Metabolic engineering of natural products for plant, animal, and human health

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and

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Molecular biology of plant natural products

We study……

- Lignin
  - Forage digestibility
- Isoflavonoids
  - Phytoalexins
  - Phytoestrogens
- Condensed tannins
  - Pasture bloat
  - Health benefits
- Triterpene saponins
  - Palatability
  - Plant defense
  - Adjuvants

We do…….

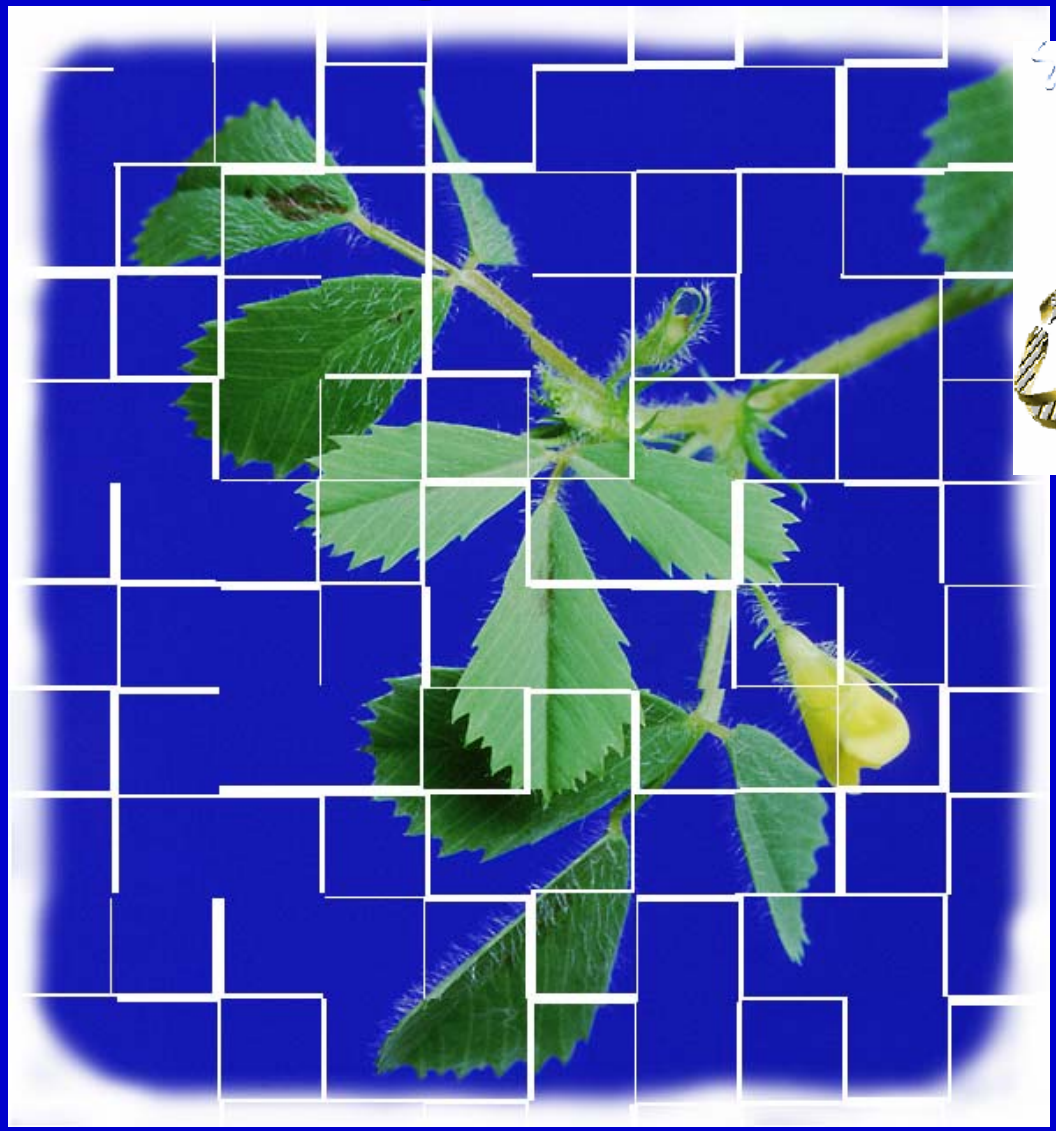
- Phytochemistry
- Enzymology
- Gene discovery through genomics
- Pathway engineering
  - Understanding pathways
  - Addressing function
  - Improving traits

Natural products from legumes

650 genera, >18,000 examined phytochemically

- Lignins
- Flavonoids
- Isoflavonoids
- Terpenoids (di-, sesqui- and tri-triterpenes)
- Alkaloids (eg. quinolizidine, dipiperidine)
- Coumarins
- Anthraquinones
- Non-protein amino acids
- Cyanogenic glycosides

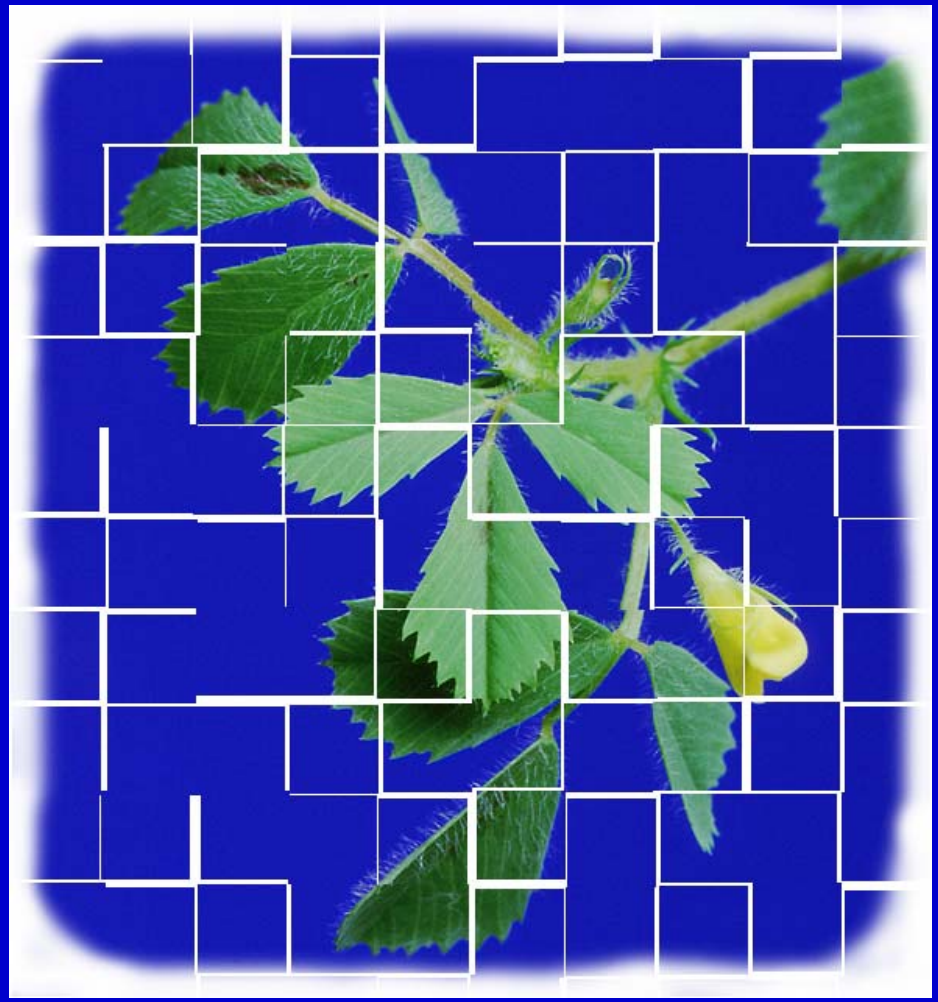
Integrated functional genomics of the model legume *Medicago truncatula*



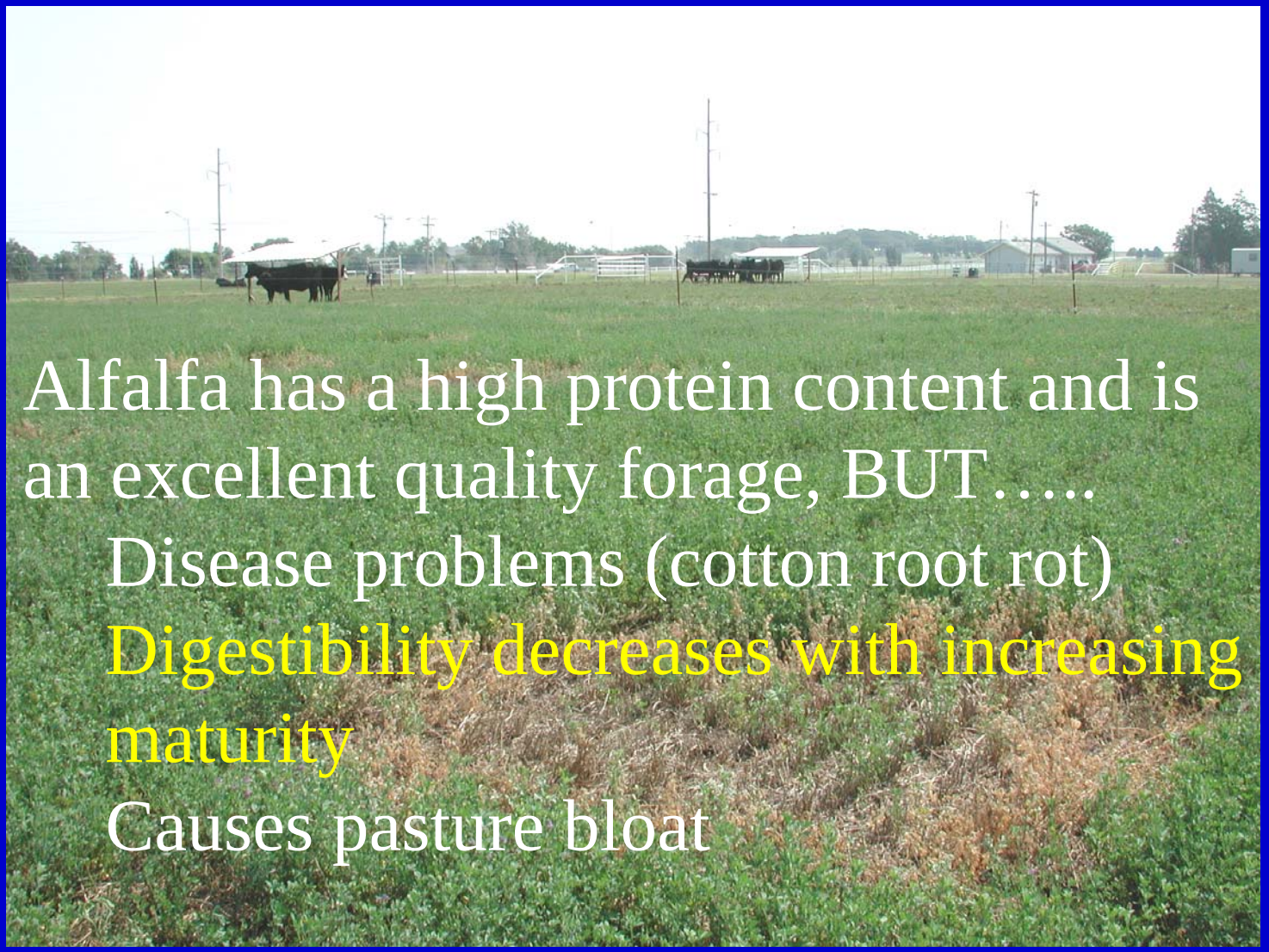
MDKLLV...

Agronomic traits in *Medicago truncatula*

- Nitrogen fixation
- Mineral nutrition
- Forage quality
  - Protein content
  - Digestibility
  - Palatability
  - Bloat
  - Silage properties
- Disease resistance
- Drought tolerance
- Grazing tolerance
- Source of health-promoting natural products



Re-designing alfalfa as the perfect forage



Alfalfa has a high protein content and is an excellent quality forage, BUT…..

- Disease problems (cotton root rot)
- Digestibility decreases with increasing maturity
- Causes pasture bloat

A structural model of lignin

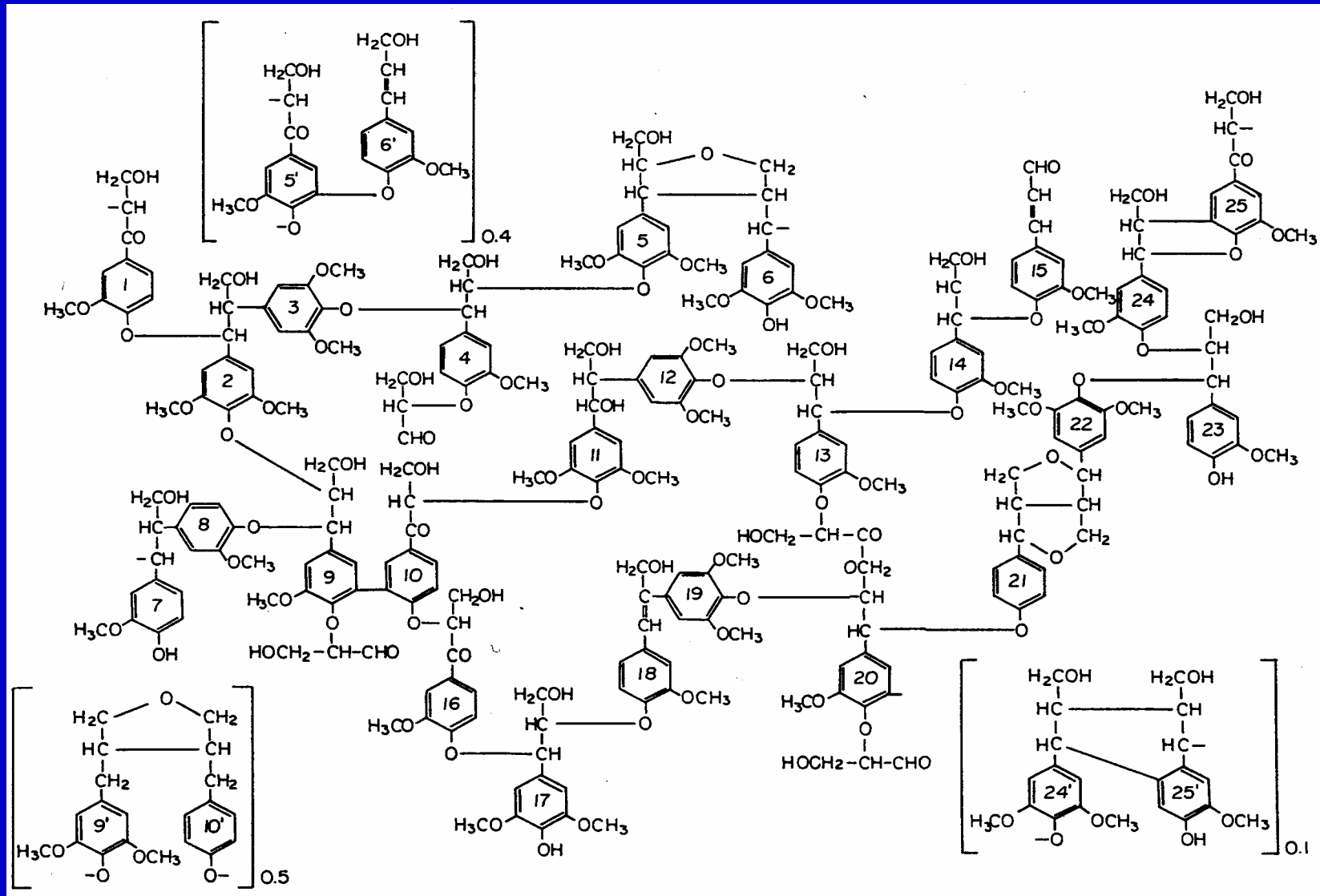
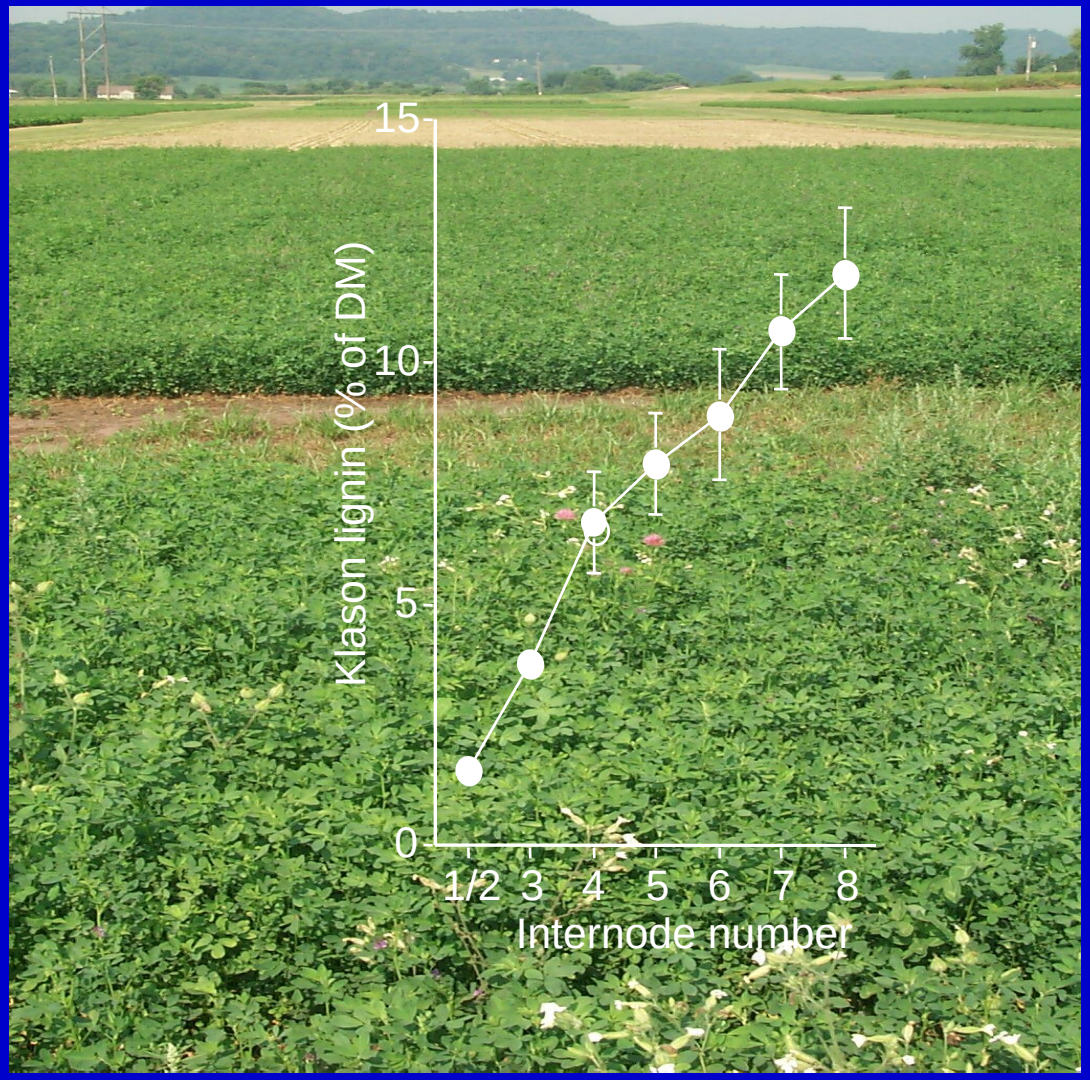
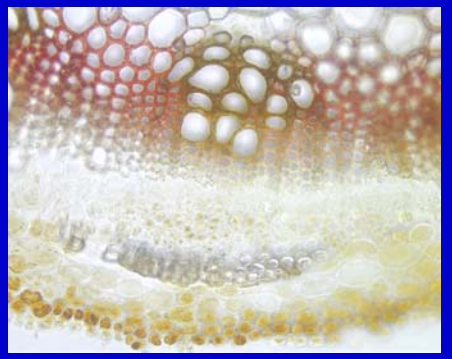


Fig. 1.2. Structural model of beech lignin. (Nimz 1974)

