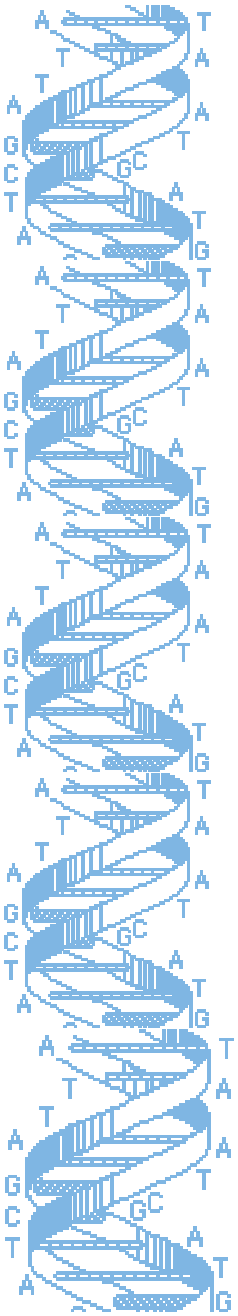


Metabolic Engineering

Plant Metabolic Engineering under Construction

Jacqueline Vanni Shanks

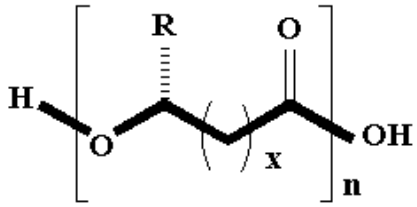
Dept. of Chemical Engineering

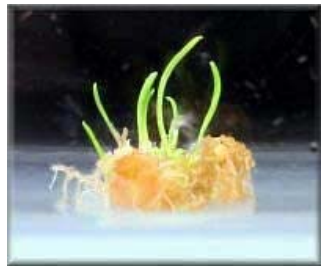
Iowa State University

Plant Potential – the six F's

- **Food for Humans**
- **Feed for Animals**
- **Fiber**

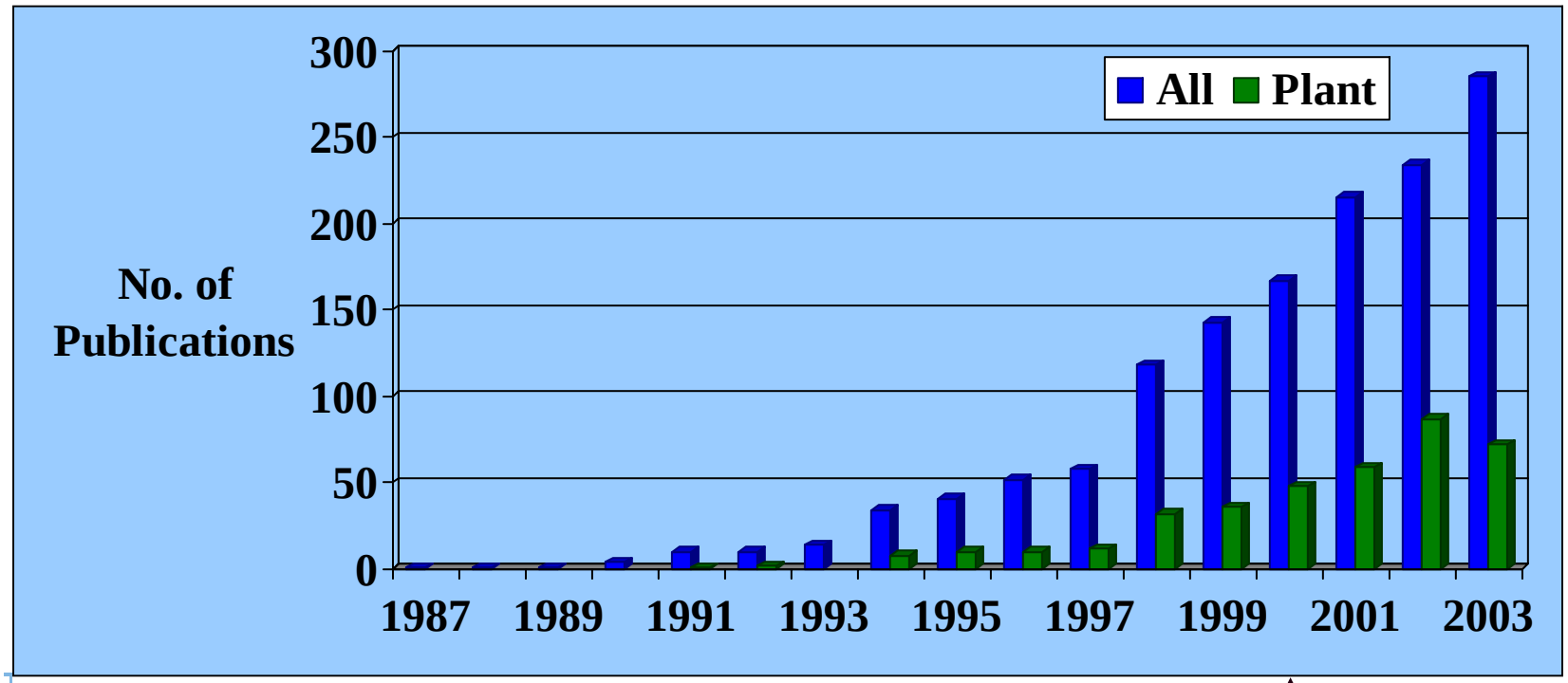
- **Fuel**
- **Pharmaceuticals**
- **Feedstocks for the Chemical Industry**





7th F - Phytoremediation

Publications* containing "Metabolic Engineering"



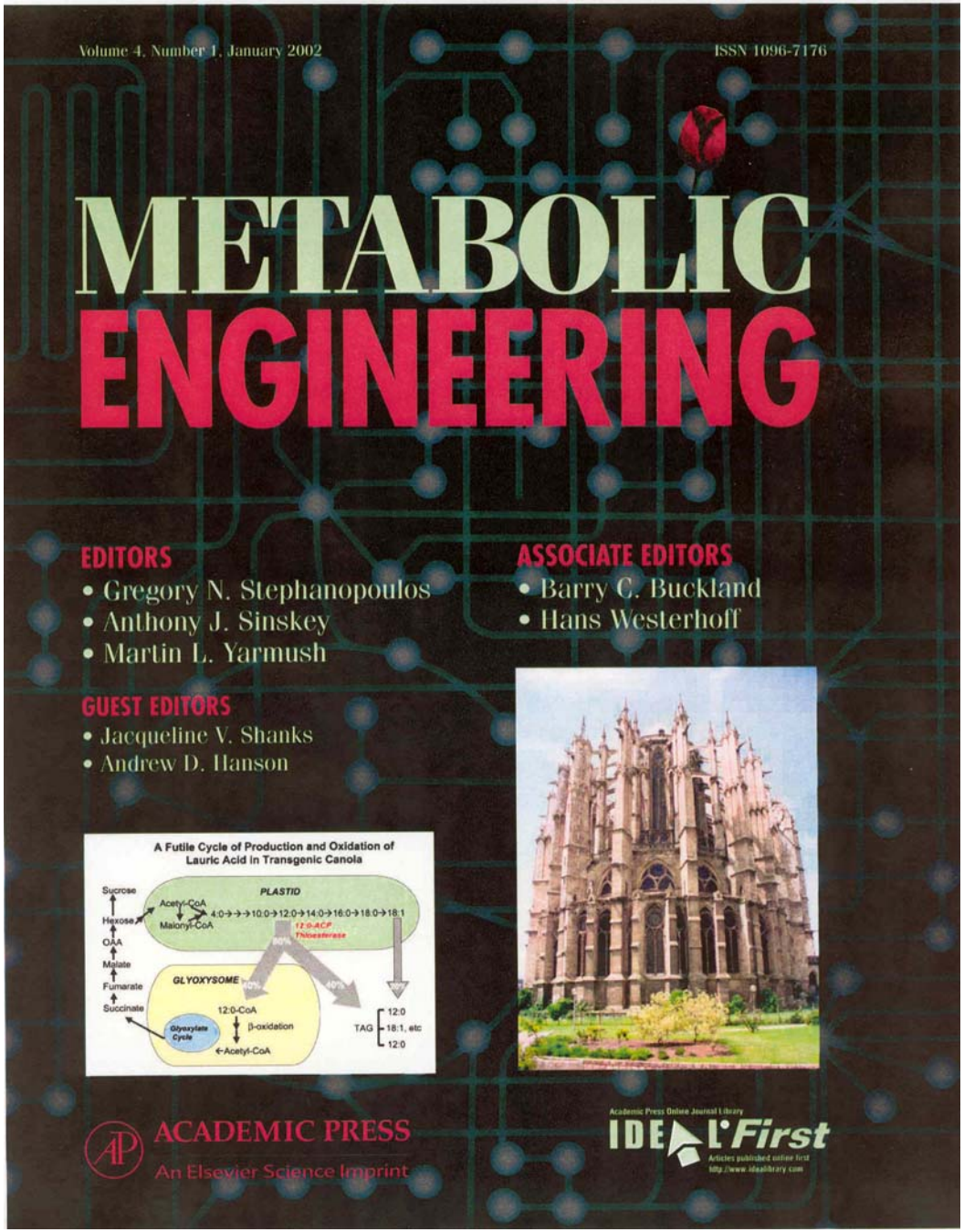
*SciFinder Scholar

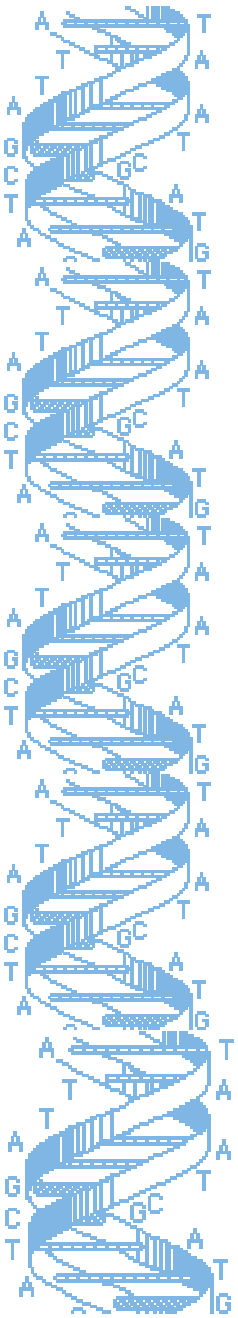
1999

Journal dedicated to Metabolic Engineering is founded

JAN 2002

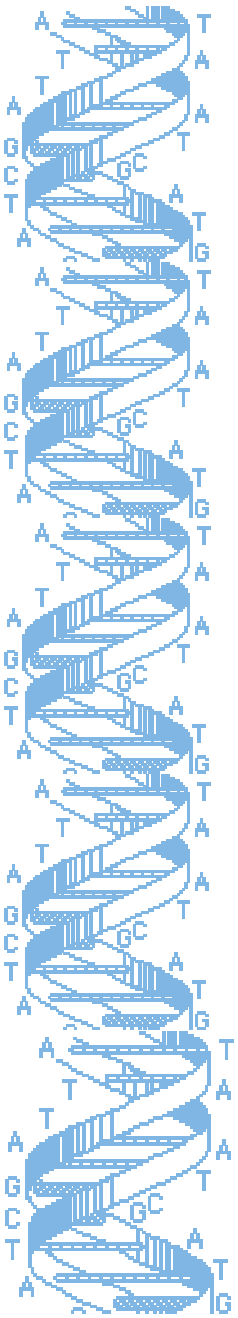
ME issue dedicated to Plant Metabolic Engineering





