DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

Developing Advanced Genetic and Synthetic Biology Tools for Improved Algae Productivity

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PEAK -

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This presentation does not contain any proprietary, confidential, or otherwise restricted information

Goal Statement

- *The **goal** of this project is to develop a process for making advanced genetic tools using: genomics, synthetic biology, high-throughput screening methods, and breeding technologies.*
- *The **outcome** of this project will be a process in which a genome sequence from any algal strain is the input and a designed set of genetic tools and high throughput screening technologies are the output.*

- *Relevance to bioenergy industry*
  - Drop-in hydrocarbon fuels can be produced directly from algae; however, it is generally acknowledged that wild type algal strains do not have the productivity, robust growth characteristics, and *value added co-products* that will allow algal biofuels to compete economically with fossil fuels.
  - Improved molecular and synthetic biology tools coupled with classic domestication processes - could enable biofuels from algae to be cost competitive with fossil fuel prices of today in as little as five to ten years.

Quad Chart Overview

Timeline

- Project start date 09/30/2017
- Project end date 12/31/2020
- Percent complete 20%

Barriers addressed

- Aft-C. Biomass Genetics and Development
- Ct-K. Developing Methods for Bioproduct Production

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded		- 0 -	$370,421	$2,629,580
Project Cost Share*		- 0 -	$60,450	$361,808

•Partners:

• Global Algae Innovations, 5%
• Triton Algae Innovations, Cost share
• Algenesis Materials, Cost share

*Only fill out if applicable. If there are multiple cost-share partners, separate rows should be used.

**Only fill out if applicable.

Objective

Developing advanced genetic tools and HT screening protocols for commercial algae production strains

End of Project Goals

- A suite of advanced genetic tools, high-throughput screening, and breeding protocols for green algae and cyanobacteria.
- Commercially relevant strains producing a high value protein co-product for TAI, and improved lipid accumulation for GAI.
- A process for developing genetic control systems that can be applied to any species of algae as they are identified by academic or commercial enterprises.

1 - Project Overview

- This project is a continuation of 10 years of DOE funded programs to develop algae as an economic platform for biofuel production

- Genetic modification of production strains will be required for improving yields and productivity, as well as allowing for the production of high-value co-products, that will enable economic biofuel production from algae

- This project will develop advanced genetic engineering tools and methods for use in commercial production strains, both algae and cyanobacteria

- The outcome of the project will be a *process* in which a genome sequence from any algal strain is the input and a designed set of genetic tools and high throughput screening technologies are the output

Project Overview – Objectives

- **Objective 1**- Develop advanced genetic tools for improved nuclear transgene expression and advanced metabolic engineering to enable economic production of biofuels from algae.
- **Objective 2**- Improve expression and secretion of recombinant proteins as co-products to improve the economics of biofuel production.
- **Objective 3**- Establish a rapid high throughput screening method for strains with improved protein production and secretion abilities.
- **Objective 4**- Develop genetic tools and genome editing strategies to enable metabolic engineering of commercially viable species of cyanobacteria.
- **Objective 5**- Develop a suite of highly controlled gene expression tools for metabolic engineering of cyanobacteria to produce methyl-branched fatty acids and wax esters.
- **Objective 6**- Developed improved targeted genome editing and transgene delivery methods for green algae to accelerate strain engineering.
- **Objective 7**- Develop *non-transgenic* improvement strategies using breeding, high throughput screening, and mutagenesis to develop new production strains.
- **Objective 8**- Develop genetic tools for commercial strains, based on learning from previous objectives, to demonstrate rapid adaptation of genetic tools to new strains.
- **Objective 9**- Grow unmodified and genetically modified strains under field conditions to determine impact of genetic modifications on biomass productivity and product yield.
- **Objective 10**- Conduct a life cycle assessment and techno-economic assessment (TEA) on GM and non-GM strain under outdoor cultivation conditions.

2 – Project Approach (Management)

Milestones and Deliverables – Key Personnel

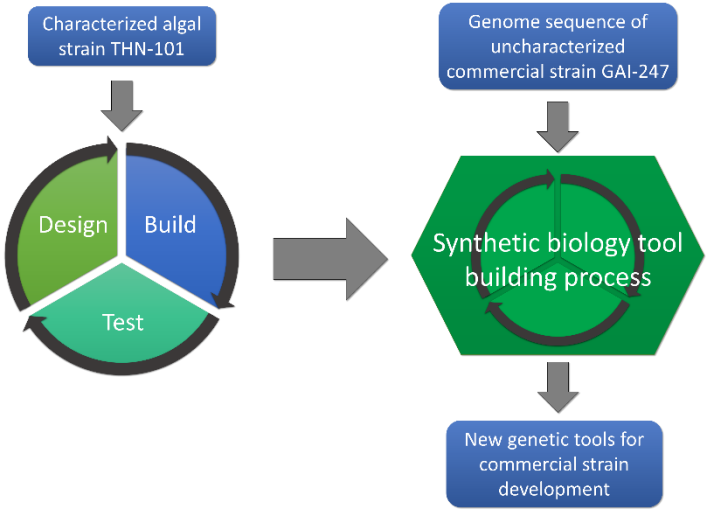
Year 1: Validate baseline genetic tool set and develop approaches for advanced genetic tool construction for algae (Mayfield) and cyanobacteria (Golden)

Year 2: Create synthetic gene control system for recombinant protein expression (Mayfield/TritonHN) and metabolic engineering of polyurethane precursors (Burkart, Golden/Algenesis). Adapt tools for commercial strain engineering

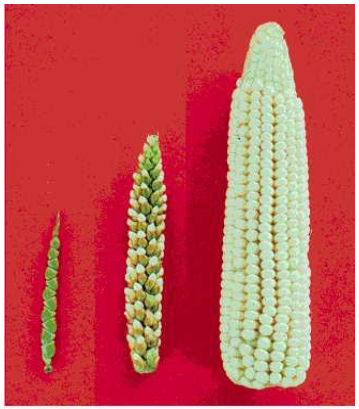
Year 3: Outdoor field trials of improved strains – GAI and Mayfield lab
TEA and LCA of GM strains to demonstrate economic and environmental improvements (UCSD & Kendal UC Davis)

2 – Project Approach (Technical)

