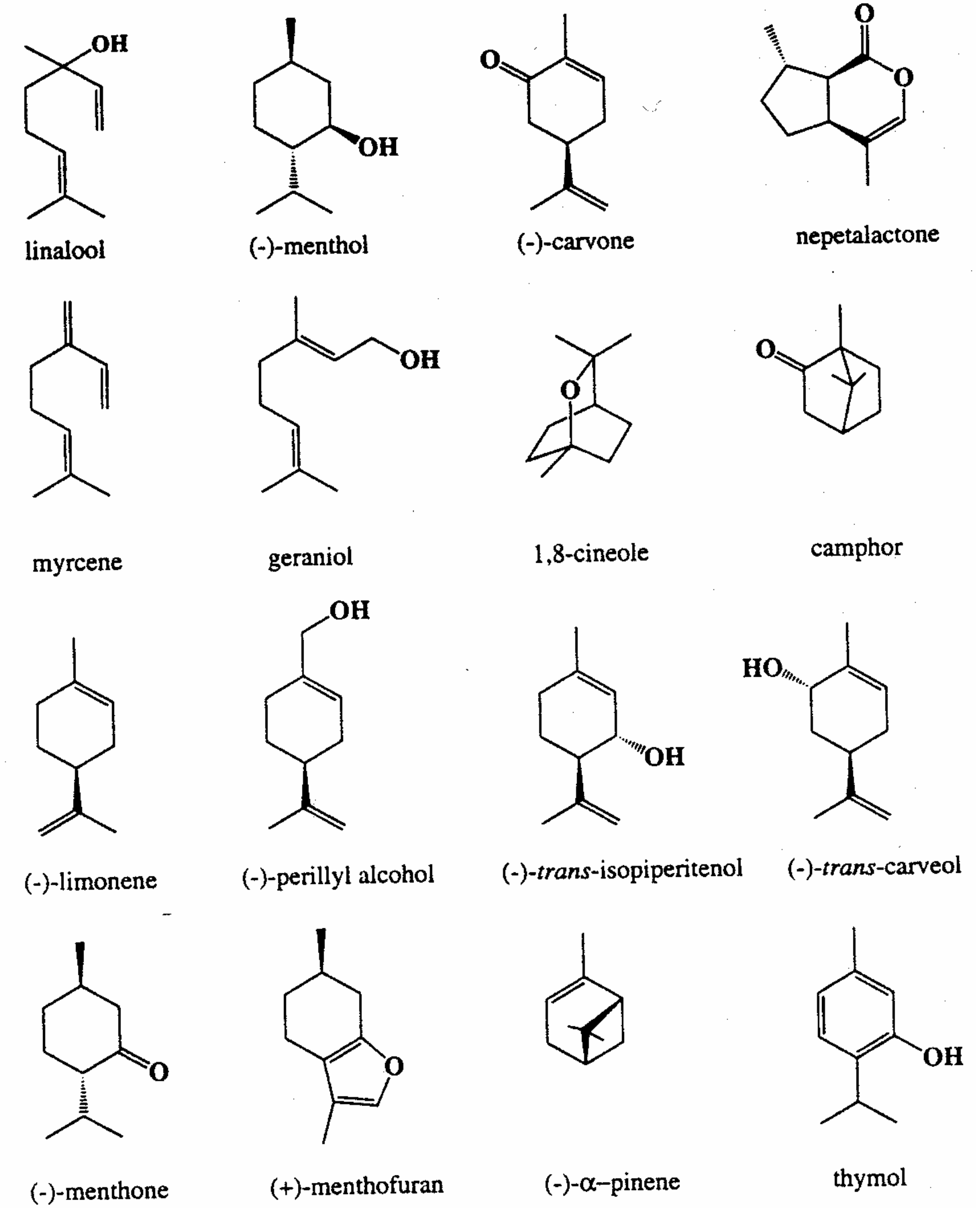


Figure 1. Structures of representative monoterpenoids.

Sesquiterpenes (C_{15})

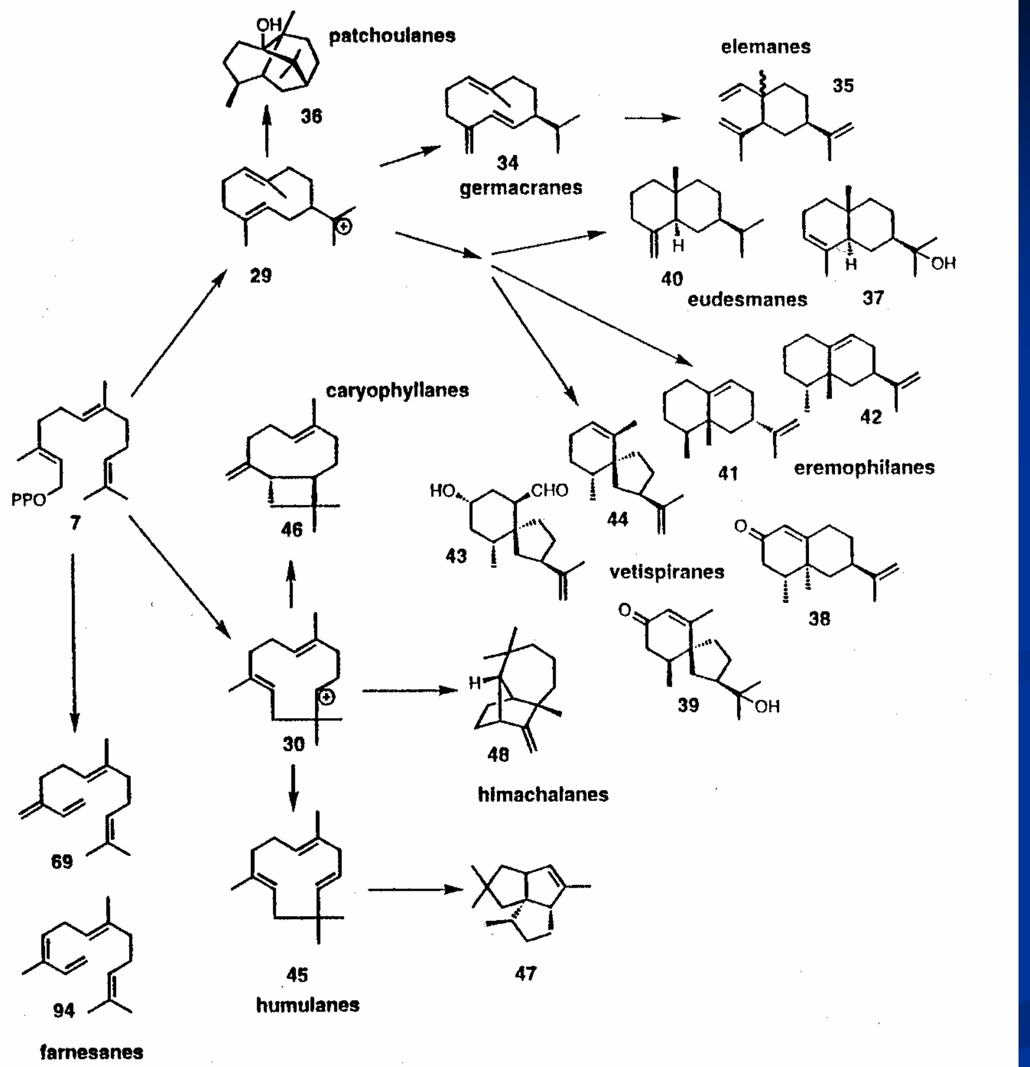
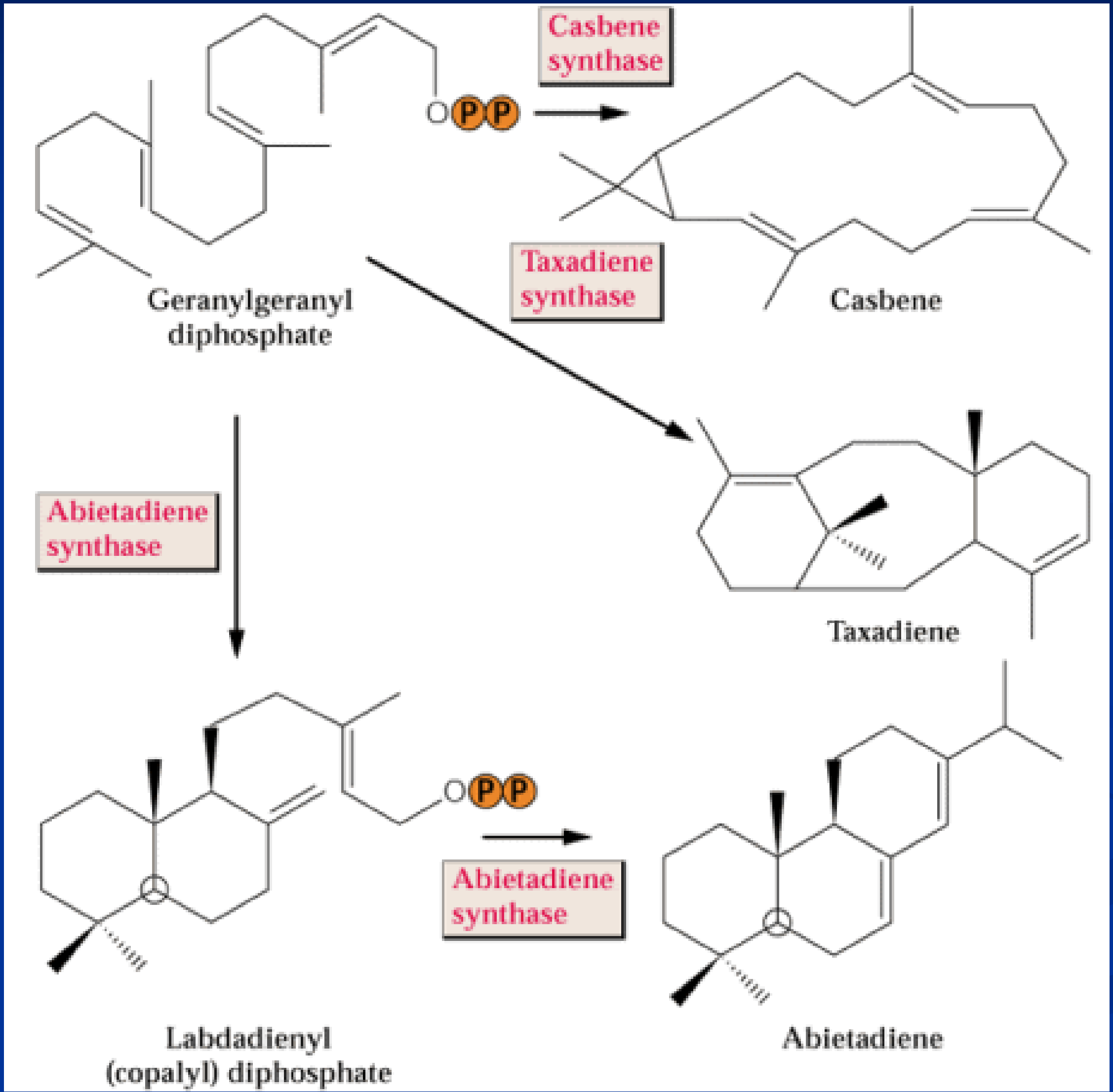
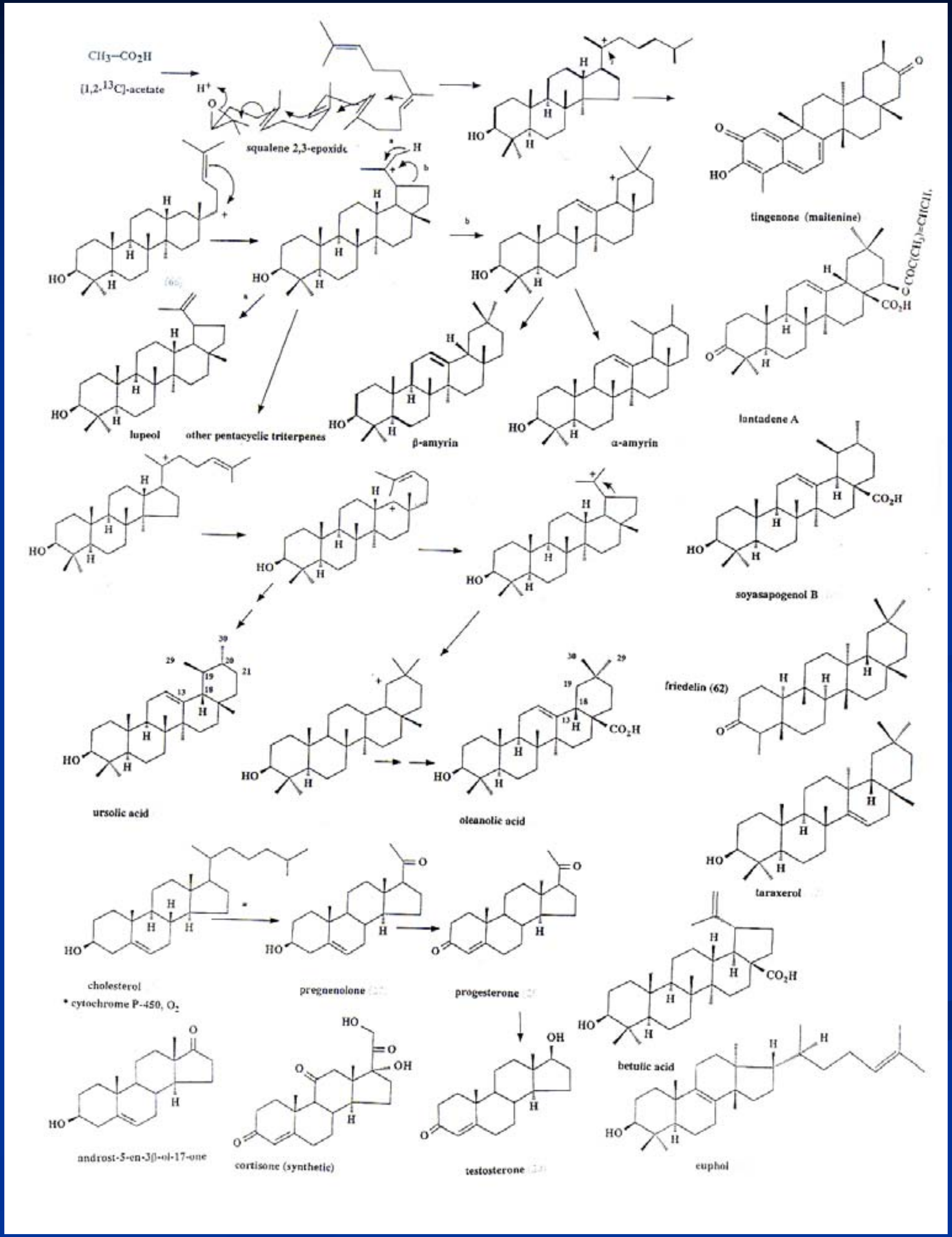


Fig. 3a. Cyclization of FPP by sesquiterpene synthases to produce different sesquiterpene skeletons: a direct cyclization of FPP

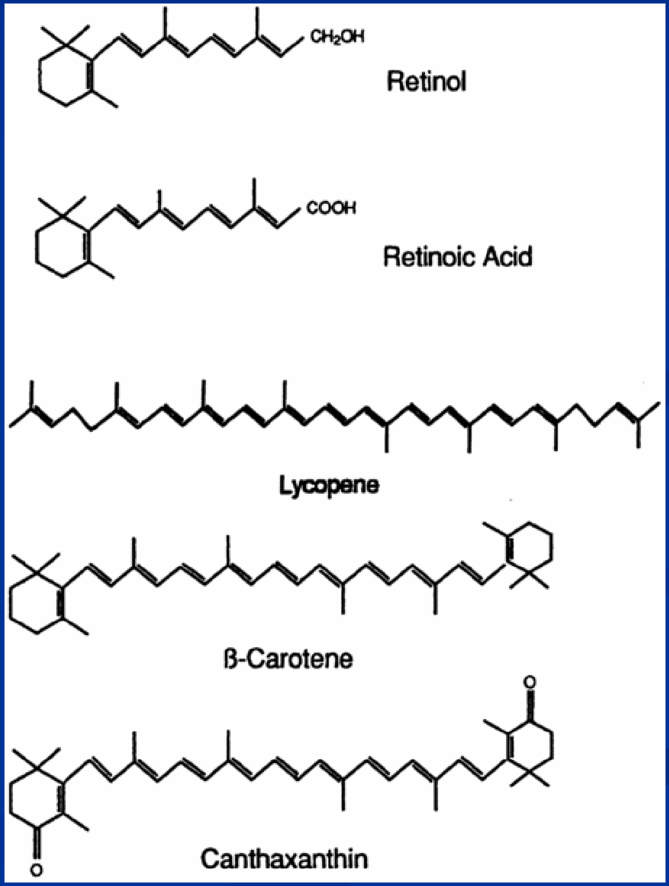
Diterpenes (C_{20})



Triterpenoids (C_{30})



Tetra-terpene / Carotenoids (C_{40})



Biosynthesis in two main compartments

- Mevalonate pathway leading to IPP in the cytosol
- The MEP pathway leading to IPP in the plastids