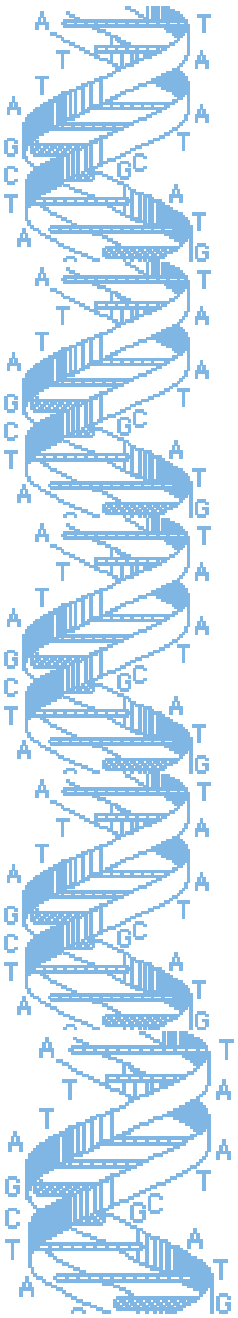
Synthesis Research Needs

- **Necessary genes cloned**
- **Transform plants with multiple genes**
- **Organ, cell-specific, or inducible promoters**



Analysis Research Needs

- **Measurement tools**
  - **protein and metabolite levels**
  - **metabolic fluxes**
  - **simultaneously and as quickly as possible**
- **Models to analyze networks**
- **Uncertainty in measurements tied to predictions/estimates**



Overproduction of Tryptophan and Indole Alkaloids in *C. roseus*

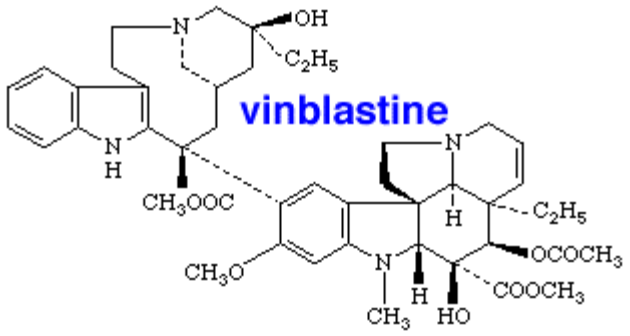
Project Number (BES 9906978, 0224600)

Jacqueline V. Shanks (Iowa State.)
Susan Gibson (Univ. of Minn.)
Ka-Yiu San (Rice)

Ganesh Sriram (ISU),Erik Hughes (Rice),
Christy Peebles (Rice), Guy Sander (ISU)
Dennis Hong (Rice & Univ. Minn*)*

Catharanthus roseus

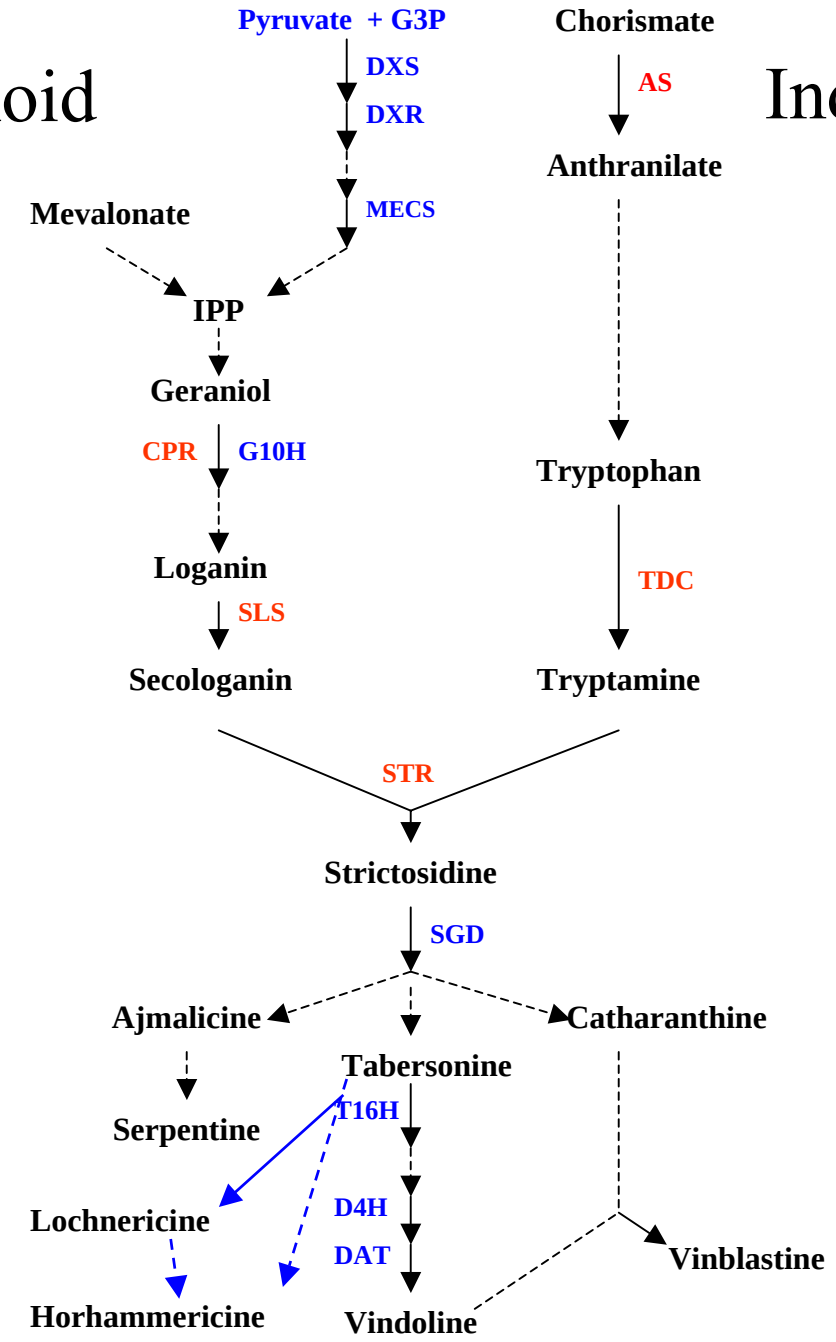
- **Therapeutic alkaloids**
  - **Vincristine & Vinblastine 10^6 $/kg**
    - **anticancer**
  - **Ajmalicine & Serpentine**
    - **anti-hypertension**
- **Alkaloids 1% (DW)**
- **Chemical or microbial synthesis unfeasible**



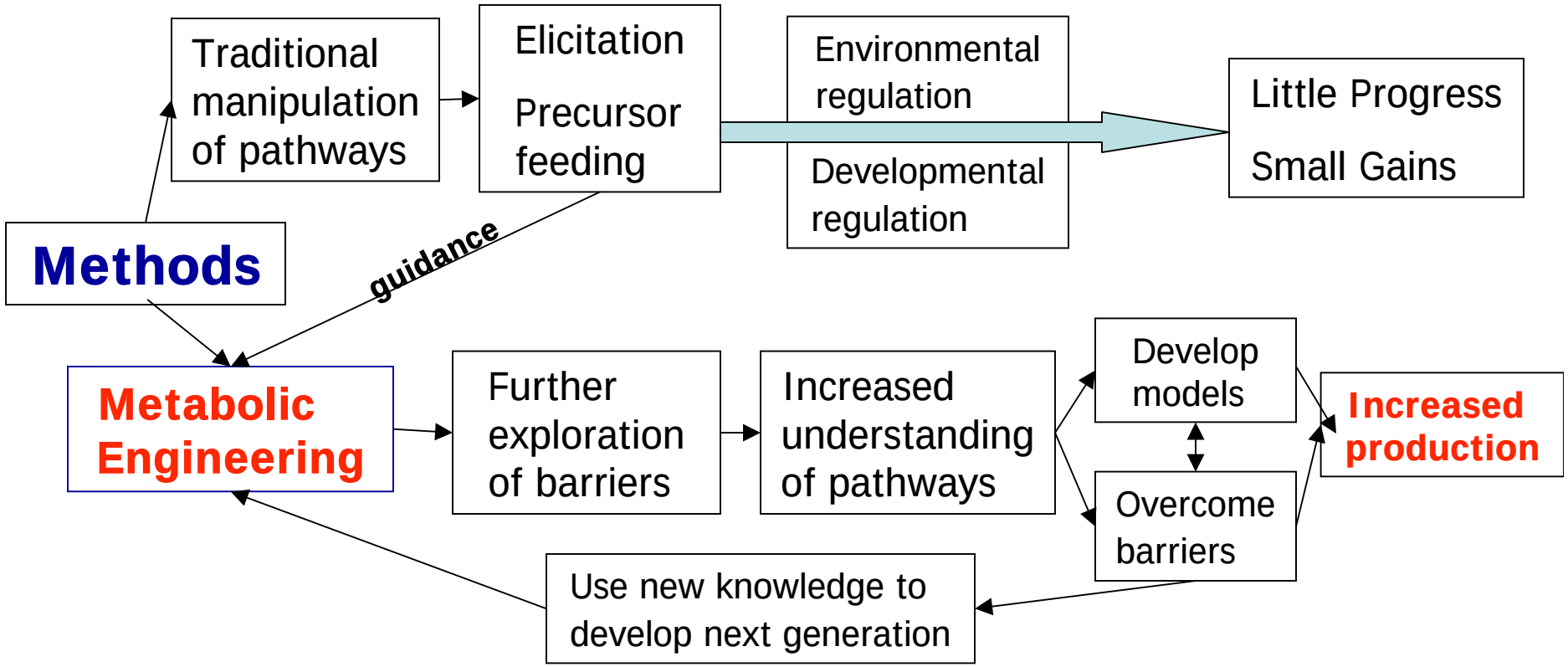
Indole Alkaloids Pathways

~1997

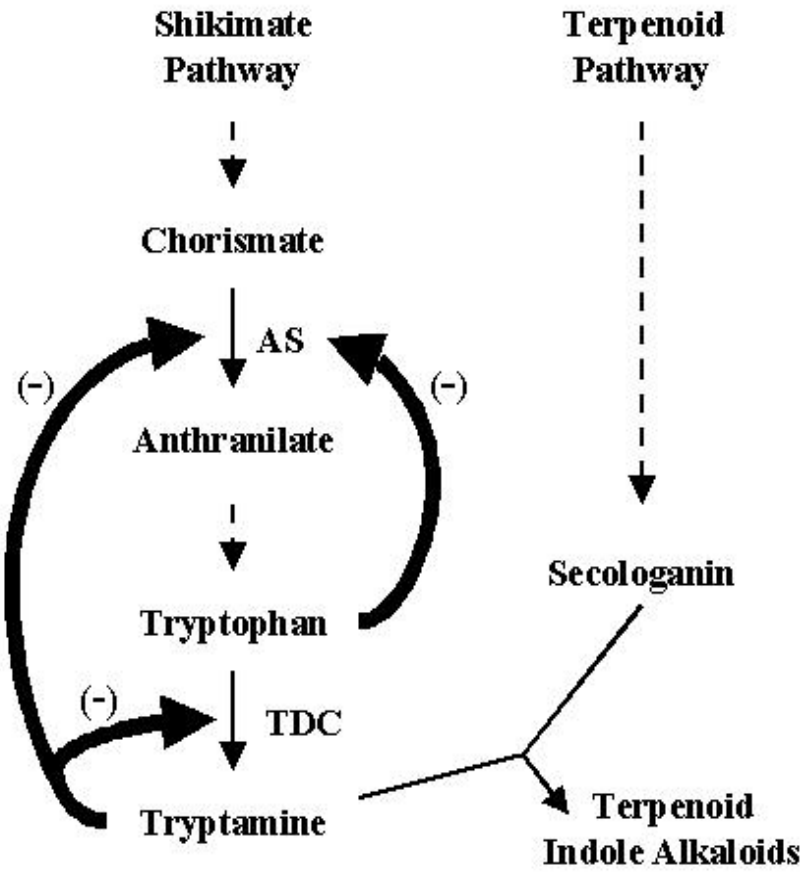
2000



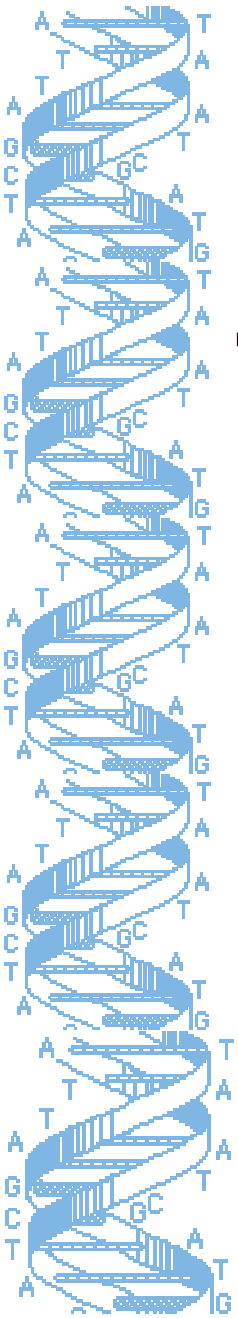
Motivation


Traditional manipulation of pathways
Elicitation
Precursor feeding
Environmental regulation
Developmental regulation
Little Progress
Small Gains
Methods
guidance
Metabolic Engineering
Further exploration of barriers
Increased understanding of pathways
Develop models
Overcome barriers
Increased production
Use new knowledge to develop next generation