Approach

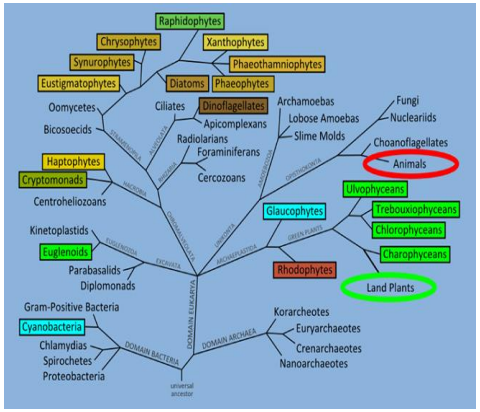


Challenge



Domestication of corn took 6,000 years

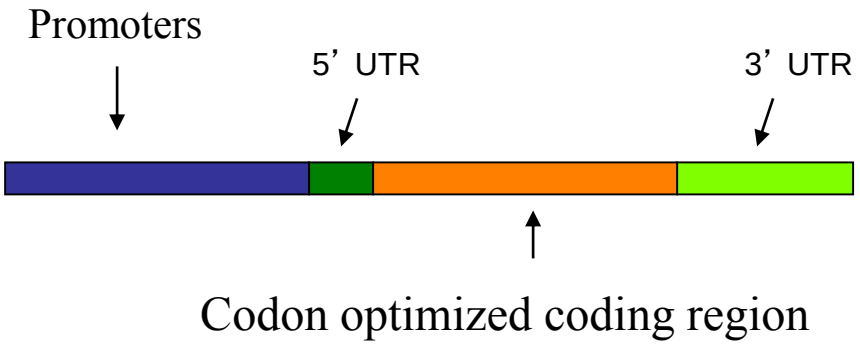
Target High Value Products



Algae are the most diverse organisms on the planet

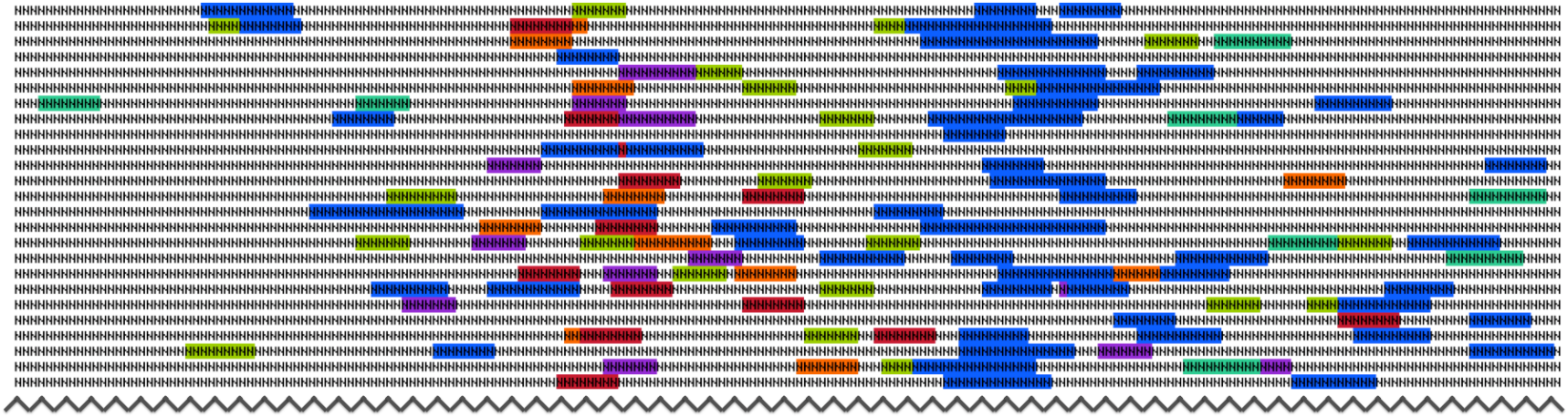
3 - Technical Progress – Genetic Tools for Microalgae

- **Improved recombinant protein expression in *Chlamydomonas***
  - Developed new vector that achieves higher expression of recombinant proteins than previous expression vectors – and much better control over gene expression

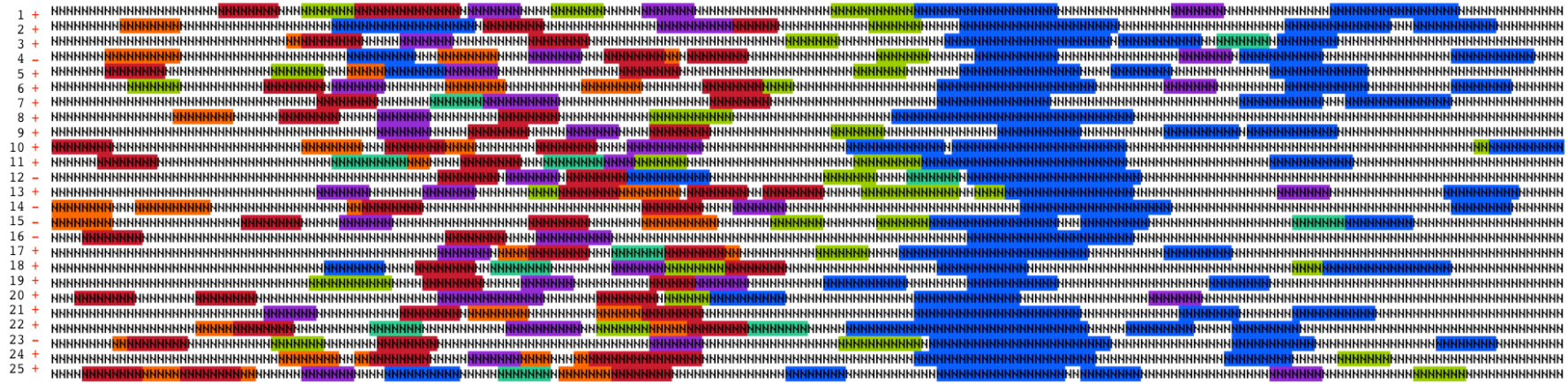


- Poor recombinant protein expression has been a problem
- Need many transformation events to get good expression
- Lack the viral promoter elements that other systems have
- Can target proteins to subcellular location including export

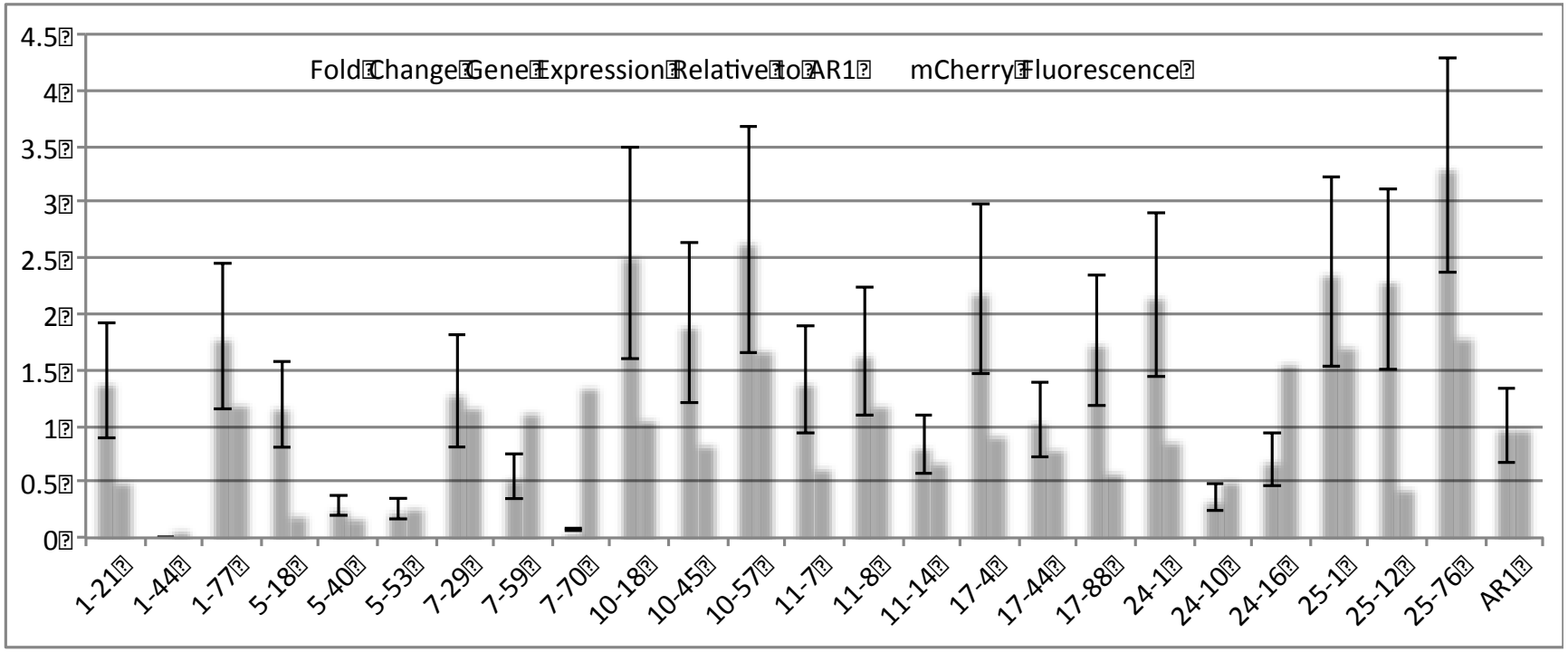
Sequences preceding highly expressed genes from *C. reinhardtii* genome



