Improving Microalgal Oil Production Based on Quantitative, Biochemical and Genetic Analyses of Metabolism

May 2013
DOE/EERE OBP Algae Program

C. Xu , J. Schwender, J. Shanklin, (PI's)
Brookhaven National Laboratory

Goal Statement

- Maximizing production of oil (triacylglycerols) in the green alga *Chlamydomonas reinhardtii*
- Main obstacle to be addressed: High lipid accumulation in algal cells can usually be triggered by nutrient deficiency. In turn, lower rates of cell growth do limit the overall oil productivity
- To identify the best target(s) to improve oil productivity, we use quantitative analysis of metabolism in combination with biochemical and genetic tools

Quad Chart Overview

Timeline

- 9/30/2010
- 9/30/2012
- 6/30/2013
- 95% completeness

Barriers

- Barriers addressed

– Ft-C. Feedstock Genetics and Development:
Improvement of algae used for biofuel production by genetic engineering.

Budget

- Total project funding
  - DOE share: $900,000
- Funding received in FY09: none
- Funding for FY10: $354,638
  Funding for FY11: $600,000
- Funding for FY2013: none
- ARRA Funding: none

Partners

- Co-PI's are Changcheng Xu, Jörg Schwender and John Shanklin, at Brookhaven National Laboratory.
- Project management: John Shanklin.

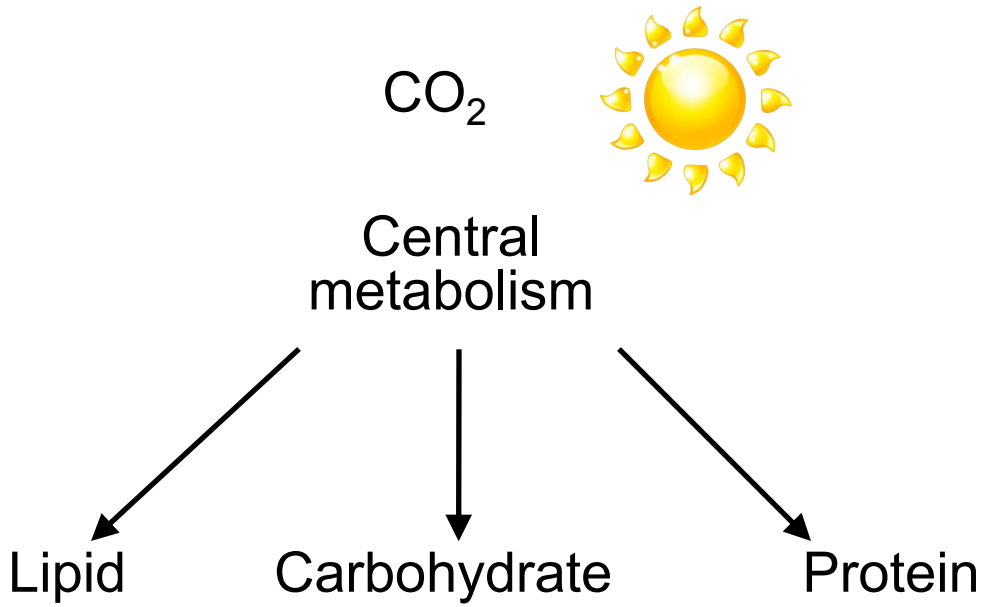
Project Overview

- Comparison of lipid- and non-lipid accumulating cultures by quantitative analysis of metabolism
- Defining the biochemical pathway and limiting factors in oil accumulation in *Chlamydomonas*.
- Genetic engineering to improve oil production

1 - Approach

To address carbon allocation and partitioning in green algae we use:

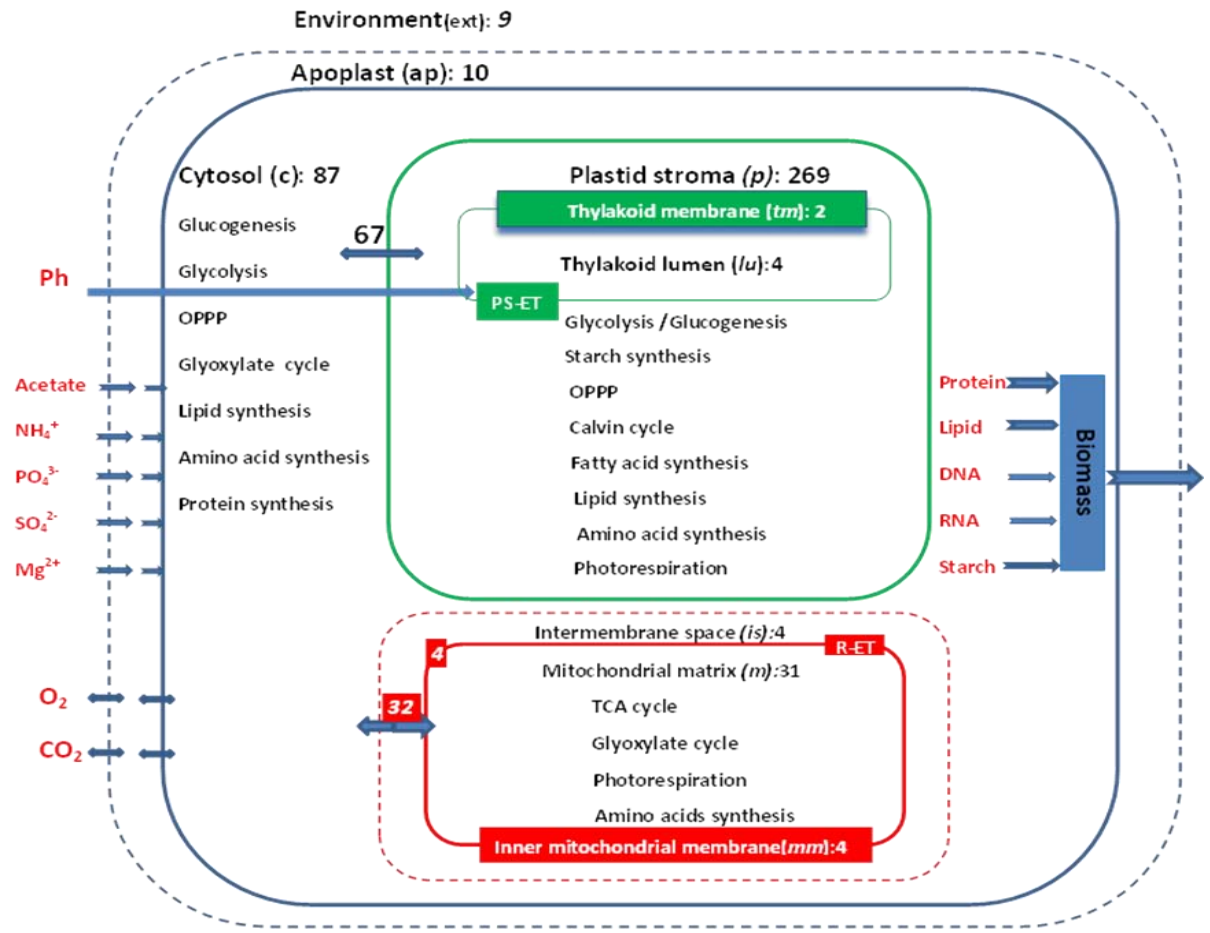
- ***Biochemical analysis***
- ***Modeling of metabolism***
- ***Metabolic engineering (genetic transformation)***



2 - Technical Accomplishments/ Progress/Results

- Built a *Chlamydomonas* Metabolic Model
- Carried out Flux Balance Analysis and Metabolic Flux Analysis
- Discovered a chloroplast pathway for oil biosynthesis in *Chlamydomonas*
- Identified carbon supply as the key limiting factor in oil accumulation in *Chlamydomonas*
- Obtained genetically engineered strains that accumulate oil under normal growth conditions
- Identified a putative stress sensor involved in oil accumulation in *Chlamydomonas*