Pathway Regulation by Anthranilate Synthase (AS)



- **AS Activity**
  - **α Feedback Inhibited by Tryptophan**
  - **β unit?**
- **Tryptophan Feeding**
  - **1.5x alkaloid levels (exponential growth)**
- **Genetic Manipulations**
  - **Feedback resistant *Arabidopsis* ASα**
    - **3x tryptophan levels**
  - **TDC from *C. roseus***
    - **Elevated tryptamine in cell culture**

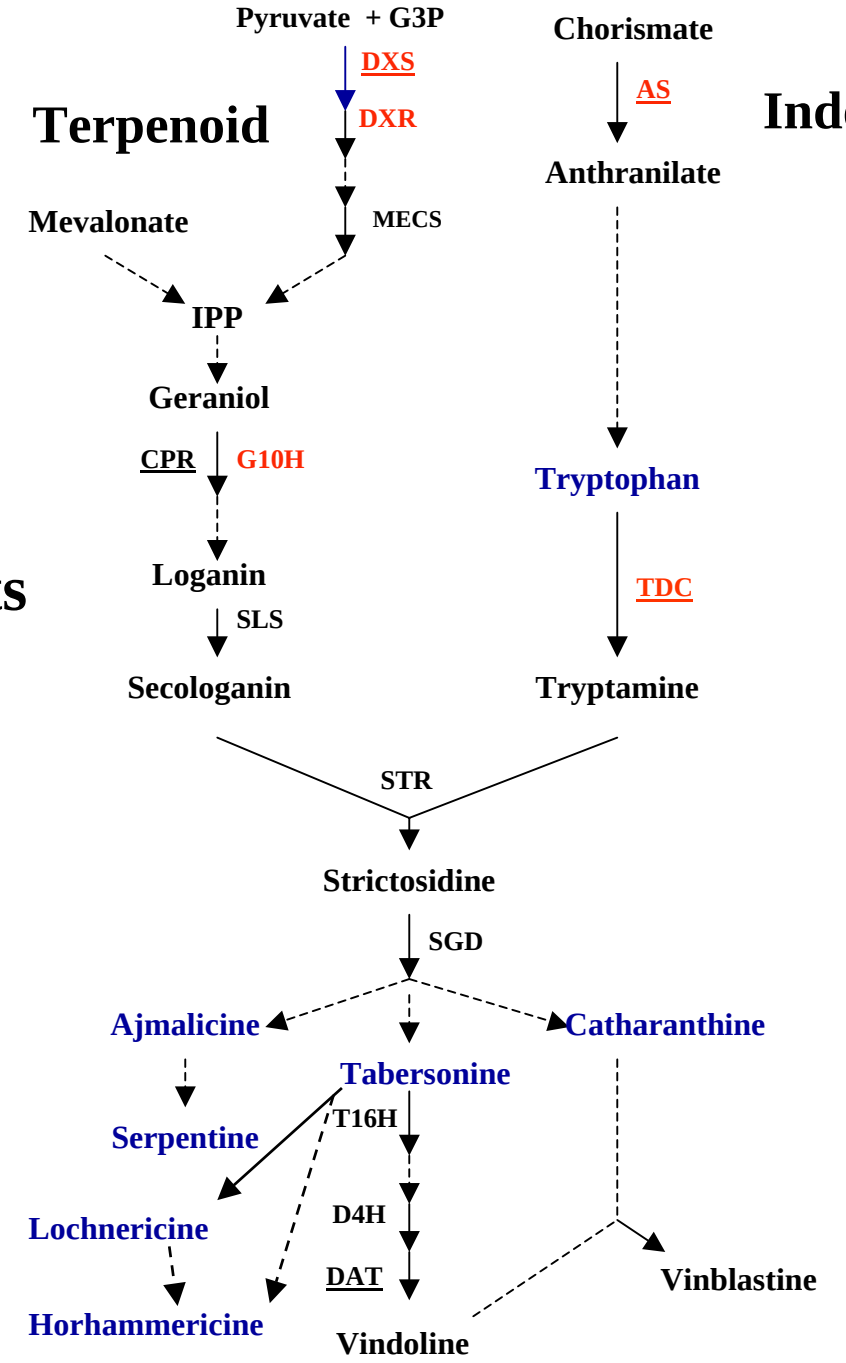


Technical Objective

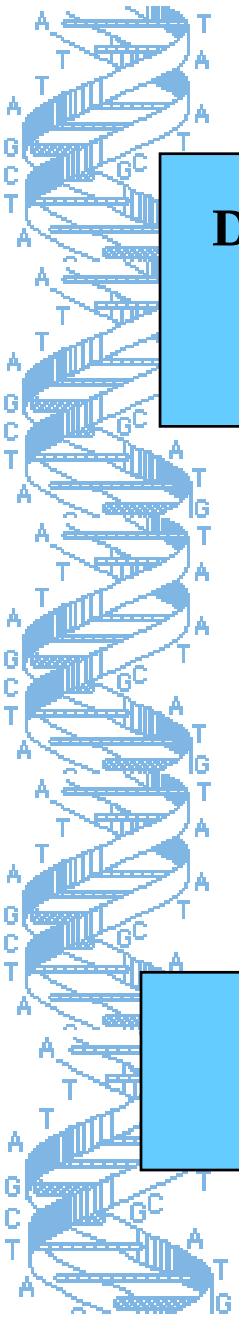
- **Engineer**
  C. roseus **hairy roots**
- **Overproduce**
  tryptophan
  indole alkaloids

Genetic targets: RED

ORCA3 induced



Technical Approach



Develop an inducible promoter system in *C. roseus* hairy root cultures

Construct and characterize transgenic hairy root lines

Metabolic characterization of 1st generation lines

NMR Flux Maps

Develop co-transformation techniques for insertion of multiple genes

Construct and characterize 2nd generation lines

Hairy root cultures

- Model system
- Transformed by Agrobacterium
- Increased genetic stability
- Fast & differentiated growth
- Higher alkaloid productivity

Clone Generation

Plasmid Construction in *E. coli*

Transgene

ATCC 15834
A. rhizogenes

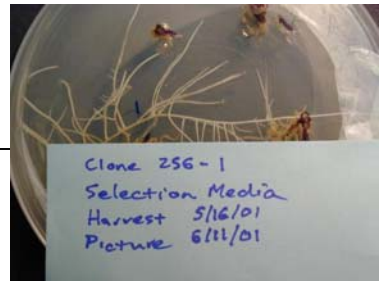
Ri

Sterile Grown Plants

(5 weeks)

Infection

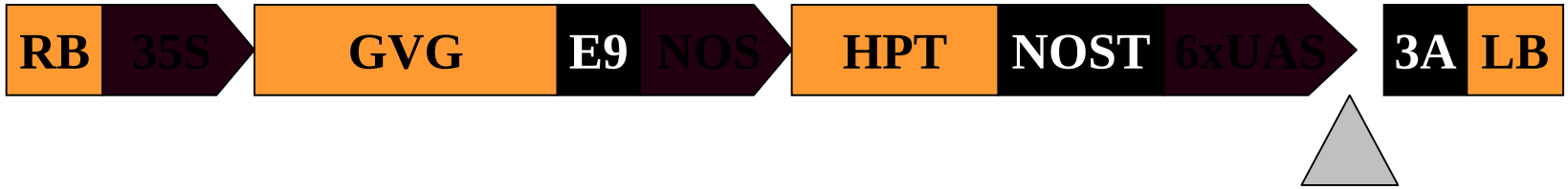
(6 weeks)



Selection Media
(6 weeks)

Adapt to Liquid Media
(12 weeks)

Inducible-Promoter System



Vertebrate Steroid Hormone Receptor System

- GVG Element

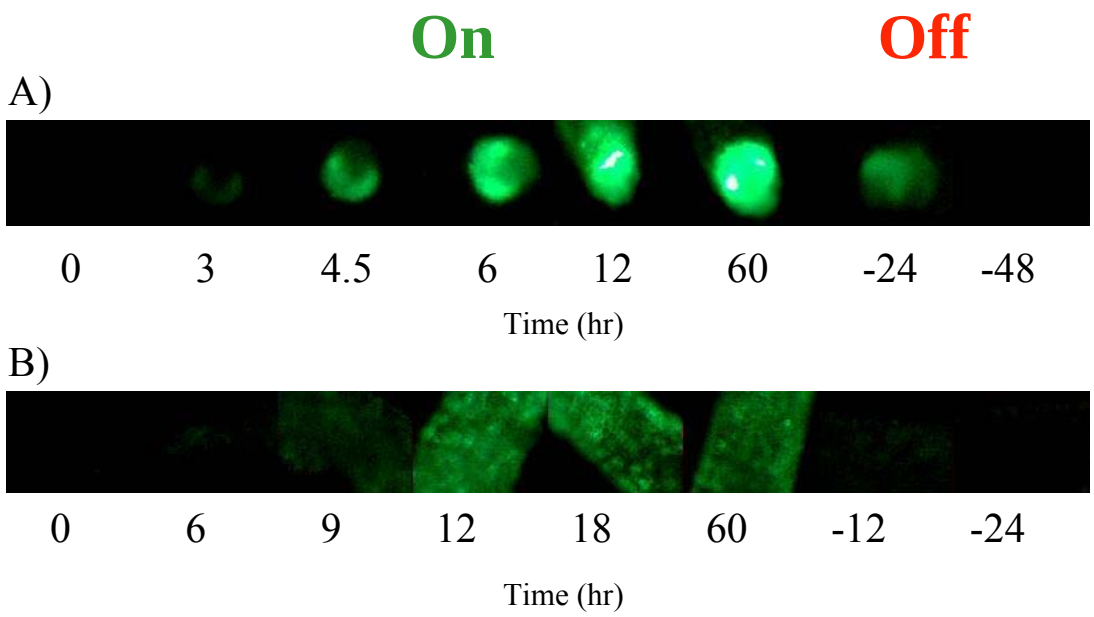
  (Glucocorticoid regulated transcription factor)