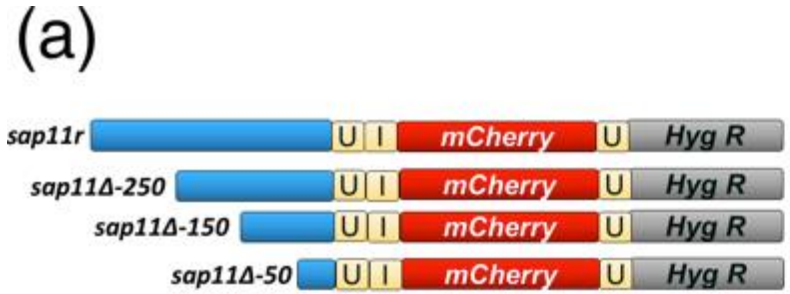
25 unique synthetic promoters were generated

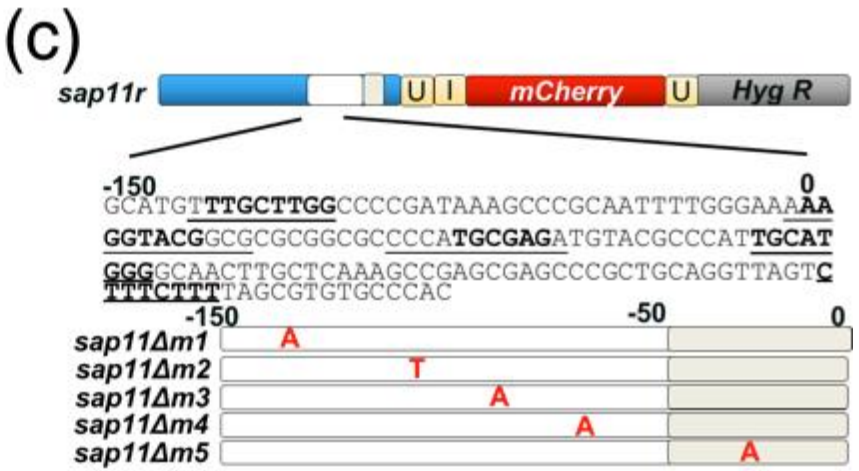


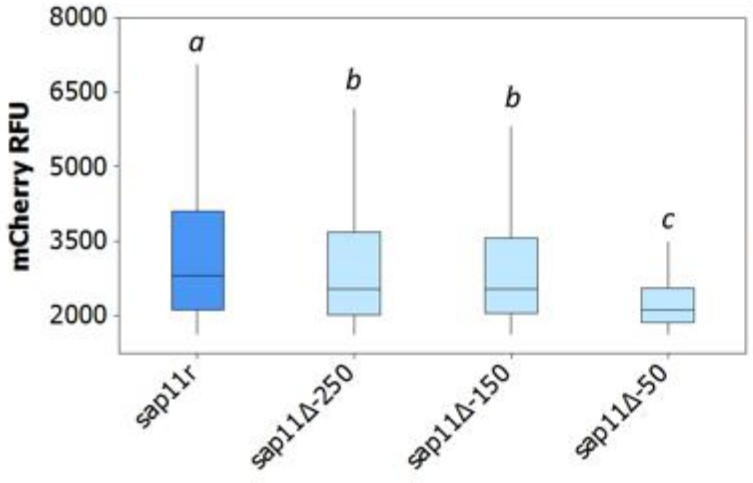
Synthetic promoters show increased transcript abundance over best endogenous promoter