Chlamydomonas Metabolic Model



We built a compartmentalized reaction network with 509 reactions (*cre509*) based on:

- *Chlamydomonas* genome (JGI), transcript, proteome data
- Comparison to various published *Chlamydomonas* genome scale models

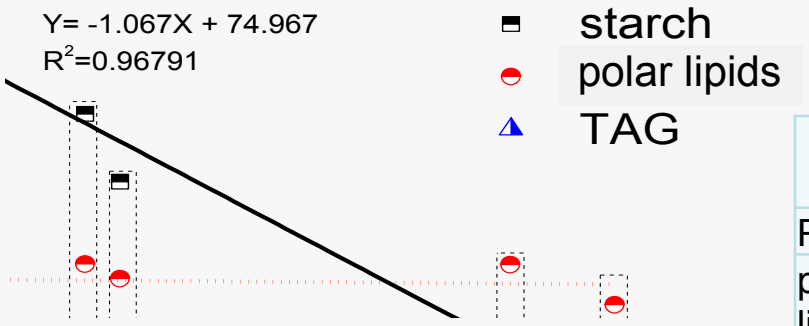
- Model for wt *CW15* = *cre509*

- Starchless mutant BAFJ5 = *cre509-S*$^-$

- While *cre509* is used for **Flux Balance Analysis (FBA)**, a derived reaction network (*cre77* reactions) is used for ^{13}C-Metabolic Flux Analysis (**^{13}C-MFA**).

Correlation of Biomass Components

Wild type *CW15*



	Protein	polar lipid	TAG	Starch
Protein	1.00	0.64	-0.90	-0.99
polar lipid	0.64	1.00	-0.90	-0.70
TAG	**-0.90**	**-0.90**	1.00	0.94
Starch	**-0.99**	-0.70	**0.94**	1.00

(Pearson correlation coefficient)

BAFJ5 starchless mutant:

	Protein	polar lipid	TAG
Protein	1.00	-0.49	-0.98
polar lipid	-0.49	1.00	0.35
TAG	**-0.98**	0.35	1.00

(Pearson correlation coefficient)

- *wt*: TAG and starch increase at the expense of protein
- *BAFJ5*: TAG increases at the expense of protein
- Polar lipids are rel. unchanged; i.e. no good correlations

Correlation Fluxes to Biomass Components

- Example: BAFJ5 vs. TAG or protein; Top 20 highest correlation coefficients.

Reactions	TAG BAFJ5
Transketolase (TK1_p,#116; TK1_c,#321)	0.972
Fructose-bisphosphate aldolase (sum of # 124,100)	-0.953
Glyceraldehyde-3-phosphate dehydrogenase (sum of # 330,122)	-0.952
phosphoglycerate kinase (PGK_p,#107)	0.952
Ribulose- bisphosphate carboxylase oxygenase (RuBisCO_p,#121)	-0.936
Phosphoribulokinase(#108)	-0.936
transaldolase (TA_p,#117;TA_c,#320	0.910
acetate kinase(ACK_p, # 492)	0.905
phosphate acetyltransferase(PAT_p,#494)	0.905
transketolase(TK2_p,#118;TK2_c,#323)	0.893
fructose-bisphosphatase(FBP_c,#90;FBP_p,#126)	-0.889
Ferredoxin-NADP+ reductase (#86)	-0.886
glutamine synthase(GS_p,#504)	-0.869
malate dehydrogenase (NADP+)(MDH_p,#338)	0.836
Photosystem I(#284), photosystem II(#87)	-0.817
H+-transporting two sector ATPase (#142)	-0.817
succinate dehydrogenase (Sdh_m,#84)	0.801
fumarate hydratase(FH_m,#85)	0.801
Ac_ap::H_c_sym, #4	0.797
phosphopyruvate hydratase(ENO_c,#325)	-0.786
acetate kinase(ACK_m, # 493)	0.785

Reactions	protein BAFJ5
Fructose-bisphosphate aldolase (sum of # 124,100)	0.990
phosphoglycerate kinase (PGK_p,#107)	-0.985
Glyceraldehyde-3-phosphate dehydrogenase (sum of # 330,122)	0.985
transketolase(TK1_p,#116; TK1_c,#321)	-0.985
Ribulose- bisphosphate carboxylase oxygenase (RuBisCO_p,#121)	0.976
Phosphoribulokinase(#108)	0.976
transaldolase (TA_p,#117;TA_c,#320	-0.962
transketolase(TK2_p,#118;TK2_c,#323)	-0.955
fructose-bisphosphatase(FBP_c,#90;FBP_p,#126)	0.954
acetate kinase(ACK_p, # 492)	-0.949
phosphate acetyltransferase(PAT_p,#494)	-0.949
Ferredoxin-NADP+ reductase (#86)	0.903
glutamine synthase(GS_p,#504)	0.892
phosphopyruvate hydratase(ENO_c,#325)	0.876
Ac_ap::H_c_sym, #4	-0.871
succinate dehydrogenase (Sdh_m,#84)	-0.862
fumarate hydratase(FH_m,#85)	-0.862
acetate kinase(ACK_m, # 493)	-0.849
phosphate acetyltransferase(PAT_m,#495)	-0.849
citrate synthase (CS_m,#105)	-0.849
citrate synthase (sum of #105,361)	-0.849

Correlation Fluxes to Biomass Components

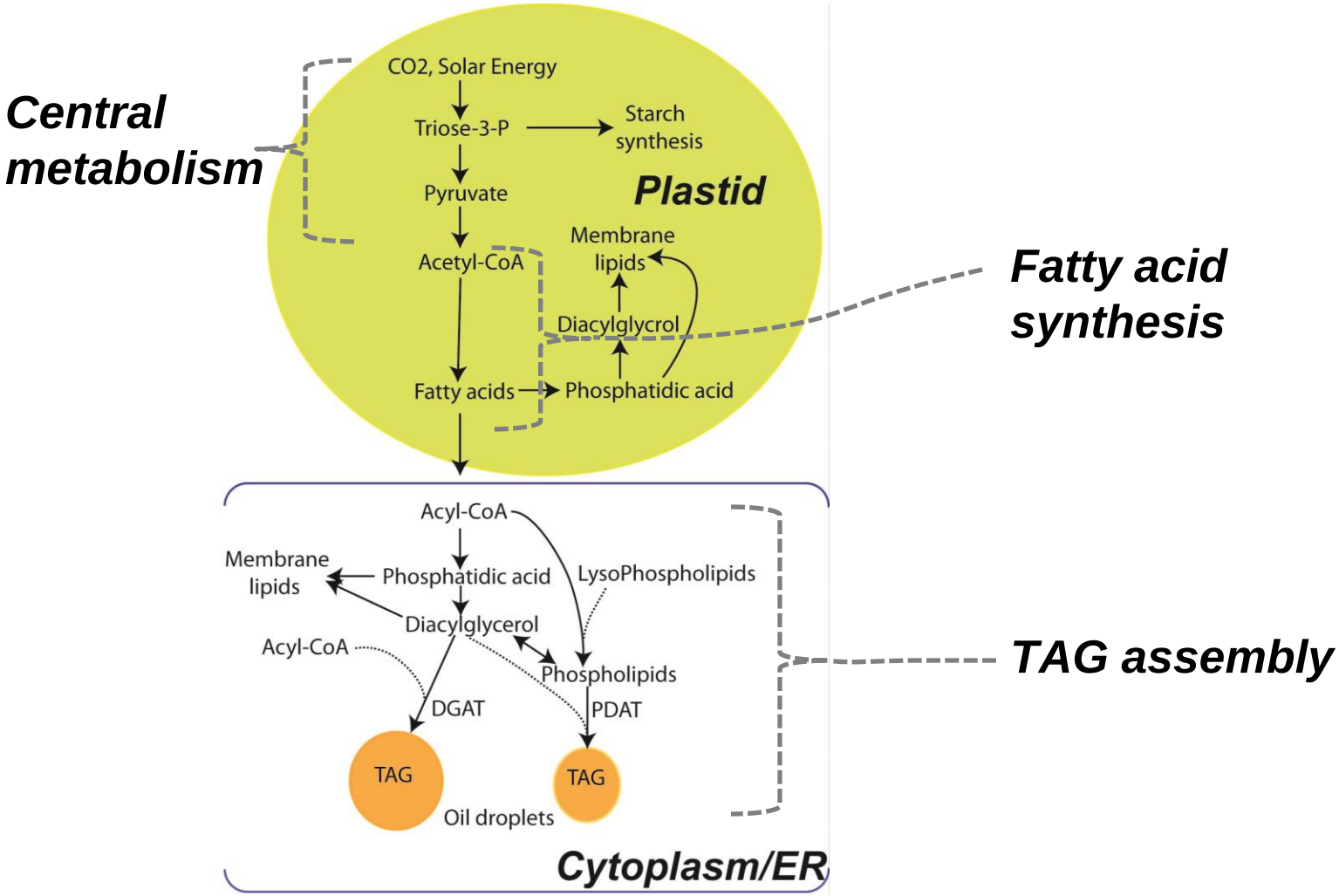
How to identify potential targets?