Biosynthesis in two main compartments

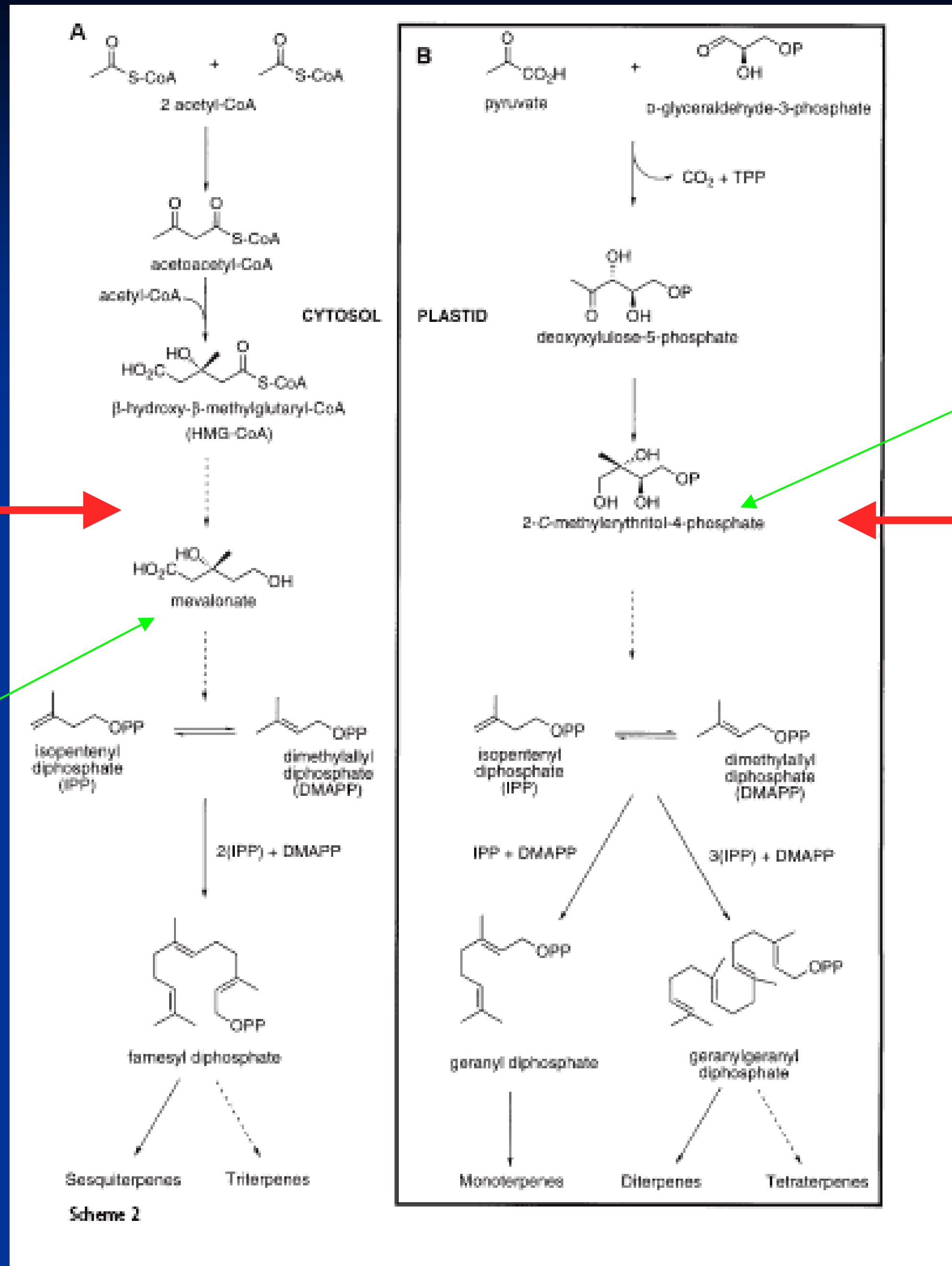
- Mevalonate pathway leading to IPP in the cytosol
- The MEP pathway leading to IPP in the plastids

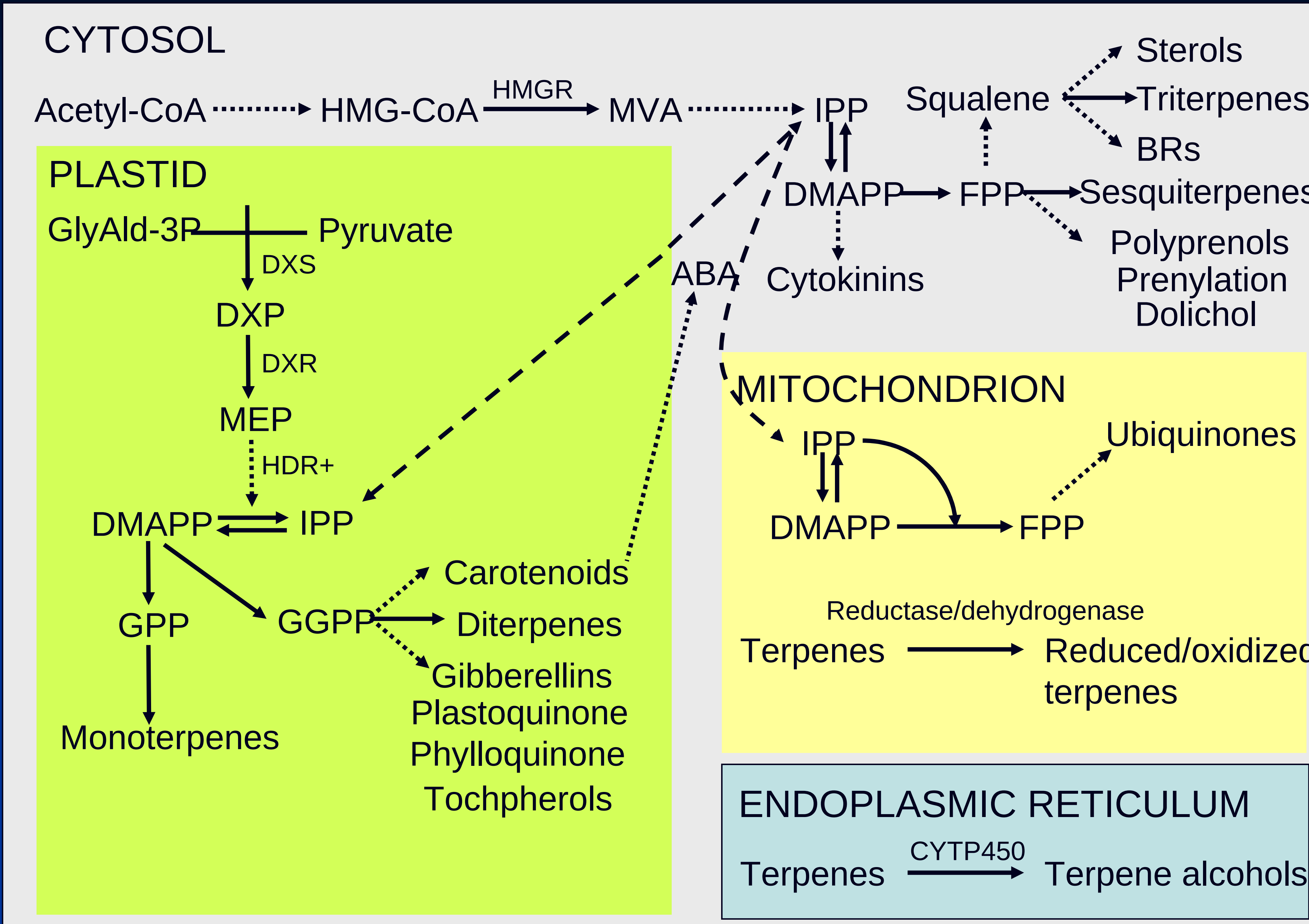
Cytosol

MEP

Plastid

Mevalonate




CYTOSOL
Acetyl-CoA
HMG-CoA
HMGR
MVA
IPP
Squalene
Sterols
Triterpenes
BRs
DMAPP
FPP
Sesquiterpenes
Polyprenols
Prenylation
Dolichol
Cytokinins
ABA
PLASTID
GlyAld-3P
Pyruvate
DXS
DXP
DXR
MEP
HDR+
DMAPP
IPP
GPP
GGPP
Monoterpenes
Carotenoids
Diterpenes
Gibberellins
Plastoquinone
Phylloquinone
Tochpherols
MITOCHONDRION
IPP
Ubiquinones
DMAPP
FPP
Reductase/dehydrogenase
Terpenes
Reduced/oxidized
terpenes
ENDOPLASMIC RETICULUM
CYTP450
Terpenes
Terpene alcohols

Biosynthesis of terpenoids

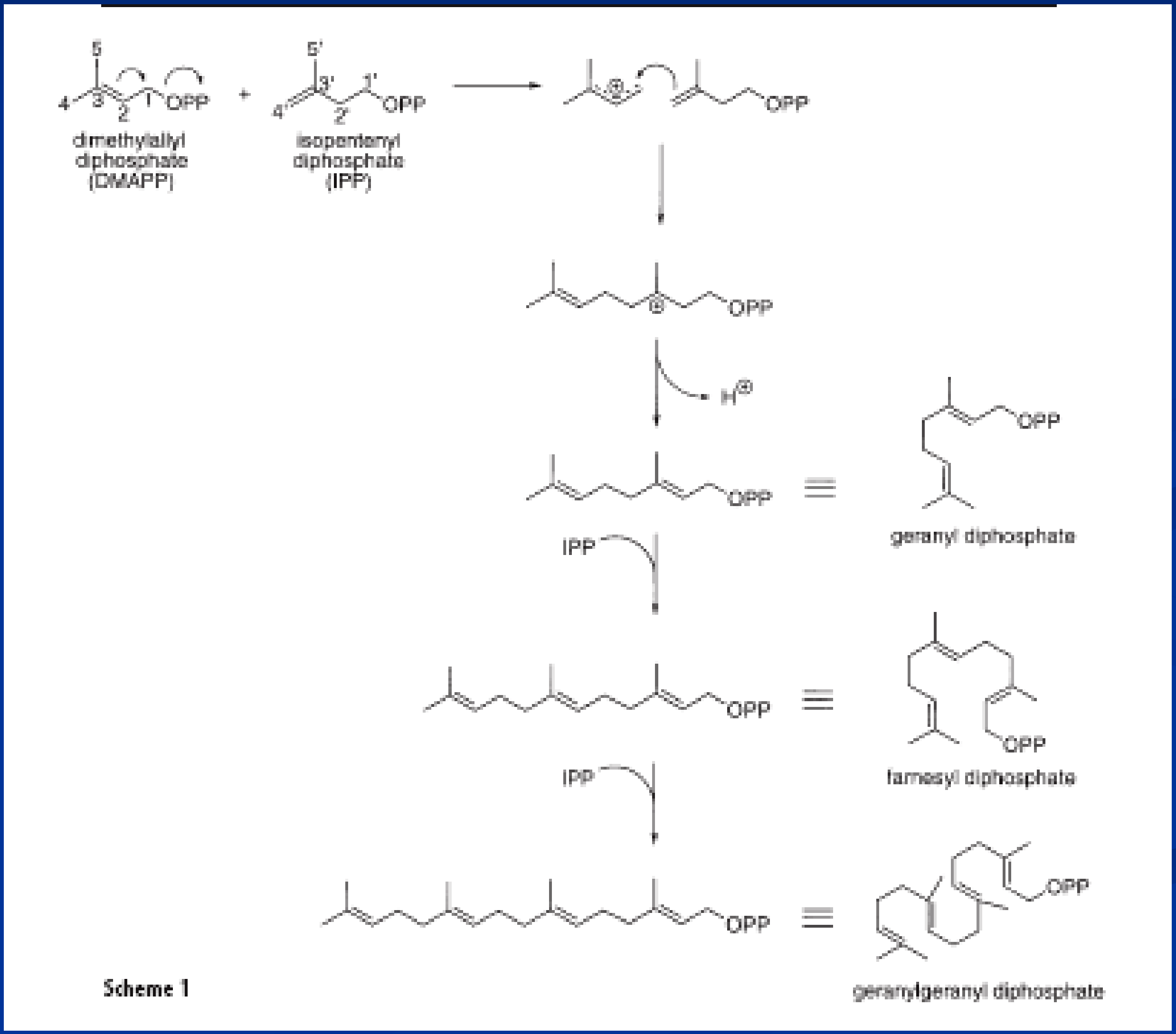
Three main reactions:

- Generating precursors by prenyltransferases (GPP, FPP,GGPP)

- Terpene synthase reactions (e.g. monoterpene synthase/cyclase)

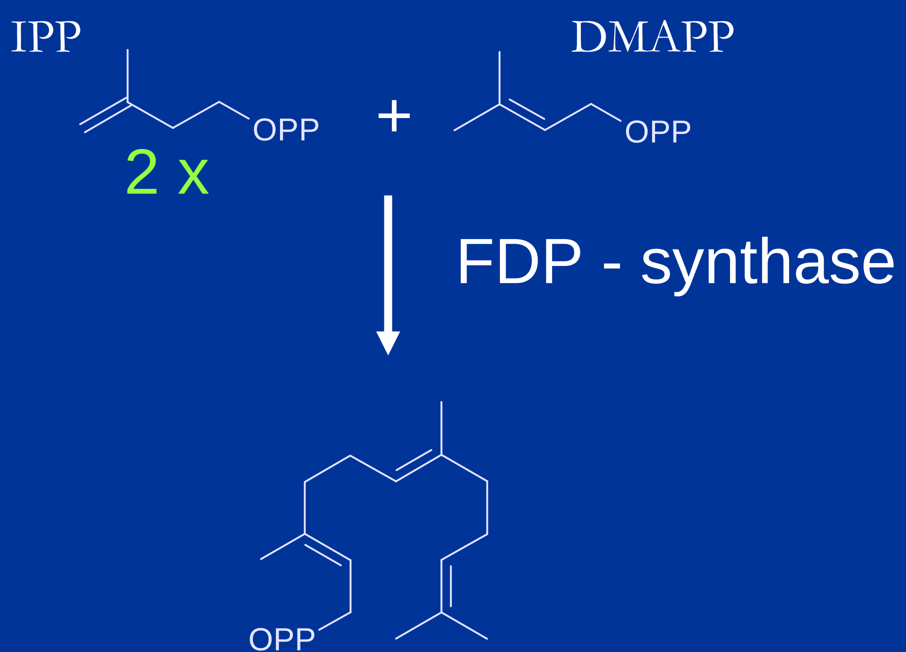
- Modification steps

Biosynthesis of Precursors (prenyltransferases)



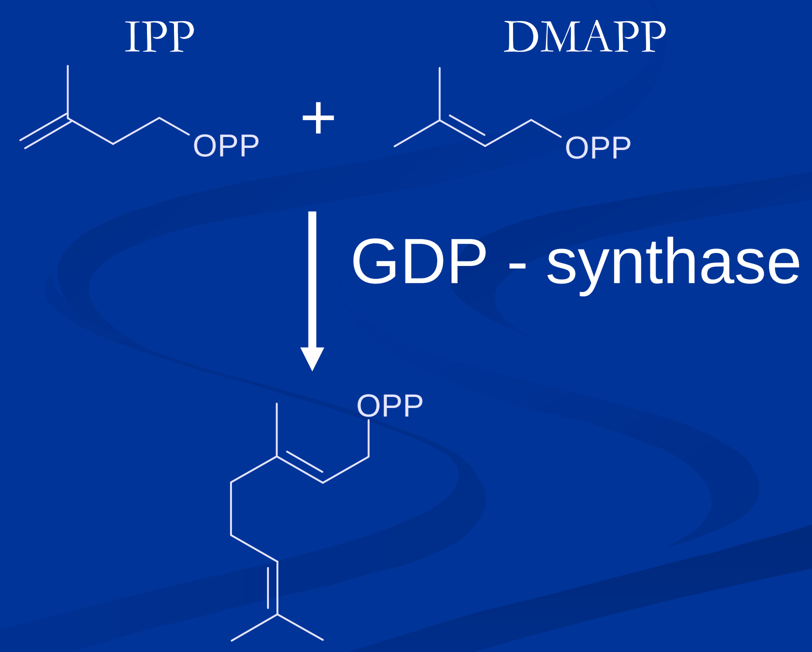
Biosynthesis of Precursors (prenyltransferases)