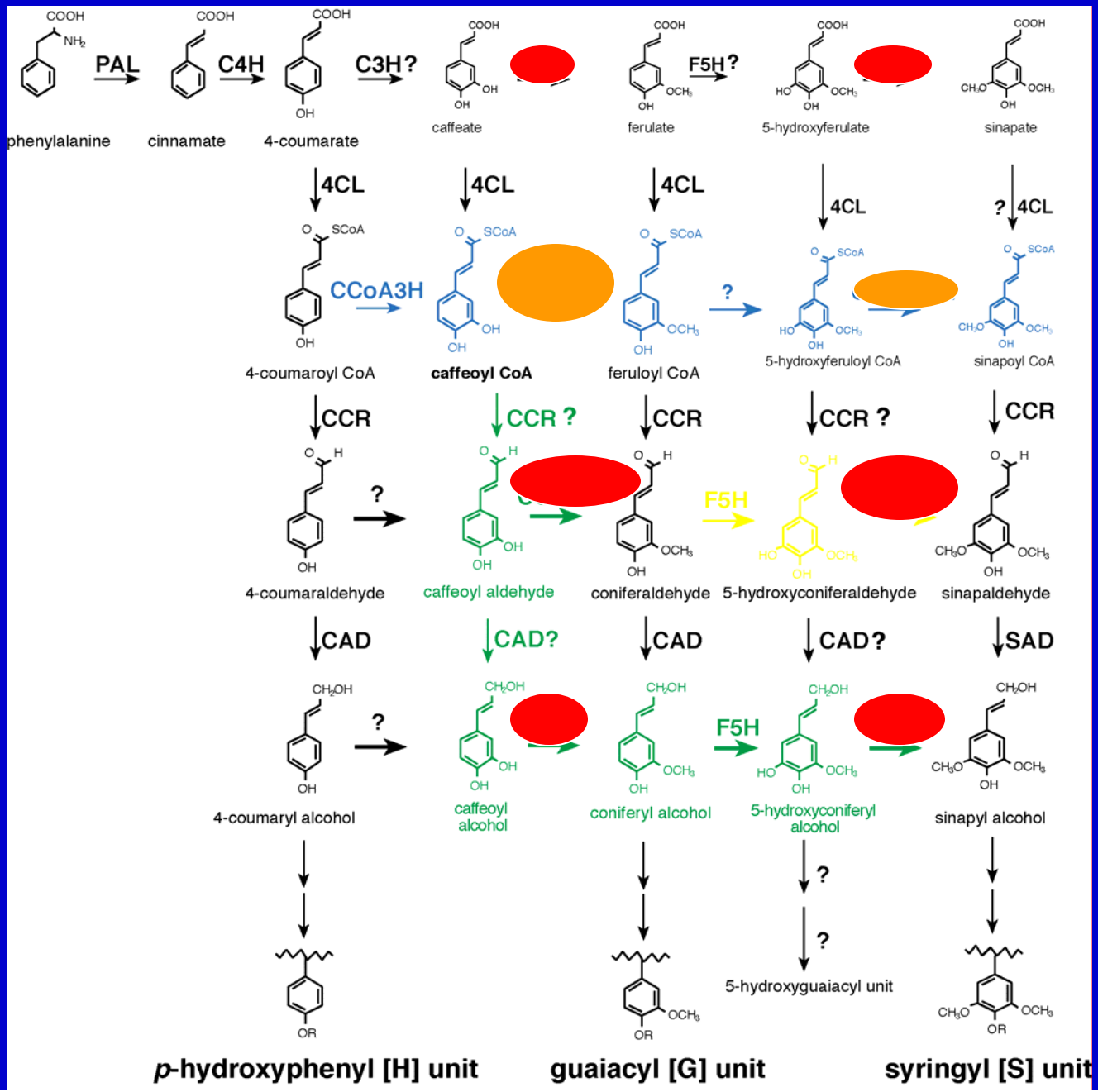
Lignin and forage digestibility

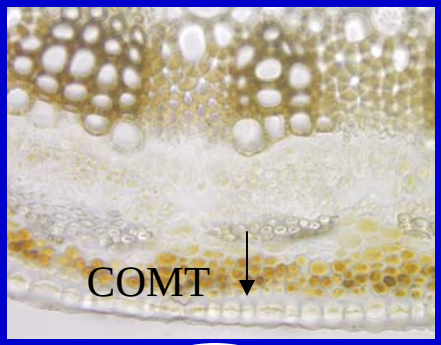


USDFRC estimates that a 10% increase in fiber digestibility would result in an annual $350 million increase in milk/beef production and = 2.8 million tons decrease in manure solids produced each year.



COMT and CCOMT: targets for lignin engineering



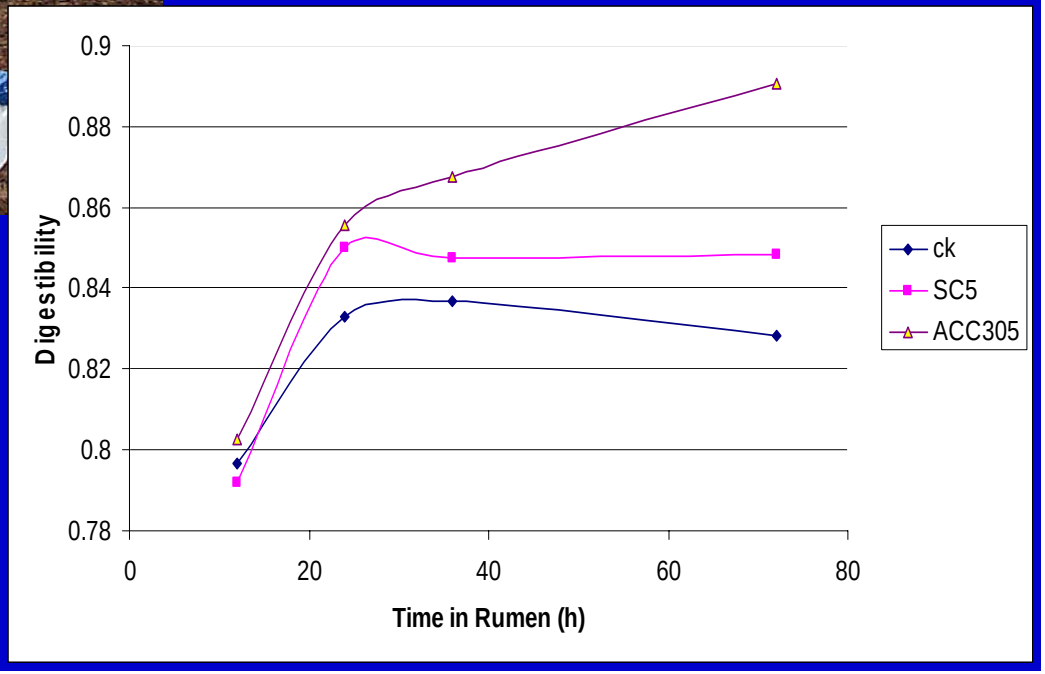


Effects of COMT and CCOMT down-regulation on lignin composition

Transgenic Lines	COMT pkat/mg	CCOMT pkat/mg	S Lignin	G Lignin	5-OHG Lignin
Wild type	5.98	22.35	158.8	305.4	0.0
COMT downregulated	1.24	22.26	8.6	246.1	8.8
CCOMT downregulated	14.39	0.78	159.1	150.2	0.0
COMT & CCOMT downregulated	0.78	5.59	54.0	223.7	0.0

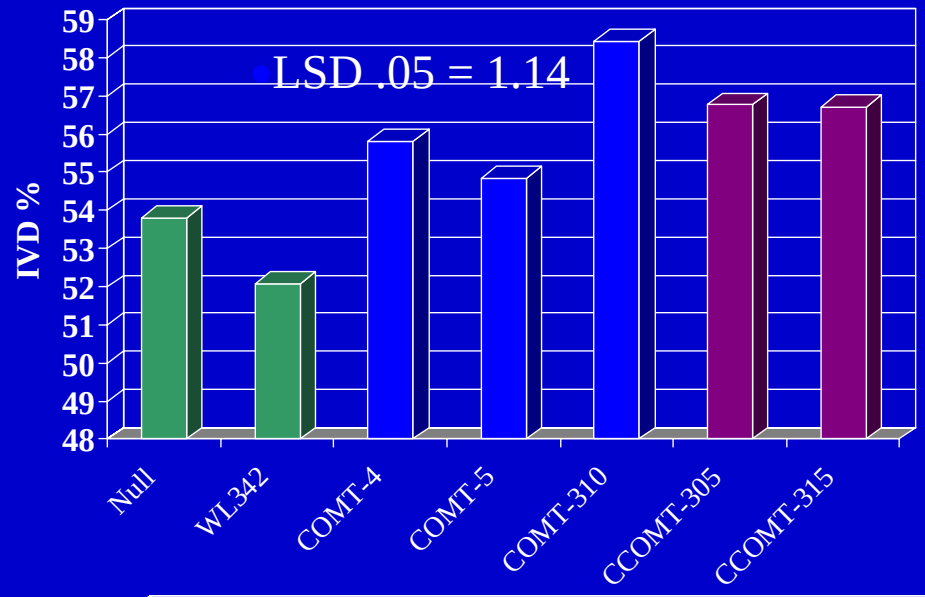
Guo et al. (2001) Plant Cell 13: 73-88

In-rumen digestibility of transgenic alfalfa forage in fistulated steers

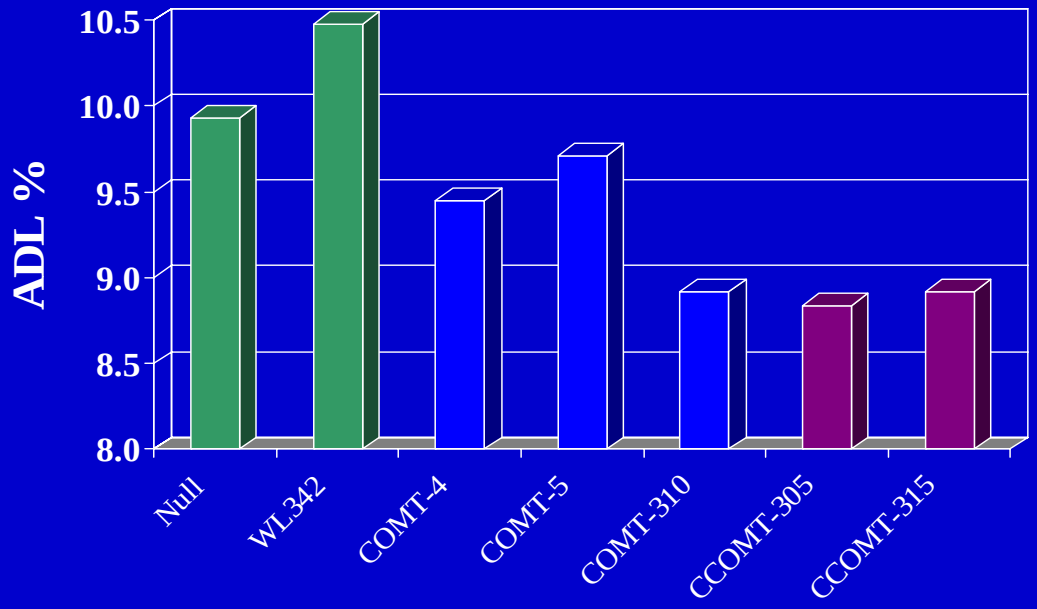


Guo et al. Transgenic Research 10, 457-464, 2001

Summary of field data (3 States)

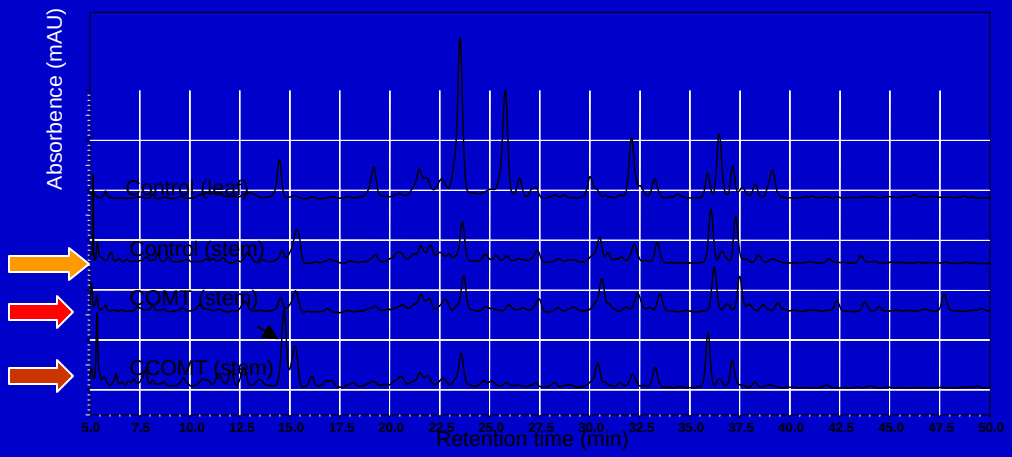


In vitro digestibility



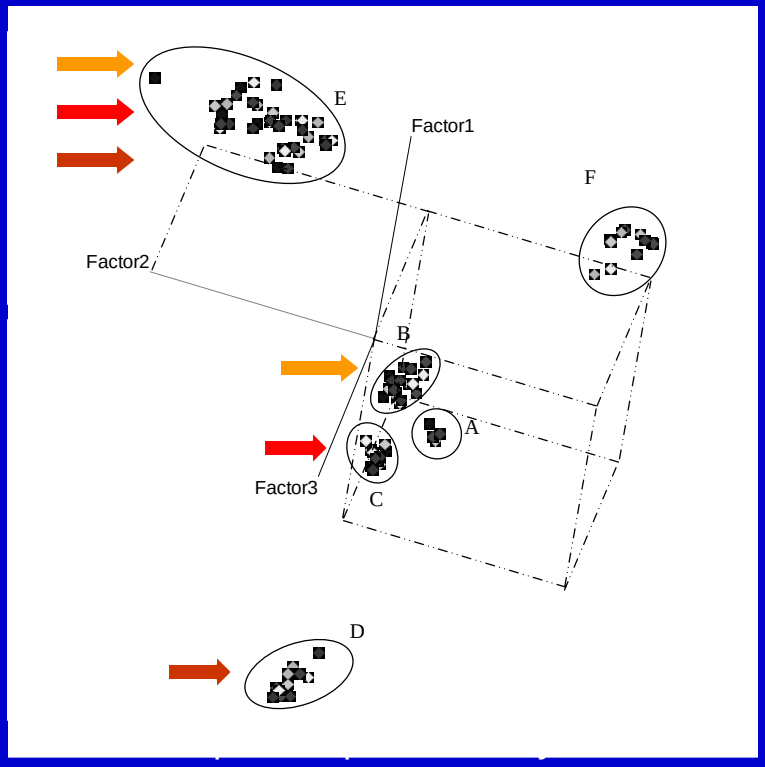
Acid detergent lignin (lower stem)

Profiling of soluble phenolics by HPLC

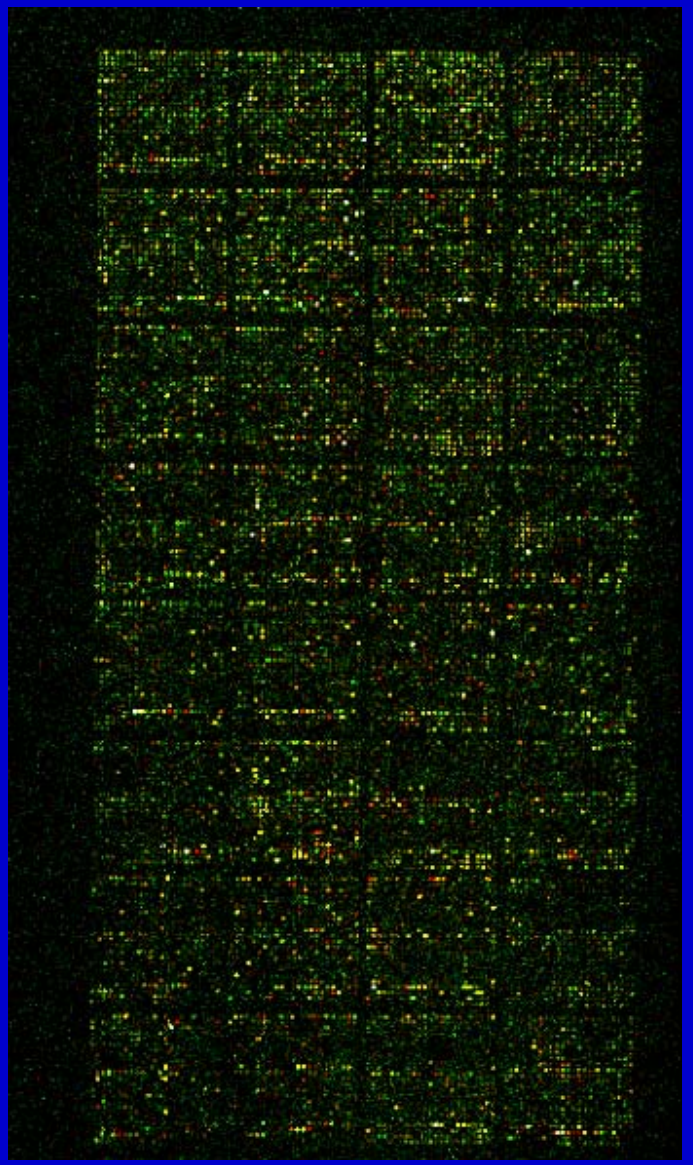


Leaf

Stem

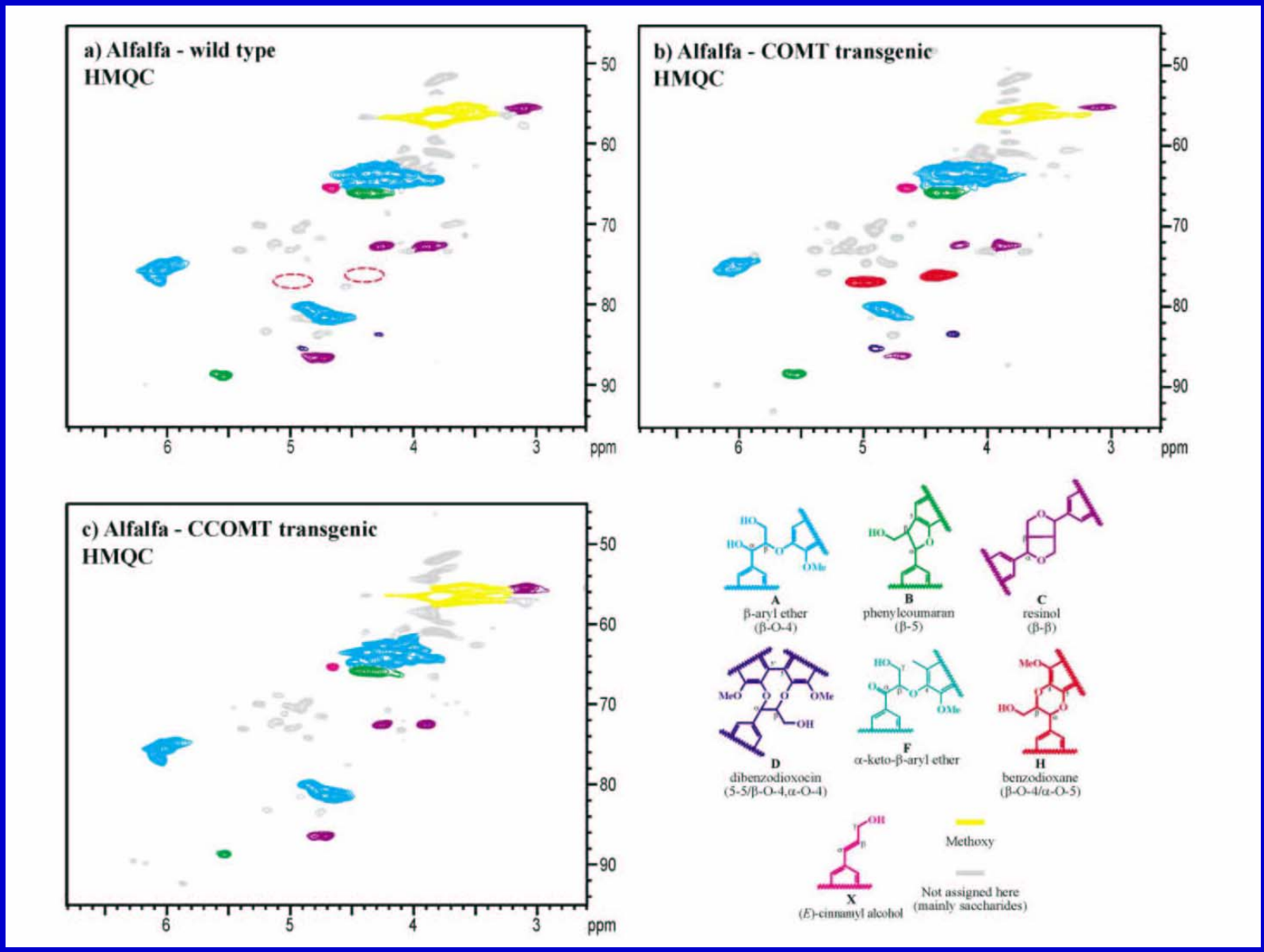


16K *M. truncatula* oligo array vs alfalfa stem RNA



Data of Fang Chen and Srinu Reddy

2-D NMR reveals novel structures in lignin from COMT down-regulated alfalfa



Marita et al. Phytochemistry, 2003

Re-designing alfalfa as the perfect forage

- Alfalfa has a high protein content and is an excellent quality forage, BUT…..
  - Disease problems (cotton root rot)
  - Digestibility decreases with increasing maturity
  - Causes pasture bloat