- Find reactions which highly correlate to both TAG and another major biomass compound, yet in an opposing manner
- Manipulation of such a reaction could lead to a tradeoff between biomass compounds.

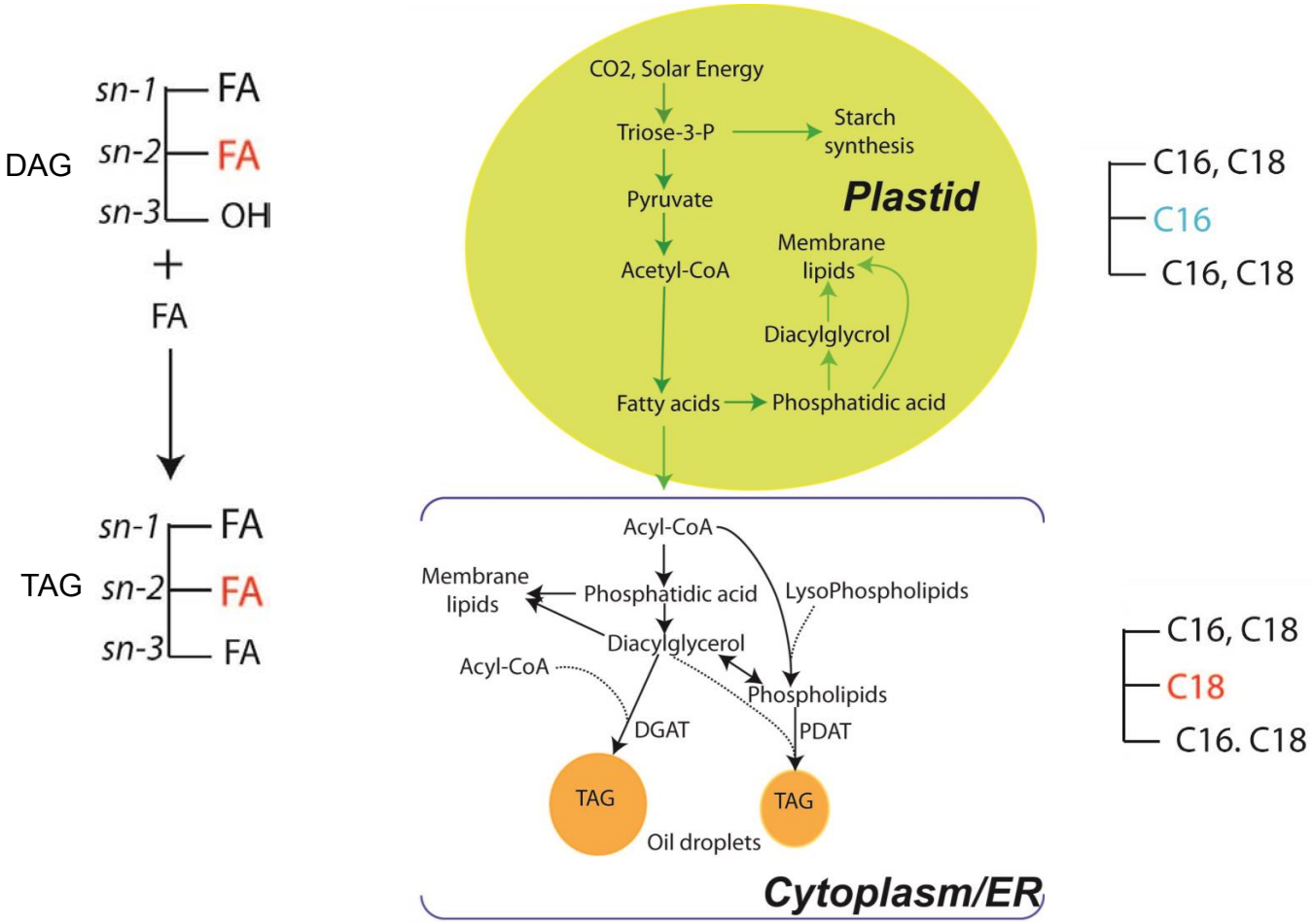
- Example: **Transketolase (EC 2.2.1.1):**

1 E4P + 1 X5P <> 1 F6P + 1 G3P

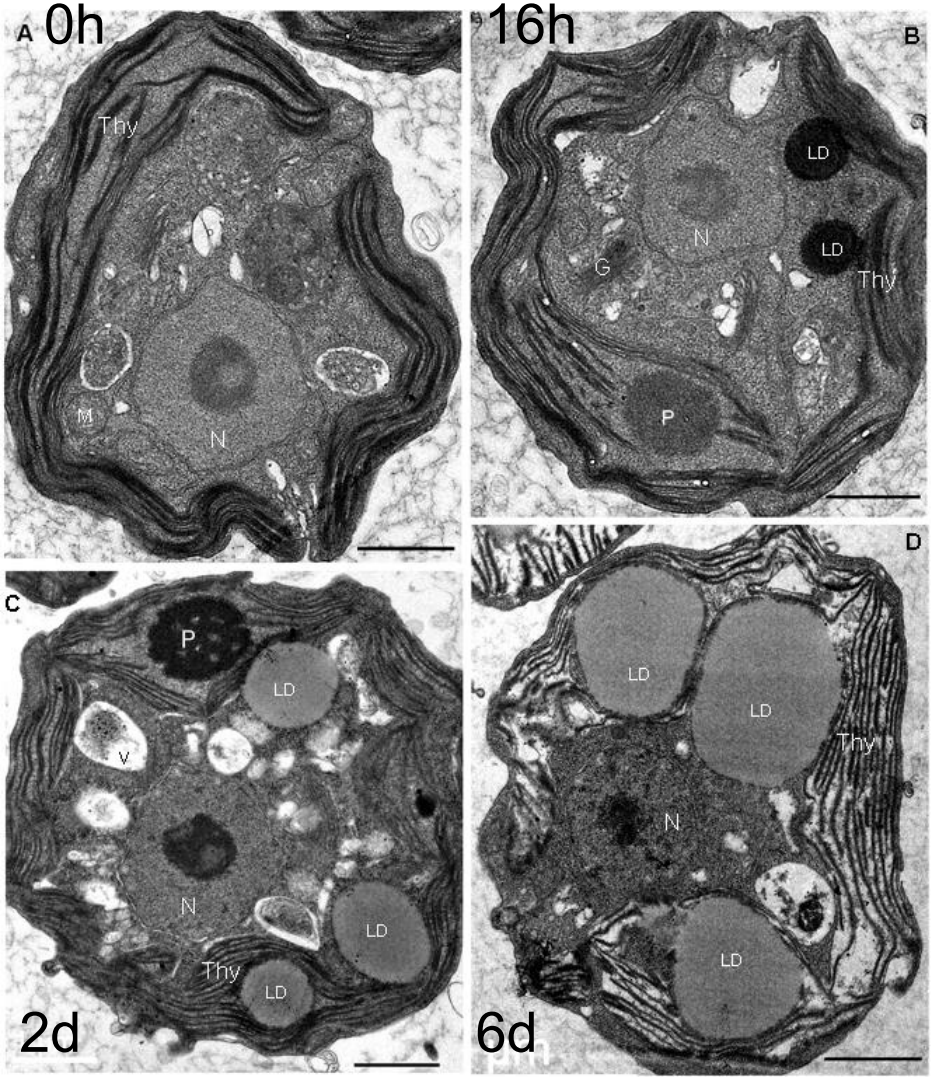
Triacylglycerol (TAG) synthesis in *Chlamydomonas*

