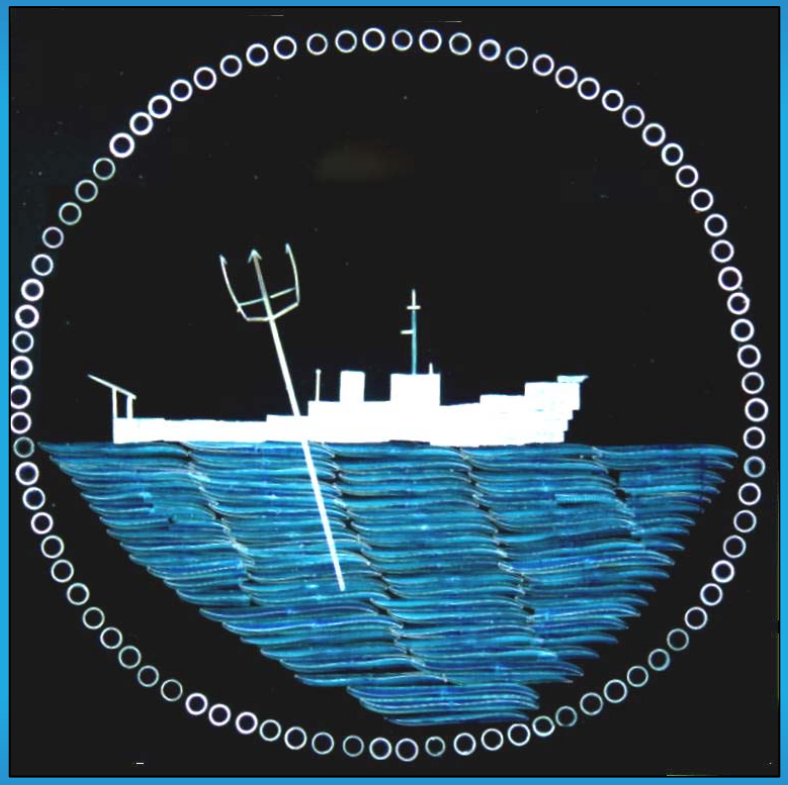
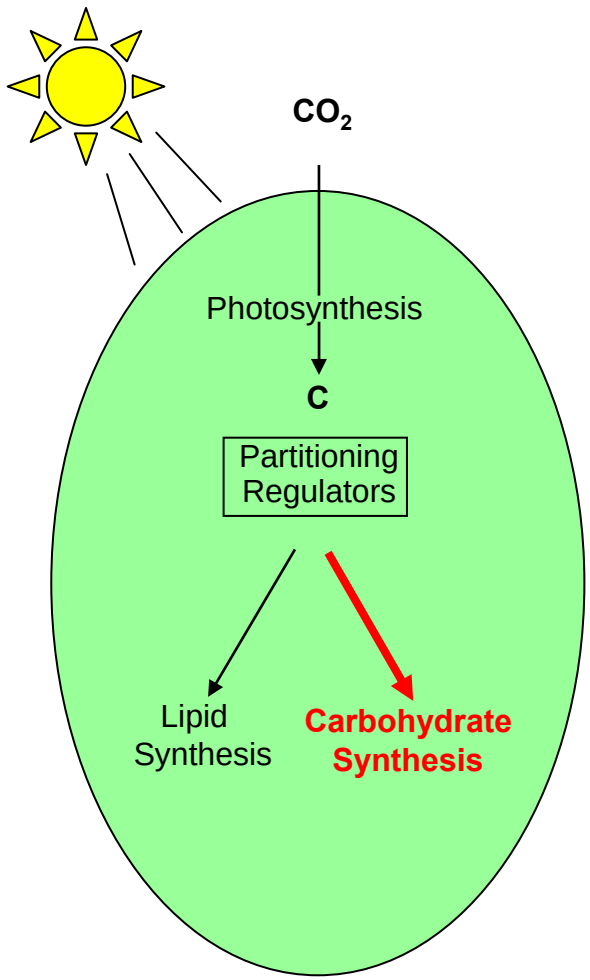
Development of Algal Genetic Tools

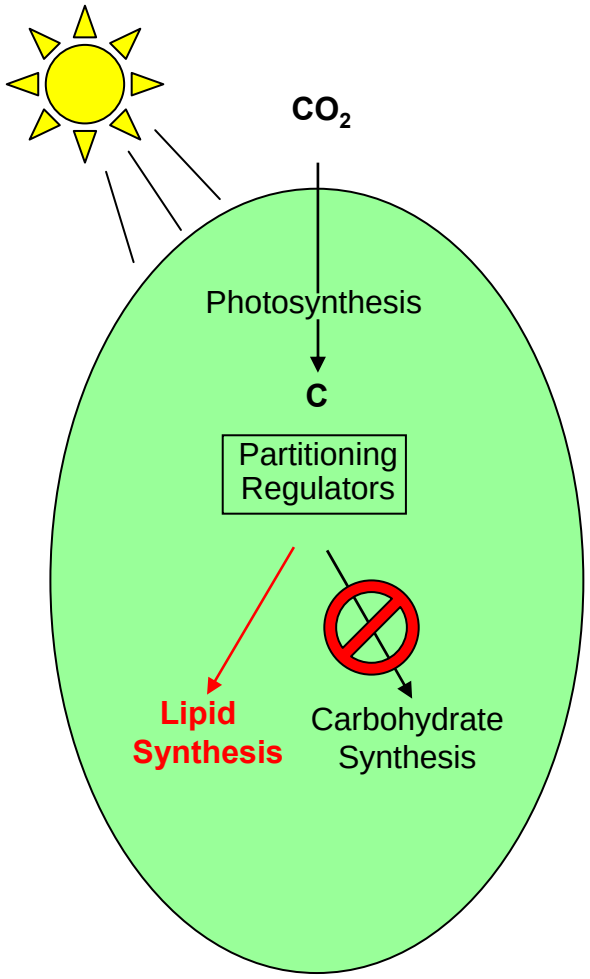


Mark Hildebrand
Marine Biology Research Division,
Scripps Institution of Oceanography, University of California, San Diego

Growth Conditions Affect Carbon Partitioning In Some Algae



Nutrient Replete
(Abundant Growth)



Nutrient Deficient
(Poor Growth)

Identification of pathway-level regulation may be especially useful in metabolic engineering to increase lipid yields under non-limiting growth conditions.

The Value of Genetic Approaches to Microalgal Biofuels Production

- Genetic approaches can aid in understanding the regulation of algal lipid metabolism and carbon partitioning under different growth conditions.

- Genetic approaches can be used to metabolically engineer or select for abundant lipid production coupled with high biomass accumulation.