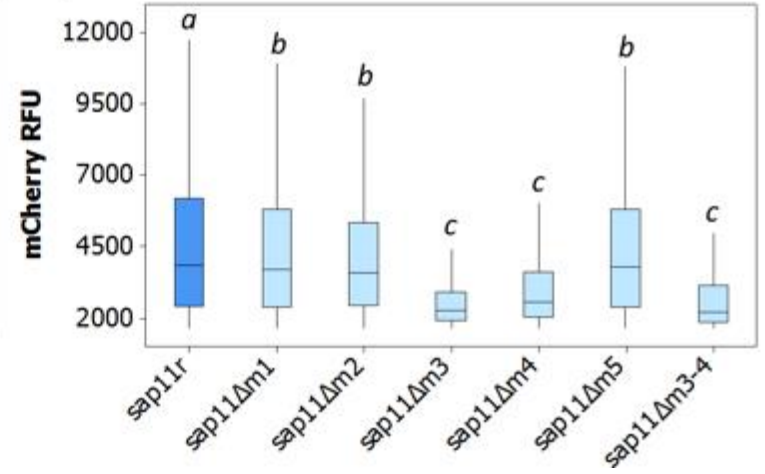


Sap11 only required 150 nt contained six elements – only 2 essential

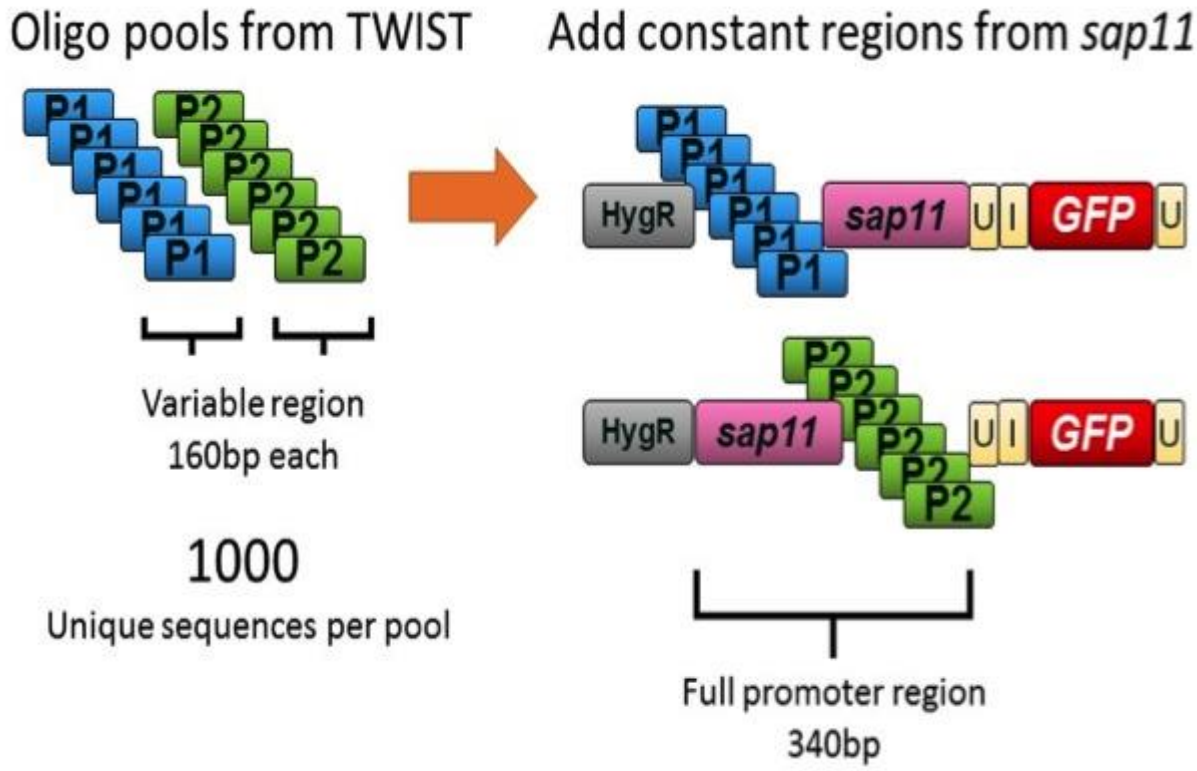






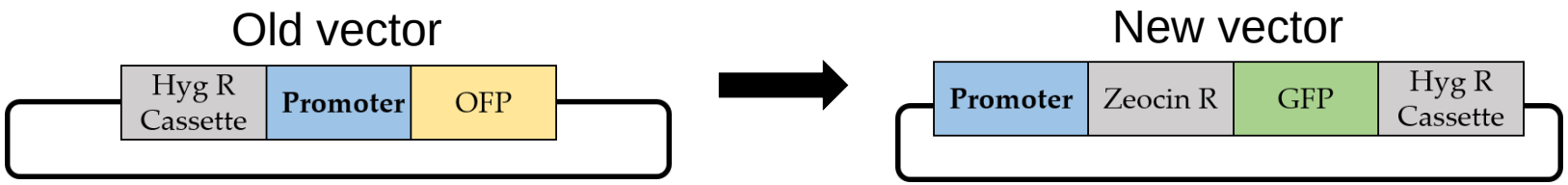


New synthetic promoter library of 2 X 1,000 members

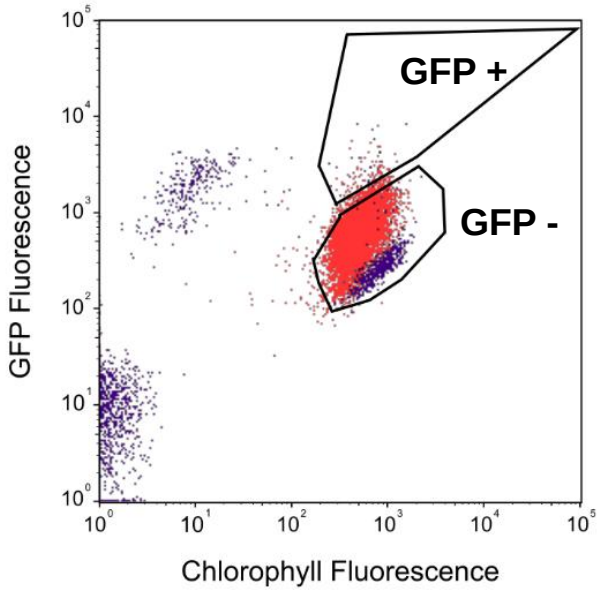


Reduced to 300 nt and 5 elements per promoter

New vector results in higher expression of GFP reporter protein

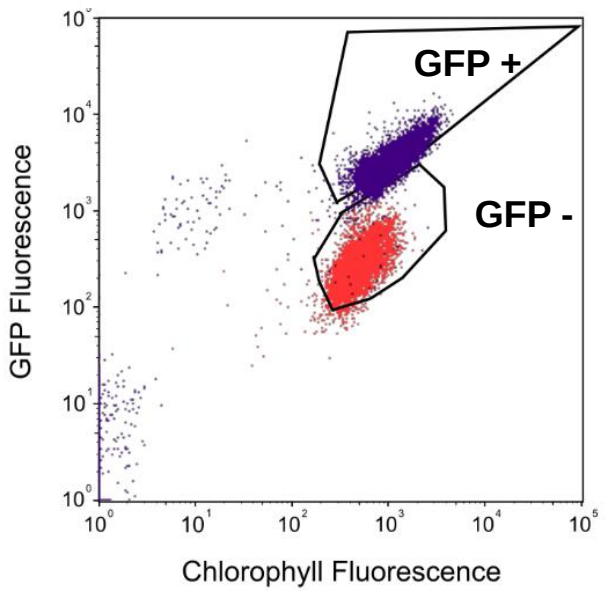


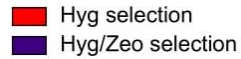
AR1 promoter



Selection	GFP +	GFP -
Hyg	1%	94.6%
Hyg + Zeo	0.22%	7.4%

RBCS2 core promoter

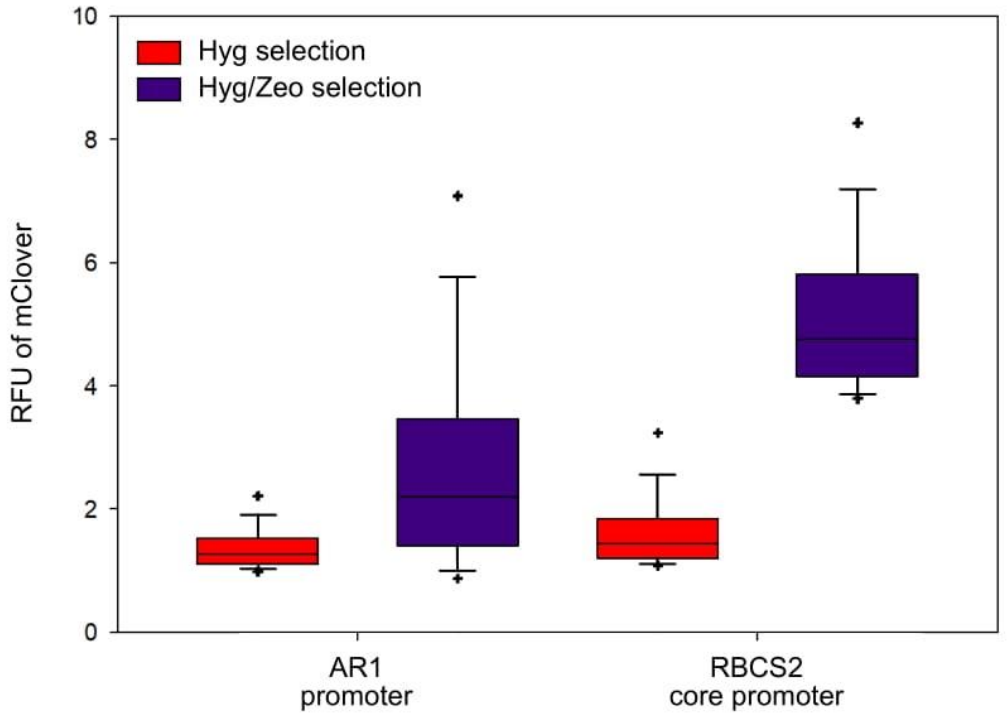




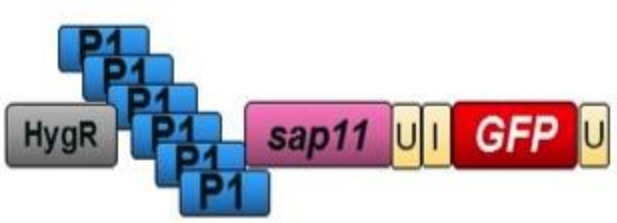
Selection	GFP +	GFP -
Hyg	0.15%	96.6%
Hyg + Zeo	64.8%	2.58%

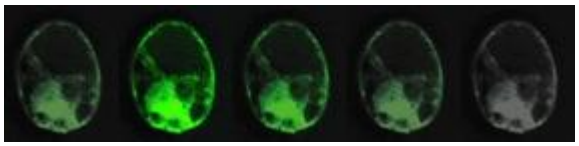
New vector results in higher expression of GFP reporter protein