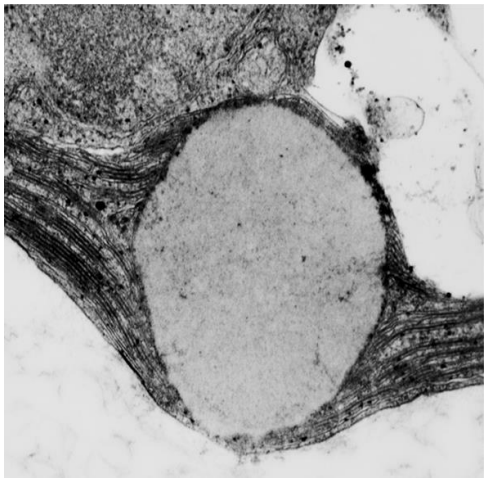


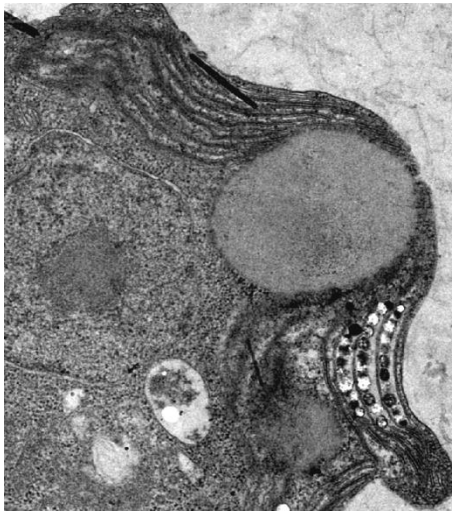
The DAG for TAG assembly has a chloroplast origin



Oil droplets are present in both the chloroplast and the cytosol in starchless mutants



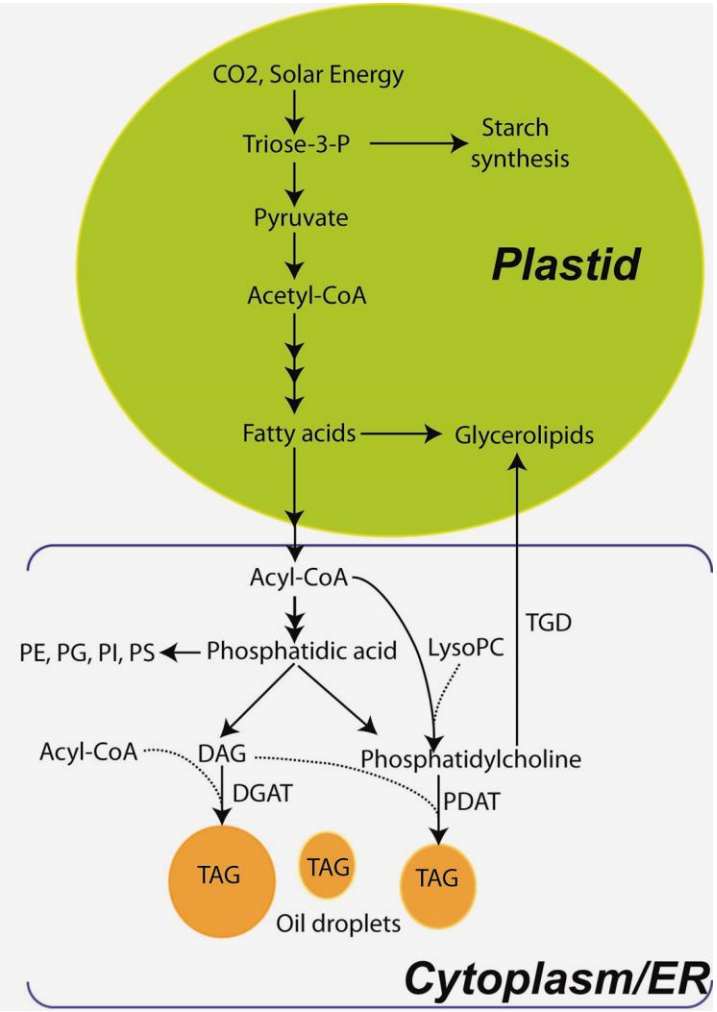




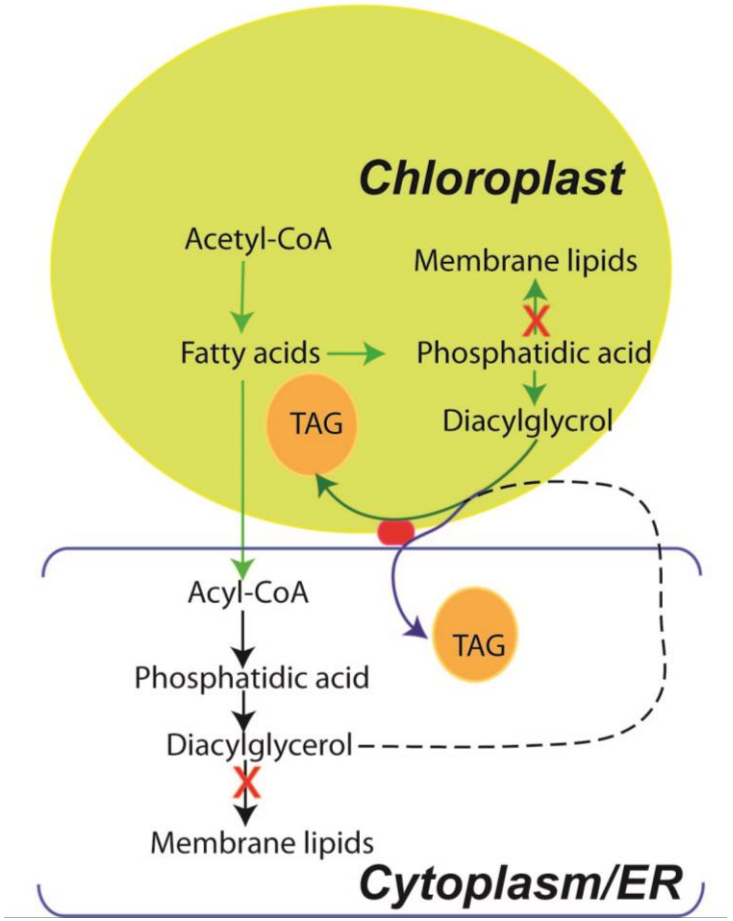
A chloroplast pathway for oil biosynthesis in *Chlamydomonas*:

A new paradigm for understanding oil synthesis in microalgae

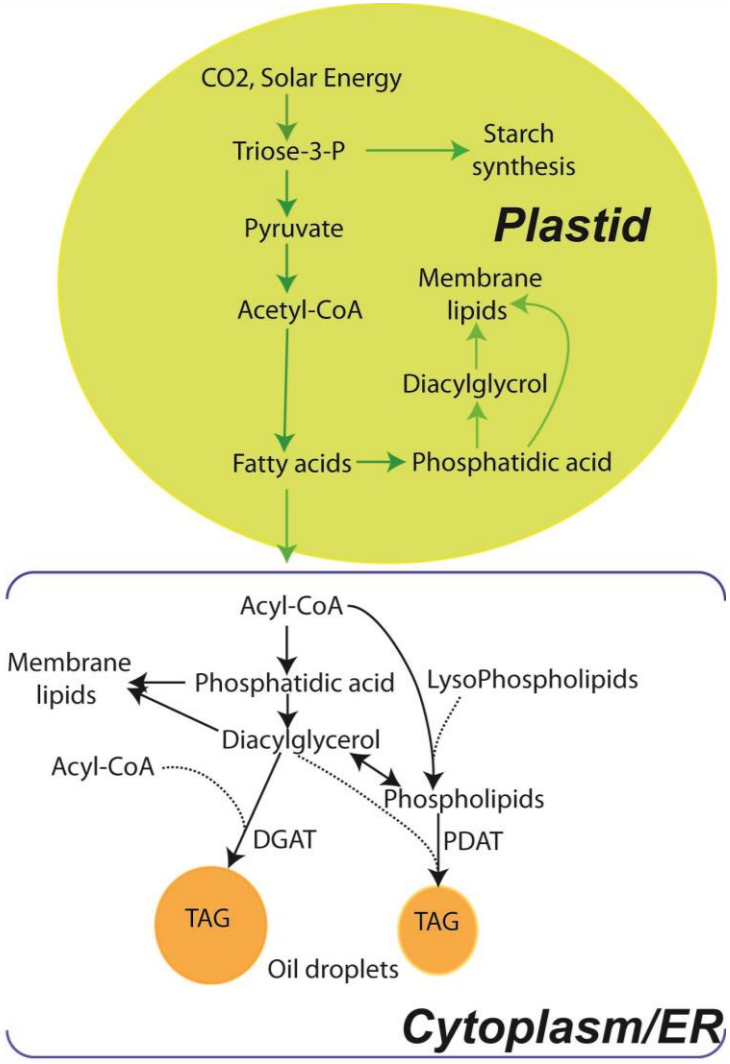
Oilseeds of plants



Chlamydomonas



Strategies for analysis of the limiting step in oil biosynthetic pathway in microalgae

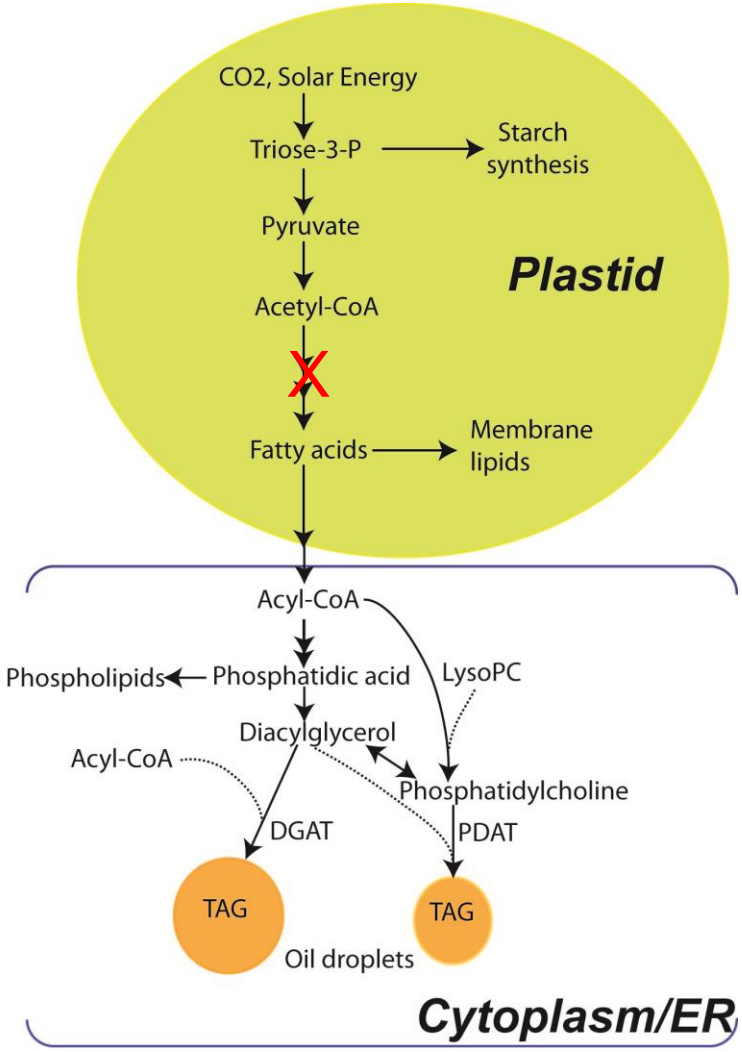


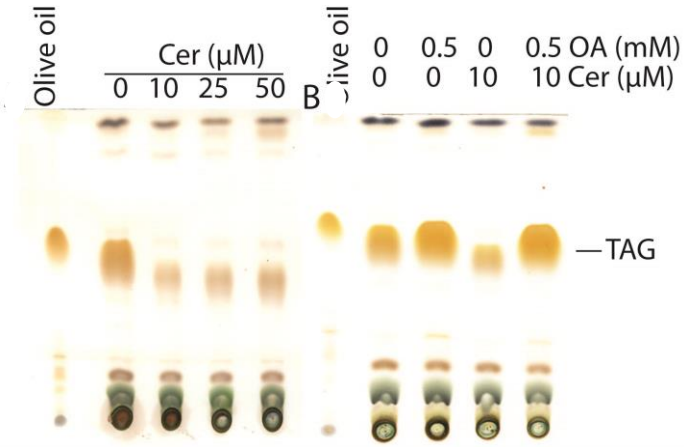
Block A
(FA synthesis)

