Aalto University
School of Chemical
Technology

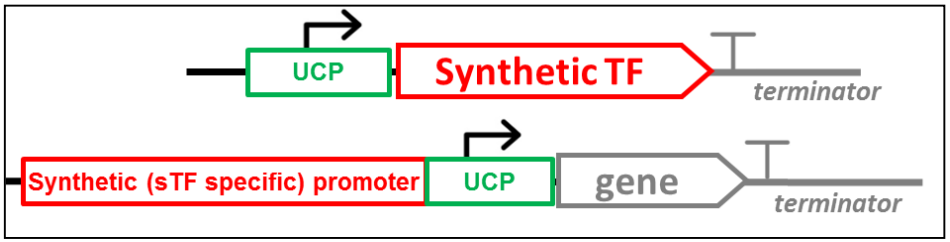
Synthetic biology

(Course CHEM-E8125), spring 2019

Synbio biotech examples

Prof. Merja Penttilä

SES
Orthogonal Synthetic Expression System for fungi



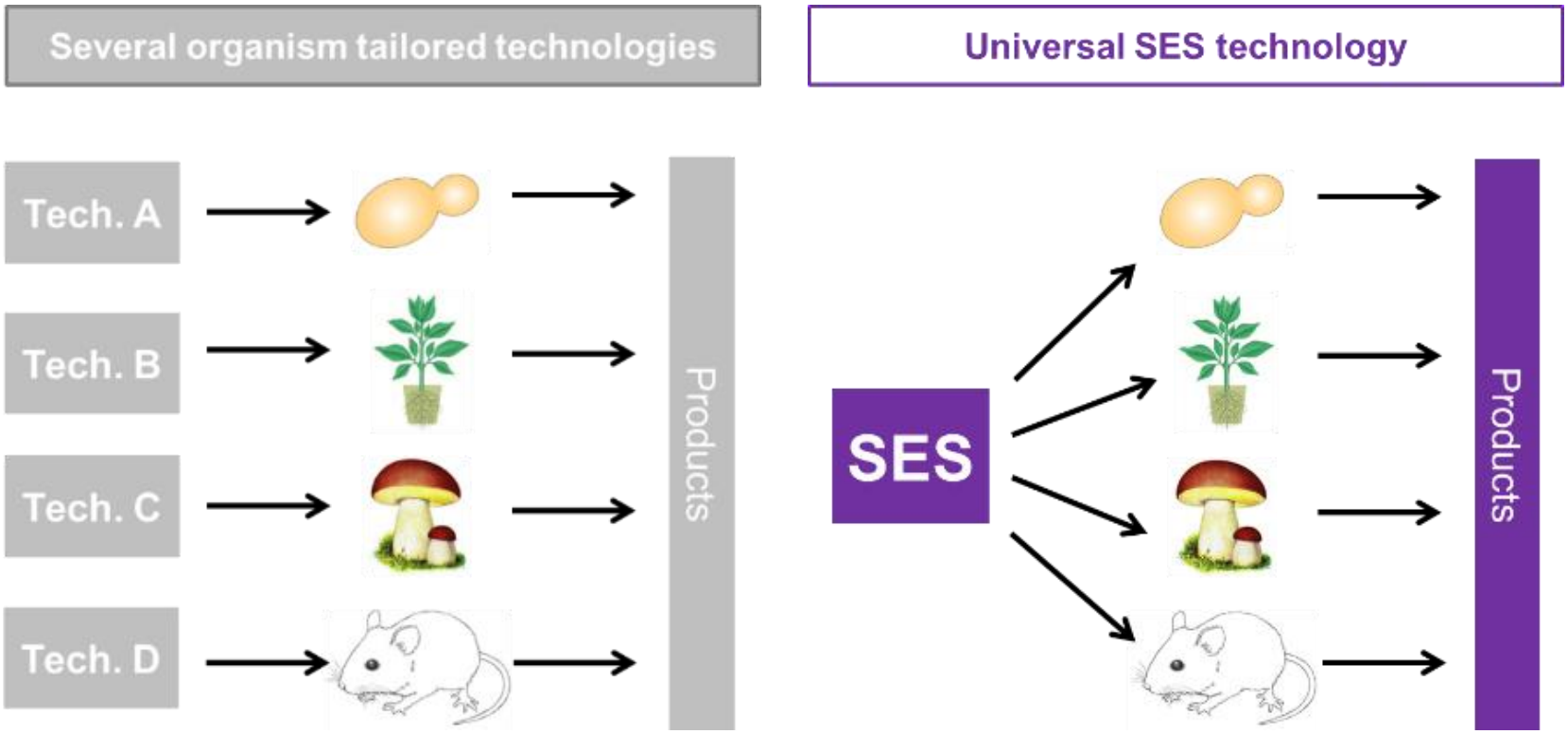
- Tunable controllable promoters, driving different expression levels
  - Constitutive, inducible or repressable
- Orthogonal, not responding to host's background regulation
  - Enables memory
- Functional over several fungal species

Anssi Rantasalo, Joosu Kuivanen, Jussi Jäntti, Dominik Mojzita /VTT

Aalto University
School of Chemical
Technology

Current situation

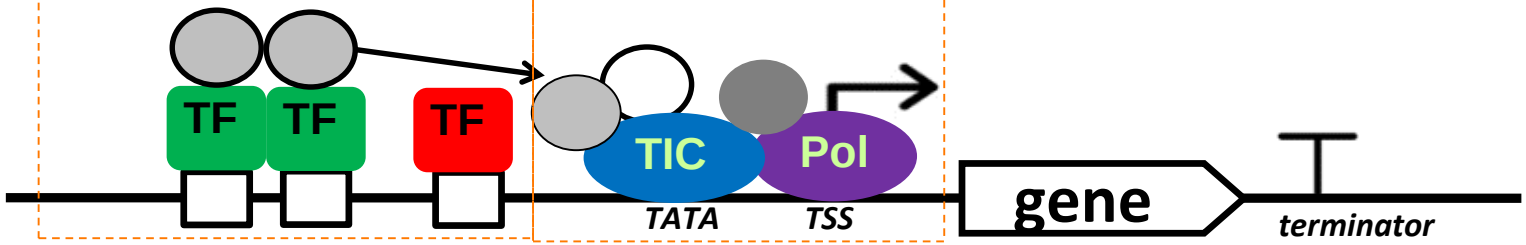
Novel approach



Eukaryotic gene expression

Constitutive or regulated

Different versions conferring different efficiency in initiation complex assembly and transactivation

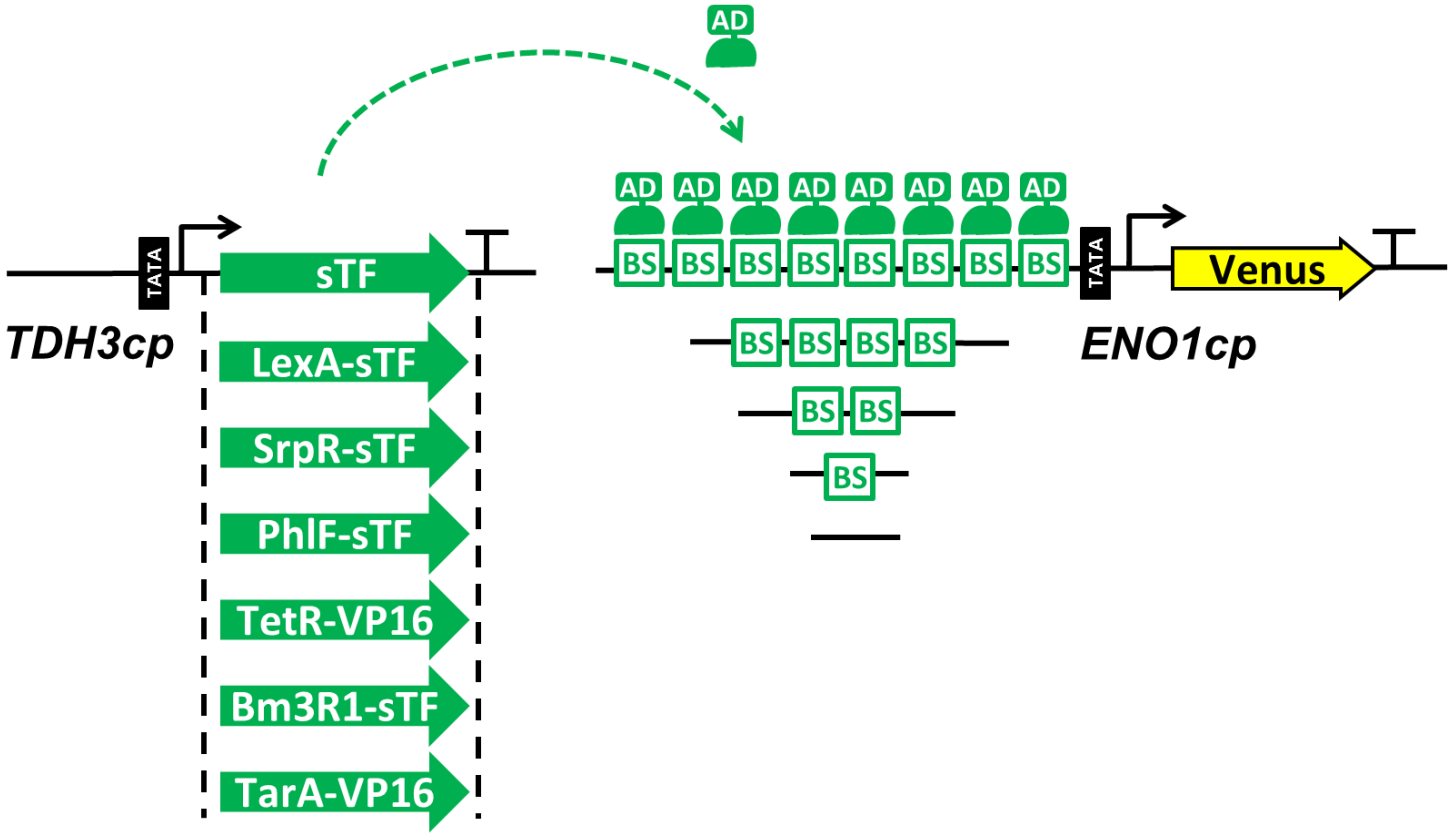


UAS/enhancer: Binding sites (RE) for specific transcription factors (TF)

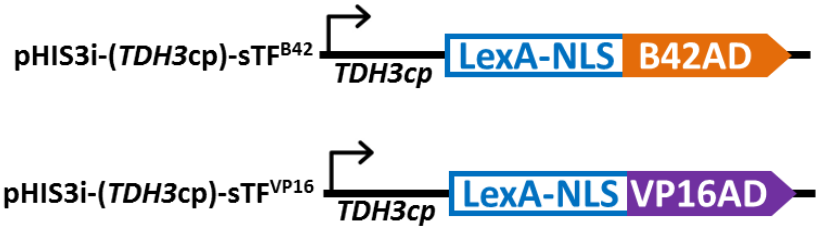
Core promoter: Binding sites for general transcription factors