Condensed tannins (proanthocyanidins)

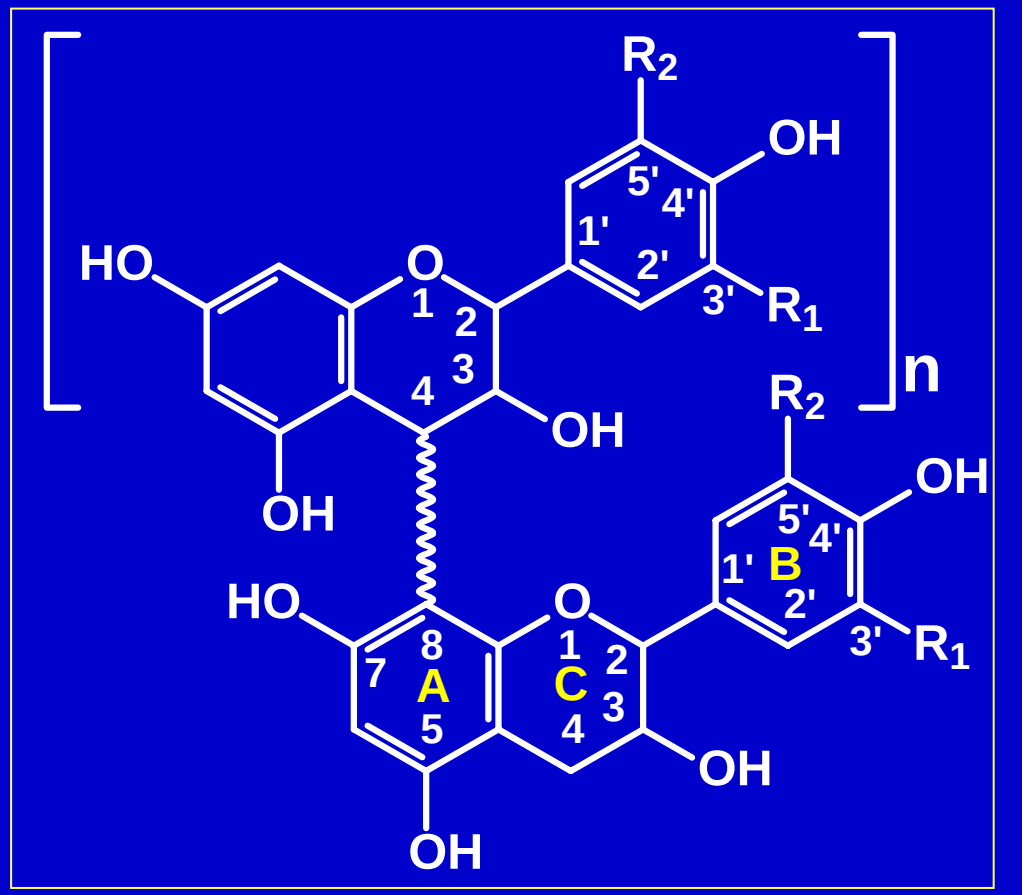
are oligomers or polymers of flavonoid (i.e.flavan-3-ol) linked by carbon-carbon bonds.

may contain 2-50 or greater flavonoid units.

differ in flavonoid units (R1, R2 =H or OH) and interflavan bond sites.

produce anthocyanidin after HCl-butanol hydrolysis

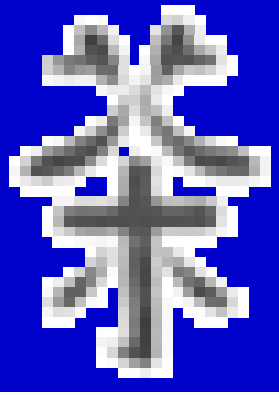
produce blue coloration with dimethylaminocinnamaldehyde



Activities of condensed tannins

- Bind to proteins, and reduce their rate of degradation (eg. tanning of leather, reduction of rumen fermentation). Help prevent pasture bloat.
  - In *Medicago* and white clover, CTs accumulate in the seed coat but not in the leaves
- Toxic to fungi, bacteria and insects.
- Antioxidant-cardiovascular benefits (polymers and monomers)
- Flavor and astringency (tea, wine, fruit juices)
- Urinary tract health (cranberry)

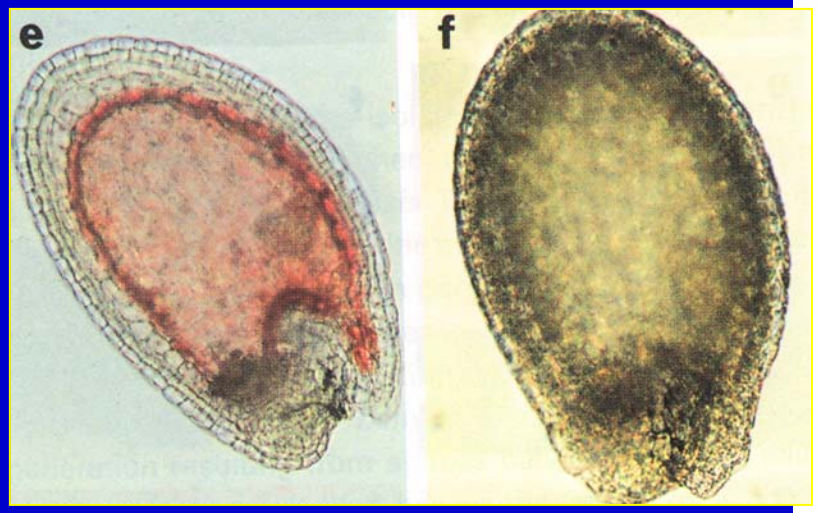

Panda Berry Tea
The Republic of Tea




VALUE SIZE
Isotonix® OPC-3™
Oligomeric Proanthocyanidins
Proanthocyanidines Oligomériques

Biosynthesis of condensed tannins and anthocyanins.