- Genetic approaches can be used to facilitate large scale processing of microalgae.

Genetic Approaches to be Considered

- **Genomics**

  Pathway-level metabolic regulation can be investigated by genome-wide surveys of gene content and mRNA responses

- **Genetic engineering**

  Alteration of gene sequence or expression should enable metabolic engineering

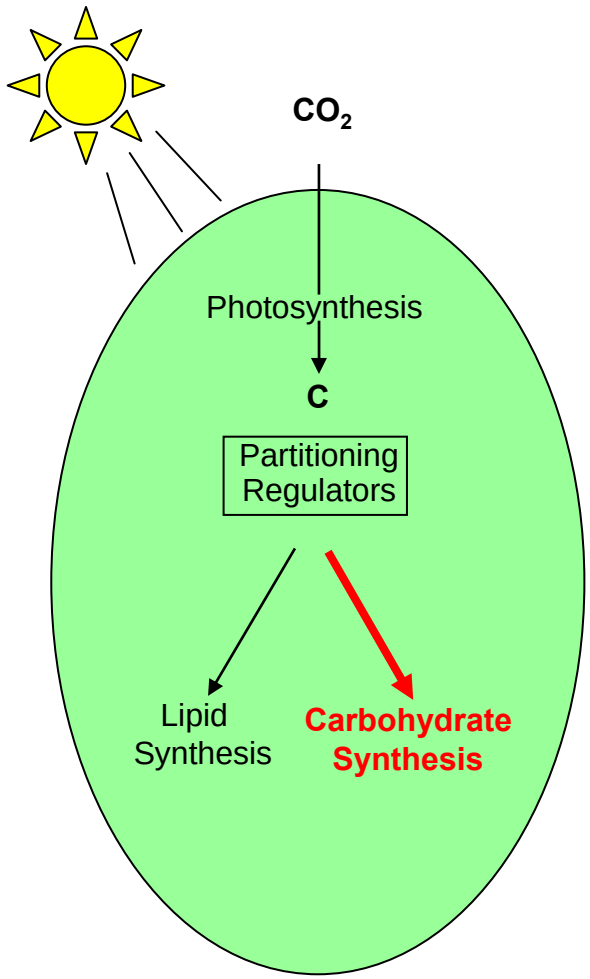
- **Directed Evolution / Selection**

  Foreknowledge of genes involved is not necessarily required, and this approach could facilitate identification of genes and regulatory steps

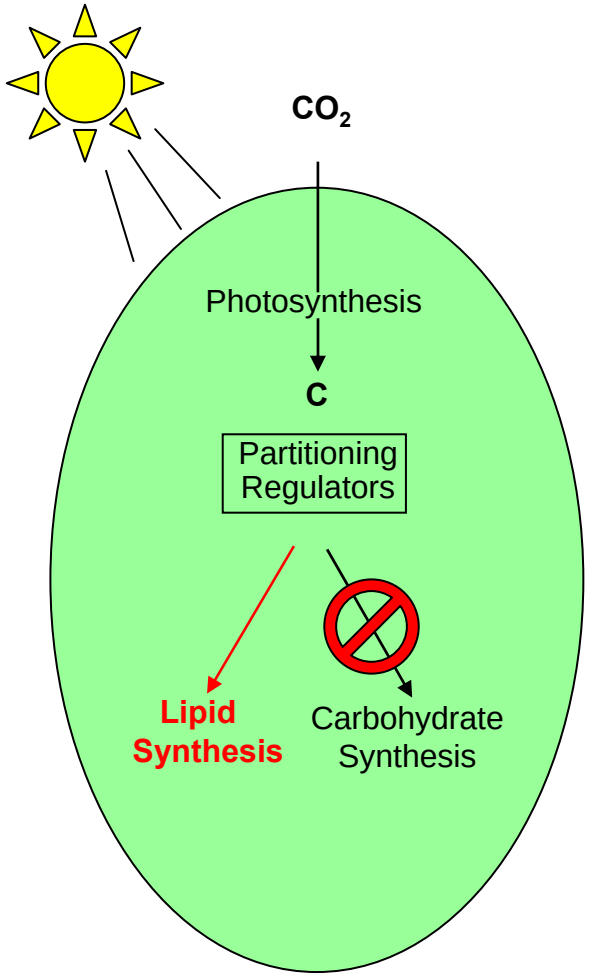
- **Sexual crossing**

  It works for crop plants, why not algae?

Growth Conditions Affect Carbon Partitioning In Some Algae



Nutrient Replete
(Abundant Growth)



Nutrient Deficient
(Poor Growth)

Characteristics of the Gene That Encodes Acetyl-CoA Carboxylase in the Diatom *Cyclotella cryptica*[a]

PAUL G. ROESSLER,[b] JANICE L. BLEIBAUM,[c]
GREGORY A. THOMPSON,[c] AND JOHN B. OHLROGGE[d]

[b]*National Renewable Energy Laboratory*
1617 Cole Boulevard
Golden, Colorado 80401

[c]*Calgene, Inc.*
1920 Fifth Street
Davis, California 95616

[d]*Michigan State University*
Department of Botany and Plant Pathology
East Lansing, Michigan 48824

INTRODUCTION

Efforts to develop renewable liquid fuels for use in transportation applications are accelerating in response to the continuing depletion of global fossil fuel reserves. Part of this effort involves the development of "biodiesel" fuel, which is a diesel fuel substitute derived from biological lipids. The primary source of biodiesel fuel at the present time is plant seed oils, which are transesterified with simple alcohols to produce fatty acid esters. These fatty acid esters function well in unmodified diesel engines, and have the advantage of being biodegradable and less polluting than petroleum-derived diesel fuel when burned.[1]

At the National Renewable Energy Laboratory, we have been investigating the use of microalgae for the production of biodiesel fuel. Microalgae have the potential to produce up to 50% of their mass as lipids and can grow in outdoor mass culture at rates approaching 50 g of dry mass/($m^2 \cdot$ day).[2] Furthermore, many species of algae grow extremely well in saline groundwater that is unsuitable for crop irrigation. As a result, our calculations suggest that microalgae could theoretically produce more biodiesel fuel than oilseed plants.

Maximal production of storage lipids typically occurs in algae only when the cells are environmentally stressed in some manner. For example, nitrogen deficiency induces lipid accumulation in many algal species.[3] Diatoms, which are microscopic algae that are characterized by the presence of silicon in the cell wall, also accumulate lipids when grown under silicon-limiting conditions.[4] Unfortunately, the growth rates of nutrient-deficient algae are greatly reduced, and so it may be necessary to genetically alter promising algal species so that lipid accumulation can be induced during normal growth modes.

