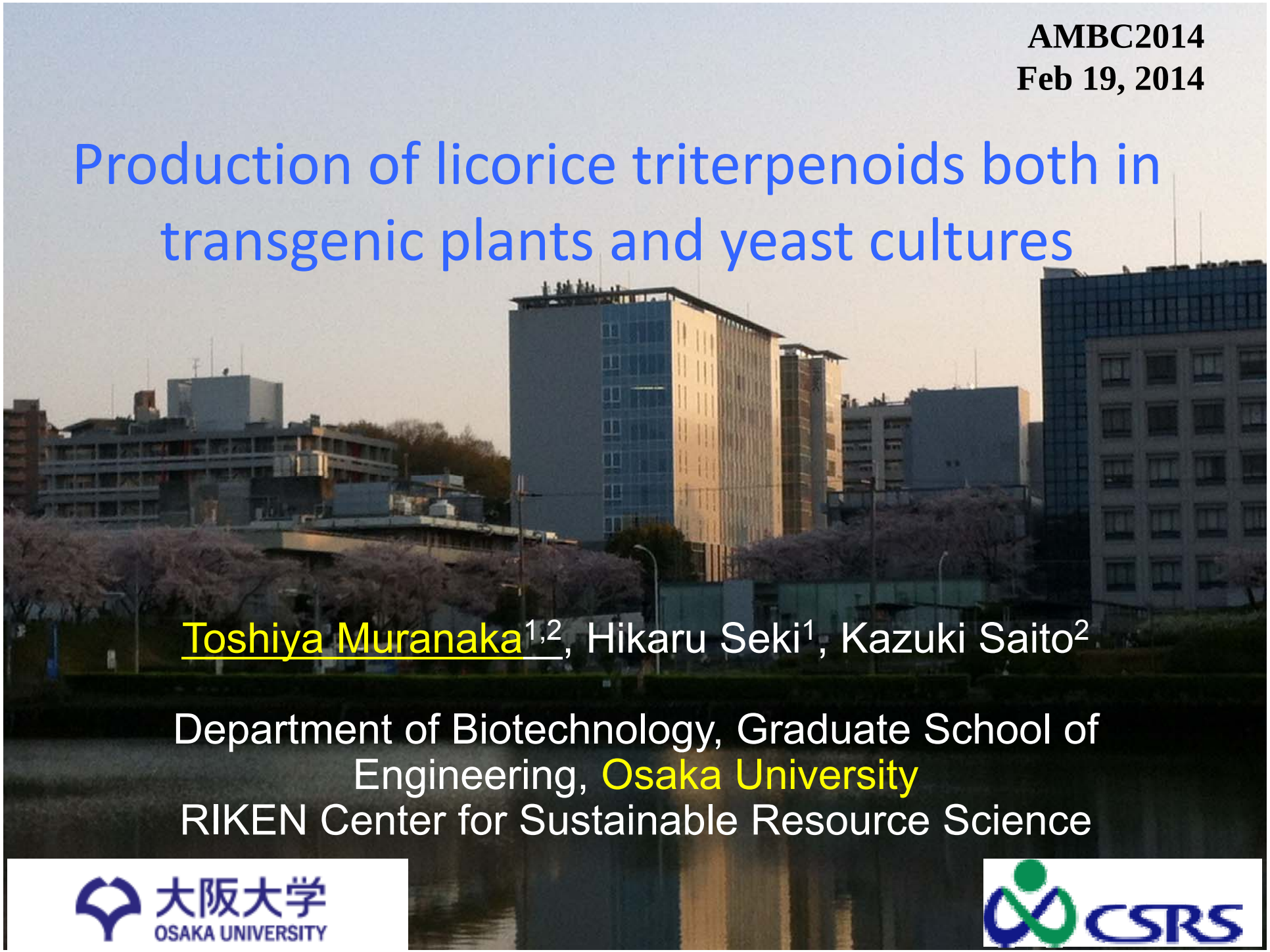


AMBC2014
Feb 19, 2014

Production of licorice triterpenoids both in transgenic plants and yeast cultures

Toshiya Muranaka[1,2], Hikaru Seki[1], Kazuki Saito[2]

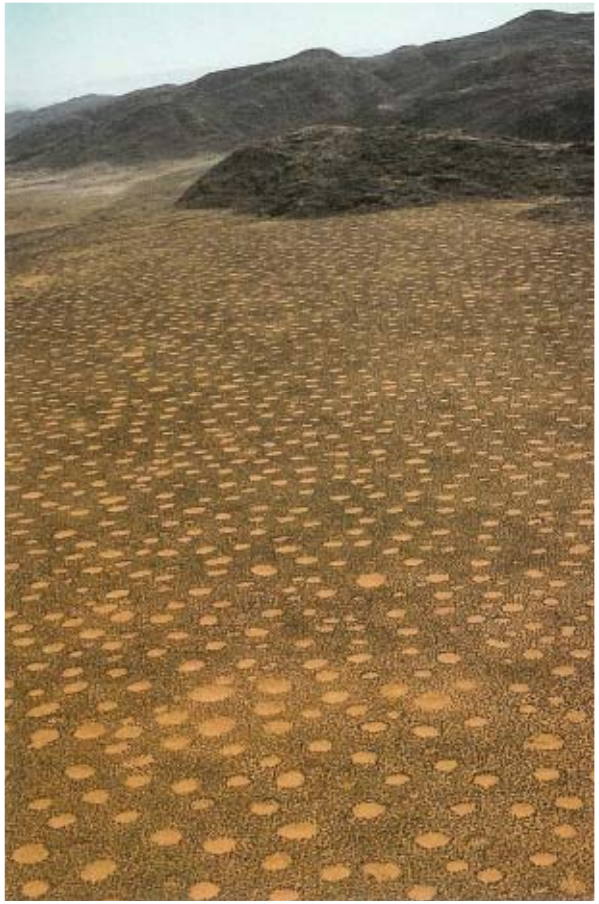
Department of Biotechnology, Graduate School of Engineering, Osaka University
RIKEN Center for Sustainable Resource Science

大阪大学
OSAKA UNIVERSITY

CSRS

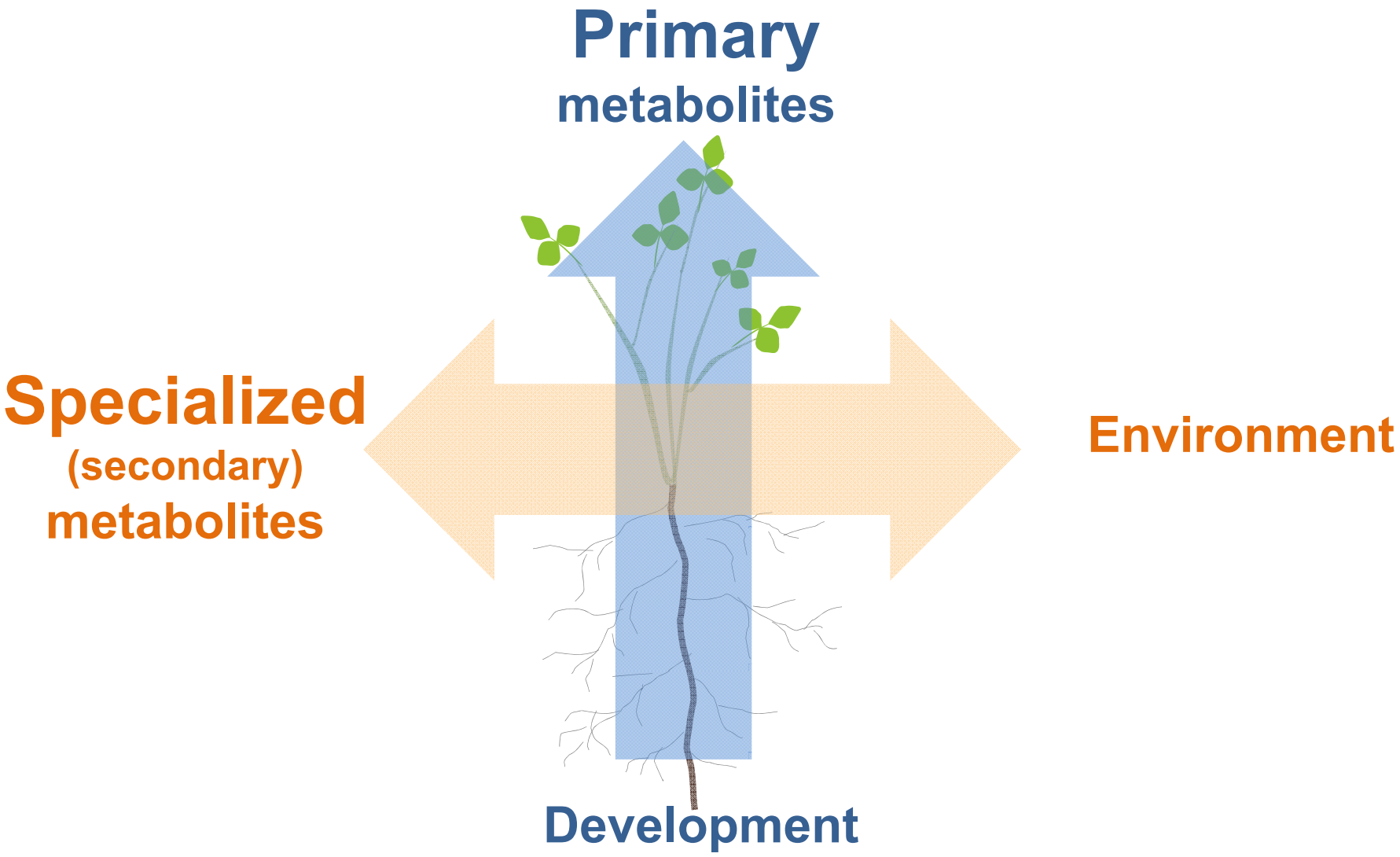






1,000,000

Plants: Metabolites



Plants: Specialized metabolites

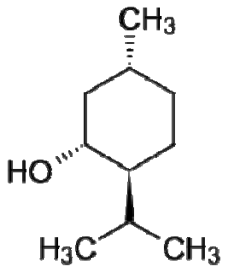
Terpenoids

Phenolic compounds

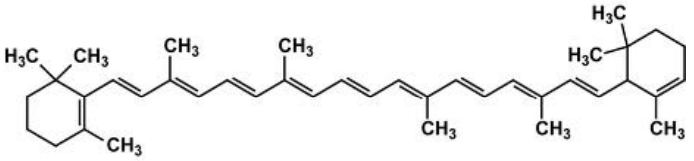
Alkaloids

1,000,000

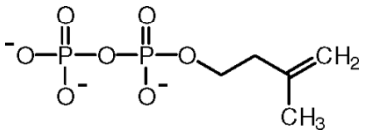
As chains of isoprene units are built up, the resulting terpenes are classified sequentially by size as mono-, sesqui-, di-, tri-, and tetra-terpenes



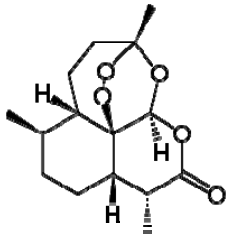
Menthol
(C5 x 2, monoterpene)



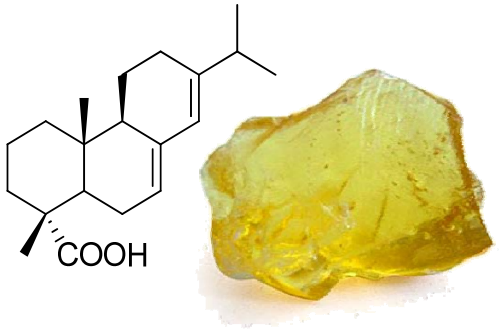
Carotene
(C5 x 8, tetraterpene)



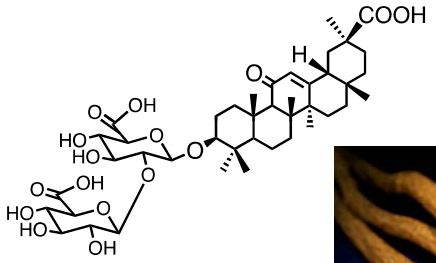
isopentenyl diphosphate (IPP)



Artemisinin
(C5 x 3, sesquiterpene)



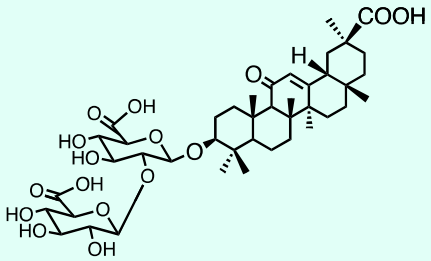
Rosin
(C5 x 4, diterpene)



Glycyrrhizin
(C5 x 6, triterpene glycoside)

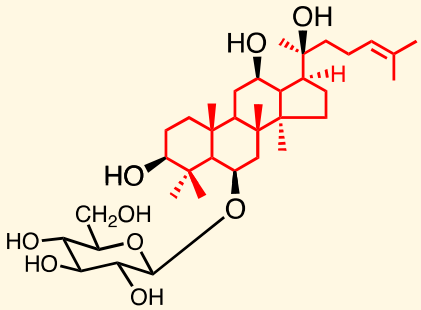
Triterpenoids include various compounds known as main active constituents of medicinal plants (crude drugs)

Glycyrrhizin (licorice, *Glycyrrhiza* sp.)



Glycyrrhizin
(hepatoprotective drugs, natural sweetener)

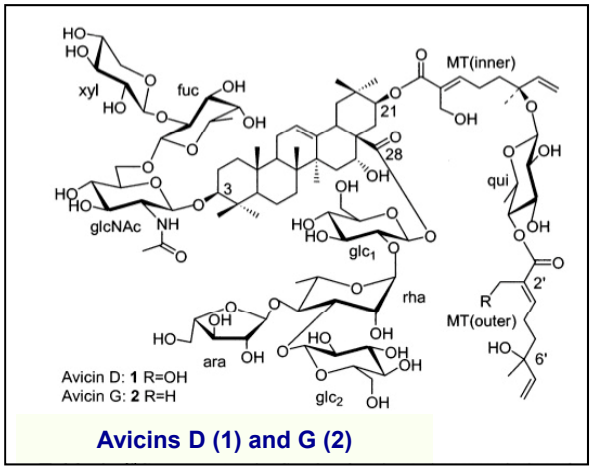
Gisenosides (*Panax ginseng*)



20(S)-ginsenoside Rh1

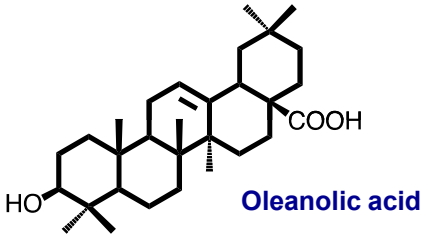
http://www.itmonline.org/arts/sars.htm

- Avicin (*Acacia victoriae*): anti-tumor activities
  (*PNAS* 98, 5821-5826, 2001)



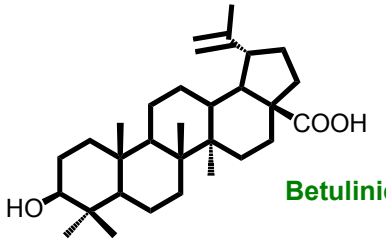
Avicins D (1) and G (2)

- Oleanolic acid: active against *Streptococcus mutans*



Oleanolic acid

- Betulinic acid: its derivative (PA-457) is being developed as an anti-HIV drug

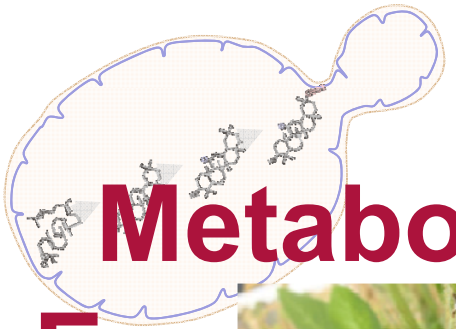


Betulinic acid

Problem: Research focus

Production *in planta;* Low or in Rare Plant

Chemical synthesis difficult



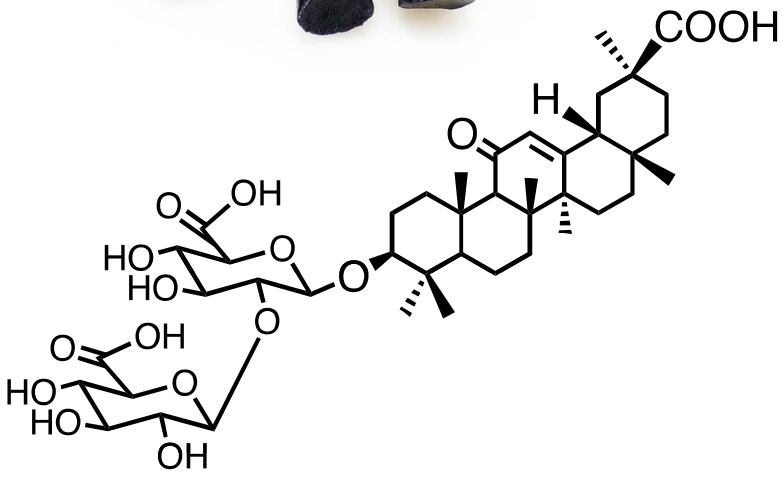
Metabolic Eng

Elucidating pathways at the molecular level will help increase the production levels within native hosts and facilitate engineering of heterologous hosts

Licorice

Glycyrrhiza spp.

ชะเอมเทศ



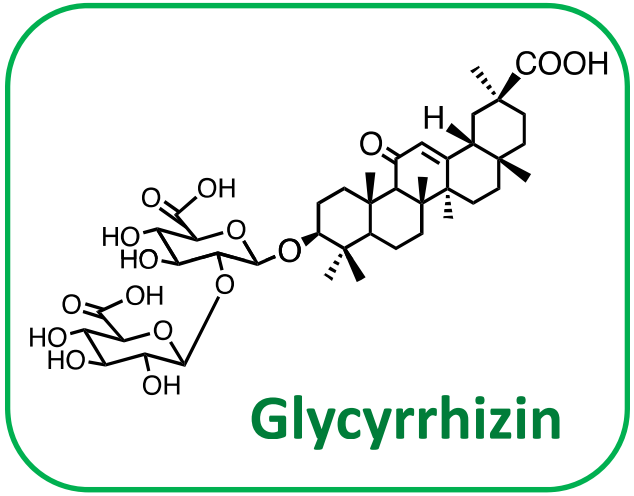
glycyrrhizin

Introduction

Licorice root and its products

- Licorice is a plant belonging to a genus of *Glycyrrhiza* in the legume family.
- The major active ingredient of licorice is **glycyrrhizin**.
- Glycyrrhizin is a **triterpenoid saponin** that is a group of natural organic compounds that are constituted by 6 isoprene units.

Licorice　Licorice roots



Glycyrrhizin

sweetener

Glycyrrhizin is 150 times sweeter than sugar.

medicinal drug

- Anti-inflammatory
- Hepatoprotective

Chinese herbal medicine

Licorice root is used in 70% of Kampo formulations.

