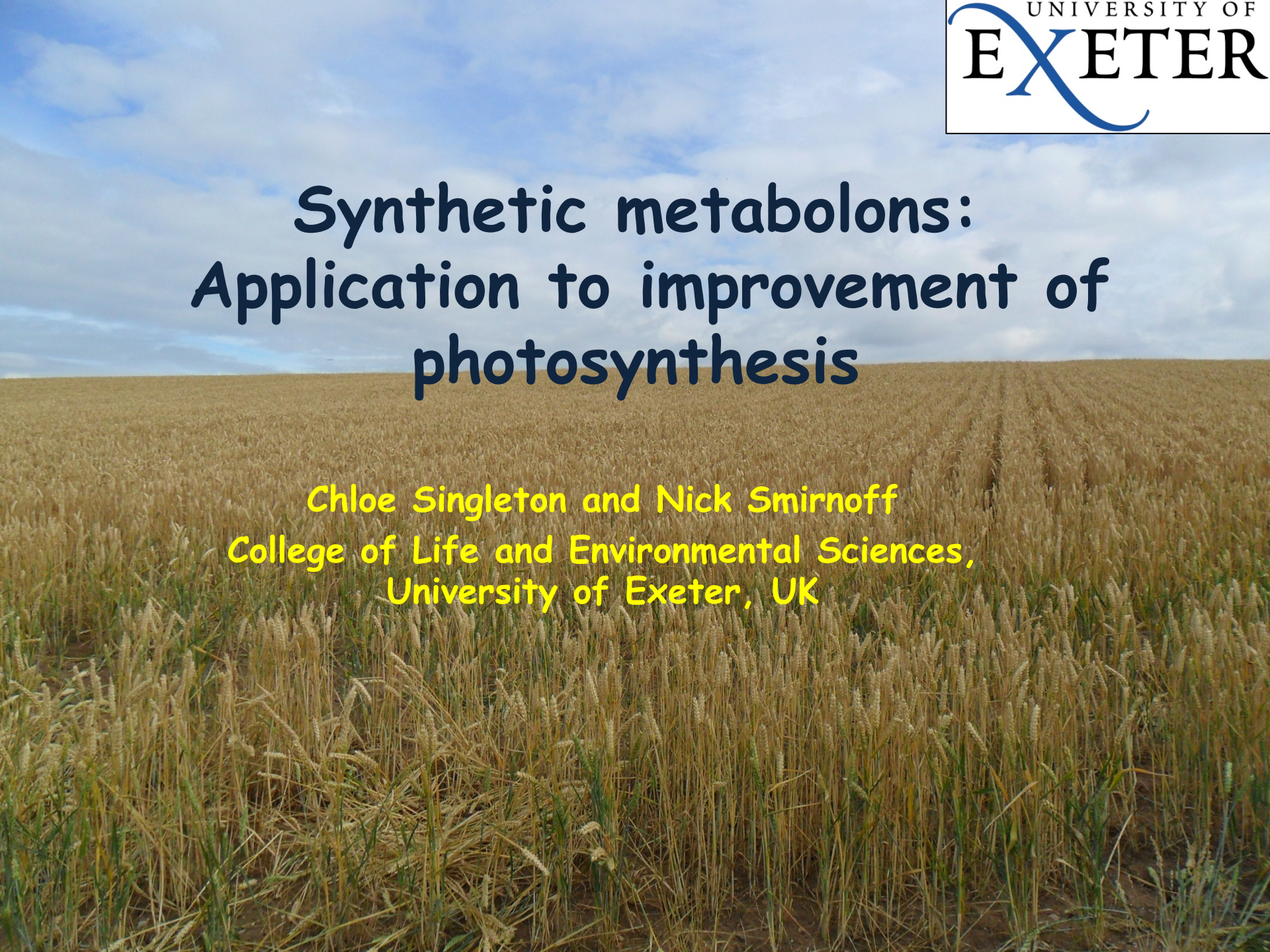


Synthetic metabolons: Application to improvement of photosynthesis

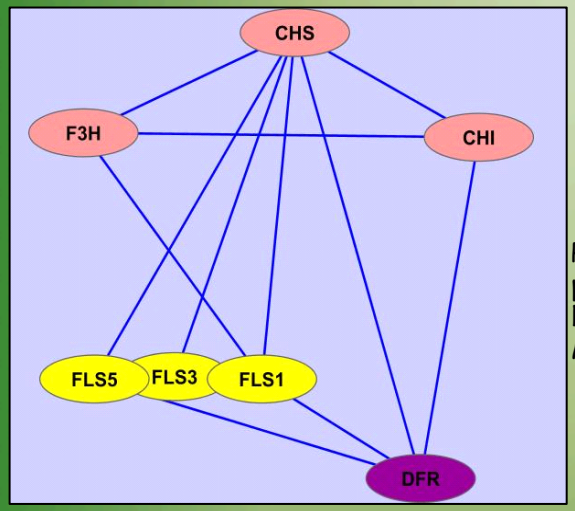
Chloe Singleton and Nick Smirnoff
College of Life and Environmental Sciences,
University of Exeter, UK

"A metabolon is a temporary structural functional complex held together by non-covalent interactions and structural elements of the cell such as integral membrane proteins and proteins of the cytoskeleton."

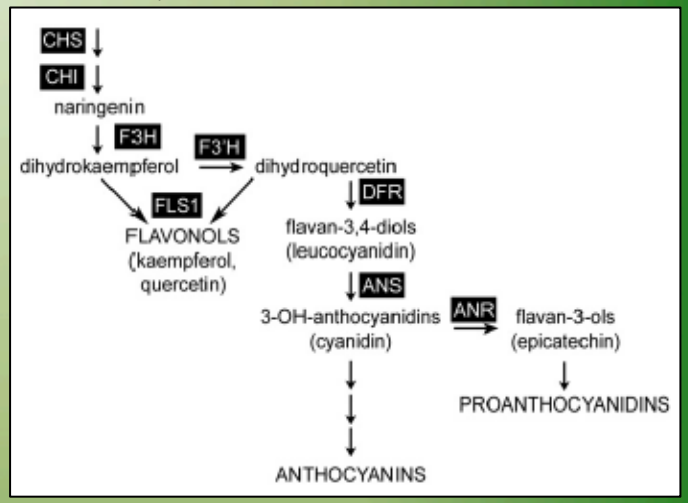
- In this talk:
  - Naturally occurring metabolons
  - Synthetic metabolons
  - Work carried out in our lab

Flavonoid biosynthesis metabolon

- Membrane-associated flavonoid enzyme complex
- Association with the cytoplasmic face of endoplasmic reticulum



Protein-protein interaction network predicted by high throughput yeast 2 hybrid analysis in Arabidopsis (Jay Moore PRESTA ideas database)



- Evidence-
  - co-purifcation[1], immunolocalization[1,3], pulldowns[2], yeast-2-hybrid[2]
  - Mutagenesis
  - More recently visualization by FRET
  - Isotope dilution method, tracking of metabolites[4,5]

1. Hrazdina (1992) in *Recent Advances in Phytochemistry*. Plenum Press, New York. 1-3
2. Burbulis and Winkel-Shirley (1999) *Proc. Natl. Acad. Sci. USA*. **96**, 12929012934
3. Saslowsky and Winkel-Shirley. (2001) *Plant J*. **27**, 37-48
4. Hrazdina and Wagner (1985) *Arch. Biochem. Biophys*. **27**, 88-100
5. Rasmussen and Dixon (1999) *Plant Cell*. **11**, 1537-1551