- Double antibiotic selection of Hygromycin + Zeocin identifies transformants with up to 3X higher GFP expression than using Hygromycin alone

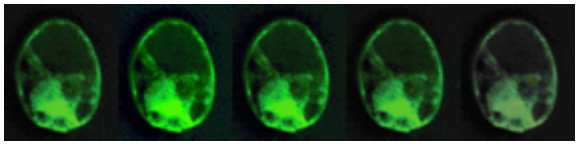


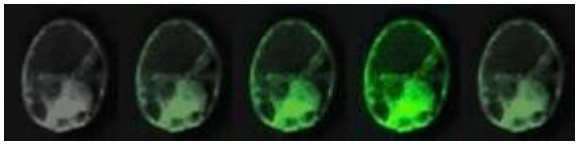
Identifying conditional promoter elements



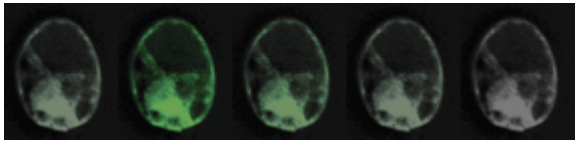


Library conditions





Minimal media



Expression of mCherry driven by synthetic promoters under light and dark growth

TAP LIGHT

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	310	1718	1267	488	835	1344	1290	419	170	215	606	679
B	1325	196	909	642	476	3792	944	681	412	1047	748	731
C	273	471	160	304	554	599	944	515	168	510	518	681
D	1258	260	642	231	214	597	1604	564	654	182	548	373
E	529	1228	686	208	451	180	653	869	664	415	567	2329
F	655	585	481	2149	1241	179	422	1236	464	471	1622	479
G	340	911	177	548	185	161	224	742	679	1538	671	326
H	1423	921	689	450	653	282	2475	544	1725	197	253	936

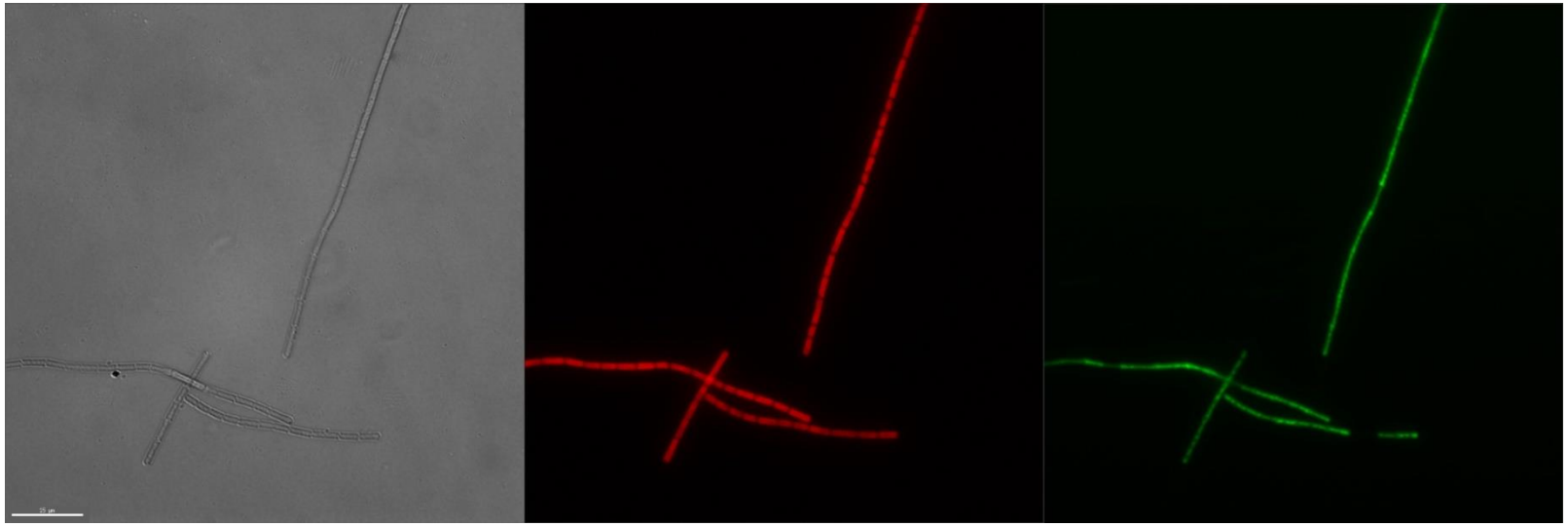
TAP DARK

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	387	1093	829	327	555	676	885	392	210	249	602	655
B	966	230	464	486	321	1716	493	404	529	756	620	612
C	341	333	275	316	950	425	481	318	238	406	426	503
D	749	229	362	250	331	448	984	357	701	256	495	552
E	376	751	674	222	365	1190	435	563	478	439	374	1540
F	445	446	418	932	732	285	2266	759	360	455	713	332
G	1280	474	217	399	265	223	271	509	444	903	499	363
H	1108	702	640	321	558	317	1375	421	1060	282	303	606

HSM LIGHT

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	430	1571	1253	589	1375	1486	1545	543	400	374	570	901
B	2074	348	973	546	478	6841	819	717	412	643	595	660
C	790	604	356	446	760	658	663	477	341	802	744	1139
D	1251	492	619	346	485	733	2113	471	699	433	689	570
E	356	1671	791	322	563	340	755	692	729	840	618	3239
F	646	455	589	1346	412	341	597	1143	601	466	1239	506
G	622	922	349	603	298	318	332	784	542	1350	818	594
H	1139	783	871	576	626	308	2201	571	2224	356	395	682

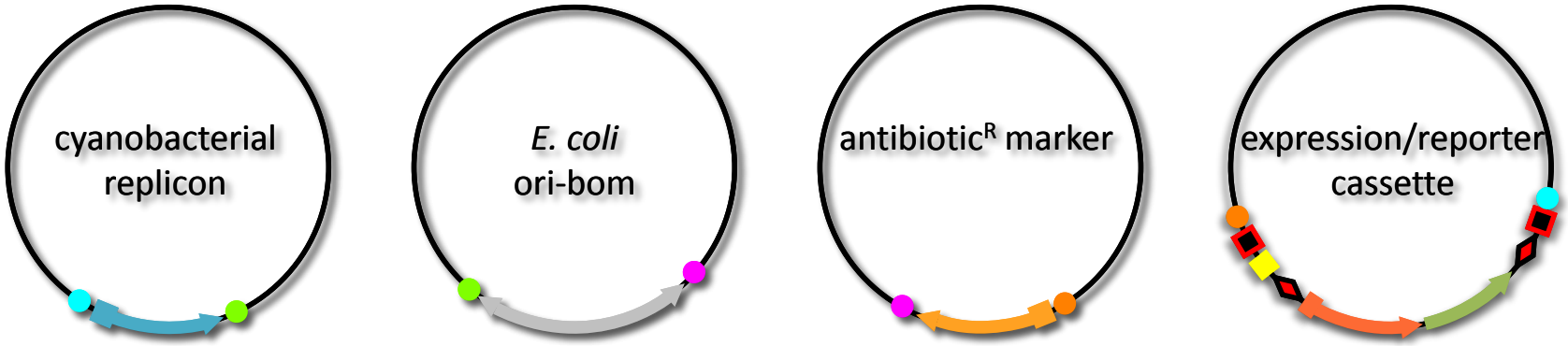
3- Technical Progress - reporter gene expression in commercial cyanobacteria J. Golden