Recombinant DNA Technology II, 1994, 721: 250-256.

Attempts to Genetically Manipulate Diatom Lipid Metabolism in the ASP

- Introduction of multiple ACCase copies into *Cyclotella cryptica* resulted in increased mRNA and ACCase activity, but not increased lipid synthesis.

- Perhaps feedback regulation of lipid synthesis pathways occurred, or carbon was not sufficiently repartitioned into lipid synthesis.

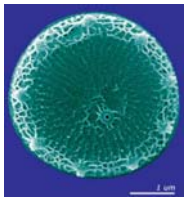
- After transformation of *N. saprophila* with the *C. cryptica* ACCase, mRNA was detected but not protein, suggesting a level of translational control.

- Ribozymes designed to cleave UGP/PMGase mRNA were introduced into *C. cryptica*, but were unsuccessful.

Better understanding of regulatory processes and better genetic manipulation tools may enable these approaches

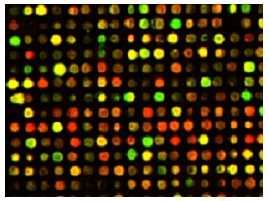
Genomics Approaches Can Facilitate Understanding of the Regulation of Lipid Synthesis at Both the Individual Gene and Pathway Levels

- Full Genome Sequence

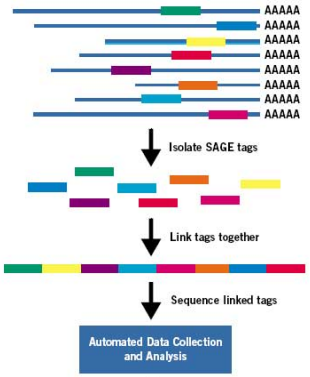


- ESTs

- Microarrays

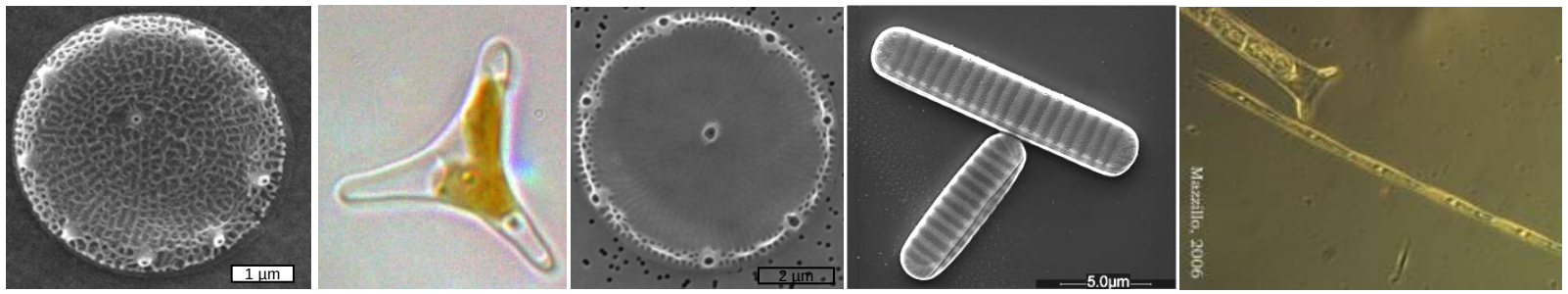


- SAGE

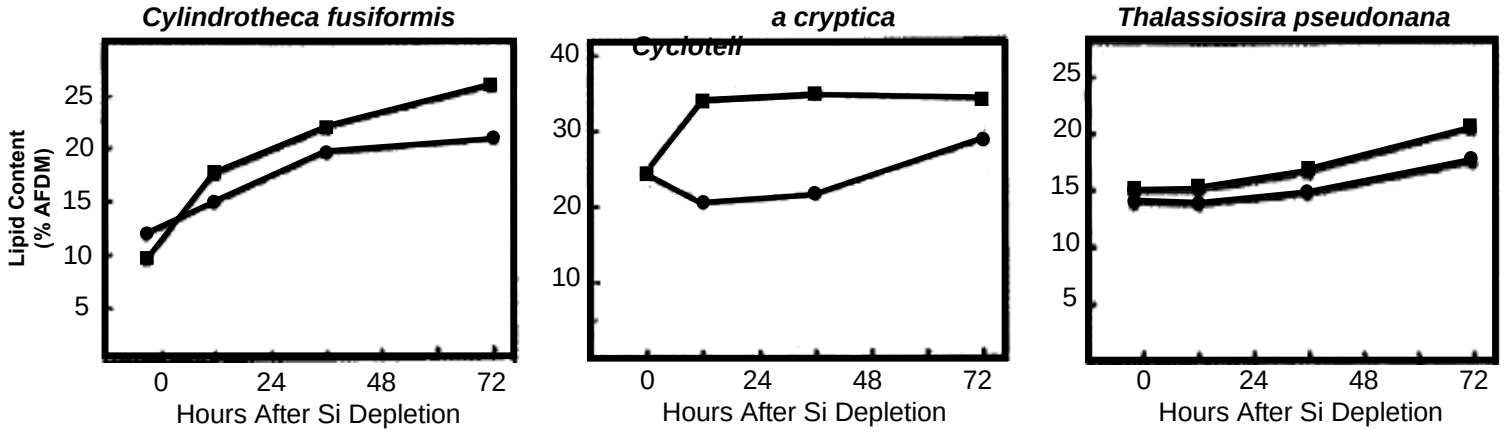


Diatom Genomes Sequenced or In Progress

- ***Thalassiosira pseudonana*** — **model centric species, small genome**
- ***Phaeodactylum tricornutum*** — **model pennate species, small genome**
- ***Thalassiosira oceanica*** — **centric species, open ocean, small genome**
- ***Fragilariopsis cylindrus*** — **pennate species, cold water environments**
- ***Pseudonitzschia multiseries*** — **pennate species, domoic acid producer**



Species-specific Effects of Si Depletion on Diatom Lipid Accumulation



Symbols: (■) Si-deficient cultures; (●) Si-replete cultures.