ban WT



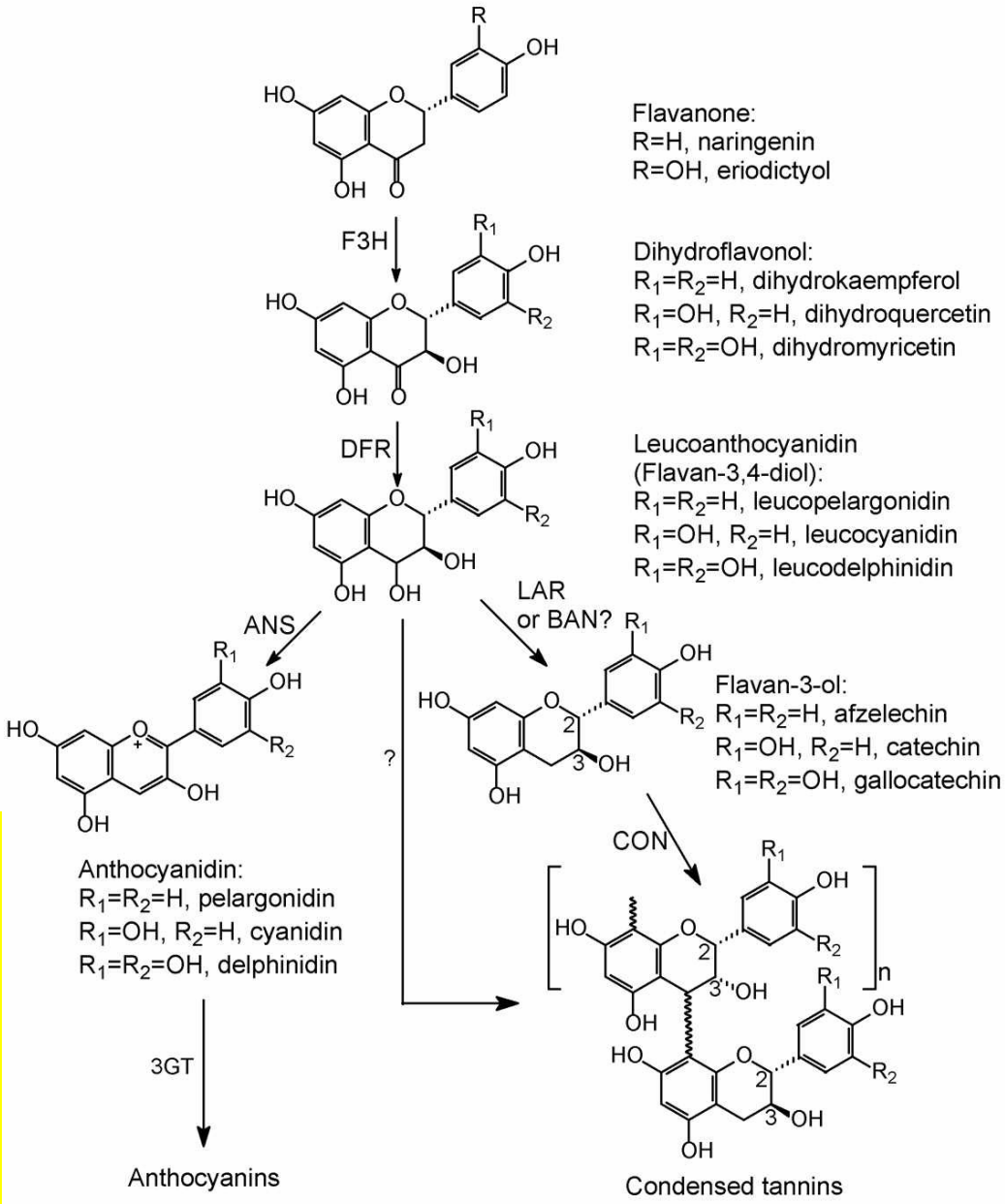


Fig.1

M.truncatula homolog of *Arabidopsis BAN* gene was identified by EST database mining -developing seed library.

```
1    M A S I K Q I E I E K K K A C V I G G T G F V A S L L I K Q L L E K G Y A V N T
1    M D Q T - L T H T G S K K A C V I G G T G N L A S I L I K H L L Q S G Y K V N T

41   T V R D L D S A N K T S H L I A L Q S L G E L N L F K A E L T I E E D F D A P I
40   T V R D P E N E K K I A H L R Q L Q E L G D L K I F K A D L T D E D S F E S S F

81   S G C E L V F Q L A T P V N F A S Q D P E N D M I K P A I K G V L N V L K A C V
80   S G C E Y I F H V A T P I N F K S E D P E K D M I K P A I Q G V I N V L K S C L

121  R A K E V K R V I L T S S A A A V T I N E L E G T G H V M D E T N W S D V E F L
120  K S K S V K R V I Y T S S A A A V S I N N L S G T G L V M N E E N W T D I D F L

161  N T A K P P T W G Y P V S K V L A E K A A W K F A E E N N I D L I T V I P T L T
160  T E E K P F N W G Y P I S K V L A E K K A W E F A E E N K I N L V T V I P A L I

201  I G P S L T Q D I P S S V A M G M S L L T G N D F L I N A L K G M Q F L S G S I
200  A G N S L L S D P P S S L S L S M S F I T G K E M H V T G L K E M Q K L S G S I

241  S I T H V E D I C R A H I F V A E K E S T S G R Y I C C A H N T S V P E L A K F
240  S F V H V D D L A R A H L F L A E K E T A S G R Y I C C A Y N T S V P E I A D F

281  L S K R Y P Q Y K V P T E F D D F P S K A K L I I S S G K L I K E G F S F K H S
280  L I Q R Y P K Y N V L S E F E E G L S I P K L T L S S Q K L I N E G F R F E Y G

321  I A E T F D Q T V E Y L K T Q G I - - - - K .        MtBAN
320  I N E M Y D Q M I E Y F E S K G L I K A K E S        AtABN
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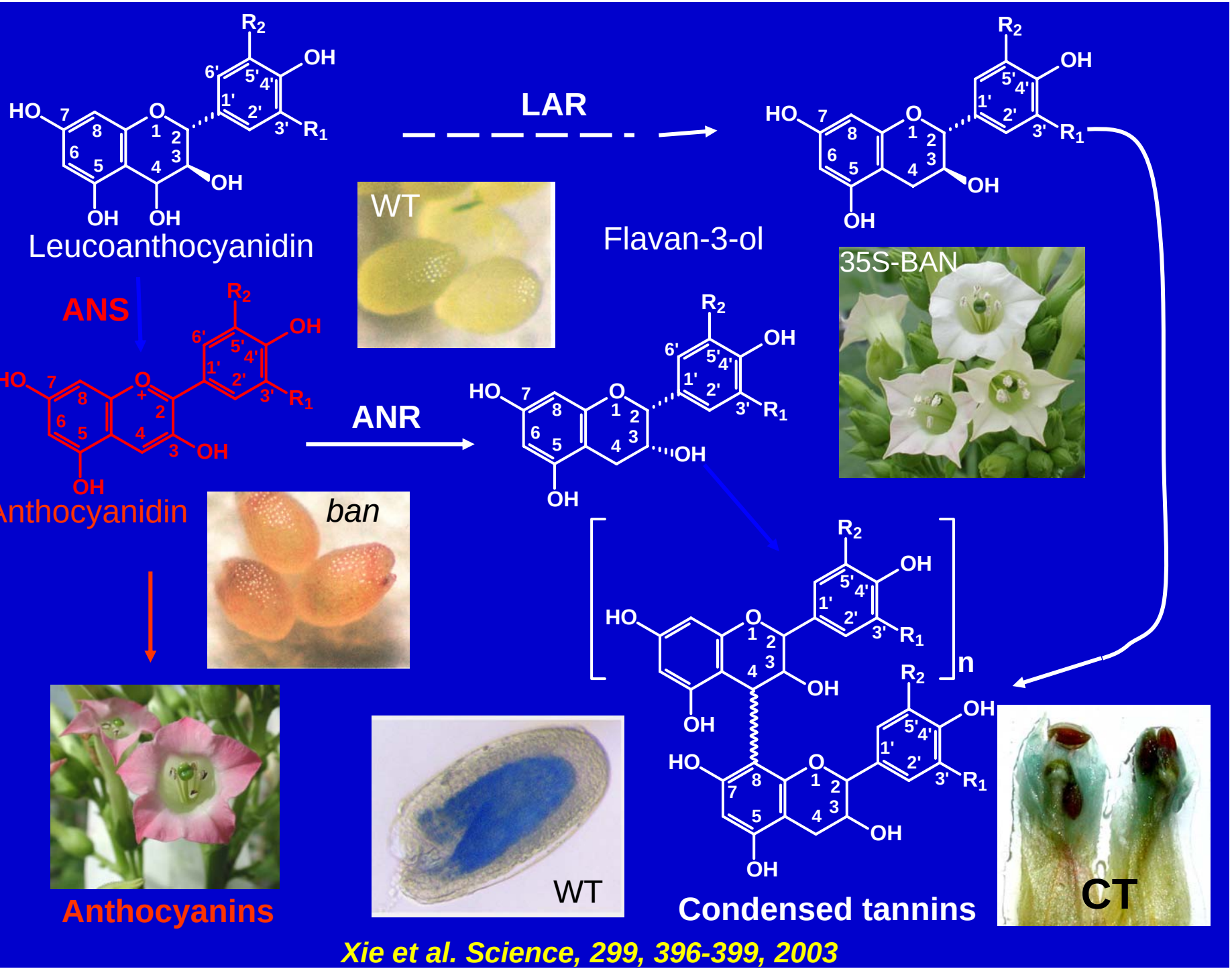
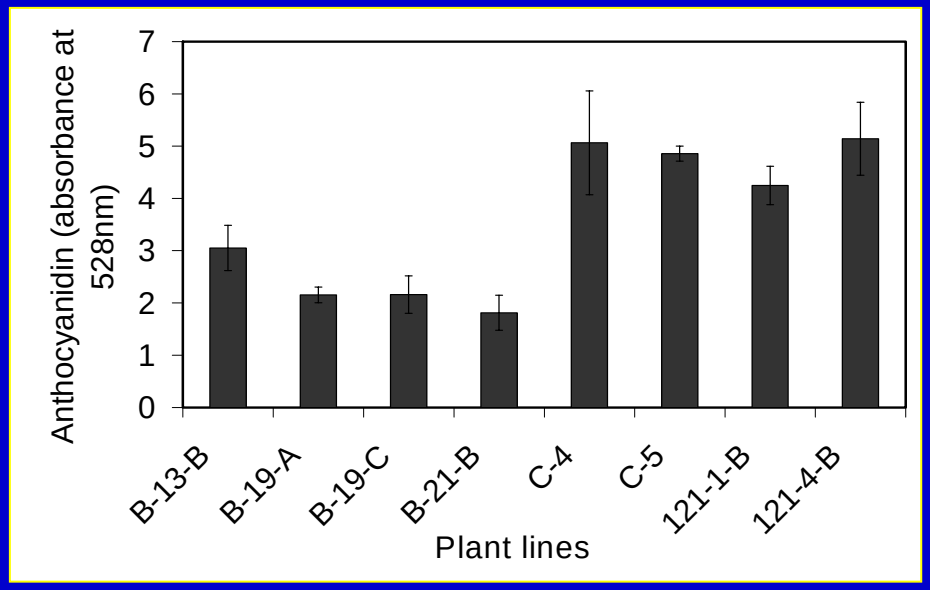
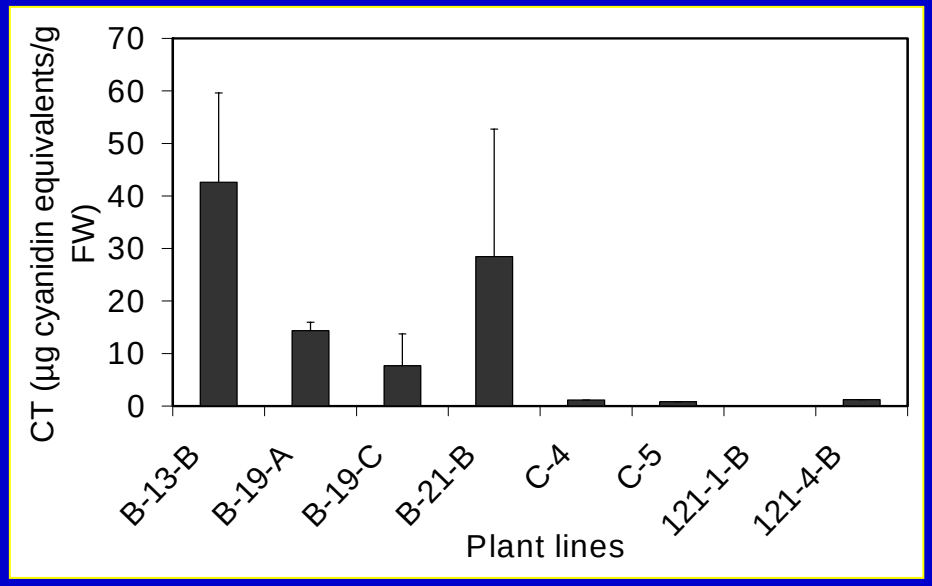

Leucoanthocyanidin
LAR
Flavan-3-ol
WT
35S-BAN
ANS
ANR
Anthocyanidin
ban
Anthocyanins
WT
Condensed tannins
CT
Xie et al. Science, 299, 396-399, 2003

Ectopic expression of MtBAN in tobacco flowers reduces anthocyanin content and results in accumulation of tannins