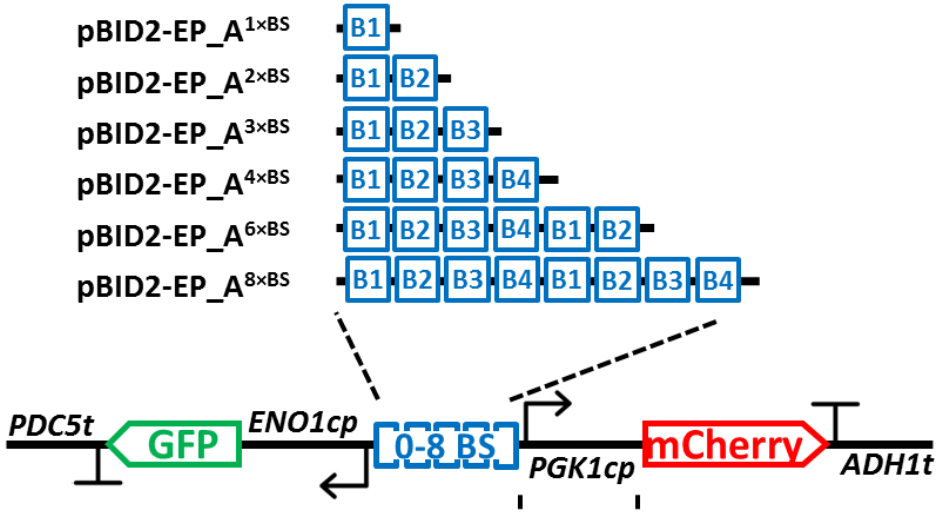
Native promoter

Synthetic gene expression system



Development of a tunable expression system for *S. cerevisiae*

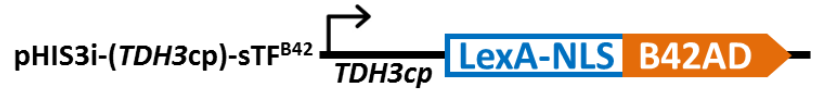


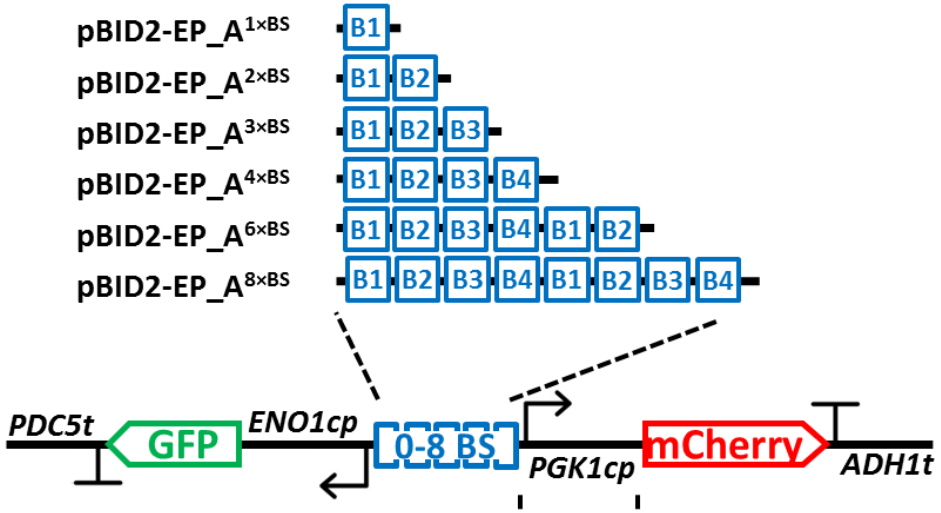


Bidirectonal synthetic promoter

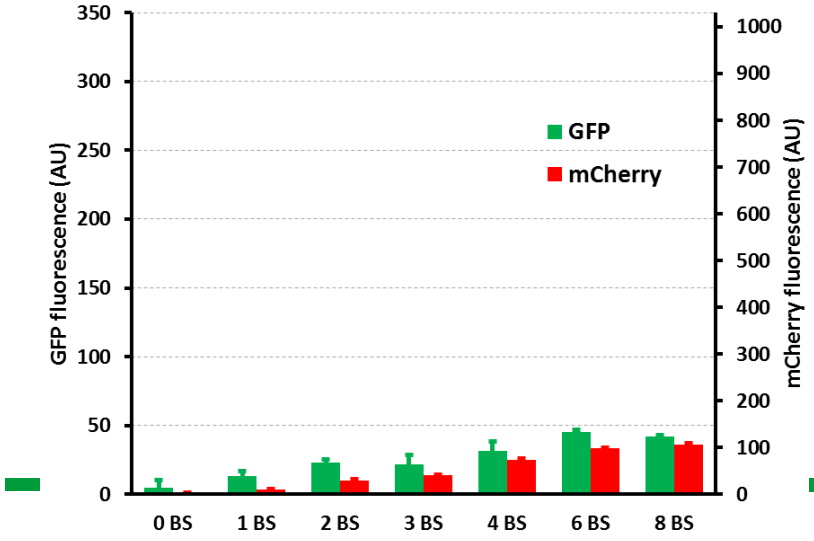
Rantasalo et al. (2016) PLoS One

Development of a tunable expression system for *S. cerevisiae*

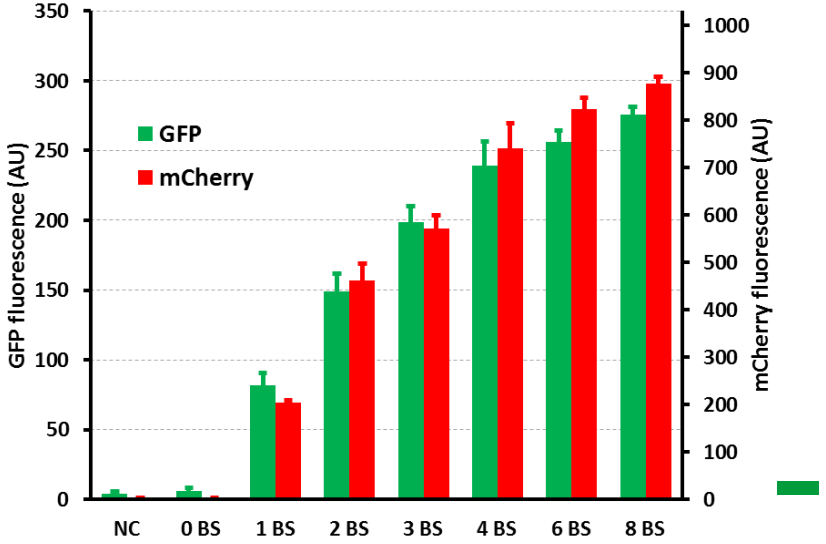




weak constitutive sTF^{B42}



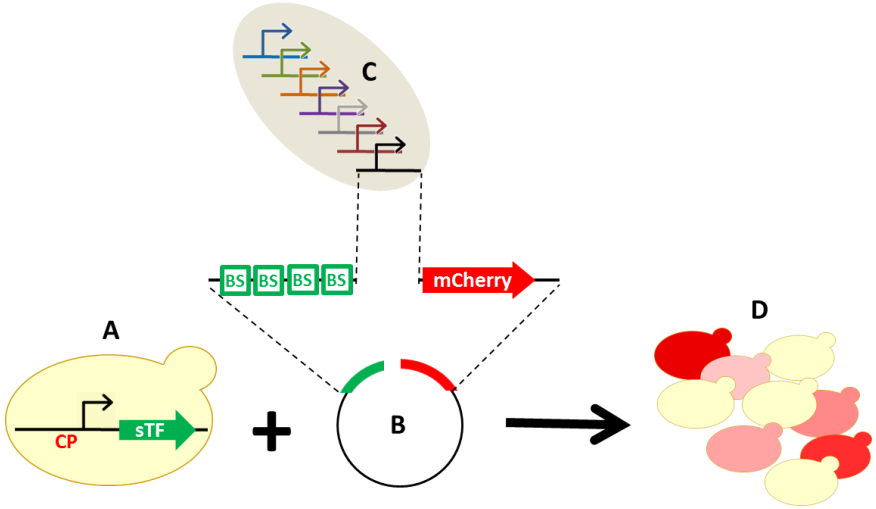
strong constitutive sTF^{VP16}



Rantasalo et al. (2016) PLoS One

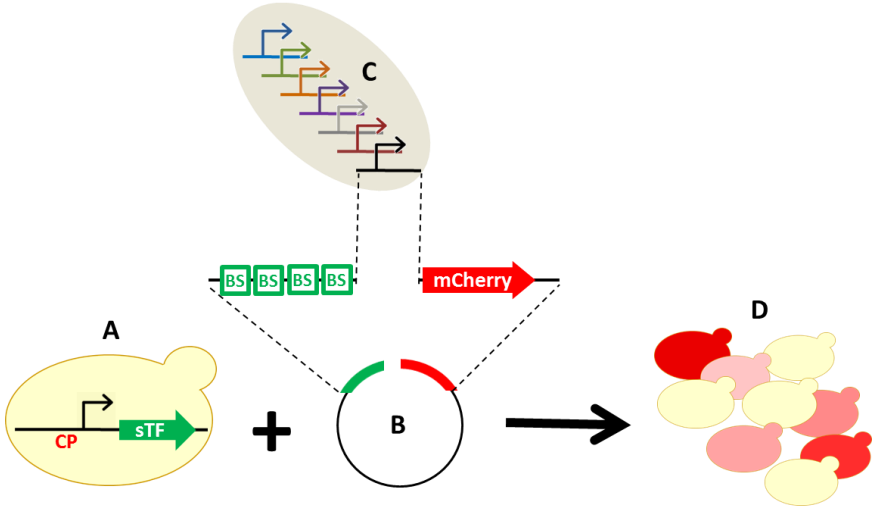
Universial core promoters for different fungi

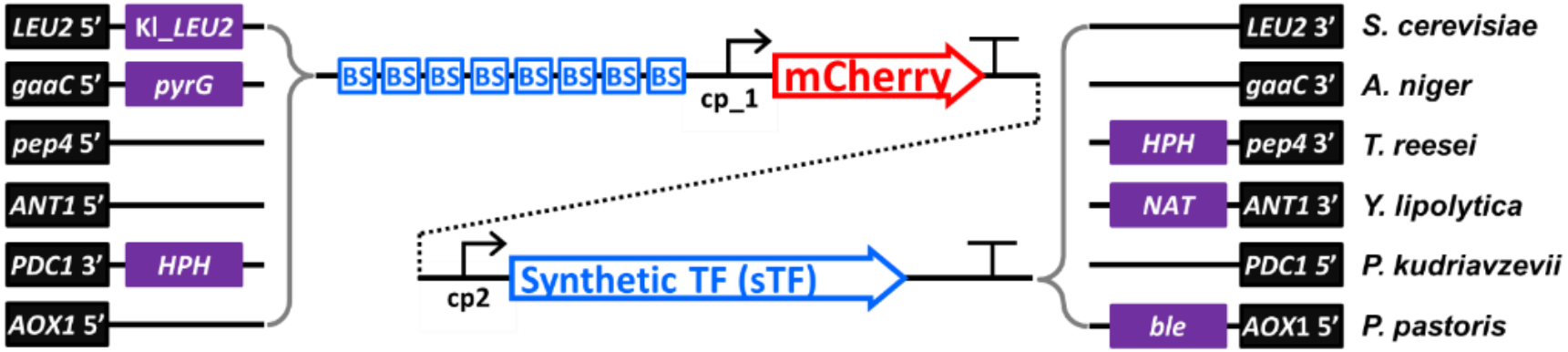
- Core promoters of highly expressed genes from various organisms (as gBlocks).
- gBlocks assembled *in vivo to a* CEN-type plasmid in a yeast strain constitutively expressing LexA-based sTF.
- Strains analyzed for red fluorescence.
- A few new strong (universal) core promoters selected.



Universial core promoters for different fungi

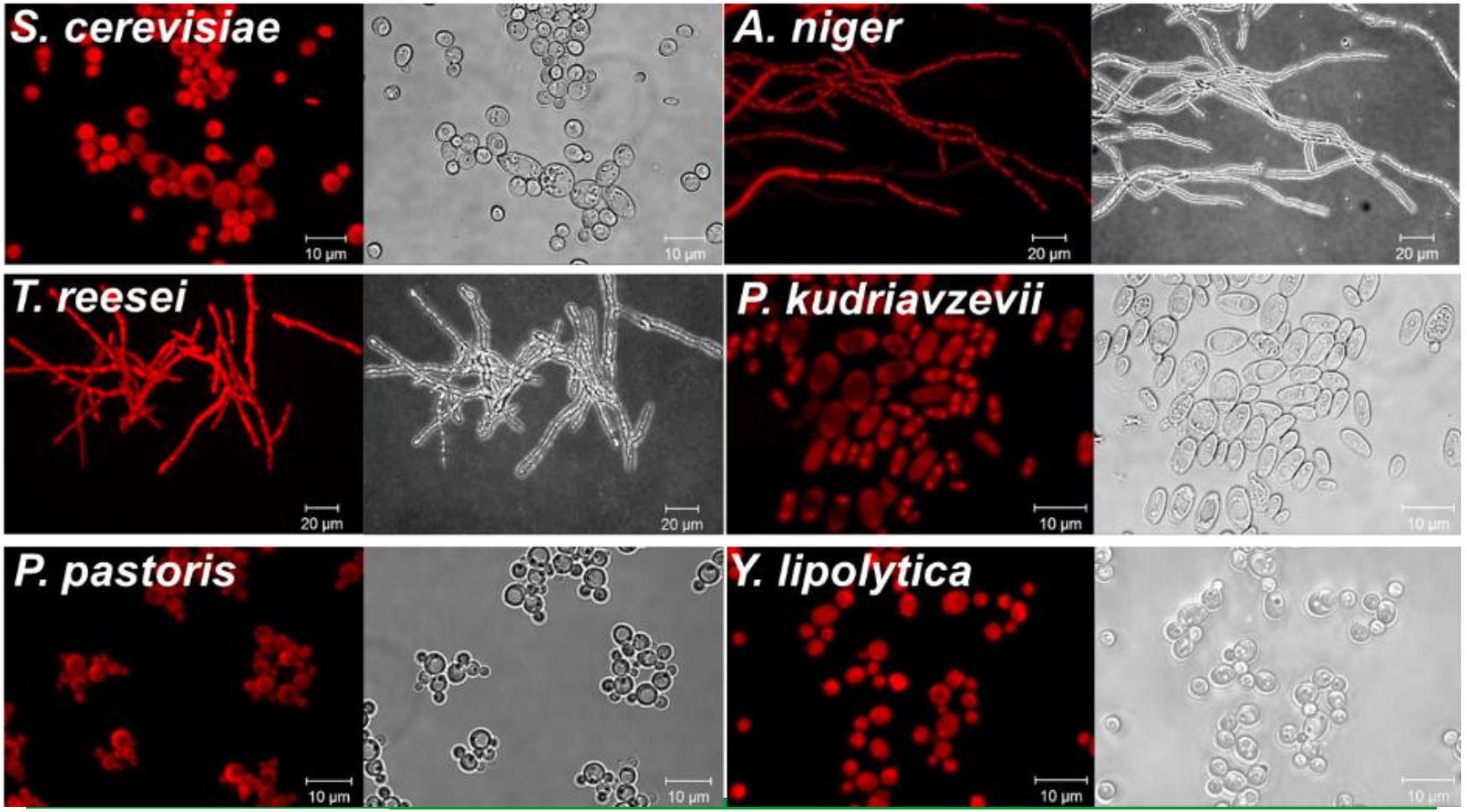
- Core promoters of highly expressed genes from various organisms (as gBlocks).
- gBlocks assembled *in vivo to a* CEN-type plasmid in a yeast strain constitutively expressing LexA-based sTF.
- Strains analyzed for red fluorescence.
- A few new strong (universal) core promoters selected.





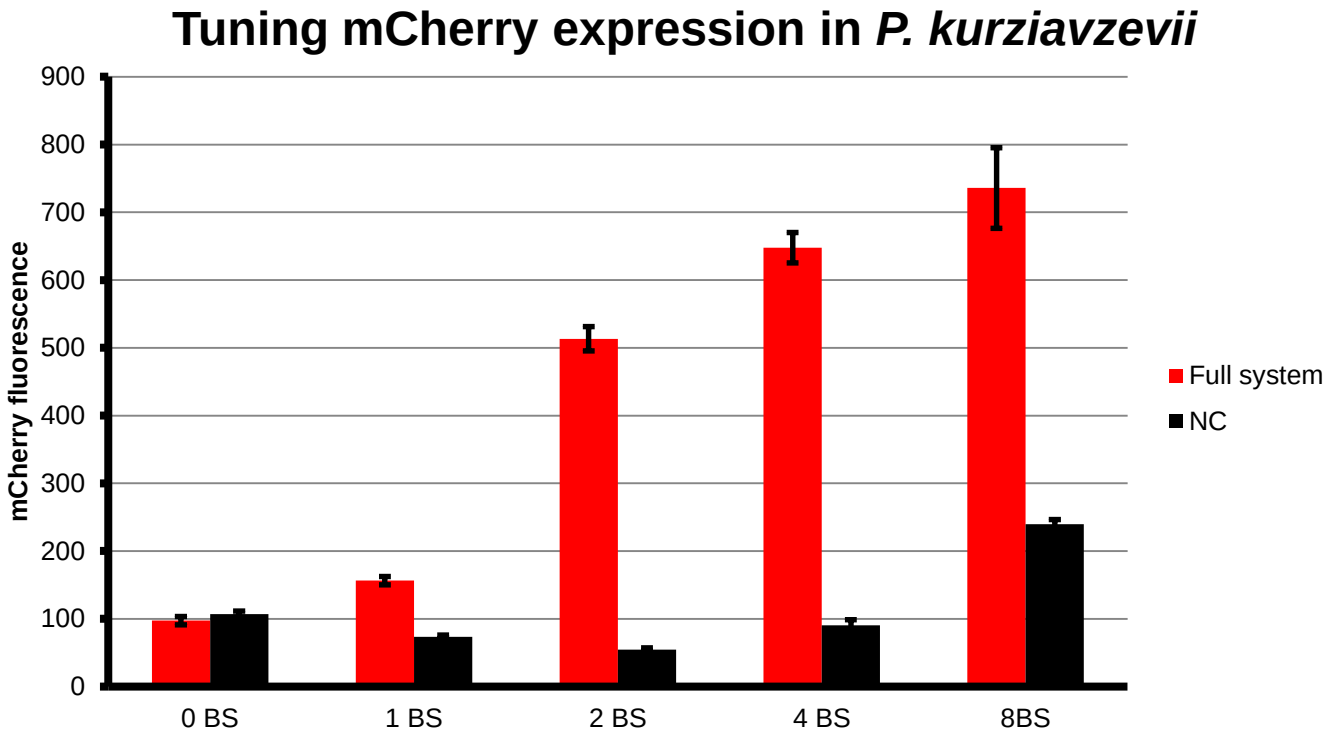
- The best performing core promoters (CP) from the screen used for the construction of transfrerable expression cassettes
- Two different CPs used for the sTF and mCherry expression

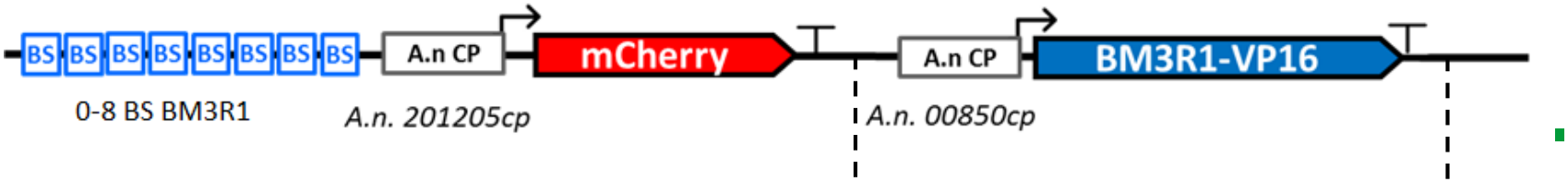
SES is functional in several fungal species



Fluorescence microscopy (mCherry)
Stable and homogenous expression in all cells/species

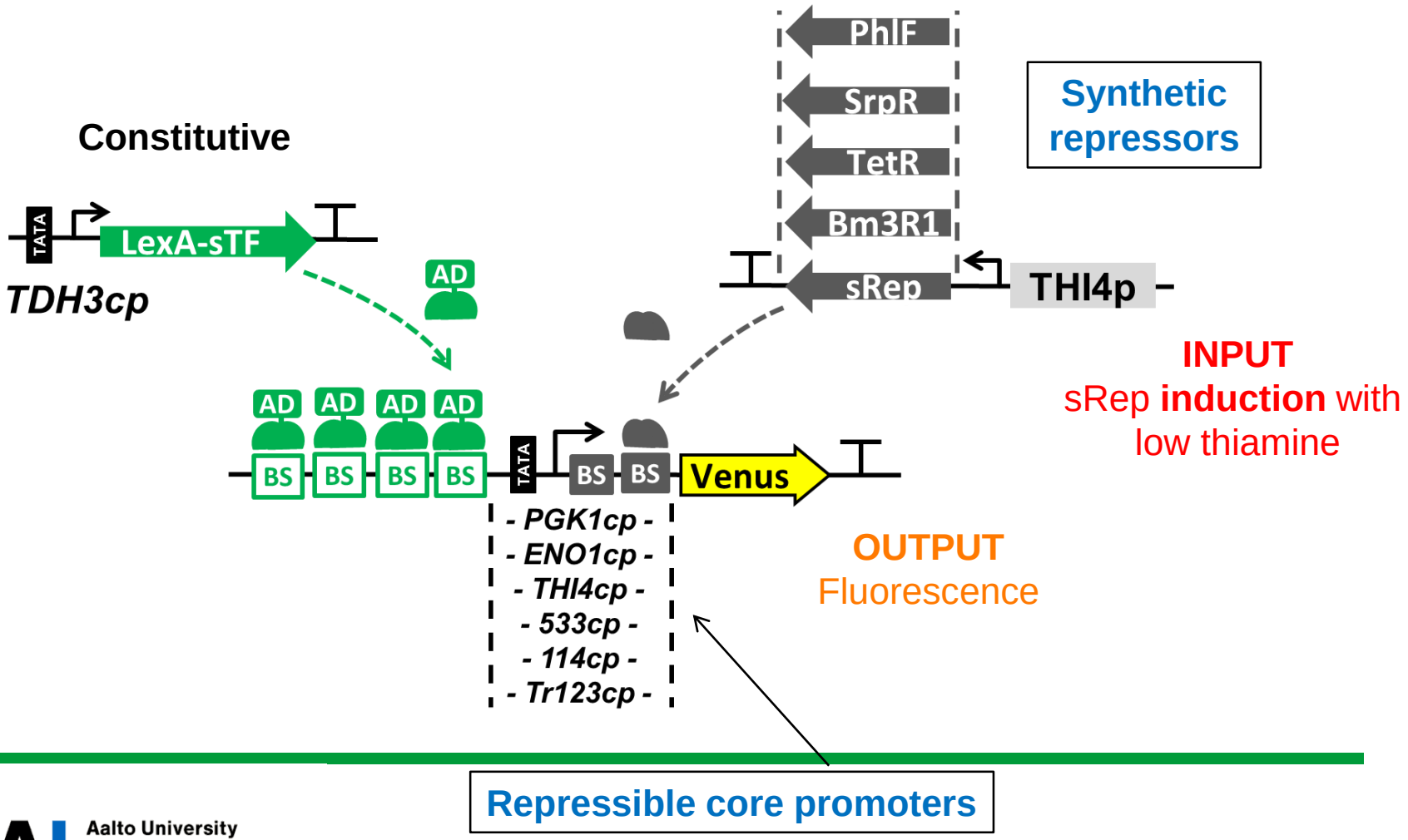
Tuning expression with SES promoters in *Pichia kurziavzevii*