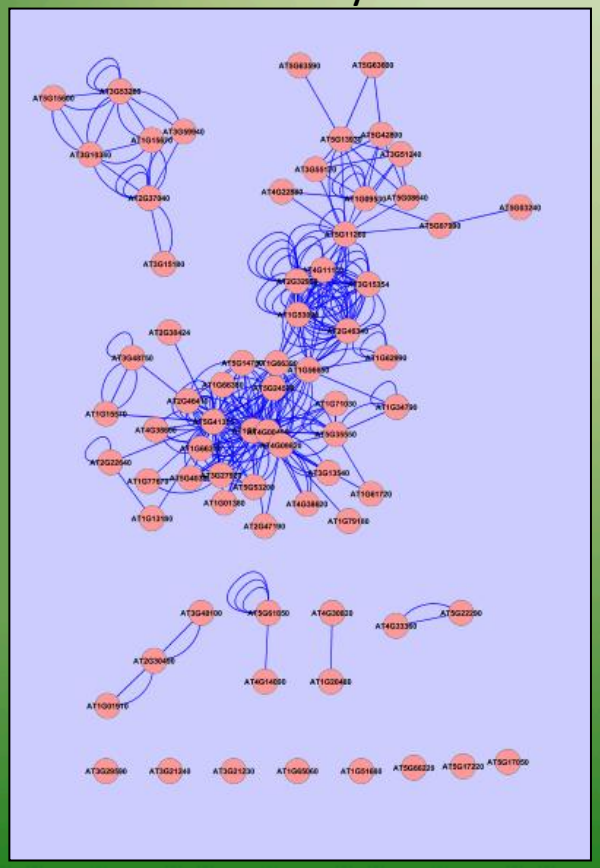
Naturally occurring metabolons

- Multifunctional enzymes- one protein, several active sites
  - Fatty acid synthase in eukaryotes
- Electrostatic interactions with surfaces of organelles
  - Glycolytic enzymes attach to surface of mitochondria in yeast and *Arabidopsis*
- Assembly on membranes, possibly in membrane microdomains (lipid rafts)
  - NADPH oxidase complex requires cholesterol-enriched microdomains for assembly
- Attachment to cytoskeleton
  - Microtubule associated enzymes from various metabolic pathways in *Arabodopsis*
- Protein-protein interactions
  - Tricarboxylic acid cycle (TCA) in *Bacillus subtilis*
- Microcompartmens
  - Cynaobacterial carboxysome

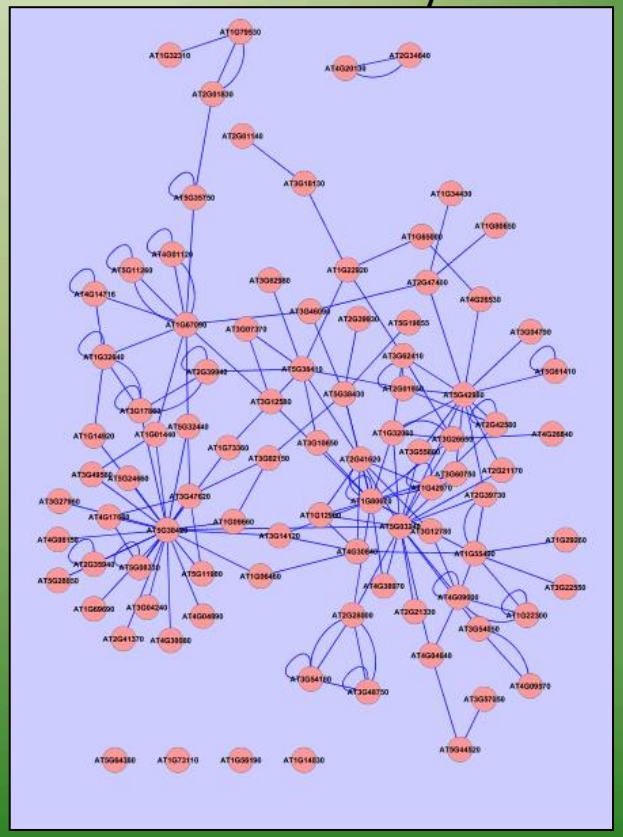
Protein-protein interaction data from IDEAs database (PRESTA)

- Preliminary survey of available Y2H, pulldown, etc. for Arabidopsis
  - Predicts physical interaction of flavonoid biosynthesis enzymes
  - But in many pathways predicts enzymes are frequently linked *via* other proteins

Flavonoid biosynthesis



Calvin-Benson cycle



Jay Moore PRESTA ideas databas

Metabolic engineering: beyond over-expressing enzymes

- It is evident that enzymes in many metabolic pathways are spatially-organised into metabolons and that such proximity has benefits

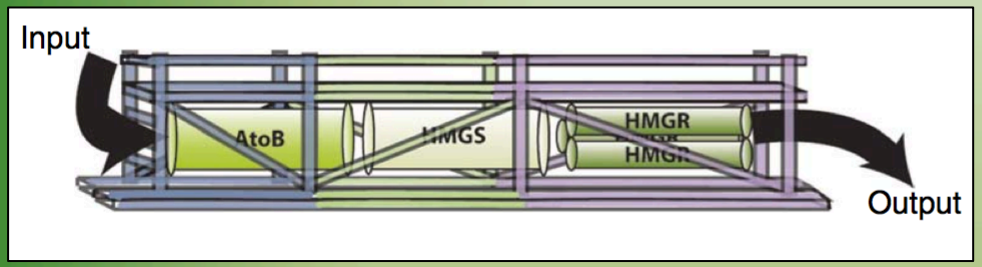
- Less enzyme needed to maintain a given flux- increased substrate concentration
- Substrate channeling
  - Potentially toxic/reactive/volatile intermediates contained
  - Pathway cross talk minimised
- Branch point control

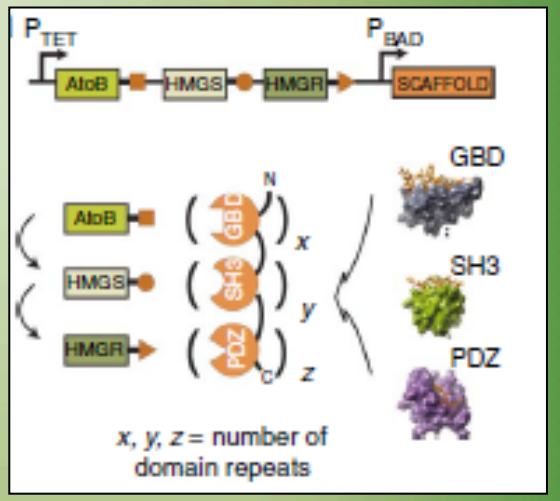
- Therefore metabolic engineering should consider the possibility of introducing synthetic enzyme complexes to improve flux