Cytosol



Farnesyl diphosphate

Plastid

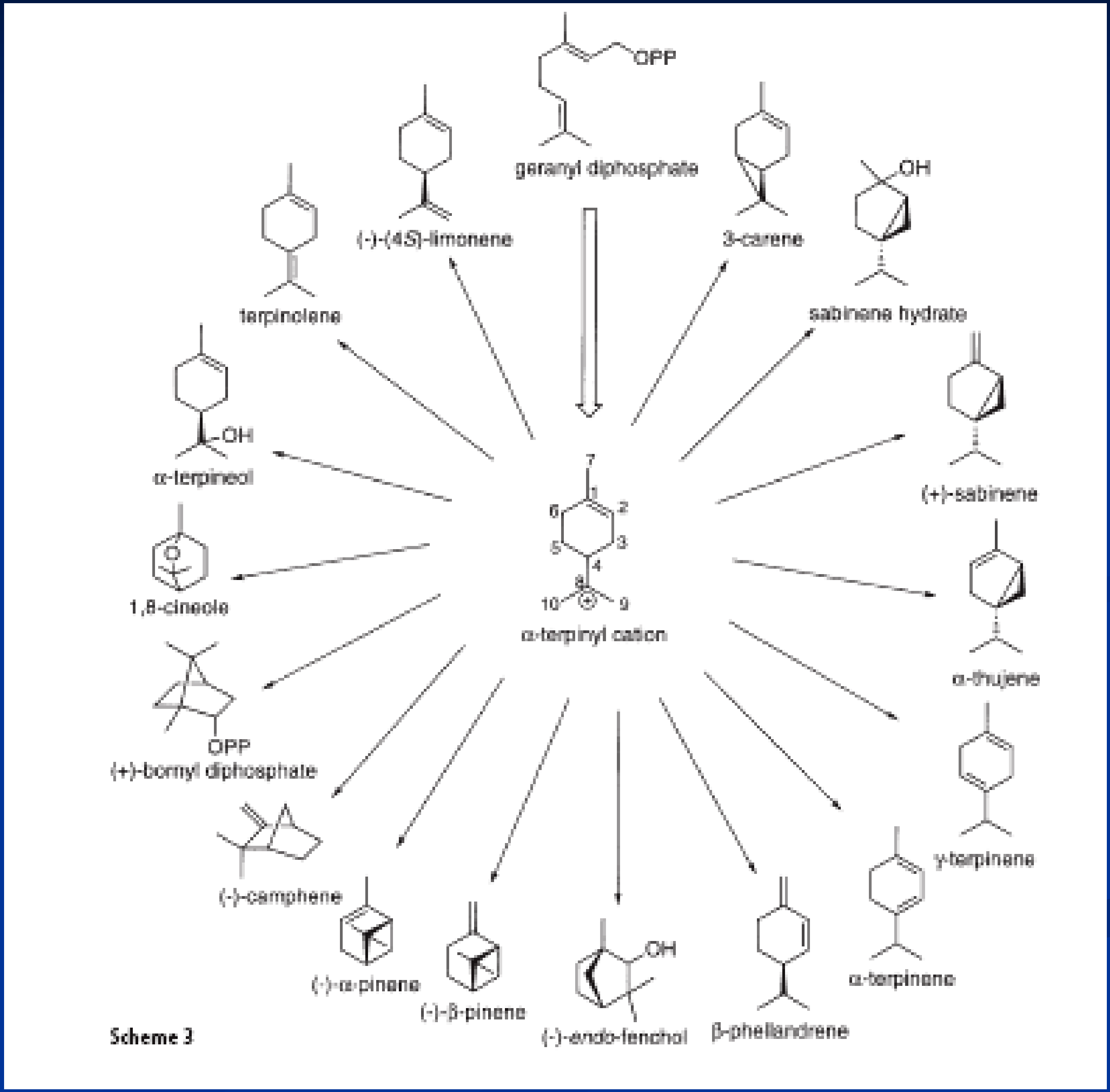


Geranyl diphosphate

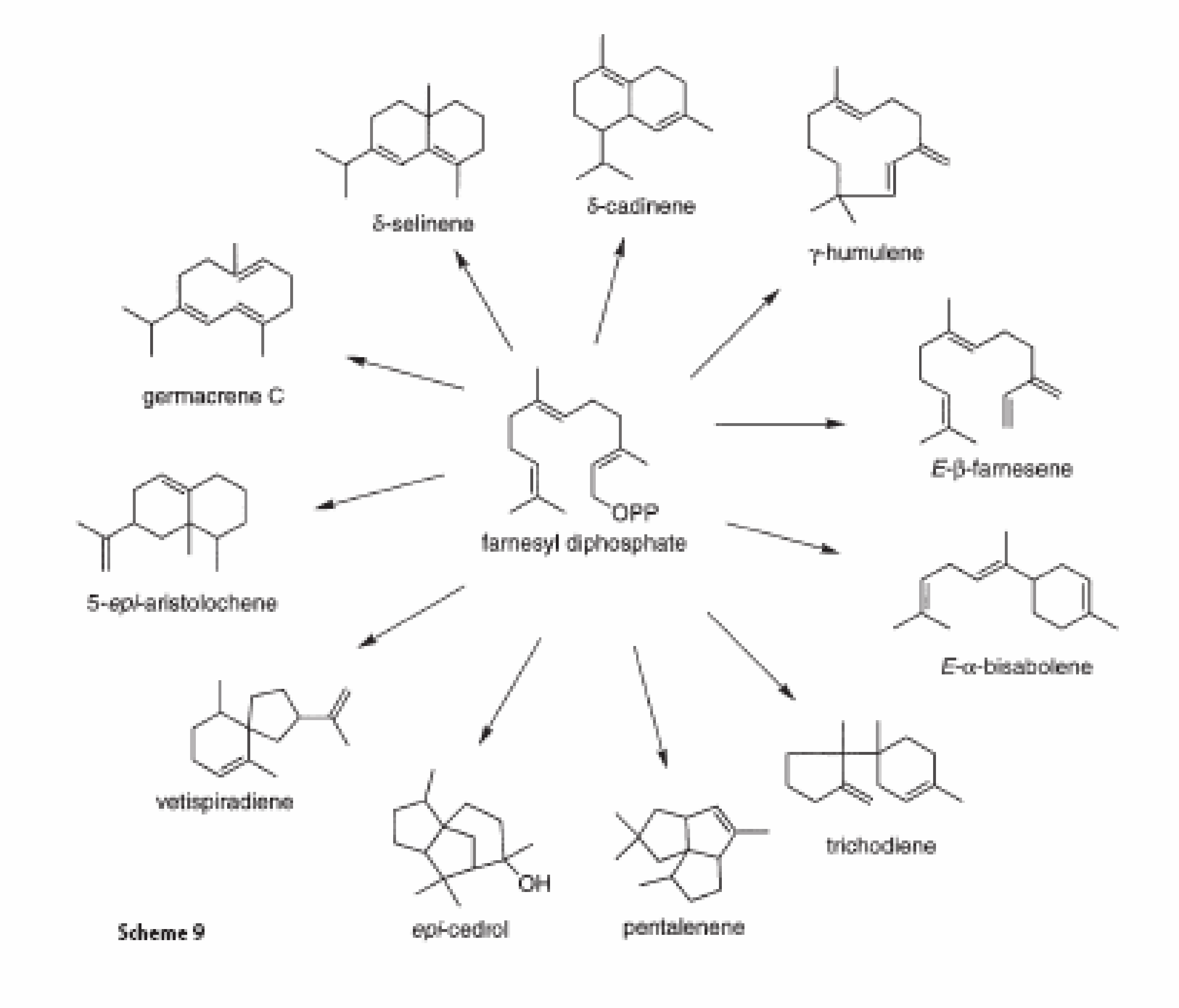
Terpene Cyclases

One enzyme…..One substrate…..Multiple products

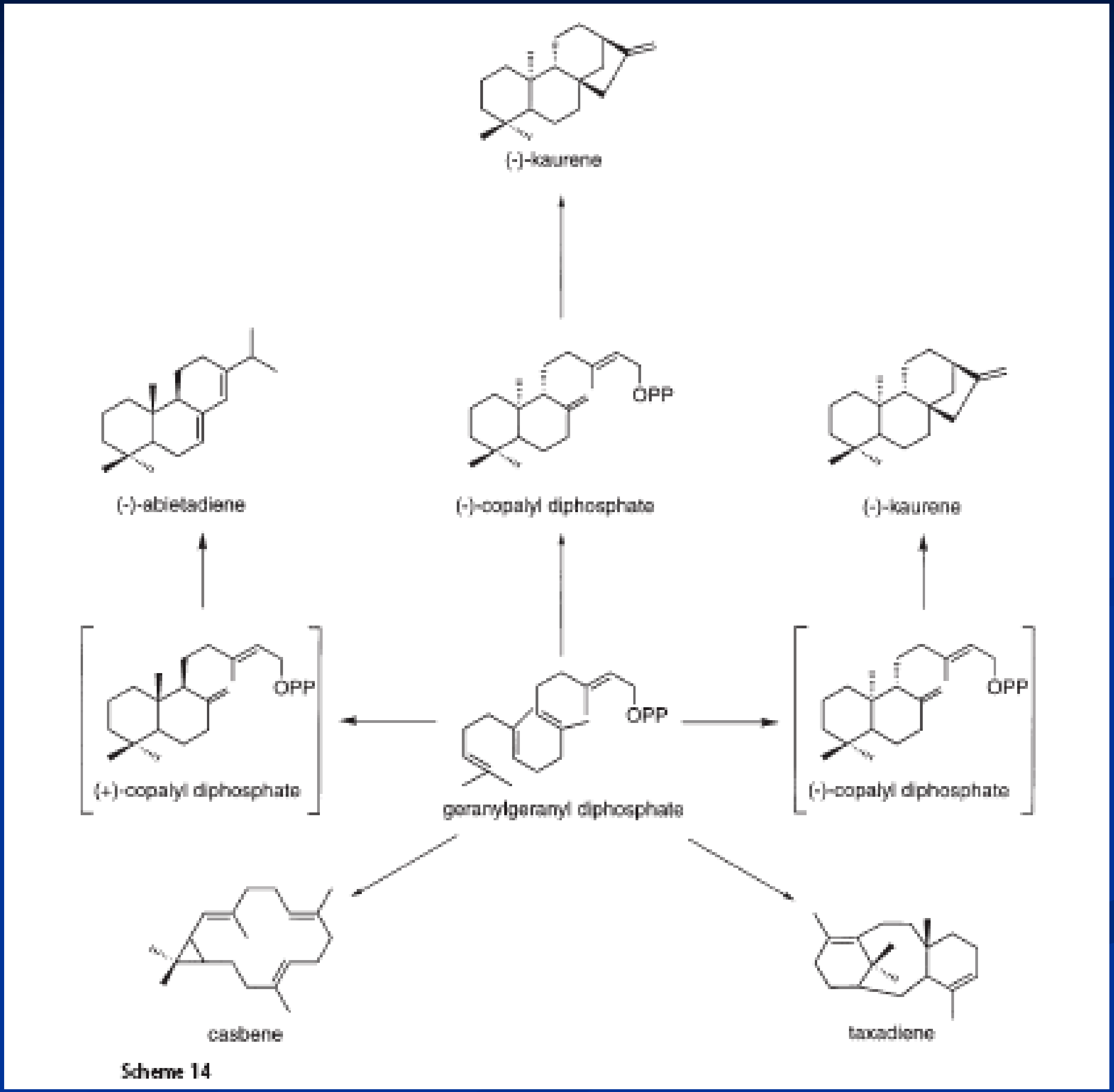
Terpene Cyclases (Mono)

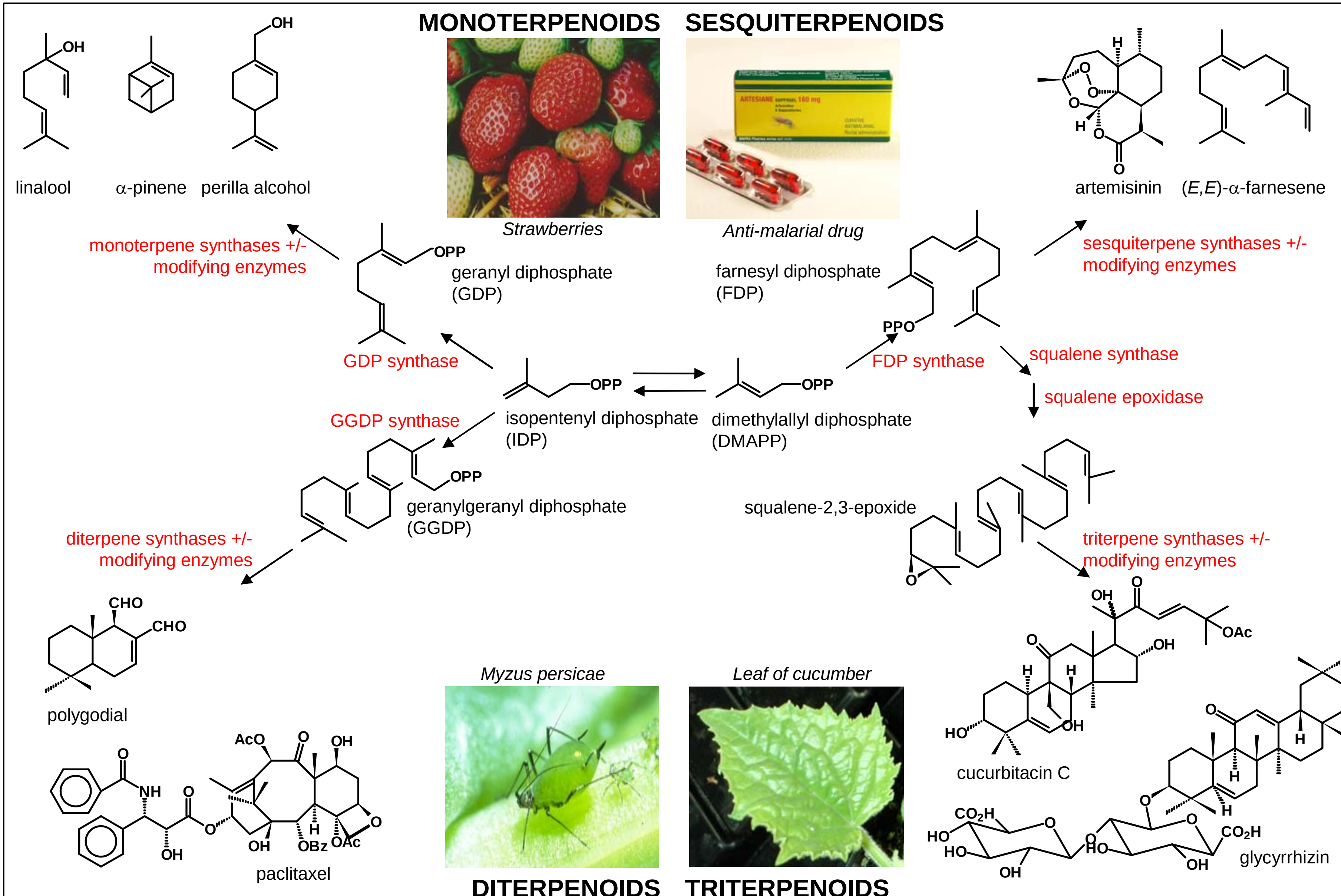


Terpene Cyclases (Sesqui-)



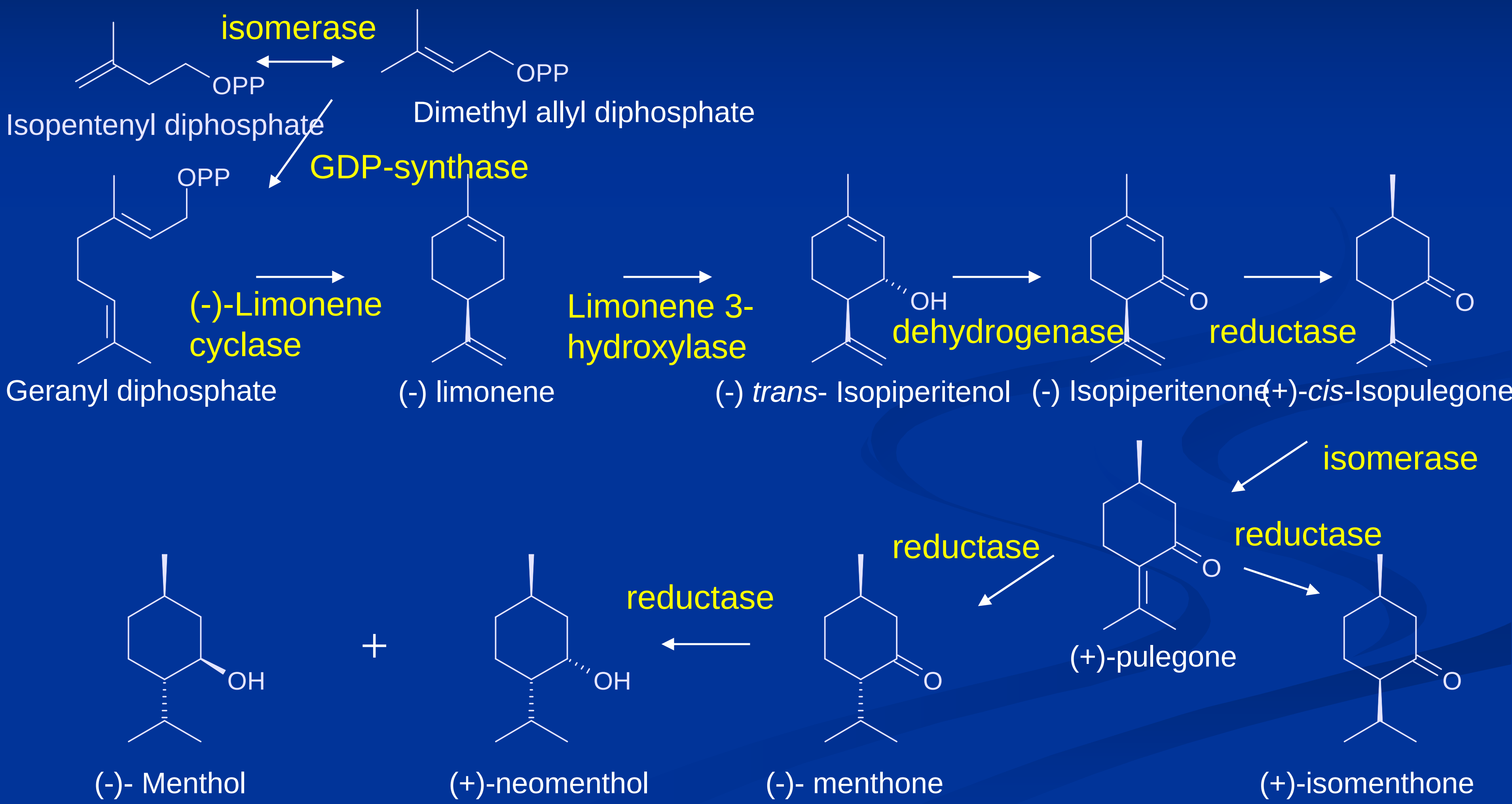
Terpene Cyclases (Diterpene)