- Inducer - Dexamethasone (DEX)

  an artificial glucocorticoid hormone

Inducible-Promoter System

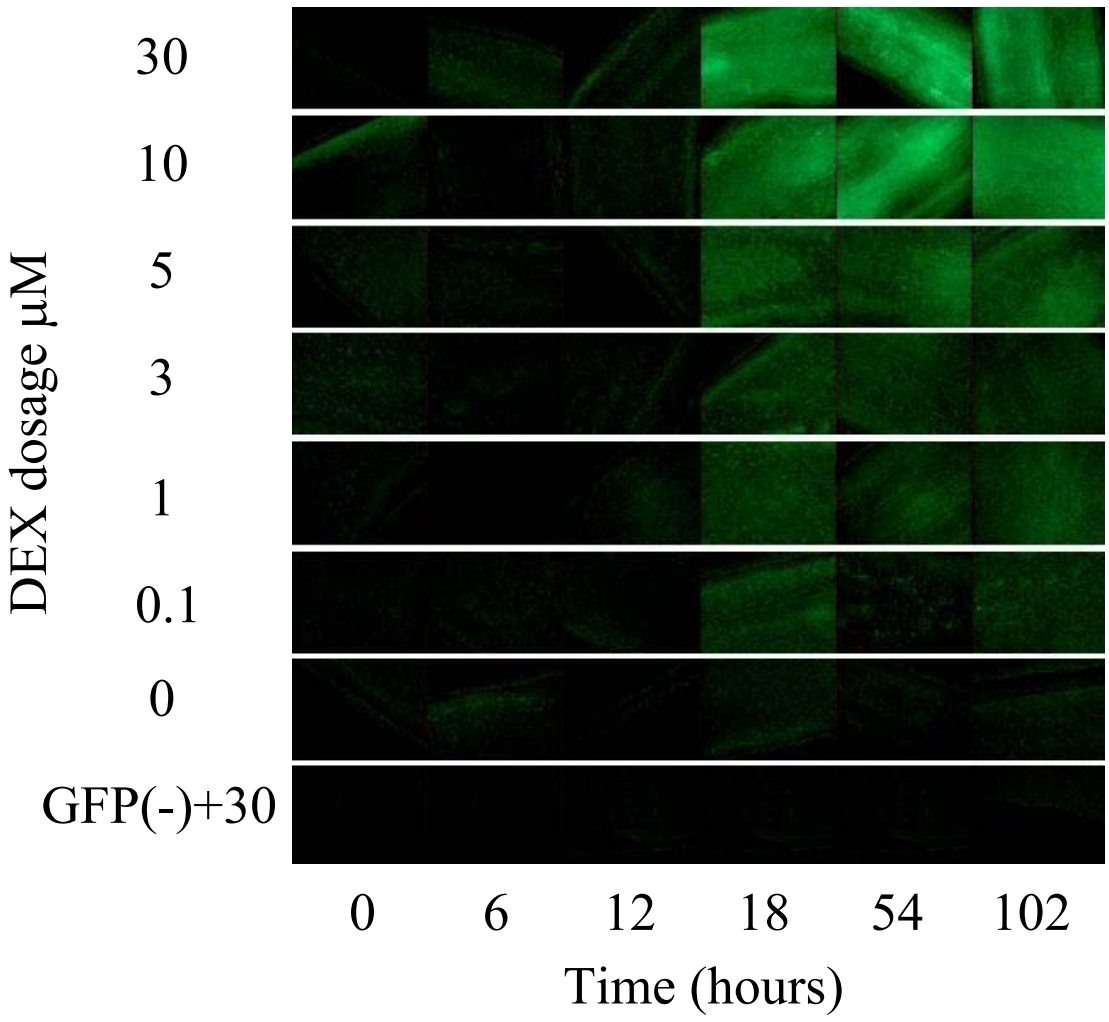
- Dexamethasone-inducible expression of green fluorescent protein (GFP)



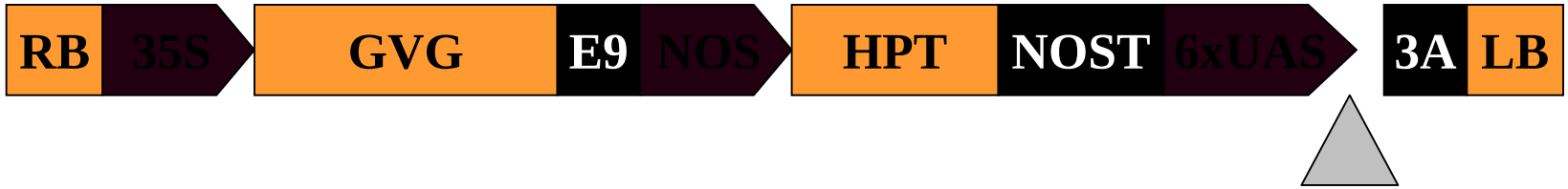
meristem region

mature region

Inducible-Promoter System



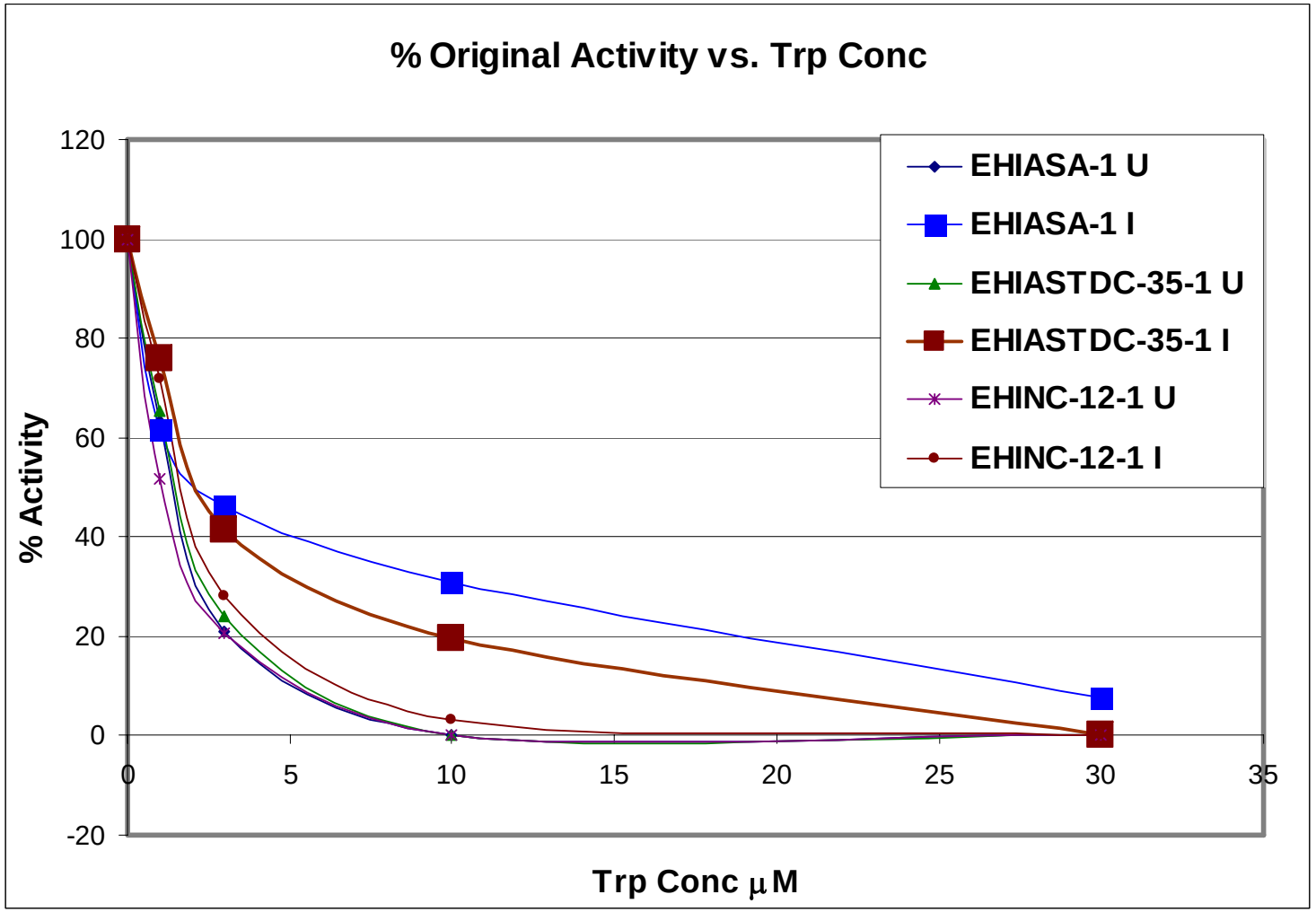
ASα and TDC Clones



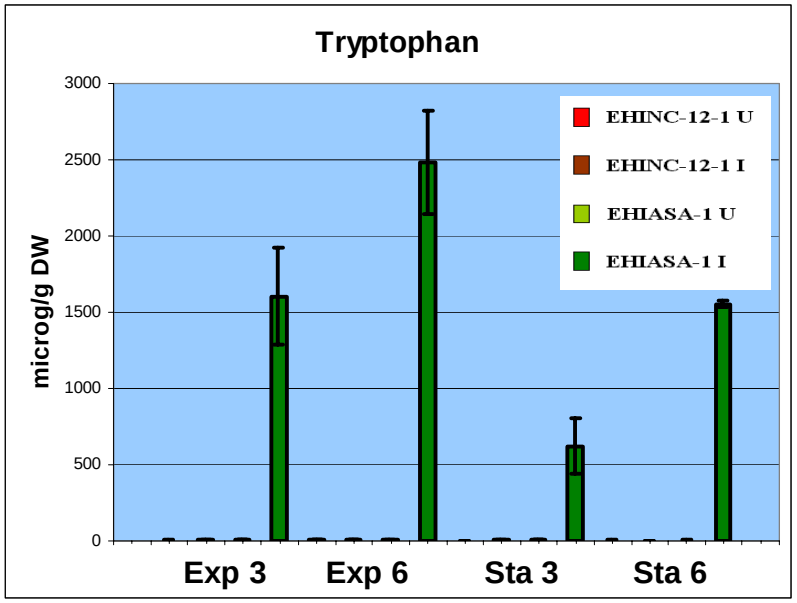
	Constitutive GVG	Inducible ASalpha	Inducible TDC
EHI**NC**-12-1	√		
EHI**ASA**-1	√	√	
EHI**TDC**-15-2	√		√
EHI**ASTDC**-35-1	√	√	√