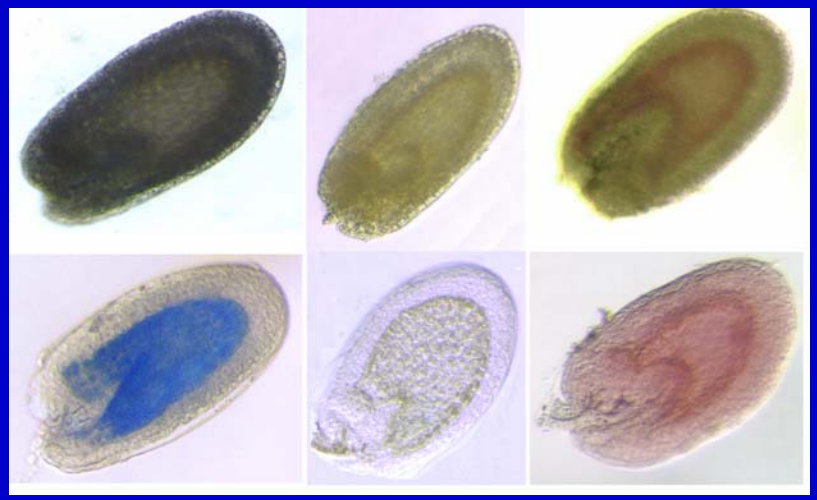


Comparison of anthocyanin content

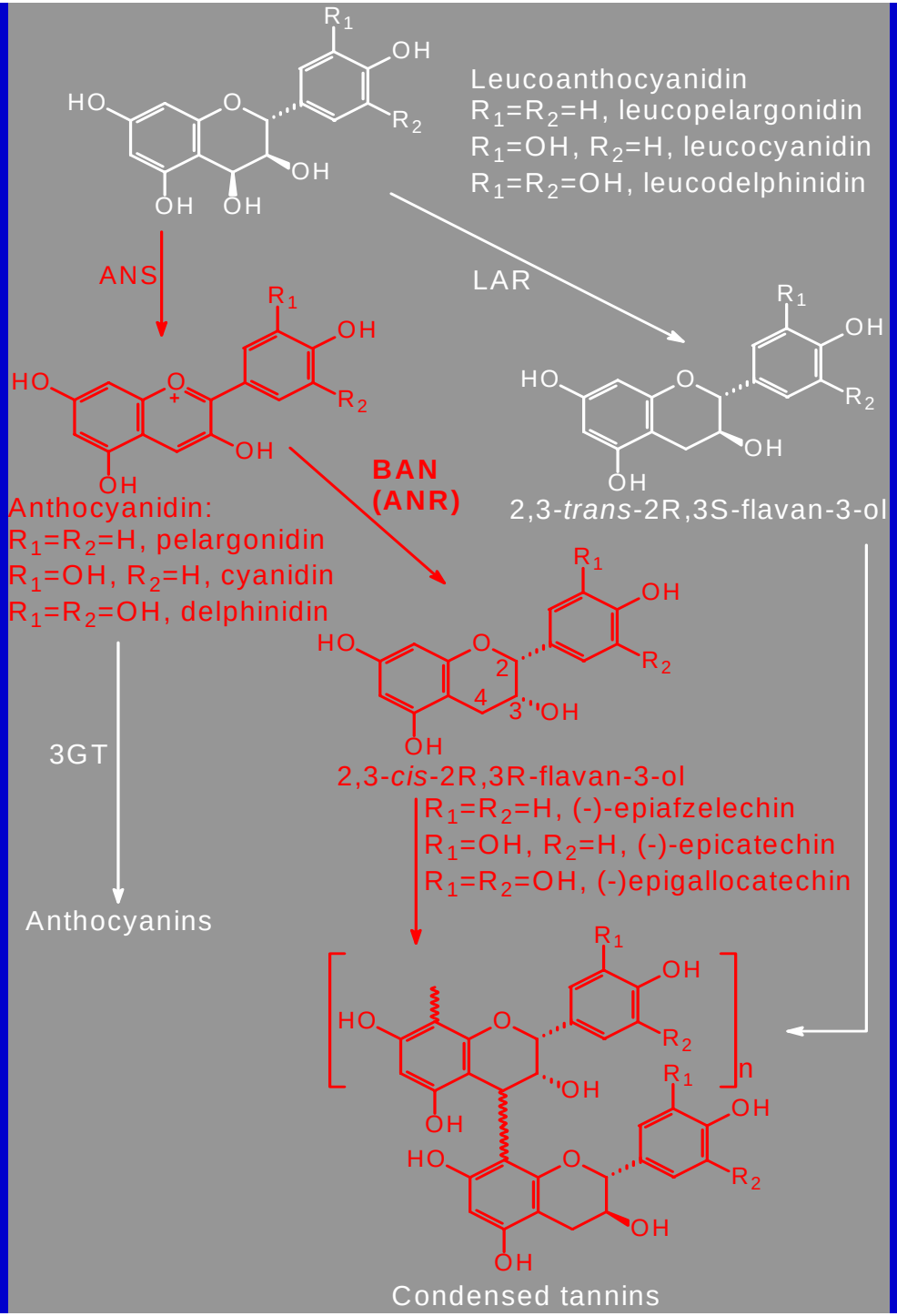


Comparison of CT content

Genetic support for new pathway to condensed tannins via BAN



Col ***tt18*** ***ban***



Species and tissue	ANR enzyme activity (pmol/min/mg protein)
Lotus corniculatus	
Leaf	721
Desmodium uncinatum	
Leaf	563
Flower	342
Pod	122
Grape	
Skin	143
Barley	
Grain	33*
Alfalfa	
Leaf	No reaction

Data of Deyu Xie

Moving tannins into alfalfa

Provision of anthocyanin substrate? Need for LAR?
Need for transporters, etc?

Anthocyanin flower pigments- natural or engineered?

WT BAN PAPIxBAN PAP1

	Anthocyanin	Epicatechin
WT	-	-
BAN	-	-
PAP1	++++++	-
PAP1xBAN	++	++

Production of epicatechin in transgenic tobacco leaves

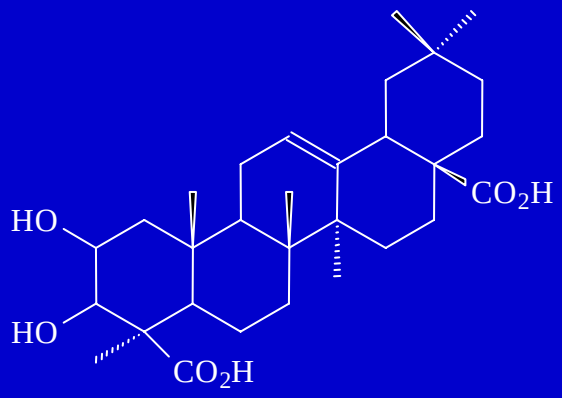
Redesigning alfalfa – triterpene saponins

- Growth inhibitory to poultry
- Anti-palatability and possibly toxic to monogastric animals
- Anti-insect
- Antifungal (avenacins in oats)
- Hypocholesterolemic
- Anticancer (desert Acacia spp.)

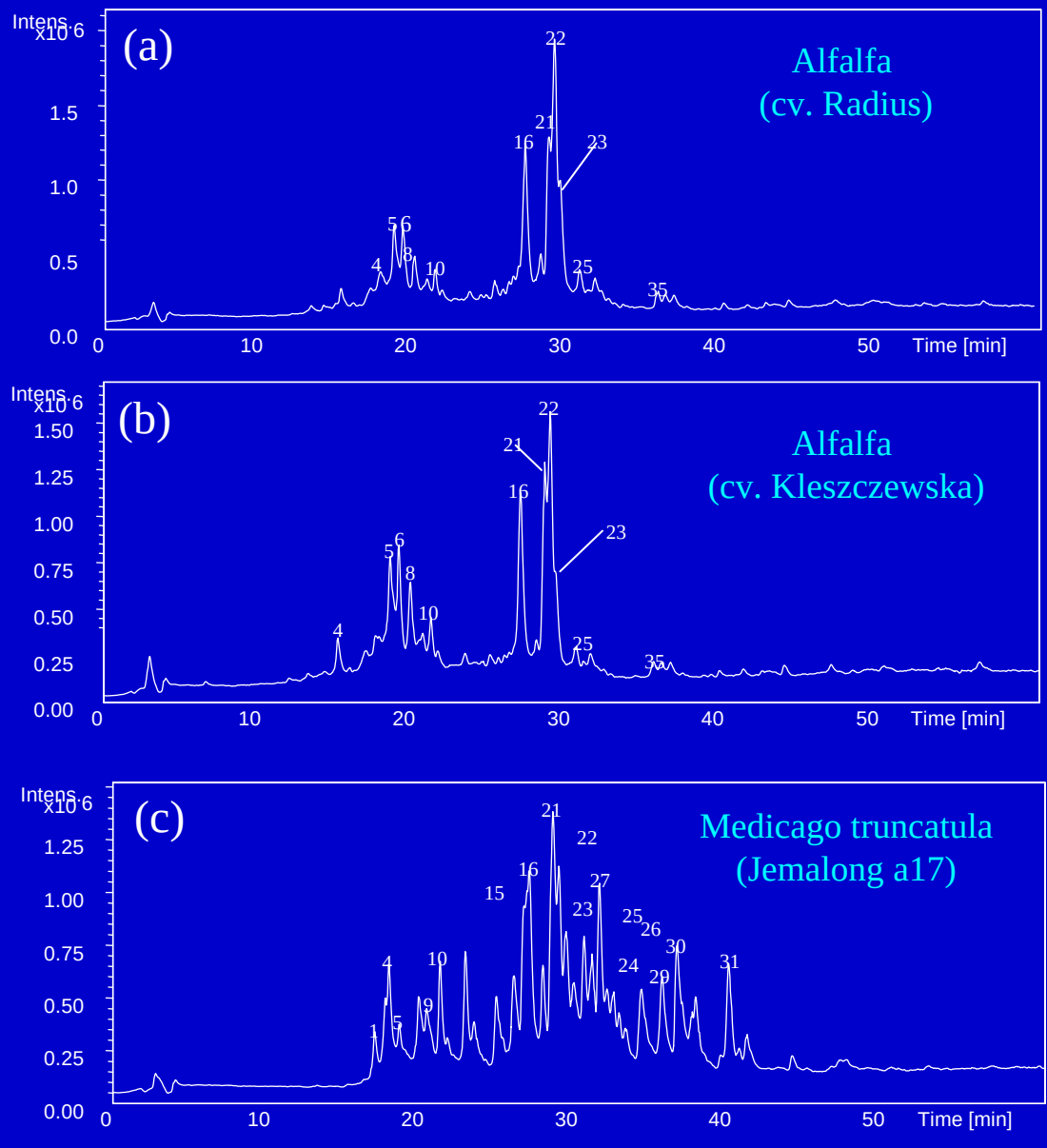
Profiling saponins in *Medicago* spp

[Sumner and Huhman, Phytochemistry, 2002]

LC/MS profiling reveals more than 30 triterpene saponins in *M. truncatula* (more complex pattern than alfalfa)

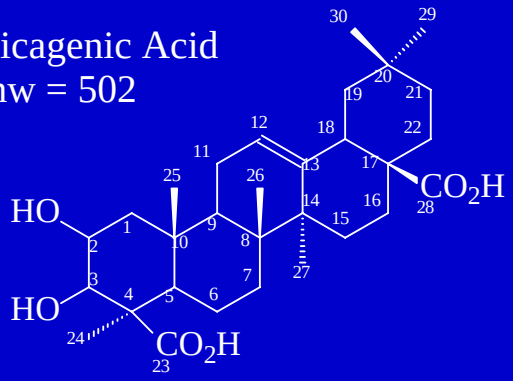


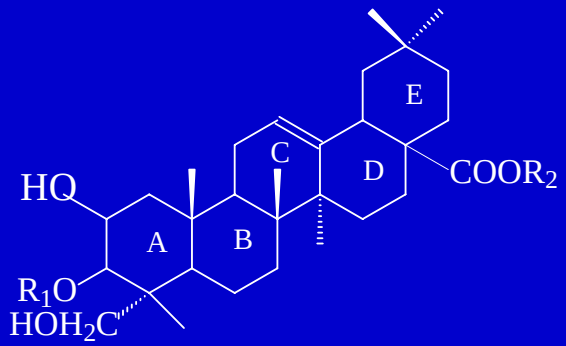
Medicagenic Acid



Saponin aglycones from *Medicago truncatula*

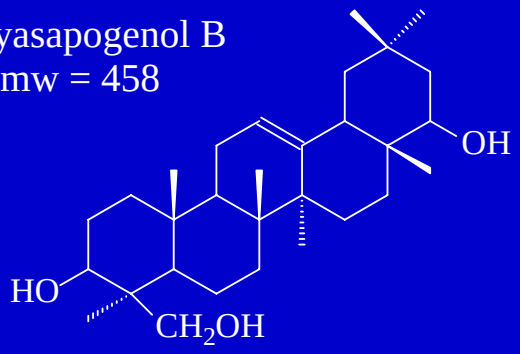
Medicagenic Acid
mw = 502

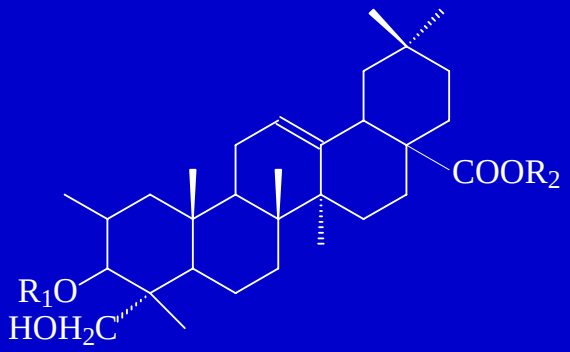




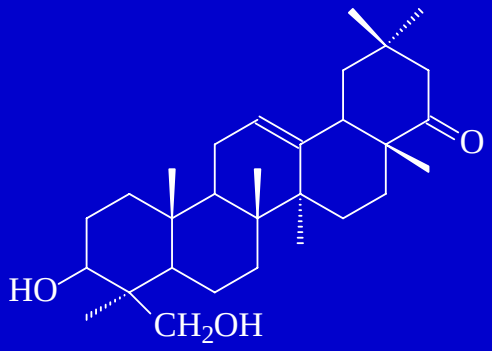
Bayogenin
mw = 488

Soyasapogenol B
mw = 458



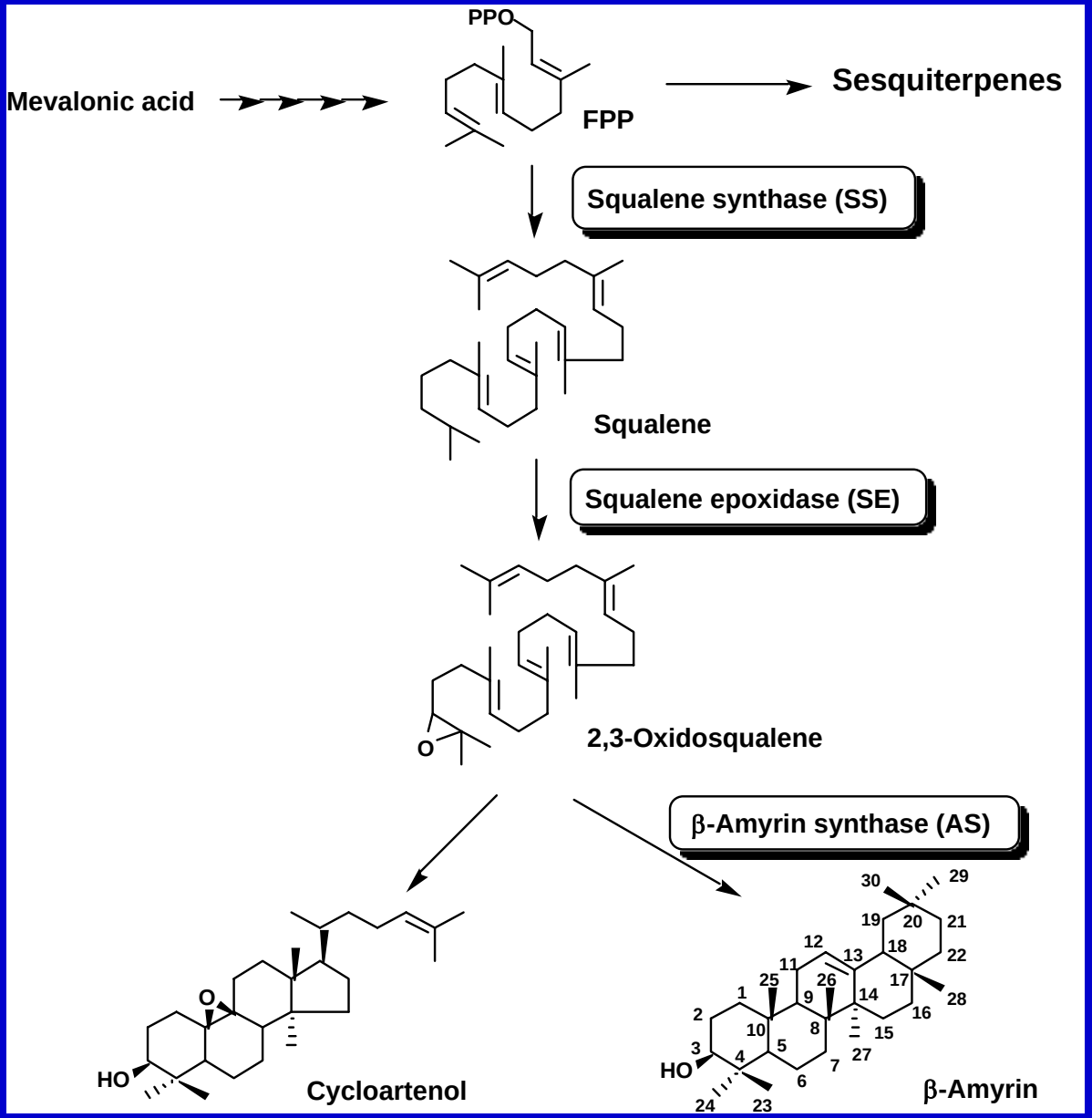


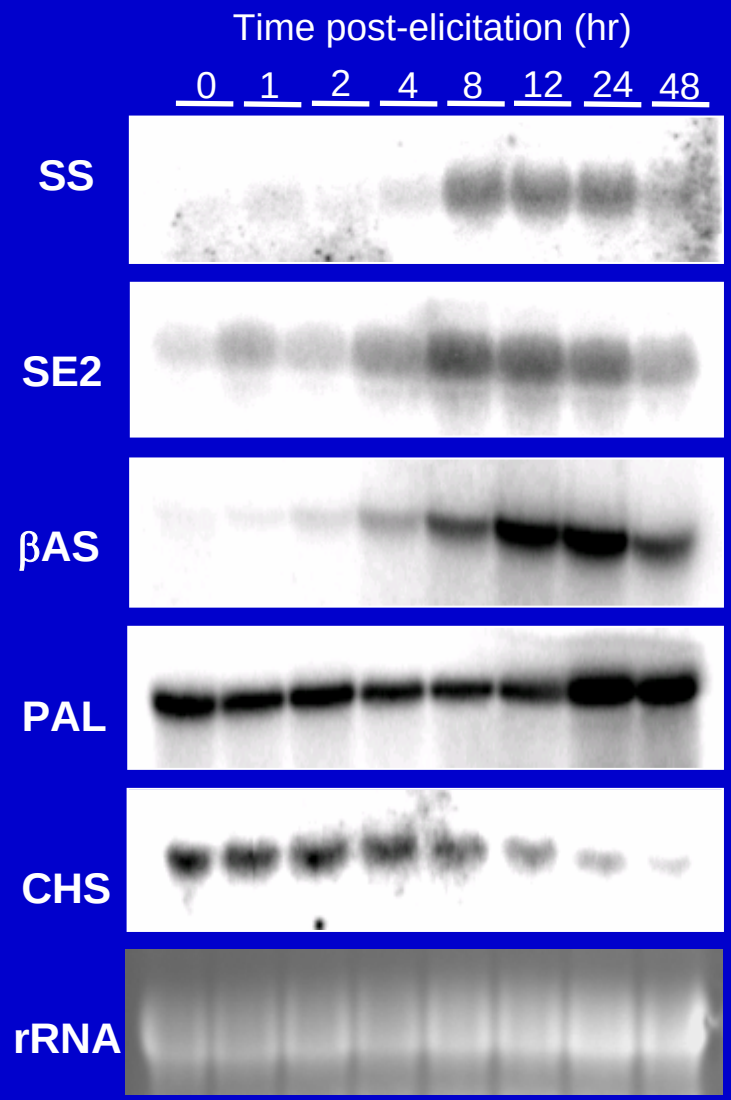
Hederagenin
mw = 472

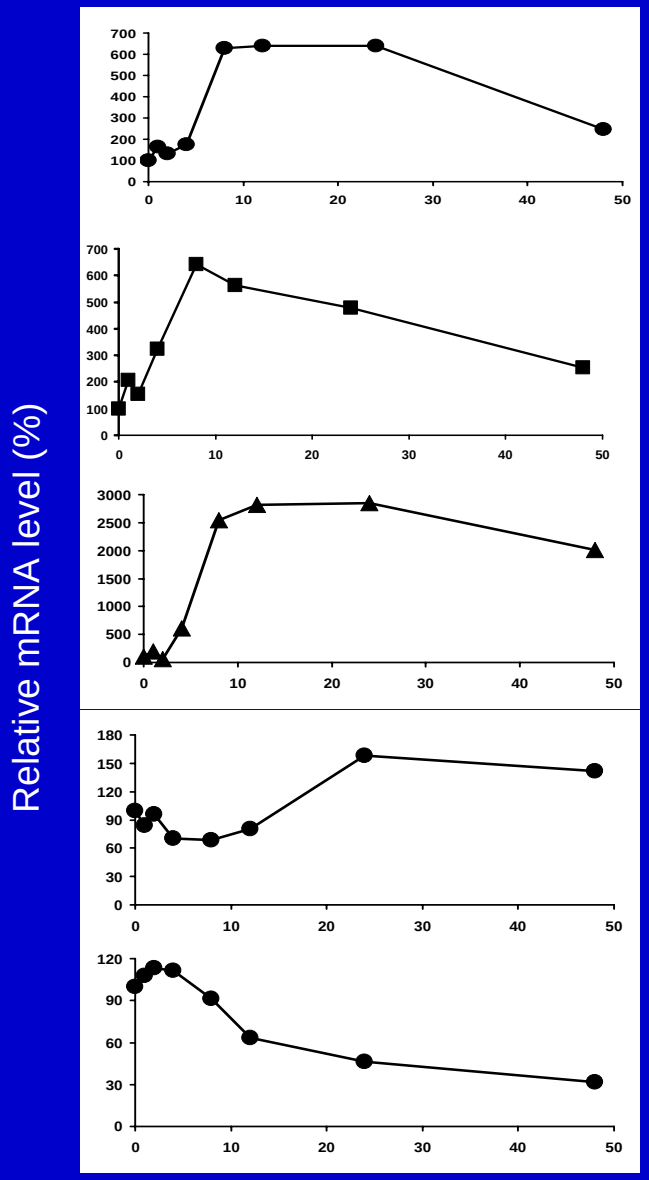


Soyasapogenol E
mw = 456

Early steps in the biosynthesis of triterpenes and sterols in plants



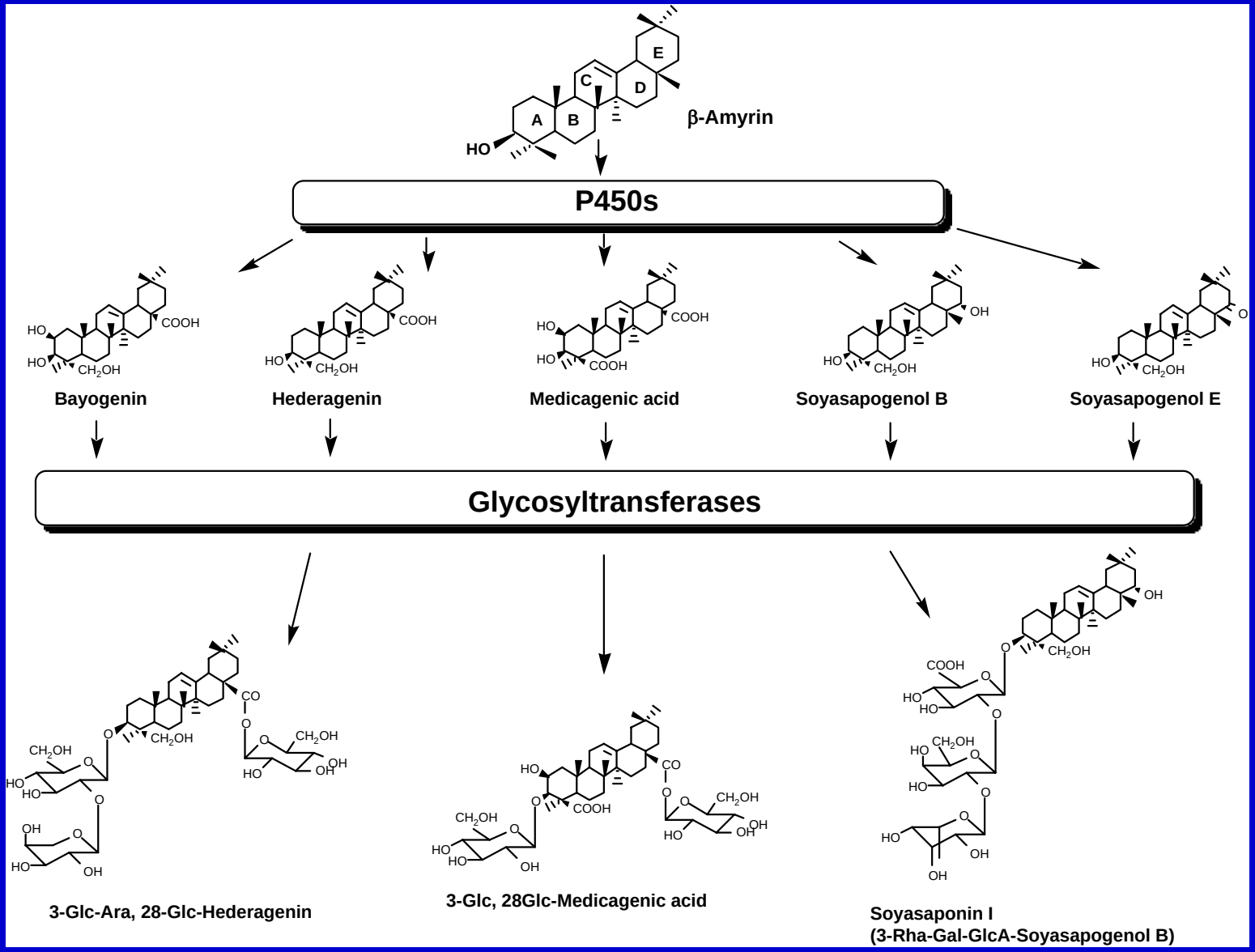




Differential responsivenes of triterpene and phenylpropanoid biosynthesis to MeJA in *M. truncatula* suspension cultures

Suzuki et al. Plant J. 32, 1033 (2002)