Glycyrrhizin is isolated from “Rare Plant”

Primary & secondary processing / isolation & extraction

Hydrolysis / purification

Licorice root

3–5 years for harvest

Potential risk of shortage

Restrictions on exports

glycyrrhizin

（Sweetener, pharmaceuticals）

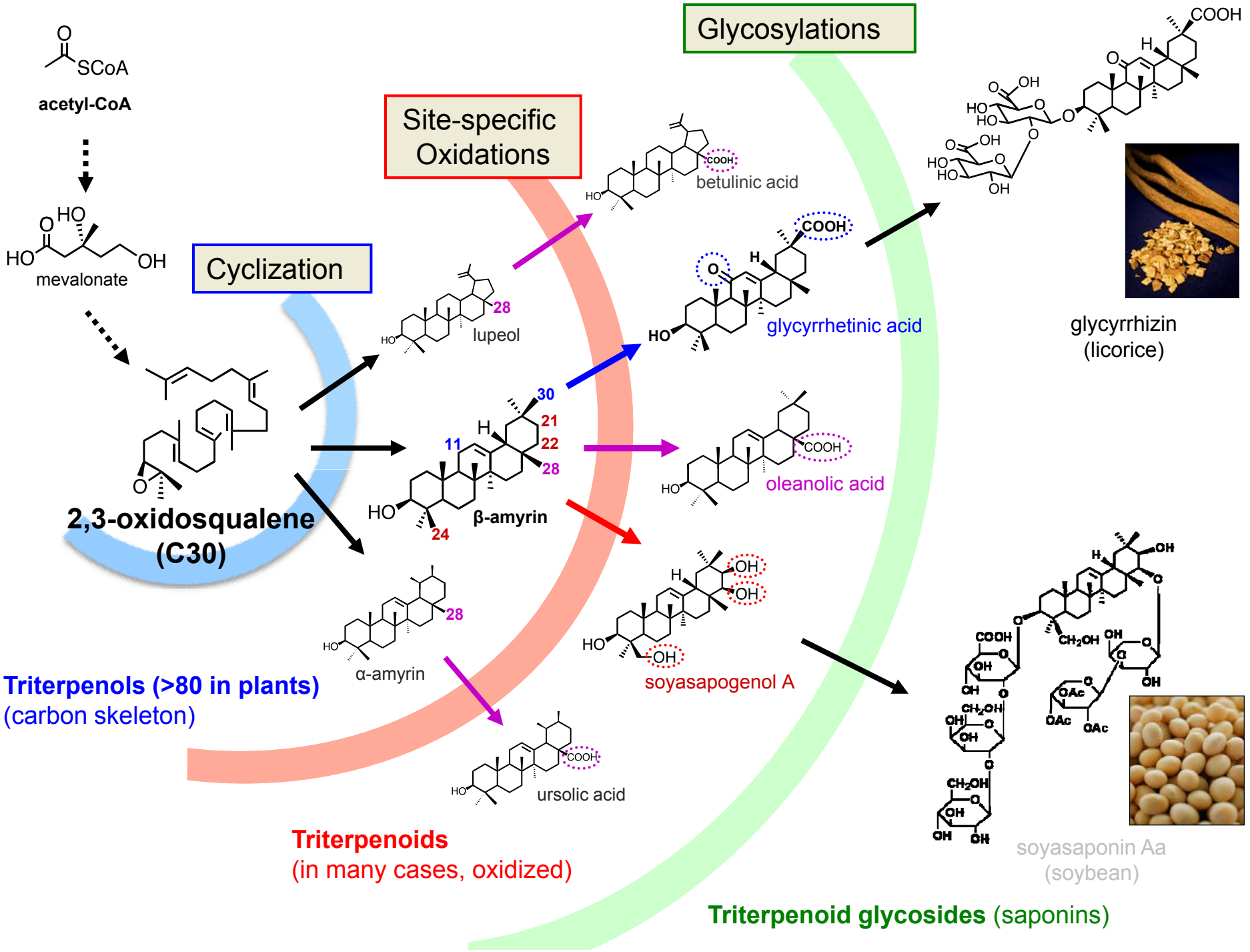
glycyrrhetinic acid

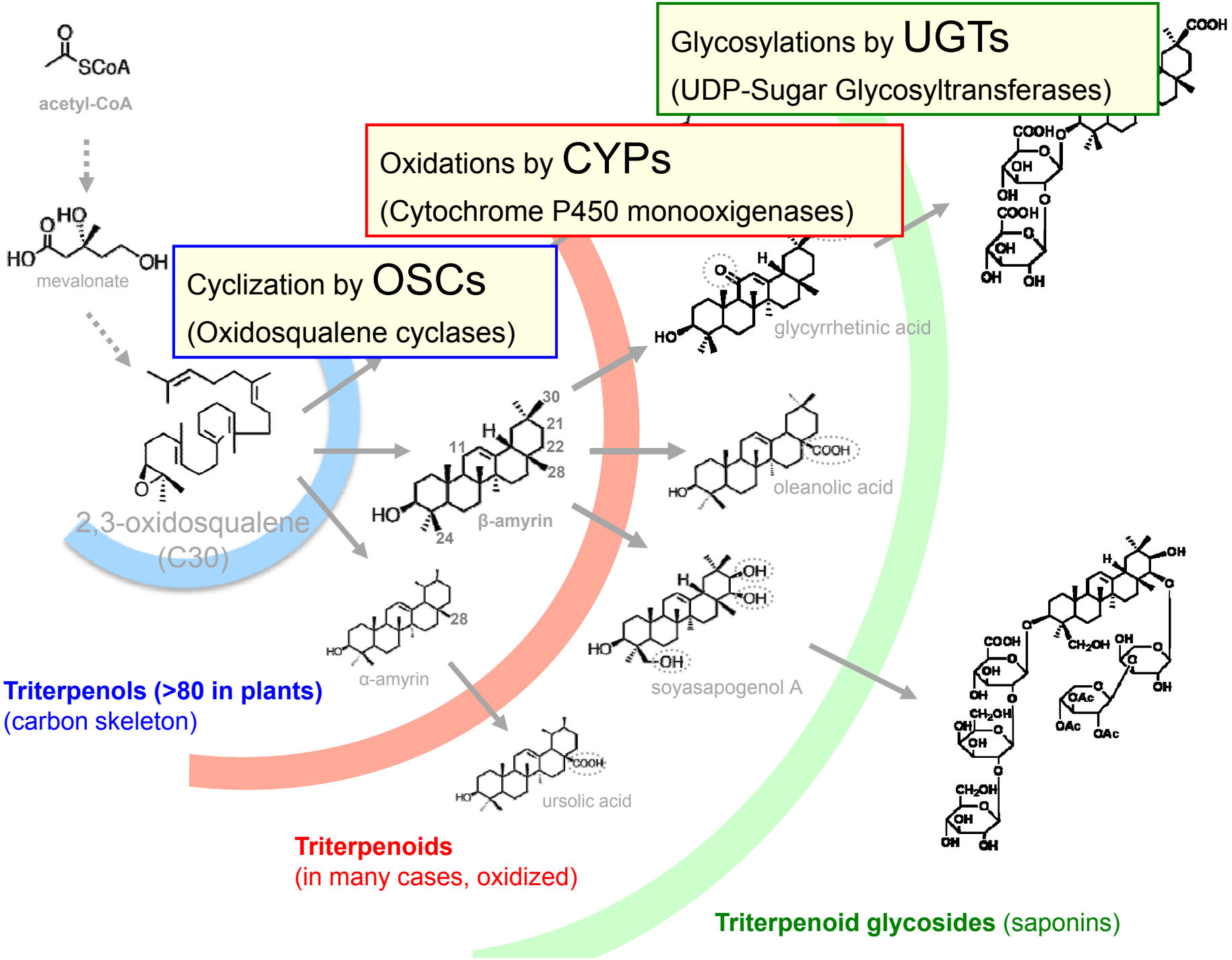
（Cosmetic, toiletry）

Market(Japan)

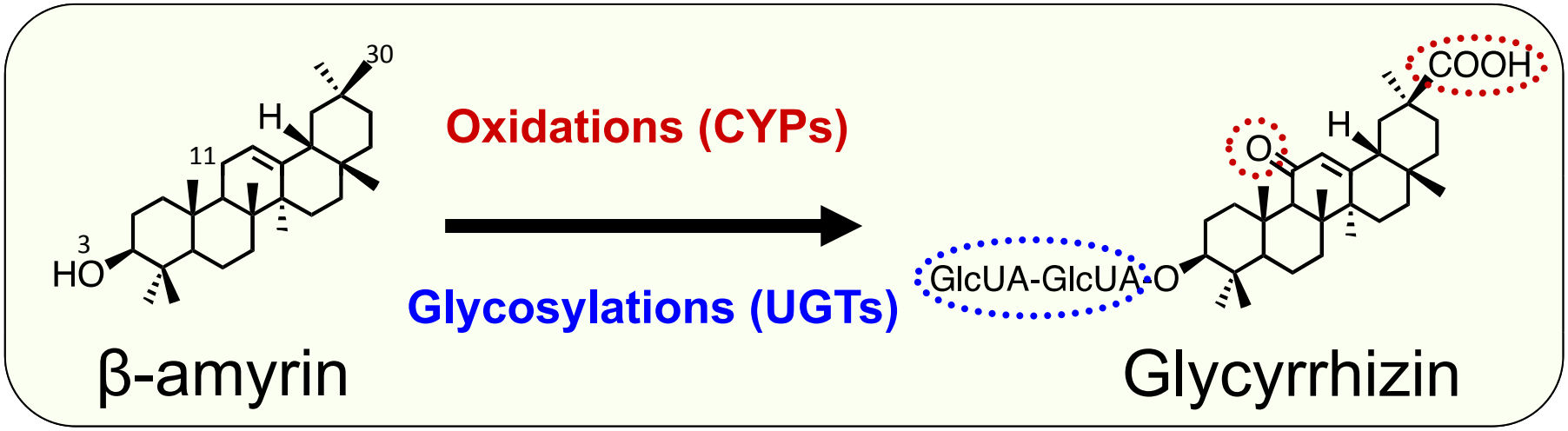
120 t, 2.4 Billion yen

30 t, 2.4 Billion yen


Glycosylations
Site-specific Oxidations
Cyclization
acetyl-CoA
SCoA
mevalonate
HO
OH
2,3-oxidosqualene (C30)
lupeol
β-amyrin
α-amyrin
ursolic acid
betulinic acid
glycyrrhetinic acid
oleanolic acid
soyasapogenol A
glycyrrhizin (licorice)
soyasaponin Aa (soybean)
Triterpenols (>80 in plants)
(carbon skeleton)
Triterpenoids
(in many cases, oxidized)
Triterpenoid glycosides (saponins)


Glycosylations by UGTs
(UDP-Sugar Glycosyltransferases)
Oxidations by CYPs
(Cytochrome P450 monooxigenases)
Cyclization by OSCs
(Oxidosqualene cyclases)
acetyl-CoA
mevalonate
2,3-oxidosqualene
(C30)
β-amyrin
α-amyrin
ursolic acid
glycyrrhetinic acid
oleanolic acid
soyasapogenol A
Triterpenols (>80 in plants)
(carbon skeleton)
Triterpenoids
(in many cases, oxidized)
Triterpenoid glycosides (saponins)

Glycyrrhizin is derived from β-amyrin, one of the most commonly found triterpenes in plants



A series of oxidative reactions at **C-11** and **C-30** positions of β-amyrin, and glycosyltransfers to C-3 hydroxyl group, are involved.

Identify relevant enzymes, CYPs and UGTs

Licorice Stolon ESTs : a resource for gene discovery in glycyrrhizin biosynthesis (Sudo *et al*., 2009)

Generation of full-length cDNA libraries from the stolons of *G. uralensis* plants

↓

Collection of approx. 56k single pass sequences, subsequently assembled into 10,372 unique sequences

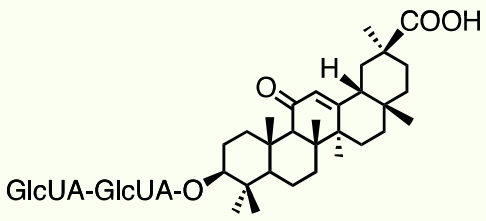
↓

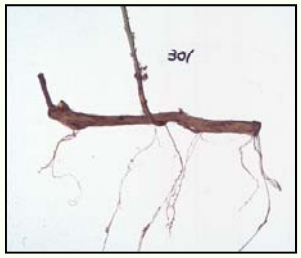
Mining of putative CYPs (ca. 40 genes) by database searches

↓

Selection of candidate P450s (highly expressed in "underground parts")

Glycyrrhizin (+)

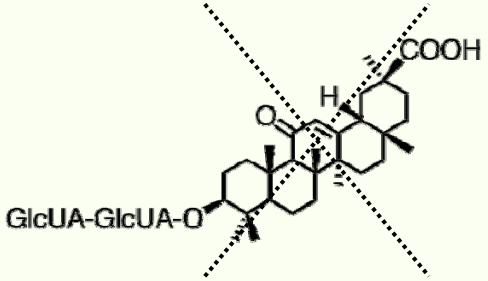




underground organs (roots/stolons)

VS

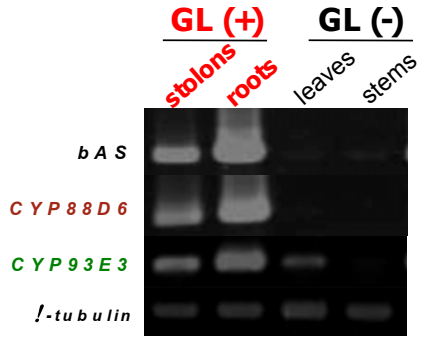
Glycyrrhizin (-)



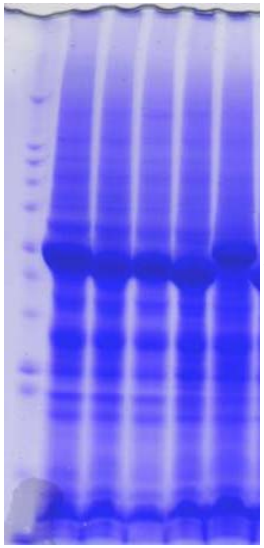
aboveground organs (leaves/stems)

In vitro enzyme assays of candidate CYPs

Selection of candidate P450s



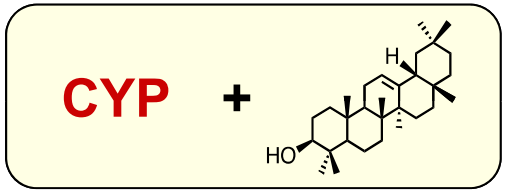
Expressed in insect cells



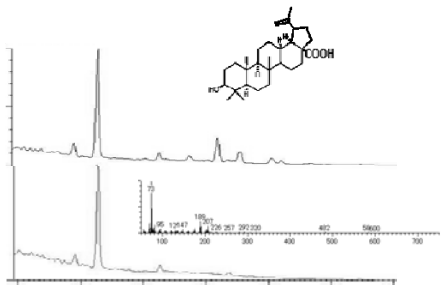
←Licorice P450s

Prepare microsome fraction (containing ER membrane)

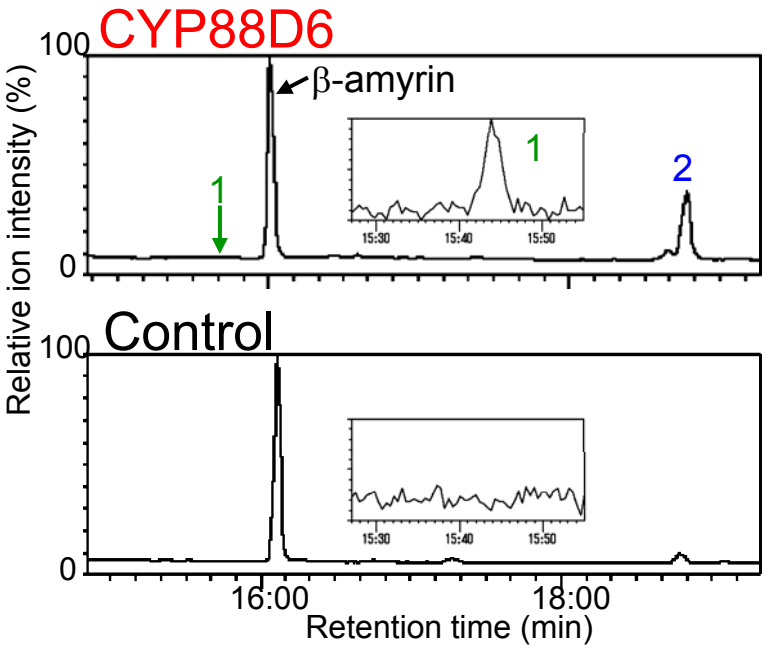
Mixed *in vitro* with β-amyrin as potential substrate

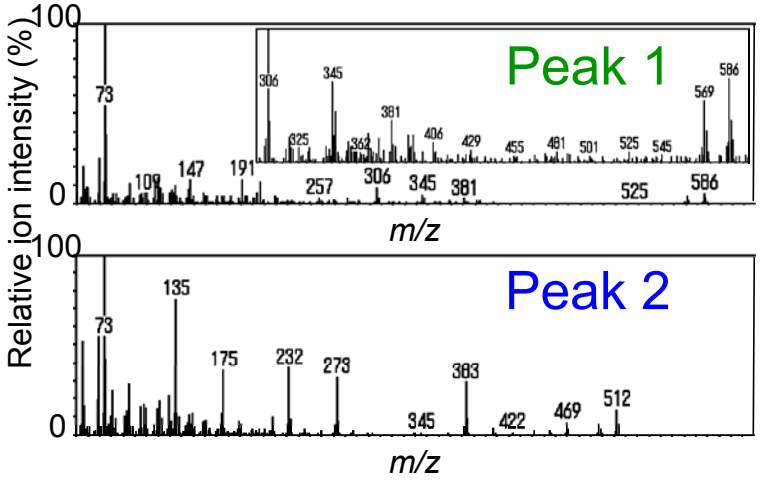


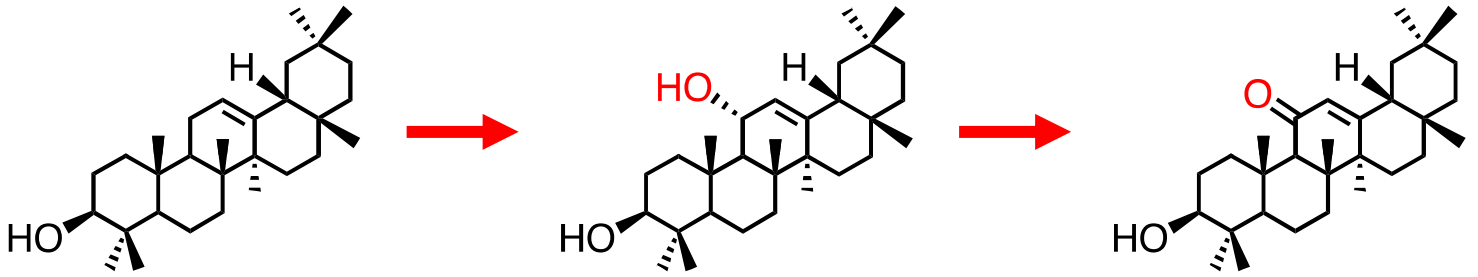
GC-MS analysis of reaction products



In vitro CYP88D6 enzymatic activity assay





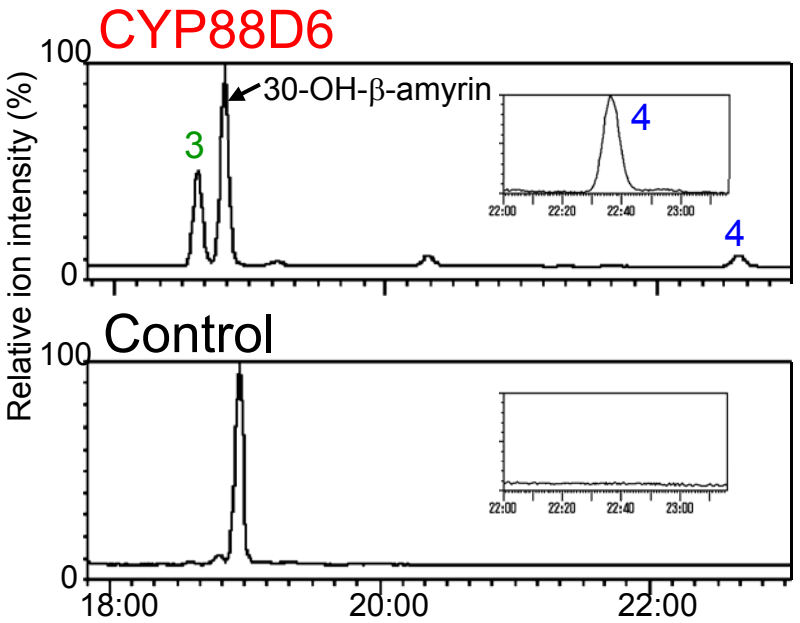


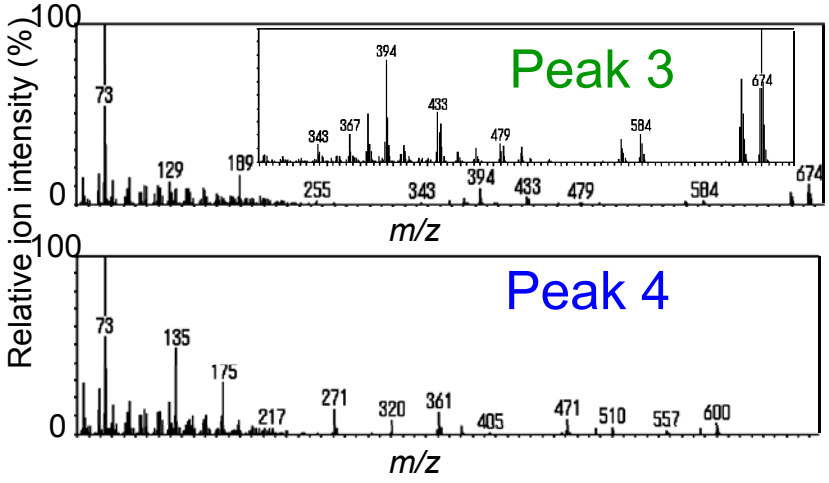
β-amyrin

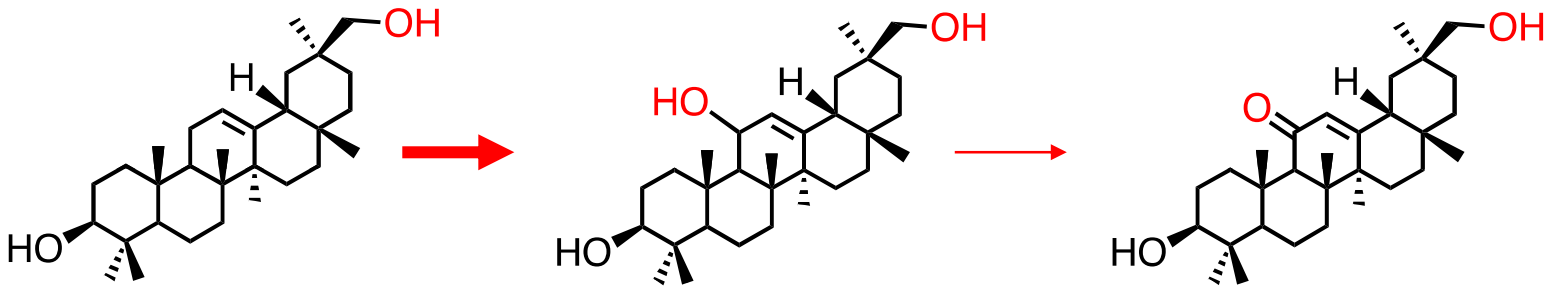
11α-OH-β-amyrin (Peak 1)