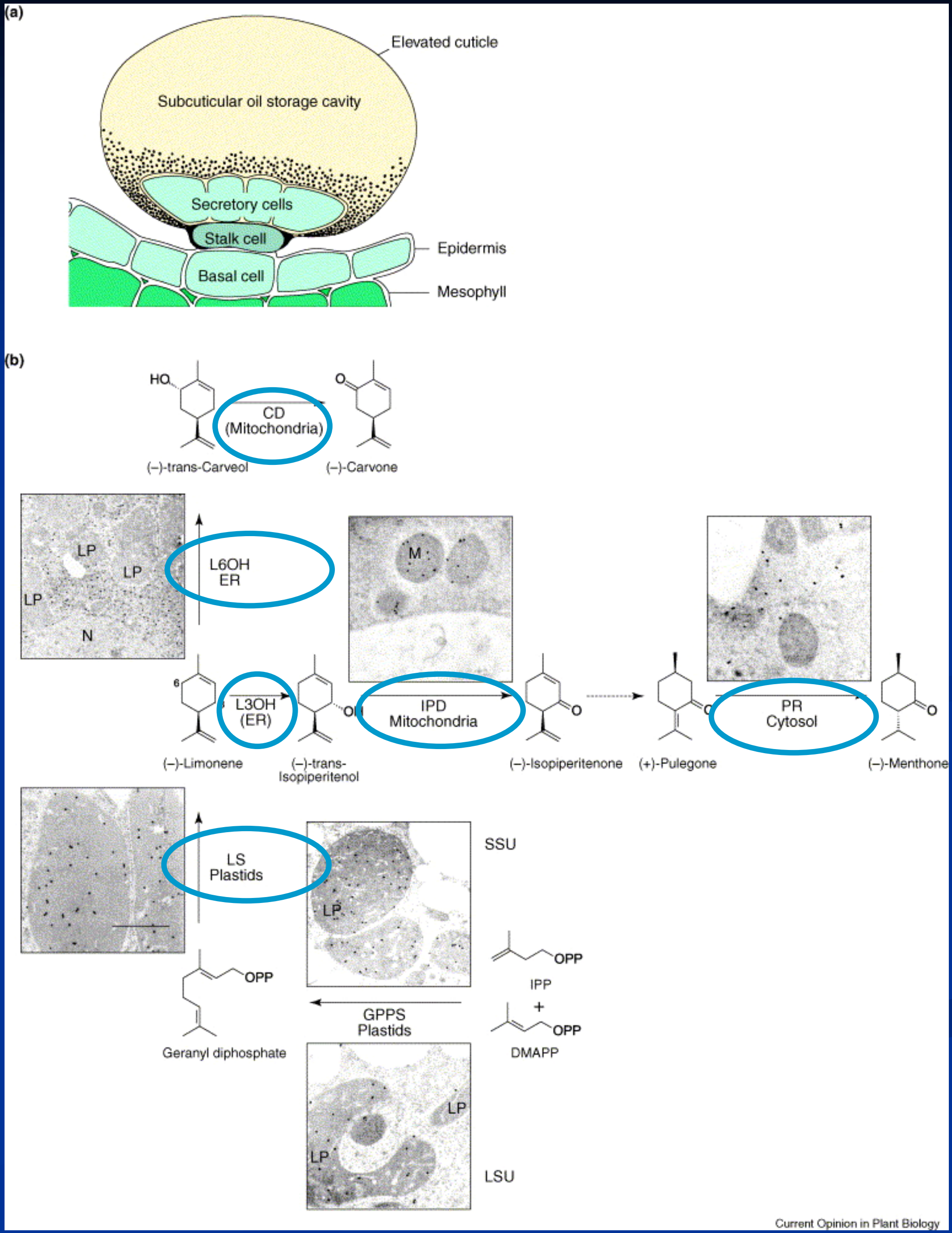


MONOTERPENOIDS
SESQUITERPENOIDS
linalool
α-pinene
perilla alcohol
Strawberries
Anti-malarial drug
artemisinin
(E,E)-α-farnesene
monoterpene synthases +/- modifying enzymes
sesquiterpene synthases +/- modifying enzymes
geranyl diphosphate (GDP)
farnesyl diphosphate (FDP)
GDP synthase
FDP synthase
squalene synthase
squalene epoxidase
isopentenyl diphosphate (IDP)
dimethylallyl diphosphate (DMAPP)
GGDP synthase
geranylgeranyl diphosphate (GGDP)
squalene-2,3-epoxide
diterpene synthases +/- modifying enzymes
triterpene synthases +/- modifying enzymes
polygodial
Myzus persicae
Leaf of cucumber
cucurbitacin C
paclitaxel
glycyrrhizin
DITERPENOIDS
TRITERPENOIDS

Modification of Monoterpene Structures



Monoterpenoid Biosynthesis in Mint



G.W. Turner and R. Croteau, *Plant Physiol* **136** (2004)

Production in Plants

Storage:

* Glandular trichomes: *Labiatae* like *Mentha*, *Cannabis*

* Cavities : Eucalypt, *Citrus*

* Resin ducts : pine trees

Production and direct emission:

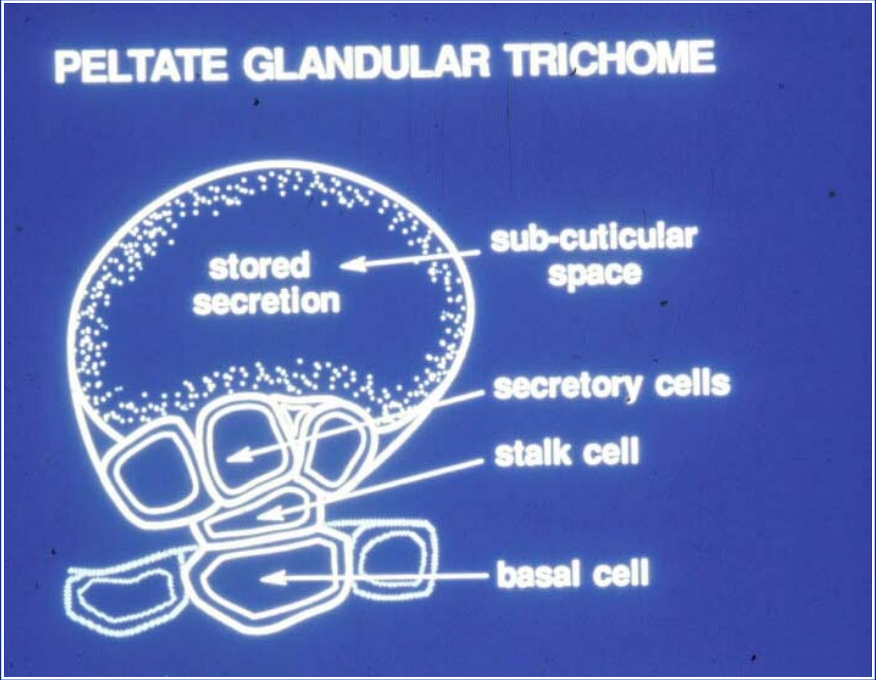
* Flowers

* Leaves

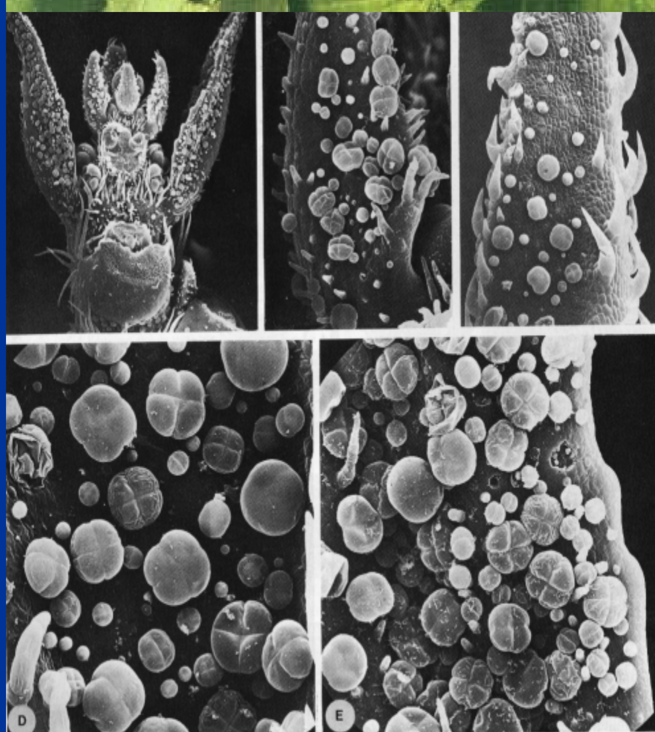
* Fruit

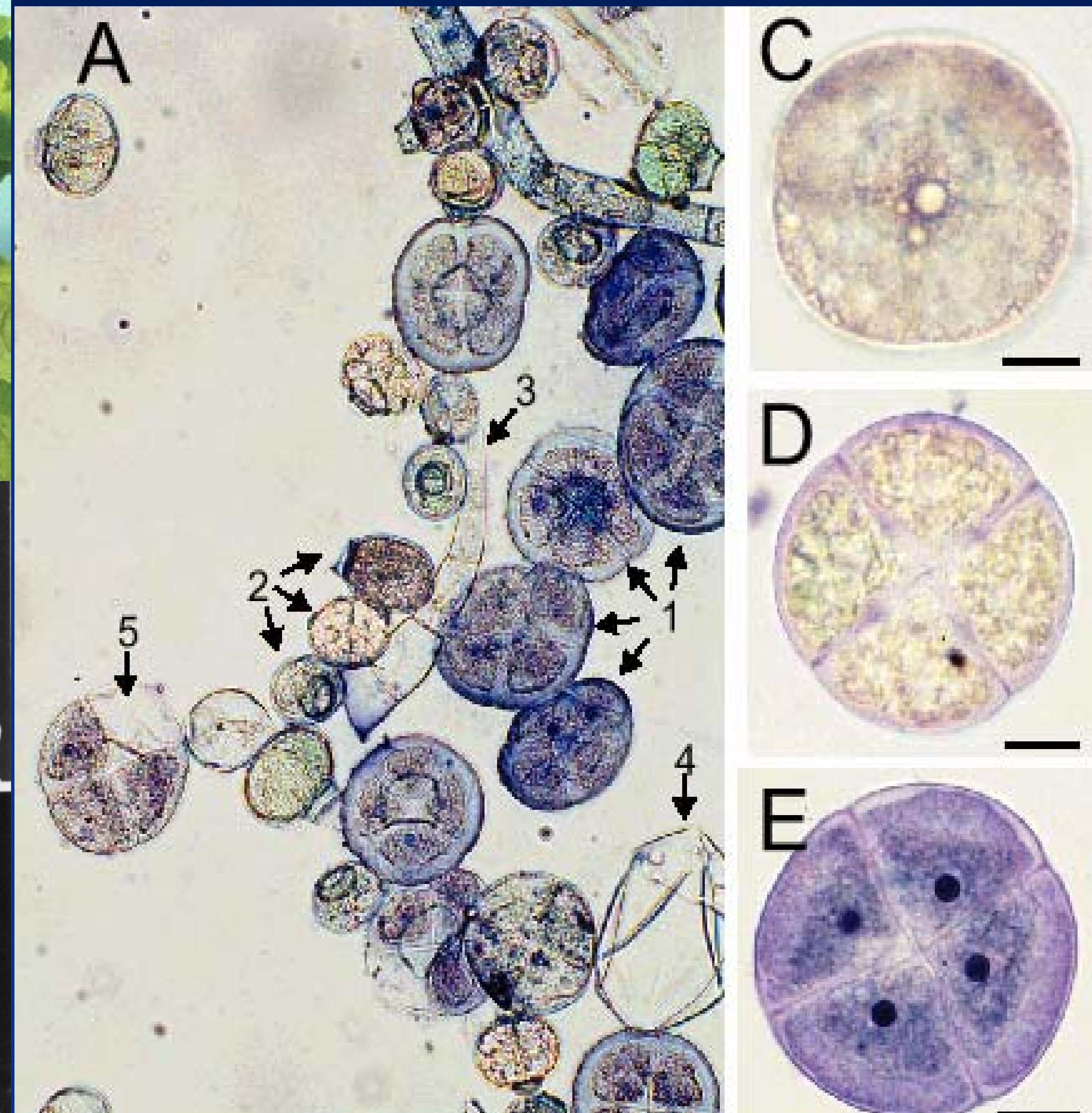
Most secondary metabolites in Basil are produced in the Peltate Glands

Peltate Glands

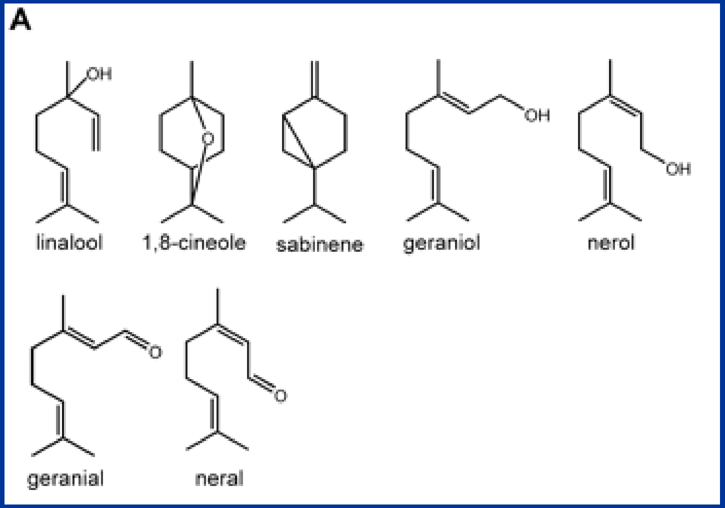


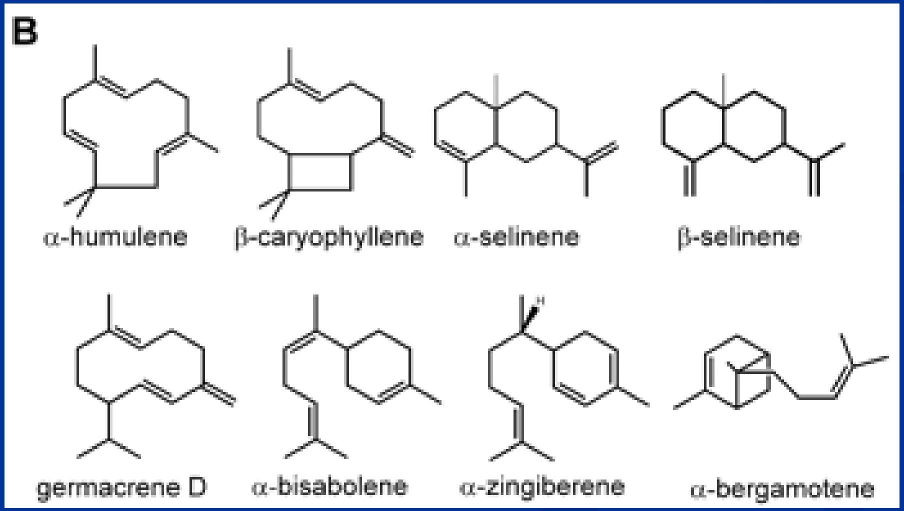
Peltate Glands Isolated From Sweet Basil





Terpenoids in Peltate Glands (Sweet Basil)