Saponin biosynthesis in *M. truncatula* involves cytochrome P450s and glycosyltransferases

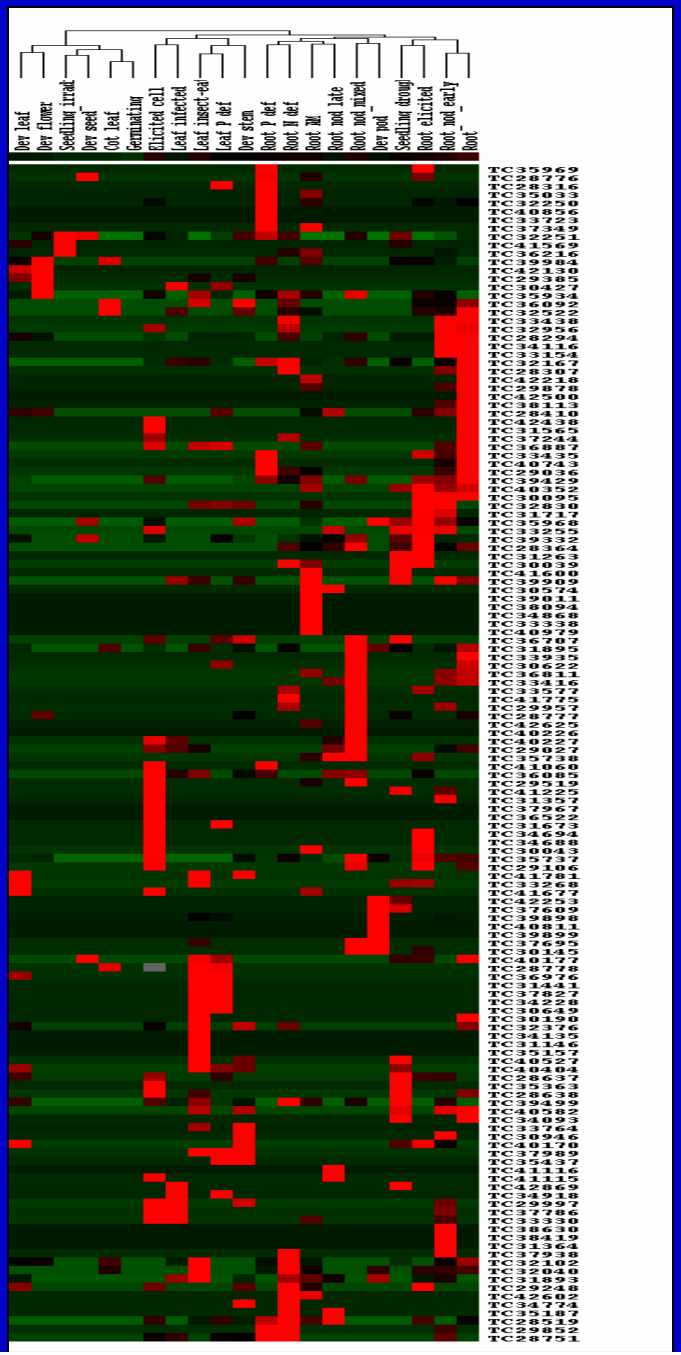


Filters for triterpene pathway gene selection

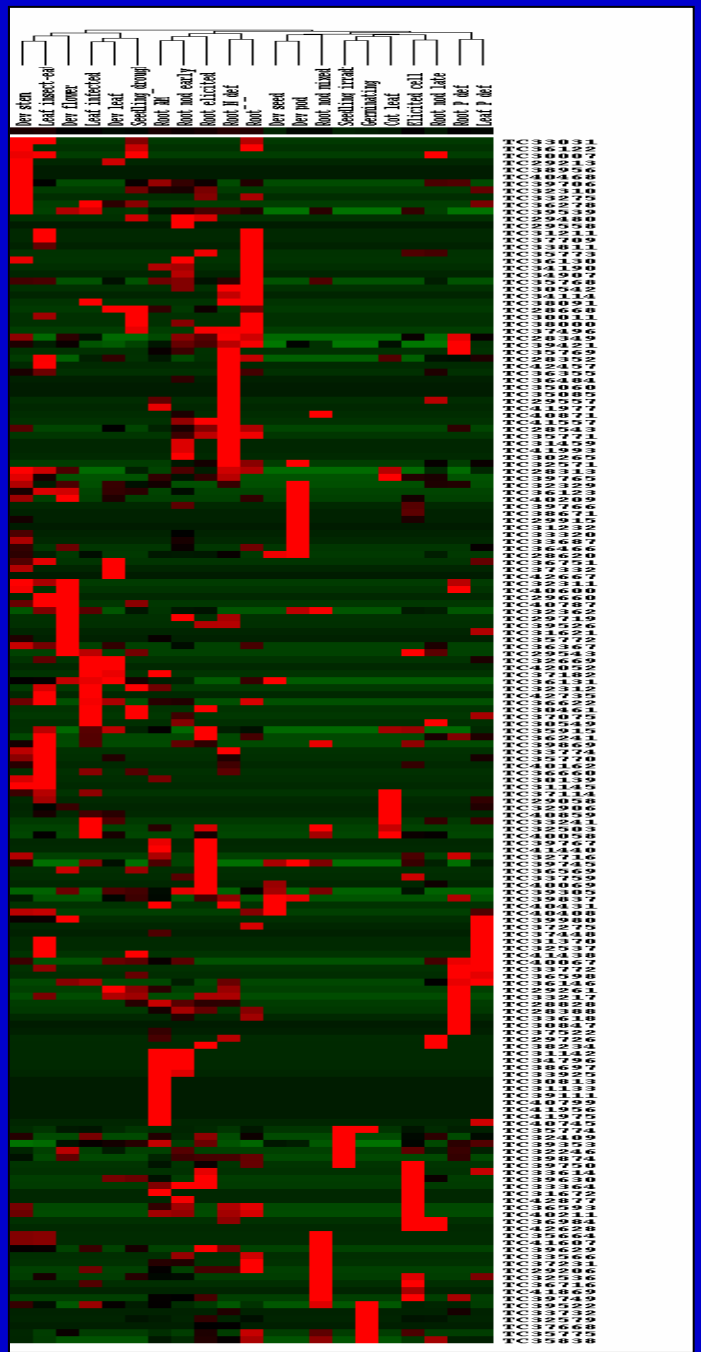
- P450 or GT involved in natural product biosynthesis
- Micro/macroarray analysis for up-regulation by methyl jasmonate
- *In silico* expression analysis: expressed in libraries where β-AS is expressed; not expressed in libraries where β-AS is not expressed.
- Induction kinetics cf β-AS (RNA gel blots)
- Functional expression in yeast (P450s) or *E. coli* (GTs)

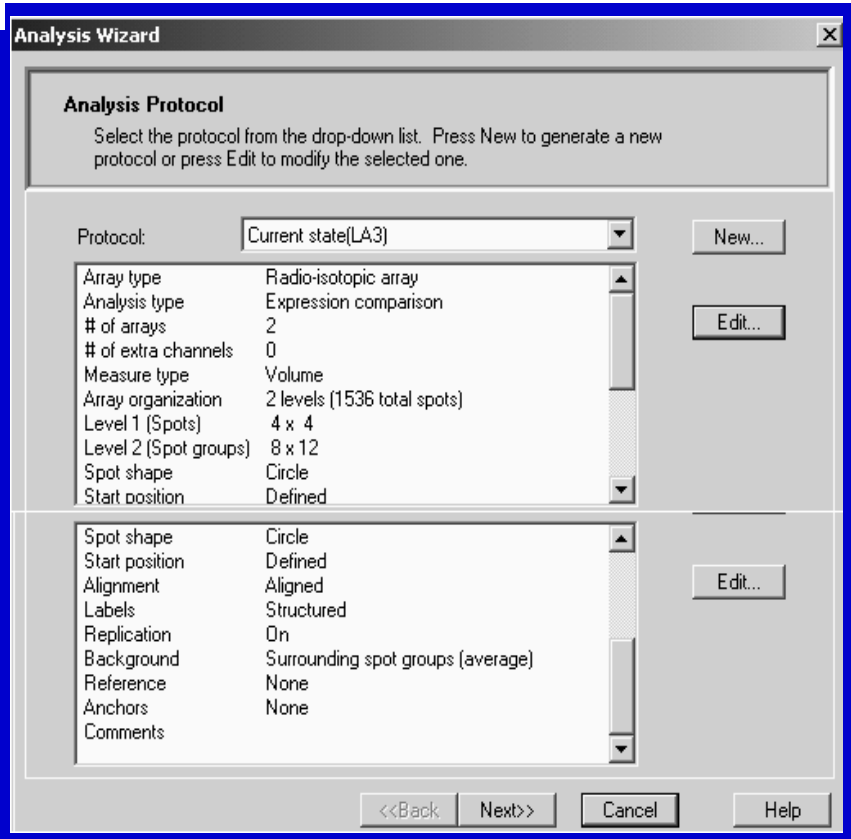
Collaboration with Seiichi Matsuda, Rice University

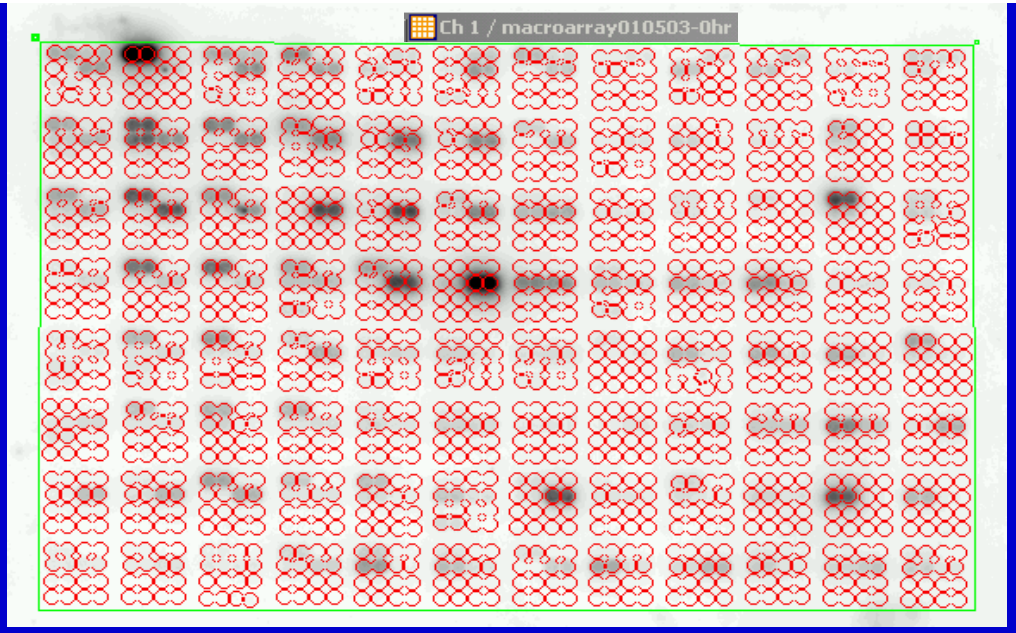
Cluster all P450 TCs

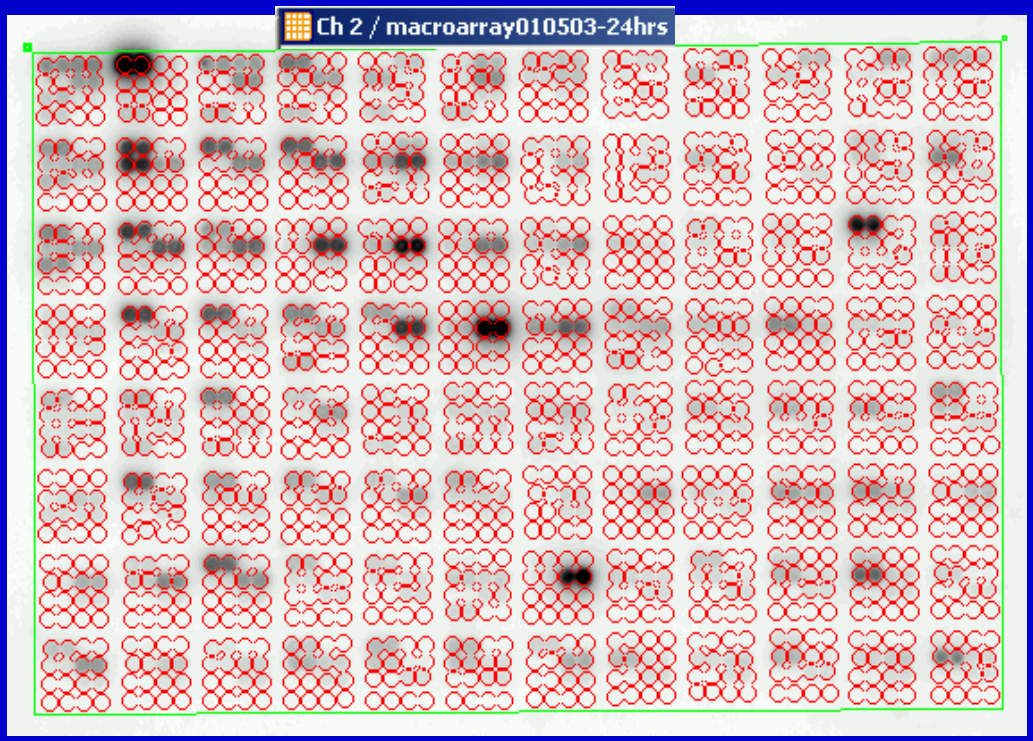


Cluster all GT TCs




Analysis Wizard
Analysis Protocol
Select the protocol from the drop-down list. Press New to generate a new protocol or press Edit to modify the selected one.
Protocol:
Current state(LA3)
New...
Edit...
Array type Radio-isotopic array
Analysis type Expression comparison
# of arrays 2
# of extra channels 0
Measure type Volume
Array organization 2 levels (1536 total spots)
Level 1 (Spots) 4 x 4
Level 2 (Spot groups) 8 x 12
Spot shape Circle
Start position Defined
Spot shape Circle
Start position Defined
Alignment Aligned
Labels Structured
Replication On
Background Surrounding spot groups (average)
Reference None
Anchors None
Comments
Edit...
<<Back
Next>>
Cancel
Help


Ch 1 / macroarray010503-0hr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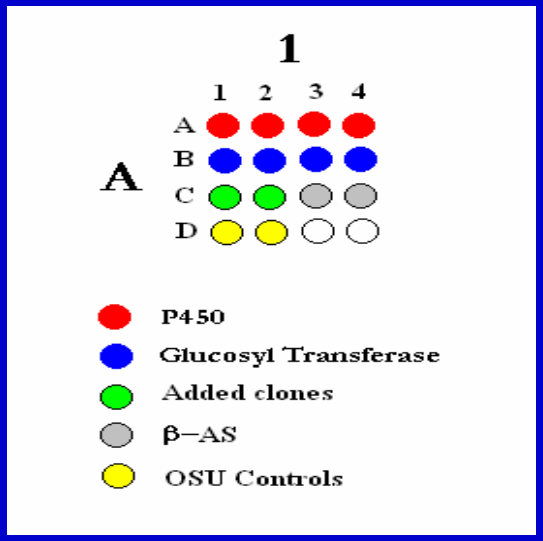
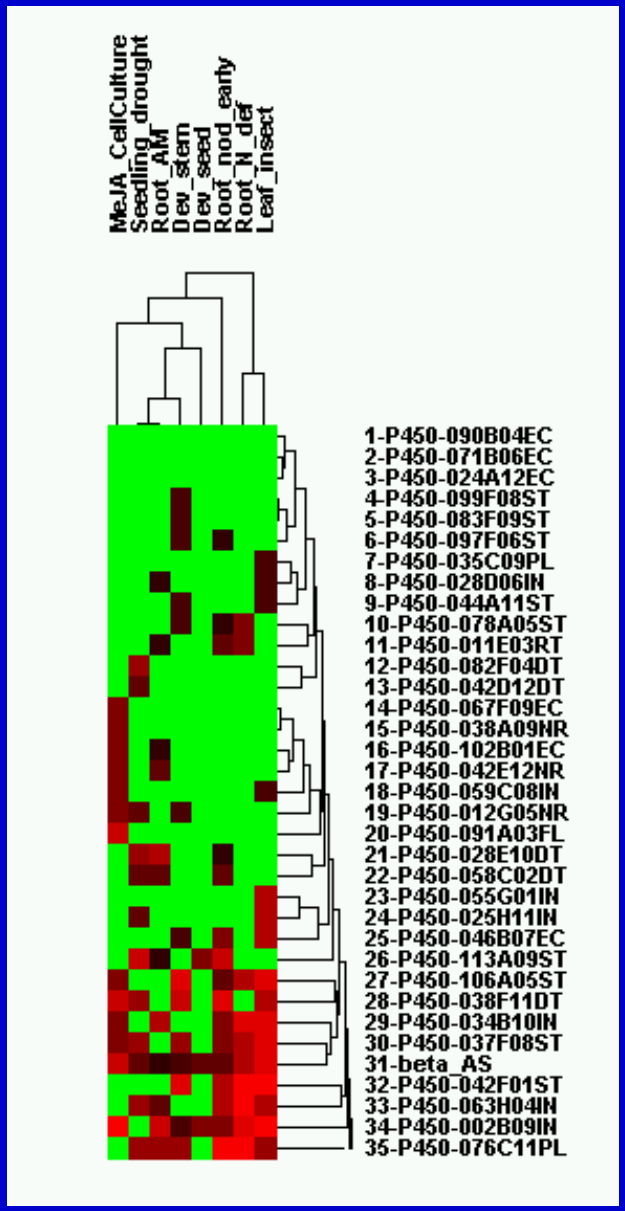
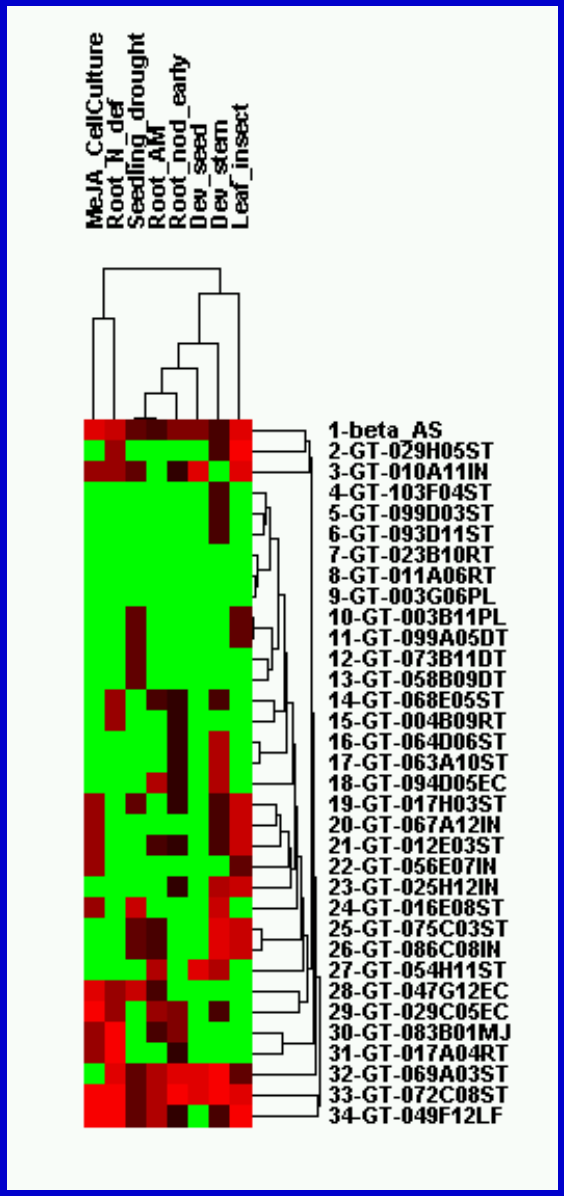

1
1 2 3 4
A
B
C
D
A
P450
Glucosyl Transferase
Added clones
β–AS
OSU Controls

Clustering P450- candidates



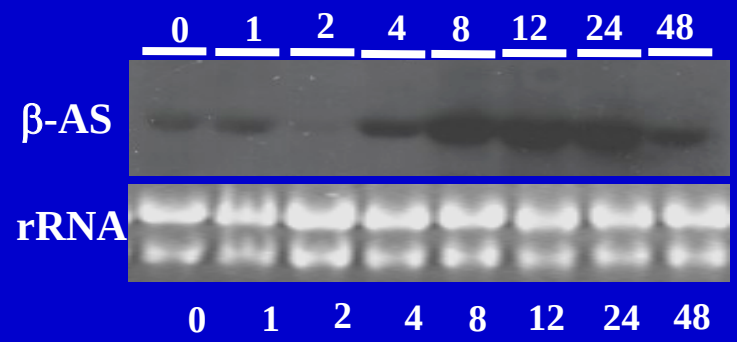
Clustering GT- candidates

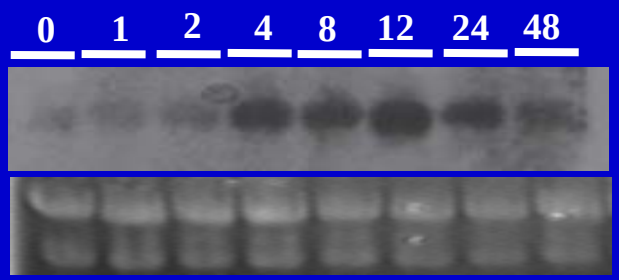


Functional characterization

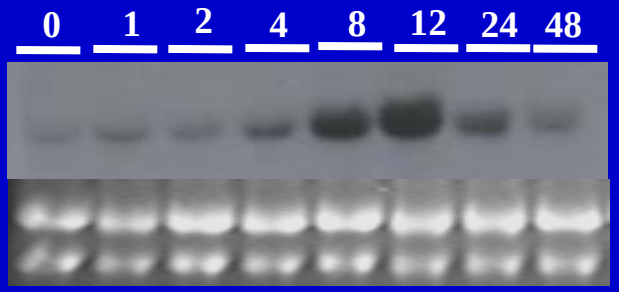
P450s- express in yeast harboring β-amyrin synthase and plant P450 reductase