Synthetic metabolons: scaffold complexes

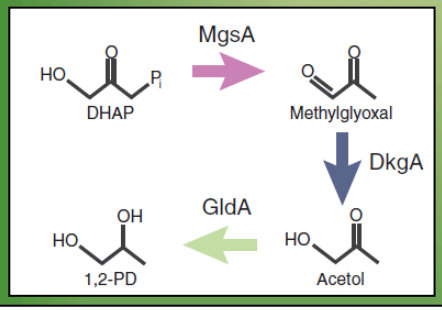
Protein scaffolds

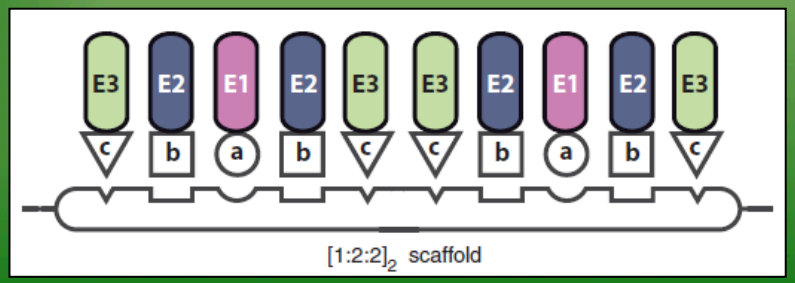
Three non-endogenous mevalonate biosynthesis enzymes scaffolded in *E. coli* using eukaryote protein-protein interaction domains





Dueber (2009) Nature Biotechnology





DNA scaffolds

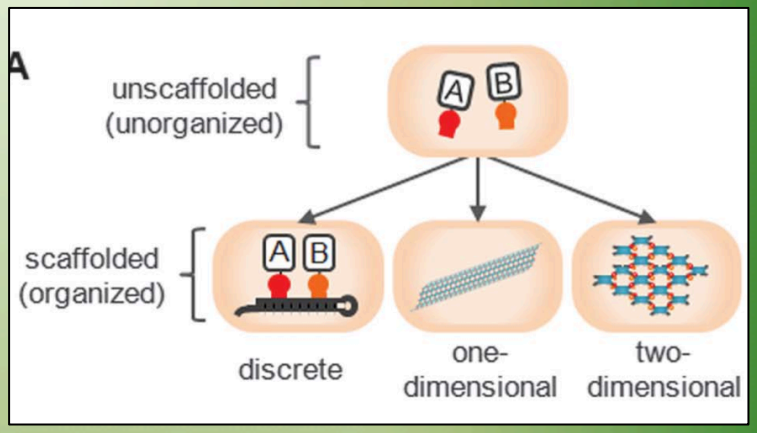
Three genes from the 1,2-propanediol biosynthetic pathway assembled on a DNA backbone using zinc finger DNA binding domains

Conrado (2011) Nuclei Acid Research

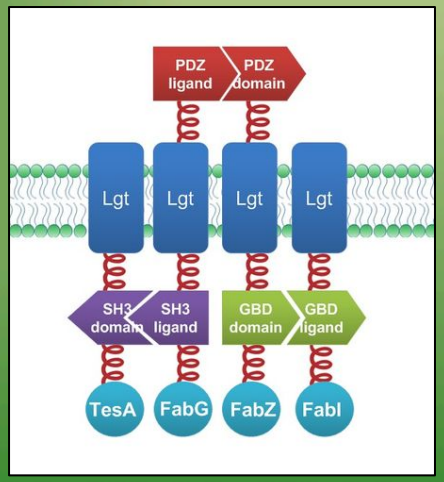
Synthetic metabolons: scaffold complexes

RNA scaffolds

Engineered RNA molecules assembled into discrete scaffolds with protein docking sites for 1D and 2D spatial organization of H_2 producing pathway



Delebeque (2011) Science



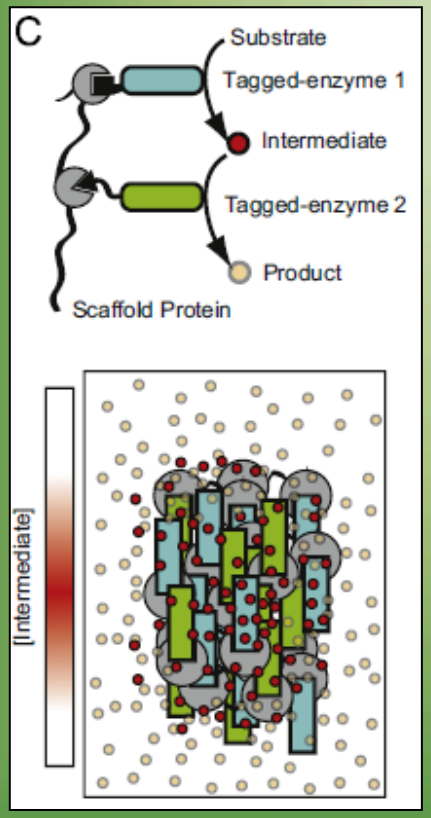
Membrane scaffolds

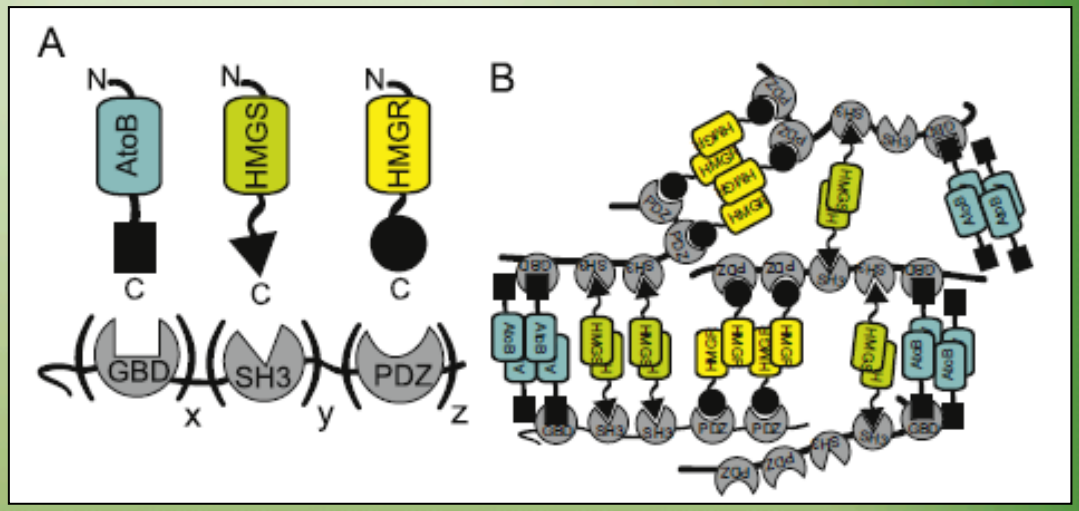
Fatty acid biosynthesis enzymes scaffolded to *E. coli* membrane

SJTU-BioX Shanghai iGEM Finalists (2012)

Synthetic metabolons: metabolite microdomains

- Minimum size?