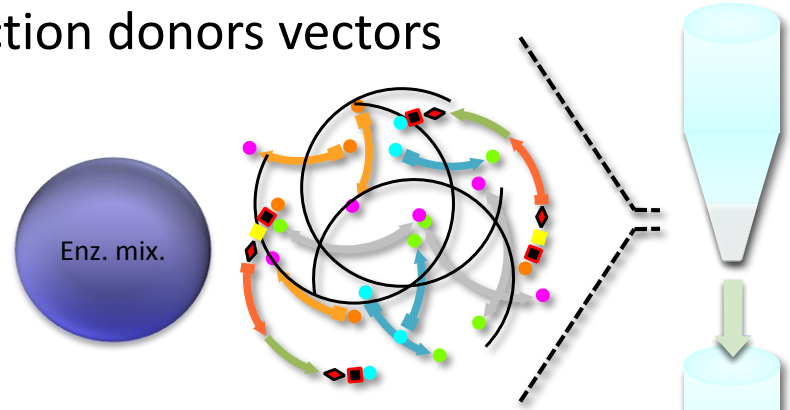
Light | Chlorophyll Fluorescence | YFP Fluorescence

- Cyanobacteria GAI-220 containing a broad host-range plasmid with a spectinomycin selectable marker and expressing a *yfp* reporter gene driven by a synthetic constitutive promoter

Assembly strategy for the modular construction of vector systems

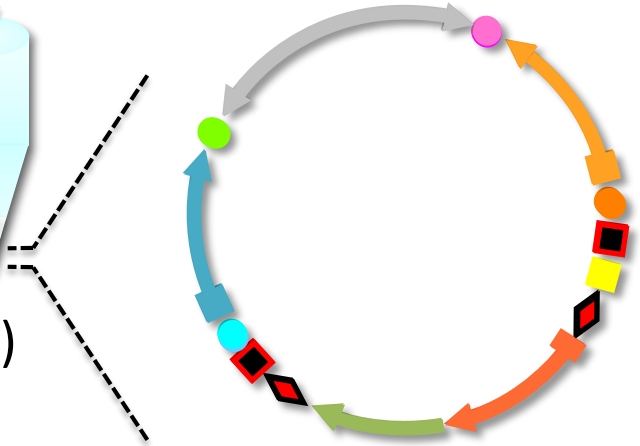


1. Selection donors vectors



2. Restriction digest

3. Column purification



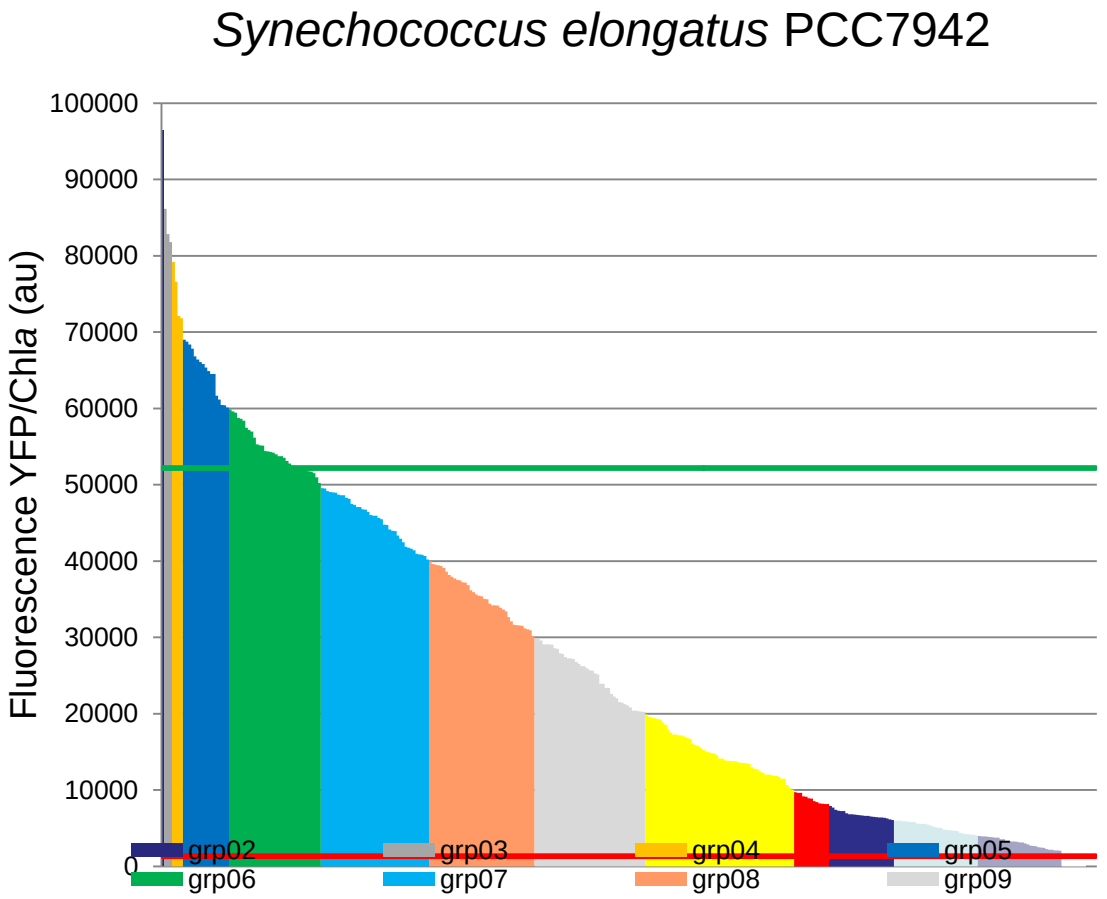
4. Assembly reaction:
Gibson or Seamless (Life Tech)

(Taton, et al. 2014
Nucleic Acids Research)

Constitutive Promoter Library

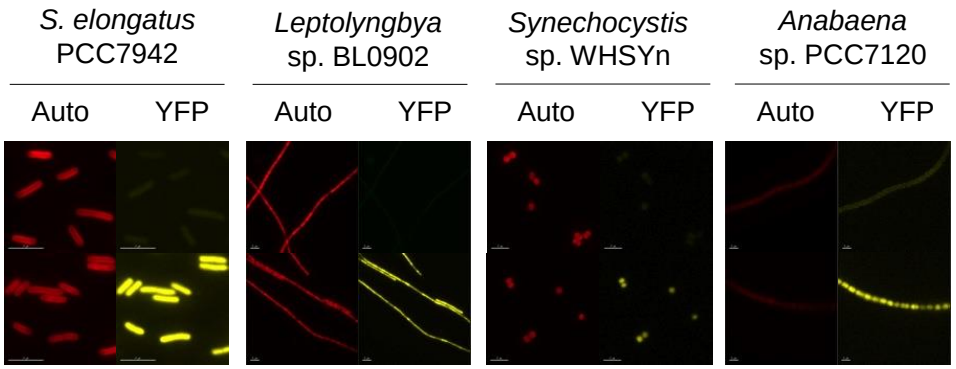
Mutagenesis of PconII promoter

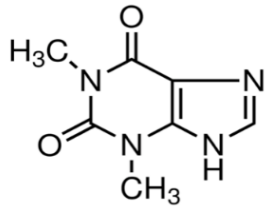
- 300 promoters first tested in *S. elongatus* PCC7942
- Cloned into RSF1010 plasmid for testing in multiple cyanobacterial strains
- Sorted into groups by strength
- Currently testing in GAI-220 for reporter gene expression