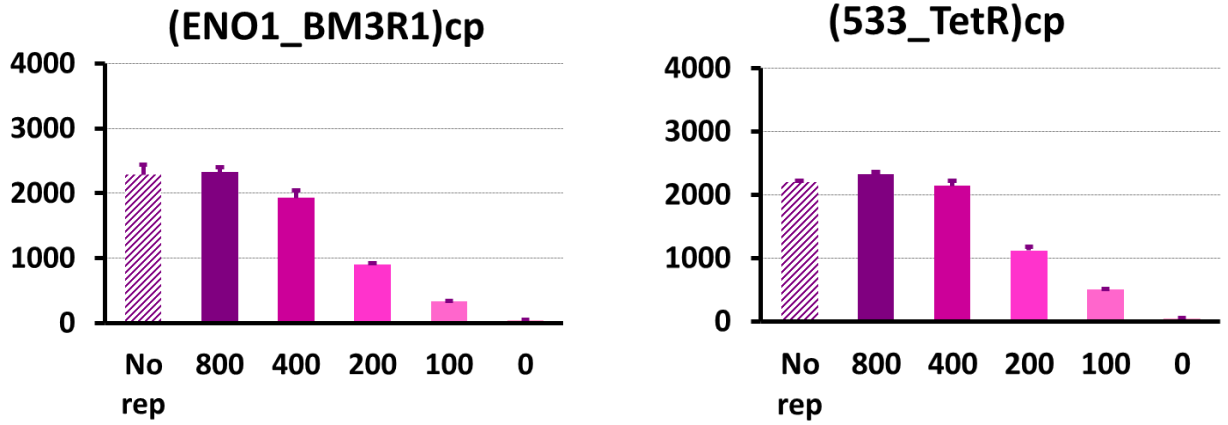
NC = without TF

Downregulation of the synthetic promoter with a synthetic repressor (sRep)

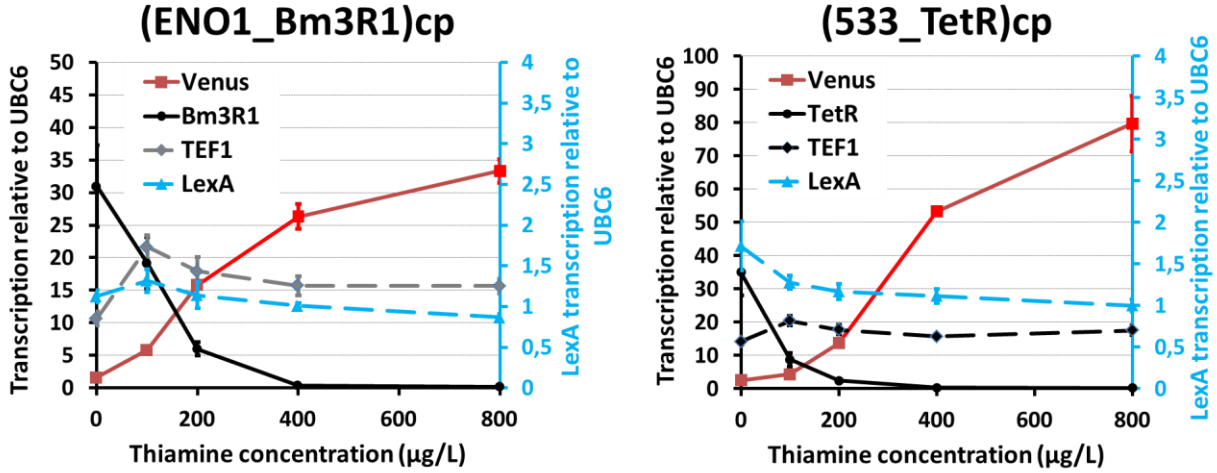


Downregulation of gene expression with synthetic repressor (sRep) in *S.crevisiae*

Fluorescence

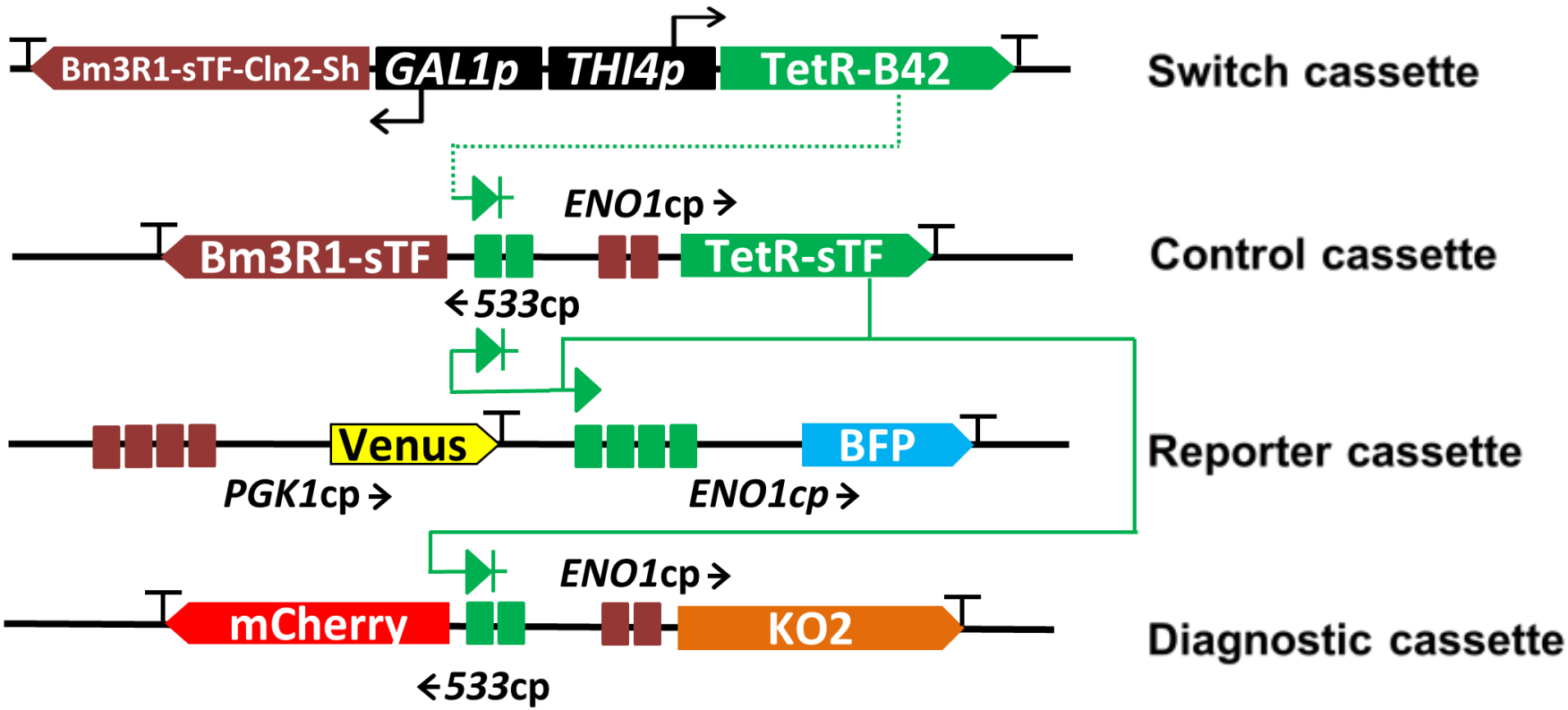


Transcription



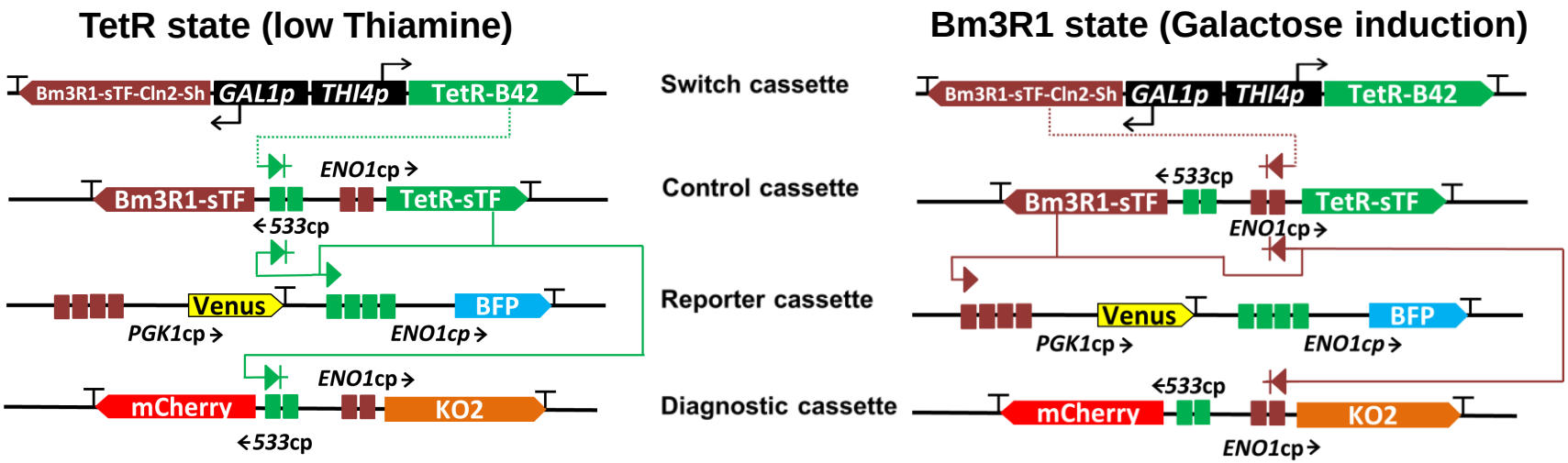
Bi-stable switch – Design

based on well-characterized orthogonal DNA parts



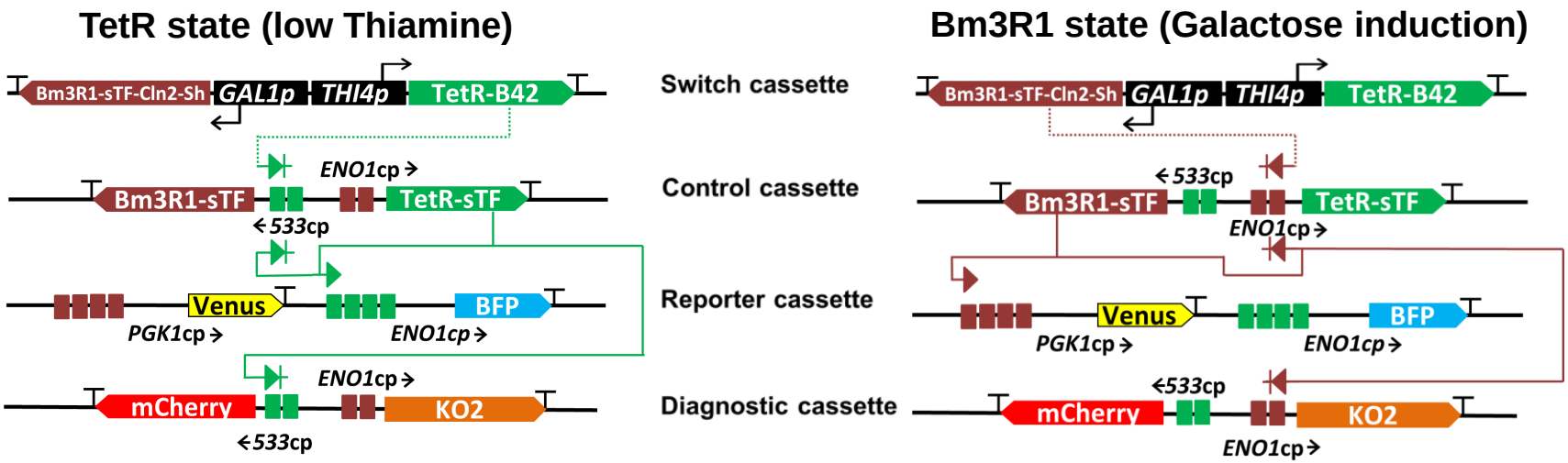
Bi-stable switch – Test

putting the system through series of tests to assess its robustness

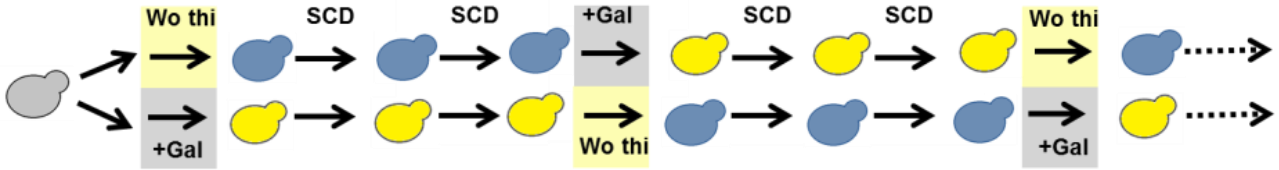


Bi-stable switch – Test

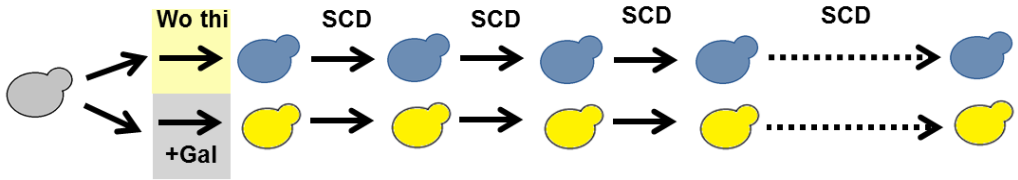
putting the system through series of tests to assess its robustness