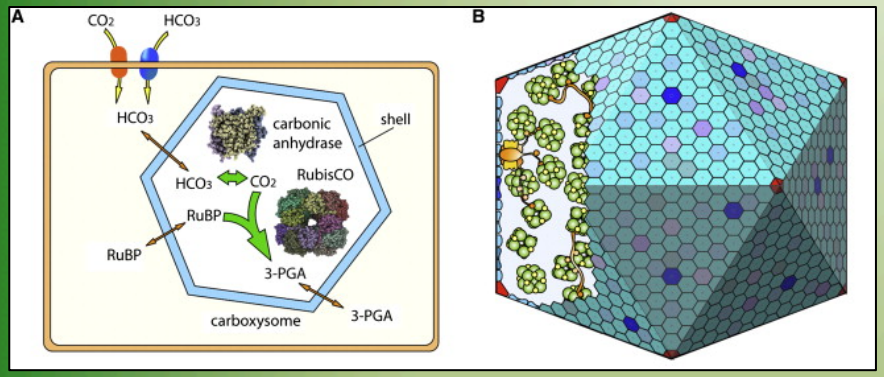
Metabolite microdomain

A region with locally elevated concentrations of metabolites that are persistent at a steady state

Lee (2012) Metabolic Engineering

Synthetic metabolons: metabolite microdomains

- Microcompartment?



Samborsaka (2012) *Cell.*

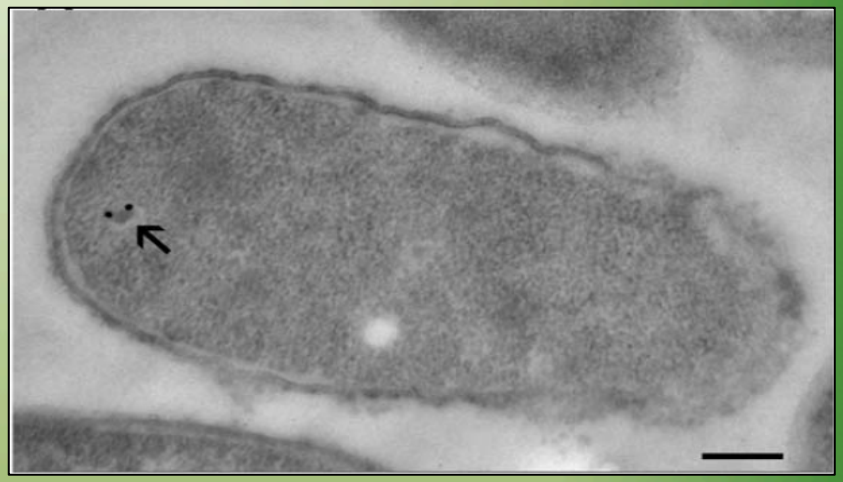


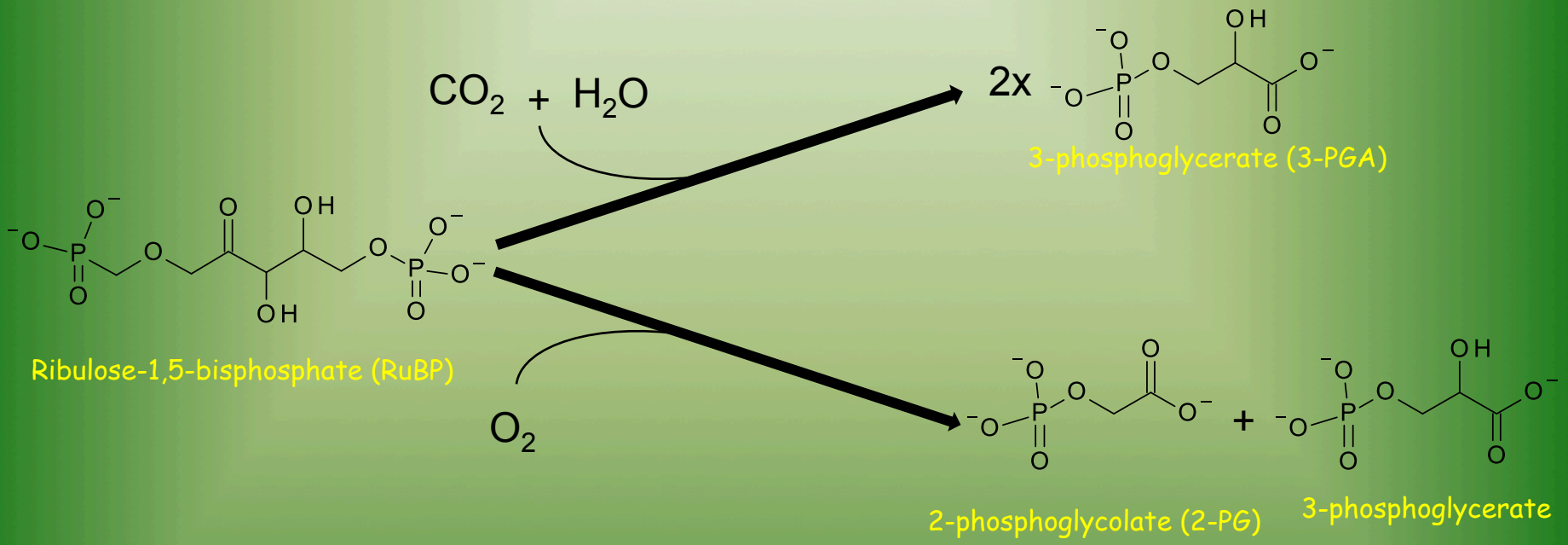
Figure 6. EutC$^{1-19}$-EGFP is sequestered in the recombinant EutSMNLK compartment. (**A**) Anti-GFP immunogold TEM of a thin section of *E. coli* JM109 cells co-expressing EutSMNLK and EutC$^{1-19}$-EGFP. Gold particles are localized to a protein shell. (Scale bar: 200 nm).

Choudray (2012) *PLOS One*

Bacterial microcompartment (BMC)

Proteinaceous shells encapsulating functionally related enzymes

Increasing the carboxylase activity of Rubisco via a synthetic protein scaffold

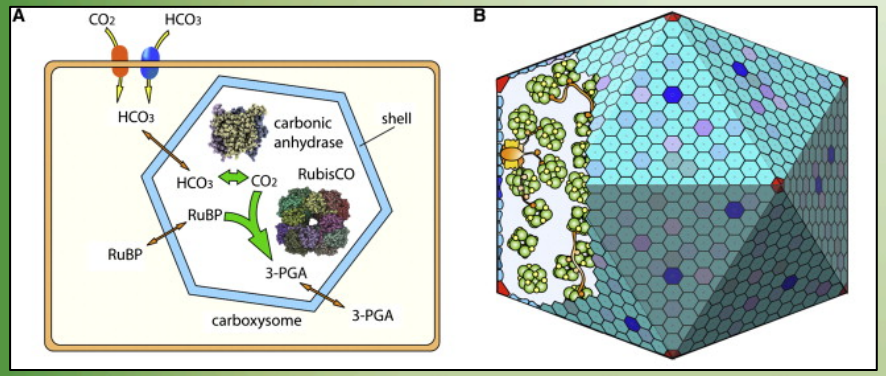


To increase carboxylation efficiency:

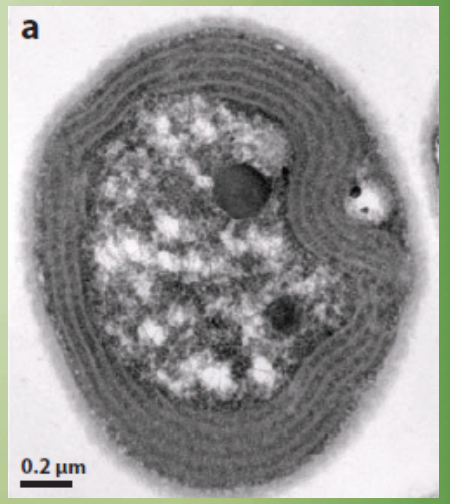
- Increase the ratio of CO_2:O_2 that reaches the active site
- **Rubisco** will be tethered to **Carbonic anhydrase (CA)** using a synthetic **"Scaffold"** polypeptide
- The system will be tested *in vitro* by over-expression and purification of the three proteins in *E. coli*

- The cyanobacterium *Synechocystis sp.* PCC 6830 GT-S will be used as a model organism to test the system *in vivo*

Synechocystis sp. PCC 6830 GT-S

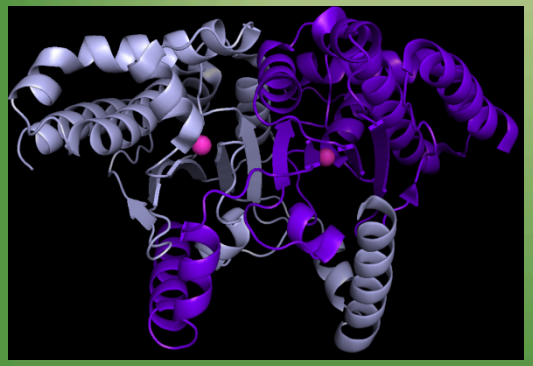


The cyanobacterial *CCM*[1]



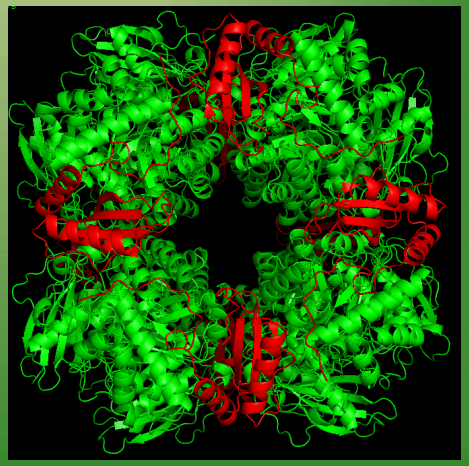
Micorgraph showing the carboxysome[2]

Carboxysome possesses:



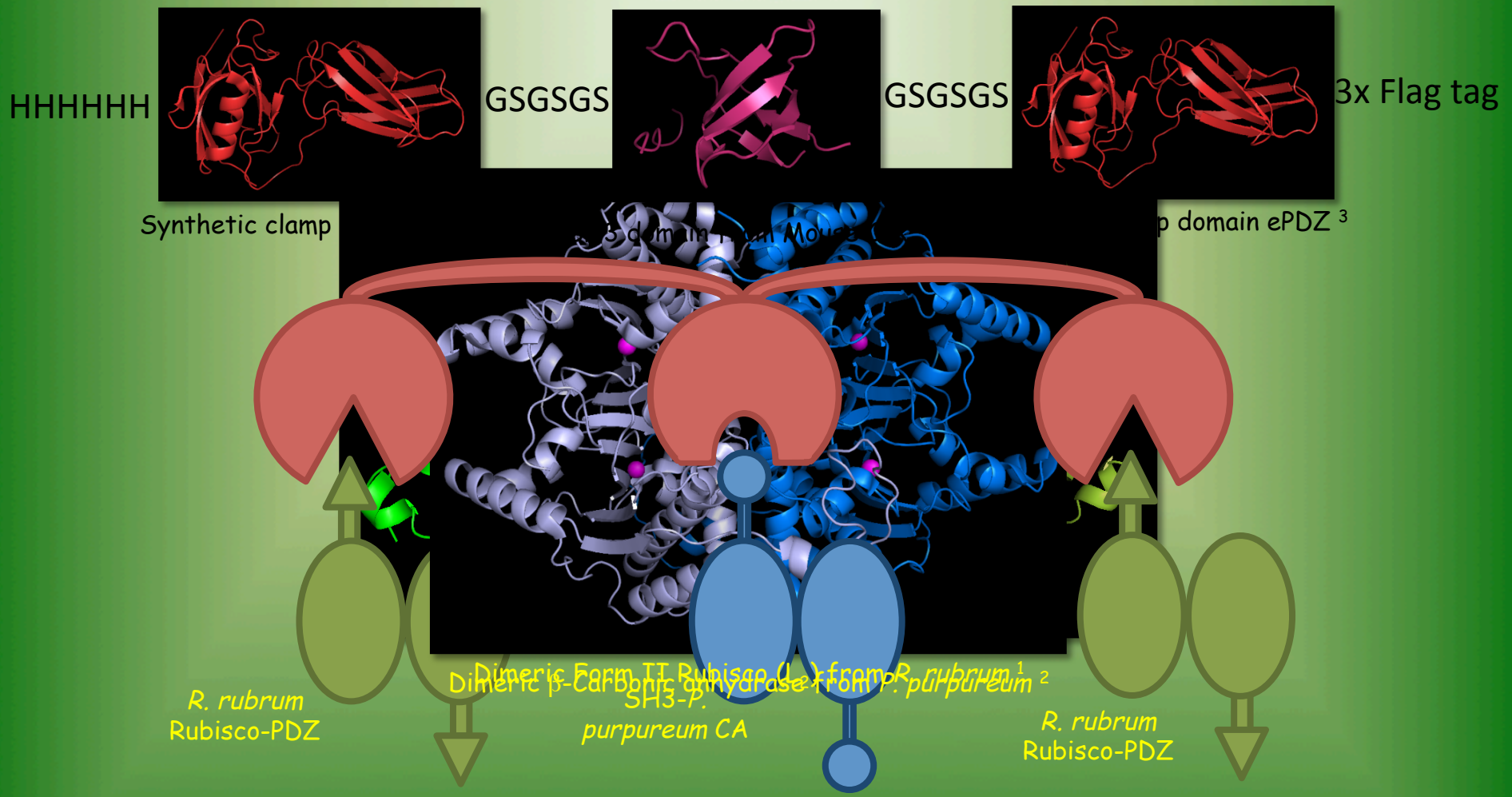
Dimeric β-Carbonic anhydrase from *E. coli* [3]

Form I Rubisco (L_8S_8) from *Synechococcus* PCC6301 [4]