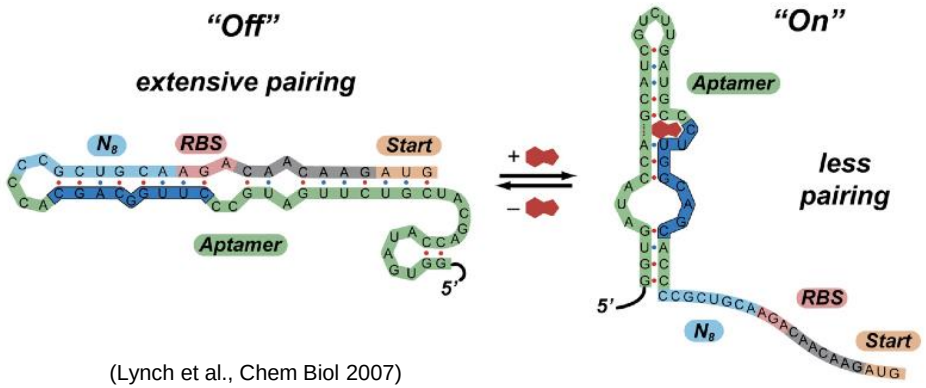


Synthetic theophylline-responsive riboswitches

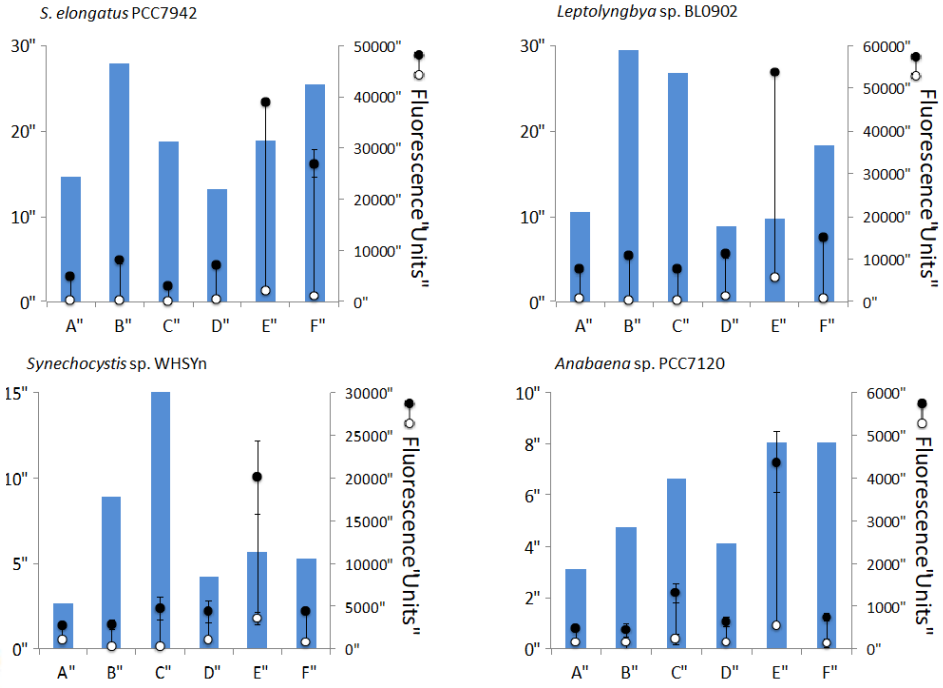
- Engineered 5' UTR of mRNA transcript
- Theophylline binding -> riboswitch conformation change allows translation
- Panel of 6 theophylline riboswitch variants (Topp et al., AEM 2010)







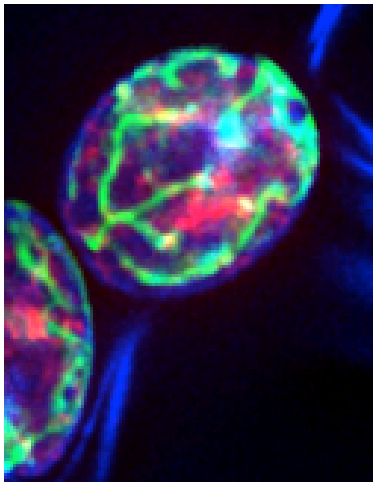
(Lynch et al., Chem Biol 2007)

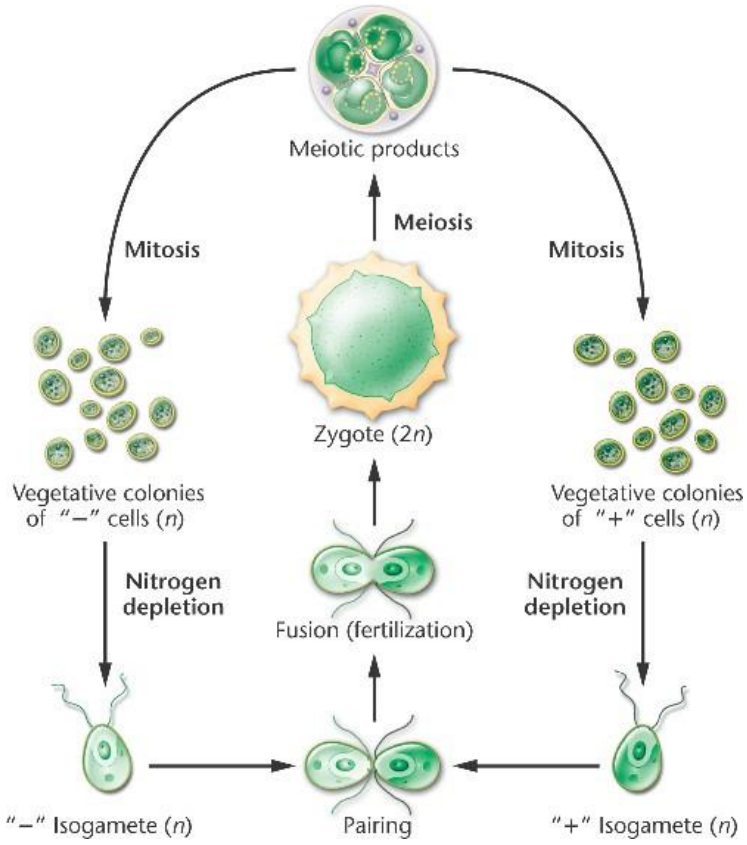


(Ma et al., 2014. Appl. Envir. Microbio.)

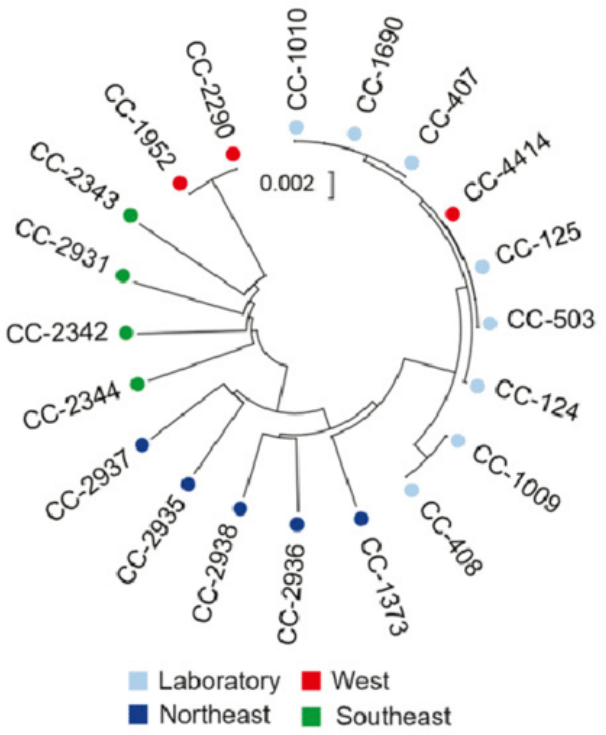
3 – Technical Progress - Genome Recombination Mating – Cell Fusion