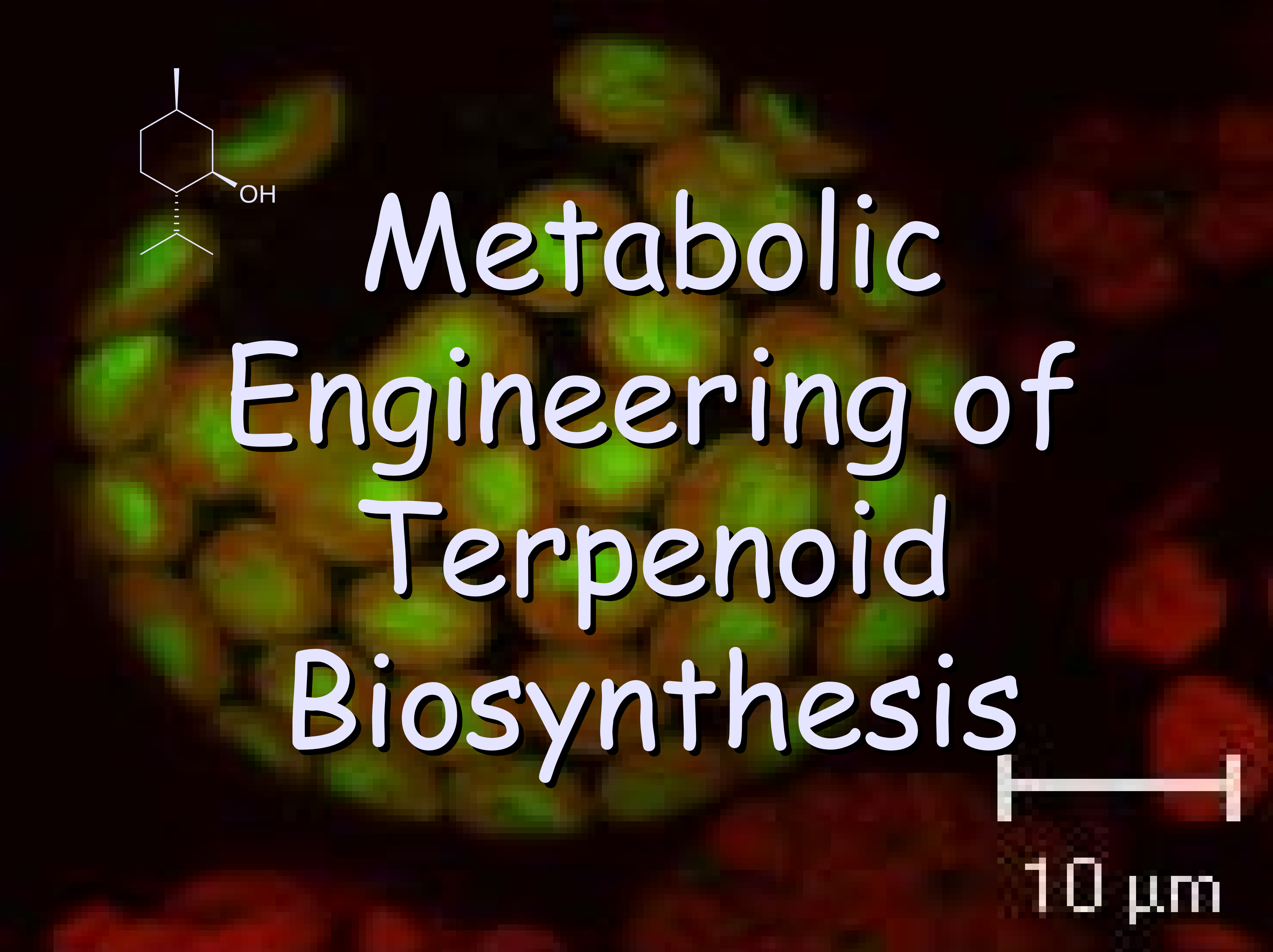




Monoterpenes

Sesquiterpenes

Metabolic Engineering of Terpenoid Biosynthesis

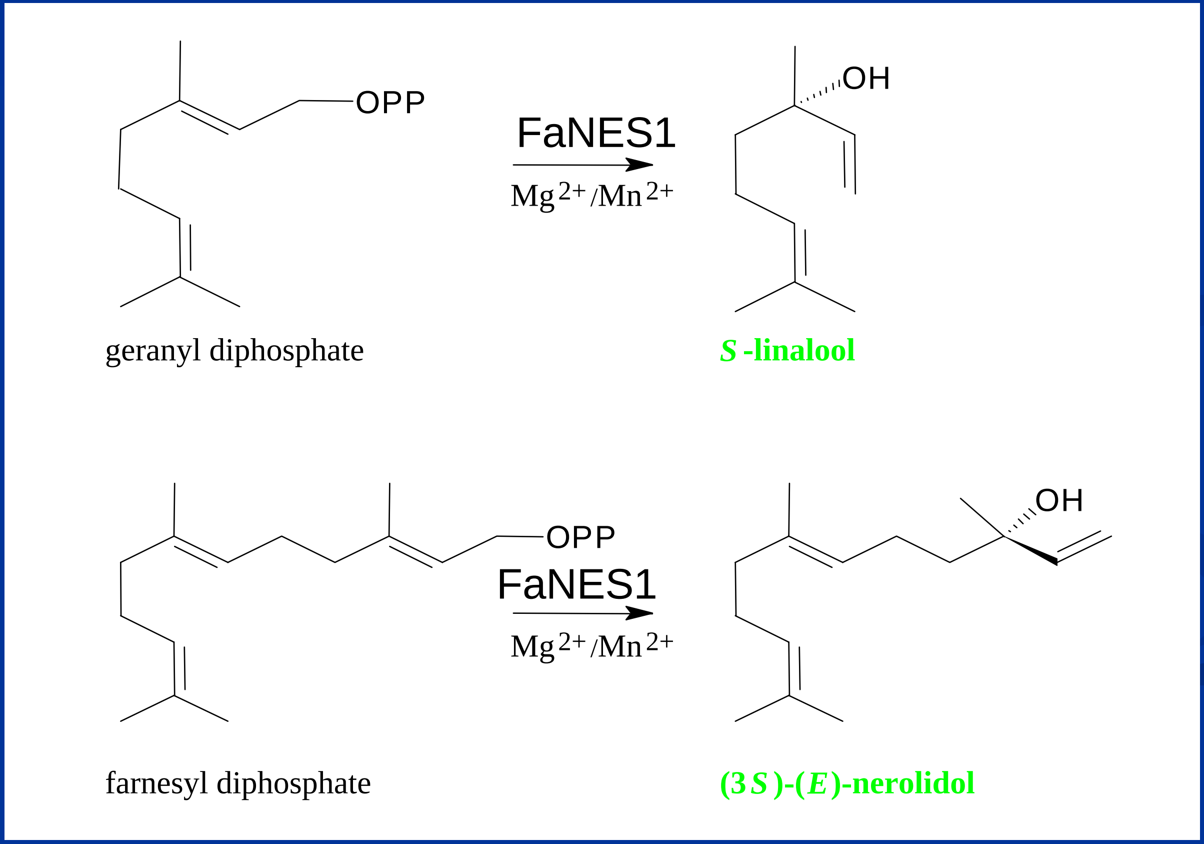


Why? Metabolic Engineering of Terpenoids in Plants

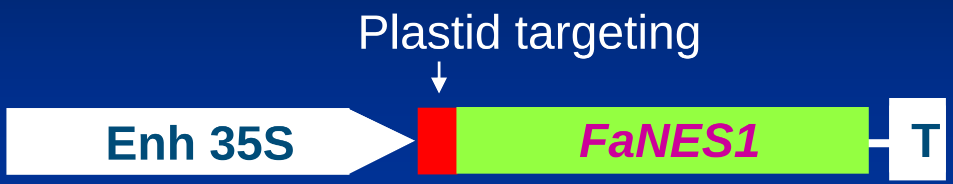
In addition, plants altered in the profile of terpenoids (and pool of precursors) make an important contribution to fundamental studies on their biosynthesis and regulation

FaNES1, a Dual Linalool / Nerolidol Synthase

Using FaNES1 allows evaluation of both mono- and sesquiterpene production



Introducing the FaNES1 Gene to Arabidopsis



Wild-type Arabidopsis leaves do not produce linalool

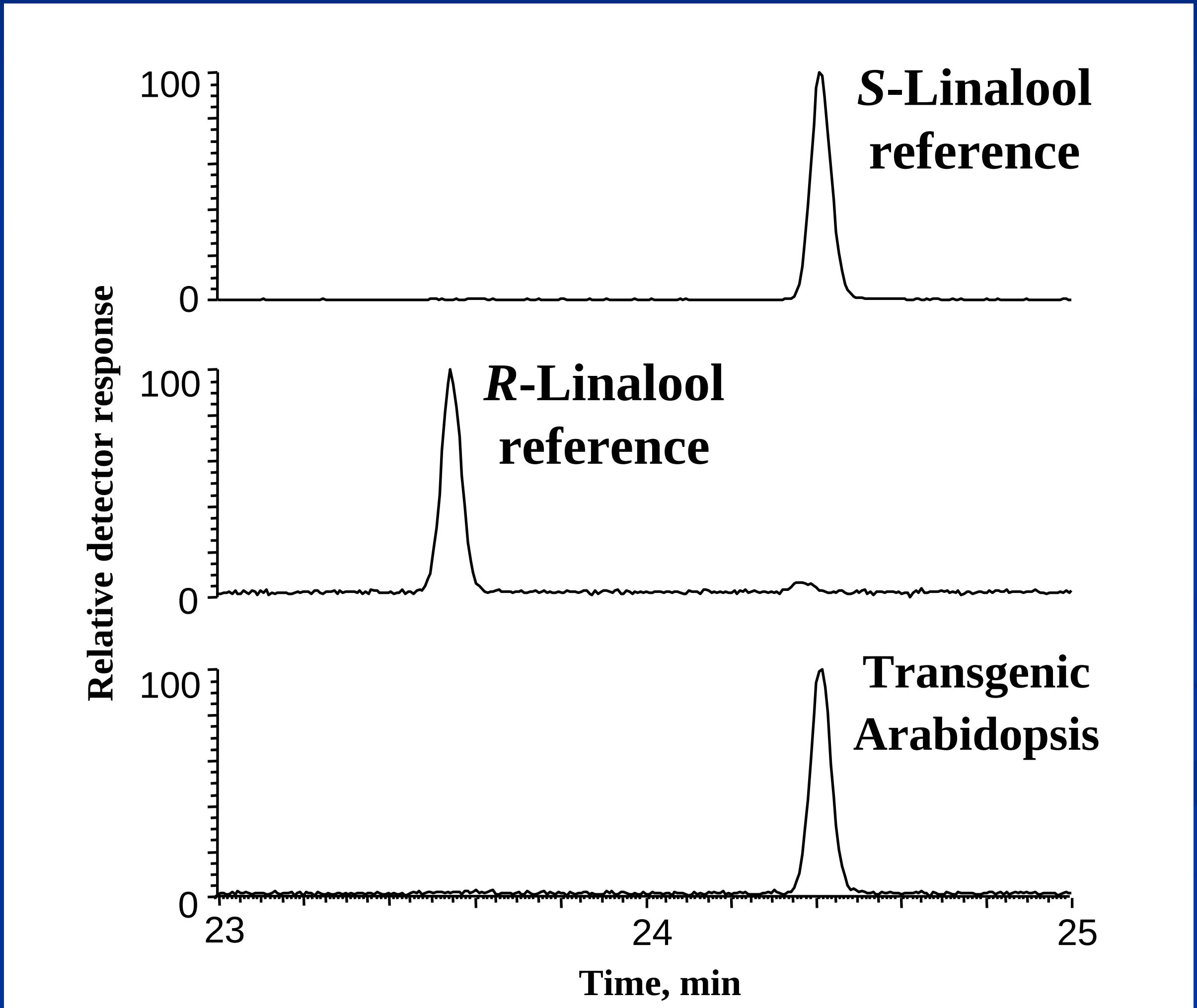
<u>Expected result</u>

monoterpenes produced in plastids:

linalool

linalool derivatives formed ?

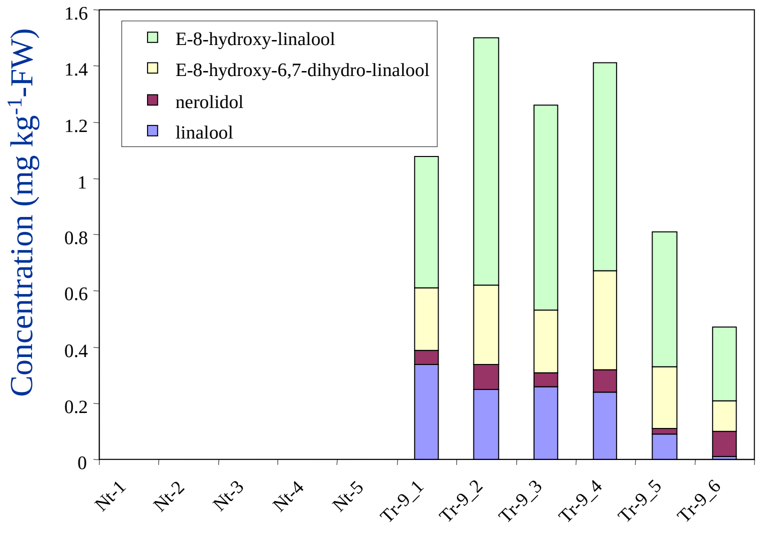
S-Linalool Formation in Leaves of Arabidopsis



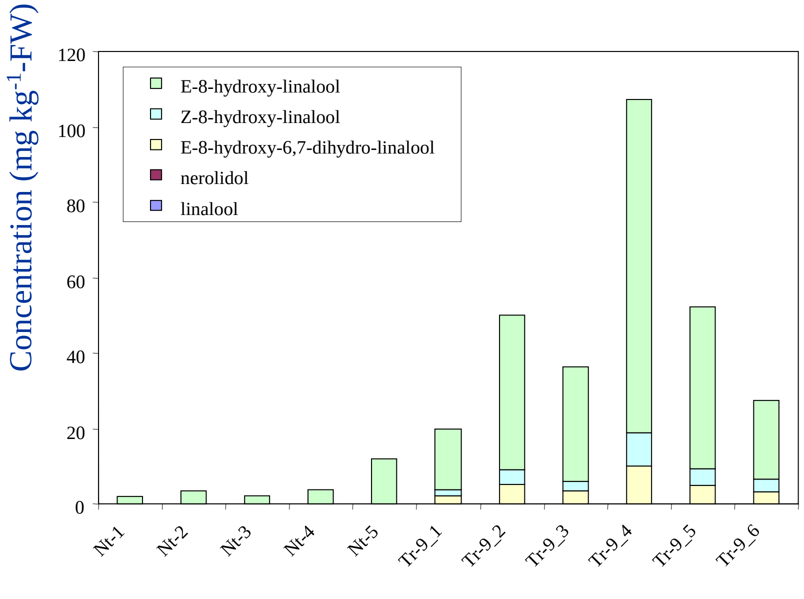
Further Modification

Free and Glycosidically Bound Terpenoids Produced by Arabidopsis

Free

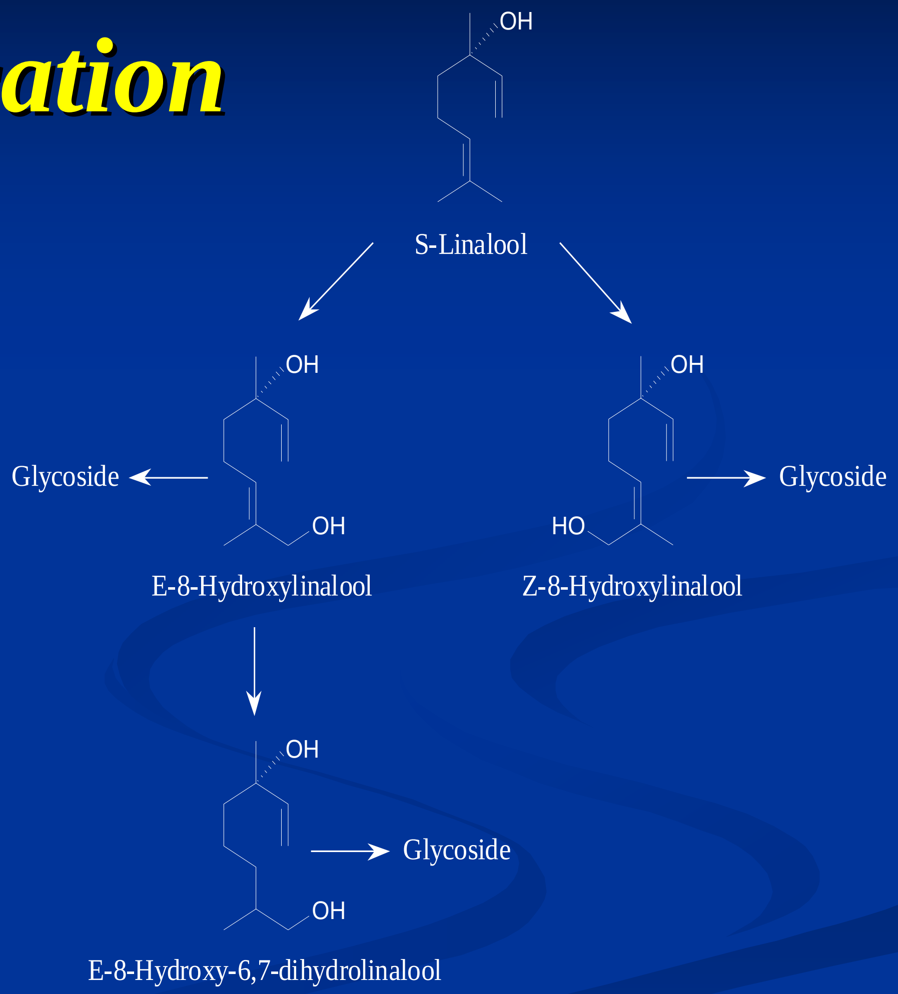


Glycosidically Bound



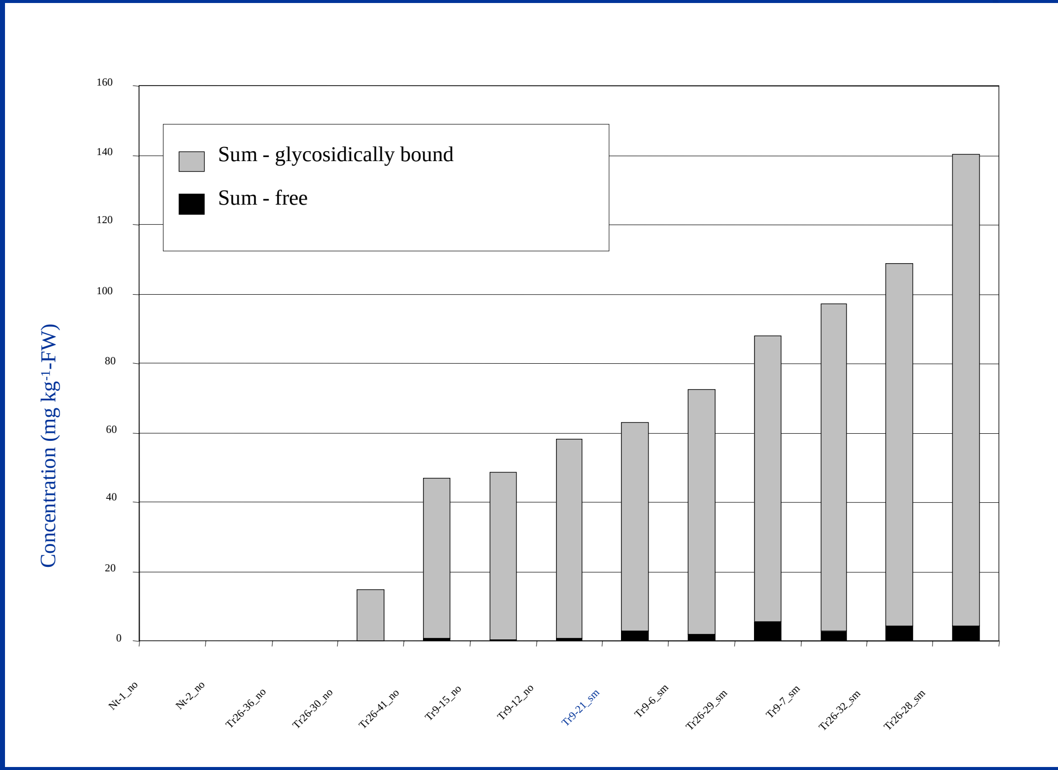
Further Modification

- Introduced product: linalool
- Modified by endogenous enzymes:
  - P450 hydroxylation (2-3)
  - Double bond reduction
  - Glycosylation (2-3)