11-oxo-β-amyrin (Peak 2)

In vitro CYP88D6 enzymatic activity assay





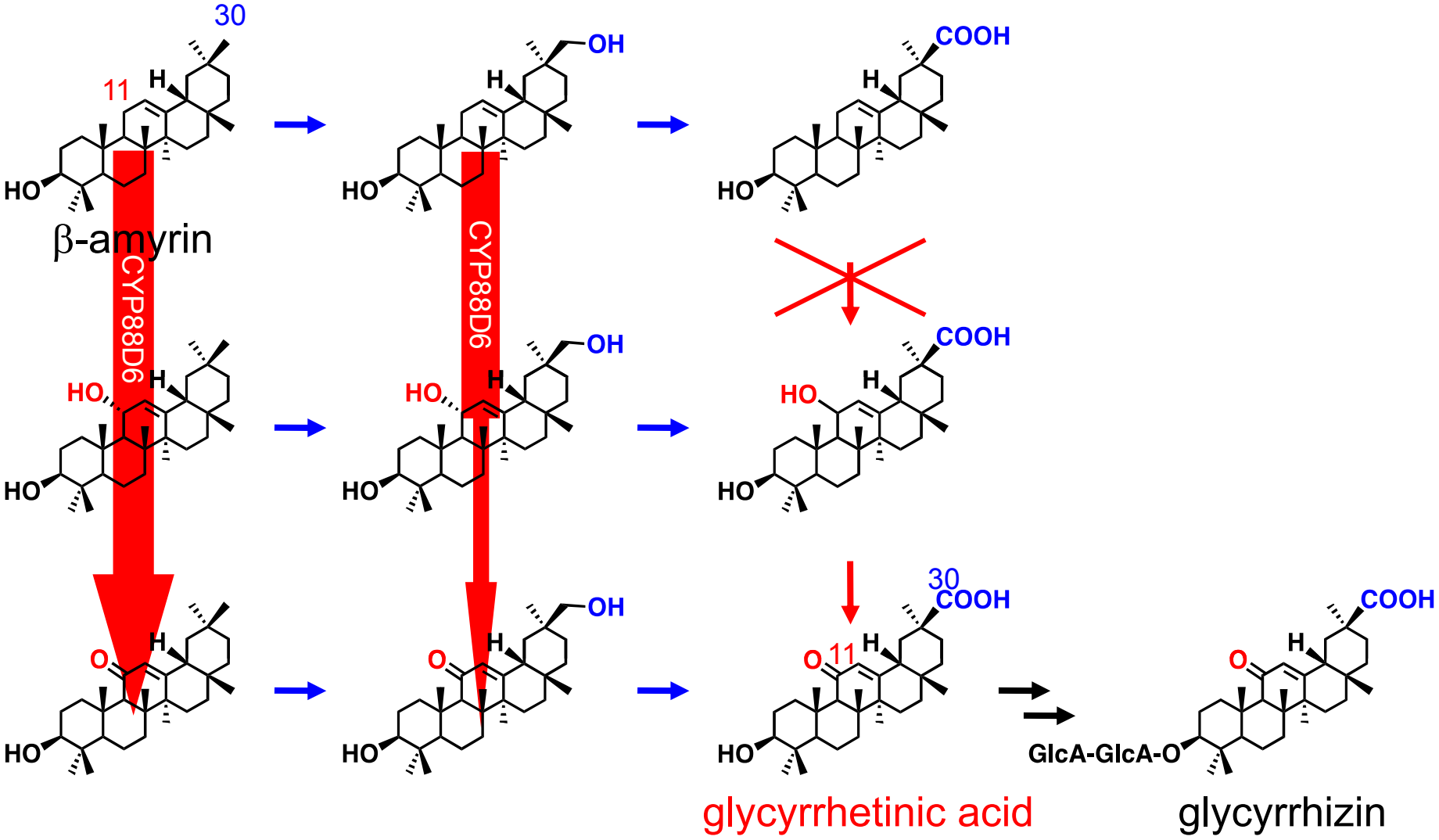


30-OH-β-amyrin

11α-30-diOH-β-amyrin (Peak 3)

30-OH-11-oxo-β-amyrin (Peak 4)

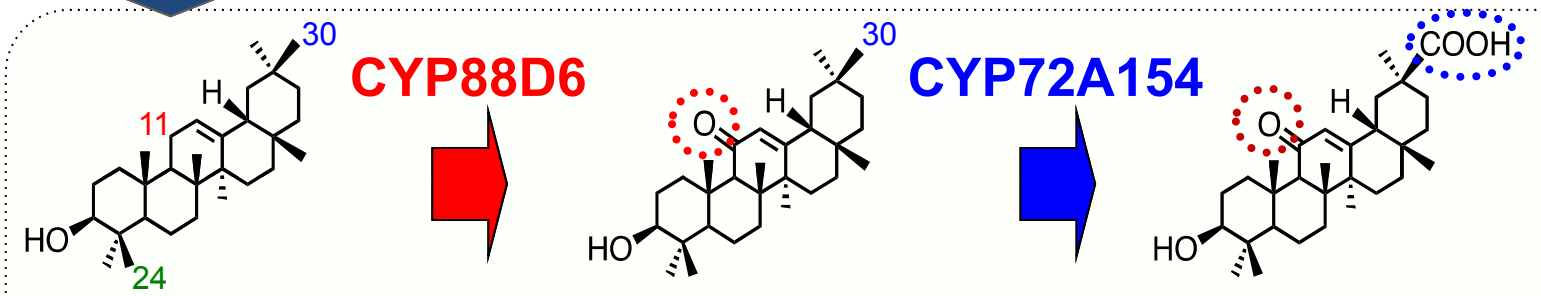
CYP88D6 catalyzes C-11 oxidation steps in glycyrrhizin biosynthesis



mevalonate

2,3-oxidosqualene

bAS



β-amyrin 11-oxo-β-amyrin glycyrrhetinic acid

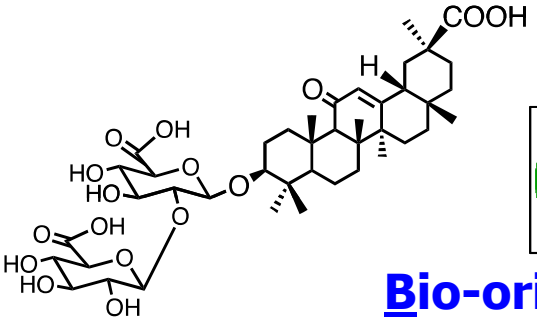
Seki *et al. PNAS* (2008)

Seki, Sawai, Ohyama *et al. Plant Cell* (2011)

Transgenic plant or microorganism?

Transgenic plant or microorganism?

- Glycyrrhizin production; in Soybean



BRAIN

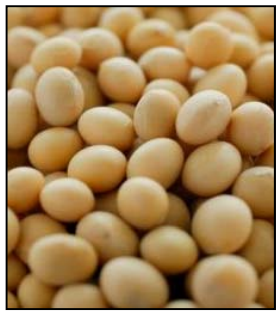
Bio-oriented Technology Research Advancement Institution (BRAIN)

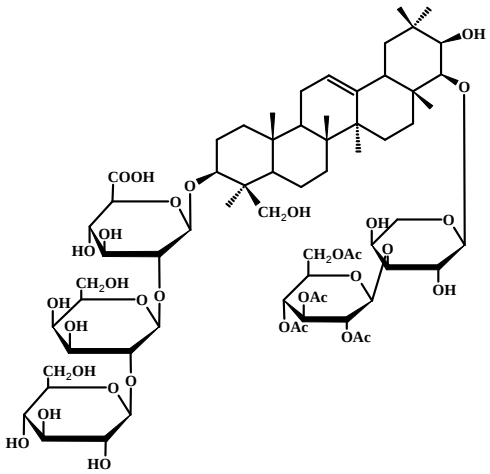
Saponins in foods (crops) are sometimes "**undesirable**"

- **Group A soyasaponins** contained in soybean seeds are recognized as undesired compound because of its unpleasant bitter taste.

- One of the goals of soybean breeding is to lower the saponin content.

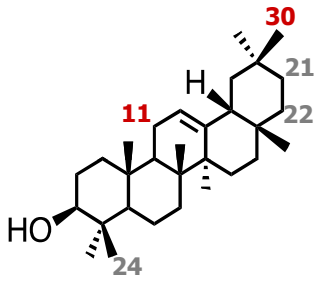
- However, the breeding programs so far have apparently not changed the content of saponins in soybean seeds substantially.





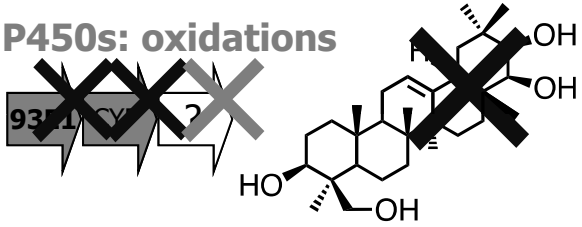
Group A soyasaponin
(triterpenoid saponin)

β-amyrin could be redirected to the heterologous pathway



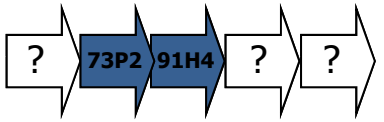
β-amyrin
(triterpene skeleton)

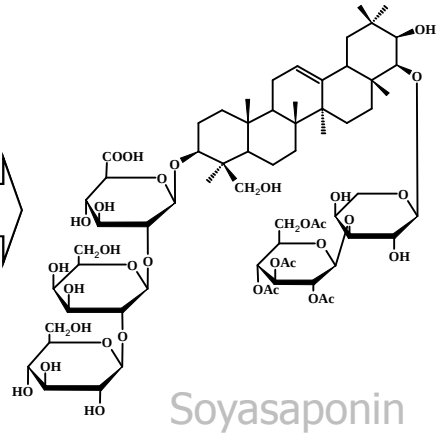
P450s: oxidations



Soyasapogenol A
(aglycone)

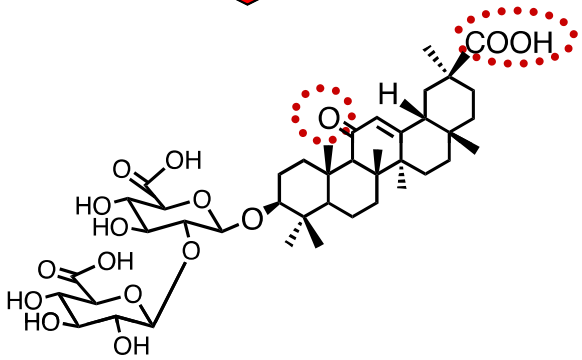
UGTs: glycosylations





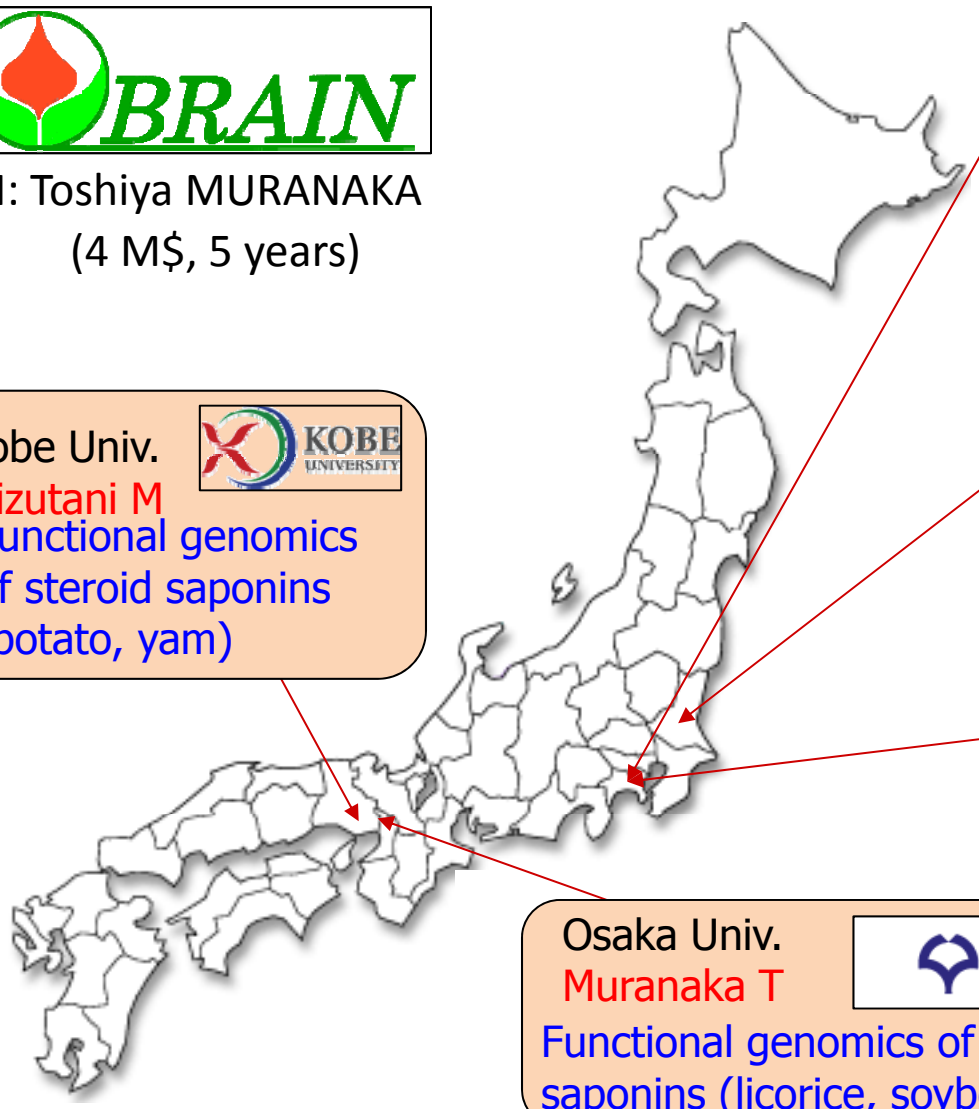
Soyasaponin

"Re-direct" β-amyrin to the heterologous glycyrrhizin pathway by recruiting glycyrrhizin biosynthetic genes (CYPs/UGTs) from licorice.



Glycyrrhizin

A research group, with research teams from universities, public institutions, and a private company



BRAIN

PI: Toshiya MURANAKA
(4 M$, 5 years)

KIRIN Co., Ltd.
Umemoto N
Transformation of potato

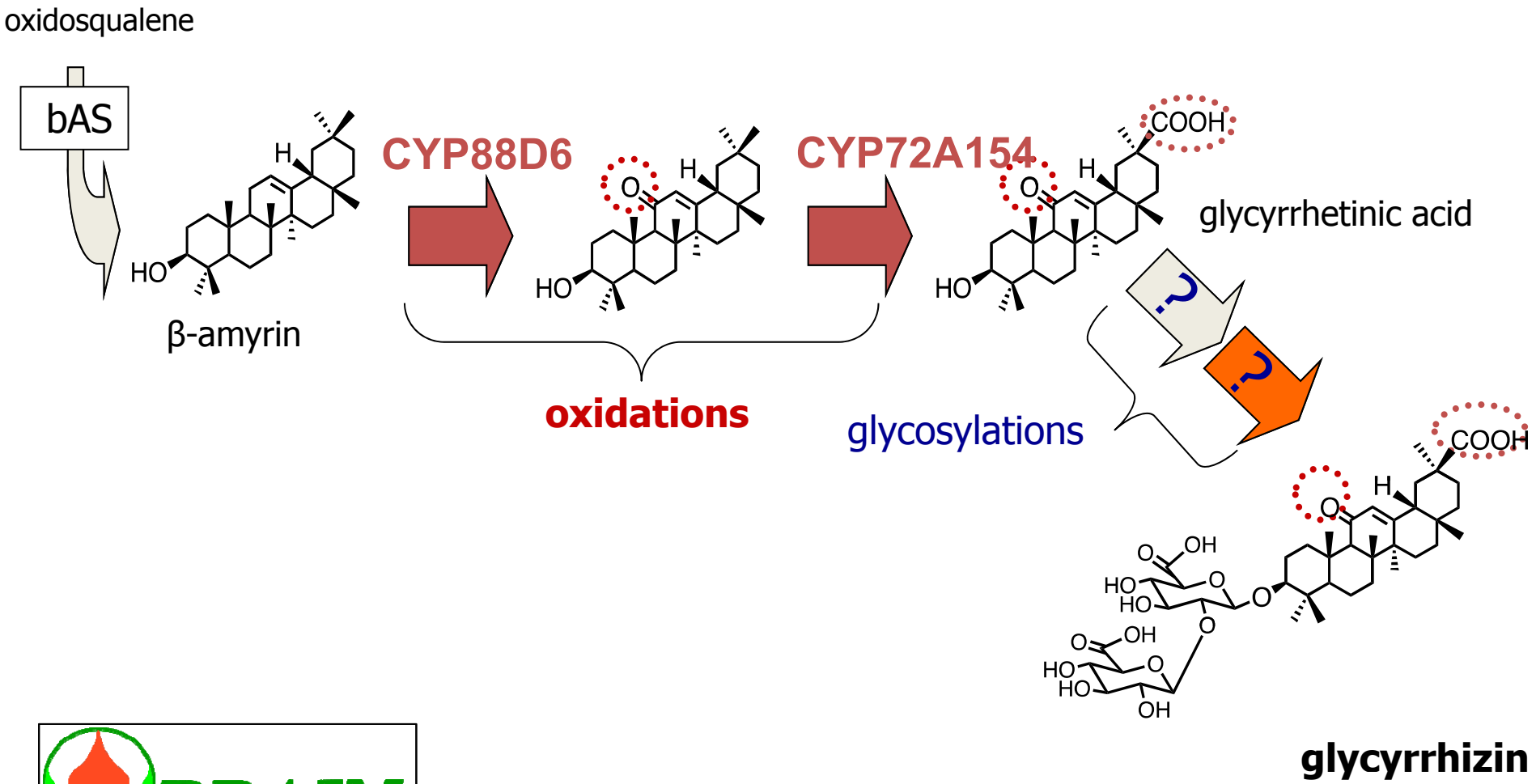
National Institute of Agrobiological Sciences
Ishimoto M
Transformation of soybean

Kobe Univ.
Mizutani M
Functional genomics of steroid saponins (potato, yam)