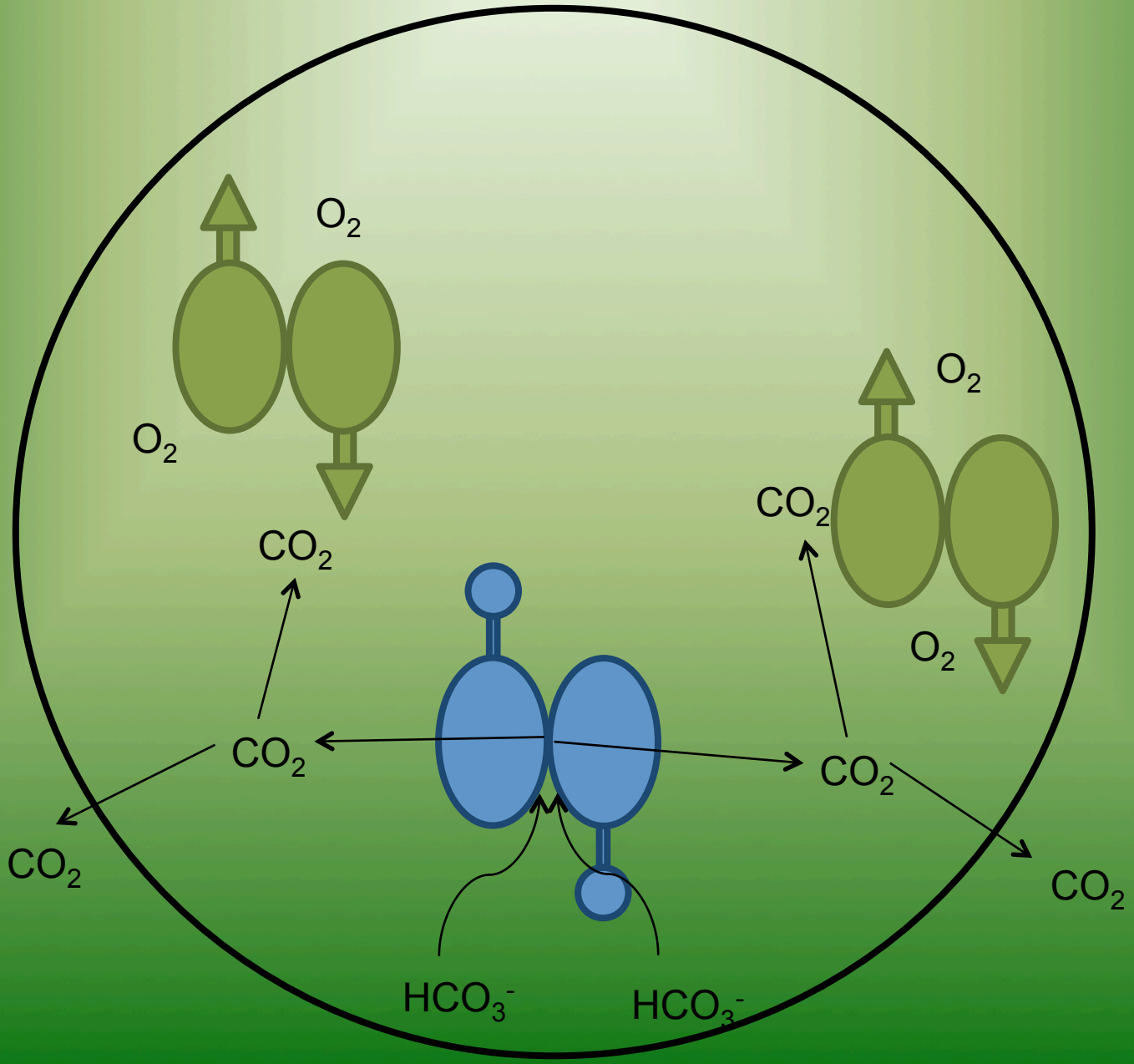
1. Samborsaka, B. & Kimber, M. (2012) *Cell.* **20**,1353-1562 778
2. Kerfield et al. (2010)
3. Cronk, J.D. et. al. (2001) *Protein Science* 10, 911-922
4. Newman, J. et. al. (1993) *Acta Crystallogr.*,Sect. D. **49**, 548-560

The Scaffold complex

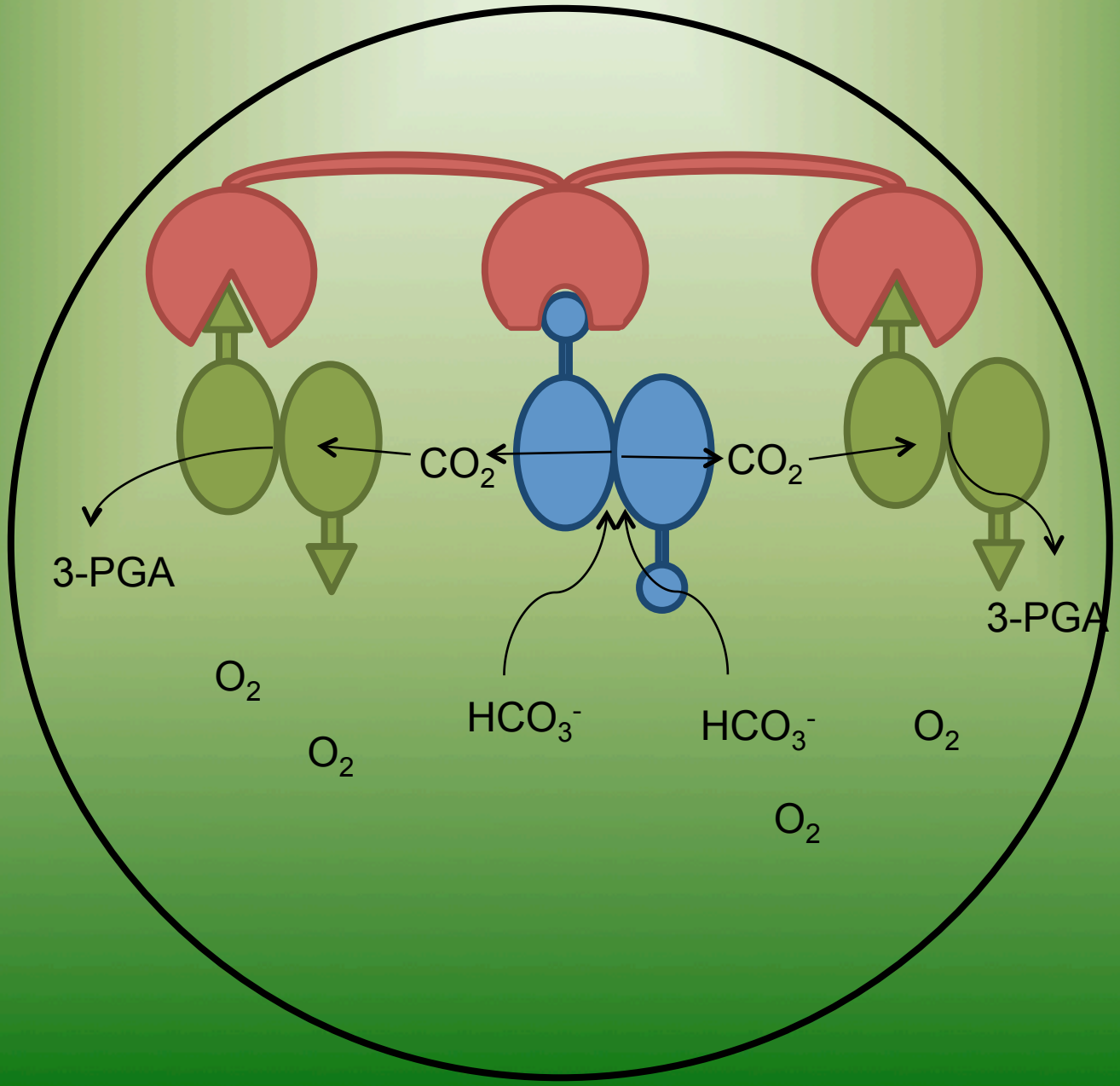


1. Lundqvist T. & Schneider, G. (1997) *J. Biol. Chem.*, **266**, 12604-12611
2. Mitsuhashi, S. et. al. (2000) *J. Biol. Chem.* 276, 5521-5526
3. Huang, J. H. et. al. (2009) *J. Mol. Biol.* **392**, 1221-1231
4. Wu, X. et. al. (1995) *Structure*, **3**, 215-226