Further Modification

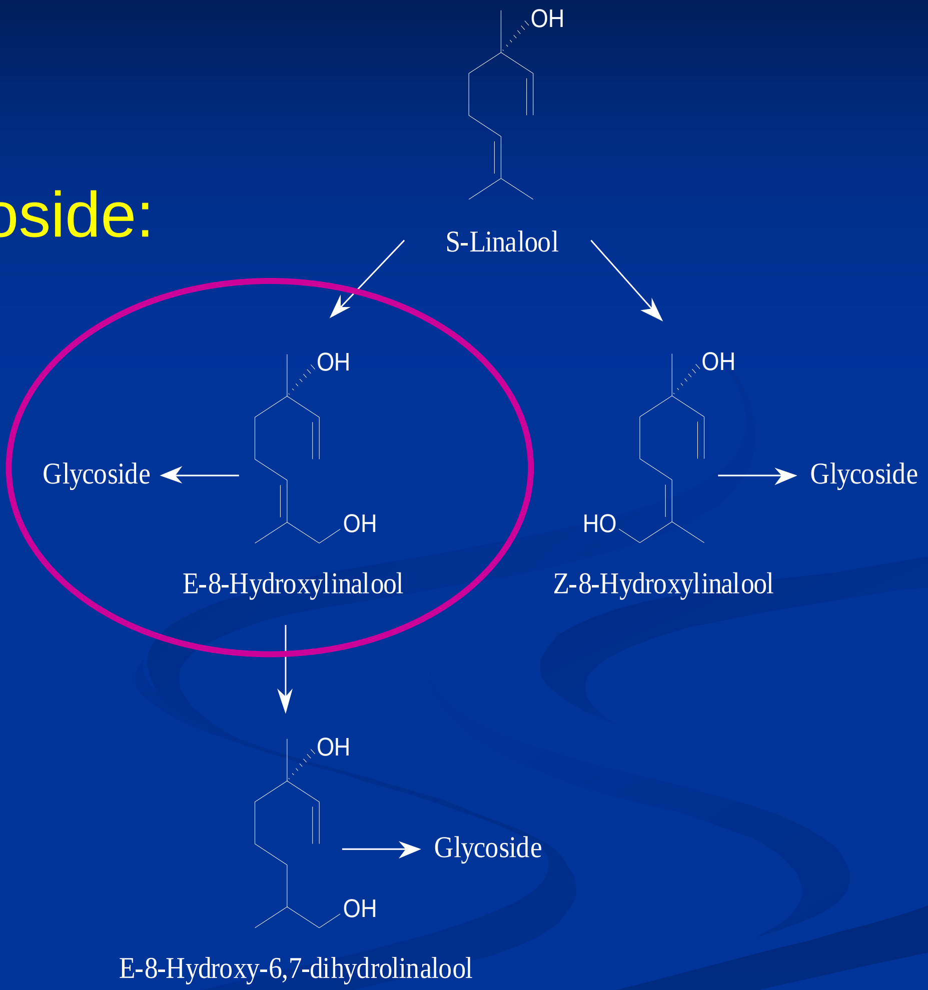
The sum of glycosylated components was in some of the transgenic lines up to 40 to 60-fold higher than the sum of the corresponding free alcohols



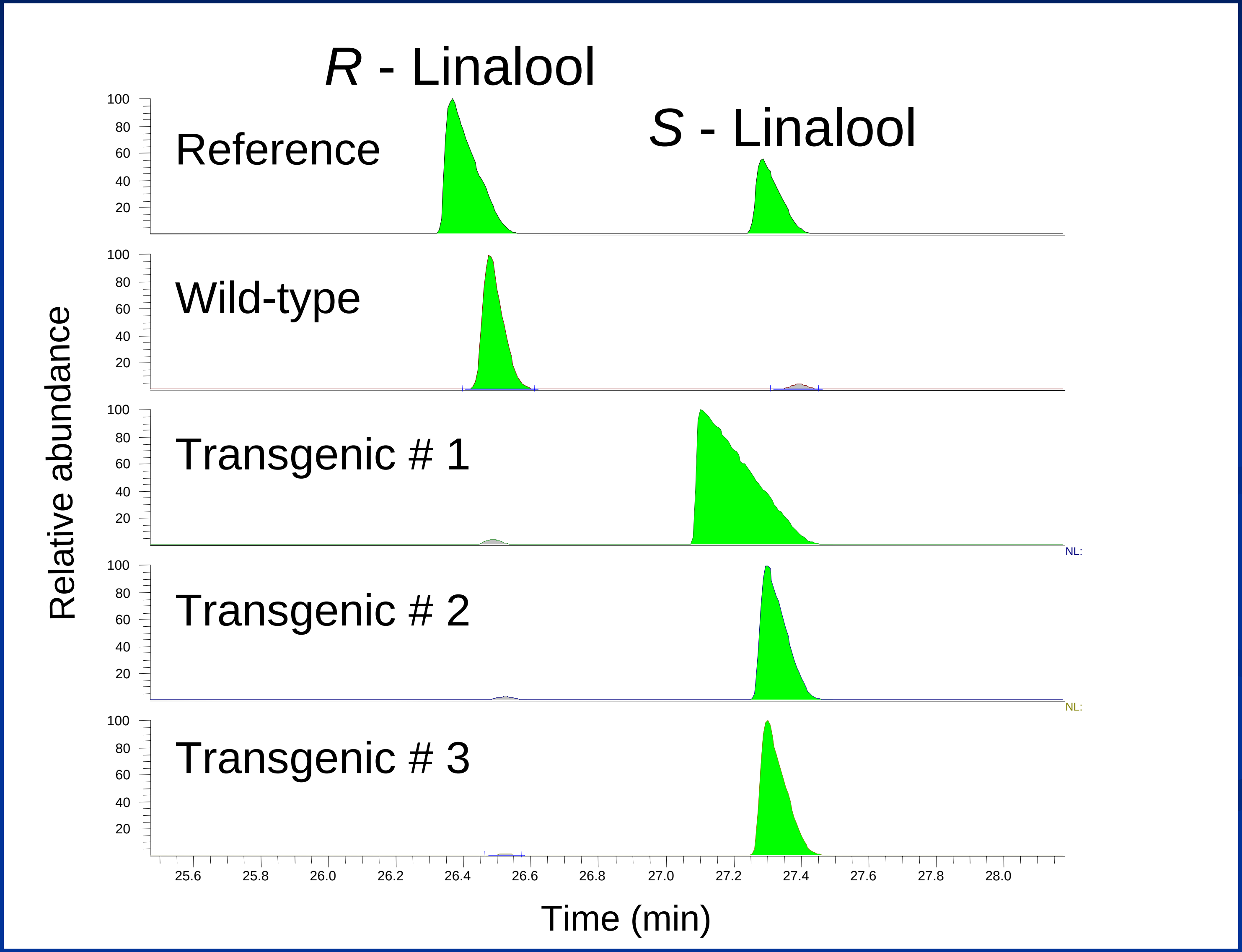
Further Modification

E-8-Hydroxylinalool and its glycoside:

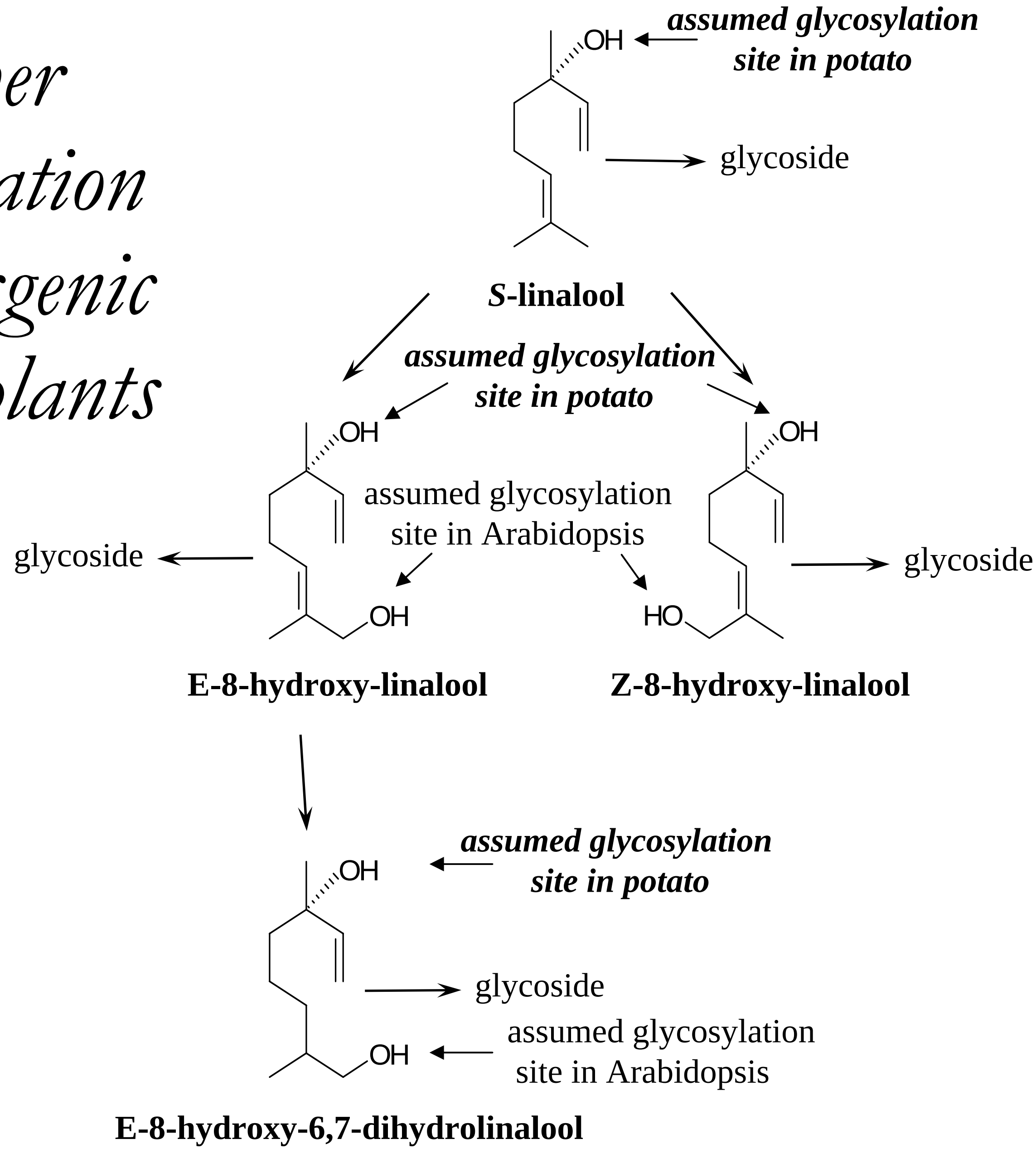
1. Produced to the highest levels in transgenic lines
2. The only component detected in leaves of wild-type plants
3. Endogenous enzymes already active and can utilize efficiently the newly introduced linalool



Potato Plants Transformed with the Same Construct



further modification in transgenic potato plants

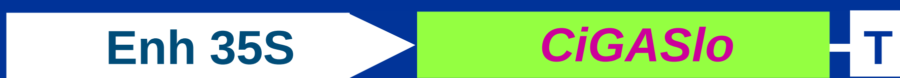


Conclusions

- In most cases the introduced metabolite could be glycosylated and/or hydroxylated
- Glycosylation could be highly efficient
- Derivatisation will be different between plant species and it will depend on the genetic make-up (i.e. activity of the endogenous enzyme)
- If the target metabolite or its derivative is already produced by the plant one should expect amplification in production but also formation of “new” metabolites (possibly metabolites that could not be detected earlier due to sensitivity of instruments)

Engineering Sesquiterpenes in Arabidopsis

Introducing the CiGASlo Gene to Arabidopsis

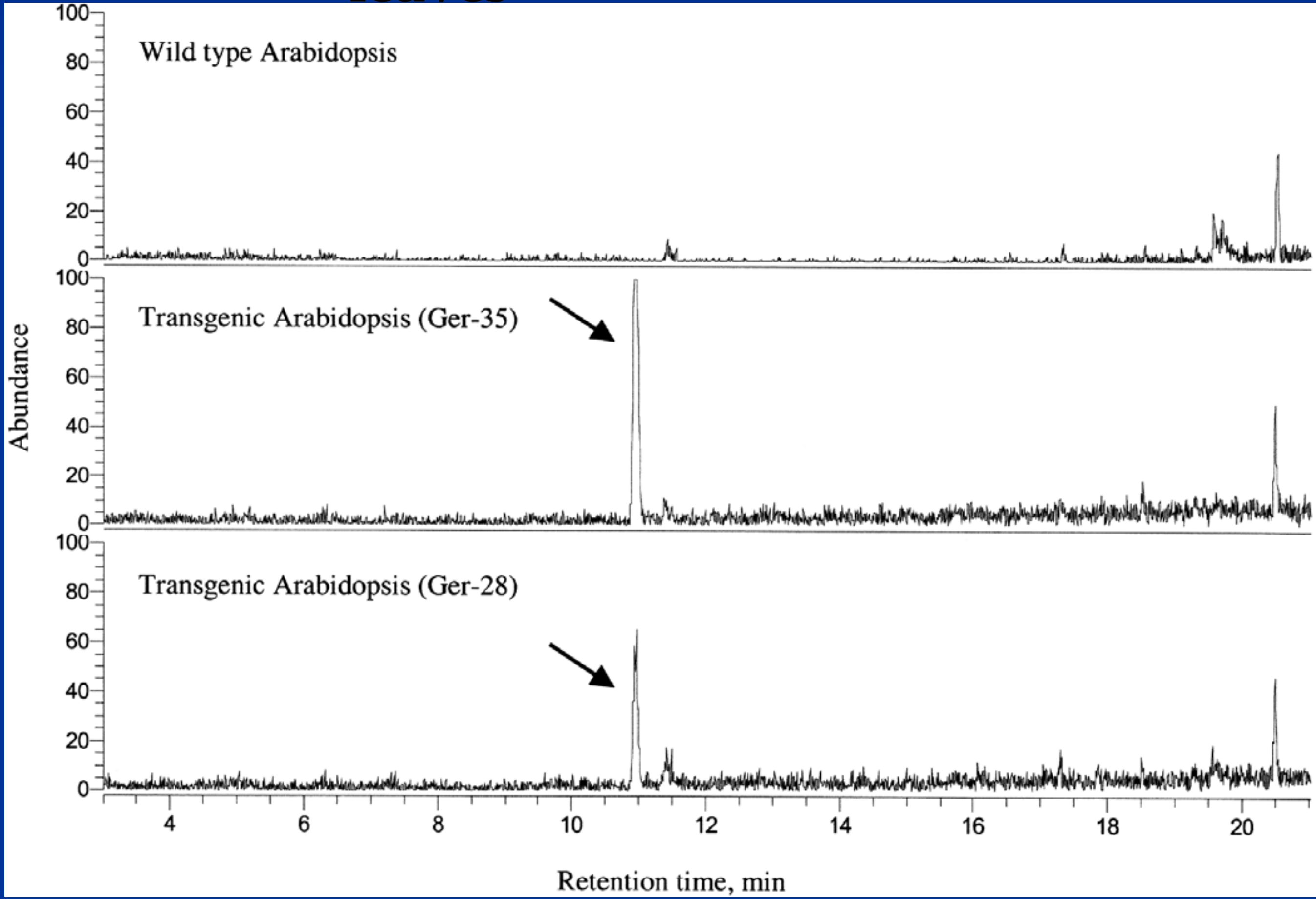


Cytosolic production of a Germacrene A synthase from Chicory

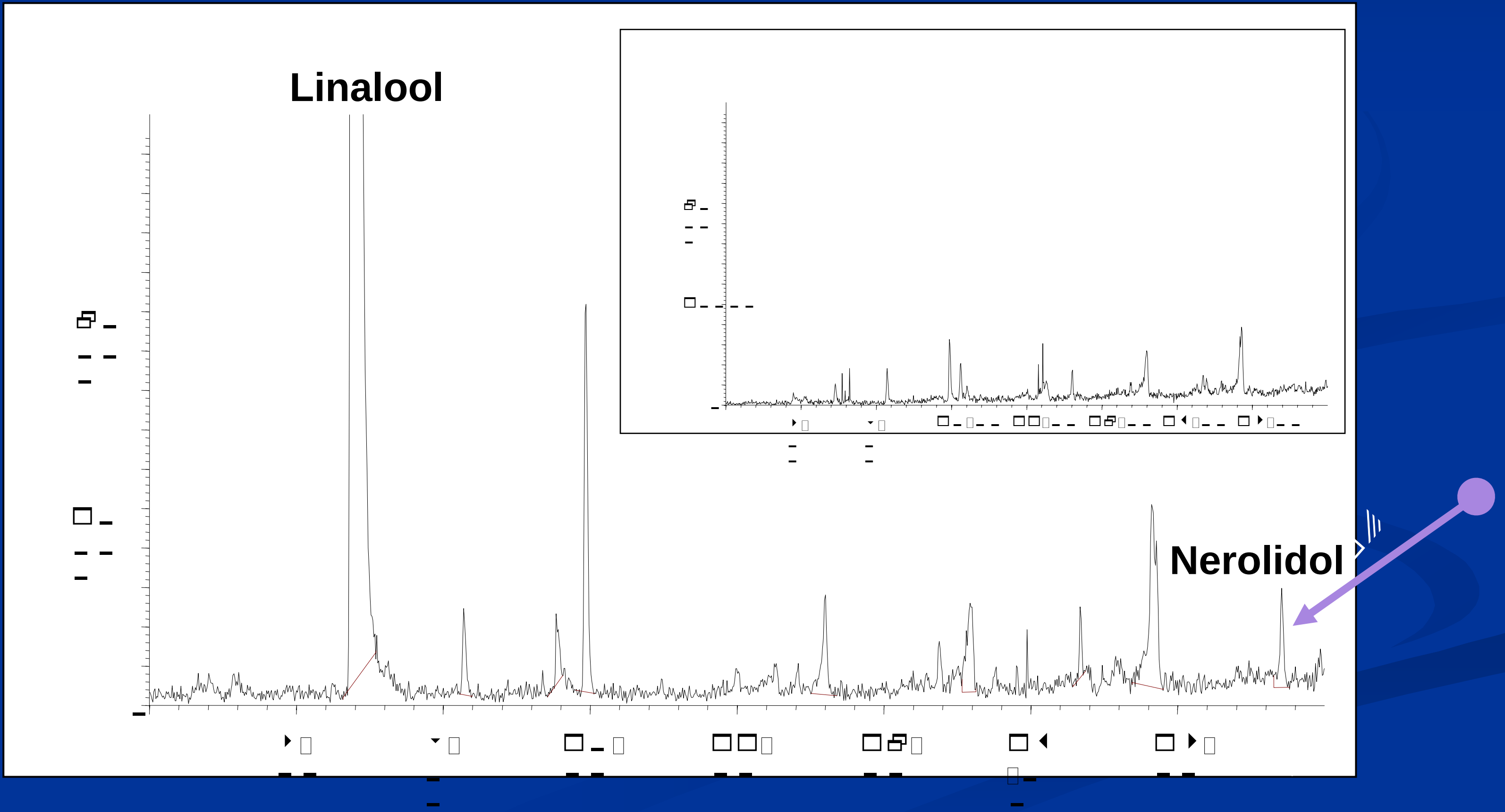
Wild-type Arabidopsis leaves do not produce Germacrene A