Repeated switches

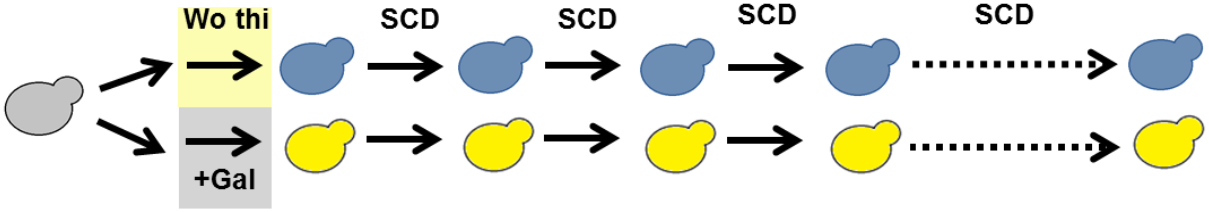


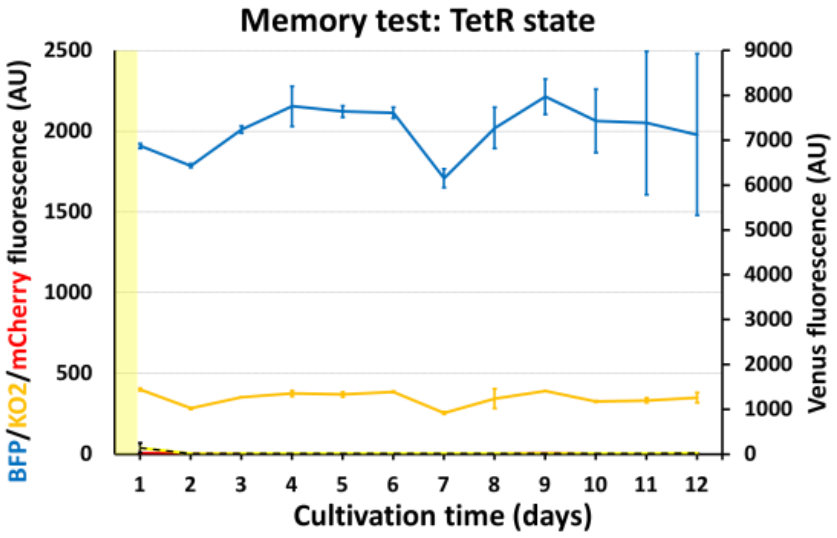
Memory

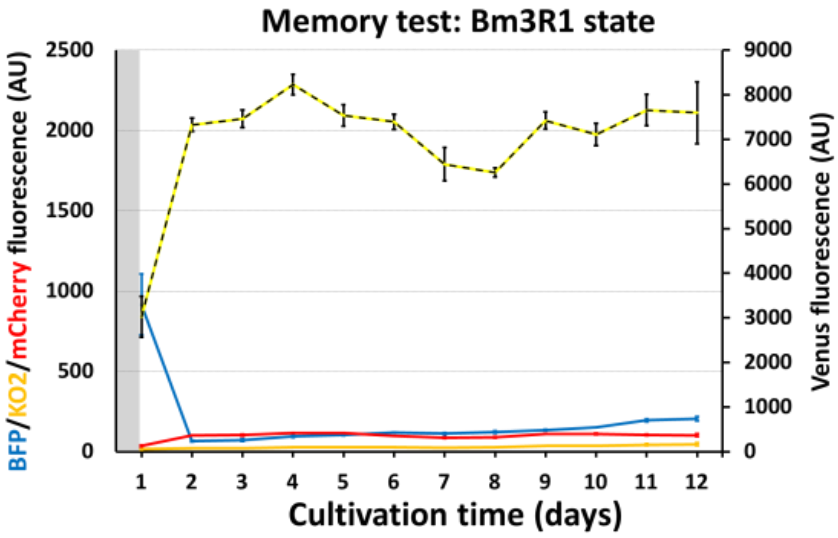


Bi-stable switch – Test

Memory

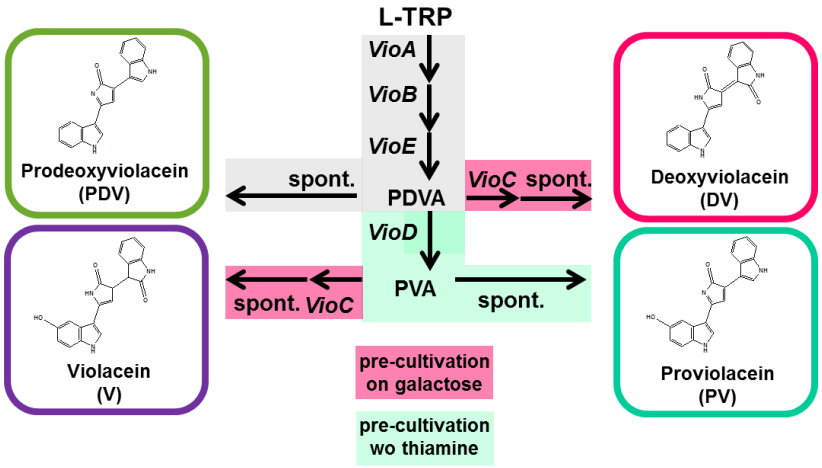


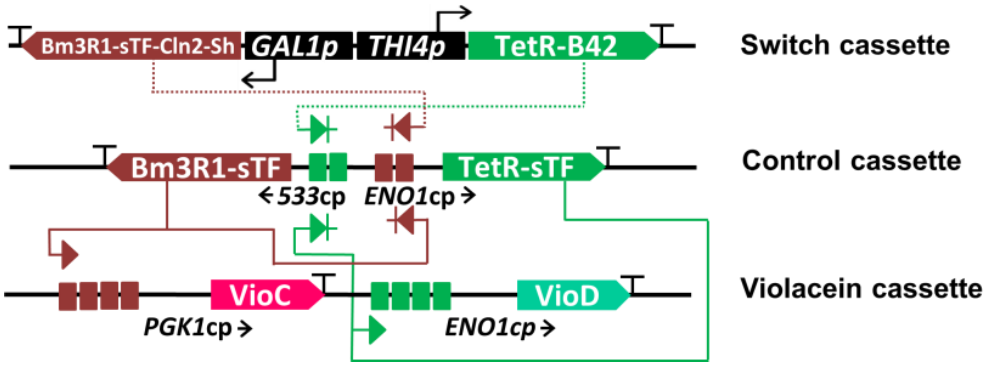


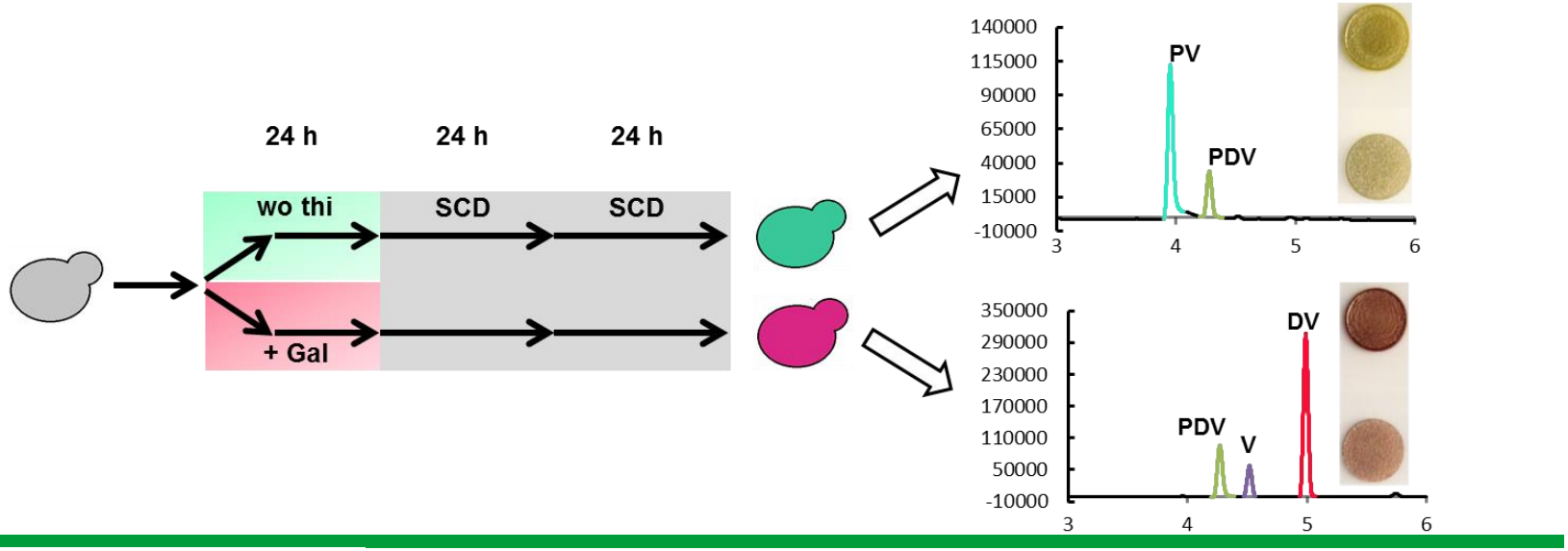


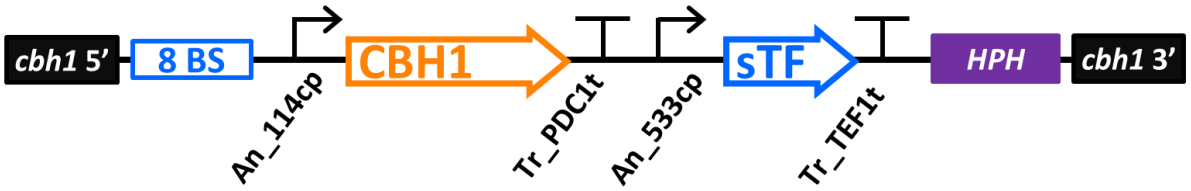
Wo thiamine With galactose SCD

Bi-stable circuit for metabolic pathway switching - Violacein pathway in *S.cerevisiae*



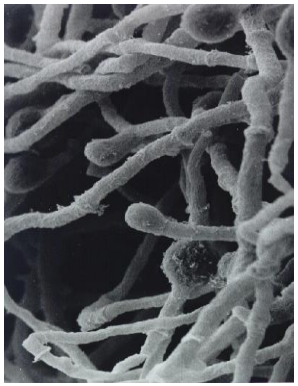






Using SES in protein production in *Trichoderma reesei*

Production on glucose enables a more pure product

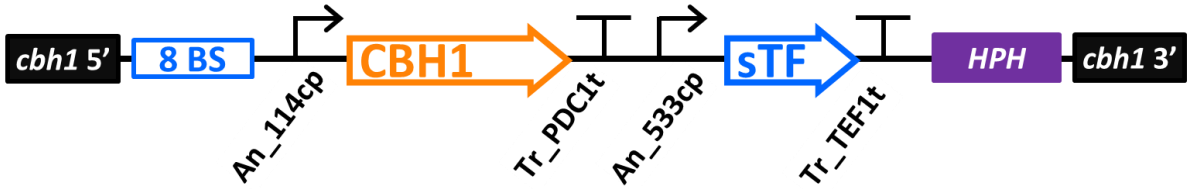


Aalto University
School of Chemical Technology

Dominik Mojzita, Mari Valkonen, Marika Vitikainen,
Chris Landowski et al, VTT

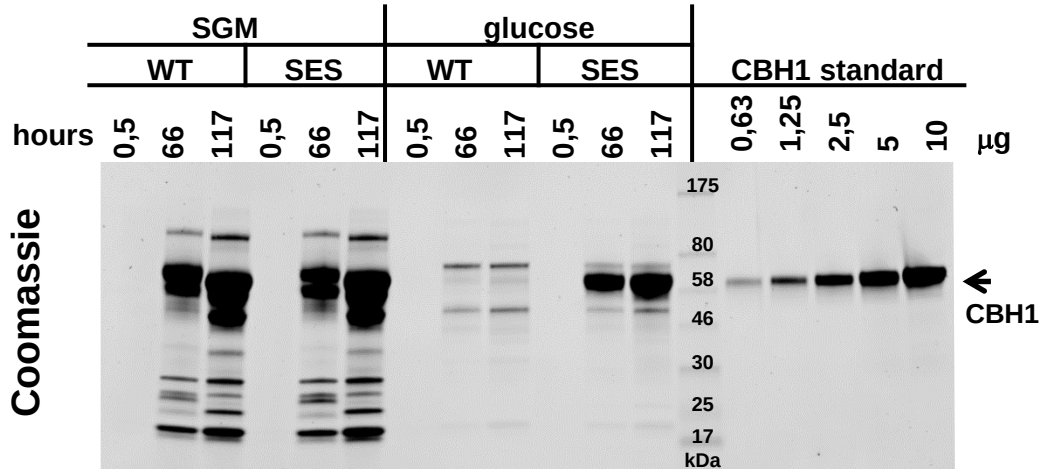
CBHI production in *Trichoderma reesei* with SES

A



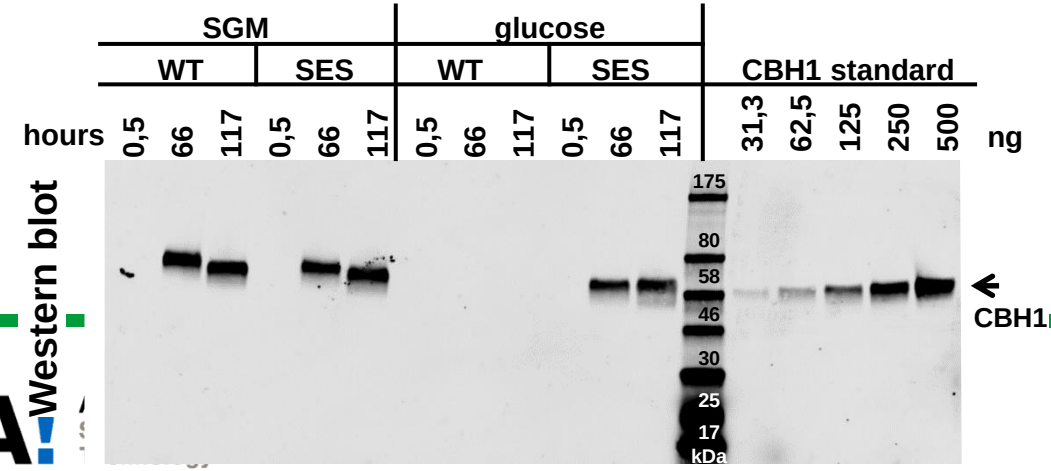
Constitutive expression

B



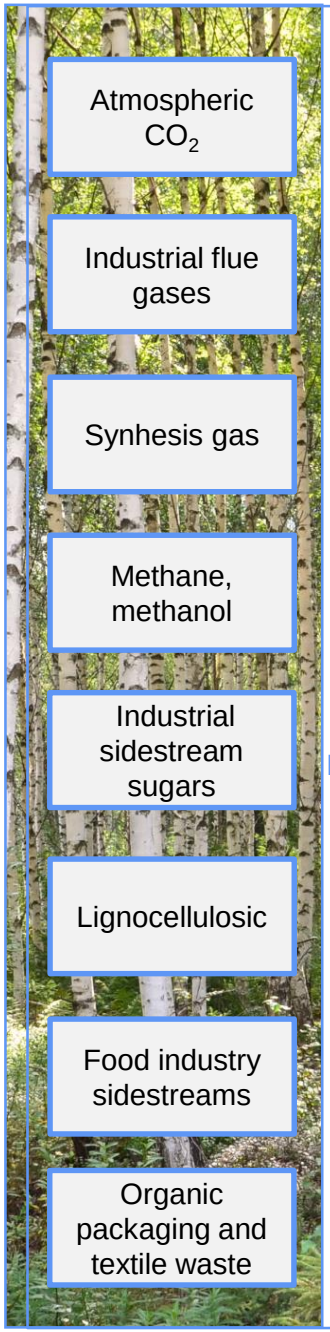
SGM	WT	0,5 h	0,6 g/L
		66 h	9,4 g/L
		117 h	17,8 g/L
	SES	0,5 h	0,6 g/L
		66 h	8,4 g/L
		117 h	17,6 g/L
Glucose	WT	0,5 h	<0,5 g/L
		66 h	0,9 g/L
		117 h	1,2 g/L
	SES	0,5 h	<0,5 g/L
		66 h	3,7 g/L
		117 h	5,9 g/L

C



SGM	WT	0,5 h	<0,1 g/L
		66 h	4,9 g/L
		117 h	4,8 g/L
	SES	0,5 h	<0,1 g/L
		66 h	4,3 g/L
		117 h	5,4 g/L
Glucose	WT	0,5 h	<0,1 g/L
		66 h	<0,1 g/L
		117 h	<0,1 g/L
	SES	0,5 h	<0,1 g/L
		66 h	3,2 g/L
		117 h	4,1 g/L

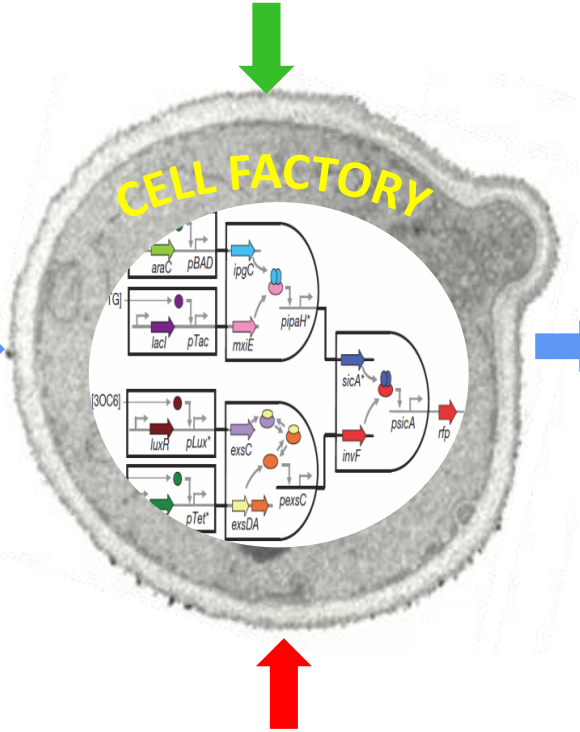
A key application potential of synbio is in circular Bioeconomy – towards a bio-based society



Biotechnology

enables a versatile use of non-fossil raw materials and unique possibilities to broaden the product range

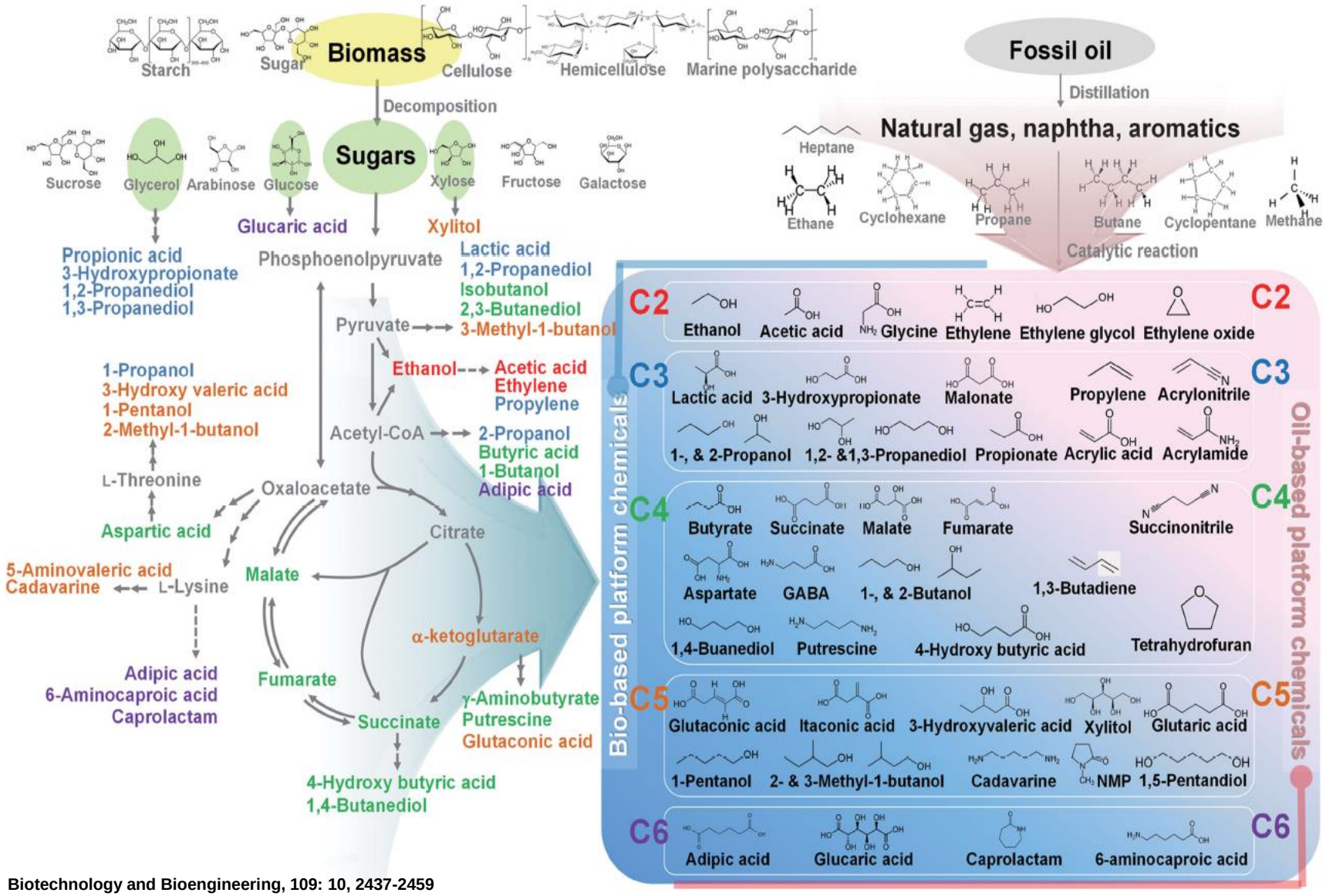
Natural synthesis power
Evolution power
Reaction specificity