GTs- express in E.coli, use trterpene aglycones as substrates





(3/8)P012G



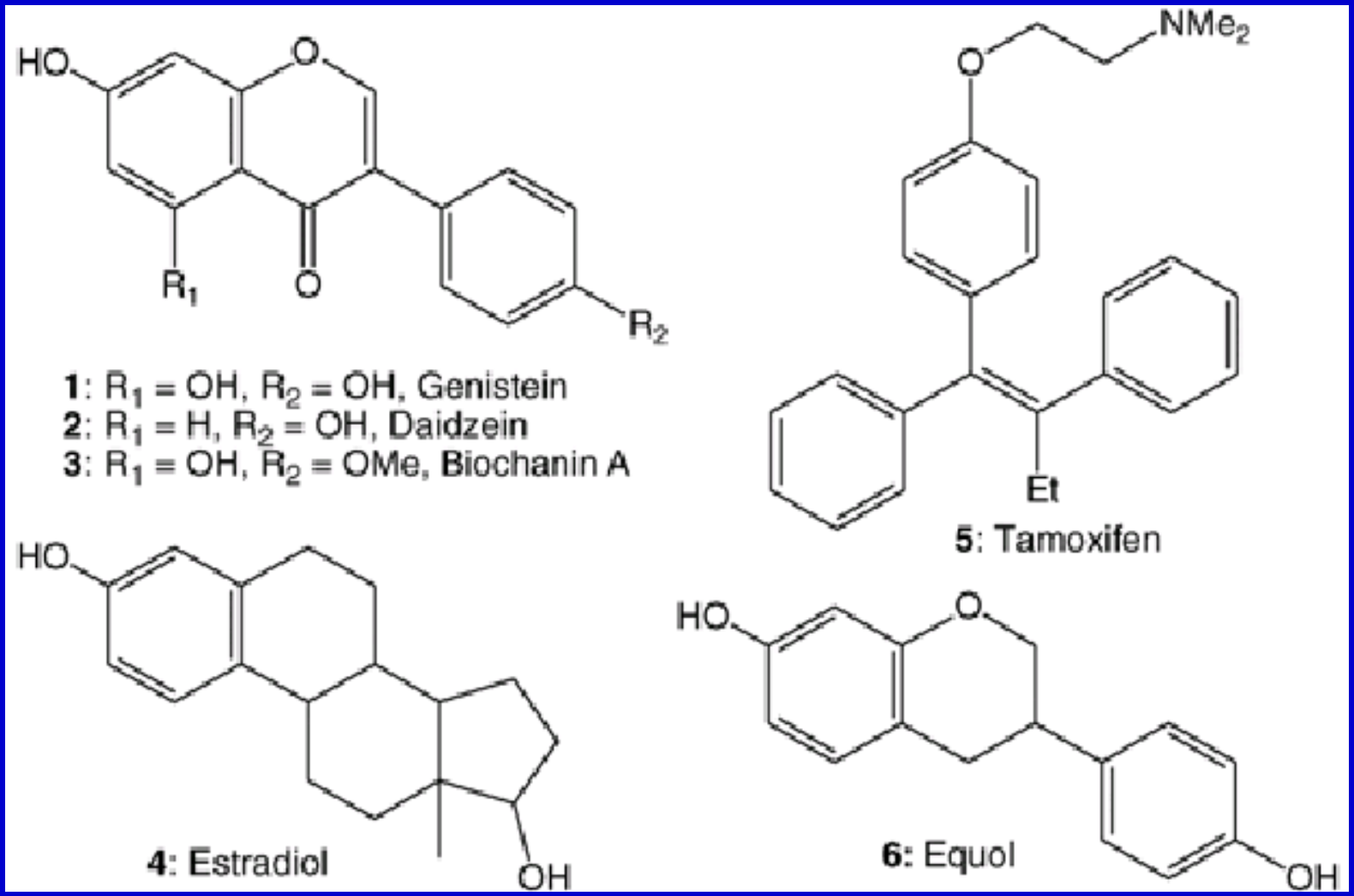
(5/8) GT068

Coordinated expression of candidate P450 and GT genes with β-amyrin synthase in MeJA-treated *M. truncatula* cell suspensions

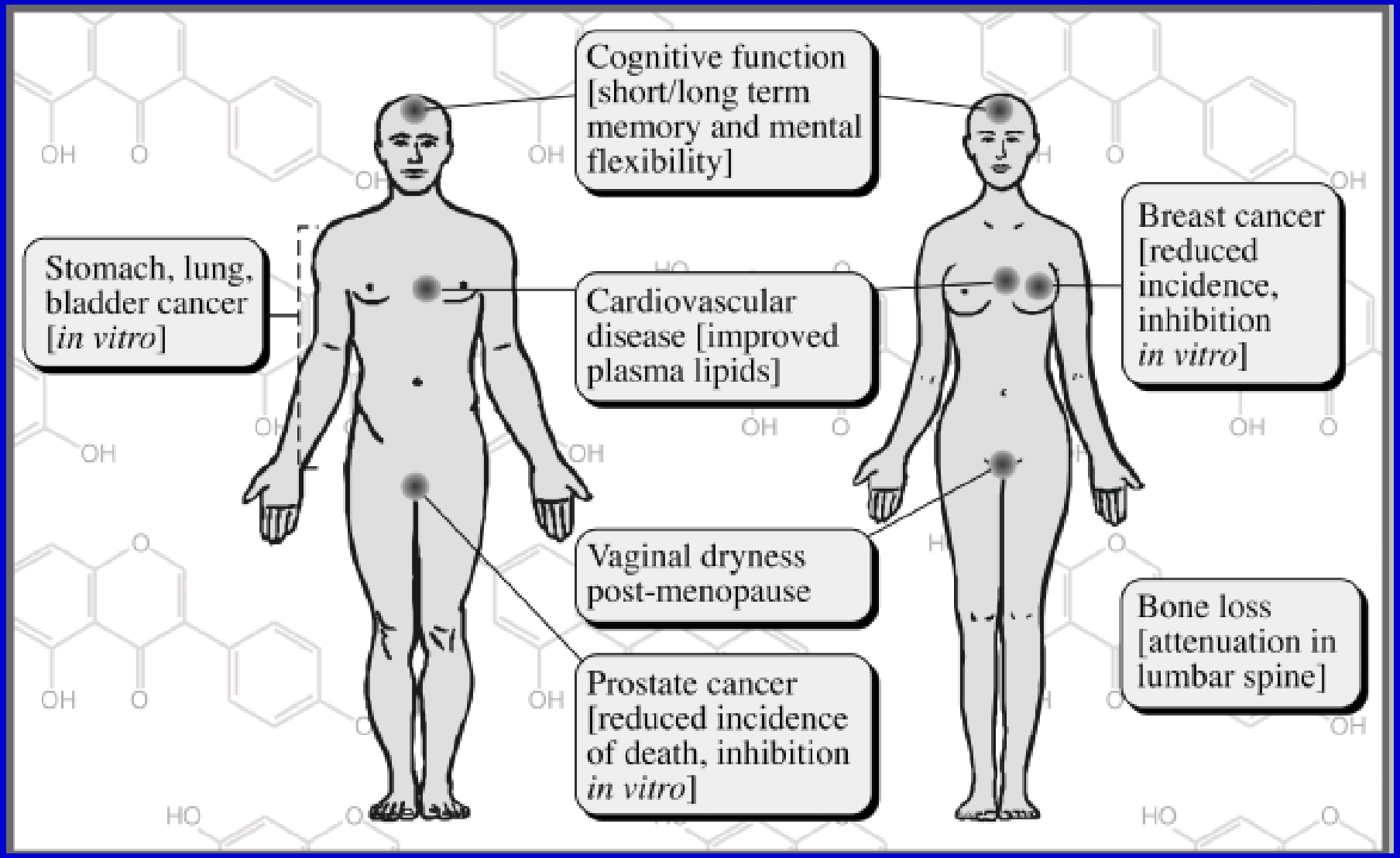
Biological activities of isoflavonoid compounds

- Antimicrobial phytoalexins
- Allelochemicals
- Anti-insect and piscicidal activity
- Nodulation gene inducers (soybean/Bradyrhizobium)
- Phytoestrogens (daidzein, genistein): anti-osteoporosis
- Anti-cancer

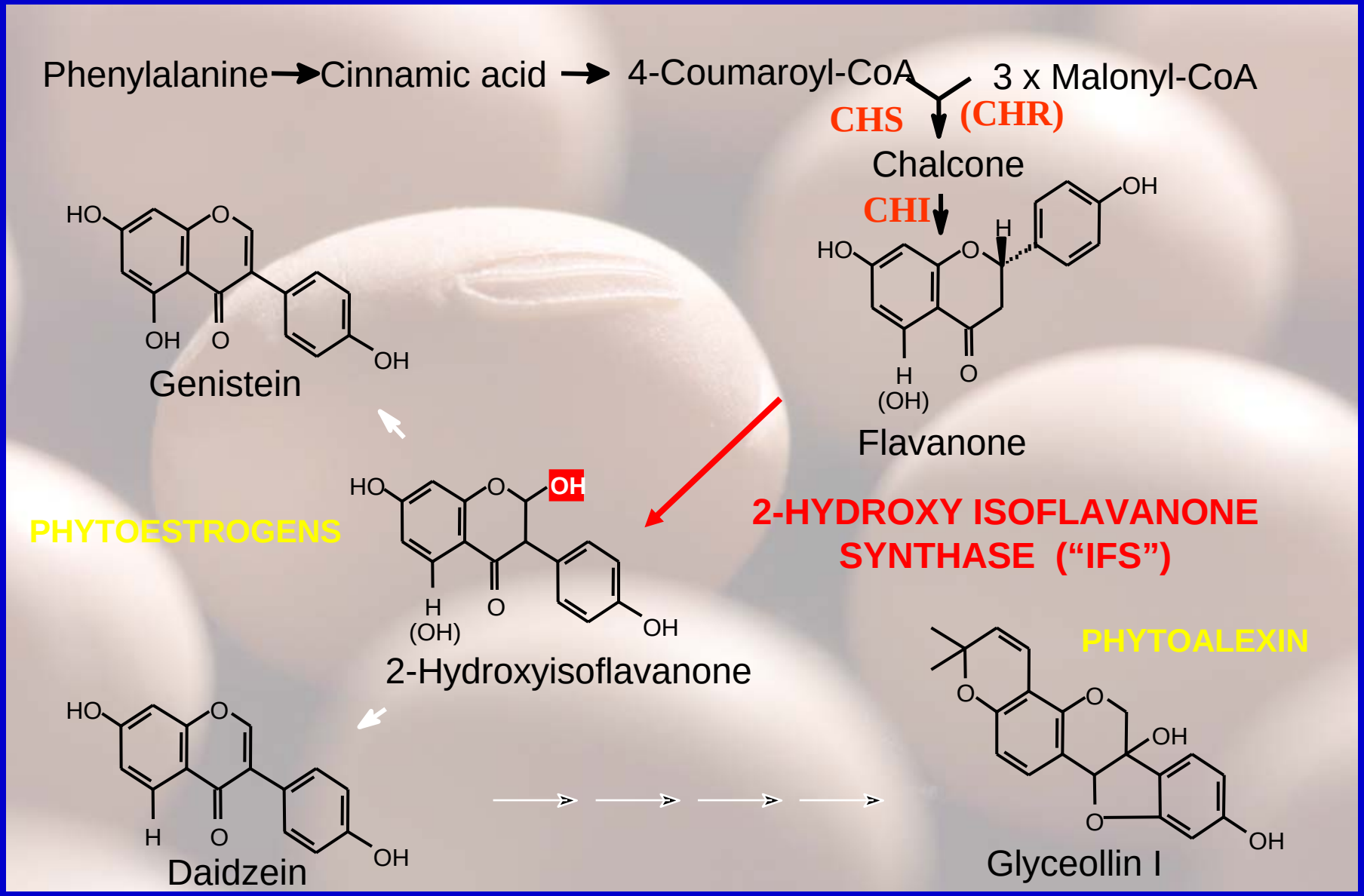
Structural similarities between isoflavone phytoestrogens and estradiol



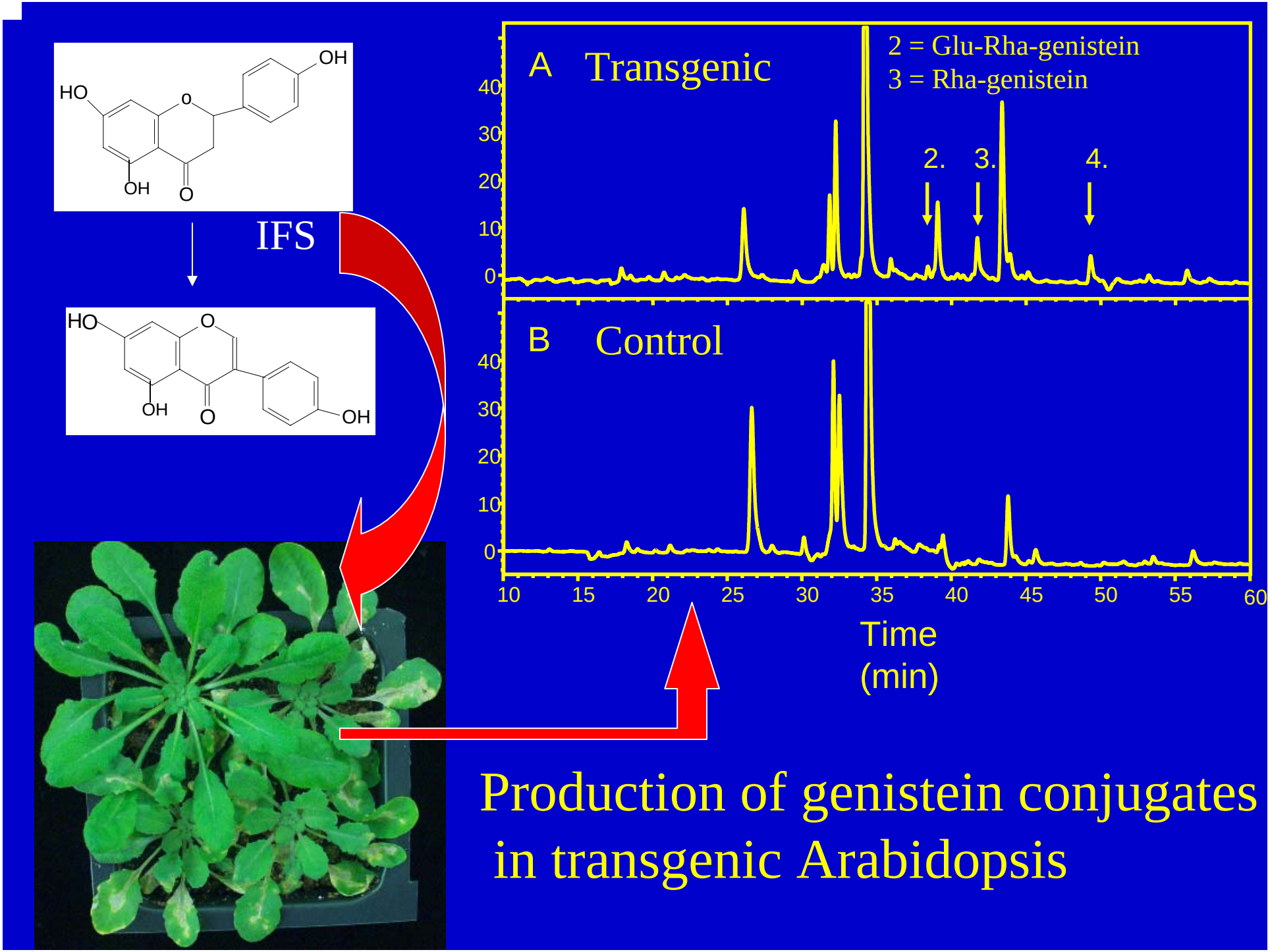
Beneficial effects of genistein on human health ?

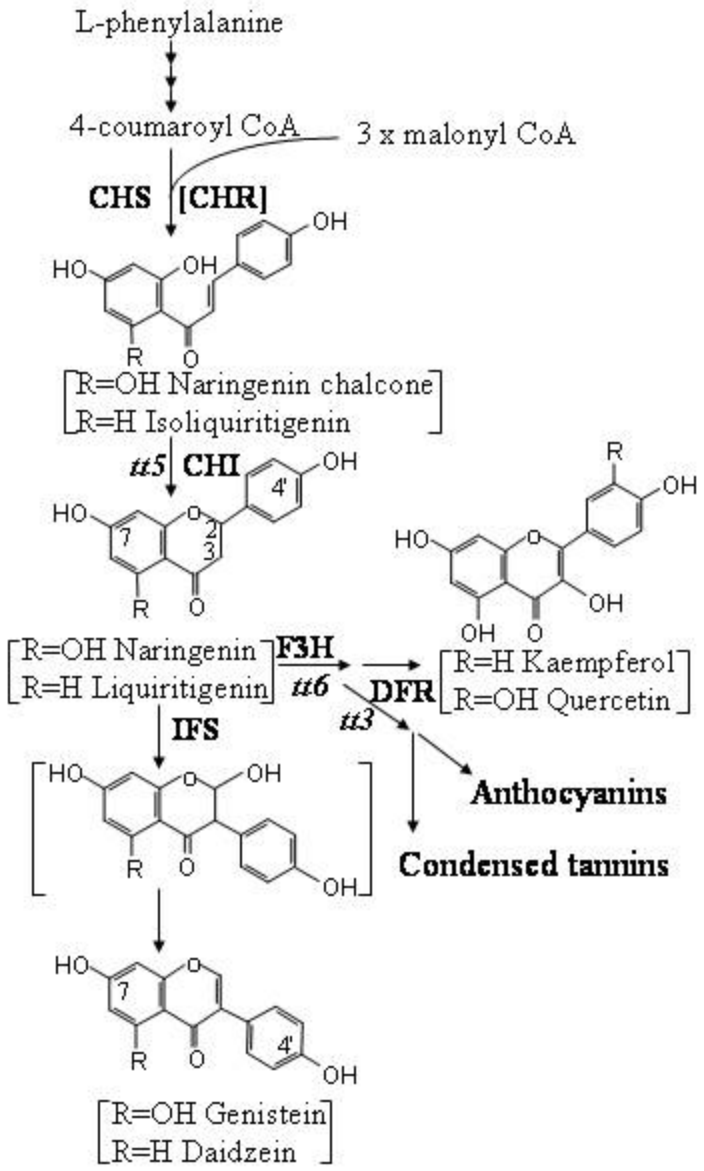


Isoflavonoid biosynthesis in soybean



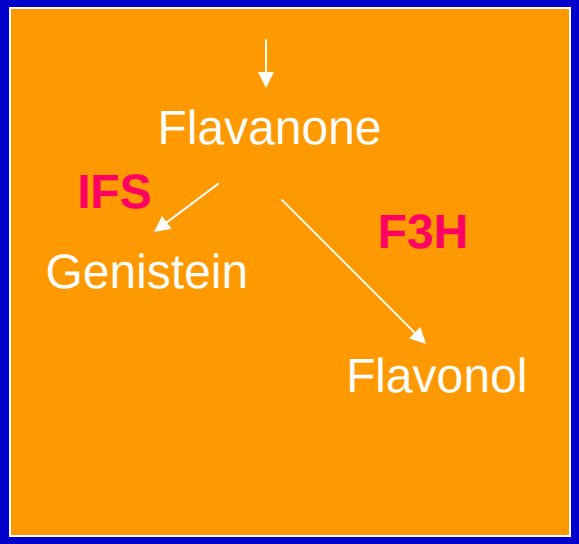
Steele et al. Arch Biochem Biophys 367: 146-150, 1999


HO
OH
O
OH
O
IFS
HO
O
OH
O
OH
A Transgenic
2 = Glu-Rha-genistein
3 = Rha-genistein
2.
3.
4.
B Control
0
10
20
30
40
10
15
20
25
30
35
40
45
50
55
60
Time
(min)
Production of genistein conjugates
in transgenic Arabidopsis

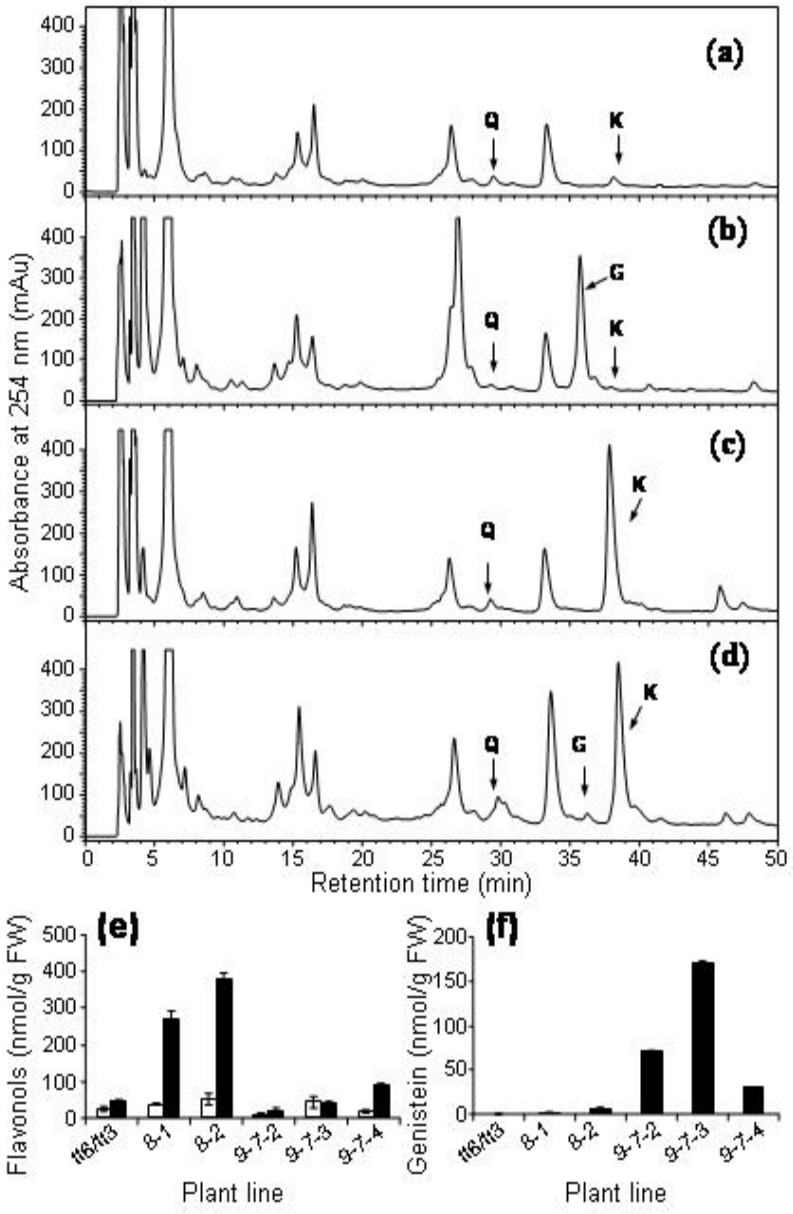

L-phenylalanine
4-coumaroyl CoA
3 x malonyl CoA
CHS
[CHR]
R=OH Naringenin chalcone
R=H Isoliquiritigenin
tt5
CHI
R=OH Naringenin
R=H Liquiritigenin
F3H
tt6
DFR
tt3
R=H Kaempferol
R=OH Quercetin
IFS
Anthocyanins
Condensed tannins
R=OH Genistein
R=H Daidzein

Successful engineering of isoflavones in Arabidopsis requires blocking flux into formation of flavonols

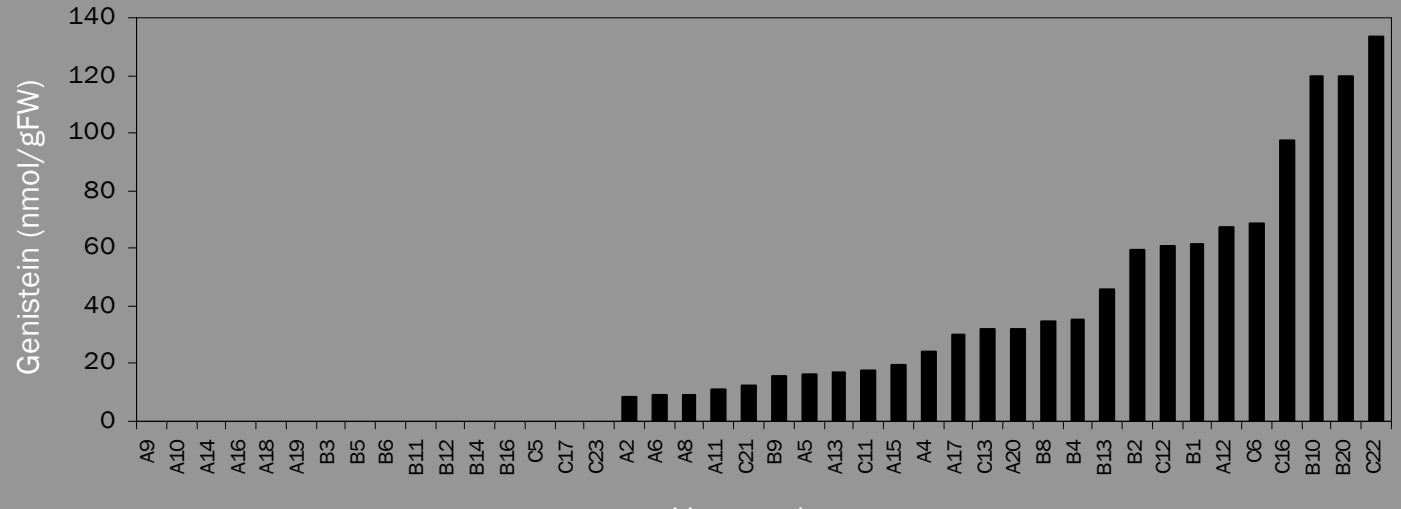
Competition between endogenous and introduced pathways

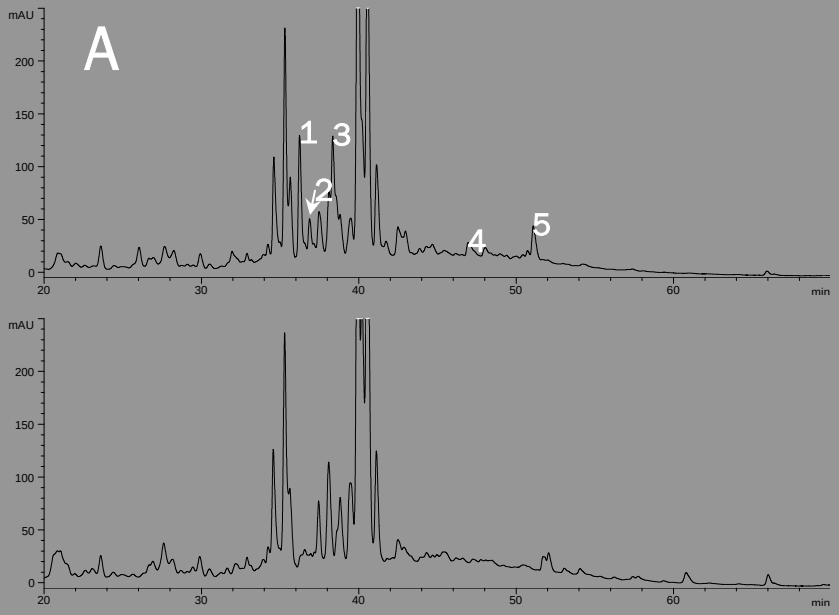


Liu et al. PNAS 99, 14778, 2002

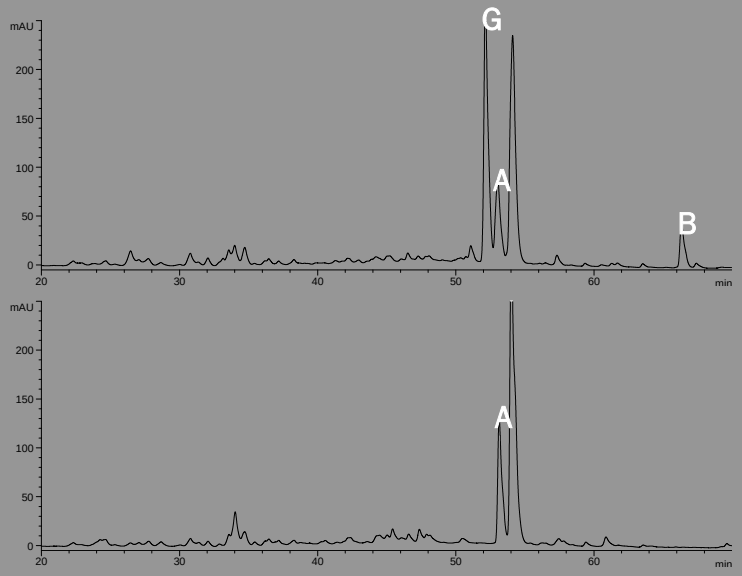


Accumulation of genistein in alfalfa leaves expressing 35S-IFS





Retention time

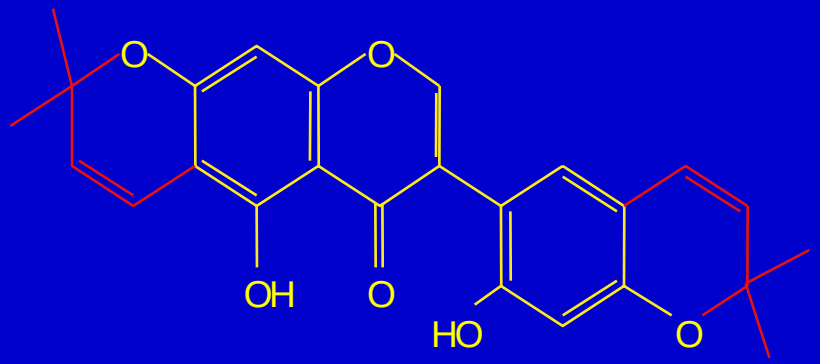


Retention time

data of Bettina Deavours

Genes for engineering complex (iso)flavonoids.

-importance of prenyltransferases

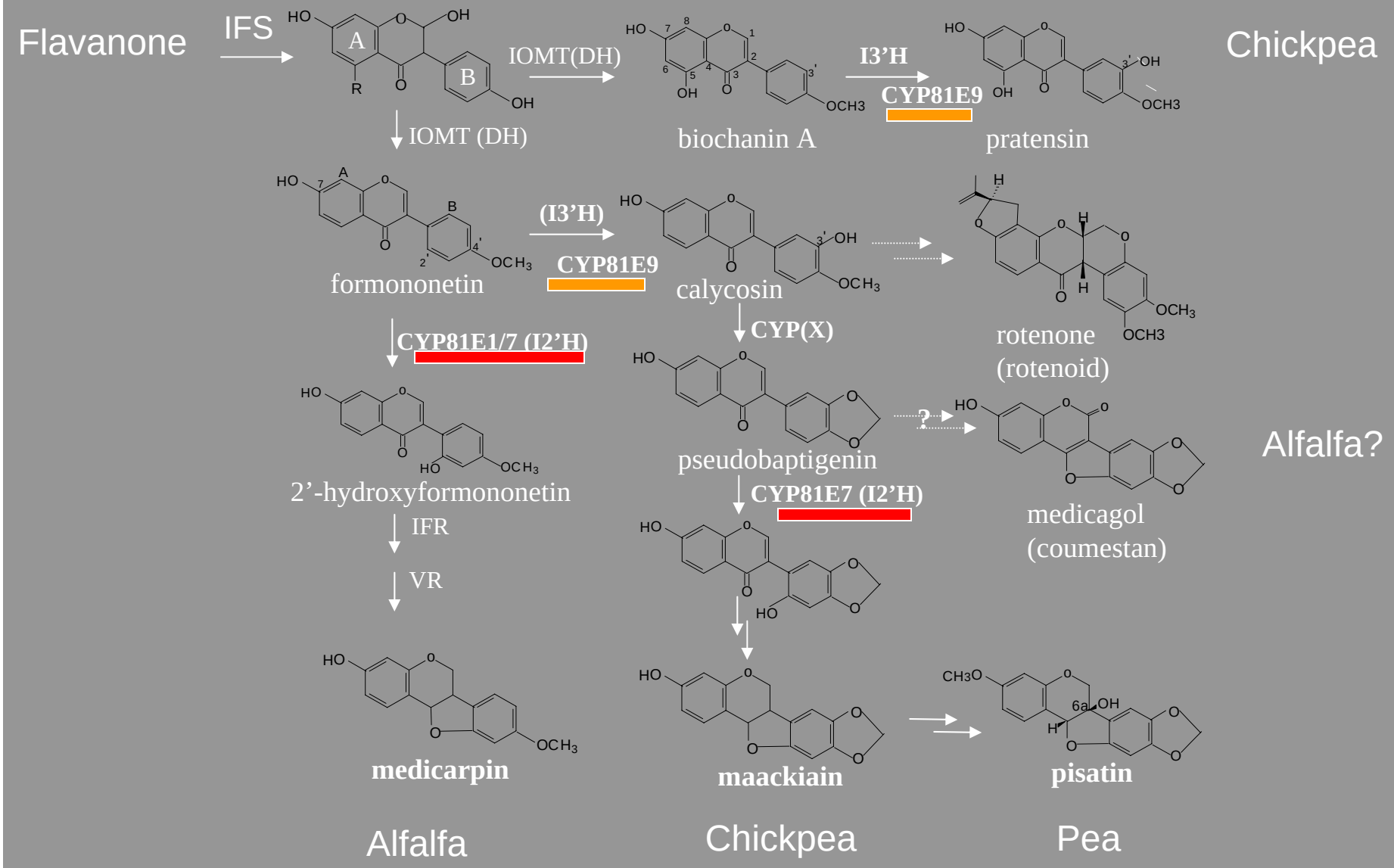


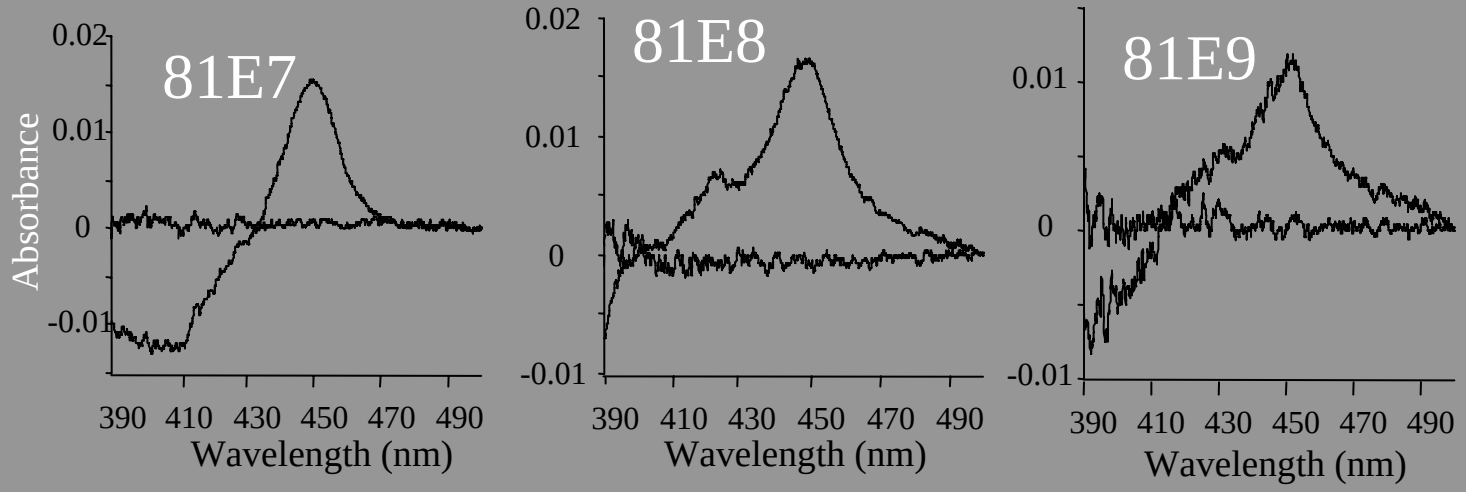