Engineering Sesquiterpenes in Arabidopsis

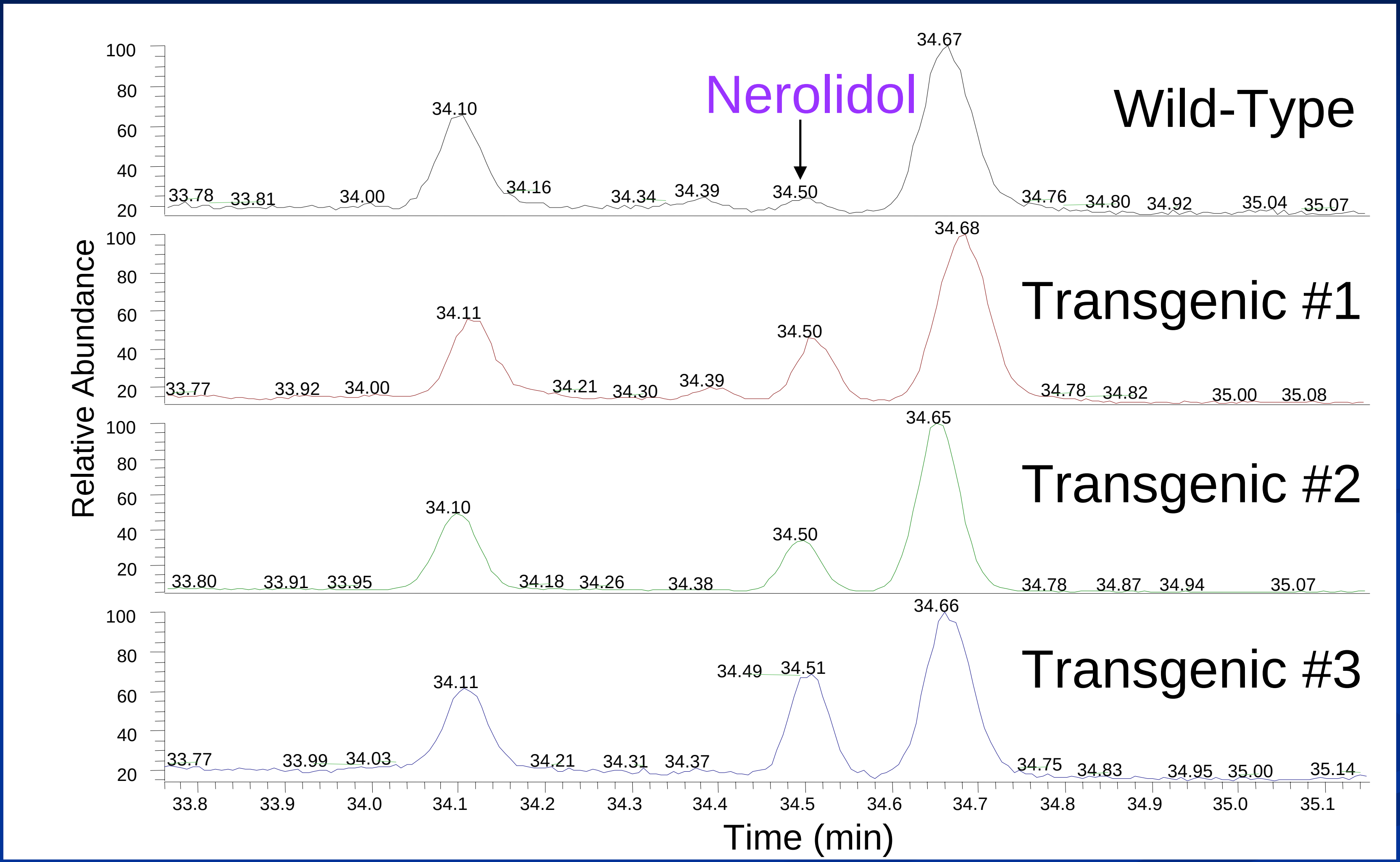
Traces of the thermal rearrangement product of Germacrene A (de Kraker et al., 1998) were detected in leaves



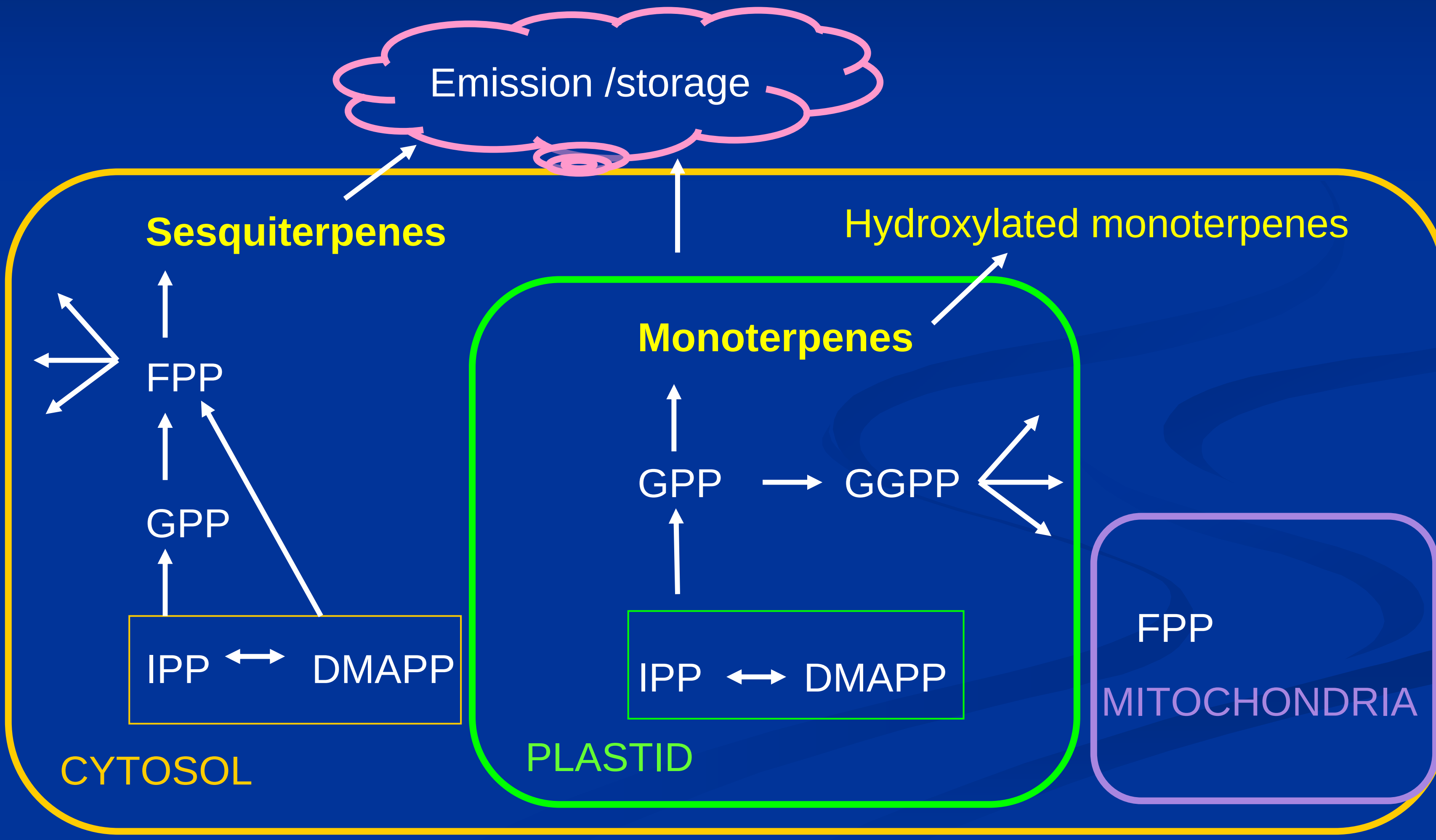
Unexpected: Sesquiterpene Production with Plastidic Targeting of FaNES1



Nerolidol is Produced at Low Level Also in Potato

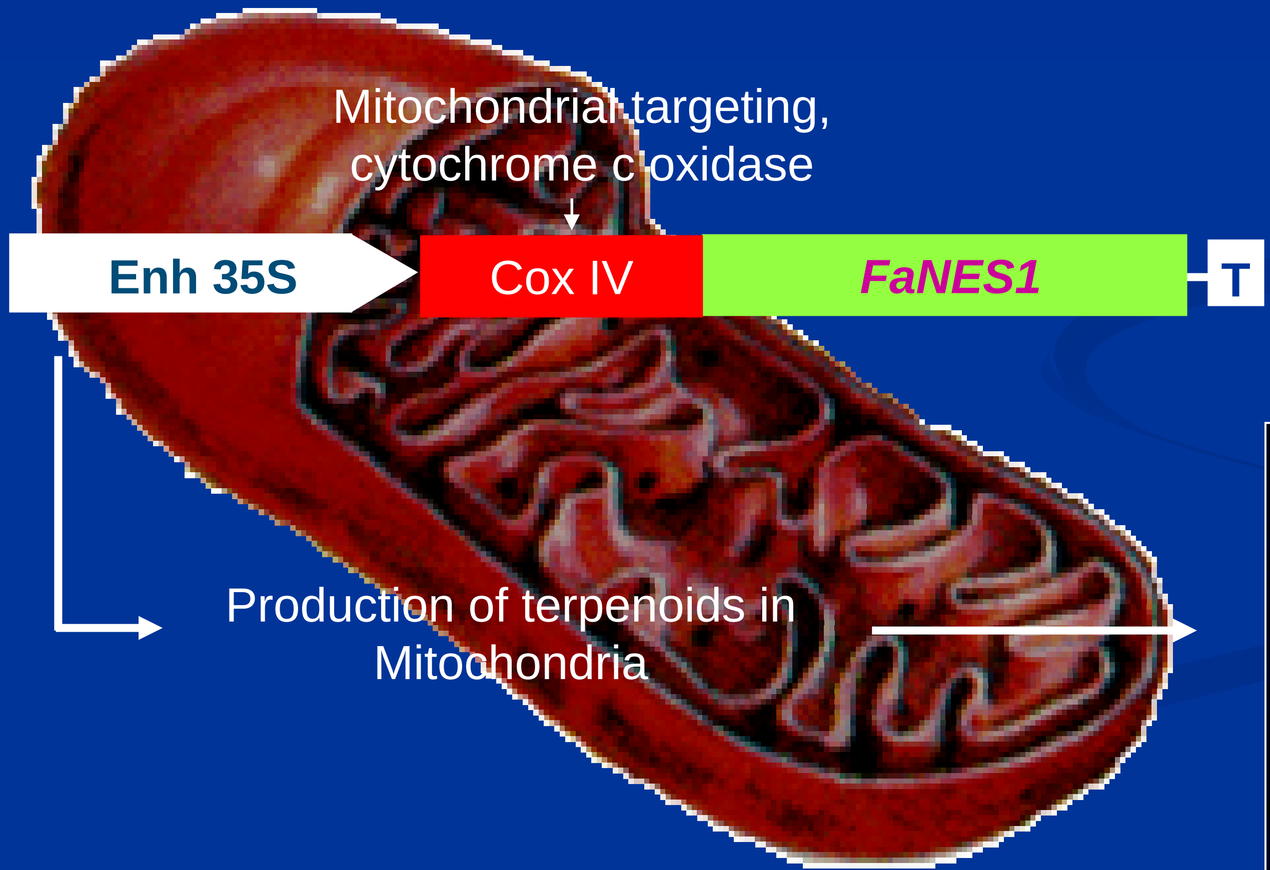


Availability of Precursor Pools?

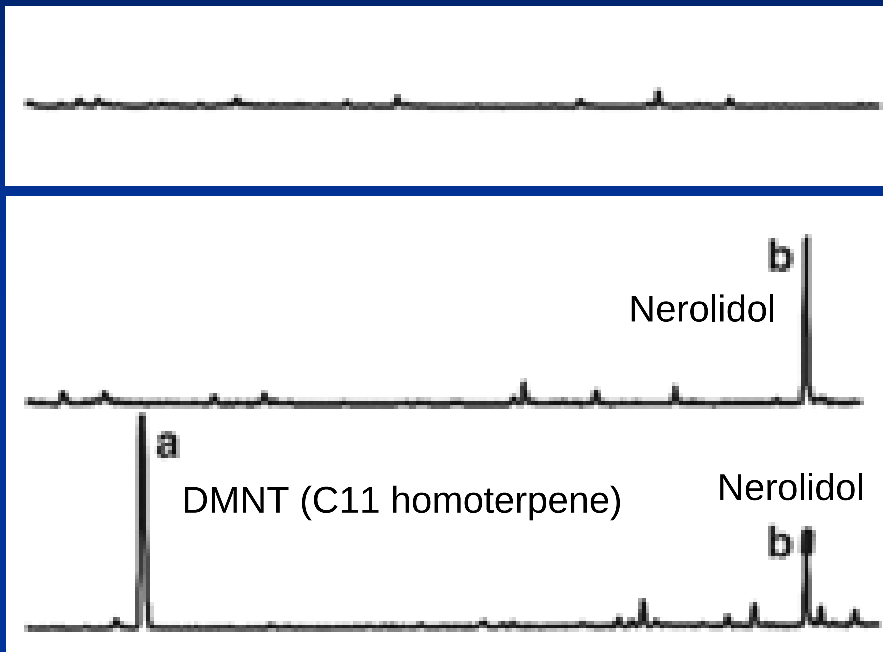


Engineering Sesquiterpenes in Arabidopsis

Introducing the FaNES1 fused to a Mitochondrial targeting signal to Arabidopsis



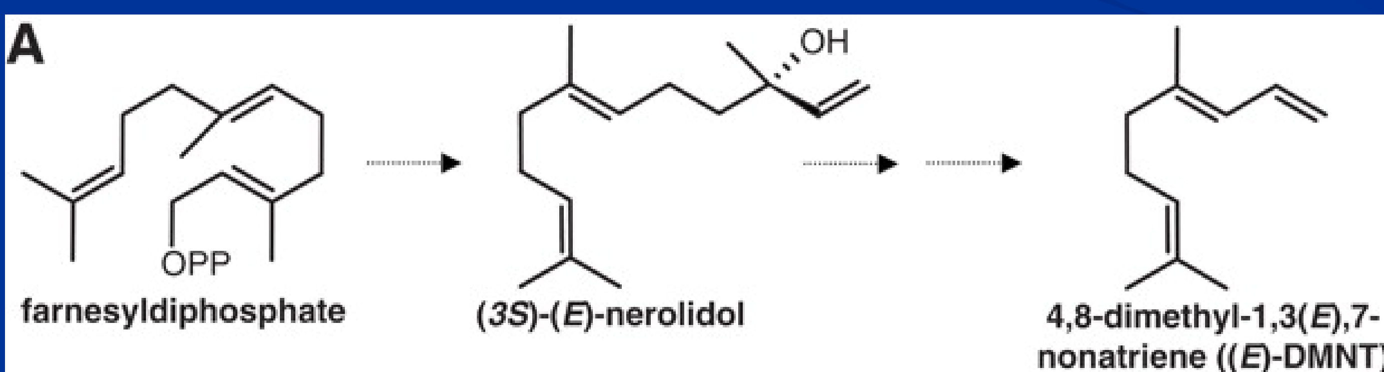
Engineering Sesquiterpenes in Arabidopsis



Undamaged
Wild-type

Transgenic undamaged
(only nerolidol)

Transgenic Undamaged
(nerolidol and DMNT)