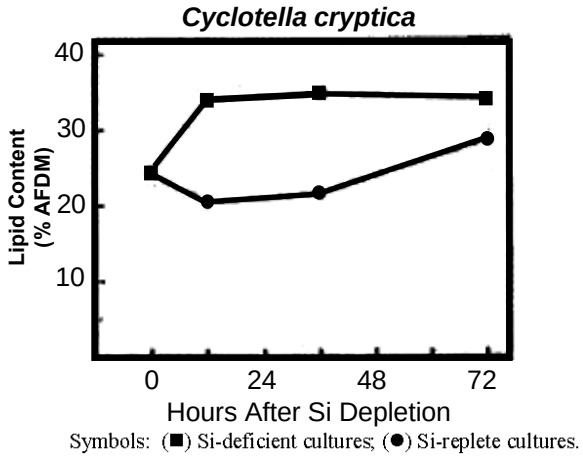
Cyclotella cryptica **accumulated more lipid, and more rapidly after Si depletion**

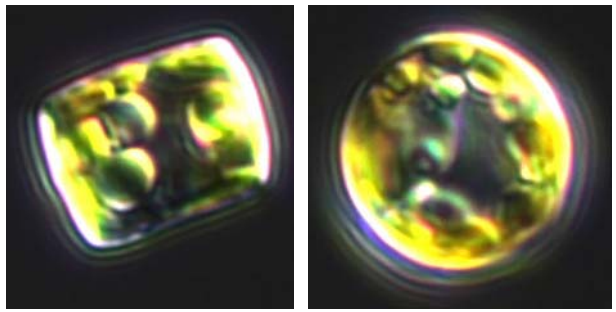
Species-specific effects are likely due to differential regulation

Comparative analysis may shed light on regulatory differences

EST / Partial Genome Sequence of *C. cryptica*

AFOSR Project Collaboration with Andy Allen at JCVI





- **Genome sizing and cell cycle stage arrest will be determined soon**
- **EST libraries during early stage of Si depletion will be generated and sequenced**
- **Also determining Si depletion response and sequencing ESTs for *T. oceanica***

Comparative analysis of transcript responses in the different genomes may shed light on regulation involved in different lipid accumulation responses

Genetic Manipulation / Gene Expression Control Approaches
AFOSR Project

1. **Develop new selectable transformation markers**
   To enable sequential addition of genes for multi-component metabolic engineering.

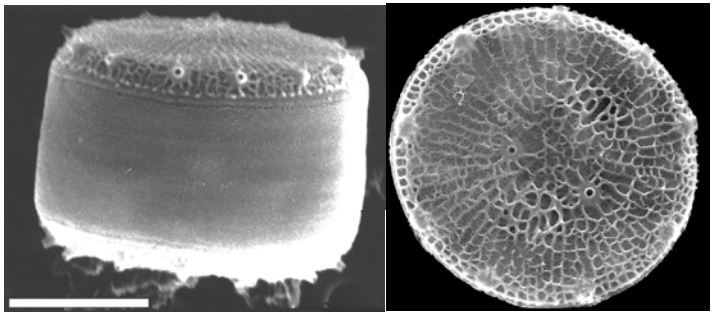
2. **Develop "universal" transformation vectors for use in a variety of species**
   To reduce the time required to construct a new vector for each species.

3. **Develop new gene expression manipulation tools**
   To enable sophisticated control over expression levels and timing.

4. **Develop new gene tagging approaches**
   To determine protein expression levels and intracellular localization.

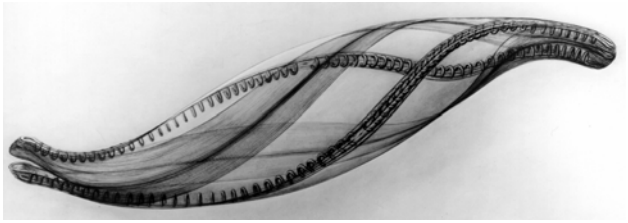
Selectable Marker Genes For Diatom Transformation

Major Study Species



Thalassiosira pseudonana

Phaeodactylum tricornutum



Cylindrotheca fusiformis

Marker Gene	Mechanism of Action	Species	Reference
neomycin phosphotransferase II (*nptII*)	Inactivates G418 by phosphorlyation	*Cyclotella cryptica, Navicula saprophila, Phaeodactylum tricorntum*	Dunahay et al. 1995, Zaslavskaia et al. 2001
sh ble	Zeocin resistance by stoichiometric binding	*Phaeodactylum tricorntum, Cylindrotheca fusiformis*	Falciatore et al. 1999, Fischer et al. 1999, Zaslavskaia et al. 2001
nat1, sat-1	Nourseothricin resistance by enzymatic acetylation	*Phaeodactylum tricornutum, Thalassiosira pseudonana*	Zaslavskaia et al. 2001, Poulsen et al. 2006
Dual selection achieved with *nptII* or *nat1* and *sh ble*		*Phaeodactylum tricornutum*	Zaslavskaia et al. 2001

We are exploring conserved mutations to antibiotic resistance in ribosomal protein genes

Promoters to Drive Selectable Marker Gene Expression For Diatom Transformation

Promoter	Species	Reference
ACCase	*Cyclotella cryptica, Navicula saprophila*	Dunahay et al. 1995
fcp	*Phaeodactylum tricorntum, Cylindrotheca fusiformis, Thalassiosira pseudonana*	Falciatore et al. 1999, Fischer et al. 1999, Zaslavskaia et al. 2001, Poulsen et al. 2006

A "Universal" Transformation Vector Would be Very Useful

We Have Successful Transformation to nat Resistance With:

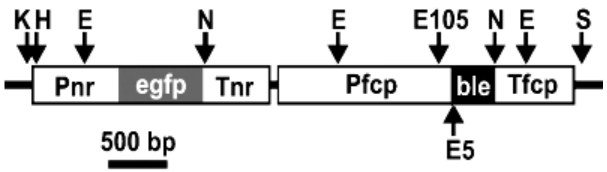
Promoter	**Species**
fcp	*T. pseudonana, T. oceanica, T. weissflogii*
ACCase:	*T. pseudonana & Nitzschia alba*
rpl41:	*N. alba*
SV40:	*T. pseudonana*

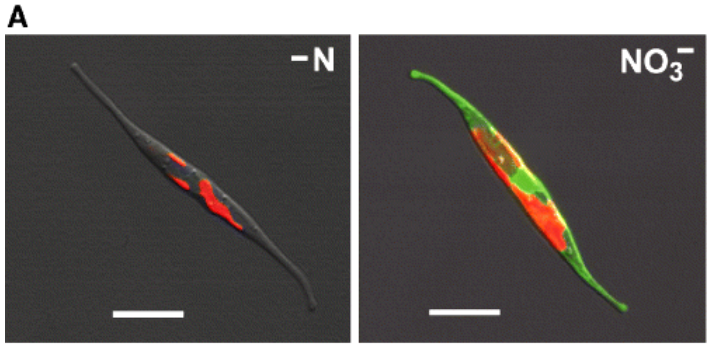
Development of New Gene Manipulation Tools