Kraussianone 1

- Component of roots of *Eriosema kraussianum*
  - South African shrublet
  - Component of “uBangalala”
  - Used by Zulus to treat impotency and infertility

    -75% activity of Viagra- PhytoViagra?

Interconversions among isoflavonoids- role of isoflavone 2'- and 3'-hydroxylases



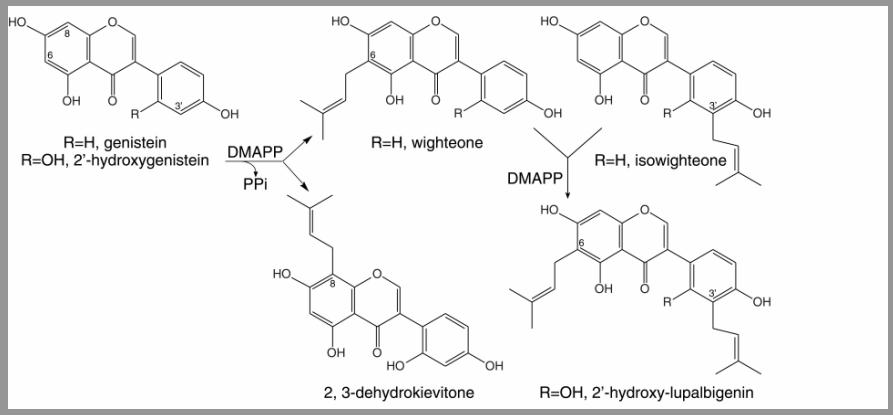


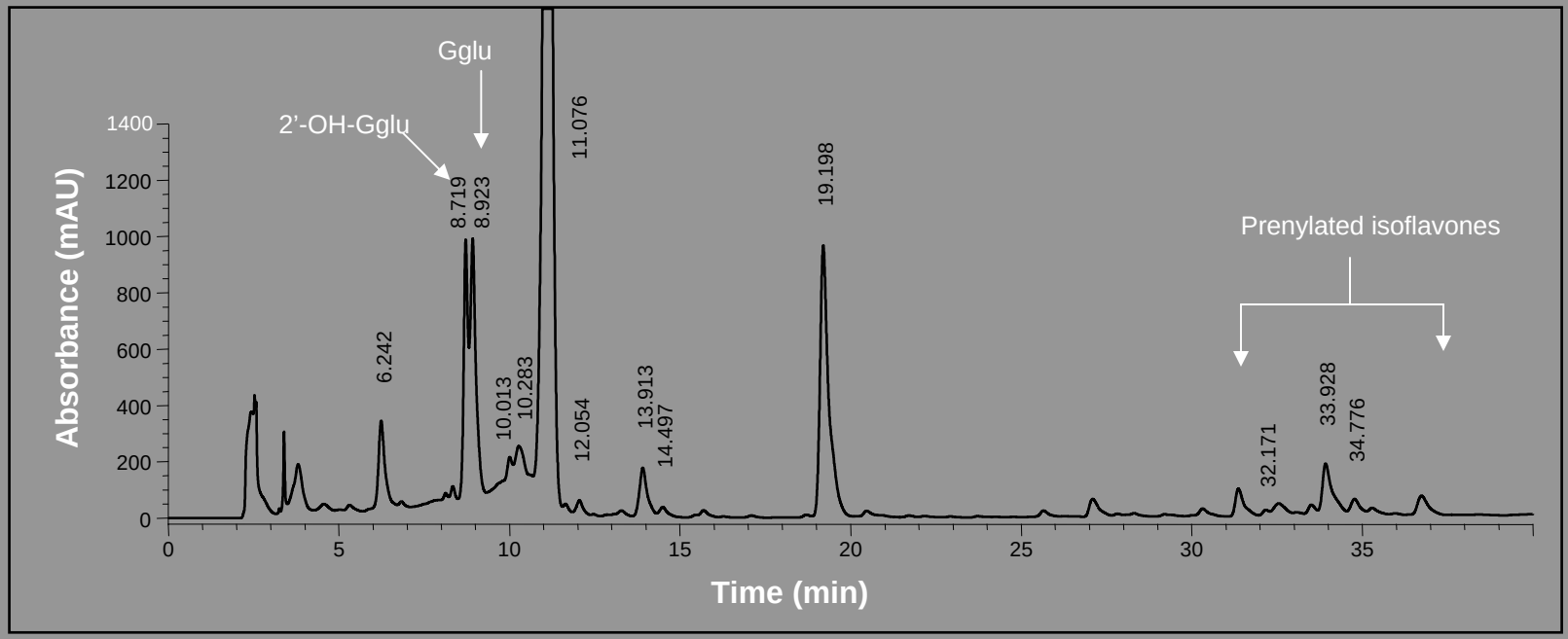
Expression of *M. truncatula* CYP81E enzymes in yeast

Liu et al. Plant Journal 36: 471-484

A genomics approach for the discovery of (iso)flavonoid prenyltransferases

Data mining of EST libraries from:
*Elicited soybean cell cultures
*Lupin roots
*Maclura pomifera fruit
*Hop peltate trichomes (at U. of York)





Production of prenylated isoflavonoids by lupin roots

Conclusions

- Legumes are a rich source of natural products that impart useful traits
- *Medicago truncatula* is an excellent model system for legume genomics
- Phenylpropanoid compounds can positively affect plant, animal and human health
- Genomics approaches can now be applied for natural product pathway gene discovery – increased efforts in structural biology will facilitate progress
- Commercial development of transgenics modified in natural product pathways (output traits) is in the pipeline
- The possibility exists to produce “isometabolic” plant lines for testing effects of plant natural products on animal health

Metabolic engineering of (iso)flavonoids for validating effects on human and animal health

PAP1

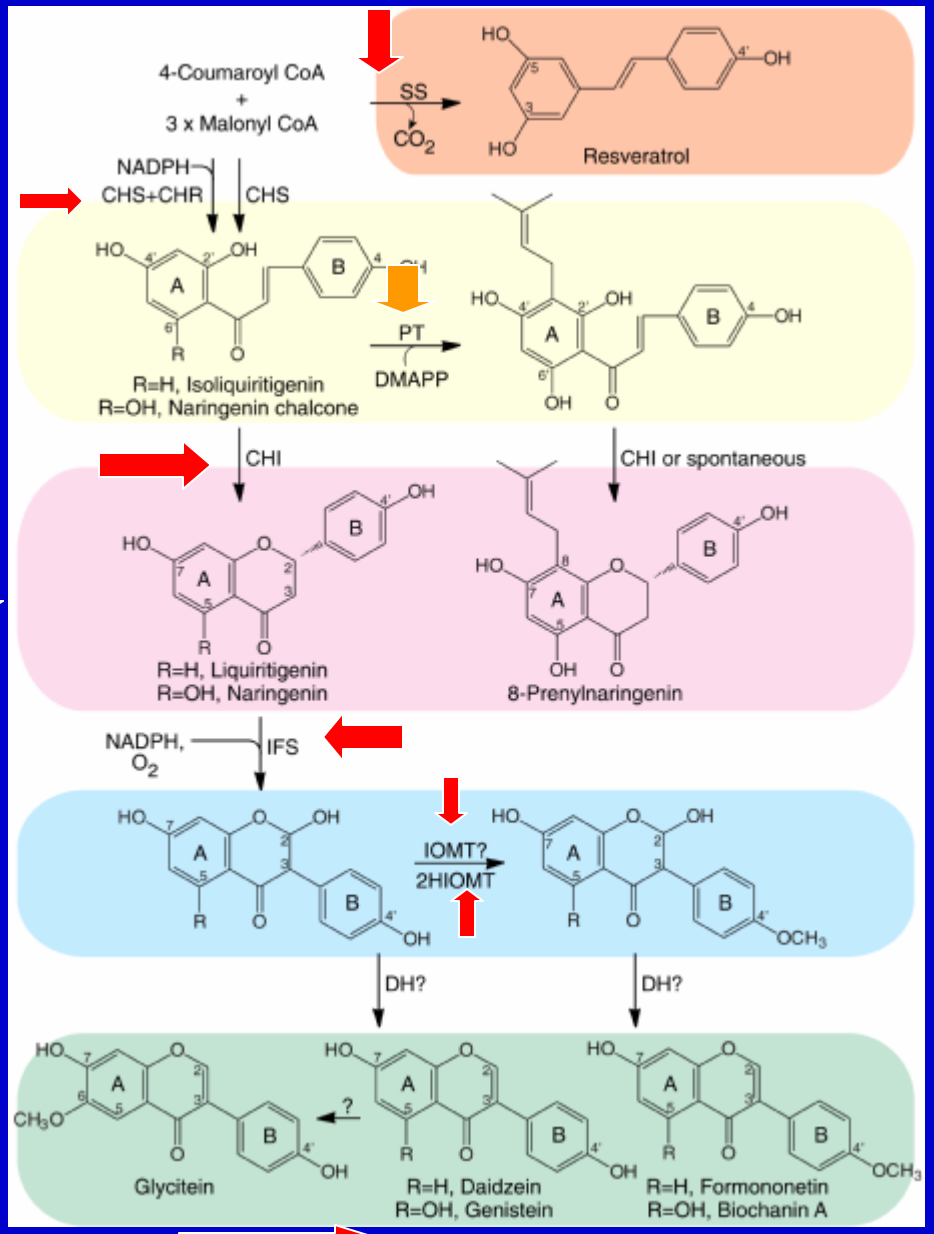
Flavonols

Anthocyanins

ANR

Epicatechin

Tannins



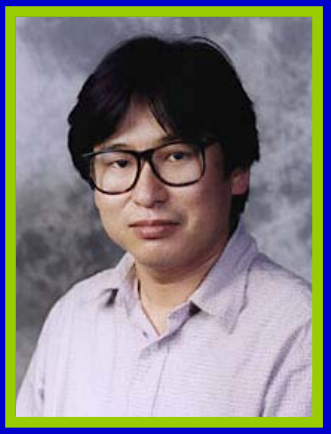
Complex isoflavonoids

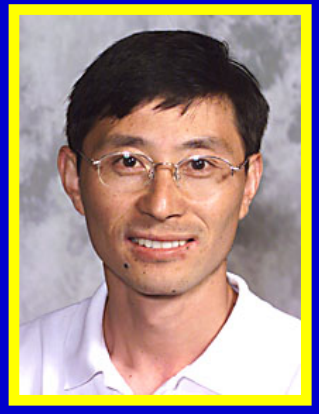










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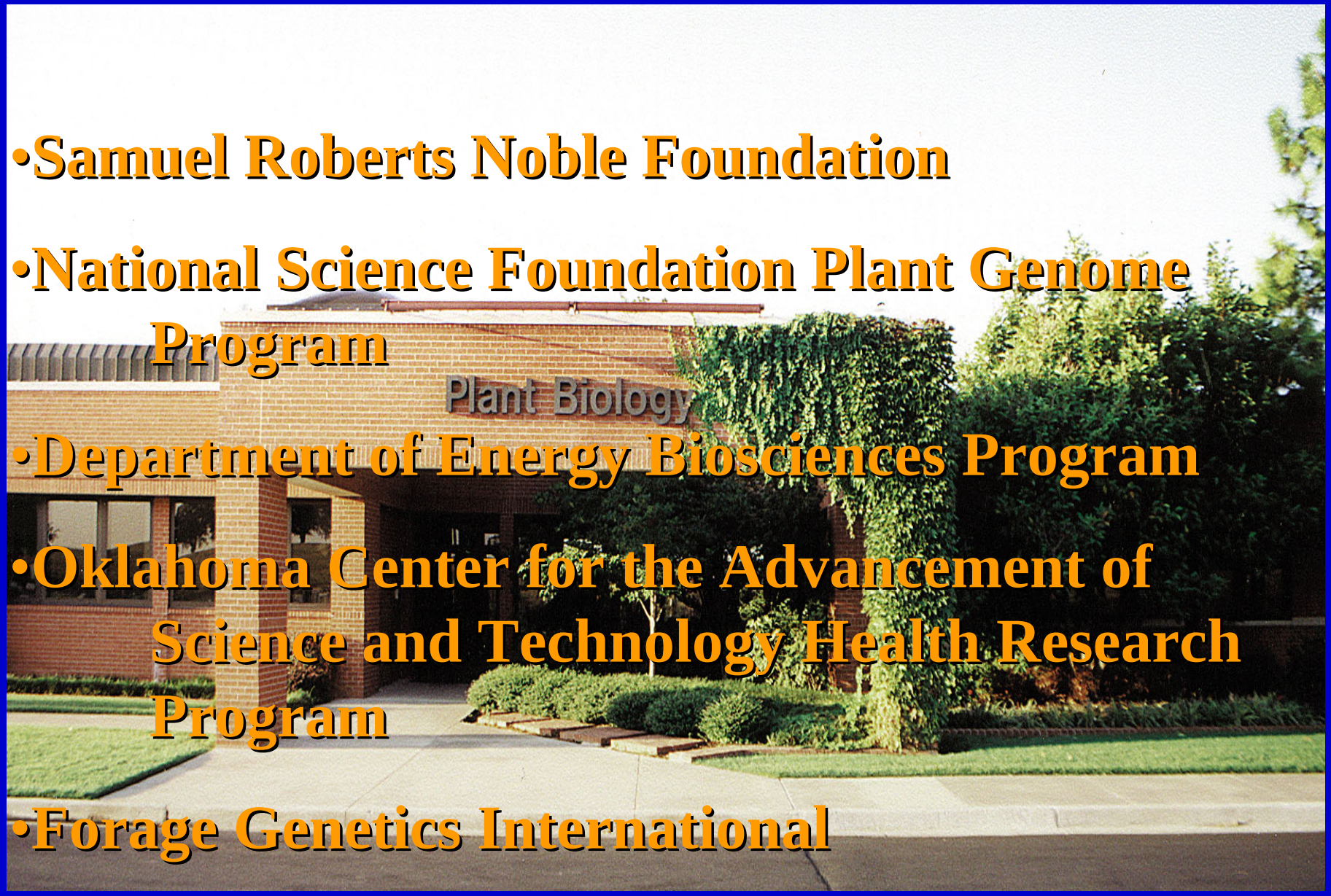
Funding from:



- **Samuel Roberts Noble Foundation**
- **National Science Foundation Plant Genome Program**
- **Department of Energy Biosciences Program**
- **Oklahoma Center for the Advancement of Science and Technology Health Research Program**
- **Forage Genetics International**