C15 sesquiterpene

C11 homoterpene

Conclusions

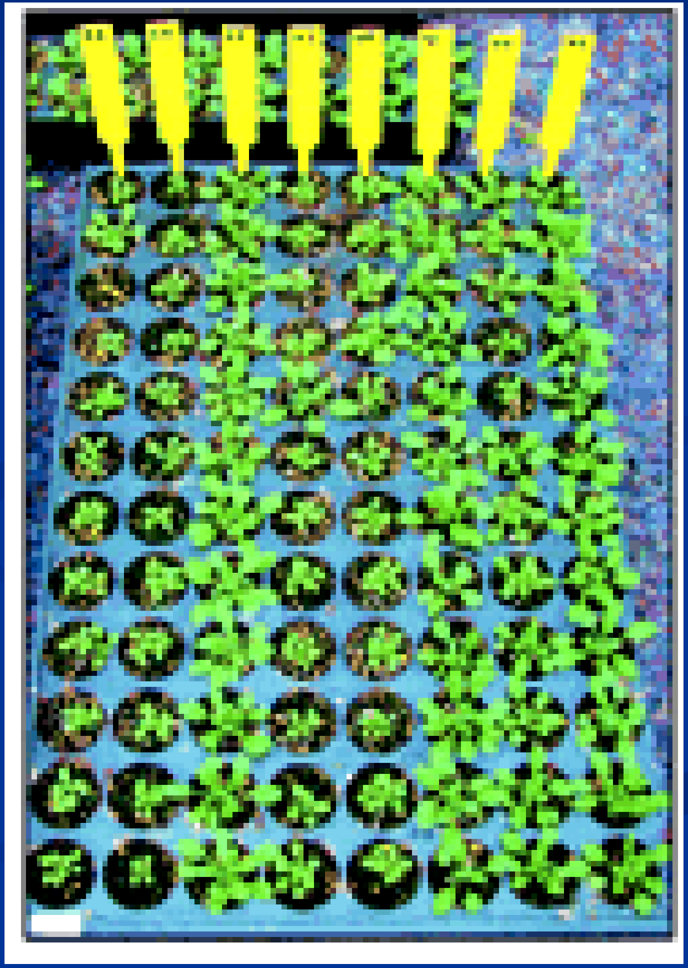
- Engineering sesquiterpene production in the cytosol compared to plastidic production of monoterpenes seems more difficult

- Targeting different cell compartments for engineering terpenoids might be a valuable tool

- Further modification of introduced terpenoid might be different in <u>each cell compartment</u>

The Cost of Terpenoid Production in Plants

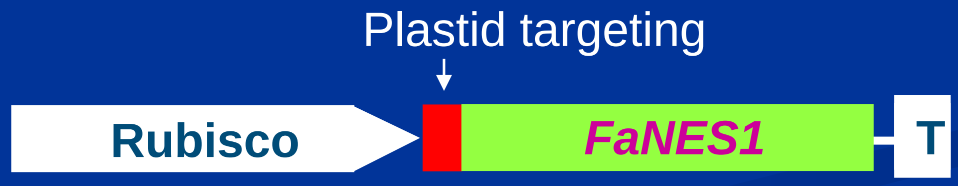
Growth retardation with constitutive over-expression of *FaNES1* in Arabidopsis



The Cost of Terpenoid Production in Plants

Constitutive over-expression of *FaNES1* in potato controlled by the Rubisco promoter

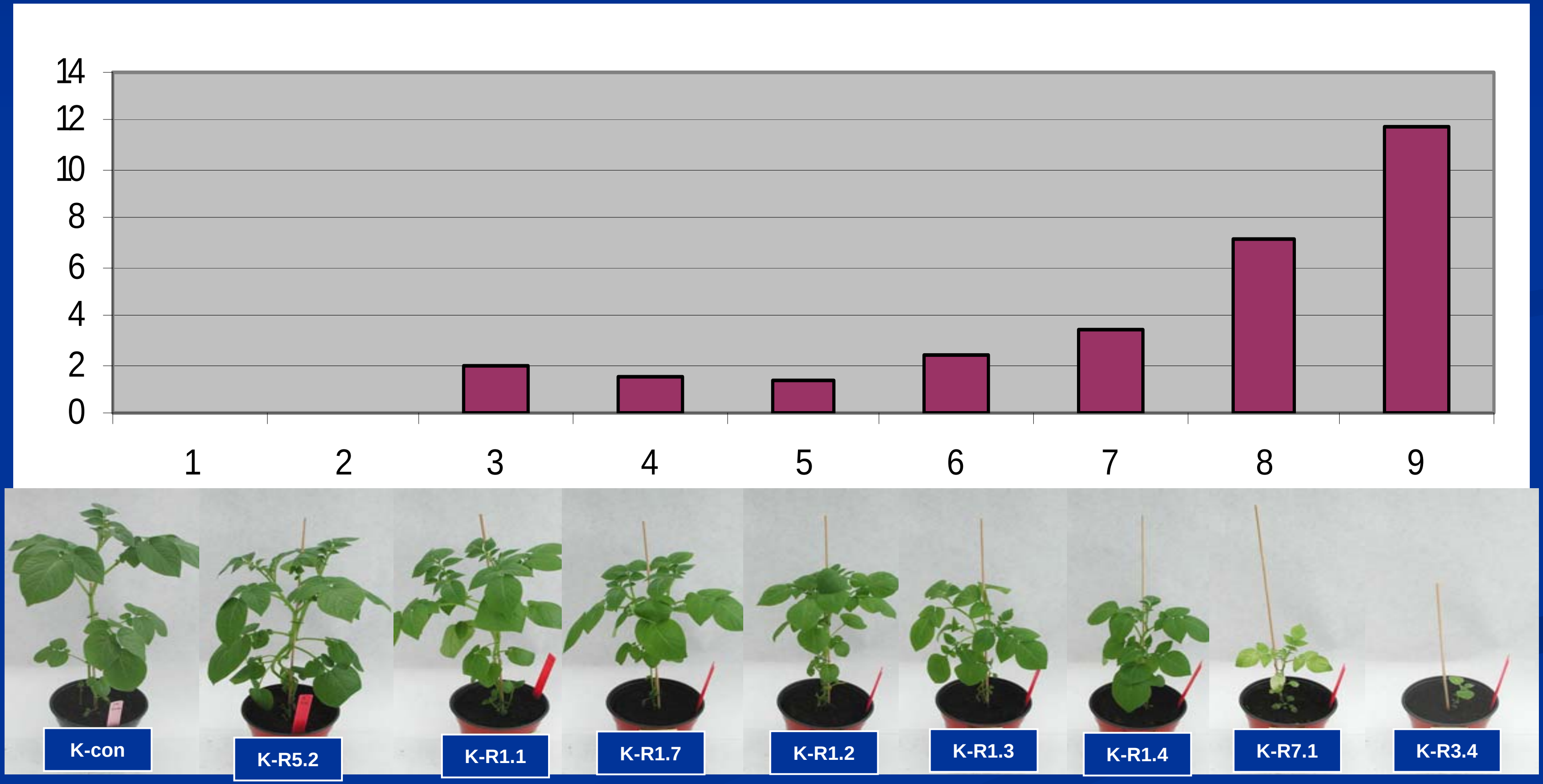
The Rubisco promoter is x 10 fold stronger than the 35S promoter



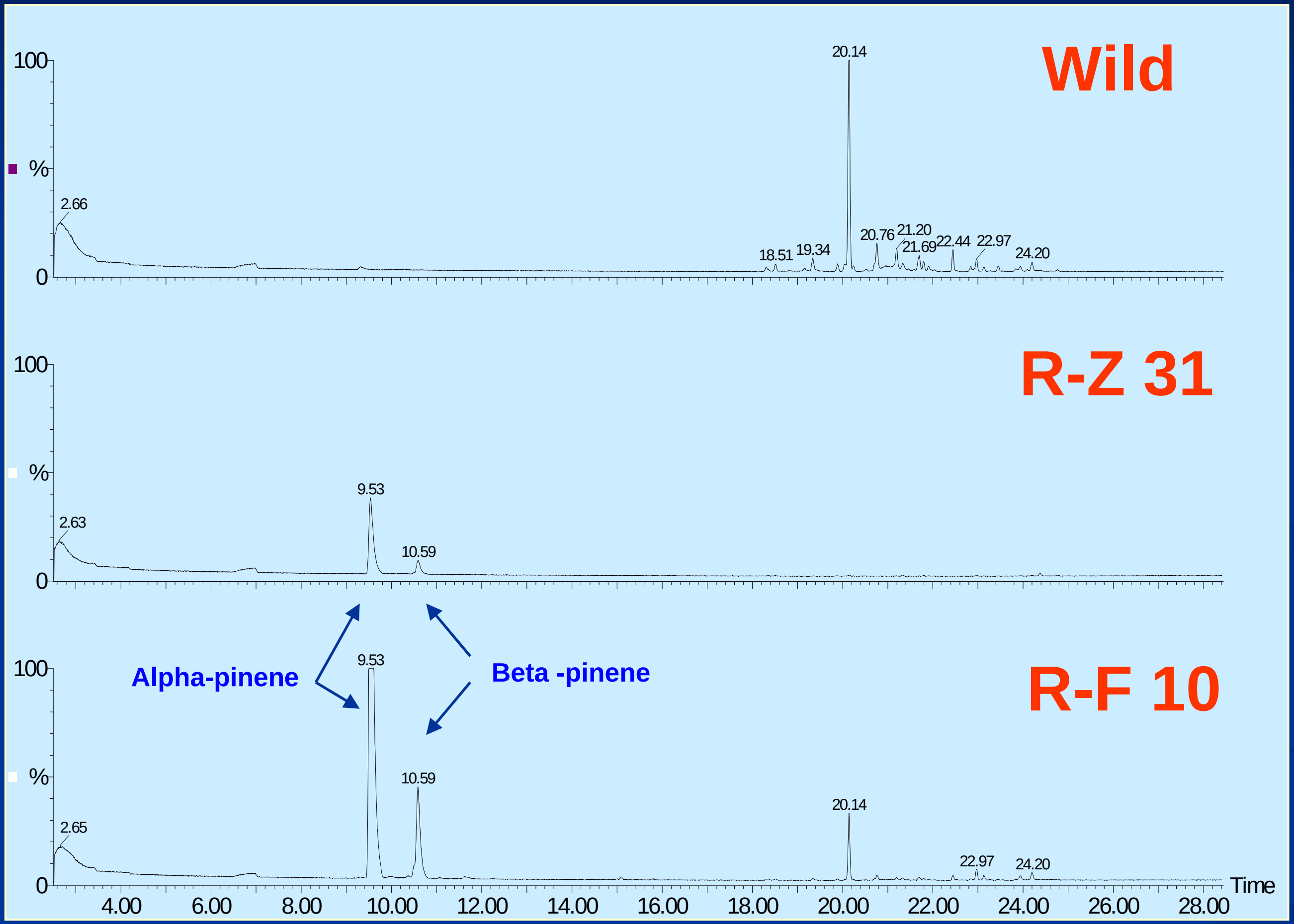
The Cost of Terpenoid Production in Plants

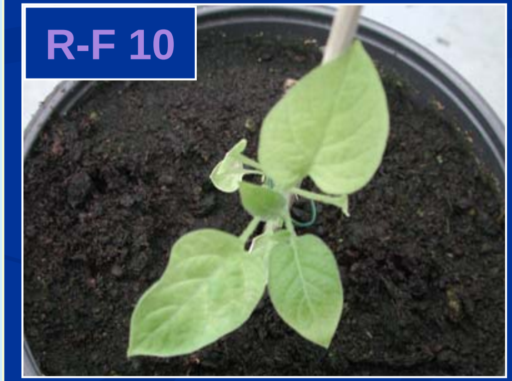
Bleaching and growth retardation with constitutive over-expression of *FaNES1* in potato

Effect of Linalool Expression on Potato Phenotype



Potato Plants Overexpressing FvPINS



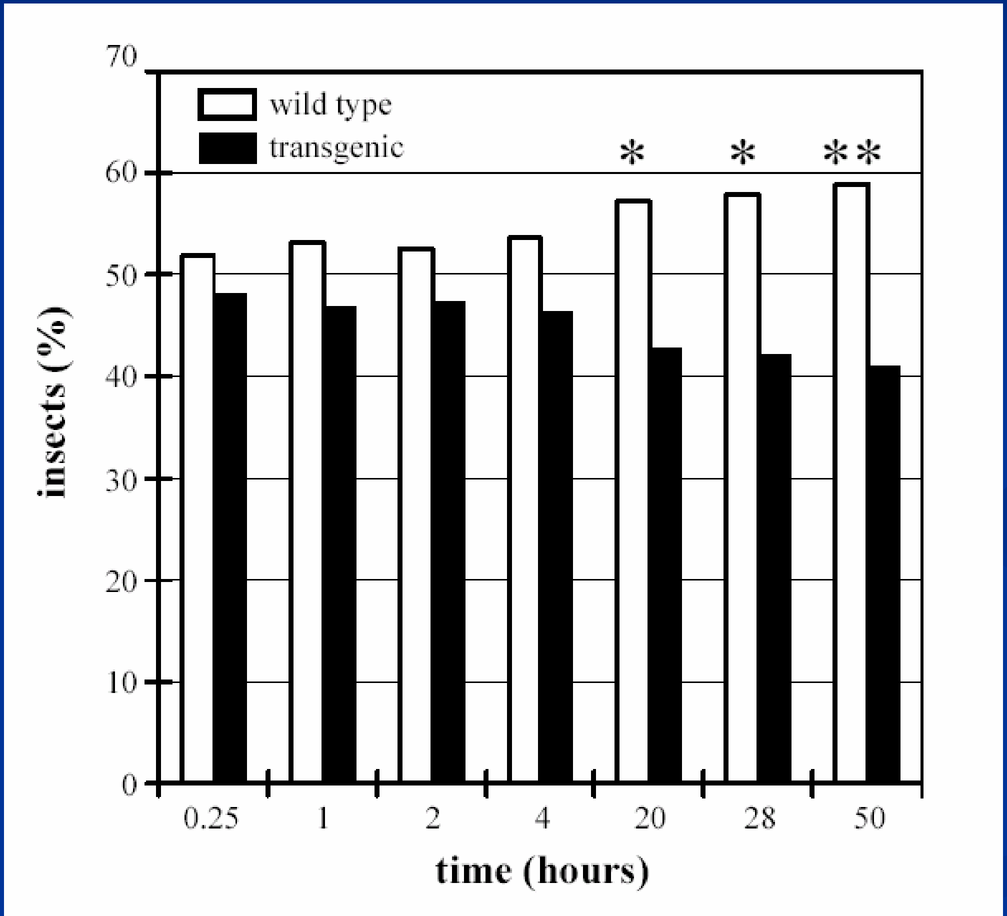


Conclusions

- Engineering with a very strong, constitutive promoter is deleterious to plants (toxicity or altered precursor pool for other pathways)

- Use of specific and/or inducible promoters for engineering terpenoids

Volatiles Produced by Trangenic plants Influence Insect Behavior

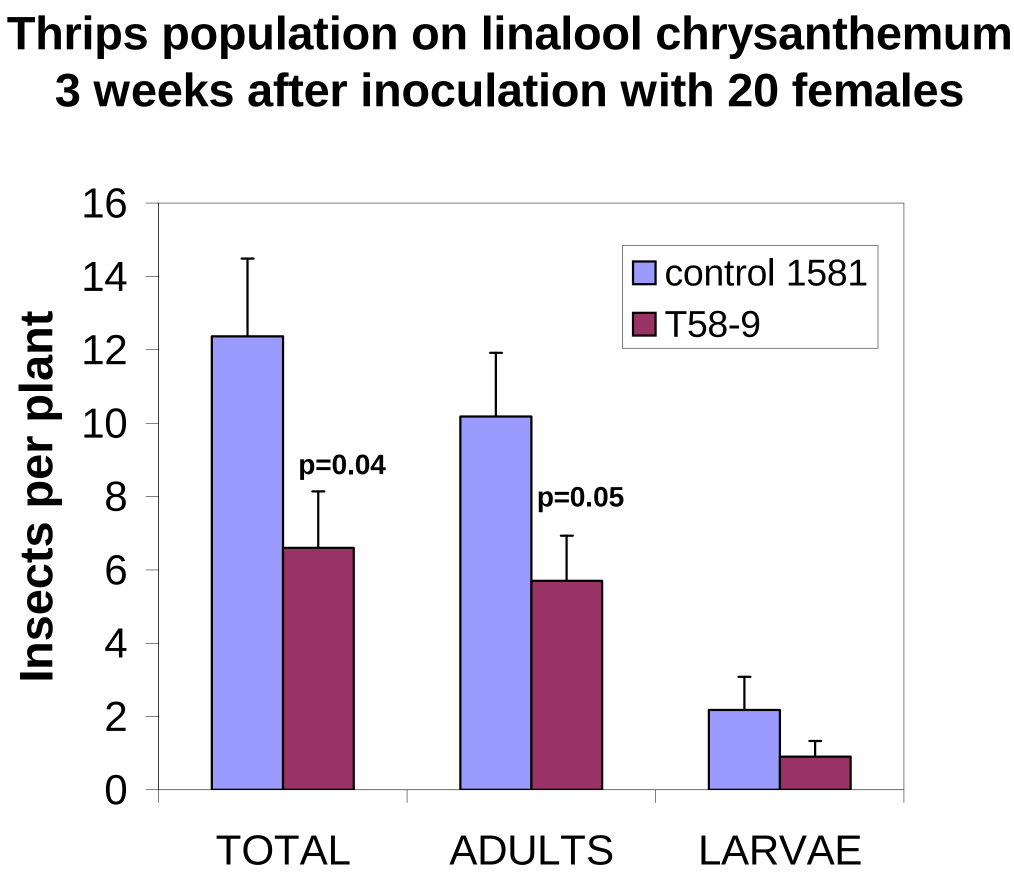


Leaves detached from transgenic Arabidopsis plants expressing the strawberry *FaNES1* gene.

Linalool deters aphids

No Choice Greenhouse Test in Perspex Hoods

- Linalool synthase chrysanthemum T58 lines
- 20 females
- N=6-13 plants per line
- 3 weeks; 22 C


Thrips population on linalool chrysanthemum
3 weeks after inoculation with 20 females
Insects per plant
16
14
12
10
8
6
4
2
0
control 1581
T58-9
p=0.04
p=0.05
TOTAL
ADULTS
LARVAE

Different Thrip Damage Phenotype in Transgenic Linalool Plants

Control

only edges

large surface

Linalool

transgenic

only spots

not at the edges

Conclusions

-Terpenoids produced by engineered plants influence insect behavior

-High levels of linalool production deters insects (aphids and thrips) in different plant species