RIKEN CSRS
Saito K
Detailed metabolic profiling analyses of engineered plants

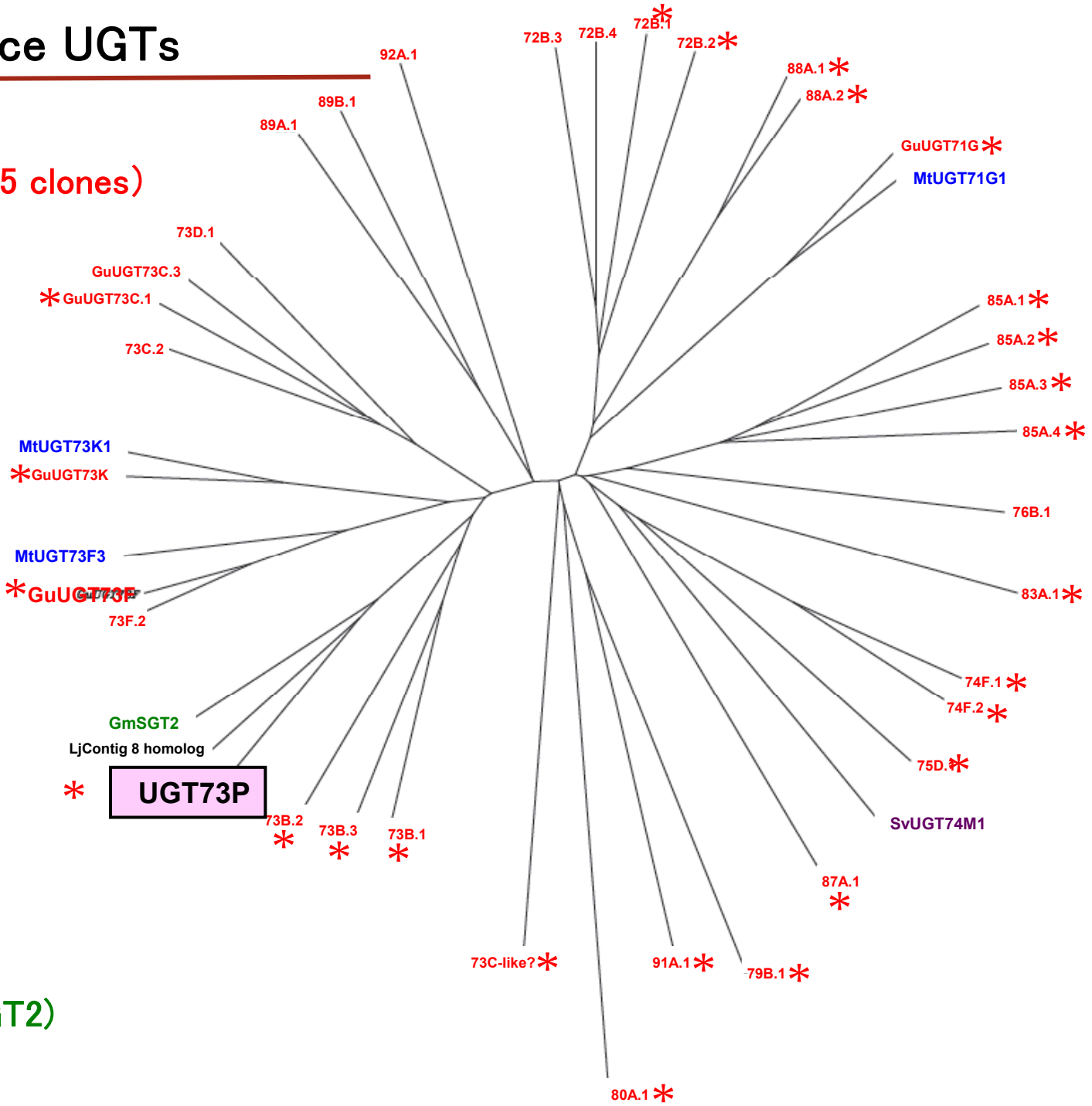
Osaka Univ.
Muranaka T
Functional genomics of triterpenoid saponins (licorice, soybean)

Two CYPs were identified in glycyrrhizin pathway



Licorice UGTs

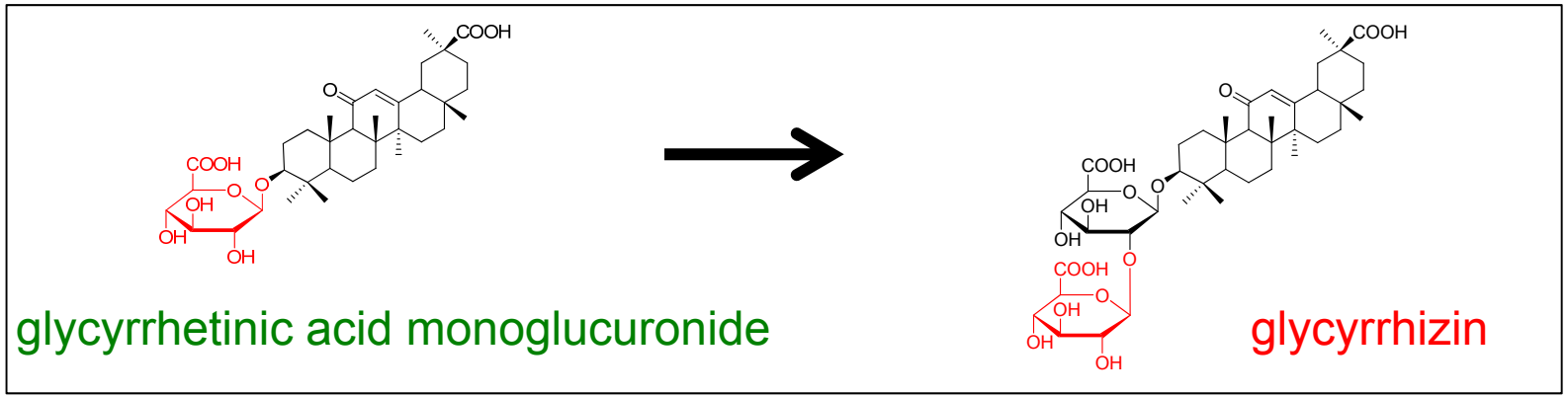
* In vitro assay (25 clones)

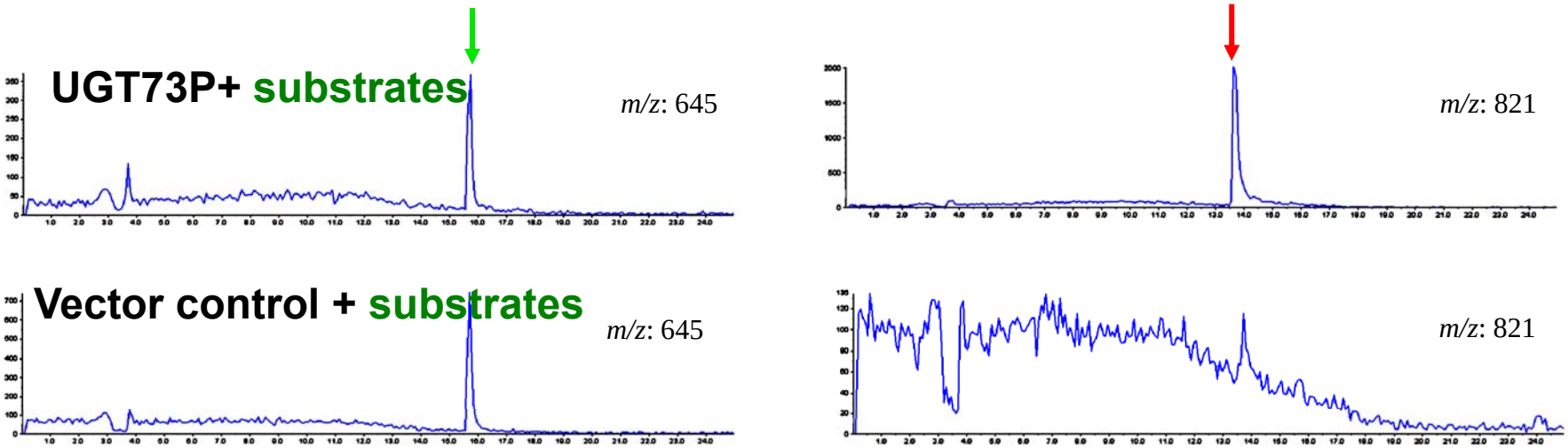


Medicago UGT

Soybean UGT (SGT2)

Isolation of UDP- glucroindase in licorice



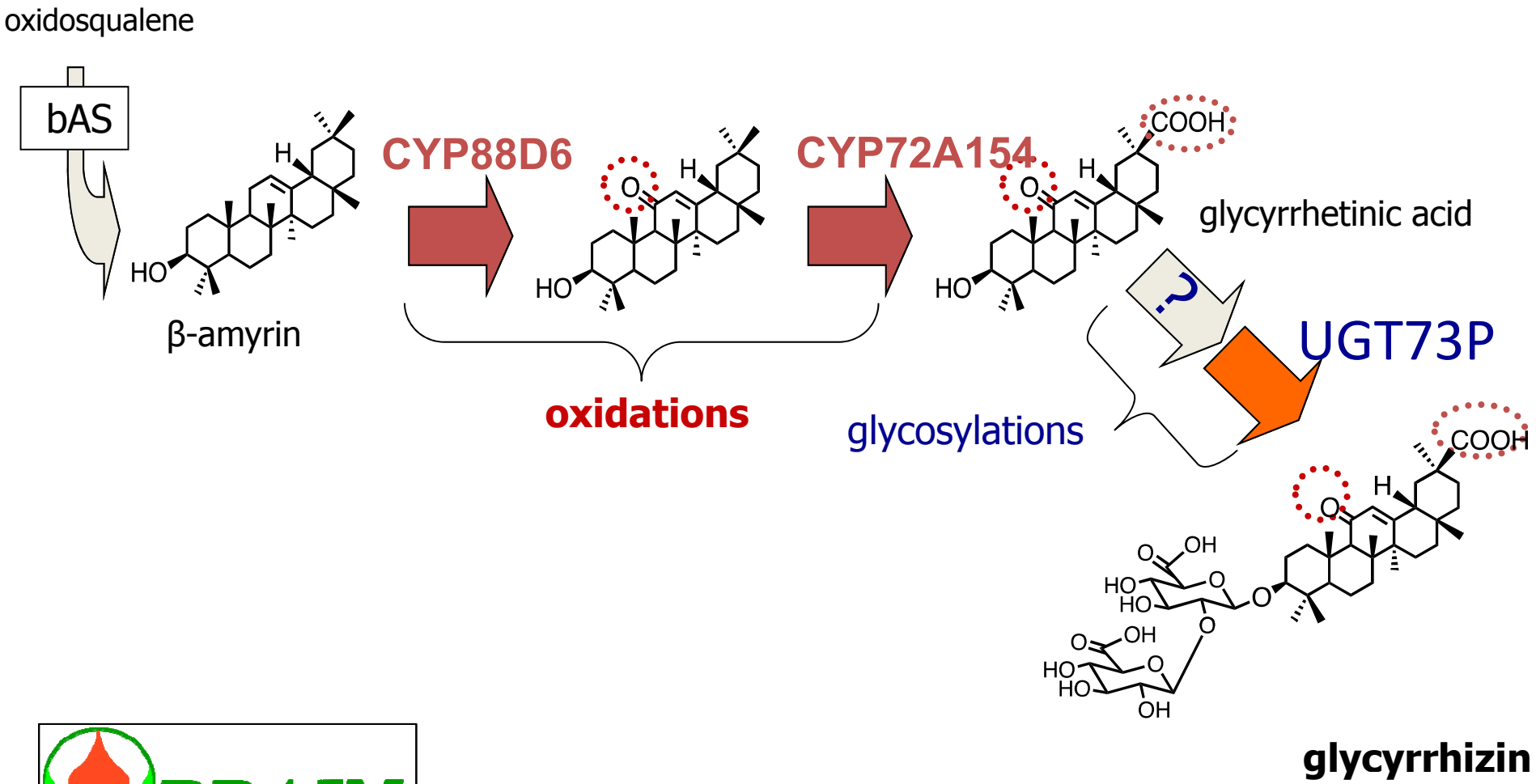


(negative mode)

In vitro assay of yeast extract with substrates (glycrrhetinic acid monoglucuronide and UDP–glucuronide).

Seki *et al*. (unpublished, Patent filed (April 2013))

Two CYPs and one UGT were identified in glycyrrhizin pathway



Progress –to isolate first UGT

Unigene (43,882 genes)

Ramilowski et al. *PCP* (2013)

BLASTX search (NCBI)

Database : All plants

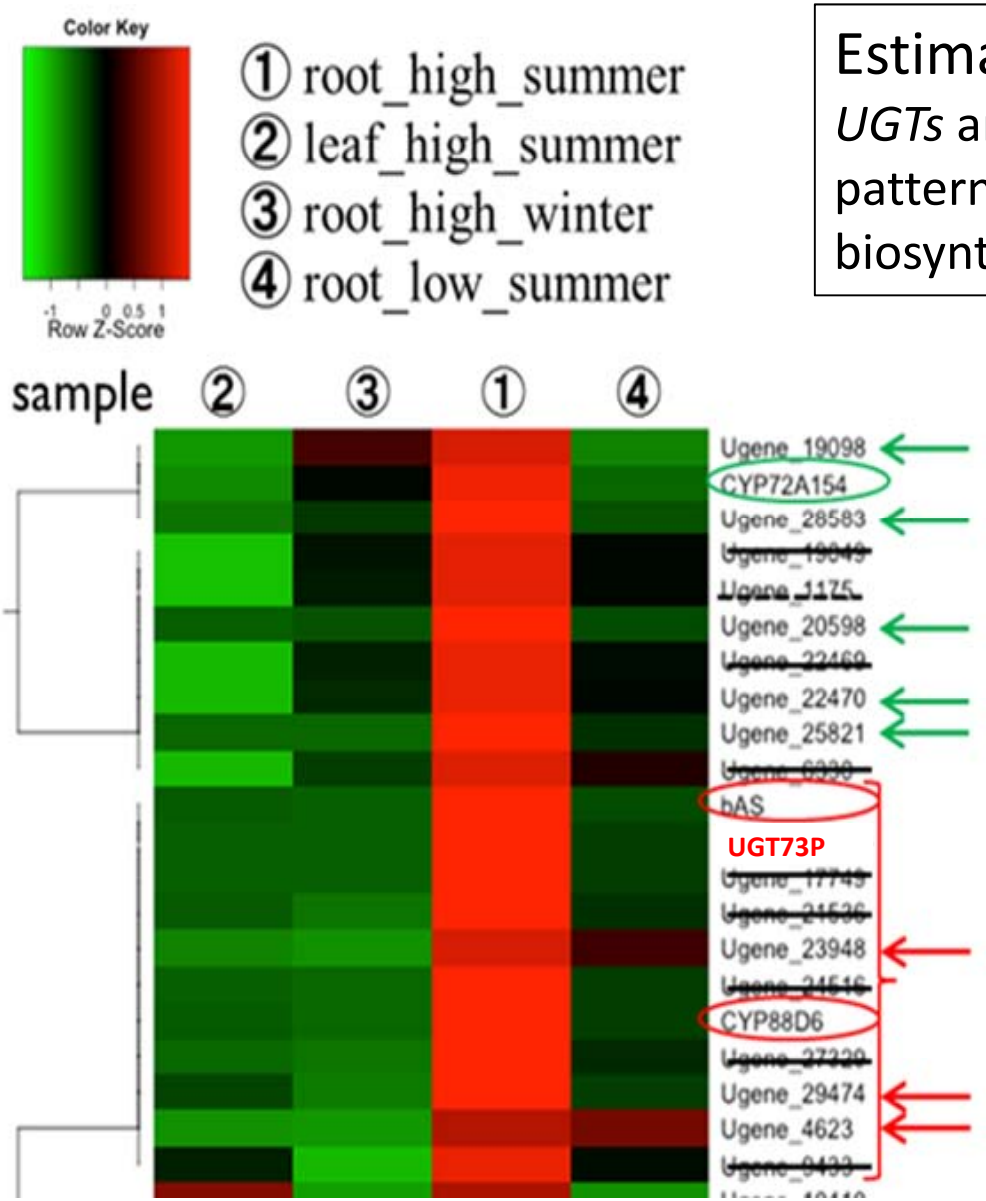
Query : Putative cDNA genes

Search UGT-like genes

⇒155 genes are applicable

UGT	71			72			73		74				75		76	78	79	80		81	82	83	84		85	87	88	89		91		92
Sub family	B	C	D	B	D	E	B	C	B	C	E	F	B	D	F	D	B	A	B	A	A	A	A	B	A	A	A	A	B	A	B	A
Hit	4	2	1	14	2	2	16	18	1	3	4	3	1	1	2	1	7	3	1	1	2	7	1	1	30	6	11	1	2	3	1	3

Progress –to isolate first UGT



Estimate

UGTs are expected to exhibit similar expression pattern to the genes involved in Glycyrrhizin biosynthesis

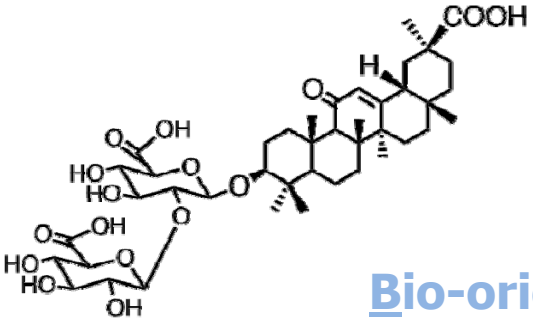
Remove the known or being examined genes (36 sequences)
: Black lines

8 genes are novel UGT-like sequence

Unigene_19098
Unigene_28583
Unigene_20598
Unigene_22470
Unigene_25821
Unigene_23948
Unigene_29474
Unigene_4623

Transgenic plant or microorganism?

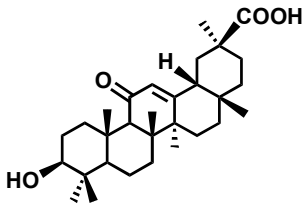
- Glycyrrhizin production; in Soybean



BRAIN

Bio-oriented Technology Research Advancement Institution (BRAIN)

- Glycyrrhetinic acid production; in Yeast



研究成果展開事業

A-STEP 研究成果最適展開支援プログラム

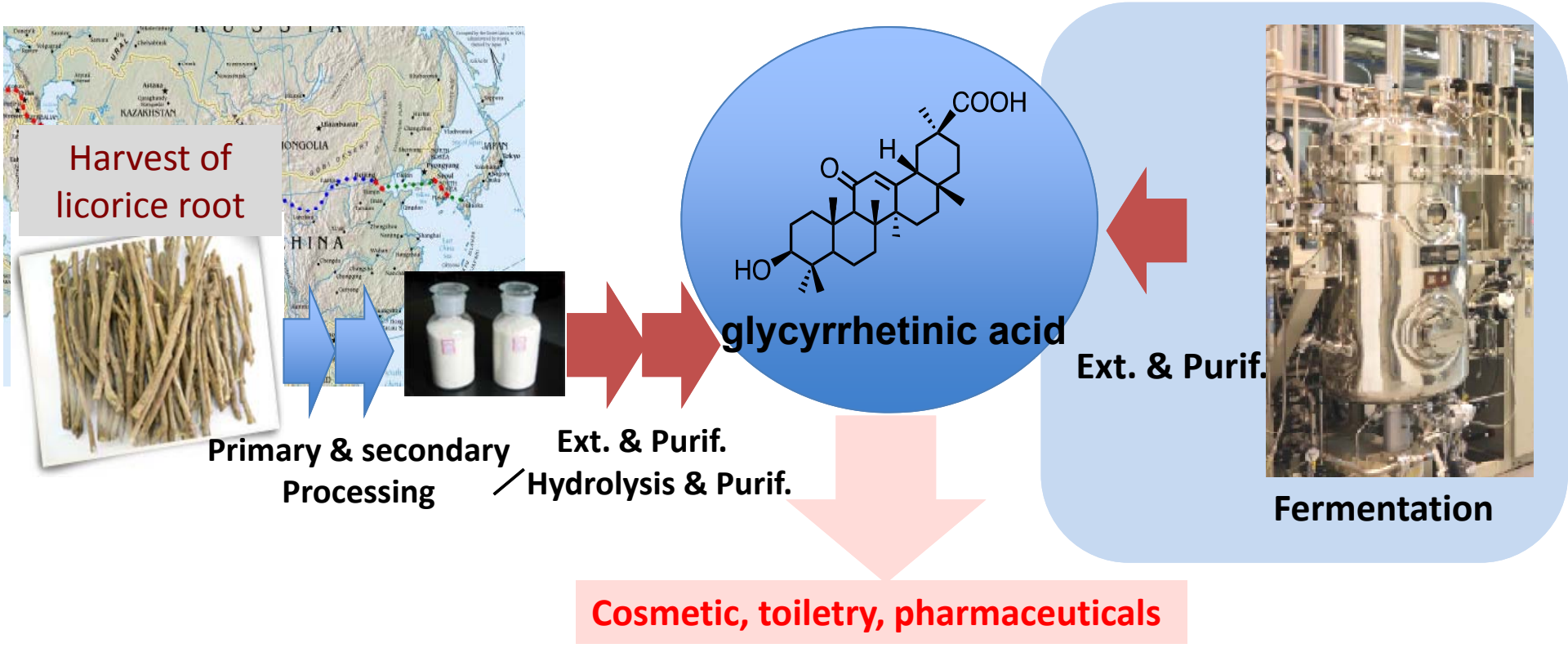
Adaptable and Seamless Technology transfer Program through targetdriven R&D

Concept of the study

研究成果展開事業
A-STEP 研究成果最適展開支援プログラム
Adaptable and Seamless Technology transfer Program through targetdriven R&D

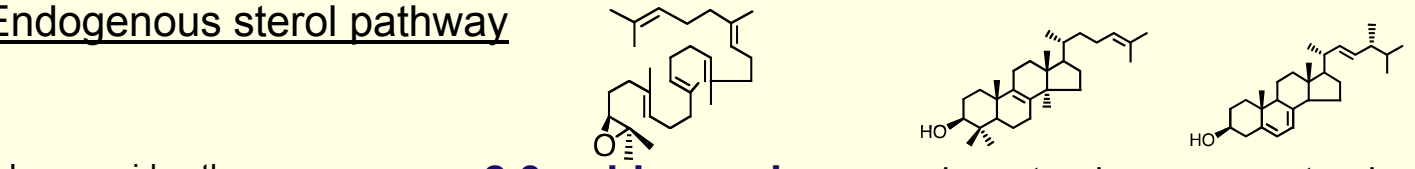
Started from Oct. 2012

Licorice, a rare plant, is dependent on 100% import from China and other countries. We aim at production of glycyrrhetinic acid and its derivatives are by yeast fermentation for stable and sustainable supply