Unscaffolded system



The Scaffold complex

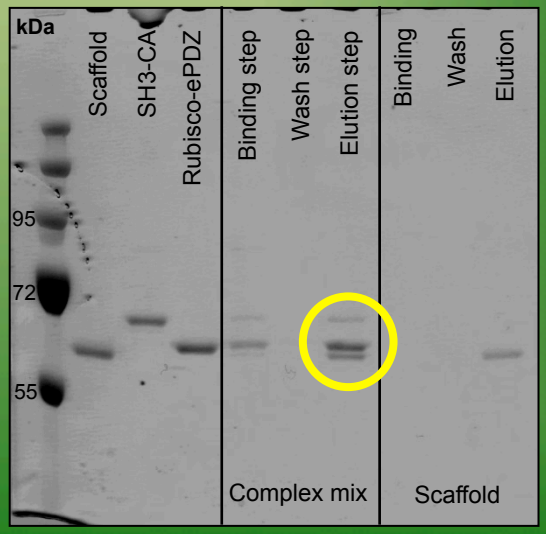


Scaffold *in vitro*

- Cloned genes into *E. coli* expression vectors
- Over-expressed *Rr* Rubisco-PDZ, SH3-*Pp CA* and Scaffold

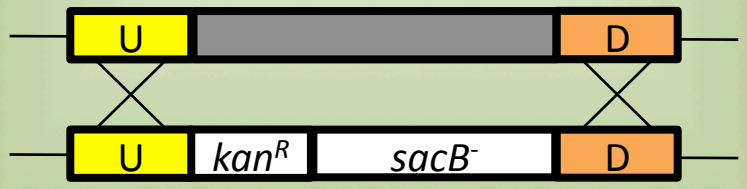
- Initial LC-MS analysis demonstrated that *Rr* Rubico-PDZ catalyses formation of 3-PGA and 2-PG from RuBP
- Electrometric assay with SH3-*Pp CA* has confirmed enzyme is active

- Verified complex formation
  - Scaffold binds to Anti-Flag® M2 Magnetic Beads (Sigma) using the Flag-tag at C-terminal
  - *Rr* Rubisco-PDZ and SH3-*Pp CA* bind to Scaffold *via* binding peptides

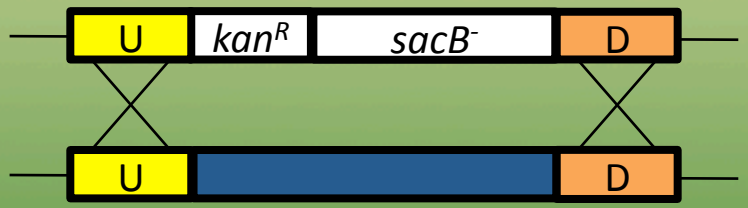


In vivo techniques

- Homologous recombination replaces target gene (*rbcL* or *ccaA*) with *kan*^{R}-*sacB*^{neg} selection cassette



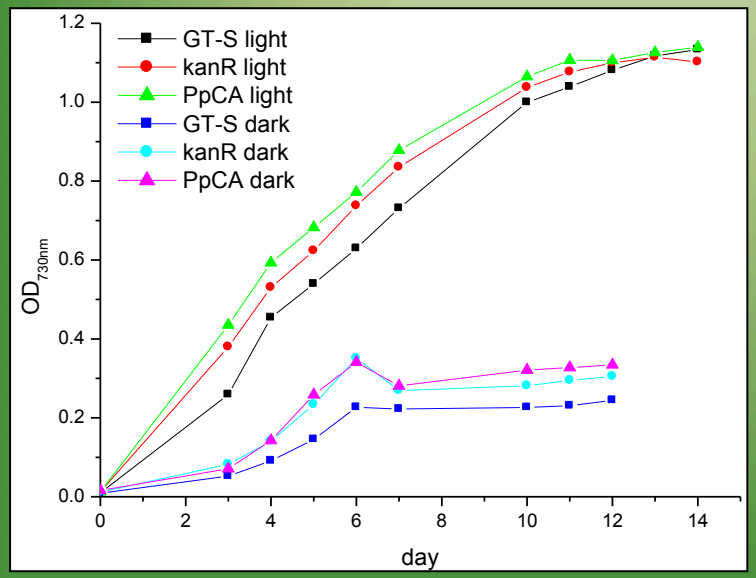
- Replace *kan*^{R}-*sacB*$^{-}$ selection cassette with either *Rr rbcL-PDZ* or *SH3-Pp ccaA*



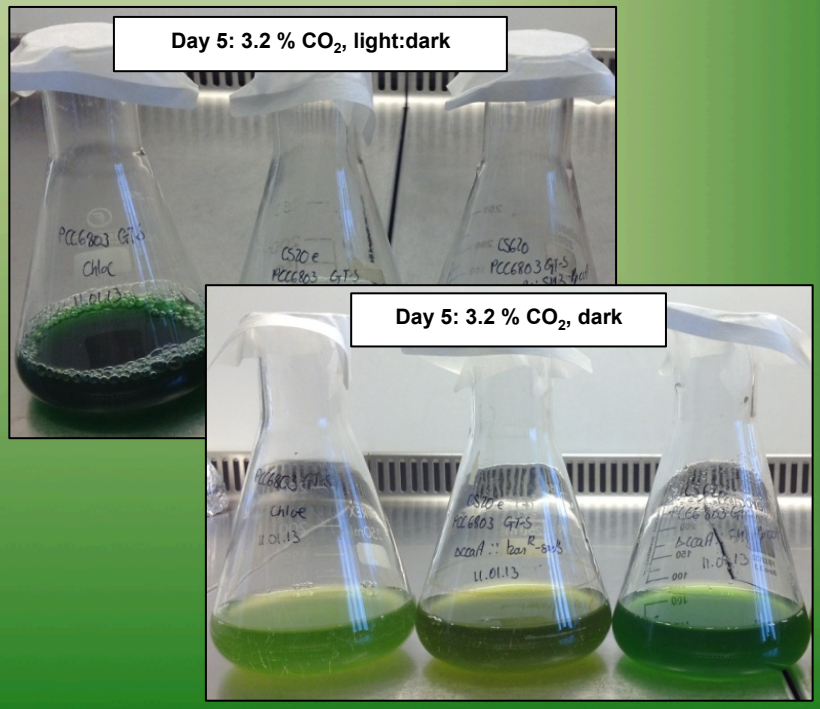
- Triparental mating introduces conjugal plasmid *pScaffold-kan*^{R}
  - Expression under control of the *rbcL* promoter

Carbonic anhydrase *in vivo*

- The *Synechocystis* CA knockout strain (*ΔccaA*::*kanR-sacB*$^-$) and our mutant *Synechocystis* SH3-*Pp* CA strain (*ΔccaA*::*SH3-Pp ccA*):
  - No significant difference in growth curve compared to WT (GT-S)
  - **Do not grow in air (data not shown)**