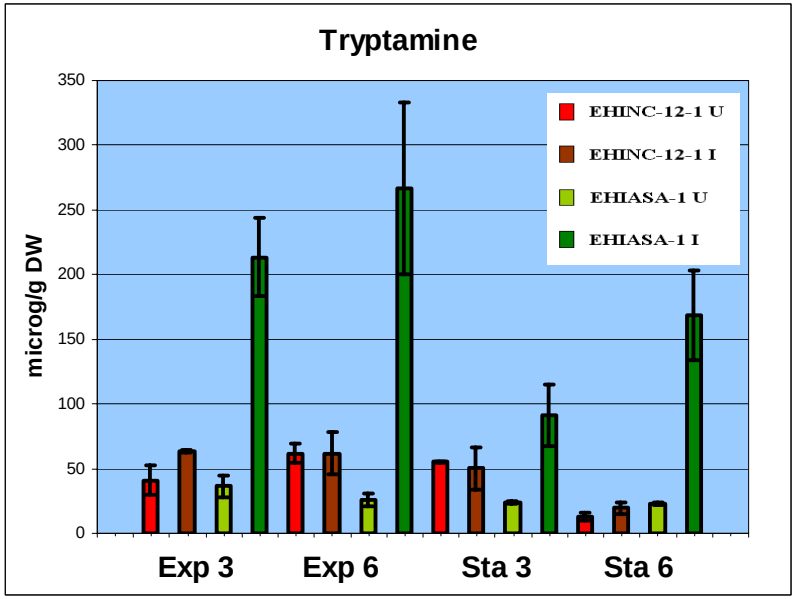
Feedback Inhibited AS

Induced 3 μM for 72 hours

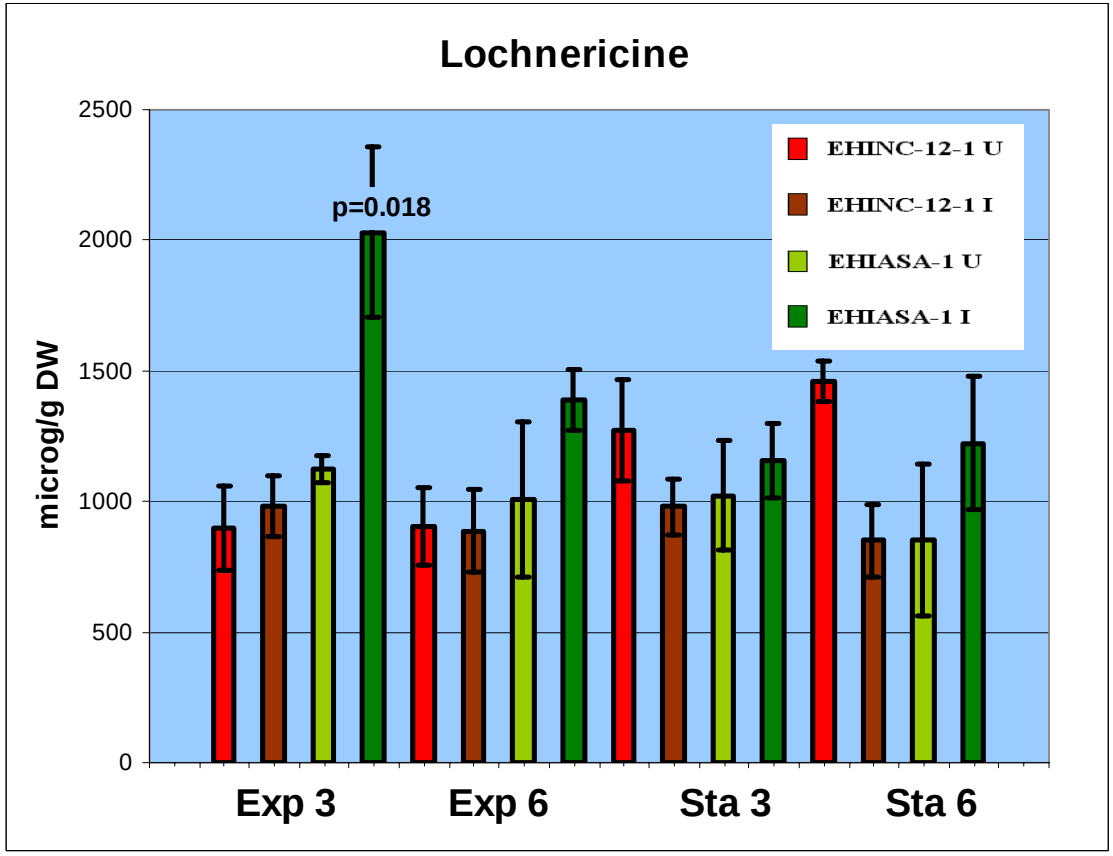


ASα line – huge increases in tryptophan and tryptamine



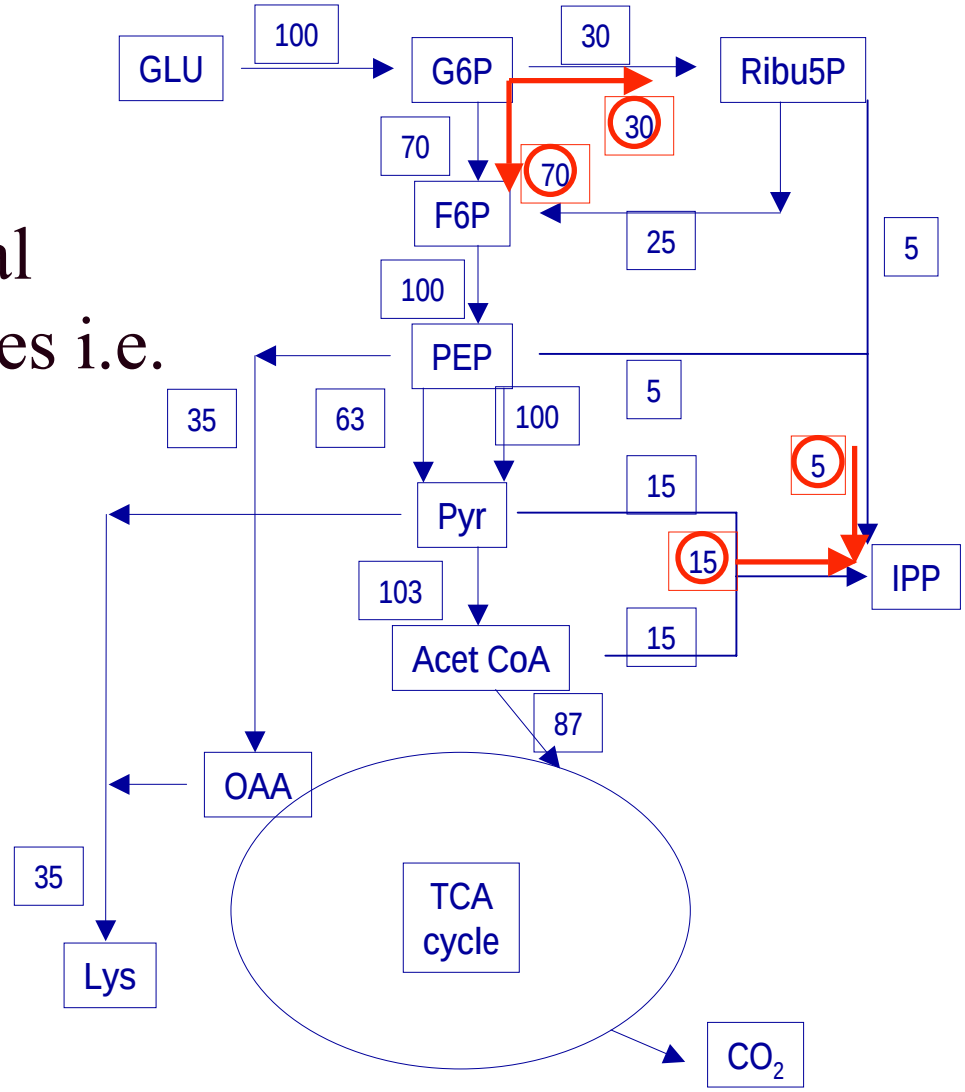


ASα line – increase in Lochnericine



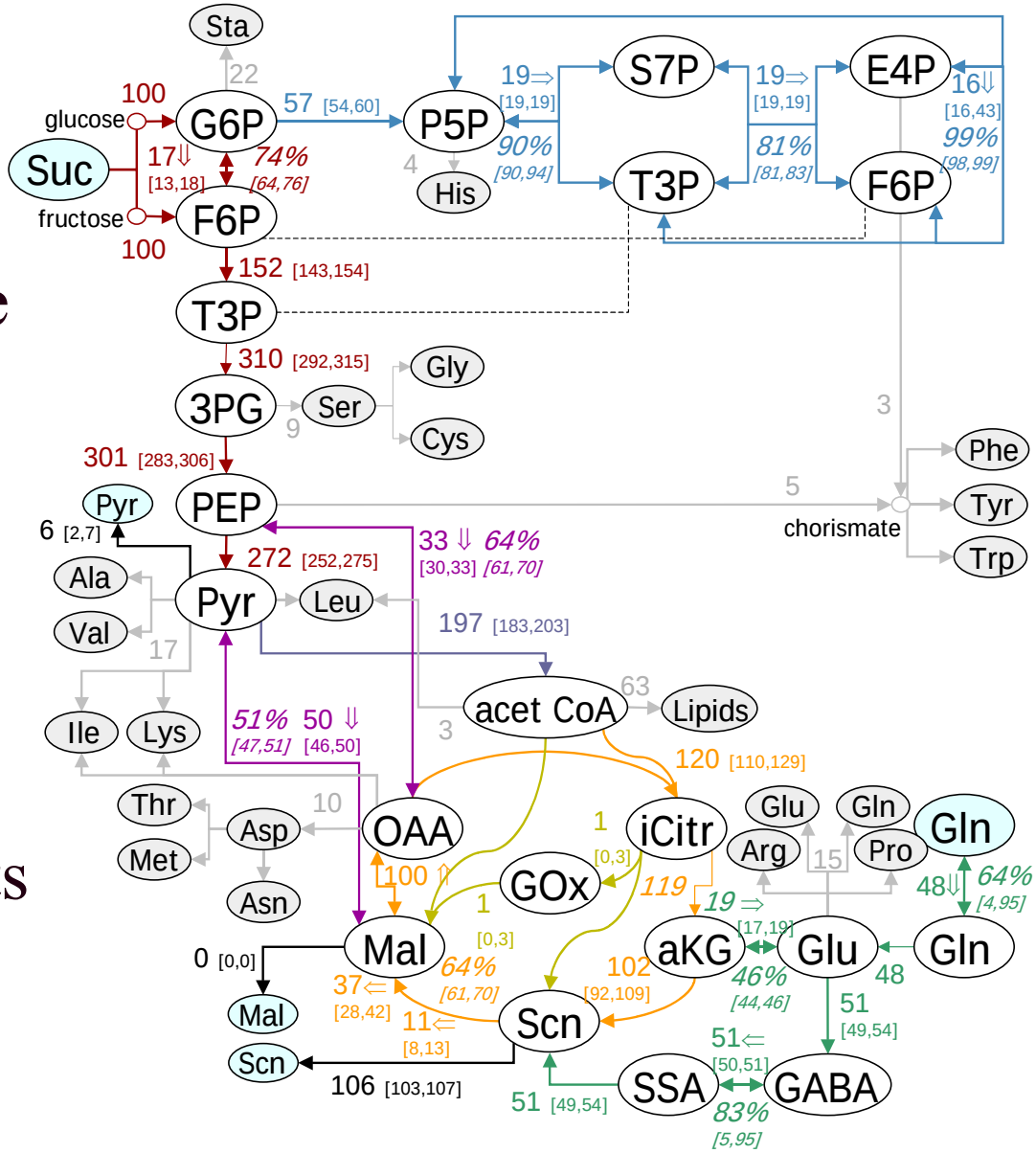
Metabolic Flux Analysis (MFA)

- NMR/GC data
  - provides additional experimental values i.e. flux ratios
  - value shown in microbial studies



Flux map

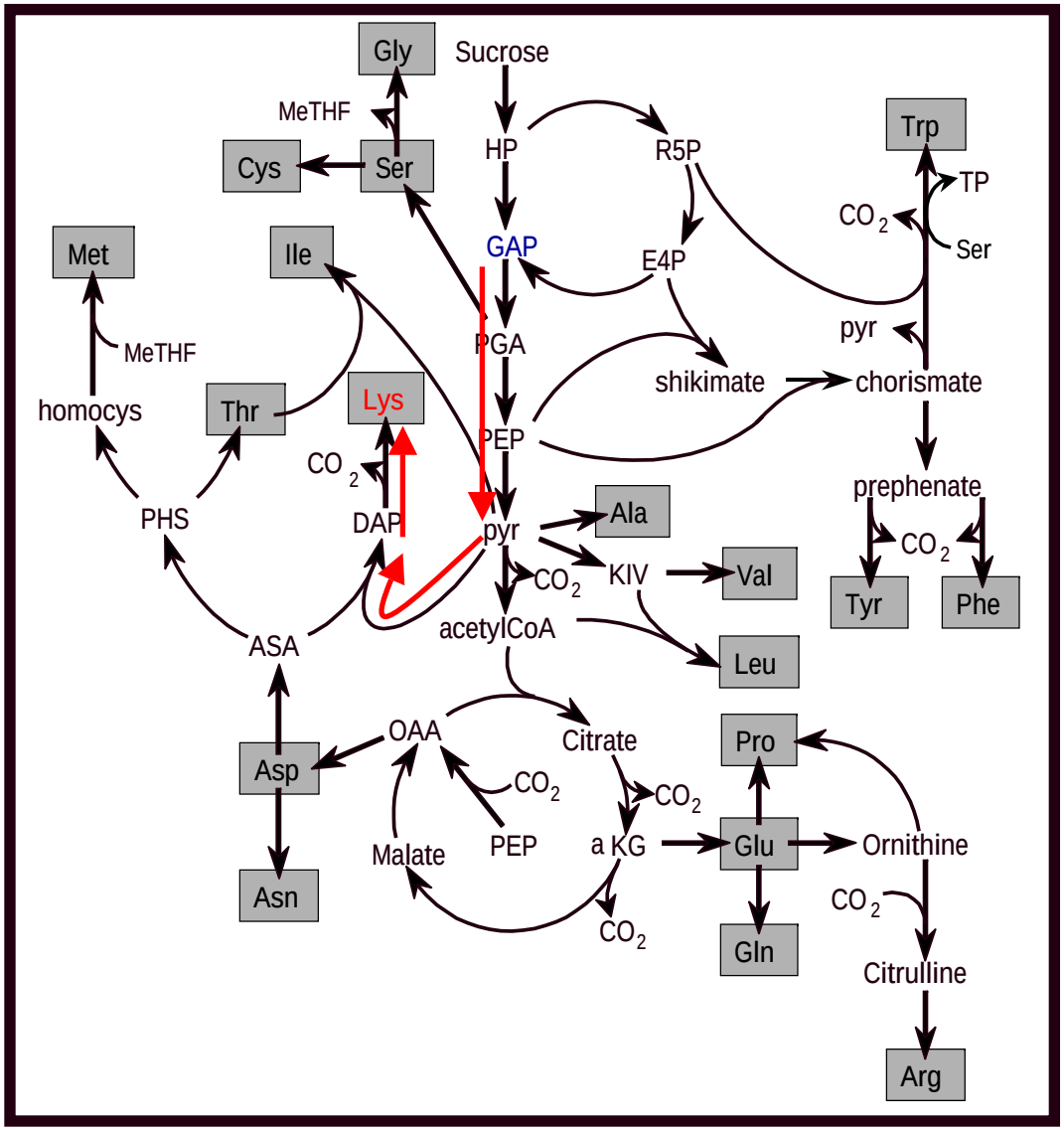
- Network Structure
  - In one or more compartments
- Flux values
- Uncertainty
- Few measurements needed
- Fast computation