V

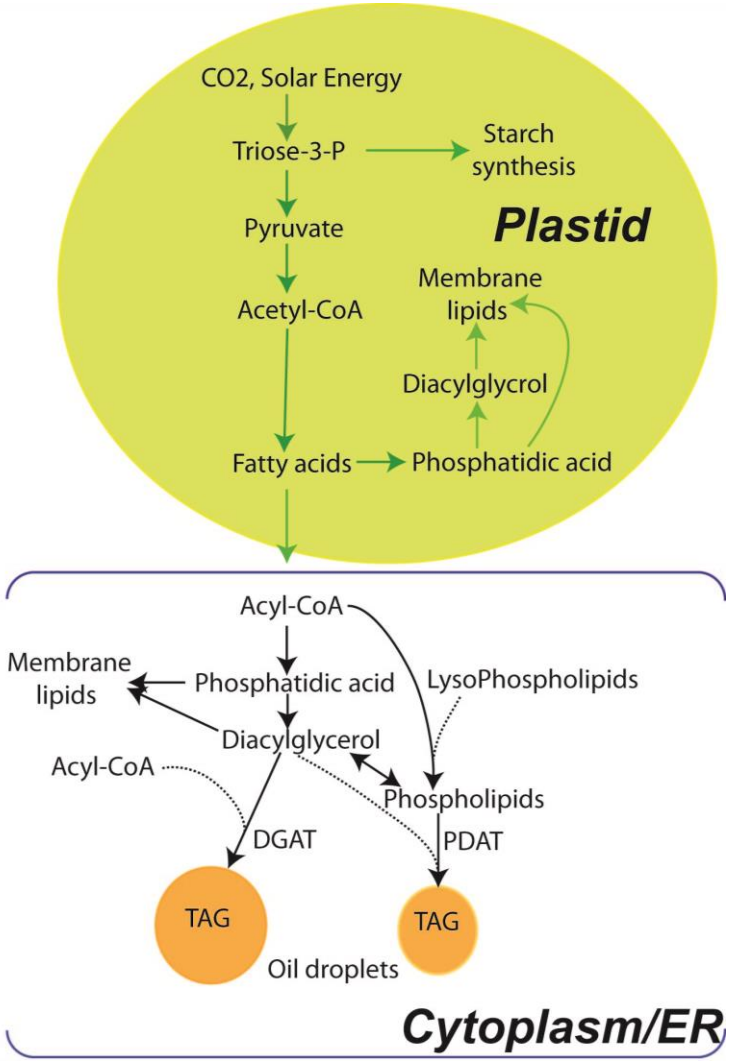
Block B
(FA utilization)

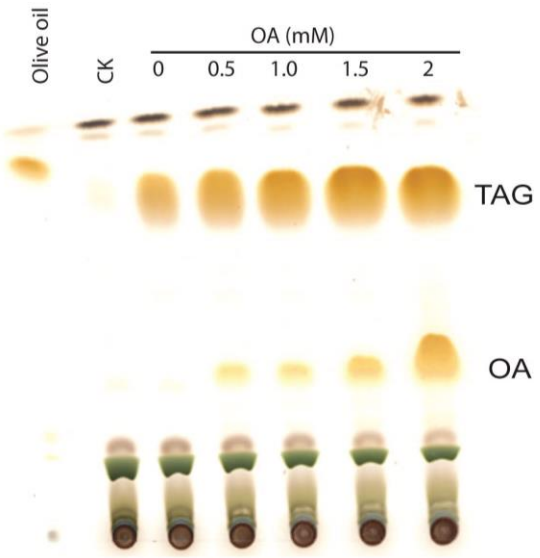
Oil accumulation in *Chlamydomonas* is dependent on *de novo* fatty acid synthesis in the chloroplast



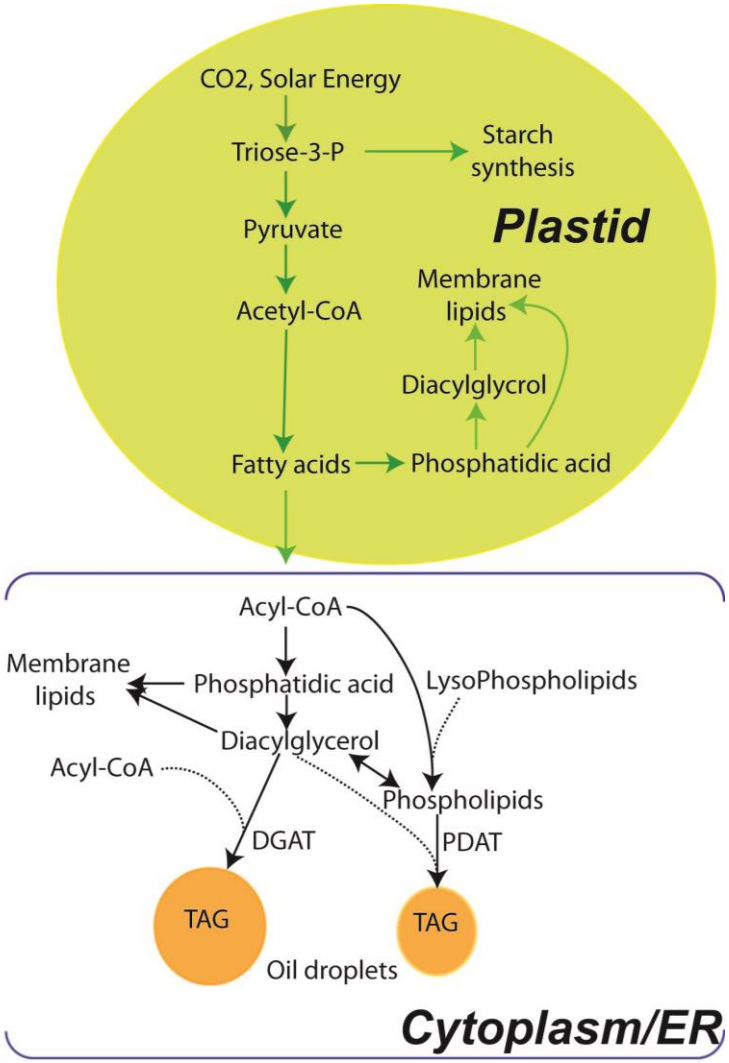


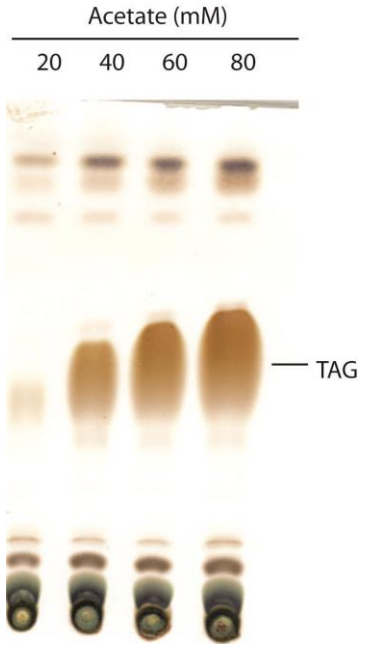
Oil accumulation is limited by fatty acid synthesis