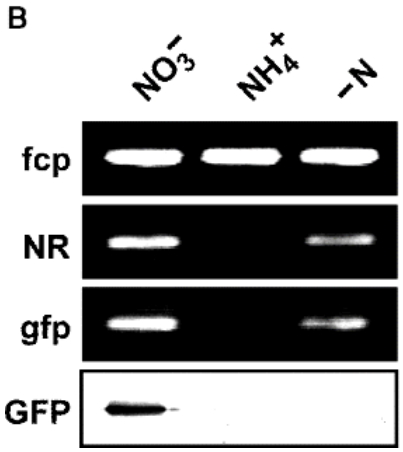
- Inducible Promoters
- Homologous Gene Replacement
- Knockout or Knockdown Approaches
- Protein Tagging

Development of New Gene Manipulation Tools: Inducible Promoters

The Nitrate Reductase (NR) Gene (Poulsen et al. 2005)

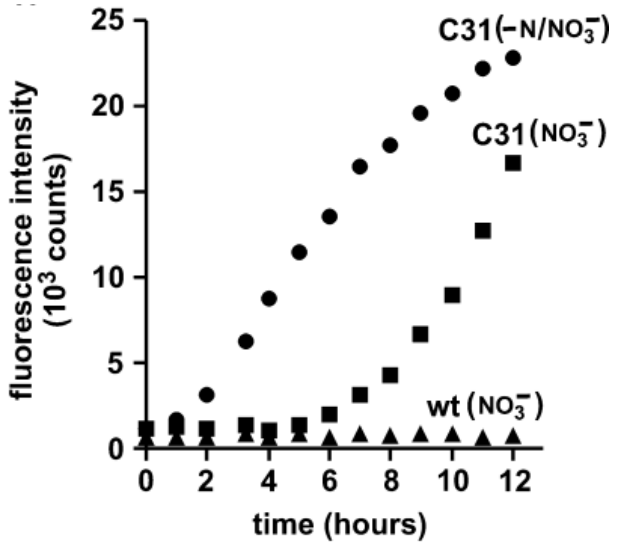




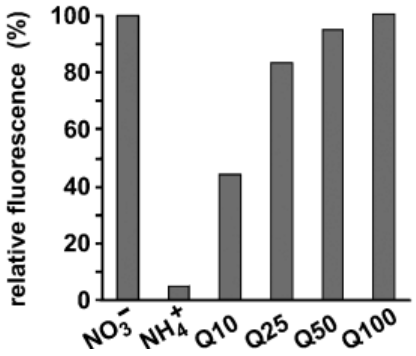


Translational Regulation

Prestarvation for N decreases response time

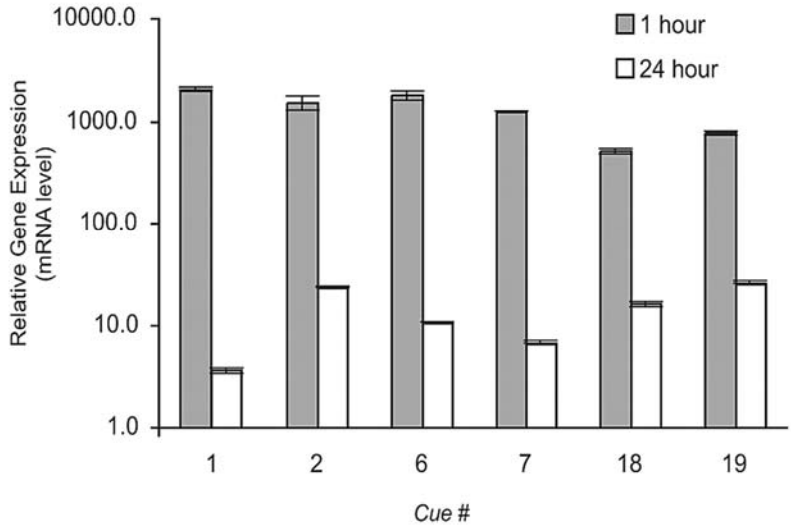


Protein expression levels are partly titratable

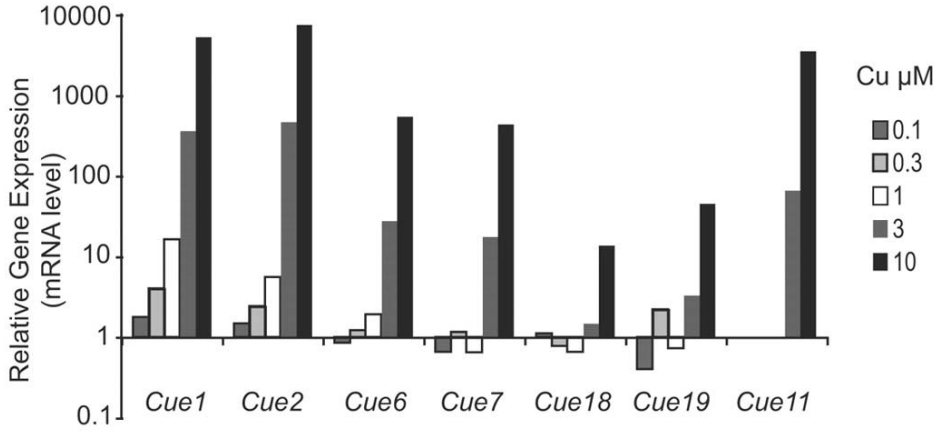


Nitrate Reductase is Light and Cell Cycle Regulated

Development of New Gene Manipulation Tools: Inducible Promoters
Copper Induced (*cue*) Genes (Davis et al. 2005)



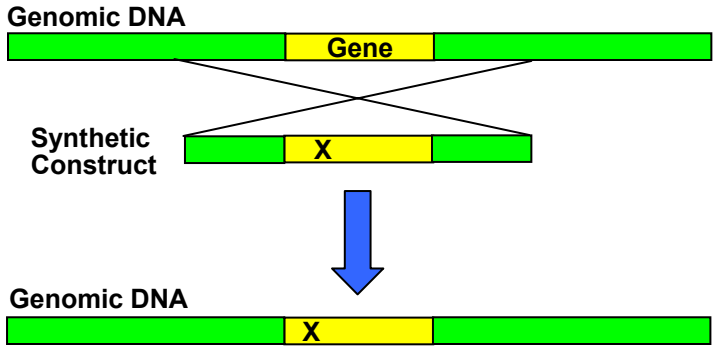
Cue Genes are Induced 1000 – fold after exposure to Cu



Cue Gene mRNA Levels are Titratable

Development of New Gene Manipulation Tools: Homologous Gene Replacement

Homologous Recombination



Homologous gene replacement can be used to insert a mutation into a gene or knock it out, since the native gene is replaced, phenotypic interference is eliminated.