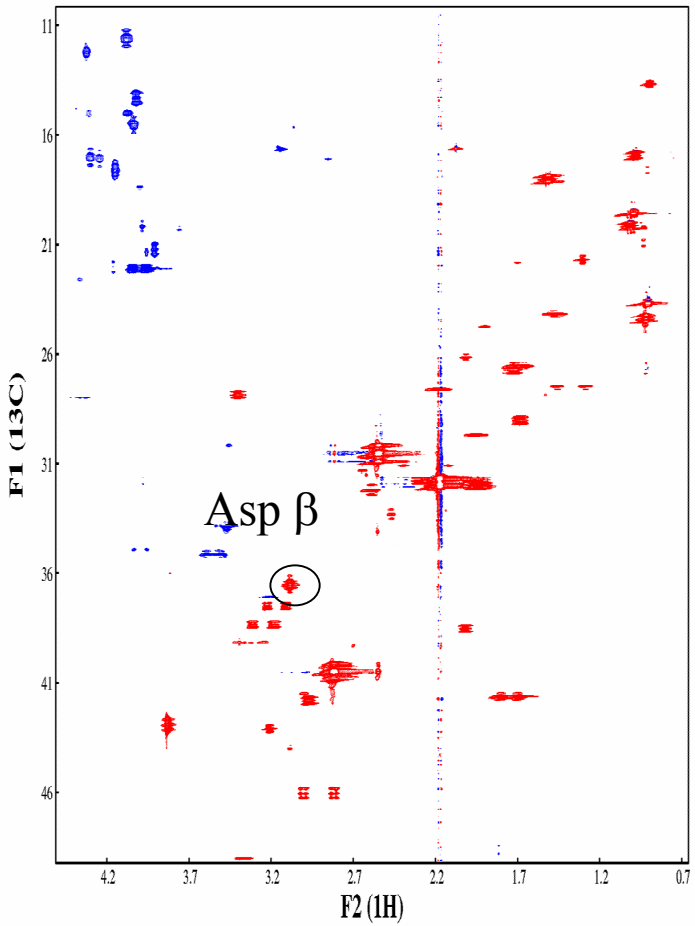
Bond-labeling experiment (BLE): rationale

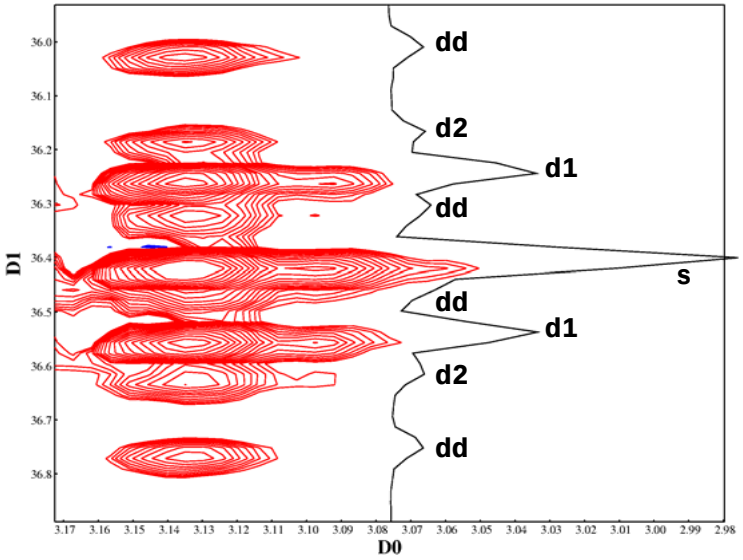
- Amino acids (AAs) reflect the structure of their precursors
- For instance, a part of the Lys molecule reflects the structure of GAP
- Thus if the ensemble of all AAs from an organism is analyzed, it will provide information about the structure of a number of central carbon metabolism precursors (which depends on metabolic flux)

How do we do this?

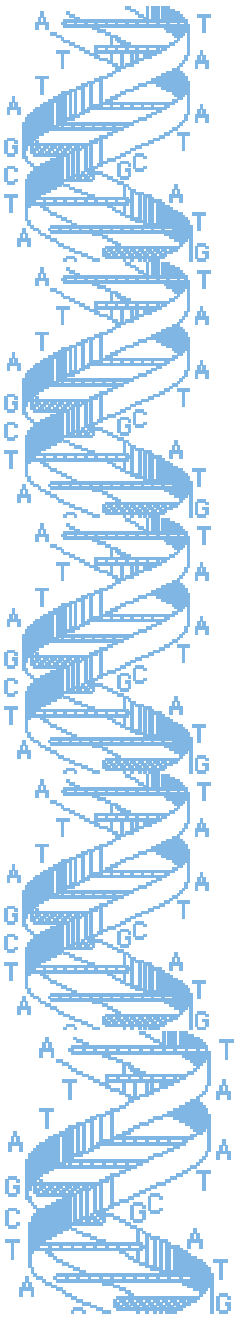


NMR spectrum of amino acids

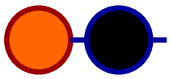
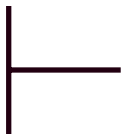




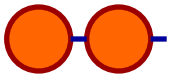
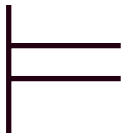
Asp β



^{13}C fine structures

^{13}C attached to ^{12}C    singlet

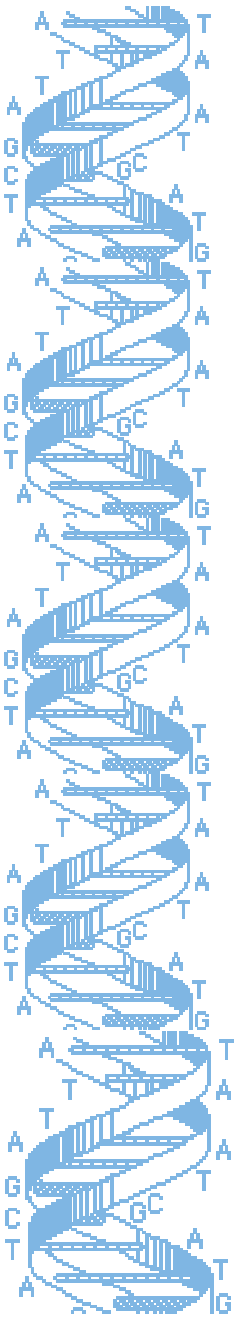
Most likely formed by a biosynthetic bond between a ^{13}C and a ^{12}C molecule

^{13}C attached to ^{13}C    doublet

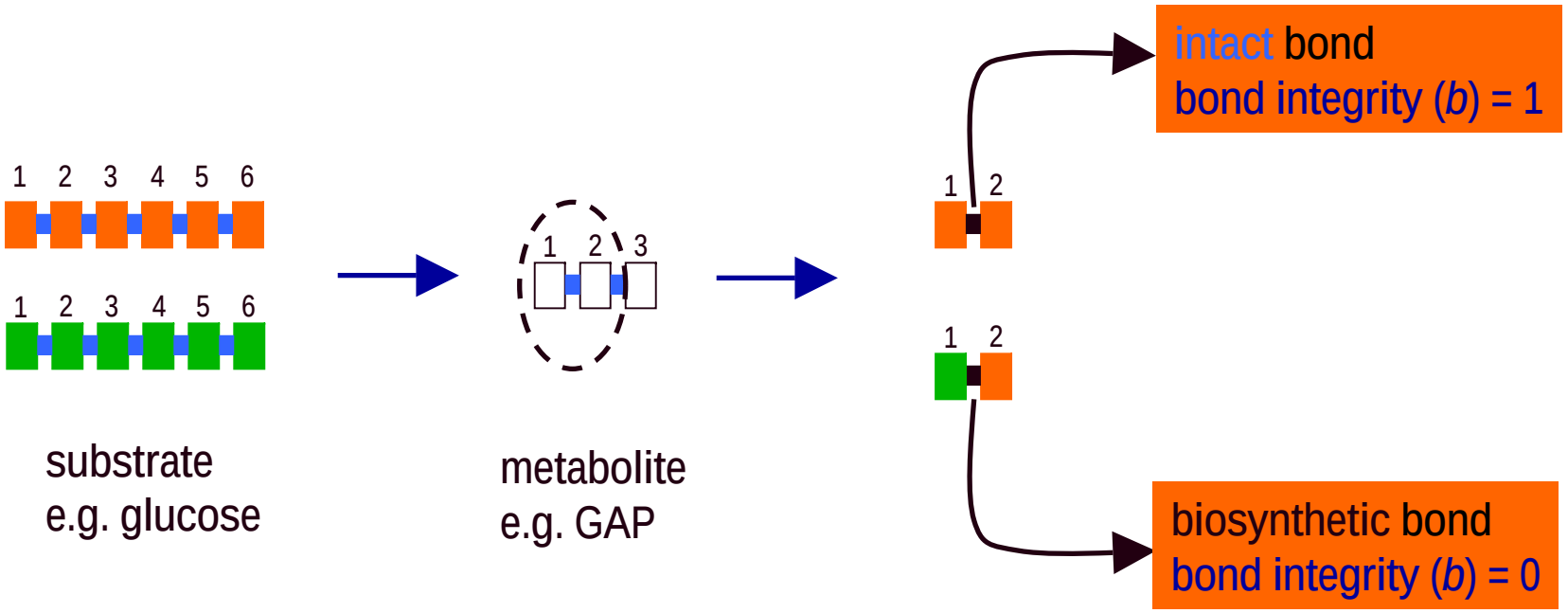
Most likely formed from an intact ^{13}C molecule

Different metabolic histories

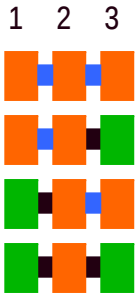
Relative abundance of doublets and singlets represents the relative concentrations of intact and biosynthetic bonds in the same metabolite molecule (e.g. different pathways)