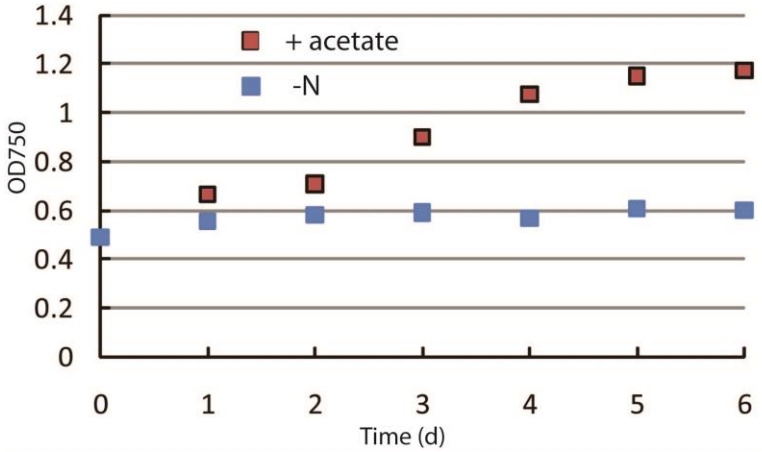




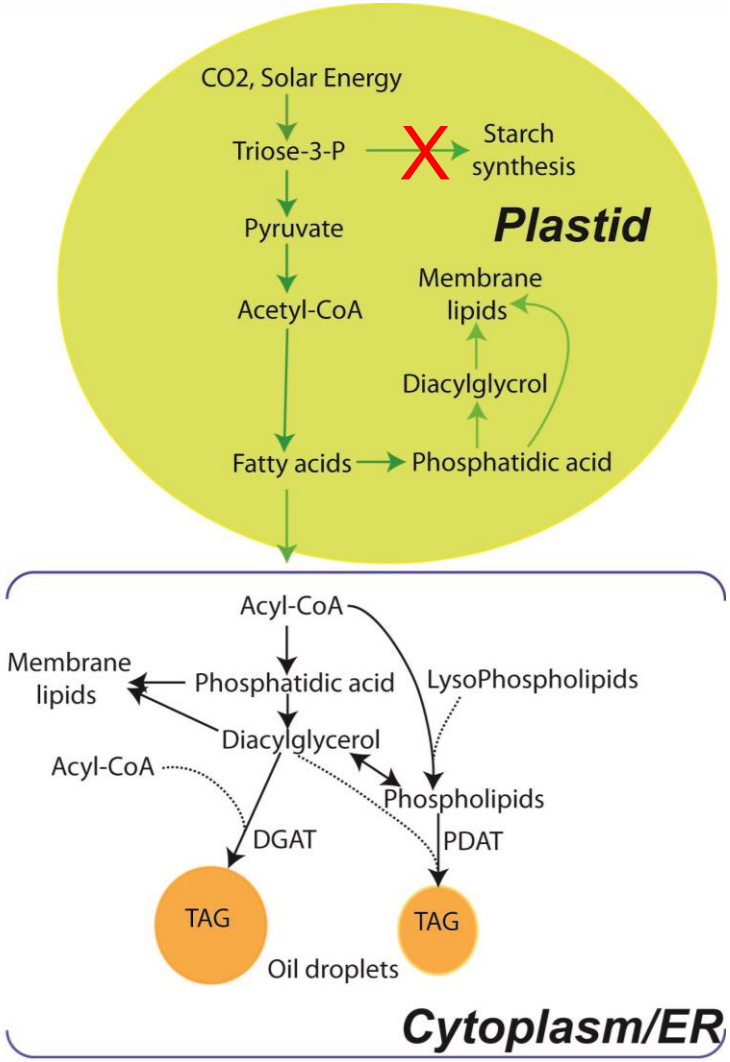
Fatty acid synthesis is limited by carbon precursor supply

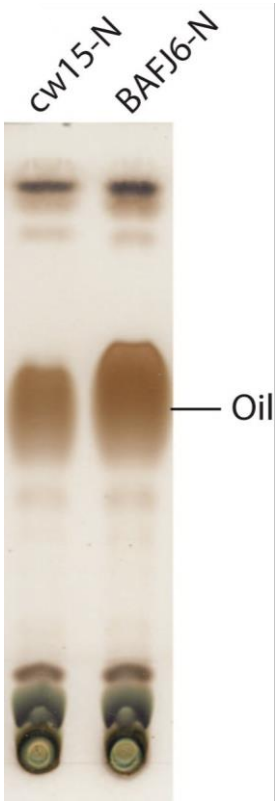




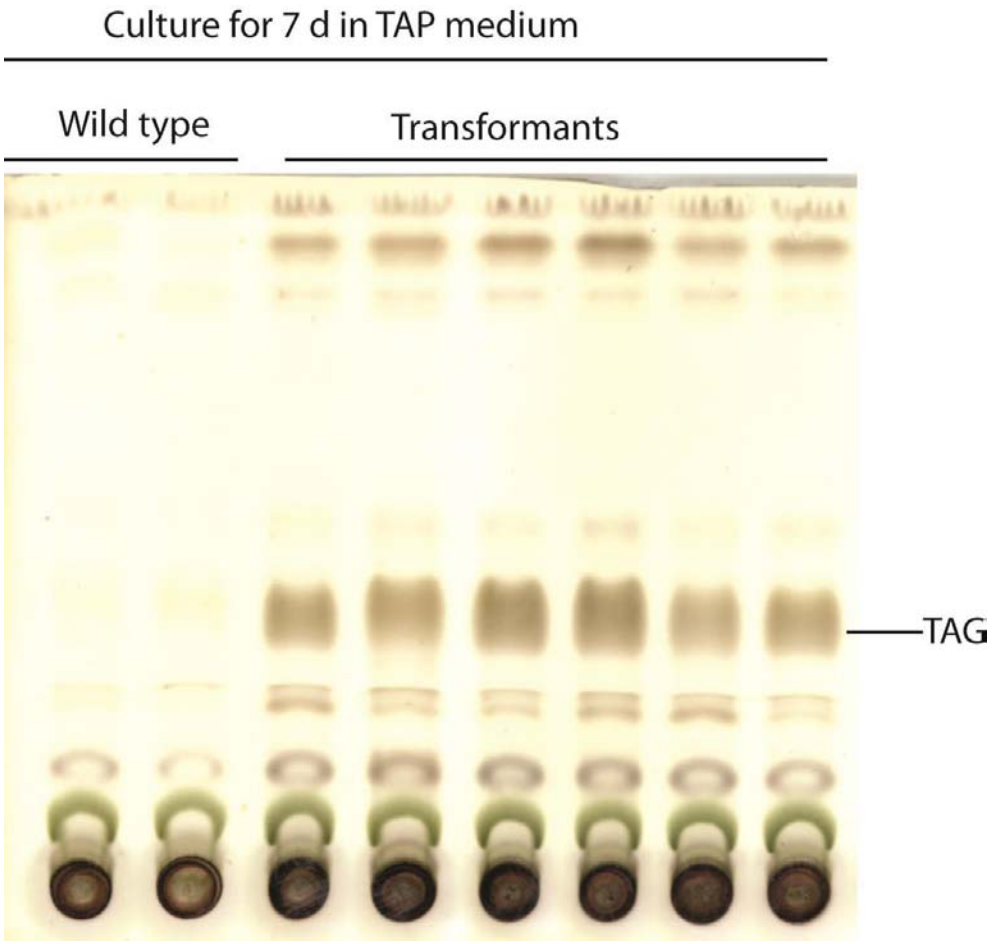


Inactivation of starch biosynthesis increases oil accumulation in *Chlamydomonas*





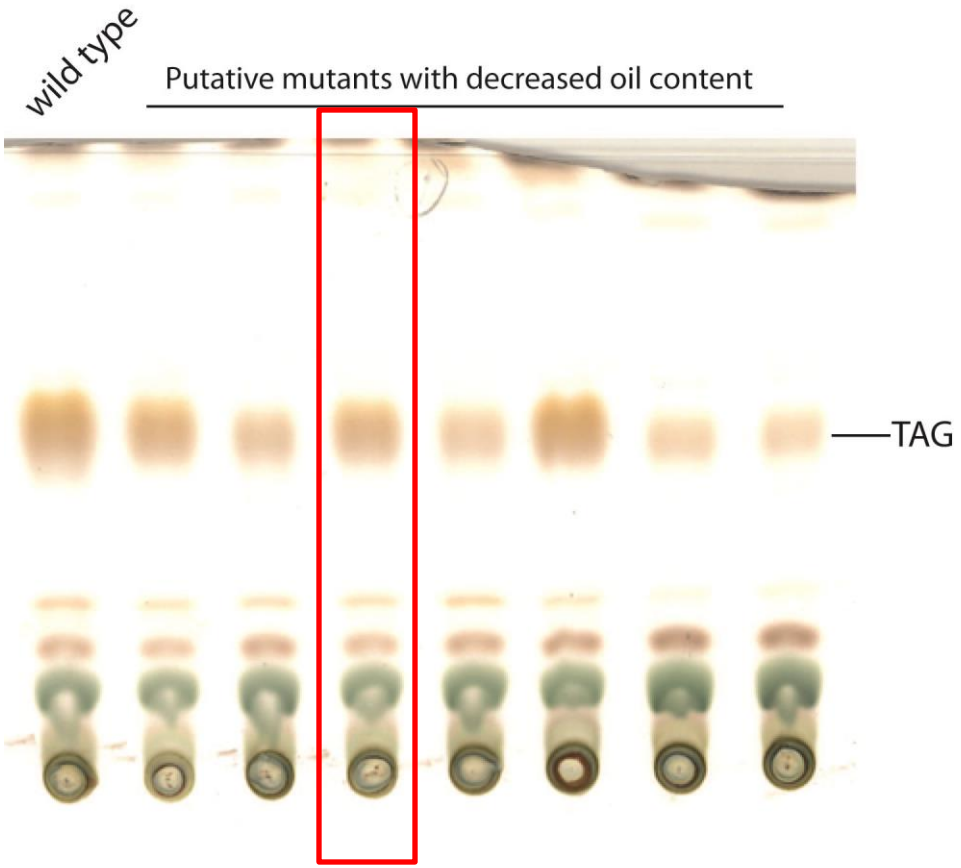
Ectopic expression of oleosin promotes oil accumulation under normal growth conditions