Current Status

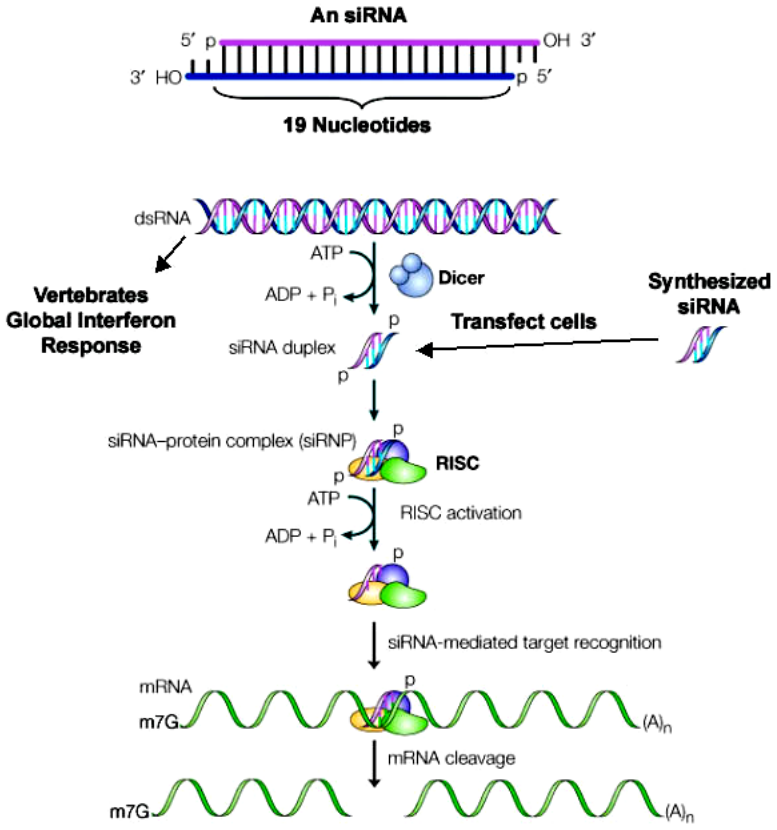
A restriction site and termination codon was inserted into a *T. pseudonana fcp* gene

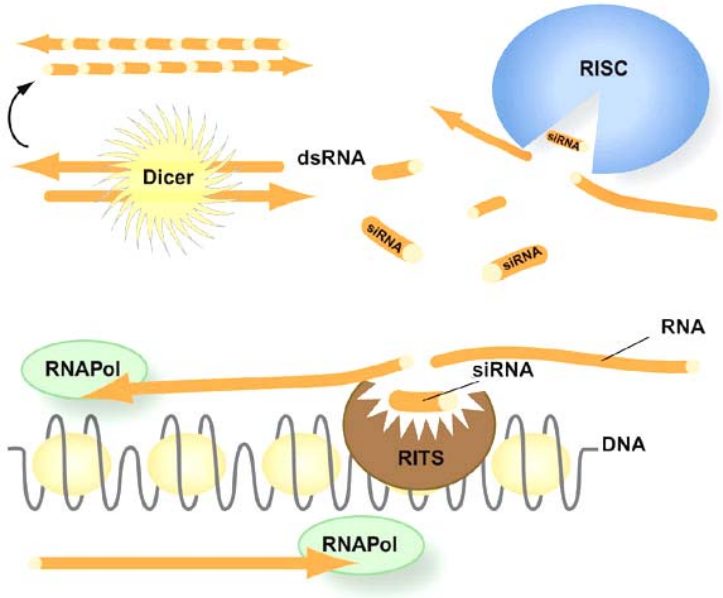
Large flanking regions were used (3 kbp)

Initial results demonstrated integration but not homologous gene replacement

Next attempt is to use single-stranded DNA

Development of New Gene Manipulation Tools: RNA Interference (RNAi)

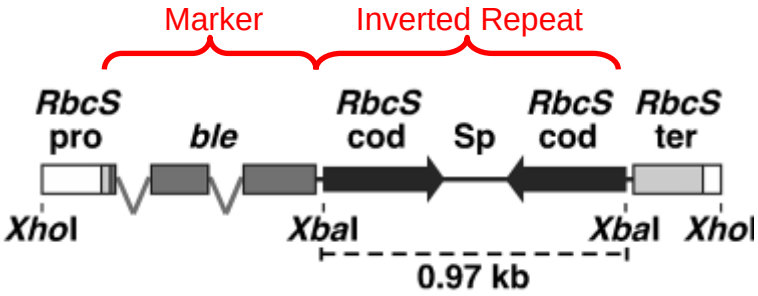




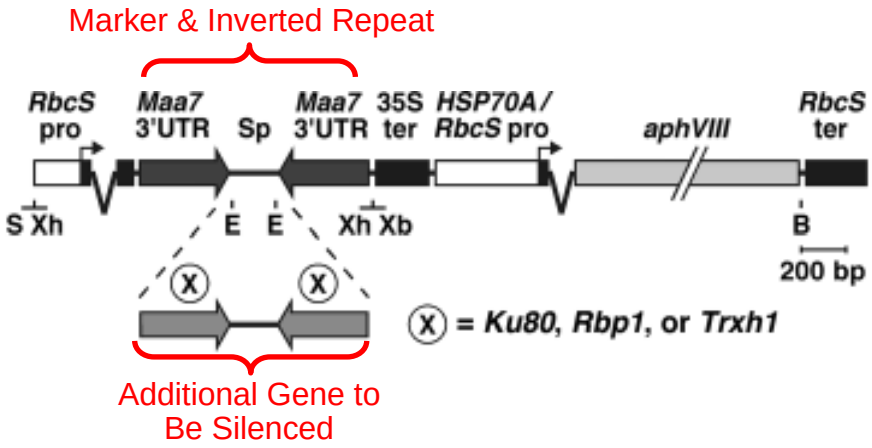
RNAi is working in *P. tricornutum* (Chris Bowler pers. commun.), we are developing for *T. pseudonana*