Bond integrity and bondomers



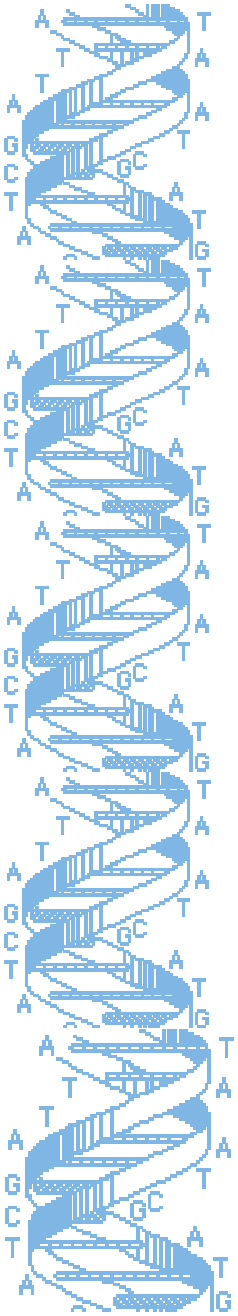
Bondmers of GAP

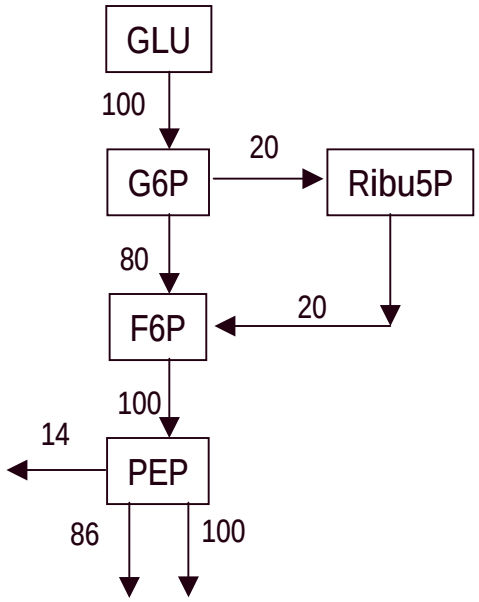


metabolite
e.g. GAP

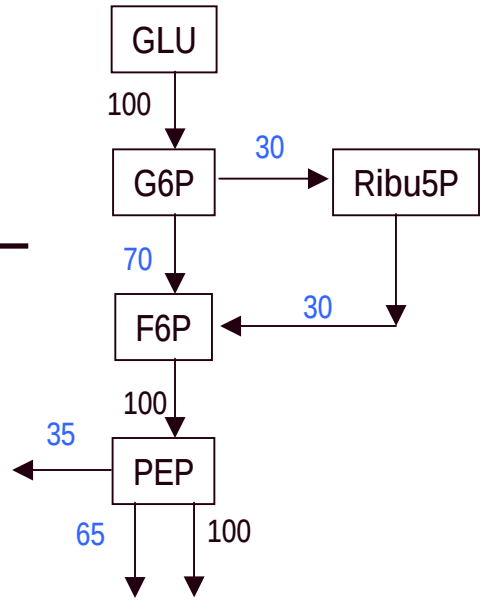
1-1, GAP_4,	$4 = 1 \times 2^1 + 1 \times 2^0 (+1)$
1-0, GAP_3,	$3 = 1 \times 2^1 + 0 \times 2^0 (+1)$
0-1, GAP_2,	$2 = 0 \times 2^1 + 1 \times 2^0 (+1)$
0-0, GAP_1,	$1 = 0 \times 2^1 + 0 \times 2^0 (+1)$

Solution strategy





repeat 1-4 till convergence



1. guess a flux distribution
2. simulate bond integrities

Mathematical model

simulated 50 81
NMR 60 99

Ala

15 23 71 68
20 05 80 90

His

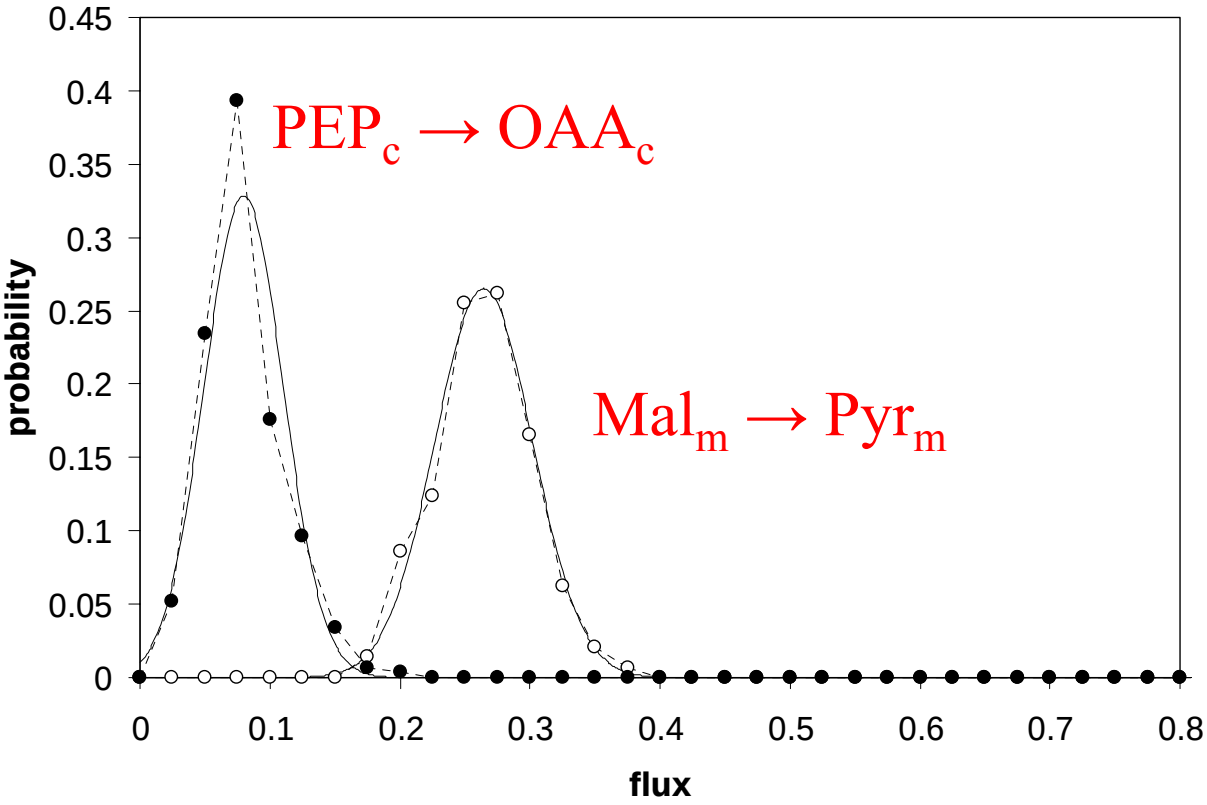
3. compare simulated and experimental bond integrities
4. refine the guesses

refined guesses shown in blue

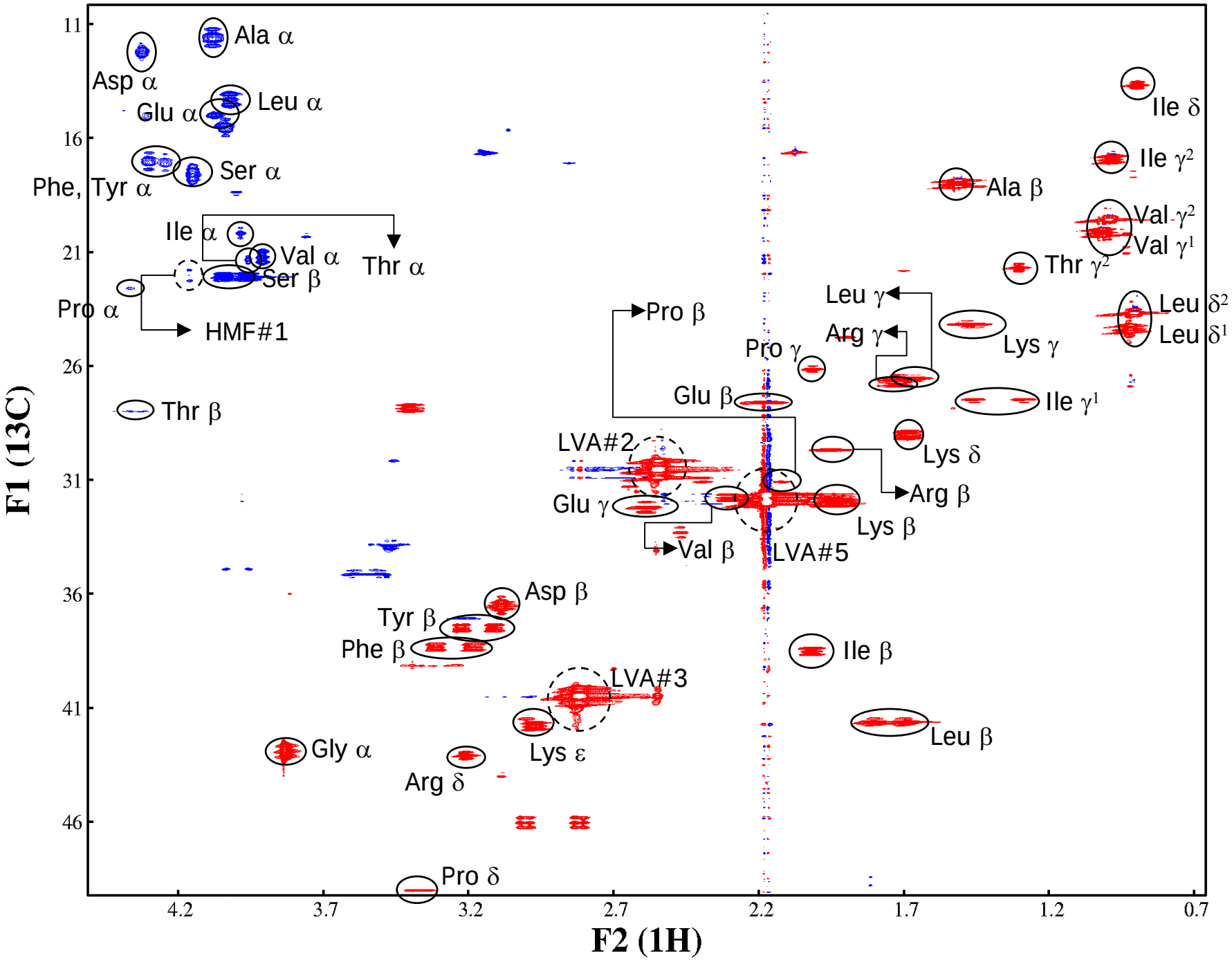
Numerical simulation methods
- direct/Monte Carlo simulation
- bond-mapping matrix method

Optimization routines
- gradient searches
- simulated annealing

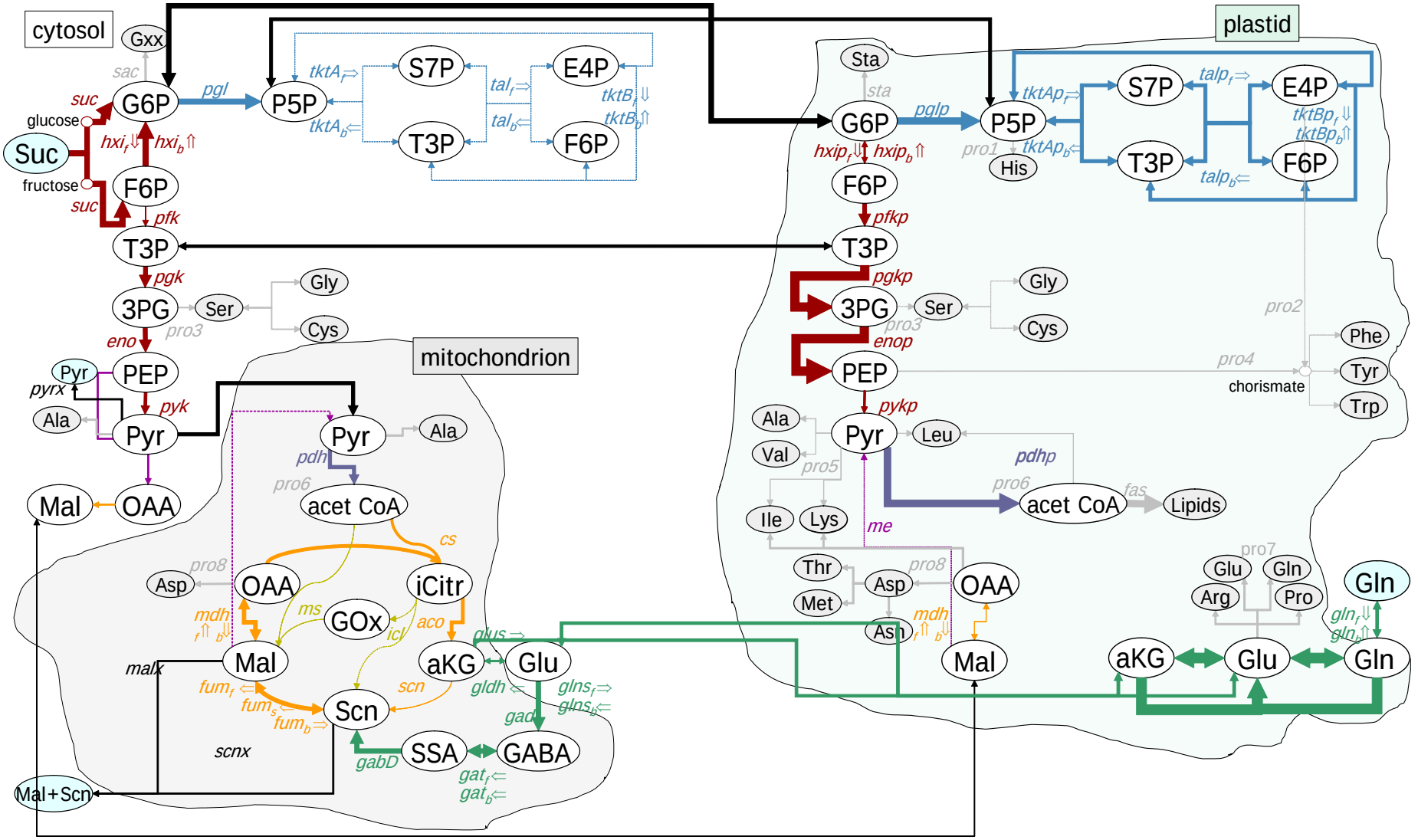
Statistical analysis of evaluated fluxes