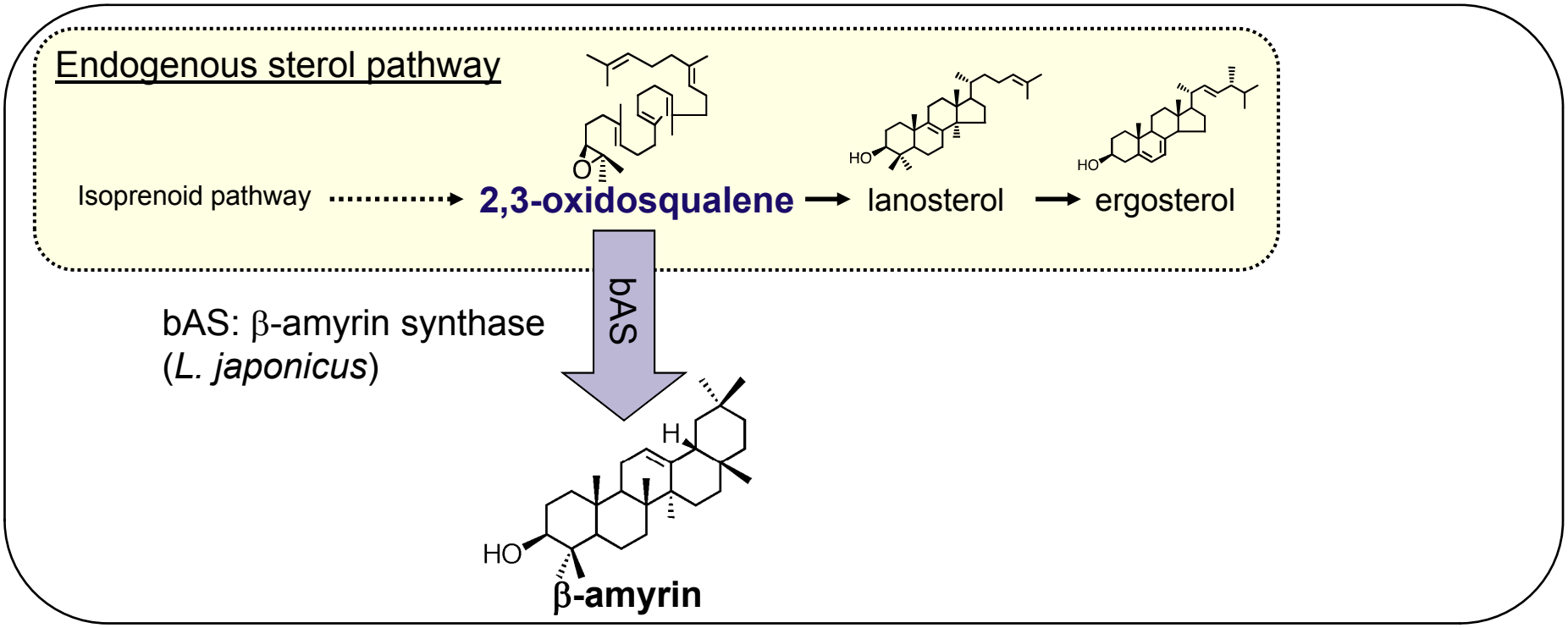


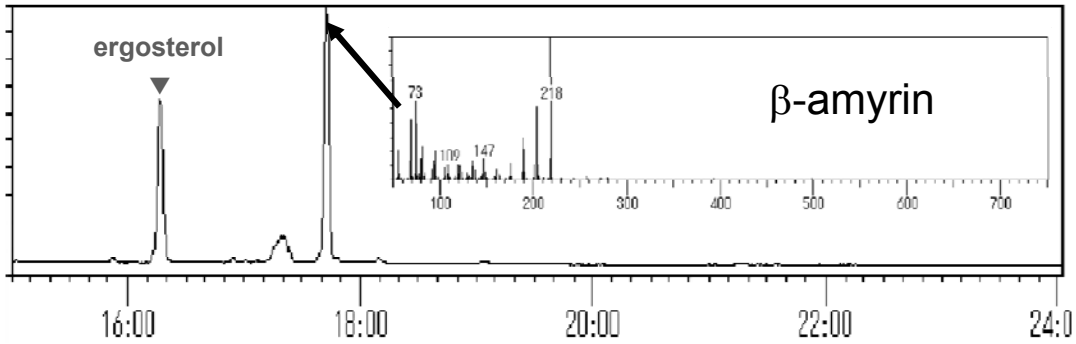
Reconstruction of the pathway for glycyrrhizin aglycone, glycyrrhetinic acid, in an engineered yeast

Endogenous sterol pathway



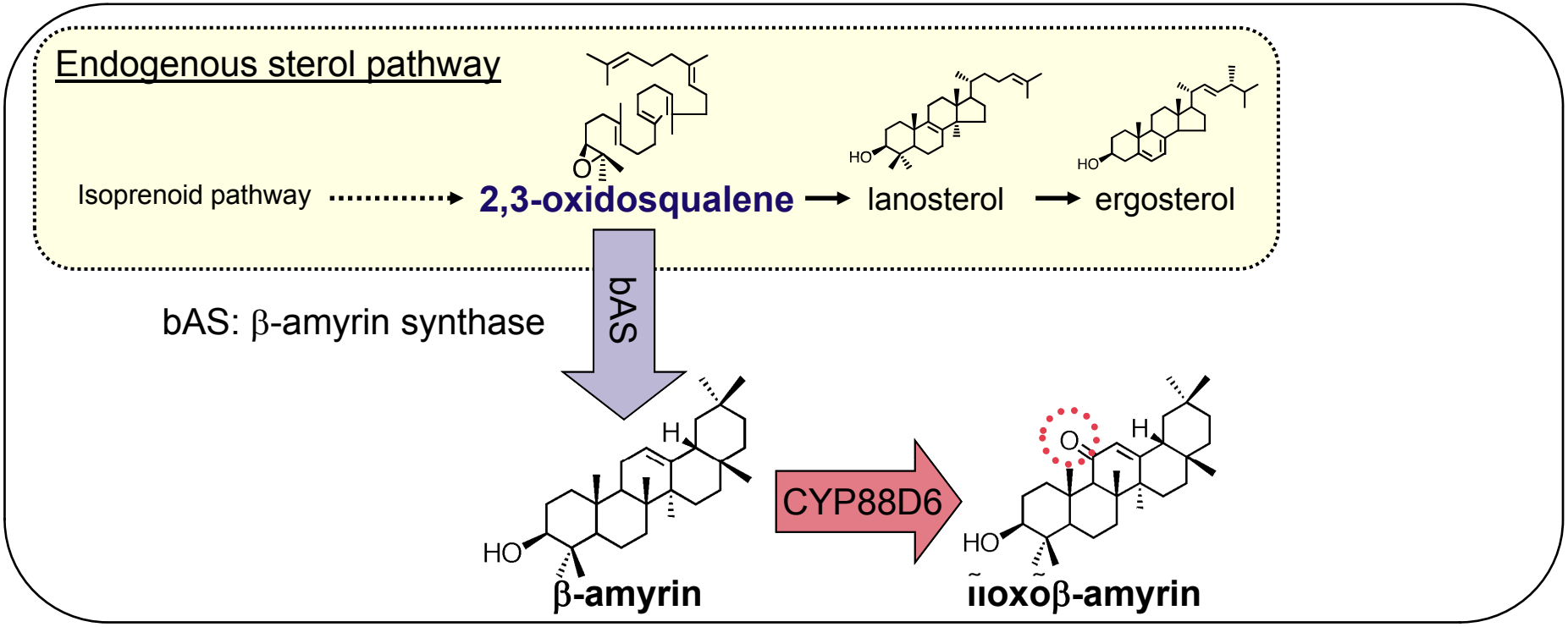
Reconstruction of the pathway for glycyrrhizin aglycone, glycyrrhetinic acid, in an engineered yeast

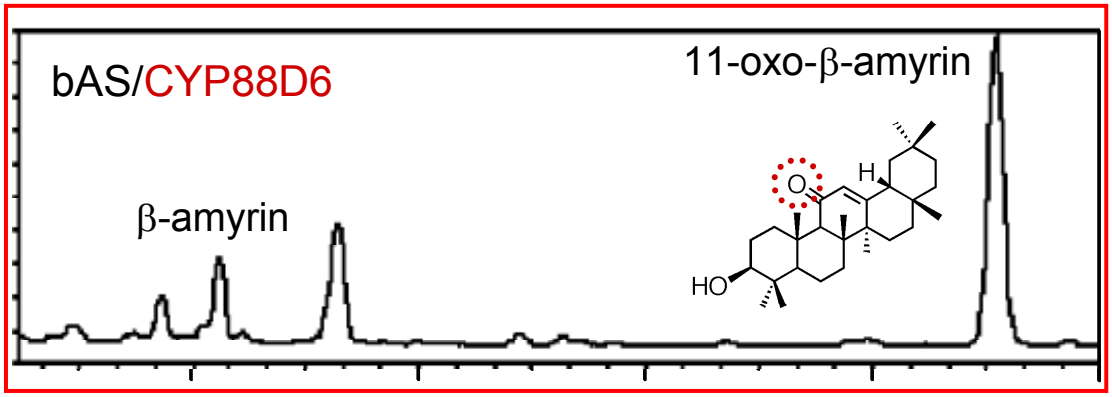




GC-MS analysis of the ethyl-acetate extract of *bAS*-expressing yeast culture

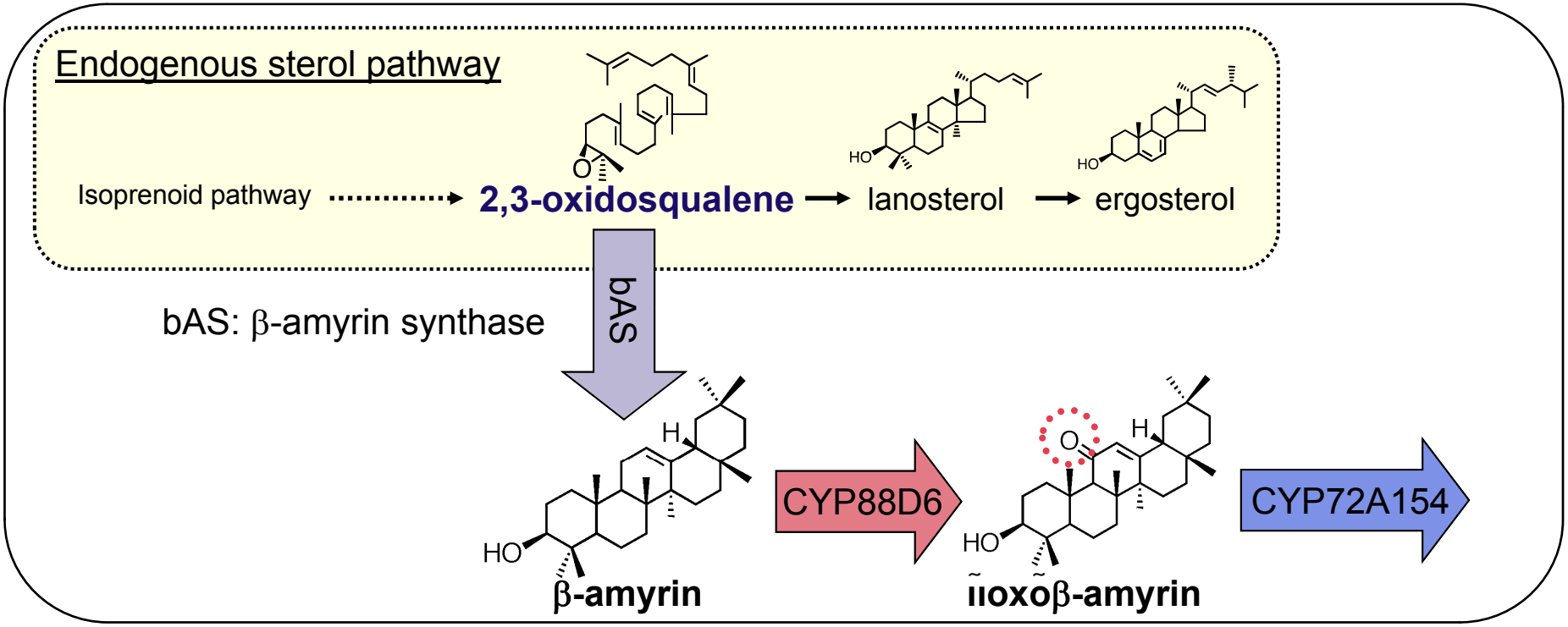
Reconstruction of the pathway for glycyrrhizin aglycone, glycyrrhetinic acid, in an engineered yeast

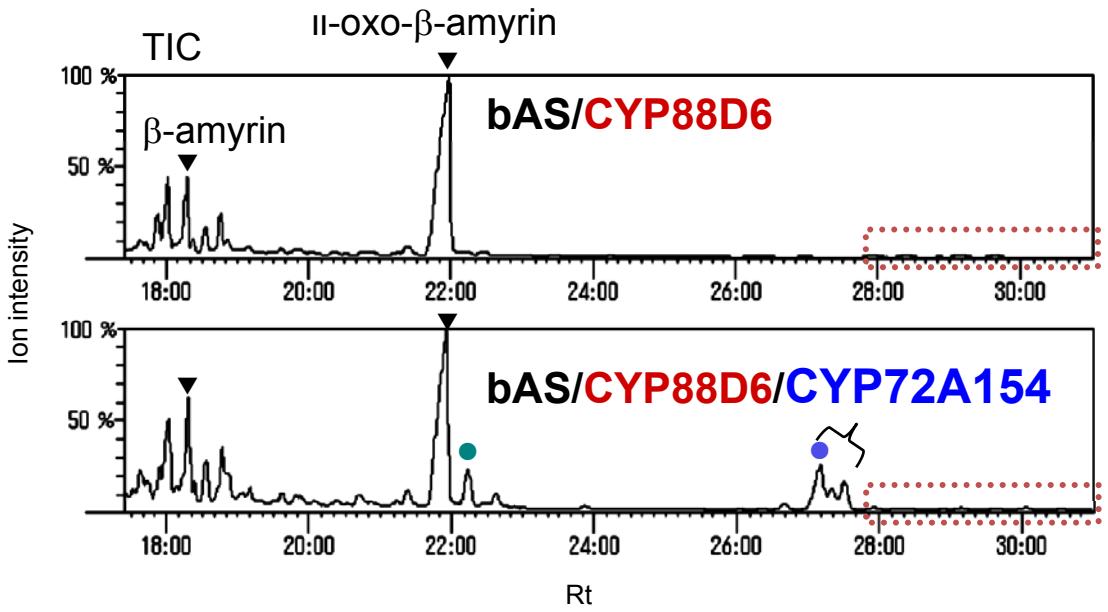


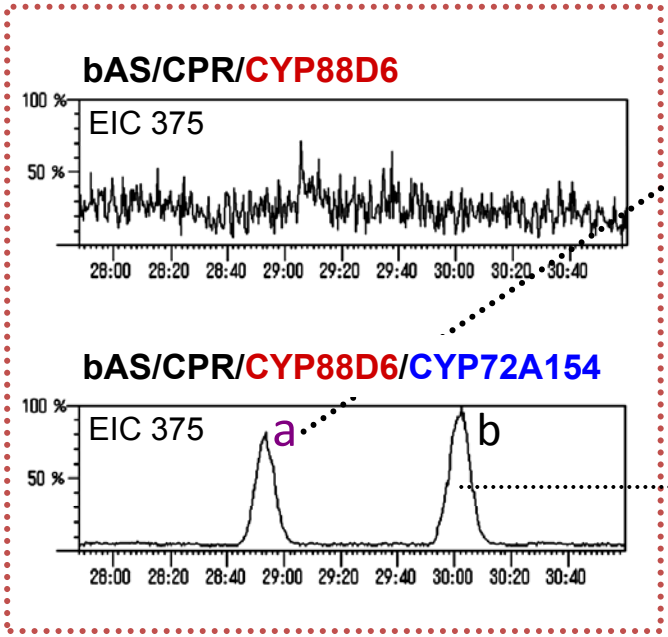


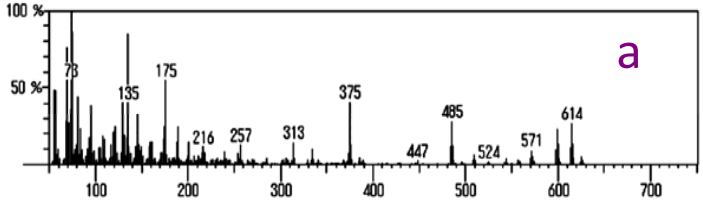
GC-MS analysis of the ethyl-acetate extract of *bAS*/*CYP88D6*-expressing yeast culture

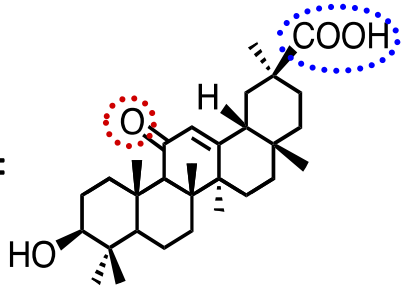
Reconstruction of the pathway for glycyrrhizin aglycone, glycyrrhetinic acid, in an engineered yeast



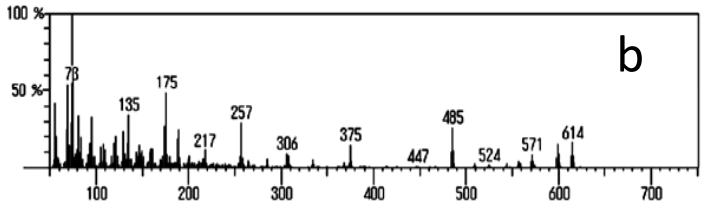


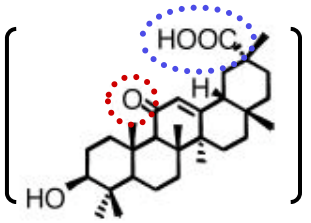






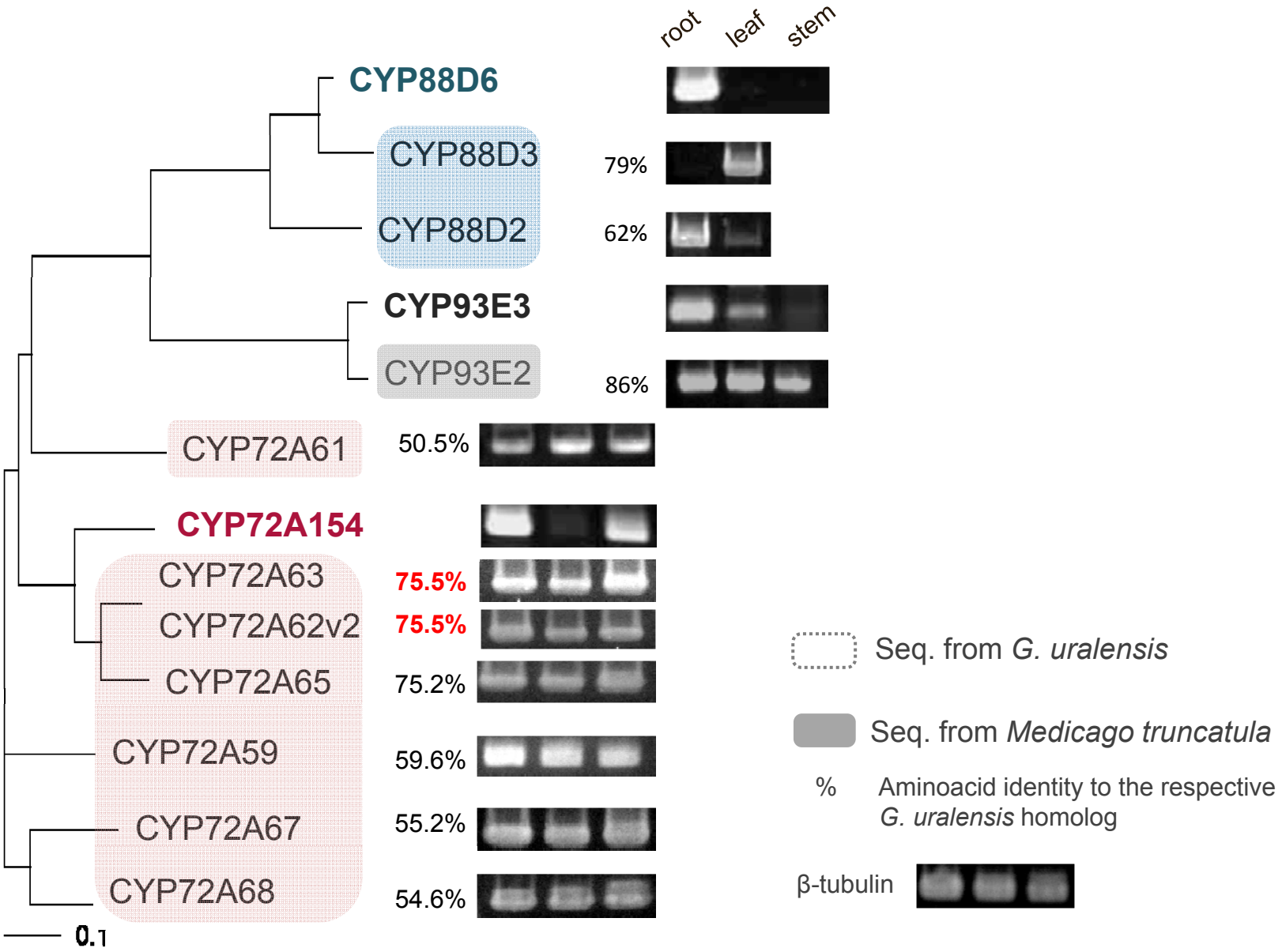
glycyrrhetinic acid (aglycone)





