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
  - Polyacetylenes-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)

Endoplasmic 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

ATP-dependant transporter
SMs sequestration into 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 / NADPH 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:**

Herbivores (insects, vertebrates) → Repellence, deterrence, toxicity

Microbes (bacteria, fungi, viruses) → Growth inhibition and toxicity

- competiting plants (inhibition of germination and seedlings growth)

Plant SMs - mixtures - variation in time, space & dev. stage

- UV-protection

Attraction →
- pollinating insects
- seed dispersing animals
- root nodule bacteria
- induced volatiles attract predatory organisms (tritrophic interactions)

M. Wink, Annual Plant Review, 1999
Examples of plant SMs and their proposed function

- Visual pollinator attractant
- Olfactory pollinator attractant
- Defense toxin
- Insect feeding deterrent
- 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.

Secondary metabolism:
- Activation of prefabricated defense chemicals
- Increase of existing defense compounds
- Induction of de novo synthesis of defense compounds (phytoalexins)

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 hydrolase) 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 laticifers 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**

<table>
<thead>
<tr>
<th>Plant defense</th>
<th>Plant taxon</th>
<th>Natural enemy</th>
<th>Counter resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxic amino acids</td>
<td>Various Leguminosae</td>
<td>Bruchid weevil</td>
<td>Modified tRNA synthase</td>
</tr>
</tbody>
</table>

- **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!!
### Major classes of secondary metabolites found in *A. thaliana*.

<table>
<thead>
<tr>
<th>Class</th>
<th>Approximate number of structures(^a)</th>
<th>Suggested functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole and indole-sulfur compounds</td>
<td>10</td>
<td>Defense against pathogens</td>
</tr>
<tr>
<td>Glucosinolates</td>
<td>35</td>
<td>Defense against pathogens and herbivores</td>
</tr>
<tr>
<td>Phenylpropanoids</td>
<td>20</td>
<td>UV protection. Defense against pathogens?</td>
</tr>
<tr>
<td>Benzenoids</td>
<td>25</td>
<td>Pollinator attractants? Defense against pathogens?</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>15</td>
<td>UV protection. Auxin transport. Seed dormancy. Defense against pathogens?</td>
</tr>
<tr>
<td>Terpenes</td>
<td>50</td>
<td>Herbivore feeding deterrents? Resistance to oxidative stress? Defense against pathogens?</td>
</tr>
<tr>
<td>Fatty acid derivatives</td>
<td>15</td>
<td>Defense against pathogens? Volatile signals?</td>
</tr>
</tbody>
</table>

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The secondary metabolism of *Arabidopsis thaliana*: growing like a weed  
John C D’Auria and Jonathan Gershenzon
**SMs in Arabidopsis**

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

* 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 (C5H8)
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

Below ground
attraction: orientation cues (non-volatile)

Above ground
attraction: fragrance (volatile)

Above ground
protection: repellents, antifeedants, predator attraction (volatile/non-volatile)

Below ground
protection: anti-microbial, antifeedant (non-volatile)
Precursors of Terpenoids
Mixed Origins of Terpenoids
Precursors (Meroterpenes)
Terpenoids - Important Molecules!

C₅ - hemiterpenes - e.g. isoprene
C₁₀ - monoterpenes - e.g. limonene
C₁₅ - sesquiterpene - e.g. abscisic acid (ABA)
C₂₀ - diterpene - e.g. gibberellin
C₃₀ - triterpene - e.g. brassinosteroids
C₄₀ - 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\textsubscript{15})

Fig. 3a. Cyclization of FPP by sesquiterpene synthases to produce different sesquiterpene skeletons: a direct cyclization of FPP
Diterpenes ($C_{20}$)
Triterpenoids
$(\text{C}_{30})$
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
Mevalonate
Plastid
Biosynthesis of terpenoids

Three main reactions:

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

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

- Modification steps
Biosynthesis of Precursors (prenyltransferases)
Biosynthesis of Precursors (prenyltransferases)

Cytosol

2x IPP + DMAPP → FDP - synthase → Farnesyl diphosphate

Plastid

IPP + DMAPP → GDP - synthase → Geranyl diphosphate
Terpene Cyclases

One enzyme.....One
substrate.....Multiple products
Terpene Cyclases (Mono)
Terpene Cyclases (Sesqui-)
Terpene Cyclases (Diterpene)
Modification of Monoterpenes

Structures

- Isopentenyl diphosphate
- Dimethyl allyl diphosphate
- Geranyl diphosphate

(-)-Limonene cyclase

GDP-synthase

Limone 3-hydroxylase

(-) trans-Isopiperitenol

(-) Isopiperitenone

(+)-cis-Isopulegone

Menthol

(+)-neomenthol

(-)-menthone

(+)-isomenthone

(+)-pulegone

(+)-isomenthone
Monoterpenoid Biosynthesis in Mint

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

Plastid targeting

Expected result
monoterpenes produced in plastids:
- linalool
- linalool derivatives formed?

Wild-type Arabidopsis leaves do not produce linalool
**S-Linalool Formation in Leaves of Arabidopsis**

![Graph showing S-Linalool and R-Linalool formation in transgenic Arabidopsis](image)
Further Modification

Free and Glycosidically Bound Terpenoids Produced by Arabidopsis

Free

Glycosidically Bound

Concentration (mg kg\(^{-1}\)-FW)

E-8-hydroxy-linalool
E-8-hydroxy-6,7-dihydro-linalool
nerolidol
linalool

Concentration (mg kg\(^{-1}\)-FW)

E-8-hydroxy-linalool
Z-8-hydroxy-linalool
E-8-hydroxy-6,7-dihydro-linalool
nerolidol
linalool
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.
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

![Graph showing relative abundance of R-Linalool and S-Linalool in reference, wild-type, and transgenic plants.](image)

- **R-Linalool**
  - Reference
  - Wild-type
  - Transgenic #1
  - Transgenic #2
  - Transgenic #3

- **S-Linalool**
  - Reference
  - Wild-type
  - Transgenic #1
  - Transgenic #2
  - Transgenic #3
further modification in transgenic potato plants

E-8-hydroxy-linalool → assumed glycosylation site in potato → glycoside

S-linalool → assumed glycosylation site in potato → glycoside

Z-8-hydroxy-linalool → assumed glycosylation site in Arabidopsis → glycoside

E-8-hydroxy-6,7-dihydrolinalool → assumed glycosylation site in potato → glycoside

assumed glycosylation site in potato
assumed glycosylation site in Arabidopsis

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)
Introducing the CiGASlo Gene to Arabidopsis

Pich 35S CiGASlo

Cytosolic production of a Germacrene A synthase from Chicory

Wild-type Arabidopsis leaves do not produce Germacrene A
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

Nerolidol

Wild-Type

Transgenic #1

Transgenic #2

Transgenic #3
Availability of Precursor Pools?

Emission /storage

Sesquiterpenes
- FPP
- GPP

Hydroxylated monoterpenes
- FPP

Monoterpenes
- GPP → GGPP
- IPP ↔ DMAPP

Cytoplasm (CYTOSOL)

Plastid (PLASTID)

Mitochondria (MITOCHONDRIA)
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)

A

farnesyl diphosphate

(3S)-(E)-nerolidol

4,8-dimethyl-1,3(E),7-
nonatriene ((E)-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 each cell compartment
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
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
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

![Graph showing insect population per plant](image)

- **TOTAL ADULTS LARVAE** per plant
- **control 1581**
- **T58-9**

- **p=0.04**
- **p=0.05**
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