Takes into account experimental error of NMR measurements


Ala α
Asp α
Glu α
Leu α
Ser α
Phe, Tyr α
Ile α
Val α
Ser β
Thr α
Pro α
HMF#1
Thr β
Pro β
Pro γ
Glu β
LVA#2
Glu γ
Val β
LVA#5
Lys δ
Arg β
Lys β
Asp β
Tyr β
Phe β
Ile β
LVA#3
Lys ε
Gly α
Arg δ
Leu β
Pro δ
Ile δ
Ile γ2
Ala β
Val γ2
Val γ1
Thr γ2
Leu γ
Arg γ
Lys γ
Leu δ2
Leu δ1
Ile γ1
11
16
21
26
31
36
41
46
4.2
3.7
3.2
2.7
2.2
1.7
1.2
0.7
F1 (13C)
F2 (1H)

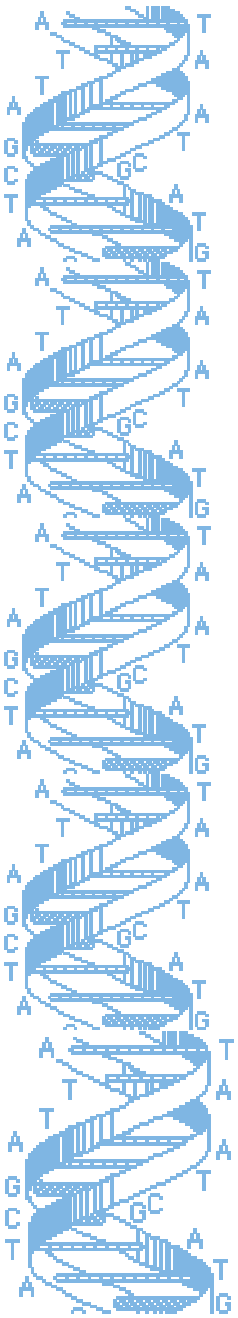
Flux Map of Soybean Embryo Metabolism



Impact

- Enhanced tryptophan → essential amino acid in crops for animal consumption
- Systems viewpoint to enhance the overproduction of medicinal metabolites in plants
- Quantitative NMR flux maps in plants are an important tool to be integrated with other systems approaches in metabolic engineering

Beauvais Cathedral



Acknowledgments

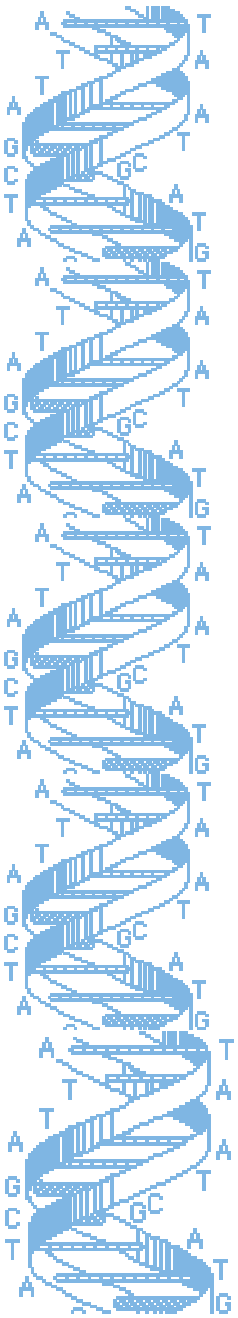
Flux Map

- Ganesh Sriram (ChE)
  - •Omar González-Rivera (ChE)
- Prof. Mark Westgate (Agronomy)
  - •Joan Peterson
  - •Ruilian Zhou
- Prof. Marty Spalding (Botany)
- Dr. Louisa Tabatabai (Protein Facility)
- Dr. Bruce Fulton (NMR Facility)

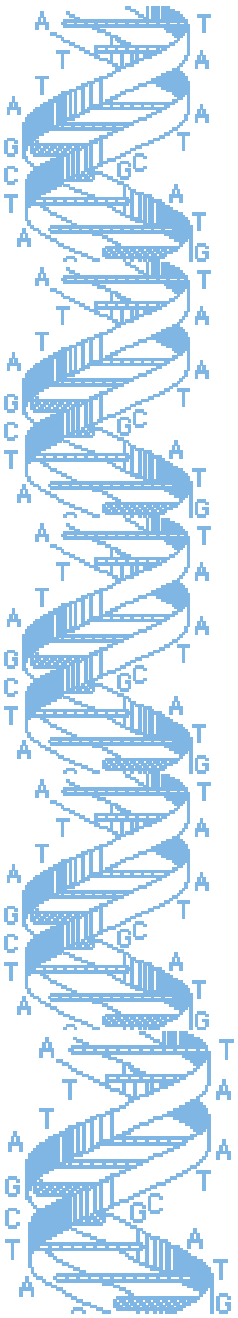
Alkaloids

- Prof. Ka-Yiu San
- Prof. Sue Gibson
- Ganesh Sriram
- Erik Hughes
- Dennis Hong
- Guy Sander
- Christie Peebles
- John Morgan
- Sushil Rijhwani
- Sundeep Vani
- Rajiv Bhadra

Funding: NSF, Plant Sciences Institute, Cargill



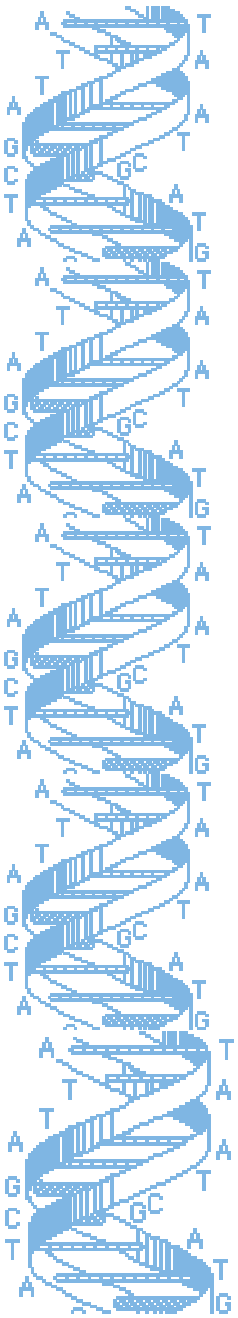
Panel Session



Improving Plants

- In silico Plant?
- Systems biology
- "Predictive" Metabolic Engineering
  - Iterative cycle of hypothesis testing

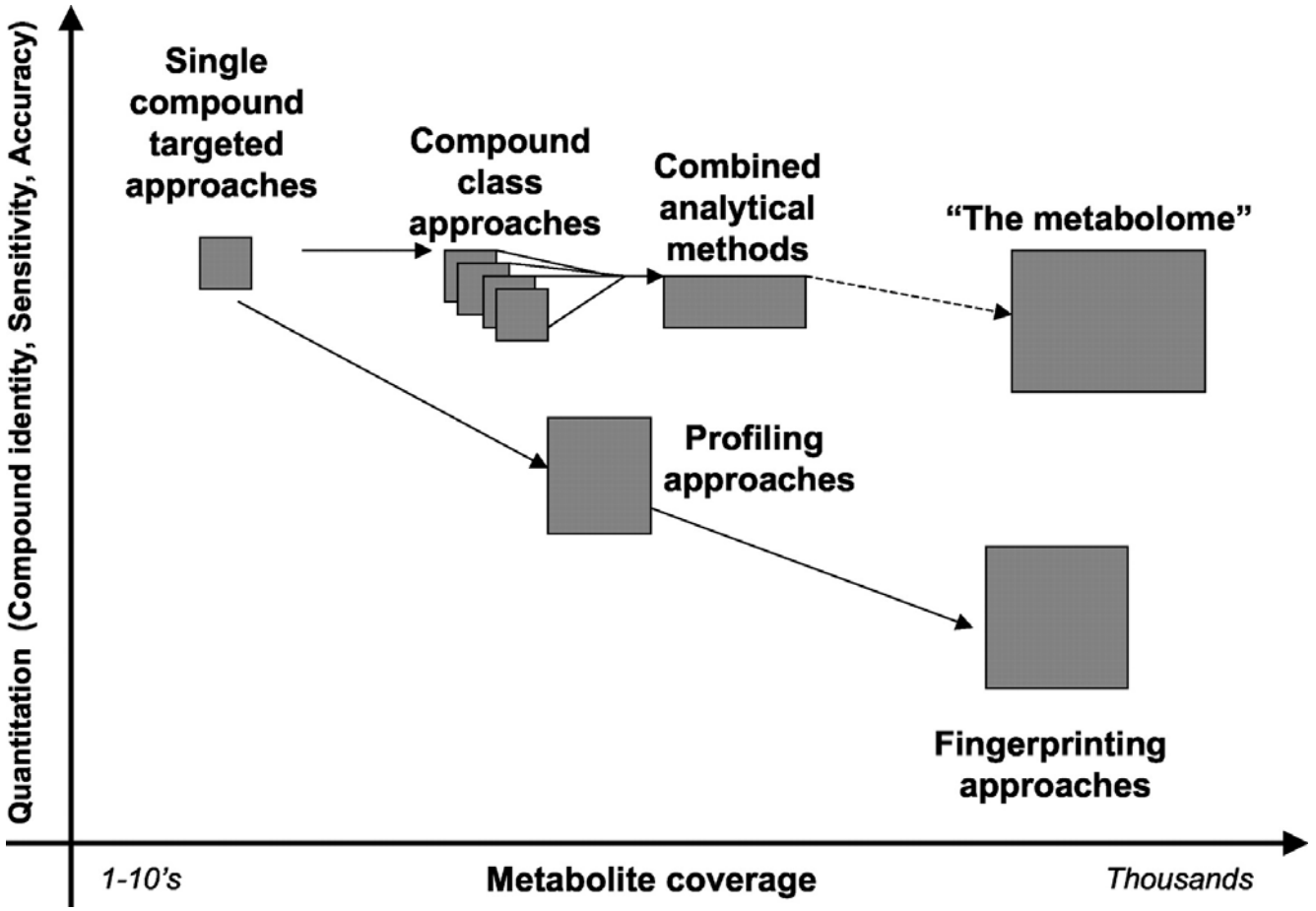
- Can we learn some basic design principles by integrating information in subsets in metabolism?



Challenges – Analysis Side

- **High-throughput techniques**
  - **more quantitative**
  - **proteomics**
  - **metabolite profiling**
  - **metabolic flux profiling**
- **Accurate databases with common language**
- **Theoretical frameworks and tools**
  - **Modeling**
  - **Computational**
  - **Statistical**
  - **Visualization**

Approaches to Metabolite Measurements



Sweetlove, Last and Fernie (2003) "Predictive Metabolic Engineering: A Goal for Systems Biology" *Plant Phys*. 132: 420-425.