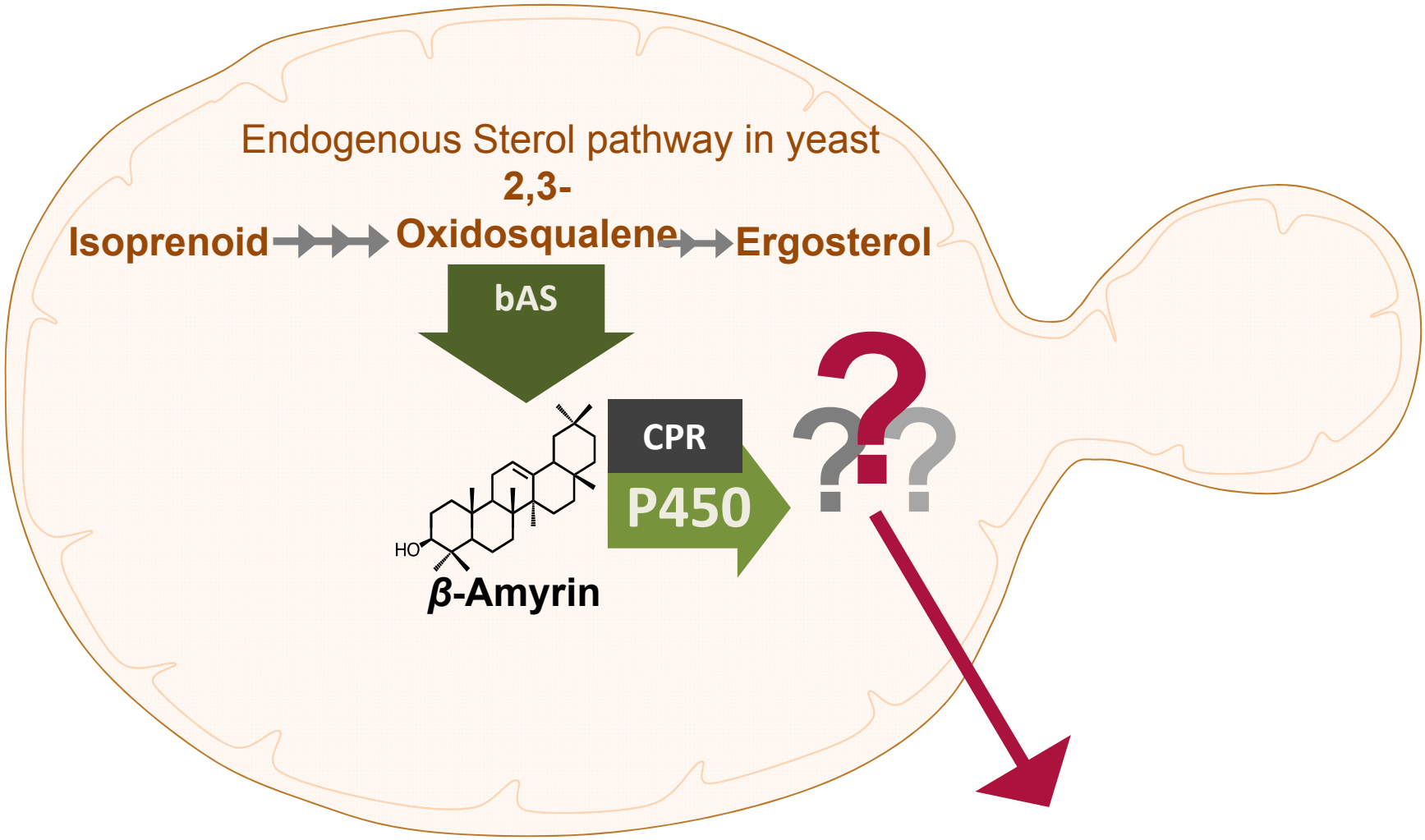
Isomer; 20-epiglycyrrhetinic acid

Homologous genes: *Medicago truncatula*

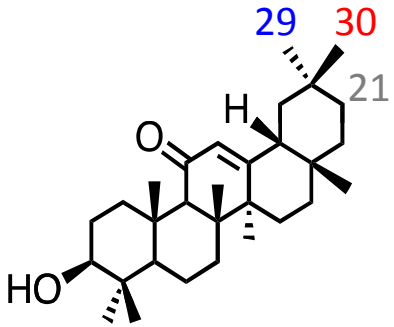


-Phylogenetic tree was generated with ClustaW program-

Generation of *bAS/CPR/P450* expressing yeast



Comparison of the function of licorice CYP72A154 and Medicago CYP72As

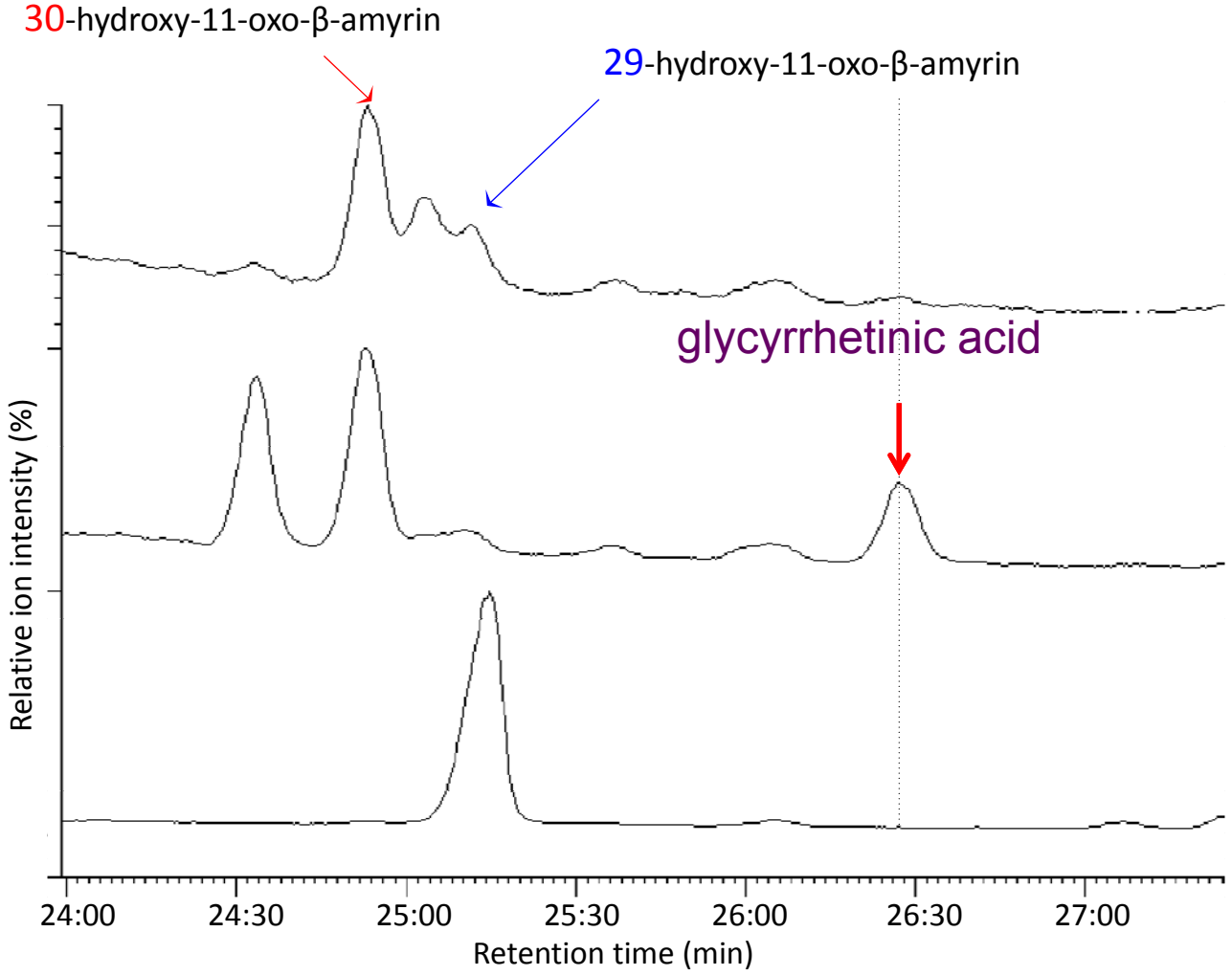


11-oxo-β-amyrin

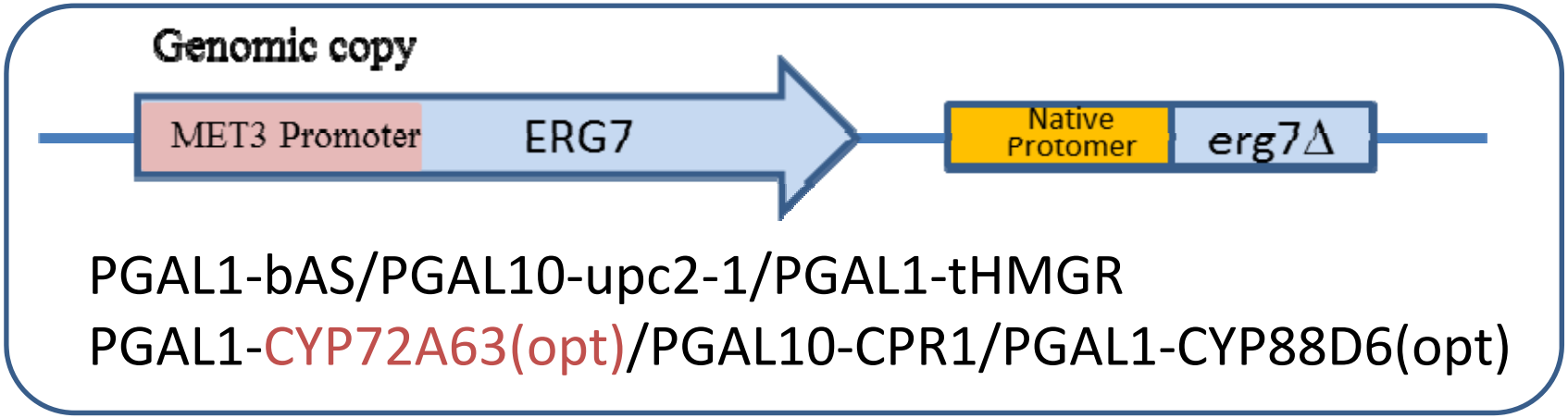
+ **CYP72A154 (licorice)**

+ **CYP72A63 (Medicago)**

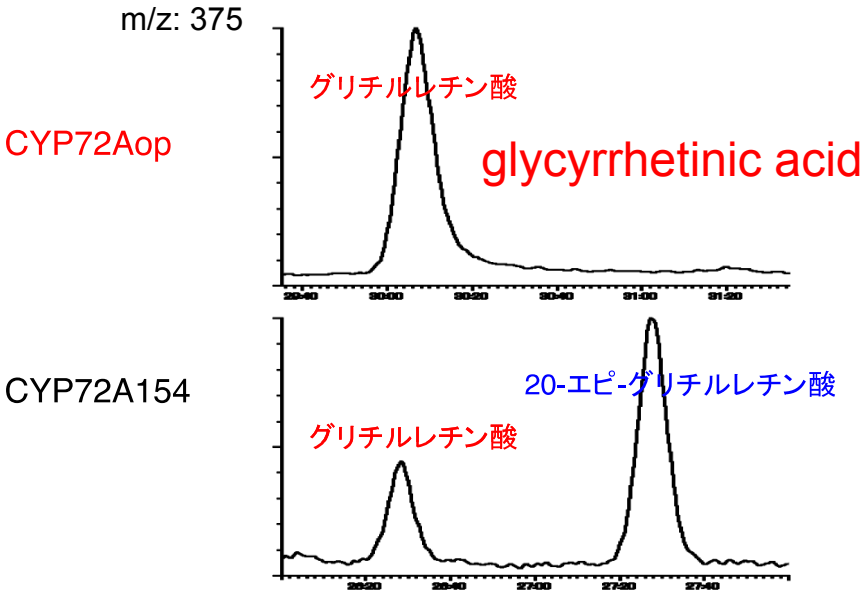
+ **CYP72A62v2 (Medicago)**

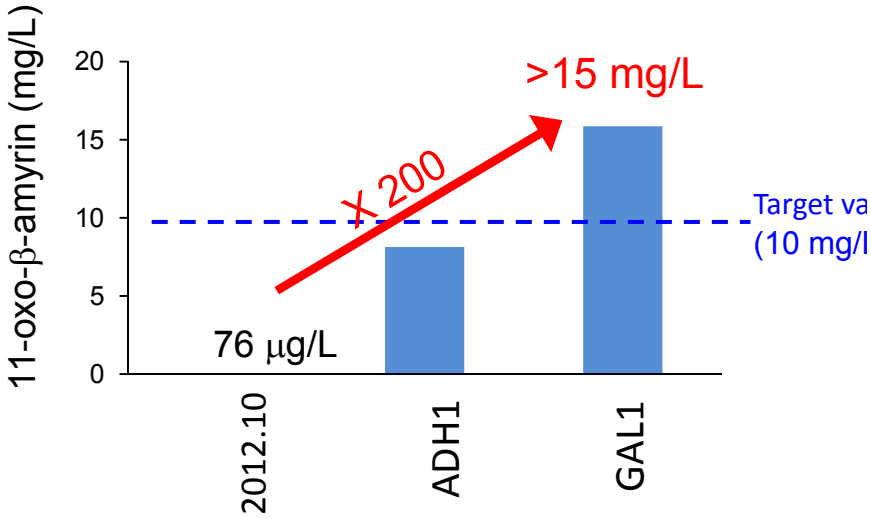


Regioselectiviy (C-30) is high in Medicago CYP72A63



PGAL1-bAS/PGAL10-upc2-1/PGAL1-tHMGR
PGAL1-CYP72A63(opt)/PGAL10-CPR1/PGAL1-CYP88D6(opt)

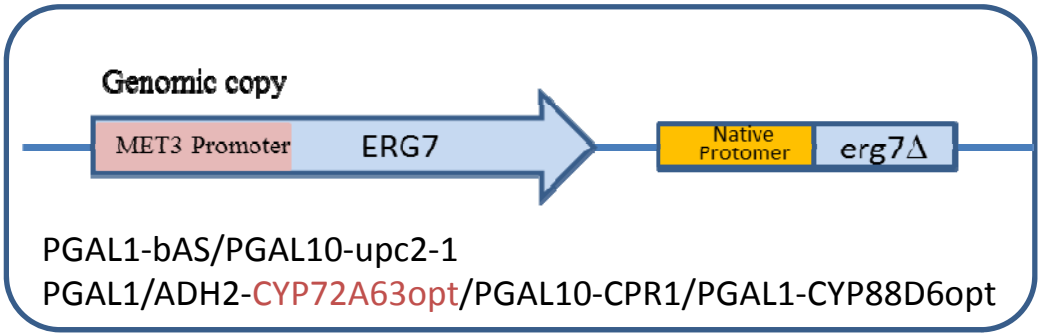


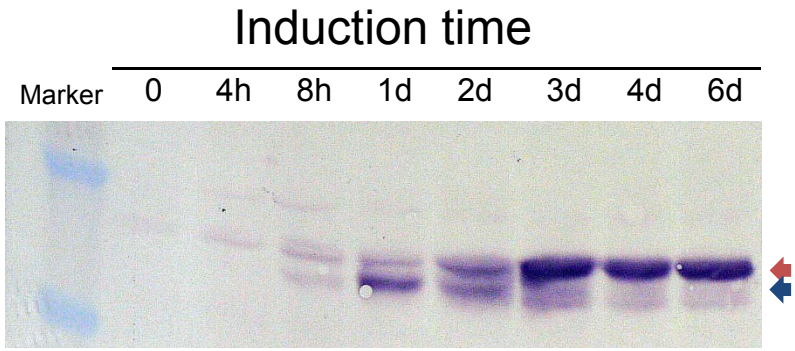


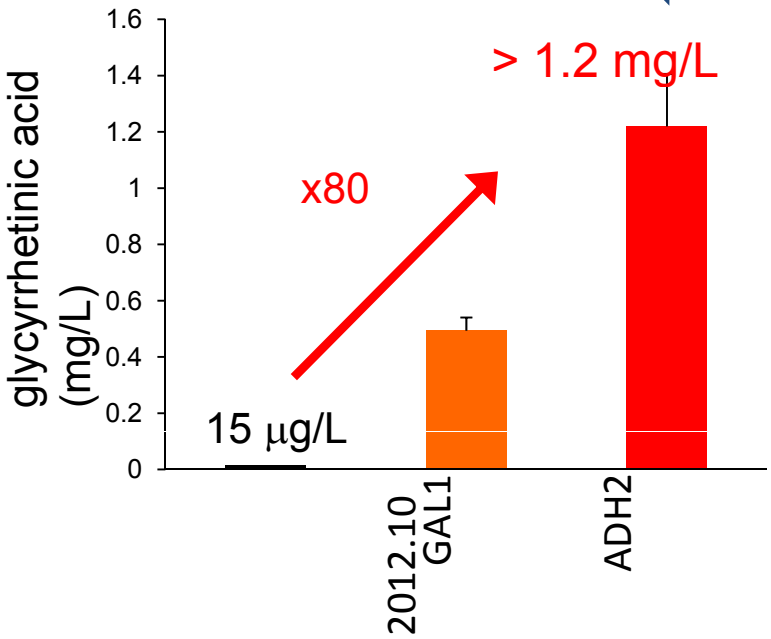
Avoid side-reaction

Production of intermediate was increased

Optimization of *CYP72A63* promoter







研究成果展開事業
A-STEP
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Adaptable and Seamless Technology transfer Program through targetdriven R&D

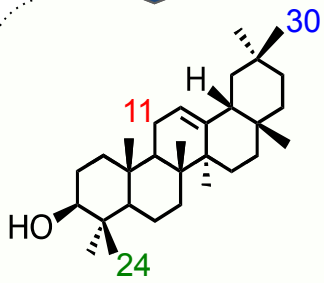
Productivity of glycyrrhetinic acid was increase

Two relevant CYPs were identified in glycyrrhizin pathway

mevalonate

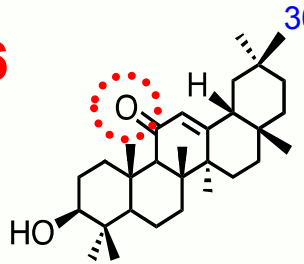
2,3-oxidosqualene

bAS



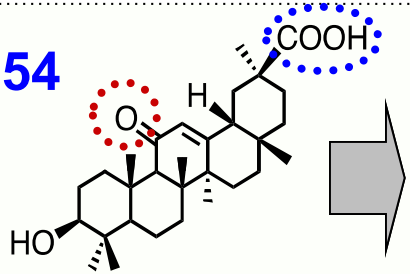
β-amyrin

CYP88D6



11-oxo-β-amyrin

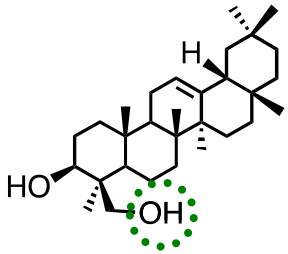
CYP72A154



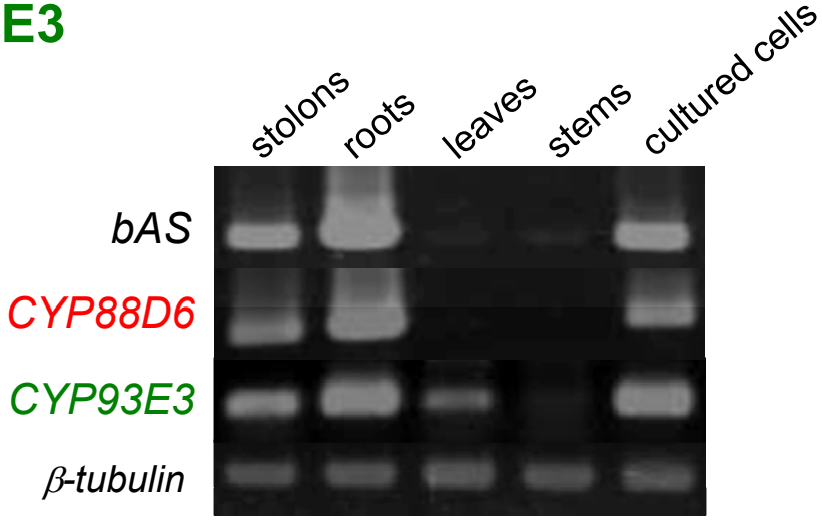
glycyrrhetinic acid (aglycone)