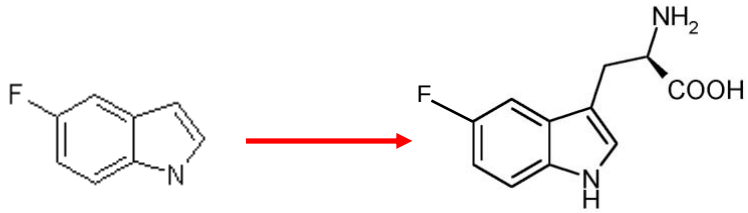
Development of RNAi in *Chlamydomonas*

Rohr et al. 2004



This construct was inefficient at RNAi





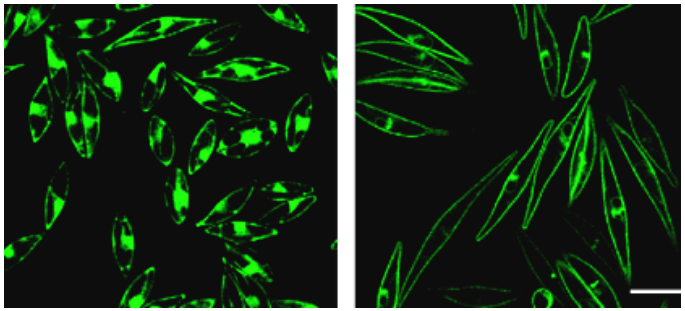
***Maa7* encodes β-tryptophan synthase which converts 5-fluoroindole to 5-flourotryptophan, a cytotoxin**

All 5-FI resistant transformants had efficient RNAi against both the *Maa7* and additional genes

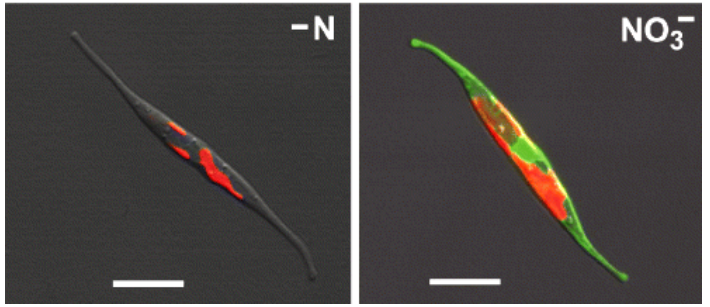
We have constructed a *Maa7* vector under control of the nitrate reductase promoter and will introduce it into *T. pseudonana*

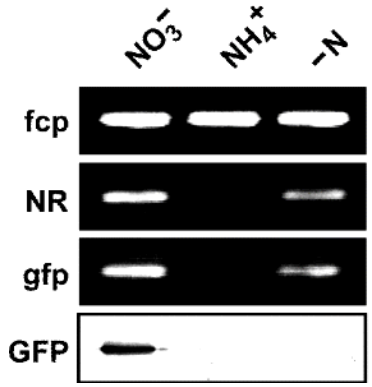
The Importance of Protein Tagging in Metabolic Engineering

Allows monitoring of protein expression



Localization

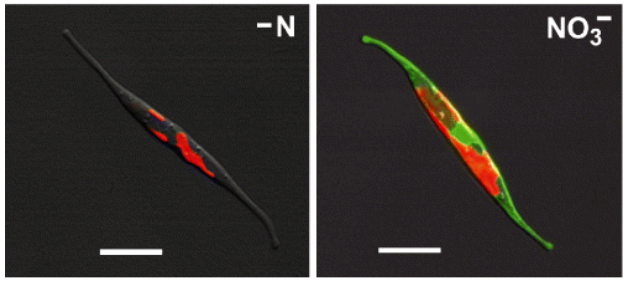




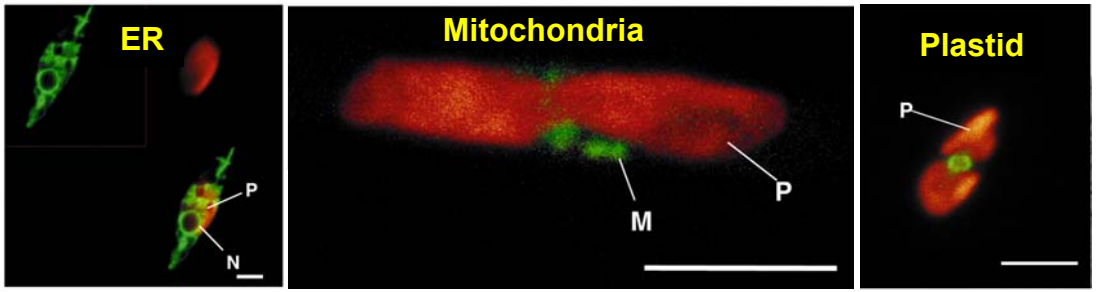
and translational regulation

Simply turning a gene “on” or “off” at the mRNA level is no guarantee of completely controlling it’s expression

Development of New Gene Tagging Approaches: What's Wrong with GFP?



Poulsen and Kröger, 2005

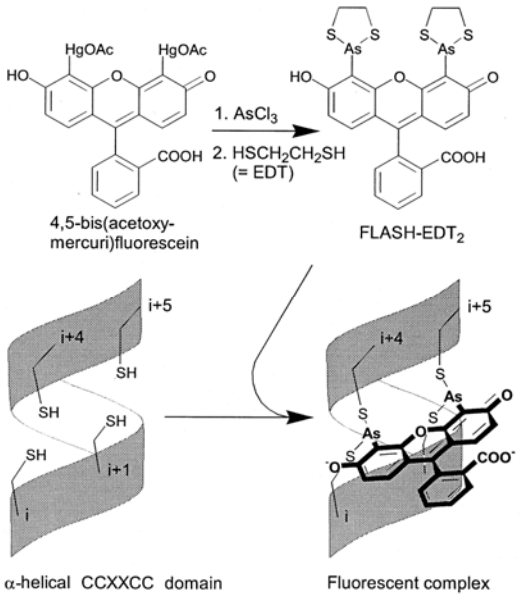


Apt et al., 2002

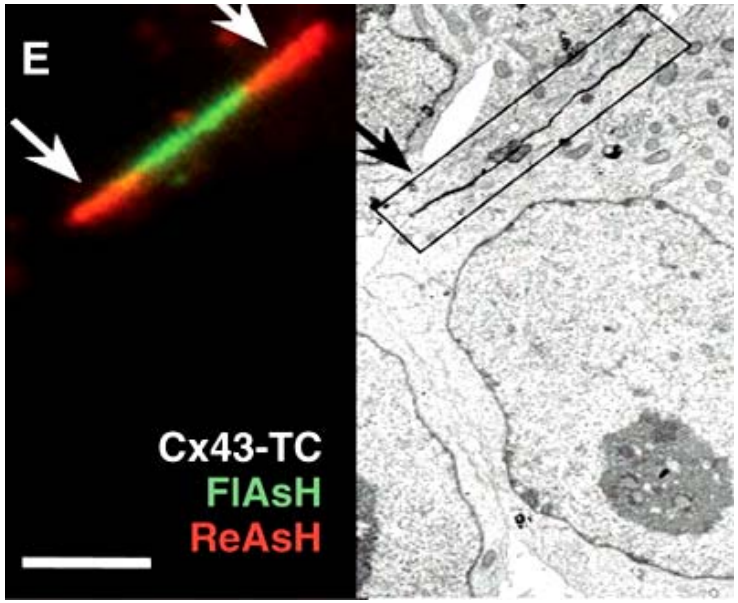
Problems - lack of fluorescence, finicky growth conditions, and localization artifacts