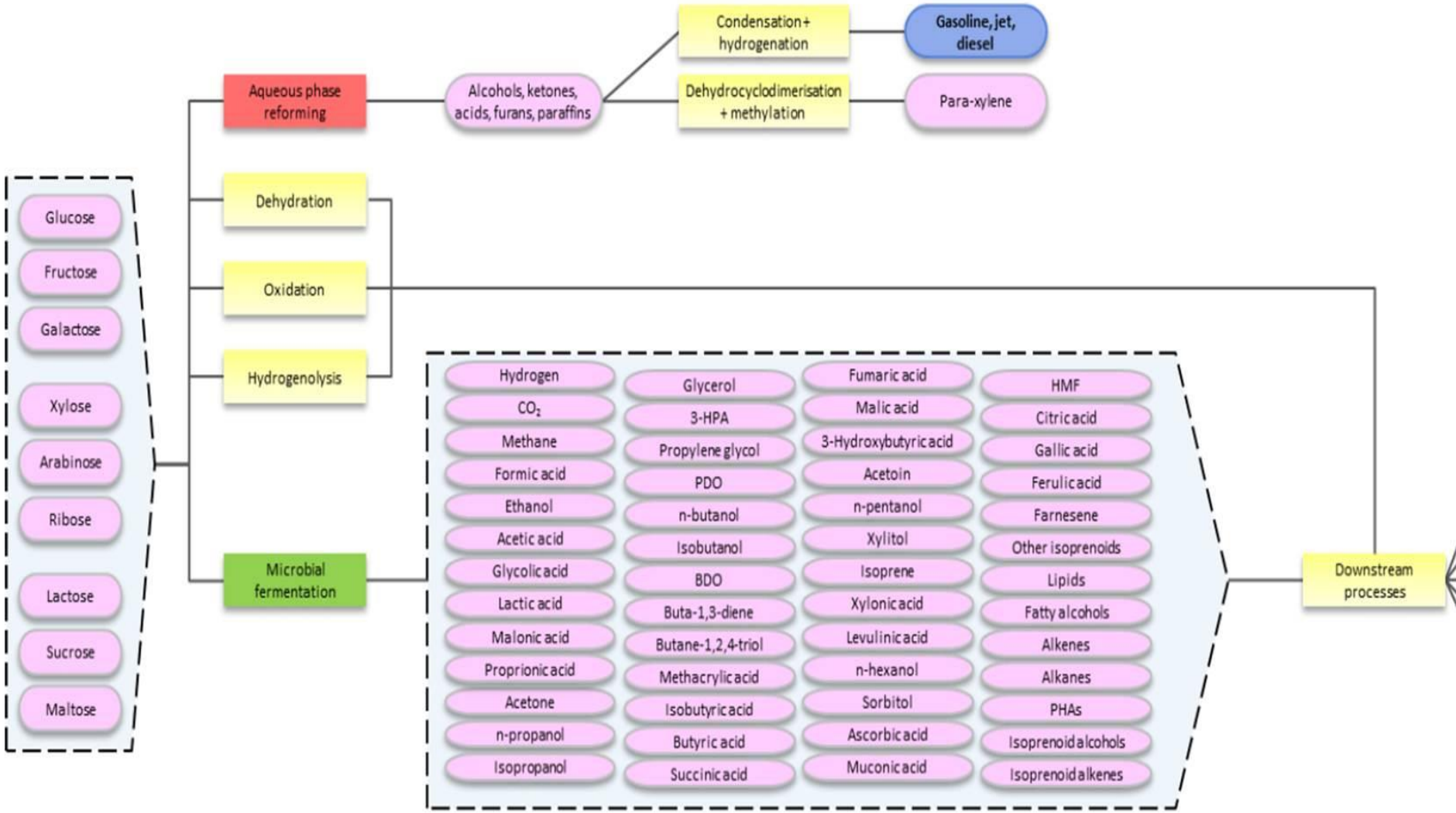
SYNTHETIC BIOLOGY

Design of cell factories based on mathematical models
Automated construction and high through-put selection of production strains
New reactions, new products, more efficient production processes

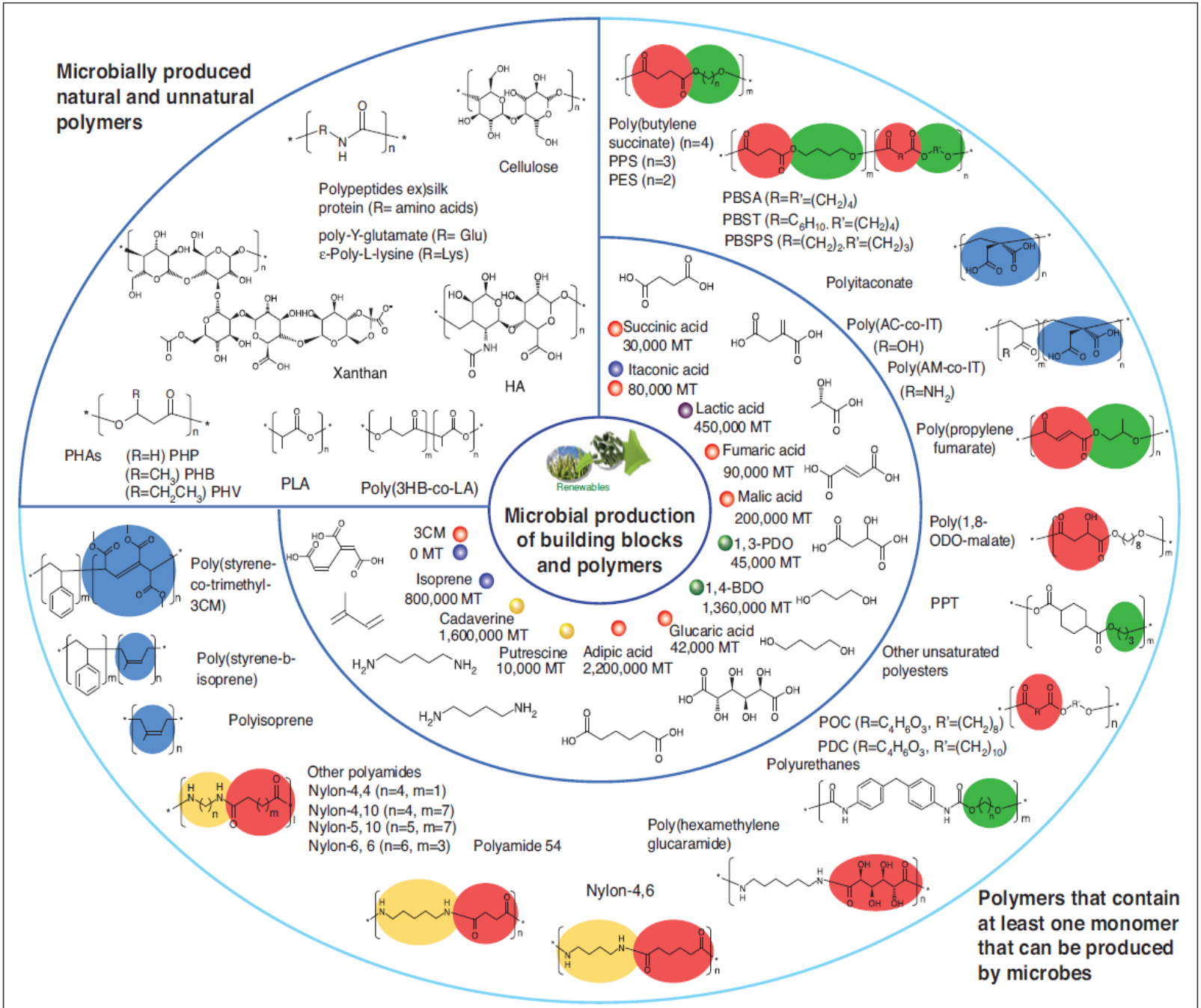
Cellular chemistry can be harnessed to produce platform chemicals and fuels that can replace those currently made from fossil resources – but also new chemicals for novel uses



Biotechnology and Bioengineering, 109: 10, 2437-2459

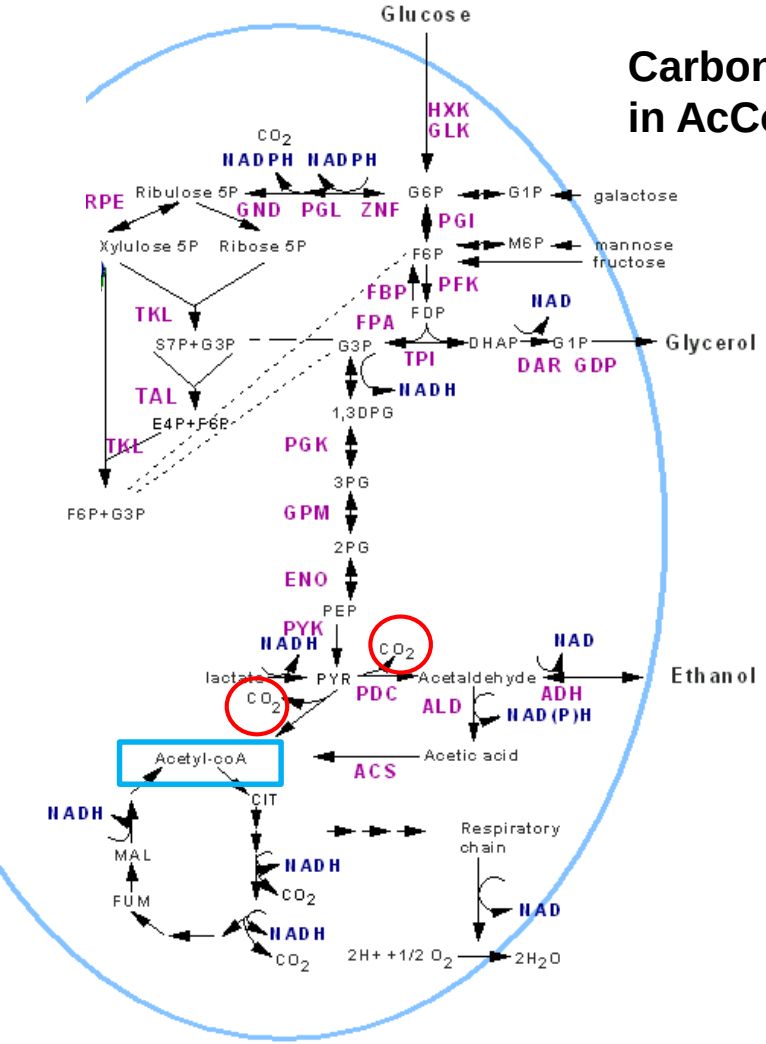


Downstream process options from sugars (the majority of which are fermentation based)



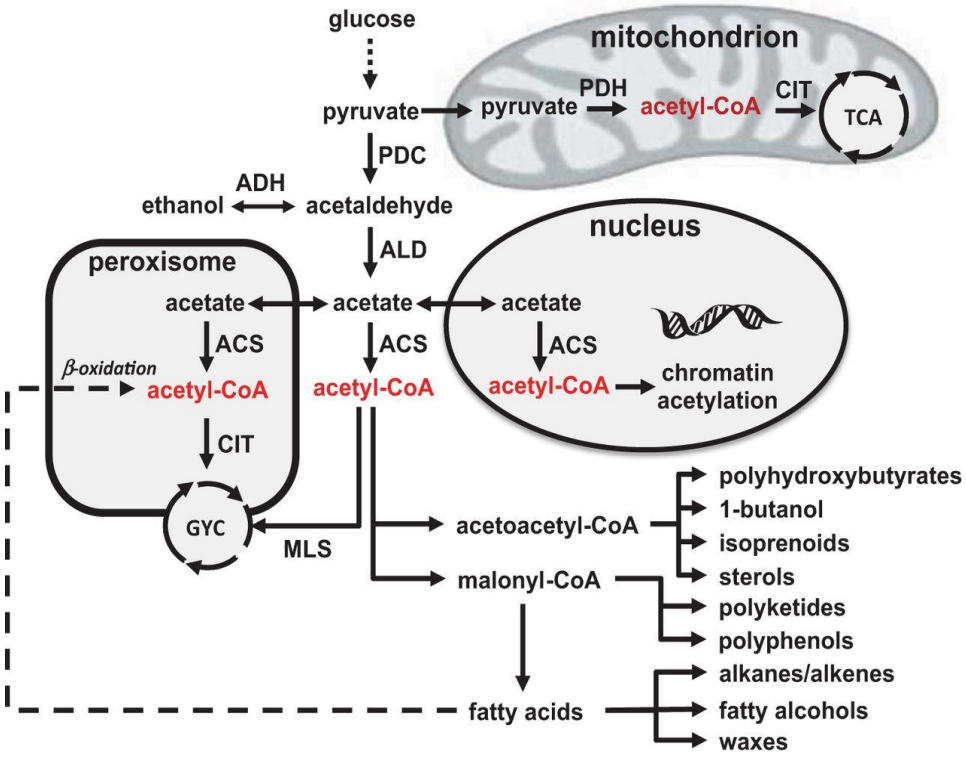
Lee et al. 2011. Curr. Opinion in Biotechn. 22: 758-767

Synthetic non-oxidative glycolysis – prevention of carbon loss in AcCoA formation -1



Carbon is lost in AcCoA formation

AcCoA is a key intermediate in product pathways

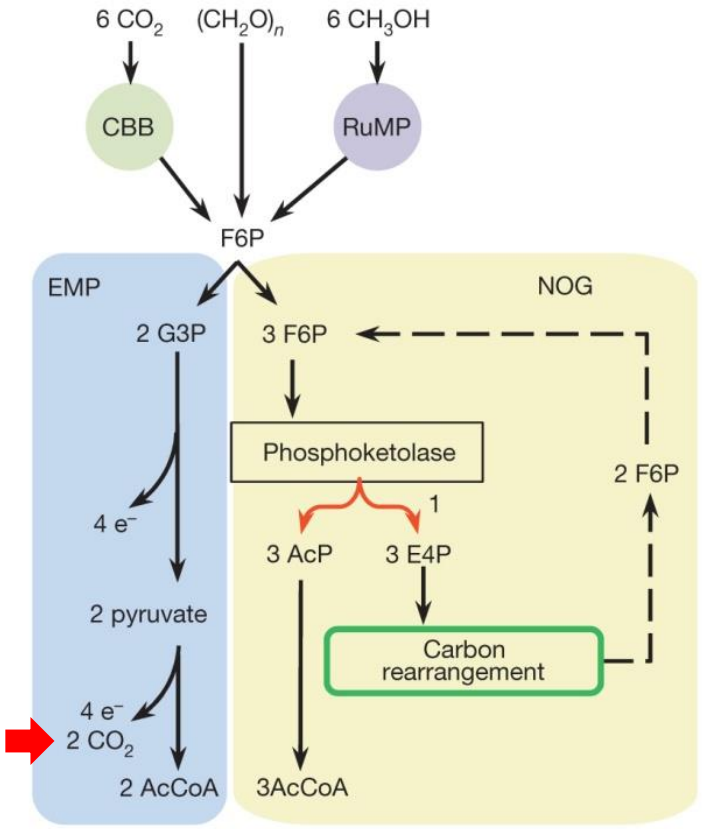


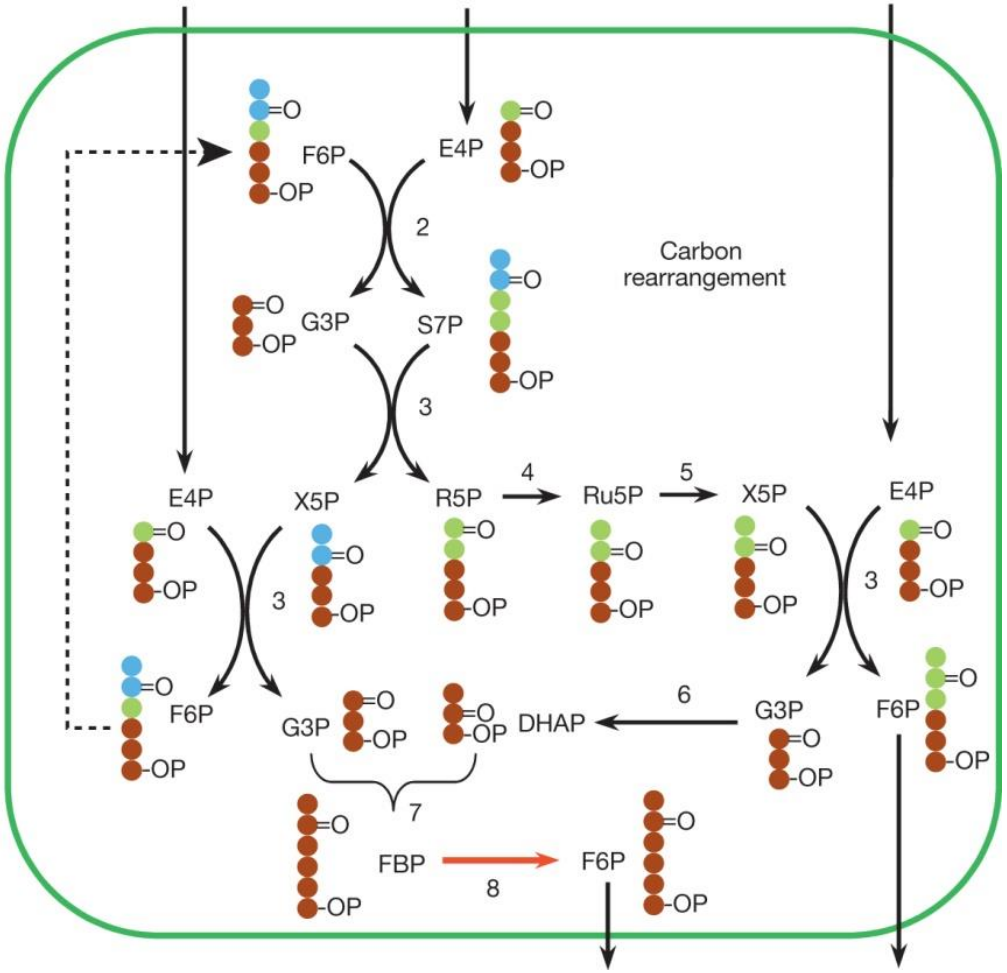
Aalto University
School of Chemical Technology

Bogorad et al. (2013). Synthetic non-oxidative glycolysis enables complete carbon conservation. Nature 502, 693-697.