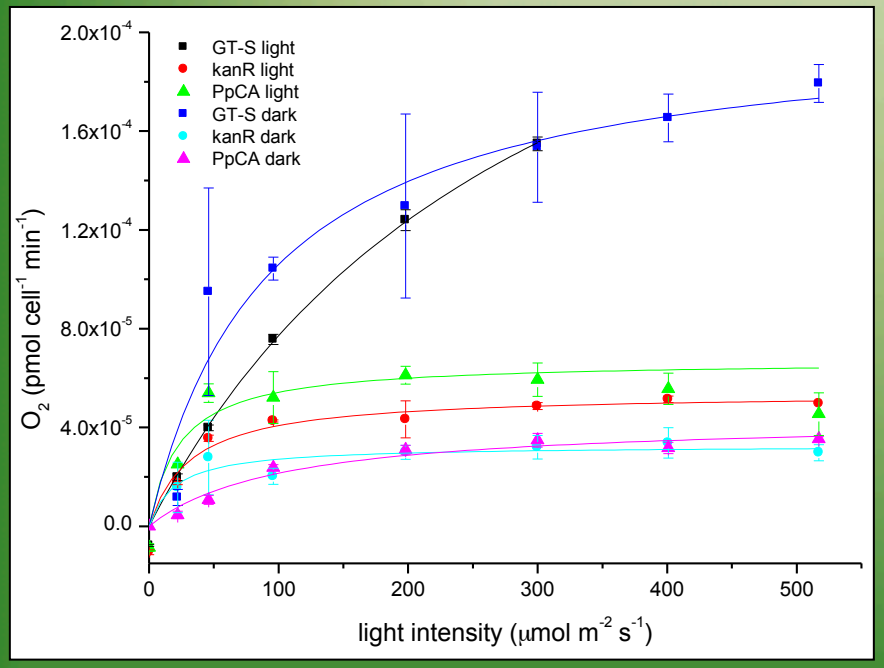


Growth conditions: 28 °C, 120 rpm, 3.2 % CO_2, 5 mM glucose ~30 μmol m^{-2} s^{-1} with either 18:6 h light:dark cycle or continuous dark



Carbonic anhydrase *in vivo*

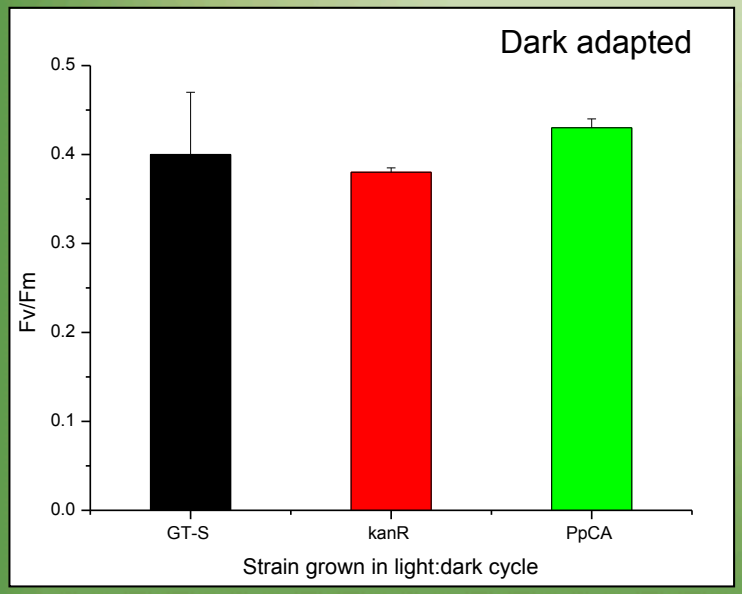
- Photosynthetic light response curves
  - O_2-electrode
- WT cultures show higher photosynthetic rates
- Both *ΔccaA* strains reach saturation at lower light intensities

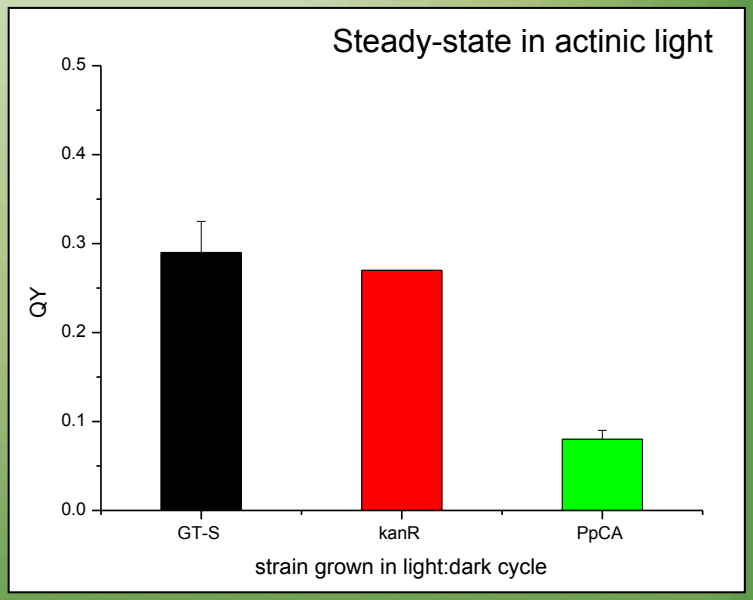


- Photosynthesis is impaired when carboxysome CA is knocked out

Carbonic anhydrase *in vivo*

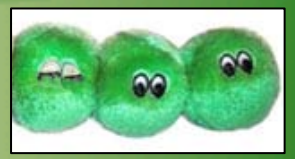
- QY- efficiency of PSII photochemistry
- Values drop significantly during actinic light stage for *ΔccaA::SH3-Pp ccA*





- Introduction of SH3-*Pp CA* into cytoplasm affects PSII photochemistry
  - A bicarbonate effect?

Rubisco &Scaffold *in vivo*



- Cannot completely knock-out native Rubisco
  - Even when *Rr*Rubisco-PDZ is simultaneously introduced with kan^R-$sacB^-$
- Therefore using a non essential gene, *Δslr0168* , to introduce *Rr rbcL*-PDZ
  - *rbc* will be replaced later
- Triparental mating has introduced Scaffold
  - Scaffold is expressed and has no significant effect on growth
  - Scaffold only detectable in high light

Next steps

- Assess the effect of scaffolding the Rubisco and *CA in vitro*
  - HCO_3^- conc., O_2 conc., presence/absence complex

- Will addition of Rubisco or Rubisco/Scaffold into cytoplasm rescue *ΔccaA* phenotype?
  - Growth in atmospheric CO_2, improved PSII chemistry?

Modelling

- We need to address the potential problem of CO_2 leakage from expression of SH3-*Pp CA* in the cytoplasm
  - Is the proximity of *CA* to Rubisco close enough to prevent CO_2 diffusion? (optimum distance between enzymes)
  - Is there a minimum size of complex required to prevent diffusion?
  - Is a protein coat (carboxysome) required?

Thank you

Nick Smirnoff

Nic Harmer

Steve Porter

Ron Yang

Mezzanine Floor lab group

Jay Moore (Warwick) and Plant Responses to Environmental Stress in Arabidopsis (PRESTA) Consortium (Warwick, Essex and Exeter)

UNIVERSITY OF EXETER

BBSRC bioscience for the future