GL

CYP93E3



soyasaponins



Seki *et al.*, *PNAS* 105 (2008) 14204-14209

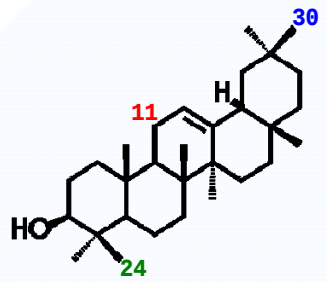
Seki, Sawai, Ohyama *et al.*, *Plant Cell* 23 (2011) 4112-4123

Three distinct CYP family are recruited in the modification of β-amyrin

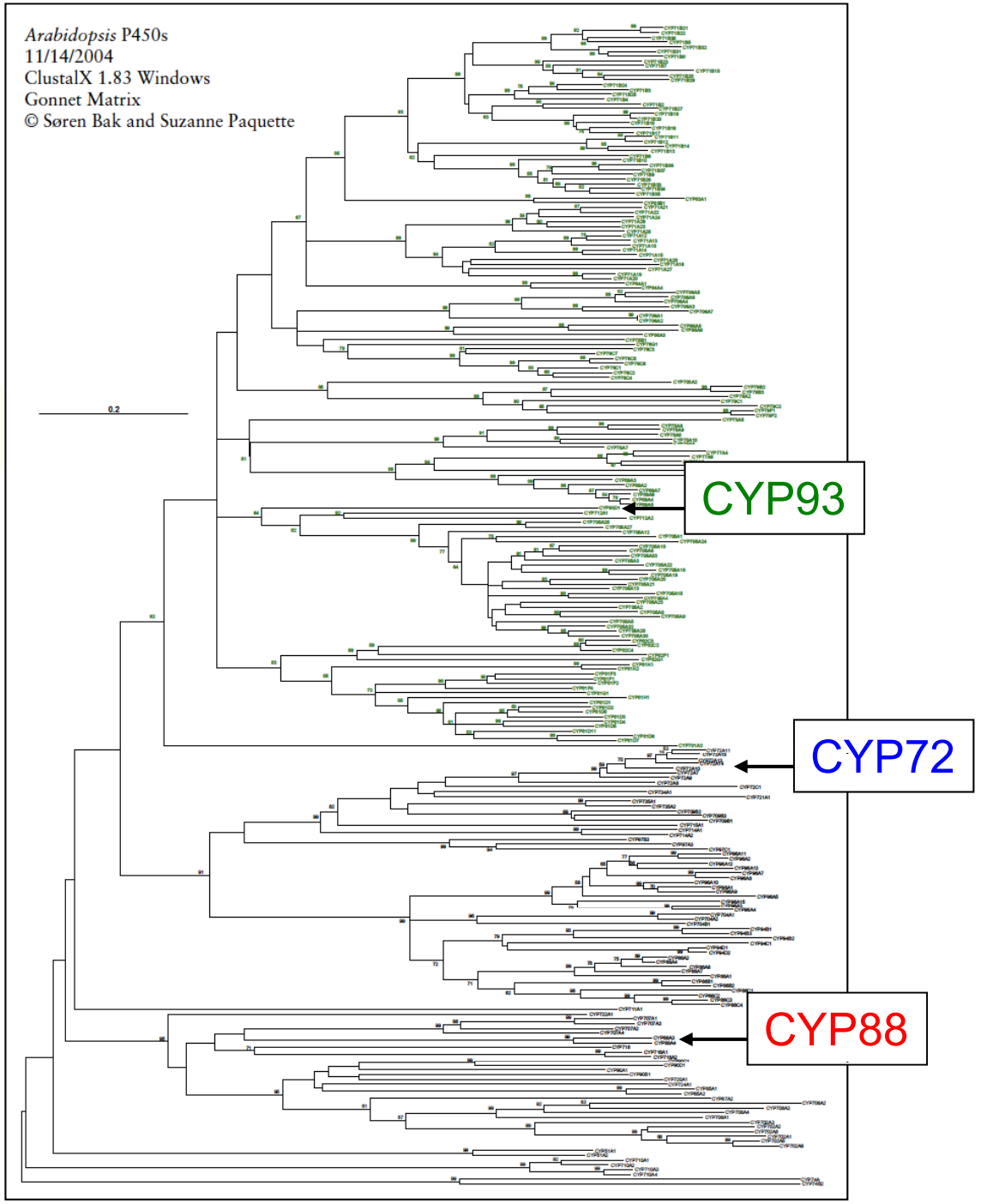
88D6 — 27% — 72A154

21% — 26%

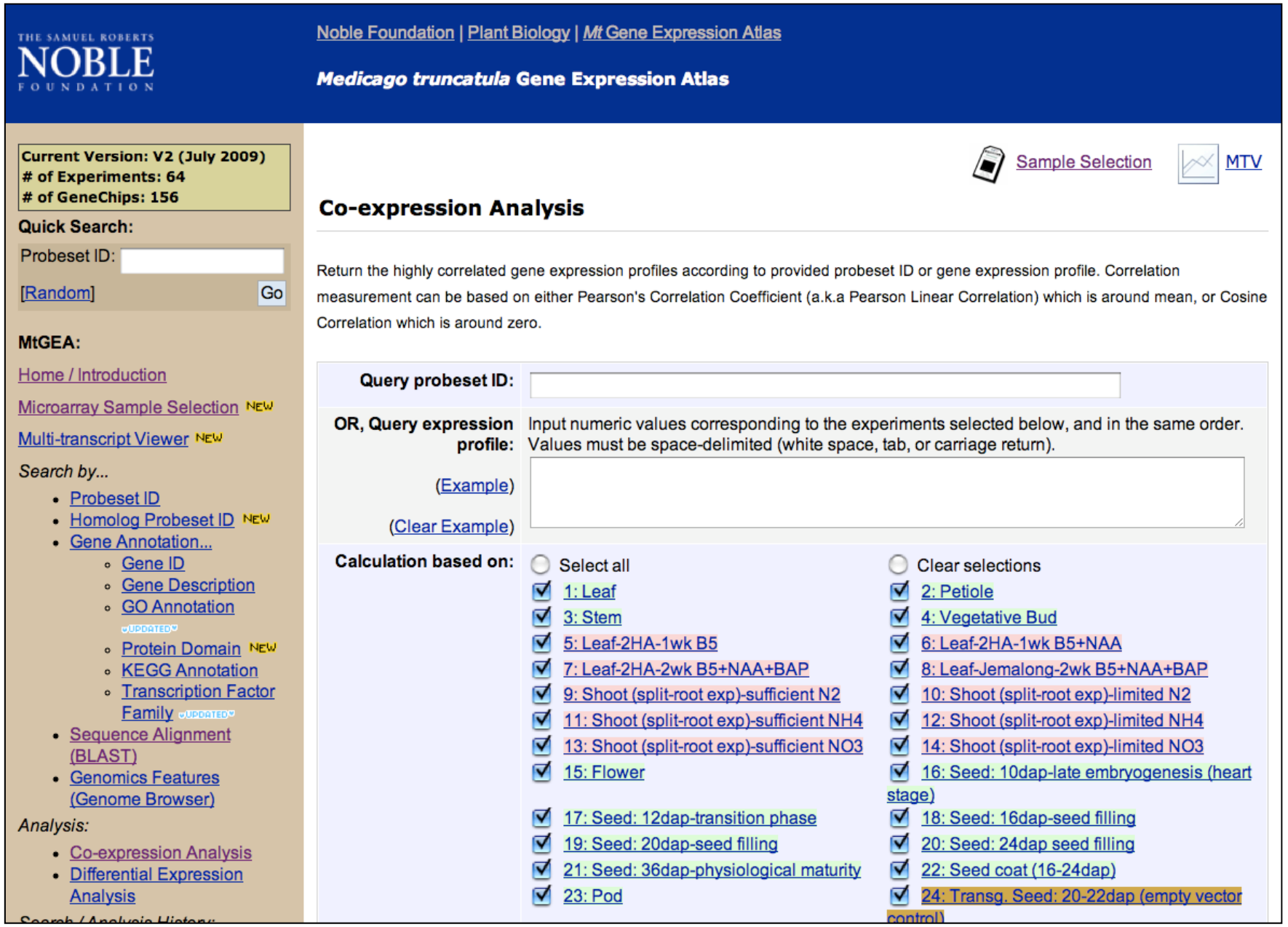
93E3



β-amyrin



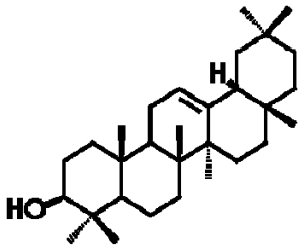
Gene co-expression analysis tool of *Medicago truncatula*



Gene co-expression analysis identified *CYPs* correlated with *bAS* (β-amyrin synthase)

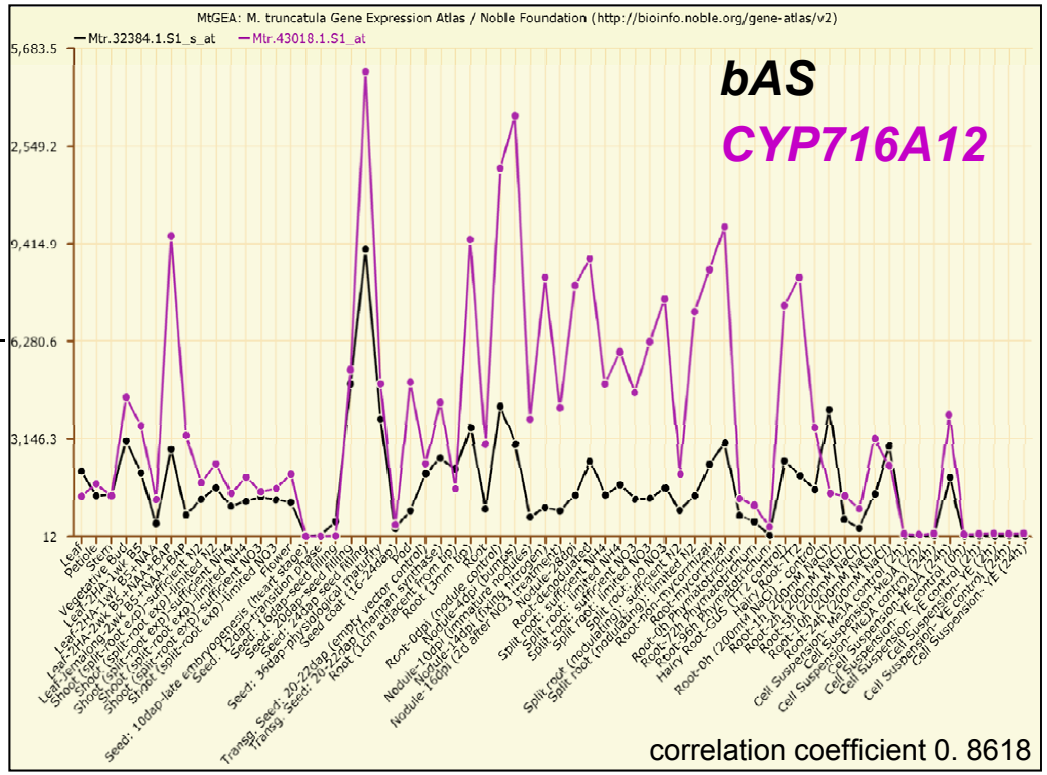
Name	Probeset	Correlation
CYP72A68	Mtr.37298.1.S1_at	0.9516
CYP72A67	Mtr.37299.1.S1_at	0.9253
CYP716A12	**Mtr.31199.1.S1_s_at**	**0.8618**
CYP704A	Mtr.49924.1.S1_s_at	0.8353
putative P450	Mtr.49925.1.S1_at	0.8219
CYP72A61	Mtr.43117.1.S1_at	0.7942
putative P450	Mtr.24735.1.S1_at	0.7734
CYP93E2	Mtr.8618.1.S1_at	0.7688

P450s correlated with bAS based on a Pearson correlation coefficient cut-off 0.7



716A12 ?

β-amyrin



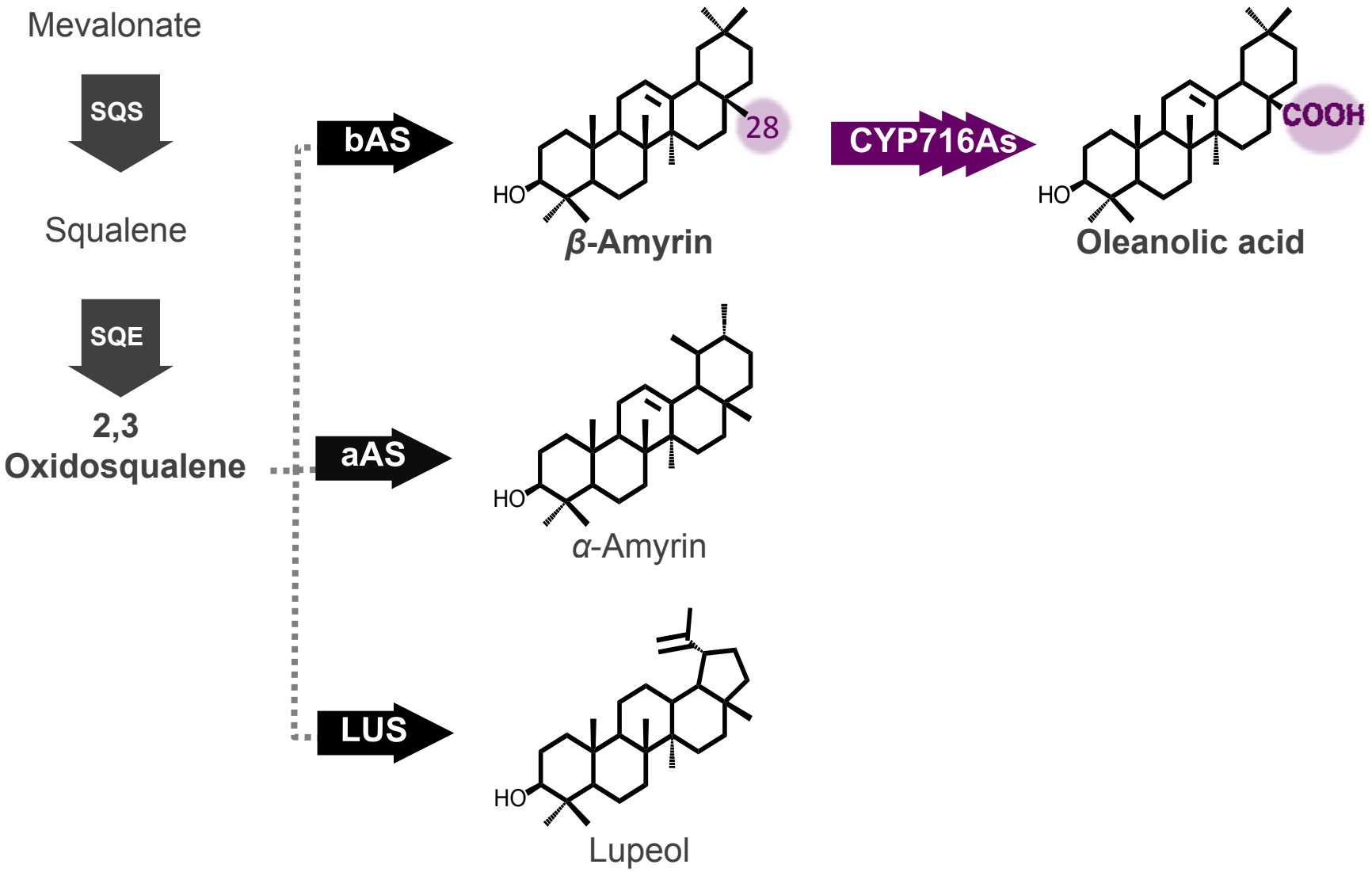
Gene co-expression analysis in *M. truncatula*

P450s correlated with *bAS*

Name	Probeset	Correlation	
CYP72A68	Mtr.37298.1.S1_at	0.9036	
CYP72A67	Mtr.37299.1.S1_at	0.9027	No detected activity
CYP72A65	Mtr.24379.1.S1_at	0.7903	
CYP716A12	Mtr.31199.1.S1_s_at	0.7525	***New***
CYP89A2	Mtr.51061.1.S1_x_at	0.6693	
CYP72A61	Mtr.43117.1.S1_at	0.5452	
CYP93E2	Mtr.8618.1.S1_at	0.4834	β-amyrin-24-oxidase

P450s correlated with bAS based on a Pearson correlation coefficient cut-off 0.4

CYP716A12 is a multifunctional P450



Fukushima *et al*., *Plant Cell Physiol*. 52 (2011) 2050-2061

CYP716A12 is a multifunctional P450

Mevalonate

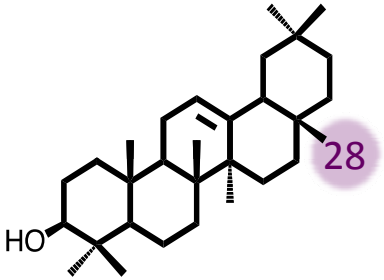
SQS

Squalene

SQE

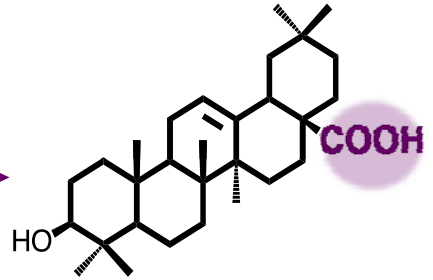
2,3 Oxidosqualene

bAS



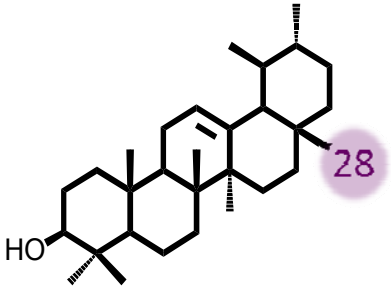
***β*-Amyrin**

CYP716As



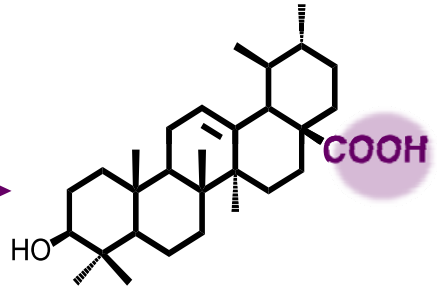
Oleanolic acid

aAS



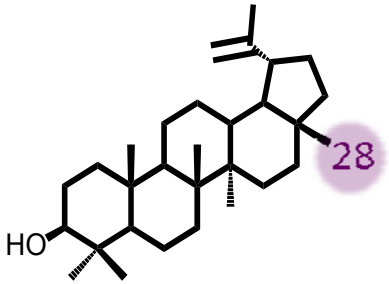
α-Amyrin

CYP716As



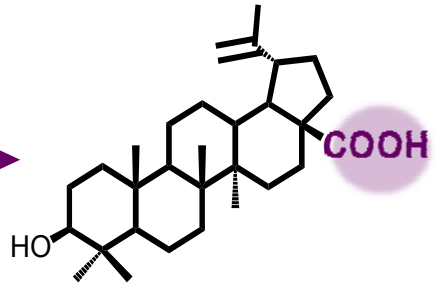
Ursolic acid

LUS

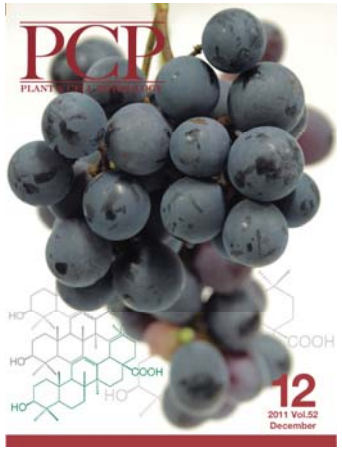


Lupeol

CYP716As

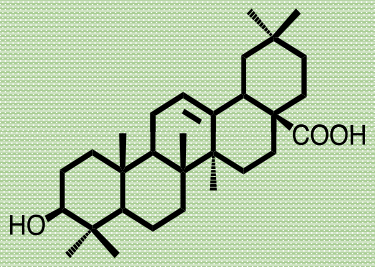


Betulinic acid



Fukushima *et al*., *Plant Cell Physiol*. 52 (2011) 2050-2061

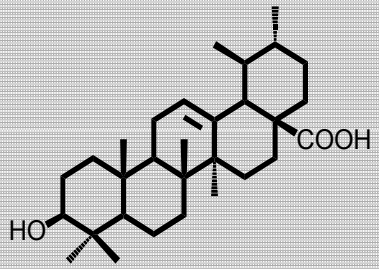
Triterpenoids similar to oleanolic acid



Oleanolic acid

Anti**tumor**
Anti**cariogenic**
Hepatoprotectiv
e
Anti-HIV

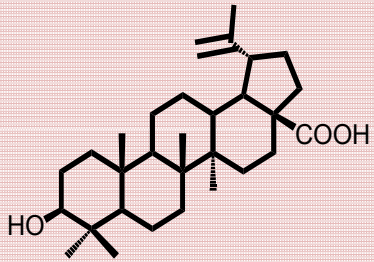
ubiquitous
>1500 species (160 families)