Synthetic non-oxidative glycolysis – prevention of carbon loss in AcCoA formation -2

C1 or sugar as carbon sources





Bogorad et al. (2013). Synthetic non-oxidative glycolysis enables complete carbon conservation. Nature 502, 693-697.

Enzyme numbers: 1, phosphoketolase; 2, Tal; 3, Tkt; 4, Rpi; 5, Rpe; 6, Tpi; 7, Fba; 8, Fbp. DHAP, dihyroxyacetone phosphate; Ru5P, ribulose 5-phosphate.

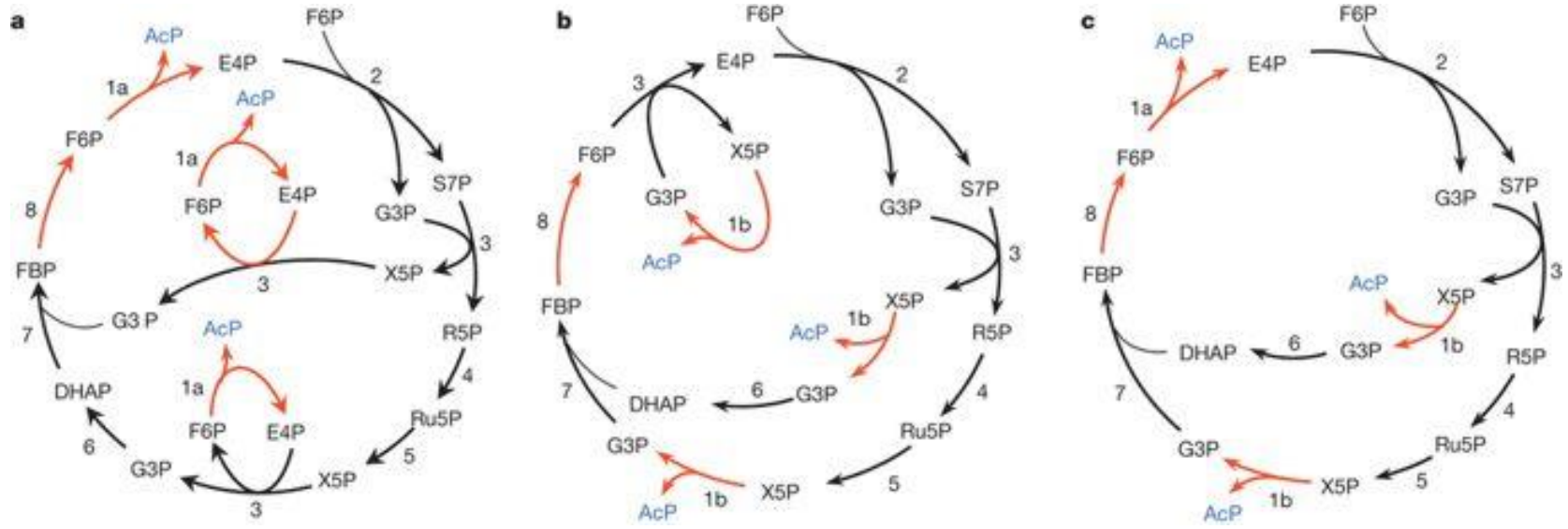
Synthetic non-oxidative glycolysis – prevention of carbon loss in AcCoA formation -3

PHOSPHOKETOLASE:

D-fructose 6-phosphate + phosphate -> acetyl phosphate + D-erythrose 4-phosphate + H_2O

D-xylulose 5-phosphate + phosphate -> acetyl phosphate + D-glyceraldehyde 3-phosphate + H_2O

D-sedoheptulose 7-phosphate + phosphate -> acetyl phosphate + D-ribose 5-phosphate + H_2O



Phosphate acetyl transferase (PTA): CoA + acetyl phosphate -> acetyl-CoA + phosphate

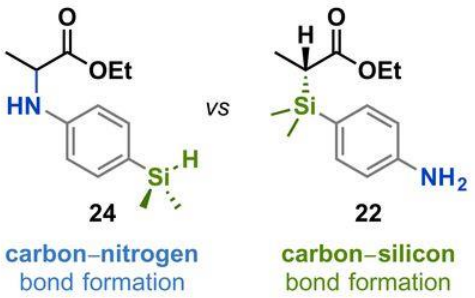
Aalto University
School of Chemical Technology

Bogorad et al. (2013). Synthetic non-oxidative glycolysis enables complete carbon conservation. Nature 502, 693-697.

Engineering for C-Si bonds

Silicon based life ?

- at least biochemicals



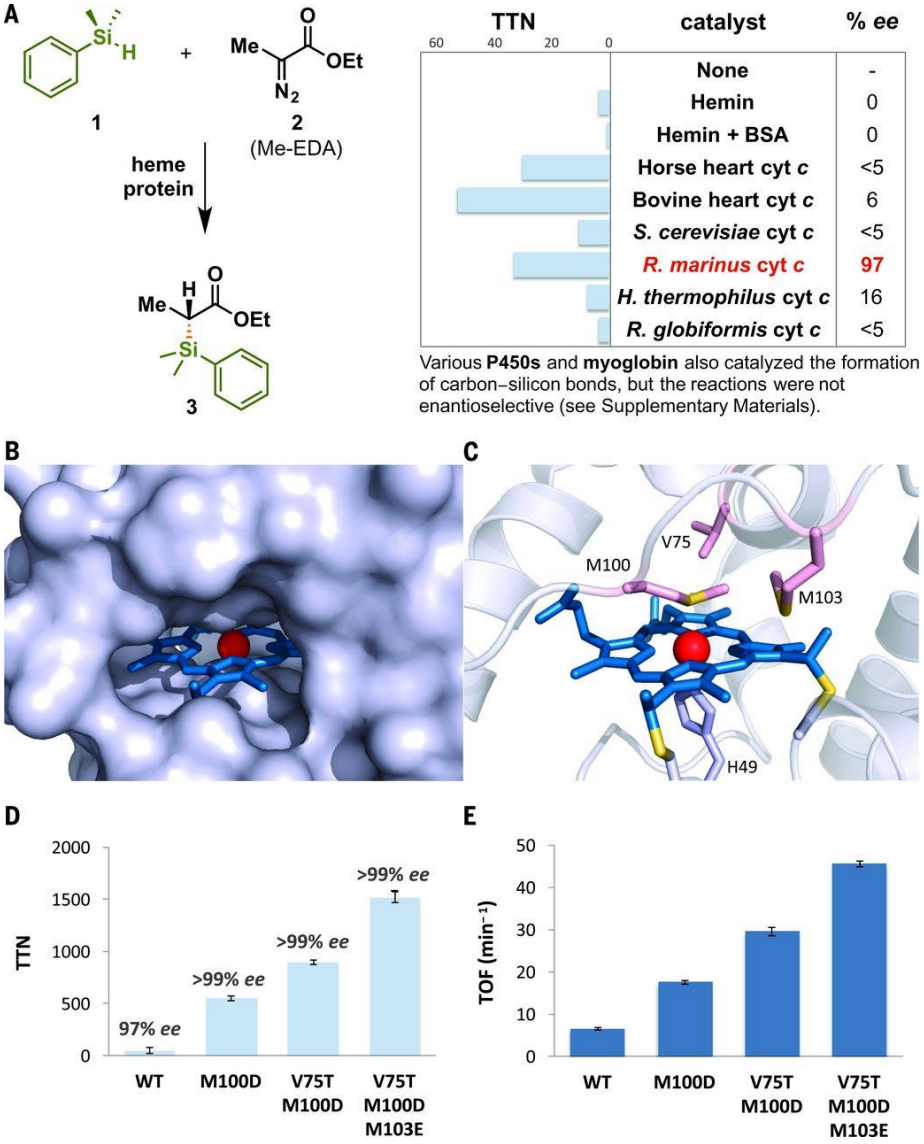
- Silicon is the second most element on Earth, after oxygen
- It is not found in biochemistry but life based on silicon (instead of carbon) has been suggested as alternative in space
- Frances Arnold and her group were able to create C–Si bonds in living *E.coli* by engineering an enzyme of *Rhodothermus marinus* from Icelandic hot springs using (only 3 rounds!) directed evolution
- Si has both metal and non-metal properties - > enzyme: cytochrome C (heme Fe^{2+}), an electron transfer protein that does not perform a catalytic function in nature
- The engineered reaction is 15-fold more efficient than with chemical catalysts with certain Si compounds

President Sauli Niinistö is giving the Millenium Technology Prize 2016 to Frances Arnold (California Institute of Technology, USA). Figure M. Penttilä

S. B. Jennifer Kan, Russell D. Lewis, Kai Chen, Frances H. Arnold. Directed evolution of cytochrome c for carbon–silicon bond formation: Bringing silicon to life. Science 25 November 2016. Vol 354 (6315). !048-1051..

Aalto University
School of Chemical Technology

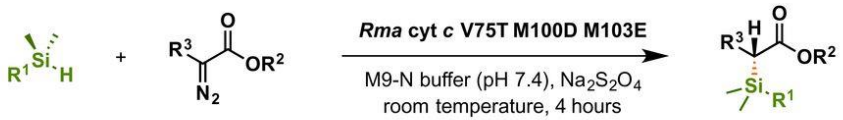
The finding could help chemists to develop new pharmaceuticals and industrial catalysts — and perhaps explain why evolution has almost completely shunned silicon.

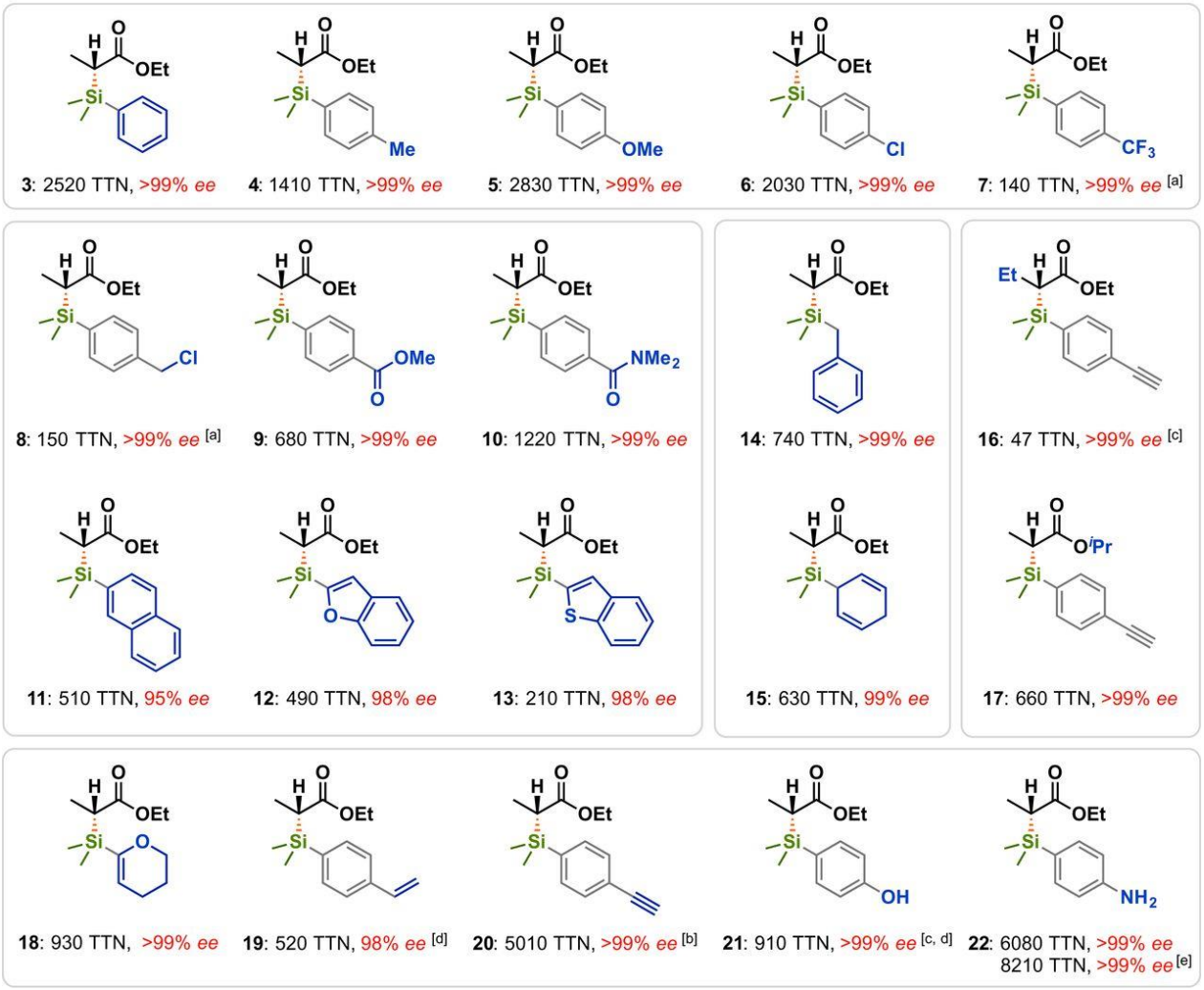


Heme protein–catalyzed carbon–silicon bond formation.(**A**) Carbon–silicon bond formation catalyzed by heme and purified heme proteins. (**B**) Surface representation of the heme-binding pocket of wild-type *Rma* cyt c (PDB ID: 3CP5). (**C**) "Active site" structure of wild-type *Rma* cyt c showing a covalently bound heme cofactor ligated by axial ligands H49 and M100. Amino acid residues M100, V75, and M103 residing close to the heme iron were subjected to site-saturation mutagenesis. (**D**) Directed evolution of *Rma* cyt c for carbon–silicon bond formation [reaction shown in (A)]. Experiments were performed using lysates of *E. coli* expressing *Rma* cyt c variant (OD_{600} = 15; heat-treated at 75°C for 10 min), 10 mM silane, 10 mM diazo ester, 10 mM $Na_2S_2O_4$, 5 vol % MeCN, M9-N buffer (pH 7.4) at room temperature under anaerobic conditions for 1.5 hours. Reactions were done in triplicate. (**E**) Carbon–silicon bond forming rates over four generations of *Rma* cyt c. Single-letter abbreviations for the amino acid residues are as follows: D, Asp; E, Glu; M, Met; T, Thr; and V, Val. TTN, total turn over number.

Aalto University
School of Chemical Technology

S. B. Jennifer Kan, Russell D. Lewis, Kai Chen, Frances H. Arnold. Directed evolution of cytochrome c for carbon–silicon bond formation: Bringing silicon to life. Science 25 November 2016. Vol 354 (6315):1048-1051.





Scope of *Rma* cyt c V75T M100D M103E-catalyzed carbon–silicon bond formation. Standard reaction conditions: lysate of *E. coli* expressing *Rma* cyt c V75T M100D M103E (OD_{600} = 1.5; heat-treated at 75°C for 10 min), 20 mM silane, 10 mM diazo ester, 10 mM $Na_2S_2O_4$, 5 vol % MeCN, M9-N buffer (pH 7.4) at room temperature under anaerobic conditions. Reactions performed in triplicate. [a] OD_{600} = 5 lysate. [b] OD_{600} = 0.5 lysate. [c] OD_{600} = 15 lysate. [d] 10 mM silane. [e] OD_{600} = 0.15 lysate.