Development of New Gene Tagging Approaches: The TC tag

E S S G S F L N C C P G C C M E P G G R



Griffin et al. 1998

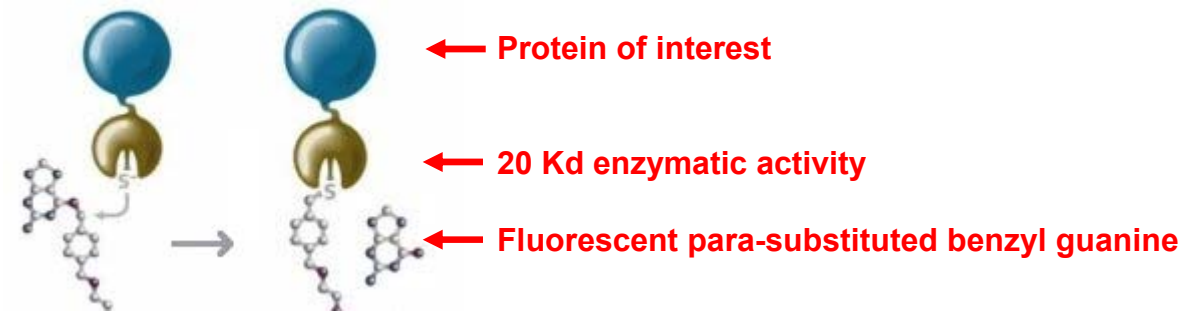


Giepmans et al. 2006

Advantages – small tag, no protein folding required, combined fluorescence and TEM imaging

Disadvantages - cells need to be fixed, only two colors, expensive

Development of New Gene Tagging Approaches: The SNAP Tag - Covalys



Advantages – multiple colors

Disadvantages – no real-time imaging, relatively large tag, requires activity

Development of "New" Gene Tagging Approaches: Antibody Epitope Tags

FLAG	DYKDDDDK
c-MYC	EQKLISEEDL
HA	YPYDVPDYA
VSV-G	YTDIEMNRLGK
HSV	QPELAPEDPED
V5	GKPIPNPLLGLDST

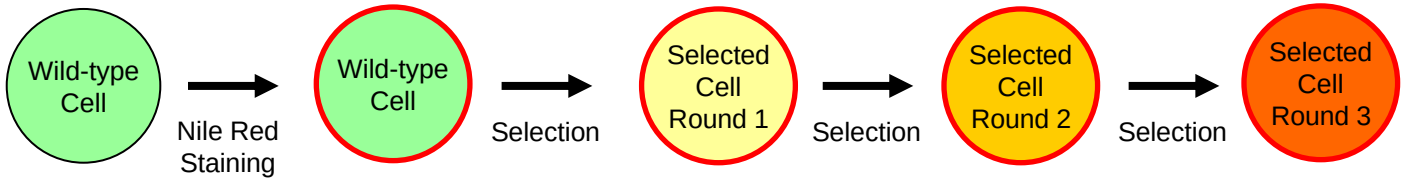
Advantages – well established technology, multiple colors

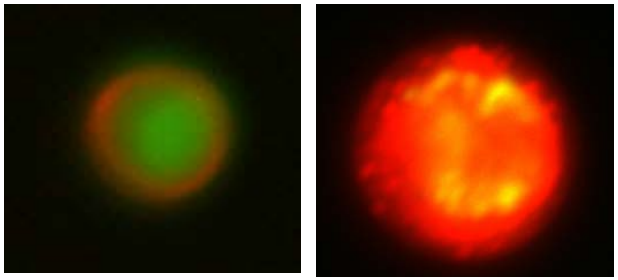
Disadvantages - cells need to be fixed

Directed Evolution / Selection Approaches

Foreknowledge of genes involved is not necessarily required

Example: increased lipid production





Nile Red Staining of Lipids

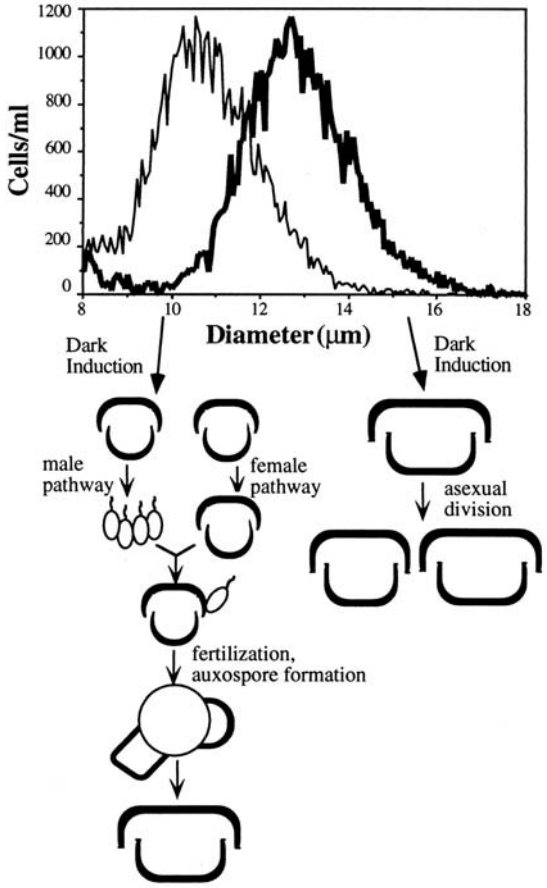
Desirable Traits

- **Abundant lipid and biomass accumulation**
- **Growth under extreme conditions (temperature, salinity, pH)**
- **Efficient light utilization**

Selection Approaches can be Coupled with Mutagenesis

- **Chemical or UV mutagenesis**
- **Tagging-based mutagenesis – enables identification of genes**
- **Genetic engineering**

Sexual Crossing Approaches – Algal Breeding



Induction of gametogenesis in *Thalassiosira weissflogii*
Armbrust 1999

Conclusions

Genetic approaches are essential to understand metabolic regulatory processes and to manipulate cellular metabolism to facilitate high lipid yields

Broad-based genetic manipulation and selection approaches should be developed for diverse algal species

Genetic approaches should not only be considered important in the initial stages of development of algal biofuels technology, but also in its continuing refinement and optimization in concert with engineering and processing needs.

Personnel Involved

Luciano Frigeri

Aubrey Davis

Jeff Carlson

Mark Hildebrand

Thanks to AFOSR / Walt Kozumbo for Funding