A forward genetic approach for dissecting lipid metabolism in *Chlamydomonas*

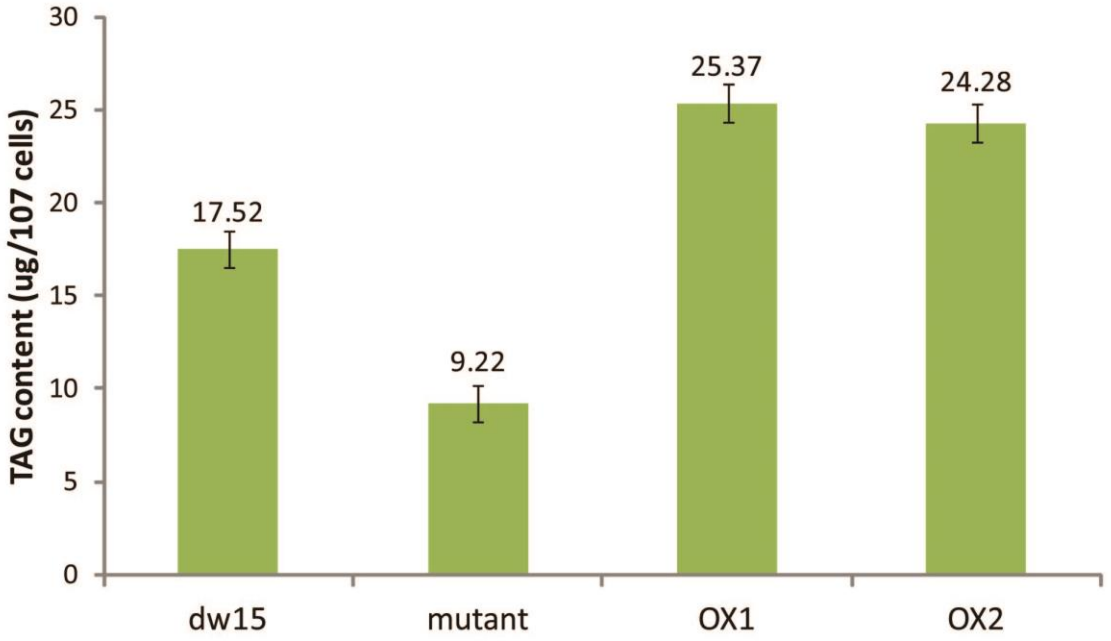
- Transformation of Chlamydomonas cells with an exogenous DNA marker to disrupt and tag any non-essential nuclear genes
- Selection of transformation
- Screening for lipid phenotypes
- Identification of the tagged genomic region by PCR-based approaches
- Complementation of the mutant phenotype with the wild type gene

Putative mutants of *Chlamydomonas* with decreased oil content



Identification of a putative stress sensor involved in the control of oil production in *Chlamydomonas*

- P2-48 encodes a putative nitrogen sensor conserved in a variety of organisms
- Overexpression of P2-48 complements the mutant phenotype and enhances oil accumulation by 2-fold in *Chlamydomonas*



3 - Relevance

- ***Increasing oil content improves the economics of biofuel production (feedstock supply).***
- ***High oil yields achieved under continuous growth allow for a continuous production process, i.e. simplify the process of feedstock production.***
- ***The tools we develop (MFA, MCA of microalgae) can be further used for directed strain improvement.***

4 - Critical Success Factors

- ***Enhancing oil accumulation under continuous growth improves overall oil productivity.***
- ***While our analysis is performed with algae growing on acetate, the results of our analysis are to be applied to algae growing autotrophically (on atmospheric CO_2). Manipulation of the targets revealed by our analysis therefore might be less relevant for autotrophically grown alga..***
- ***If we are successful, Chlamydomonas might not be the most suitable species for mass oil production. A succesful engineering approach might have to be applied to another algal strain like Chlorella.***

Future Work

FY 2013 milestones:

1.1 Further characterize P2-48 mutant

1.2 Transform oleosin into oil production relevant strains such as *Nannochloropsis* and *Chlorella*

1.3 Transform the putative nutrient sensor into *Nannochloropsis* and *Chlorella*.

1.4 Characterize other mutants at biochemical and genetic level

2.1 Perform metabolic flux analysis on transgenic strains overaccumulating oil

Summary

- **A *Chlamydomonas* Metabolic Model is established**
- **Oil accumulation in *Chlamydomonas* is limited by carbon precursor supply rather than enzymes involved in fatty acid synthesis and oil assembly**
- **Overexpression of oleosin enhances oil accumulation under normal growth conditions, thus potentially uncoupling oil accumulation from growth inhibition**
- **A putative stress sensor involved in the control of oil accumulation has been identified by a genetic approach. The potential importance of this sensor in oil production deserves further investigation**

Additional Slides

Responses to Previous Reviewers' Comments

- All reviewers except reviewer 3 clearly put strongly in doubt the choice of *Chlamydomonas* as the organism to be used in the project. Yet, we believe we can clearly document the relevance of the project
- First of all, in the algal biofuels roadmap (http://www1.eere.energy.gov/biomass/pdfs/algal_biofuels_roadmap.pdf), p. 10, under "Selecting Algal Model Systems for Study", it is outlined that "Species with sequenced genomes and transgenic capabilities are the most amenable to investigating cellular processes since the basic tools are in place."
- Efficient transformation of green algae is not trivial and transformation protocols for *Chlamydomonas* are already in practice in our lab
- *Chlamydomonas* was also chosen because a highly detailed metabolic models have been published for this organism
- Mutant screen and subsequent gene identification may not be possible in strains other than *Chlamydomonas*
- we expect that the results and methods generated in these studies with *Chlamydomonas* will have a translational effect on our ability to optimize oil accumulation in commercial production strains

Publications and Presentations

- Fan J, Xu. C. Pathway for oil biosynthesis in Chlamydomonas in response to high acetate. Poster presentation at the third Gordon Research Conference on Plant Lipid Metabolism, Structure and Function in Galveston, Texas, Jan 27, 2012-Feb1, 2013.
- Fan J, Yan C, Andre C, Shanklin J, Schwender J, Xu C. Oil accumulation is controlled by the availability of carbon precursors for fatty acid synthesis in *Chlamydomonas reinhardtii.* Plant Cell Physiol. 2012 Aug;53(8):1380-90.
- Fan J, Andre C and Xu C. A chloroplast pathway for the de novo synthesis of triacylglycerol in *Chlamydomonas reinhardtii*. FEBS Lett. 2011 Jun 23;585(12):1985-91.
- Yan C, Fan J and Xu C. Analysis of oil droplets in microalgae. Methods in Cell Biology, under review.