Can be used already for *in vitro* enzymatic catalysis. Will take some time to make larger scale production with cells possible?

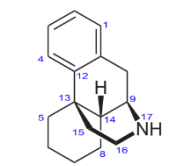
Aalto University
School of Chemical Technology

S. B. Jennifer Kan, Russell D. Lewis, Kai Chen, Frances H. Arnold. Directed evolution of cytochrome c for carbon–silicon bond formation: Bringing silicon to life. Science 25 November 2016. Vol 354 (6315):1048-1051.

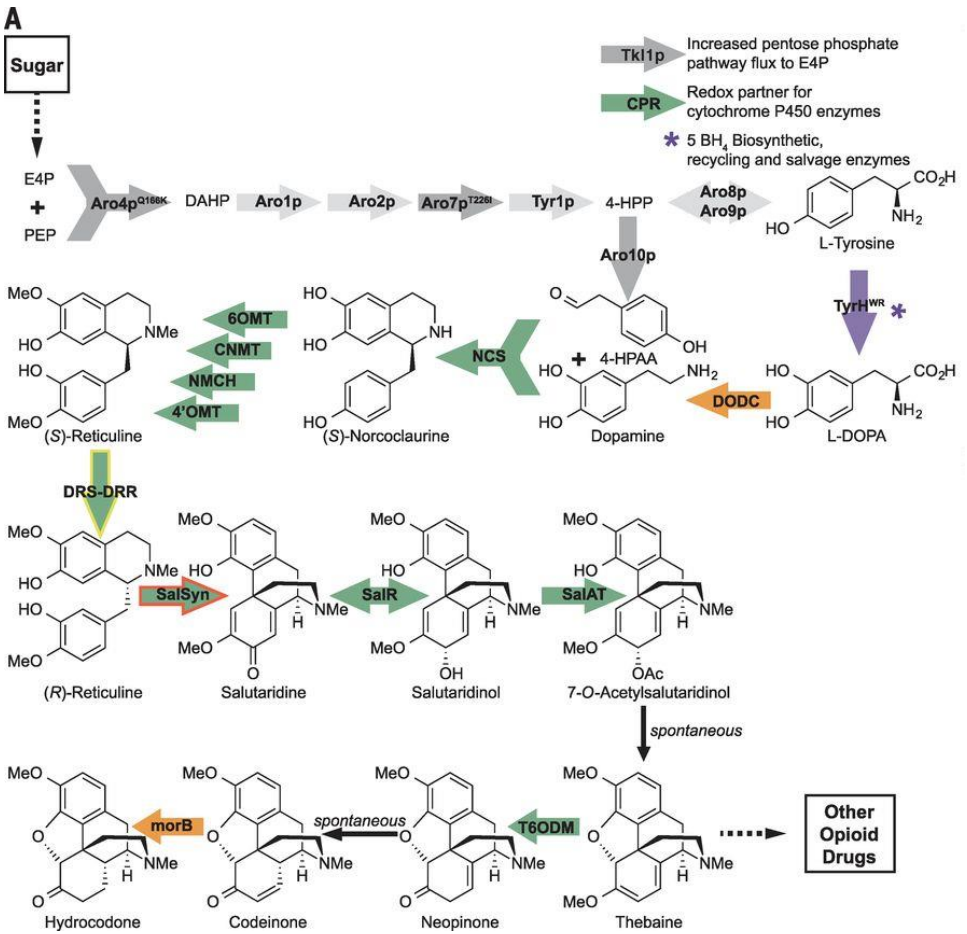
Synthetic pathway and strain optimization for opioid synthesis in yeast

Stephanie Galanie et al. Science 2015;349:1095-1100

- Enzyme engineering for correcting the processing and increasing the activity of the key pathway cytochrome P450
- Expression of 21 heterologous enzymes from plants, mammals, bacteria, and yeast (color codes)
- Overexpression of two native yeast enzymes
- Deletion of one native yeast gene



MORPHINAN



Biosynthetic scheme for production of thebaine and hydrocodone from sugar. Thebaine is a starting material for many opioid drugs through biosynthetic and semisynthetic routes. Block arrows indicate enzyme-catalyzed steps. Light gray arrows, unmodified yeast enzymes; dark gray arrows, overexpressed and modified yeast enzymes; purple arrows, mammalian (*Rattus norvegicus*) enzymes; orange arrows, bacterial (*Pseudomonas putida*) enzymes; green arrows, plant (*Papaver somniferum*, *P. bracteatum*, *Coptis japonica*, *Eschscholzia californica*) enzymes. Yellow outline highlights DRS-DRR; red outline highlights engineered SalSyn. E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-d-*arabino*-2-heptulosonic acid 7-phosphate; 4-HPP, 4-hydroxyphenylpyruvate; 4-HPAA, 4-hydroxyphenylacetaldehyde; BH_4, 5,6,7,8-tetrahydrobiopterin; Tkl1p, transketolase; CPR, cytochrome P450 reductase; Aro4p^{Q166K}, DAHP synthase; Aro1p, pentafunctional *arom* enzyme; Aro2p, bifunctional chorismate synthase and flavin reductase; Aro7p^{T226I}, chorismate mutase; Tyr1p, prephenate dehydrogenase; Aro8p, aromatic aminotransferase I; Aro9p, aromatic aminotransferase II; Aro10p, phenylpyruvate decarboxylase; TyrH^{WR}, feedback inhibition–resistant tyrosine hydroxylase (mutations R37E, R38E, W166Y); DODC, l-DOPA decarboxylase; NCS, (*S*)-norcoclaurine synthase; 6OMT, norcoclaurine 6-*O*-methyltransferase; CNMT, coclaurine *N*-methyltransferase; NMCH, *N*-methylcoclaurine hydroxylase; 4′OMT, 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase; DRS-DRR, 1,2-dehydroreticuline synthase-1,2-dehydroreticuline reductase; SalSyn, salutaridine synthase; SalR, salutaridine reductase; SalAT, salutaridinol 7-*O*-acetyltransferase; T6ODM, thebaine 6-*O*-demethylase; morB, morphinone reductase.

Cannabinoid synthesis in yeast

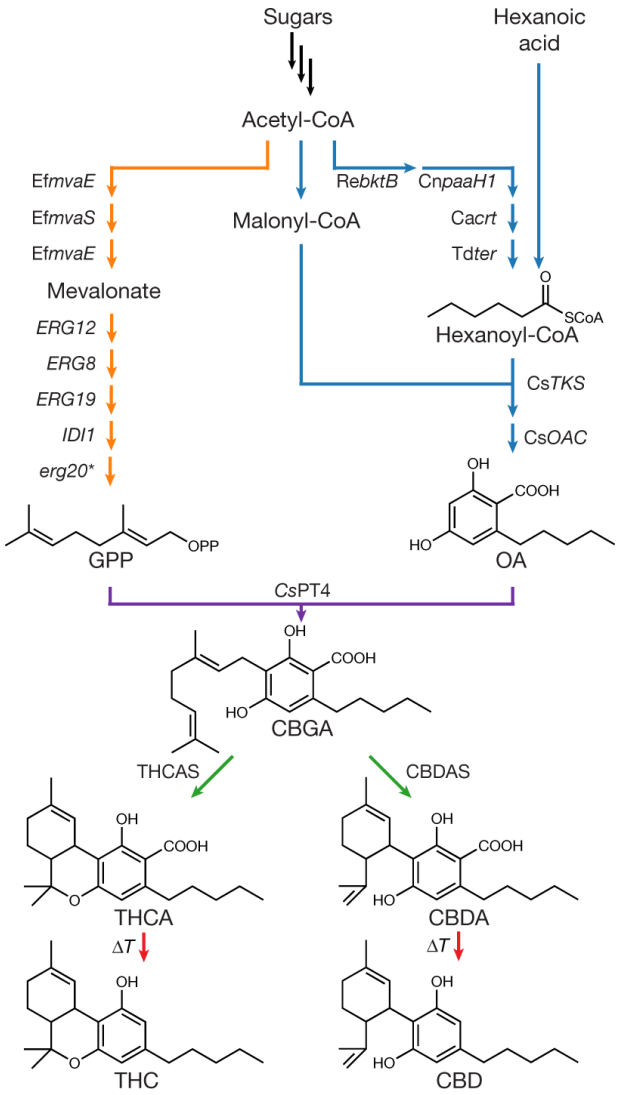
Nature 2019, vol 567:123

Complete biosynthesis of cannabinoids and their unnatural analogues in yeast

Xiaozhou Luo[1,15], Michael A. Reiter[1,2,15], Leo d'Espaux[3,12], Jeff Wong[3,12], Charles M. Denby[1,13], Anna Lechner[4,5,14], Yunfeng Zhang[1,6], Adrian T. Grzybowski[1], Simon Harth[3], Weiyin Lin[3], Hyunsu Lee[3,7], Changhua Yu[3,5], John Shin[3,4], Kai Deng[8,9], Veronica T. Benites[3], George Wang[3], Edward E. K. Baidoo[3], Yan Chen[3], Ishaan Dev[3,4], Christopher J. Petzold[3] & Jay D. Keasling[1,3,4,5,10,11]*

Synthetic pathway required for efficient precursor (hexanoyl-CoA) production

Introduced also a gene for a previously undiscovered enzyme with geranylpyrophosphate:olivetolate geranyltransferase activity (CsPT4) (known natural producer gene gave no activity)



Read this article

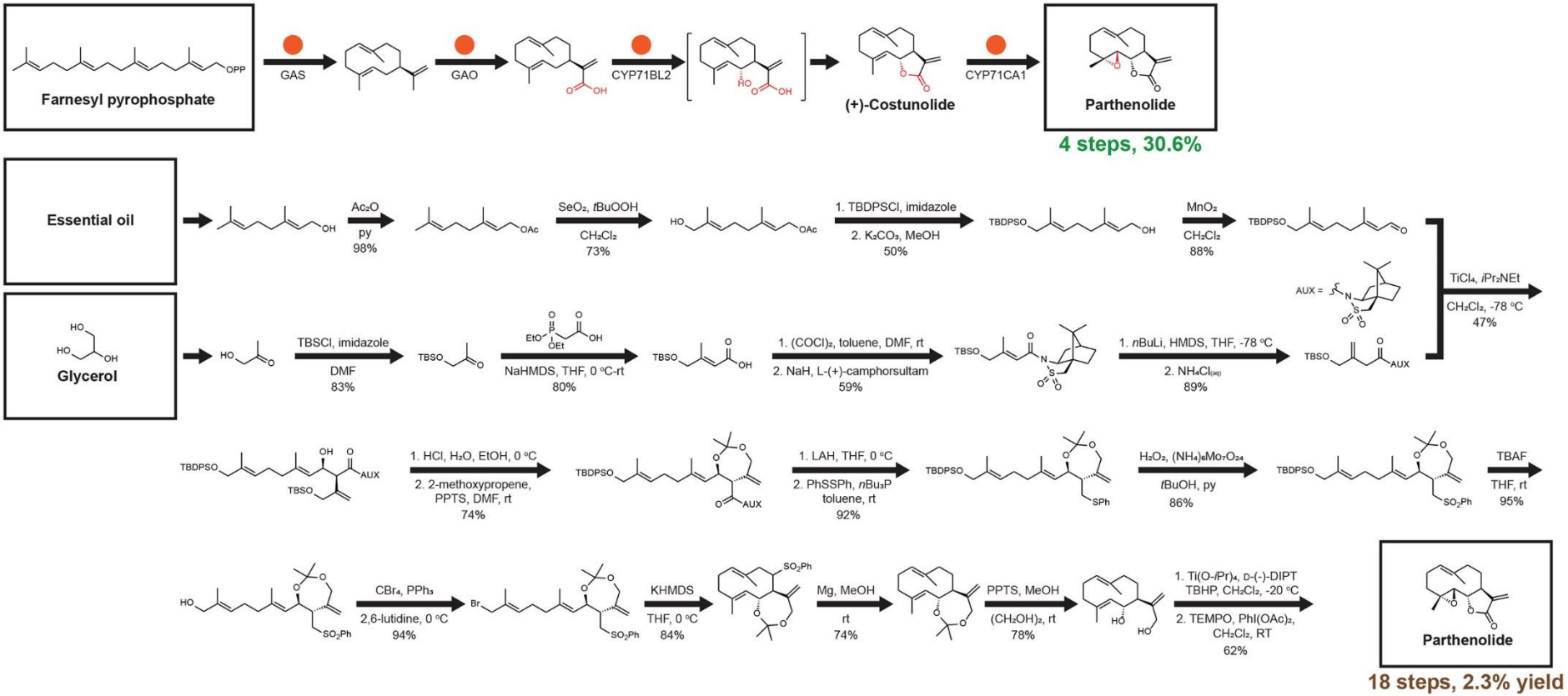
Retrosynthetic design of metabolic pathways to chemicals not found in nature

Geng-MinLin, Robert Warden-Rothman & Christopher A.Voigt

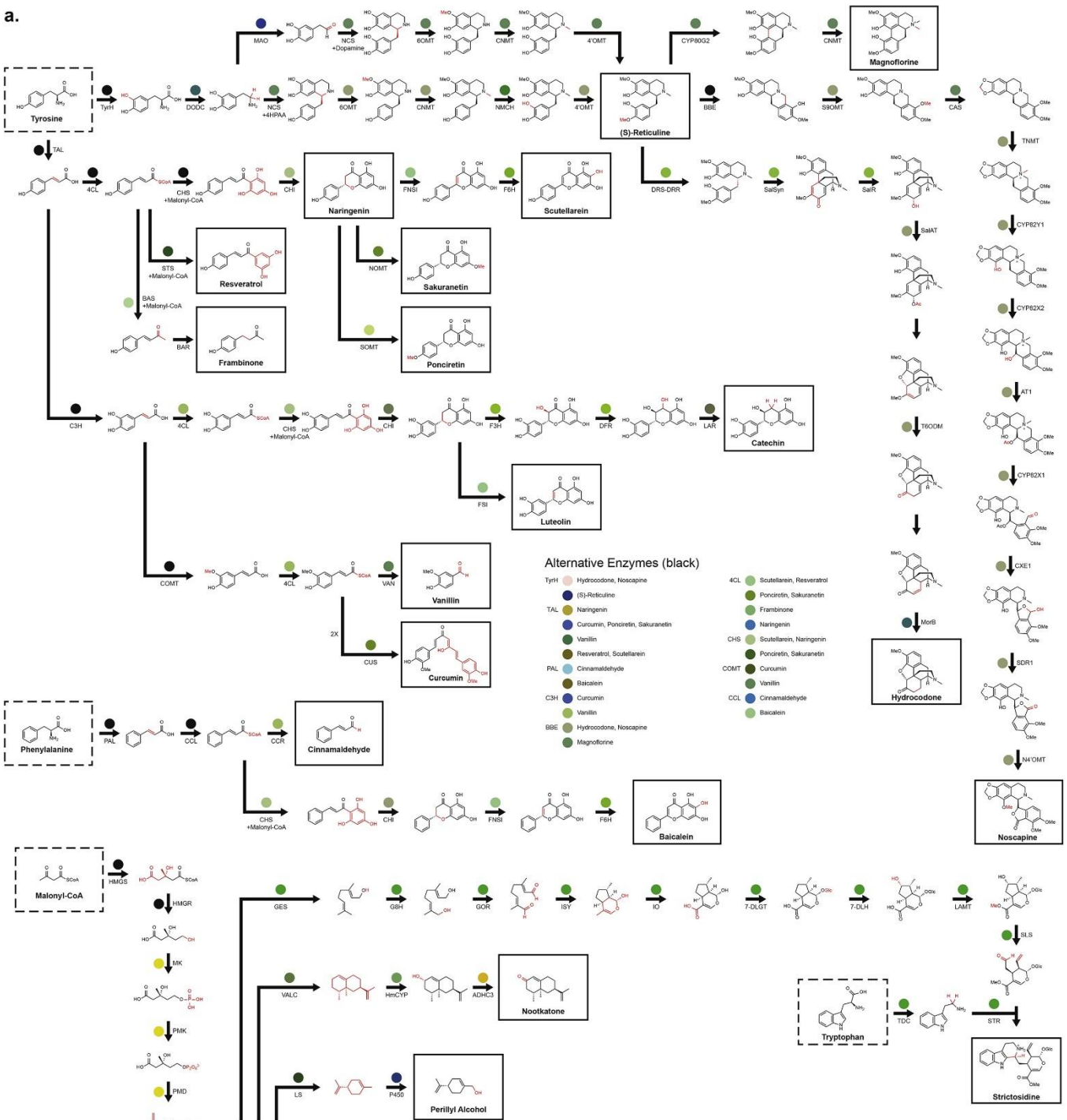
Current Opinion in Systems Biology, in press on line