Ursolic acid

Anti**tumor**
Anti**cariogenic**
Analgesic
Diuretic
Anti**diabetic**

ubiquitous



Betulinic acid

Anti**tumor**
Anti**cariogenic**
Anti-HIV
Anti**fungal**
Anti**malarial**
Anti-**HSV-1**

ubiquitous

- OSCs

β-amyrin synthase (**bAS**, Lotus)

Lupeol synthase (LUS, licorice)

α-amyrin synthase (**aAS**, olive)

δ-amyrin synthase (**dAS**, tomato)

- P450s

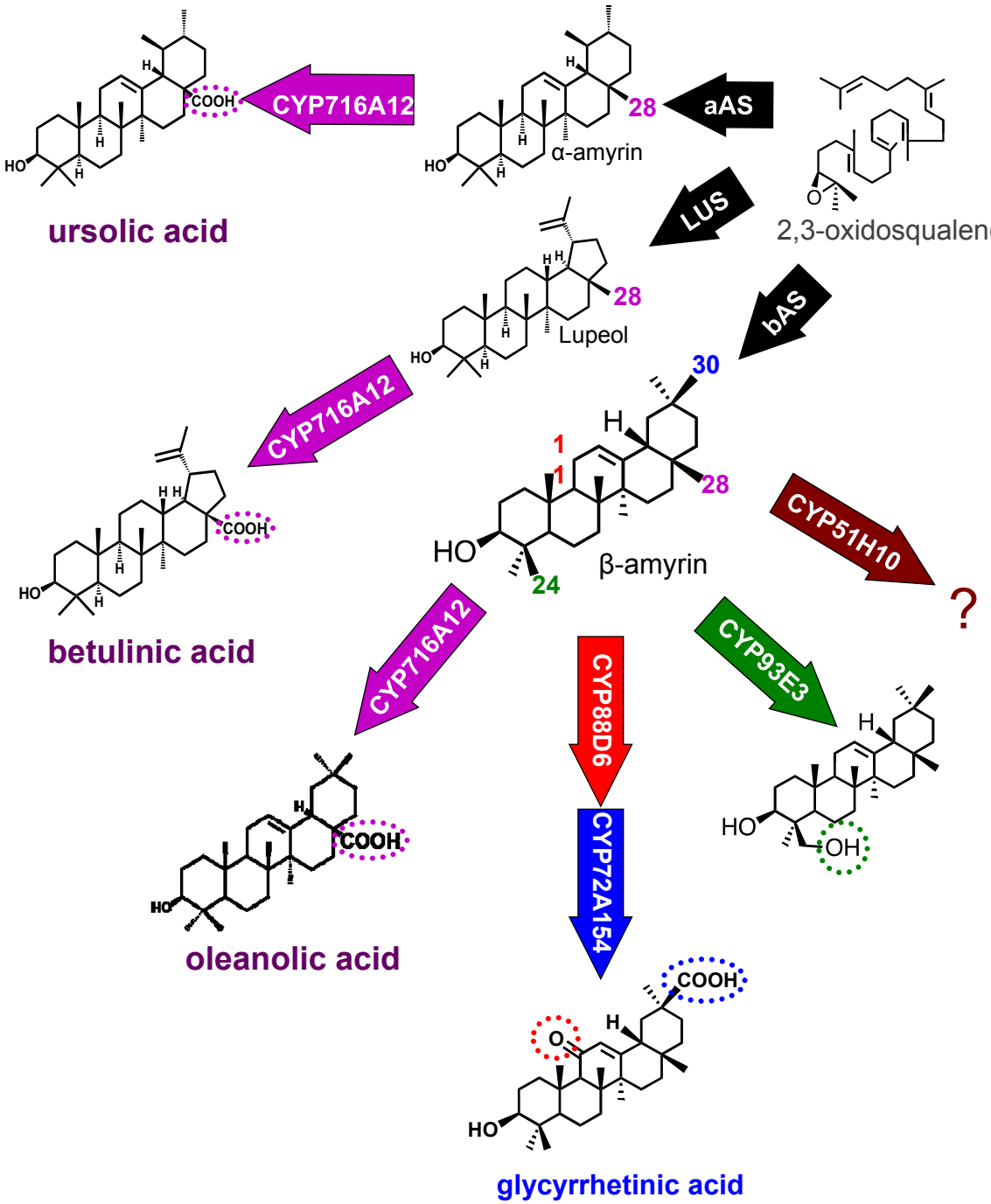
CYP88Ds (legume specific)

CYP93Es (legume specific)

CYP72As (common in plants)

CYP716As (common in plants)

CYP51Hs (monocot specific)



Previously…

*P450*s correlated with *bAS*

Name	Probeset	Correlation
CYP72A68	Mtr.37298.1.S1_at	0.9036
CYP72A67	Mtr.37299.1.S1_at	0.9027
CYP72A65	Mtr.24379.1.S1_at	0.7903
CYP716A12	Mtr.31199.1.S1_s_at	0.7525
CYP89A2	Mtr.51061.1.S1_x_at	0.6693
CYP72A61	Mtr.43117.1.S1_at	0.5452
CYP93E2	Mtr.8618.1.S1_at	0.4834

P450s correlated with bAS based on a Pearson correlation coefficient cut-off 0.4

No detected activity

Gene co-expression analysis in *M. truncatula*

CYP93E2 + CYP72A61

*P450*s correlated with *CYP93E2*

Name	Probeset	Correlation
CYP72A61	Mtr.43117.1.S1_at	**0.898**
CYP72A61	Mtr.43118.1.S1_at	0.746

P450s correlated with bAS based on a Pearson correlation coefficient cut-off 0.6

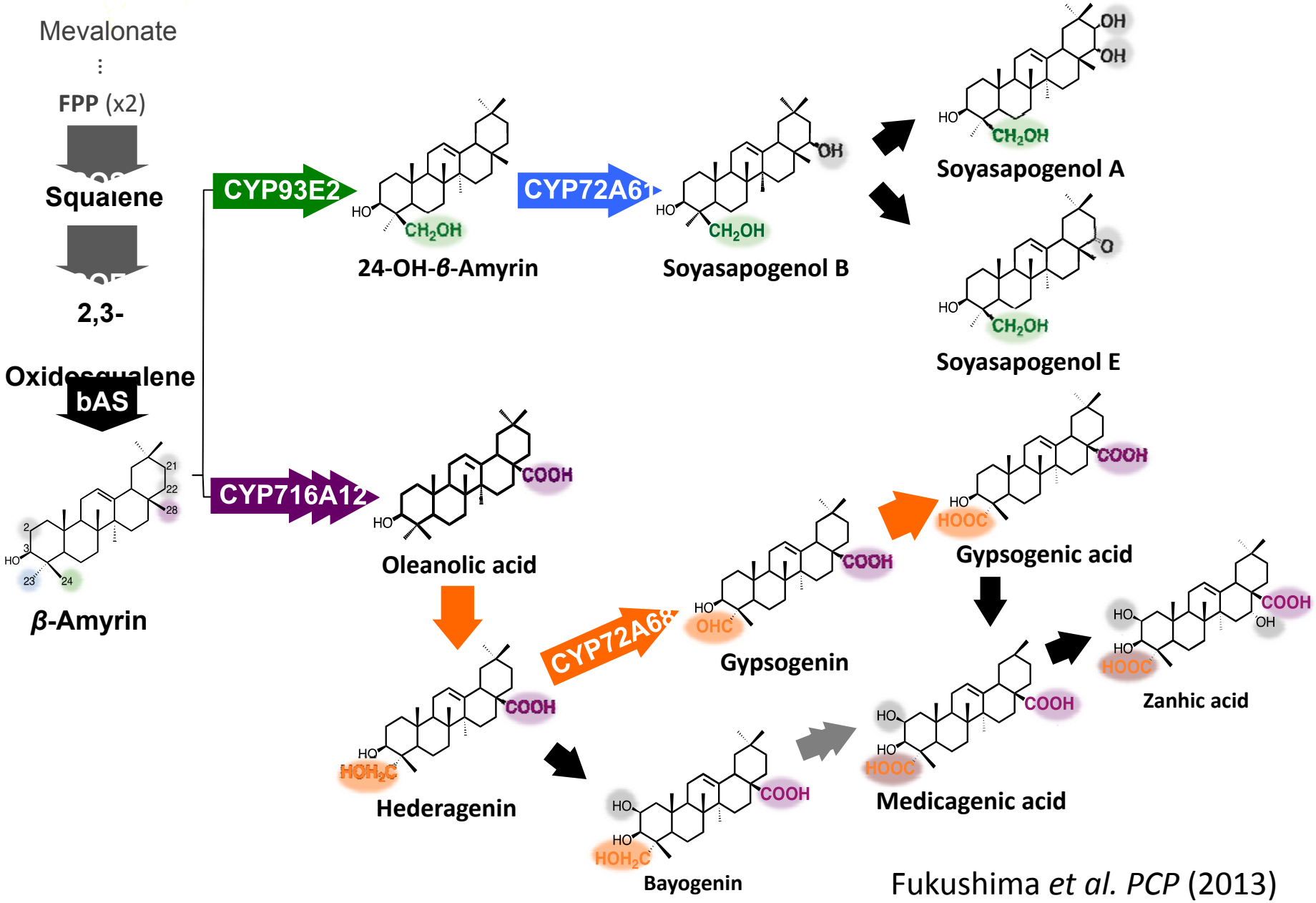
CYP716A12 + CYP72A68

CYP716A12 + CYP72A67

*P450*s correlated with *CYP716A12*

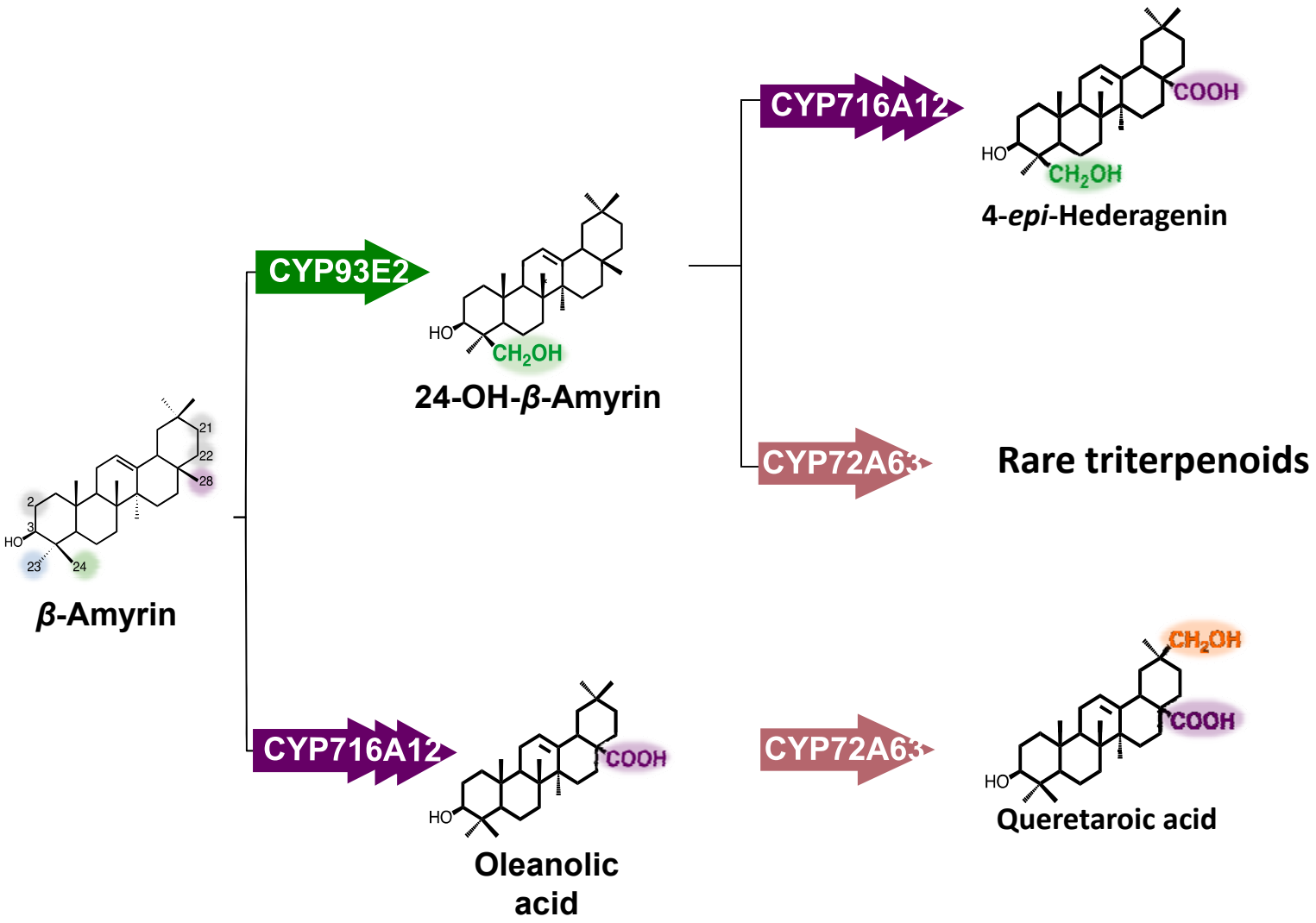
Name	Probeset	Correlation
CYP72A68	Mtr.37298.1.S1_at	**0.798**
CYP72A67	Mtr.37299.1.S1_at	**0.698**

Conclusions (in this work)



Fukushima *et al. PCP* (2013)

Conclusions (in this work)



Fukushima *et al. PCP* (2013)

Summary

Engineered plant/yeast system:

Production of useful triterpenoids

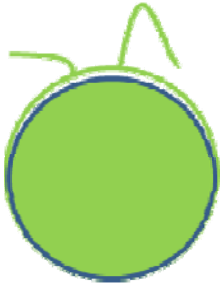
- Glycyrrhetinic acid: CYP88D6, CYP72A154/72A63
- Oleanolic acid, betulinic acid, ursolic acid: CYP716A12

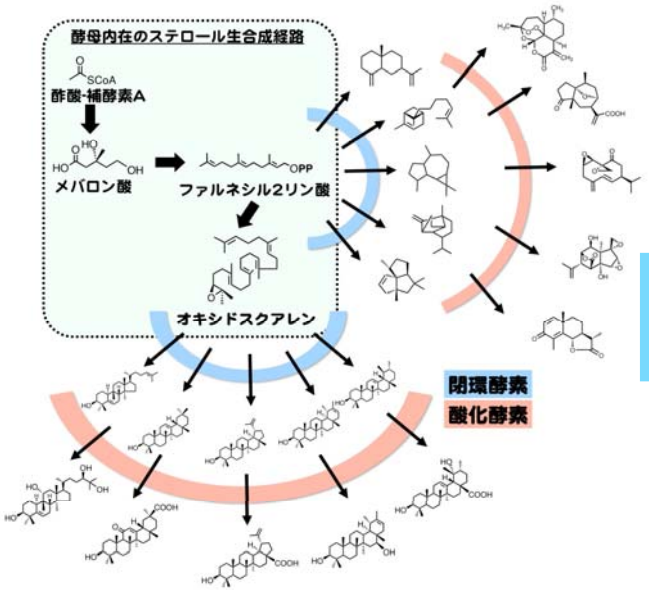
Combinatorial biosynthesis

combination of P450s

Production of rare natural product in sufficient amount and/or to generate non-natural products, to be investigated their potential biological activities

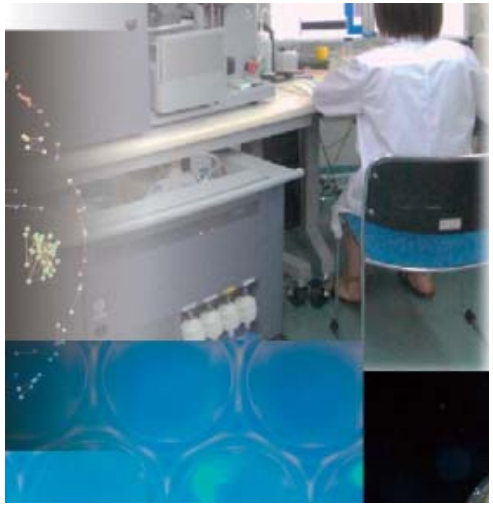
Diversity of genes for specialized metabolism(OSC, P450, UGT etc)





Combinatorial biosynthesis

Large scale production of useful terpenoids



Terpenoid factory

Non-natural/rare terpenoid library
→drug discovery, new functional food

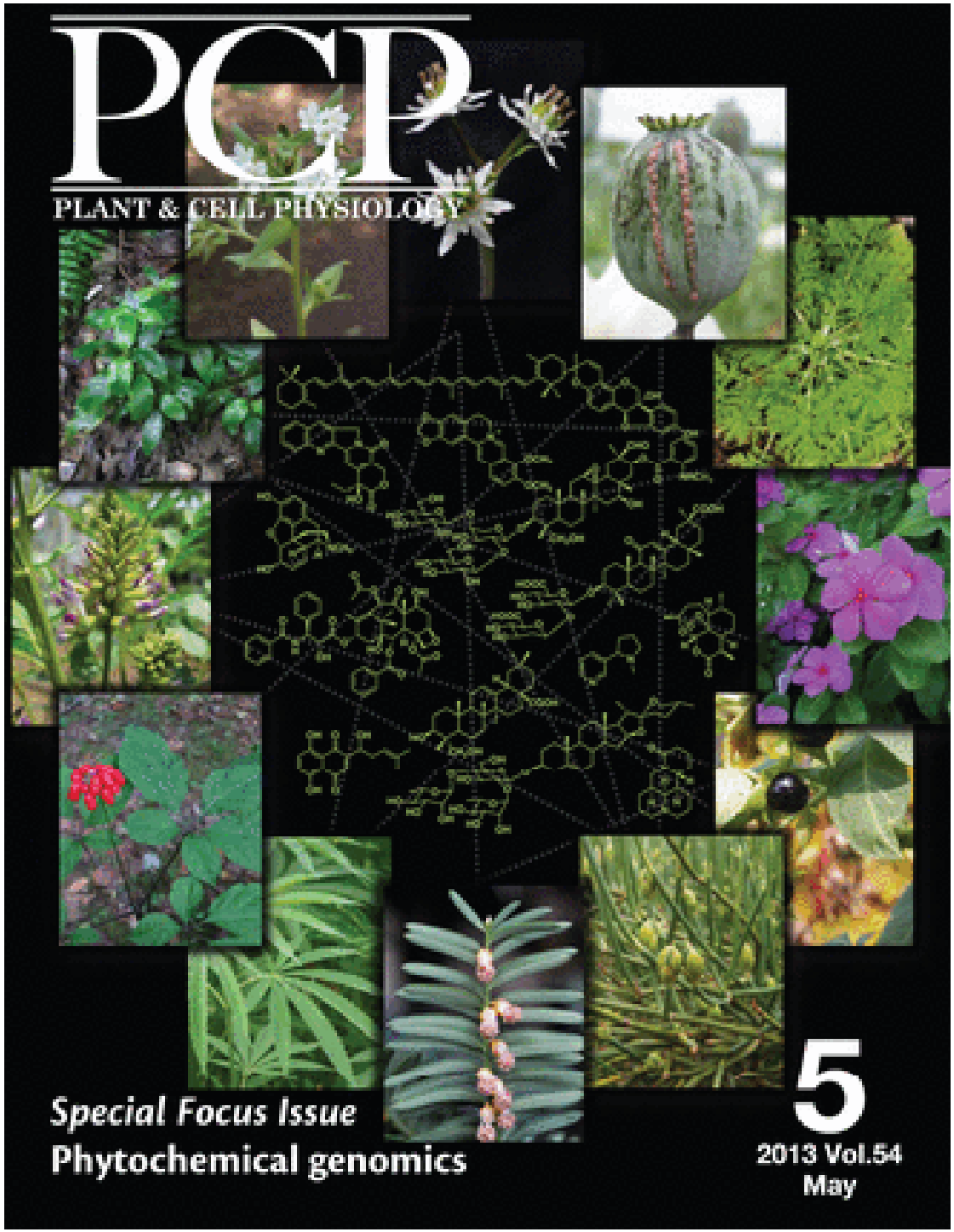
Summary 2

- The production of abietadiene was verified in *LAS*-expressing strain by GC-MS analysis.
  This is the first case of *in vivo* production of plant-derived diterpene in engineered green algae.

- These results suggest that *C. reinhardtii* could be a suitable host for the biotechnological production of abietic acid and other useful diterpenes.

大阪大学
OSAKA UNIVERSITY

Muranaka lab



PCP Special Focus Issue:
Phytochemical genomics
(2013)
(ed.) Muranaka and Saito