https://doi.org/10.1016/j.coisb.2019.04.004

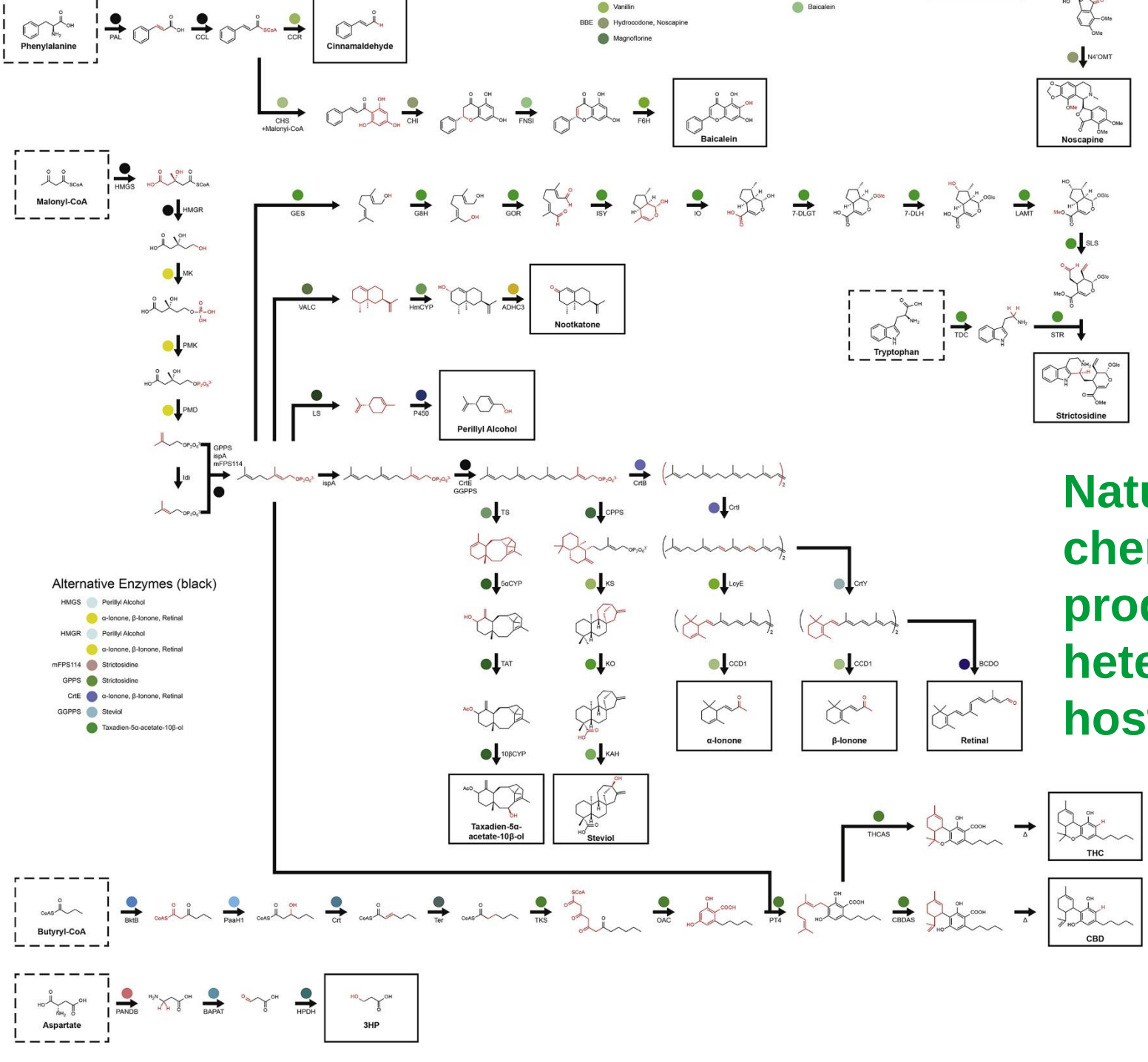
Biochemistry vs. Chemistry



Comparison of metabolic (from FPP) and chemical routes to parthenolide. The pathway has been identified and transferred from its native organism (*Tanacetum parthenium*) to yeast and the theoretical yield of the biosynthetic route is shown (0.306 g/g **glucose**).

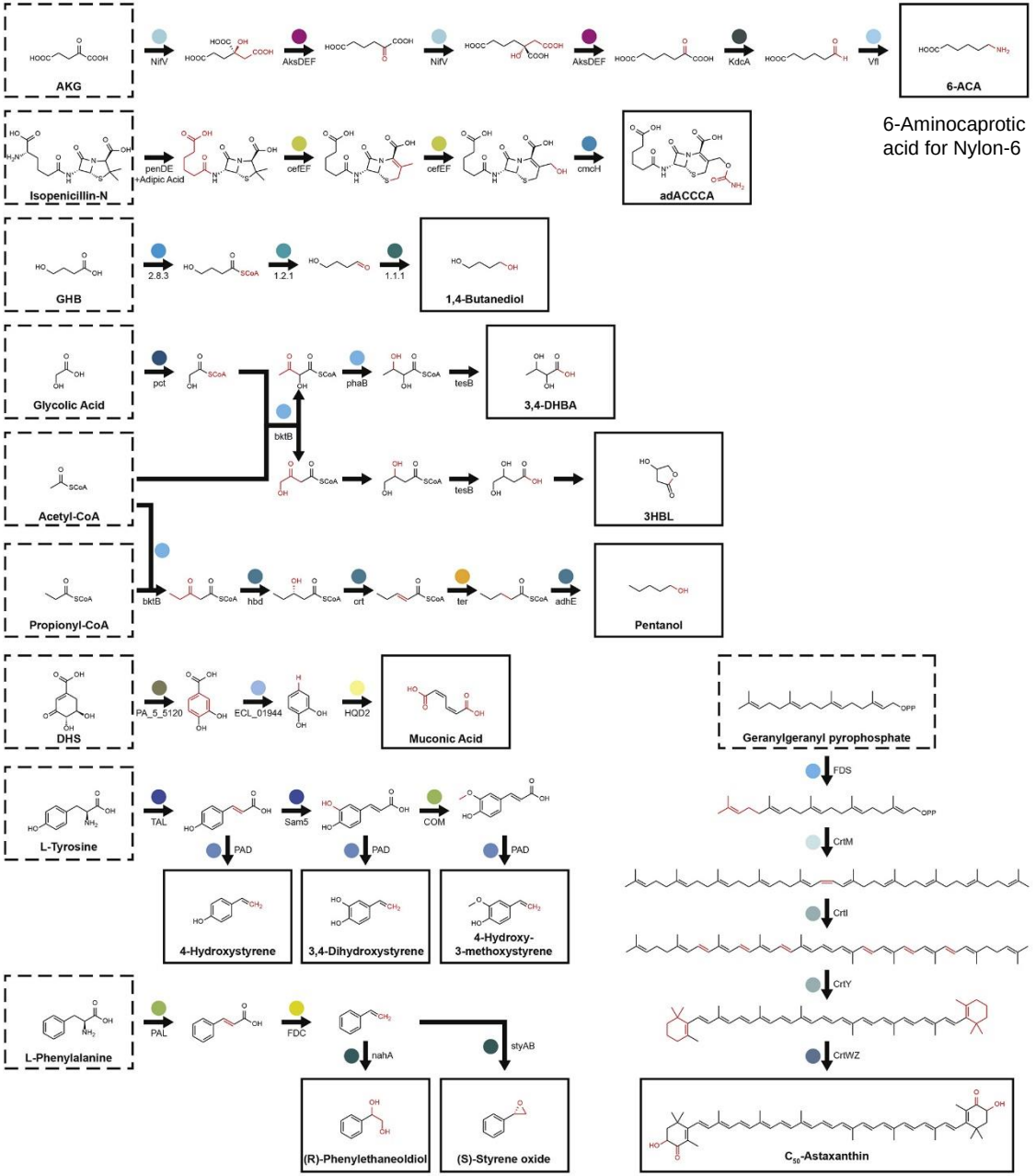


Natural chemicals produced in a heterologous host



Natural chemicals produced in a heterologous host

Retrosynthesis for xenobiotic compounds, not found in nature



Enzyme Source Organisms

Archea
- *Methanococcus aeolicus*

Fungi
- *Acremonium chrysogenum*
- *Candida albicans*
- *Pichia pastoris*
- *Podospora anserina*
- *Rhodotorula rubra*
- *Rhodotorula toruloides*
- *Saccharomyces cerevisiae*

Bacteria
- *Acinetobacter calcoaceticus*
- *Azotobacter vinelandii*
- *Bacillus cereus*
- *Bacillus amyloliquefaciens*
- *Brevundimonas sp. SD212*
- *Clostridium acetobutylicum*
- *Clostridium beijerinckii*
- *Cupriavidus necator*
- *Enterobacter cloacae*
- *Erwinia herbicola*
- *Escherichia coli*
- *Geobacillus stearothermophilus*
- *Lactococcus lactis*
- *Marine bacterium HF10_19P19*
- *Megasphaera elsdenii*
- *Micrococcus luteus*
- *Mycobacterium HXN 1500*
- *Pantoea ananatis*
- *Porphyromonas gingivalis*
- *Pseudomonas putida*
- *Pseudomonas sp. VLB120*
- *Ralstonia eutropha*
- *Rhodococcus ruber*
- *Saccharothrix espanaensis*
- *Sphingomonas sp. HXN-200*
- *Staphylococcus aureus*
- *Streptomyces castaneoglobisporus*
- *Streptomyces clavuligerus*
- *Streptomyces coelicolor*
- *Streptomyces maritimus*
- *Synechococcus sp.*
- *Treponema denticola*
- *Vibrio fluvialis*

Animals
- *Gallus gallus*
- *Rattus norvegicus*
- *Tribolium castaneum*

Plants
- *Abies grandis*
- *Arabidopsis thaliana*
- *Camellia sinensis*
- *Cannabis sativa*
- *Catharanthus roseus*
- *Coptis japonica*
- *Cucumis sativus*
- *Cucurbita maxima*
- *Cupressus nootkatensis*
- *Desmodium uncinatum*
- *Eschscholzia californica*
- *Glycine max*
- *Glycyrrhiza echinata*
- *Hyoscyamus muticus*
- *Lactuca sativa*
- *Medicago sativa*
- *Mentha spicata*
- *Nicotiana tabacum*
- *Oryza sativa*
- *Papaver bracteatum*
- *Papaver somniferum*
- *Petroselium crispum*
- *Petunia hybrida*
- *Populus euramericana*
- *Rubus idaeus*
- *Prunus sp.*
- *Scutellaria baicalensis*
- *Solanum tuberosum*
- *Stevia rebaudiana*
- *Taxus brevifola*
- *Taxus canadensis*
- *Taxus cuspidata*
- *Vanilla planifolia*
- *Vitis vinifera*
- *Zea mays*

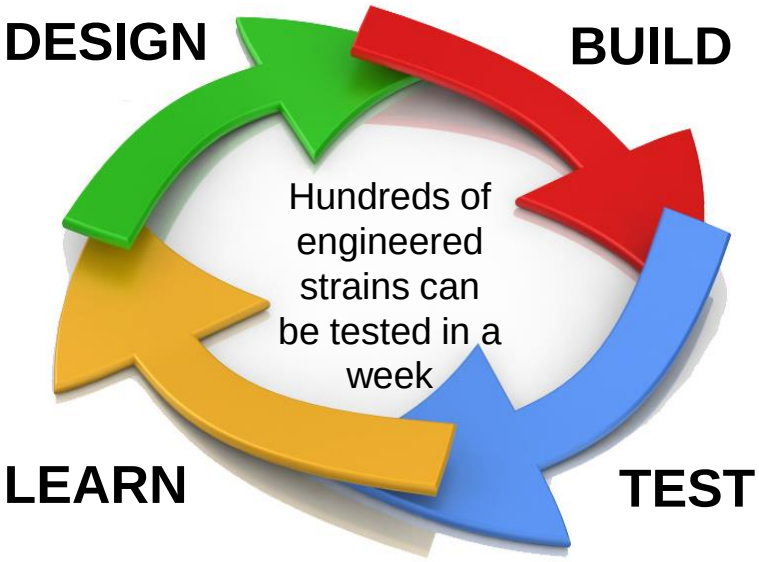
Protists
- *Euglena gracilis*

Living Factories

Automated strain engineering

Computational recepies for the robot to carry out Build and Test phases

Full automation of strain construction and cultivation



Design
Production strains and their parts are designed using computational tools

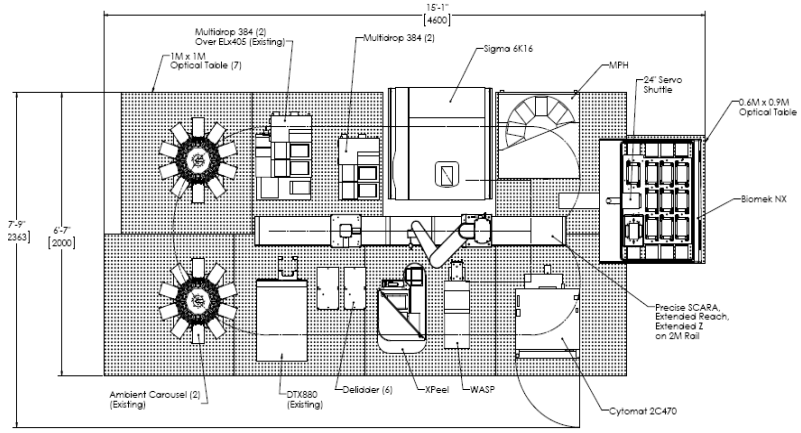
Construction of production strains
Synthetic DNA is delivered to the cells using genome editing tools such as CRISPR.

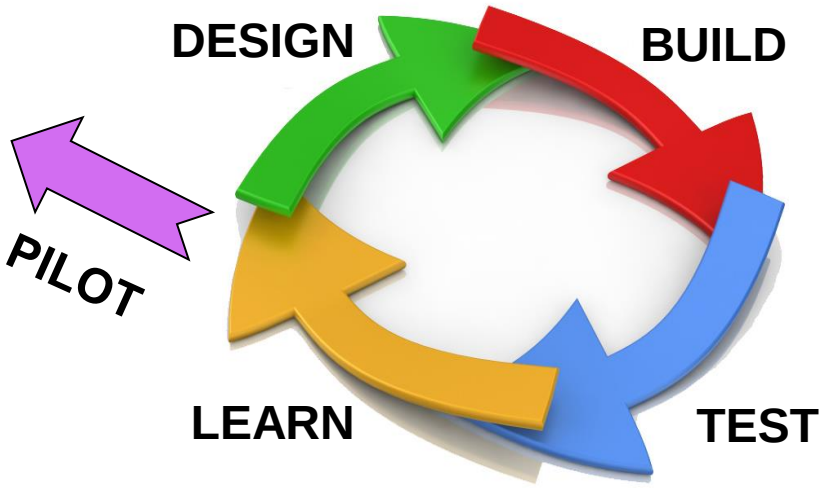
Analysis and decisions
Machine learning algorithms can help the researcher to analyse and understand measured data.

Cultivation and measurement
Robots are cultivating the strains and carry out measurements. The results are automatically stored in databases.

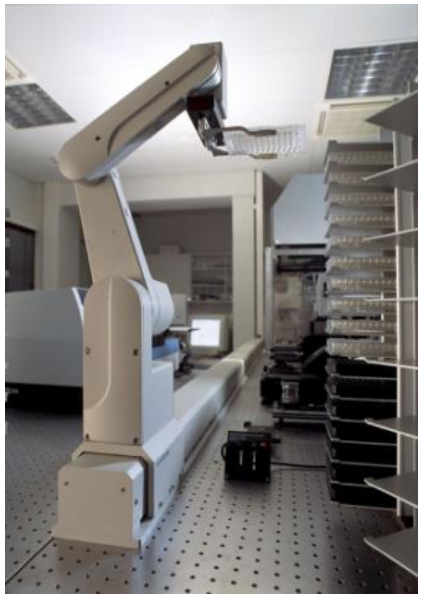
Aalto-VTT national Bioeconomy infrastructure: From synthetic biology to piloting

A versatile computing platform for design, prediction and analysis





A robotic platform for efficient DNA assembly, transformation and strain screening



Controlled parallel bioreactor systems with automated sampling and analytics

Living Factories

Industrial Biotechnology Platform at VTT

Experimental

Synthetic DNA

Synbio promoters, sensors etc

Automated DNA assembly and strain construction

Strain collections for optimal hosts

Genome editing tools, e.g. CRISPR

Systems biology (genome, mRNA, protein, metabolite, fluxes, single cell analytics, sensors)

Protein engineering & HTS

Automated HT pheno- and genotypic screening

Strain mutagenesis & evolutionary engineering

Automated bioreactor culturing & on-line analytics

Process integration (SHF, SSF, consolidated)

Gas fermentations

Batch, fed-batch, continuous fermentations

C1 raw materials

Piloting (50, 300, 1200 L)

DSP

”Waste”

Biomass pretreatment & fractionation

Standardised/flexible experimental protocols

Design → **Build** → **Analyse** → **Optimise** → **Pilot** → **Product** ← **Raw material**

Databases and flowsheets

Cost-effective product – Thermodynamics (ThdMD), Reaction database (ReGraphD), Flux/Titer/Rate/Yield prediction

Optimal host – Metabolic model construction (CoReCo), Metabolic pathway analysis (AntND)

Optimal pathway & engineering strategy – Metabolic (Bayesian/Dynamic FBA) and Kinetic modelling, Bioinformatics (SeqSear

Novel products & reactions – RetroSMARTS, Enzyme function/promiscuity prediction, Mutagenesis prediction (Machine learnin

Up and down scaling feasibility – Kinetic/dynamic MFA and process model integration

Optimal production medium – Chemical speciation, DoE, Response surface modeling

Process design – Flowsheet/process modelling

Sustainability - TEA, LCA, ILU

Computational

FROM PROCESS FEASIBILITY TO DESIGN