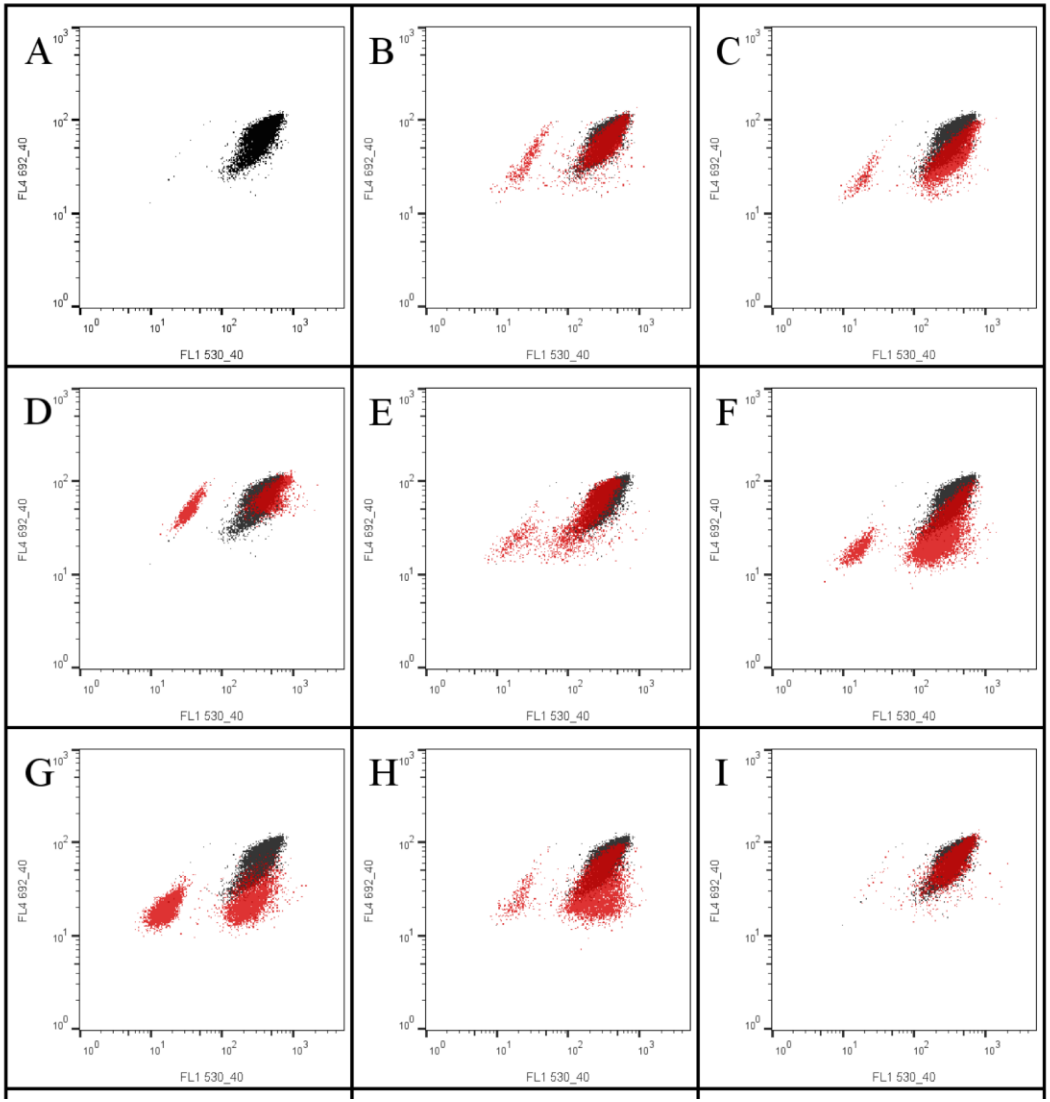
1. Done through sexual recombination or protoplast fusion to introduce genomic variation and create unique progeny
2. Shuffles multiple genes (or variants) in the genome and combines multiple traits into a single line



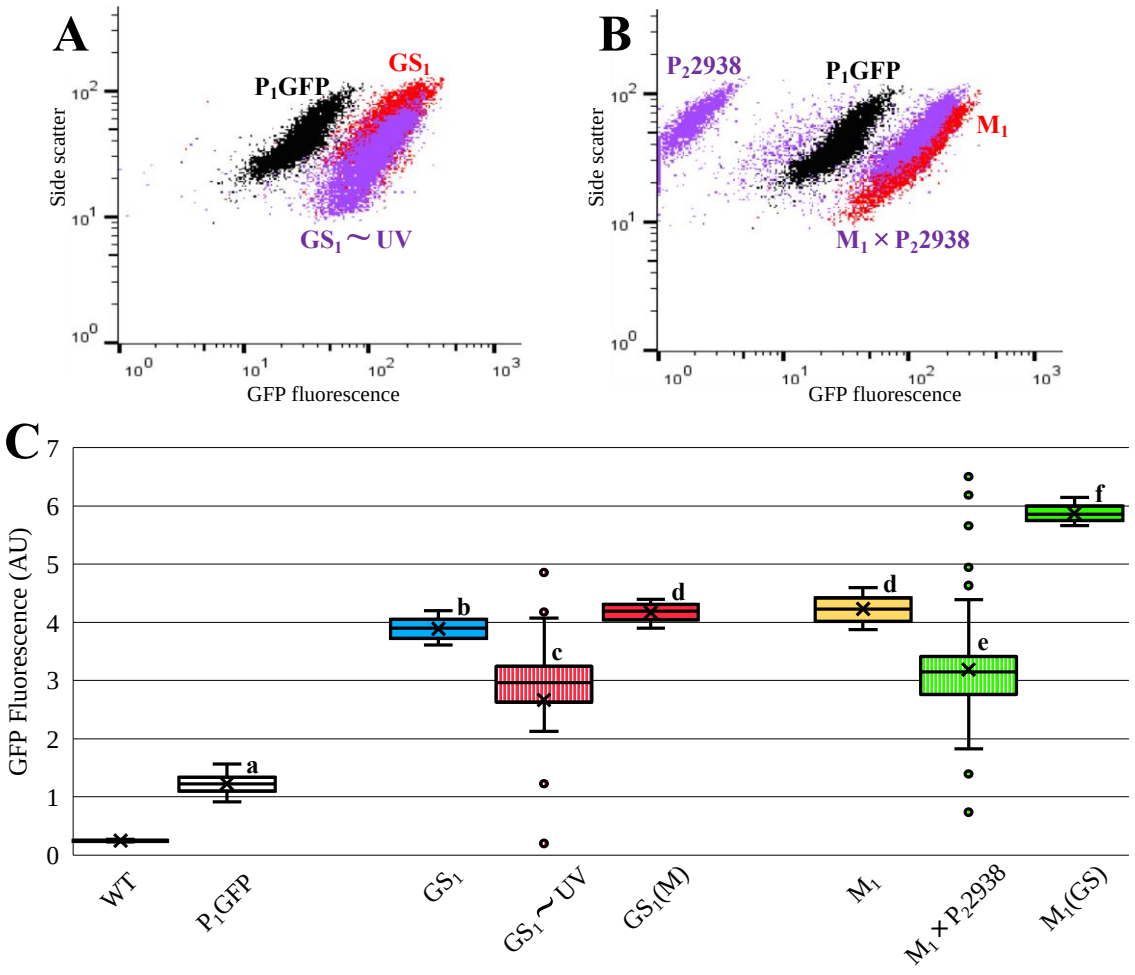


Parent GFP mt+ strain (A) crossed with diverse set wt *C. reinhardtii* isolates

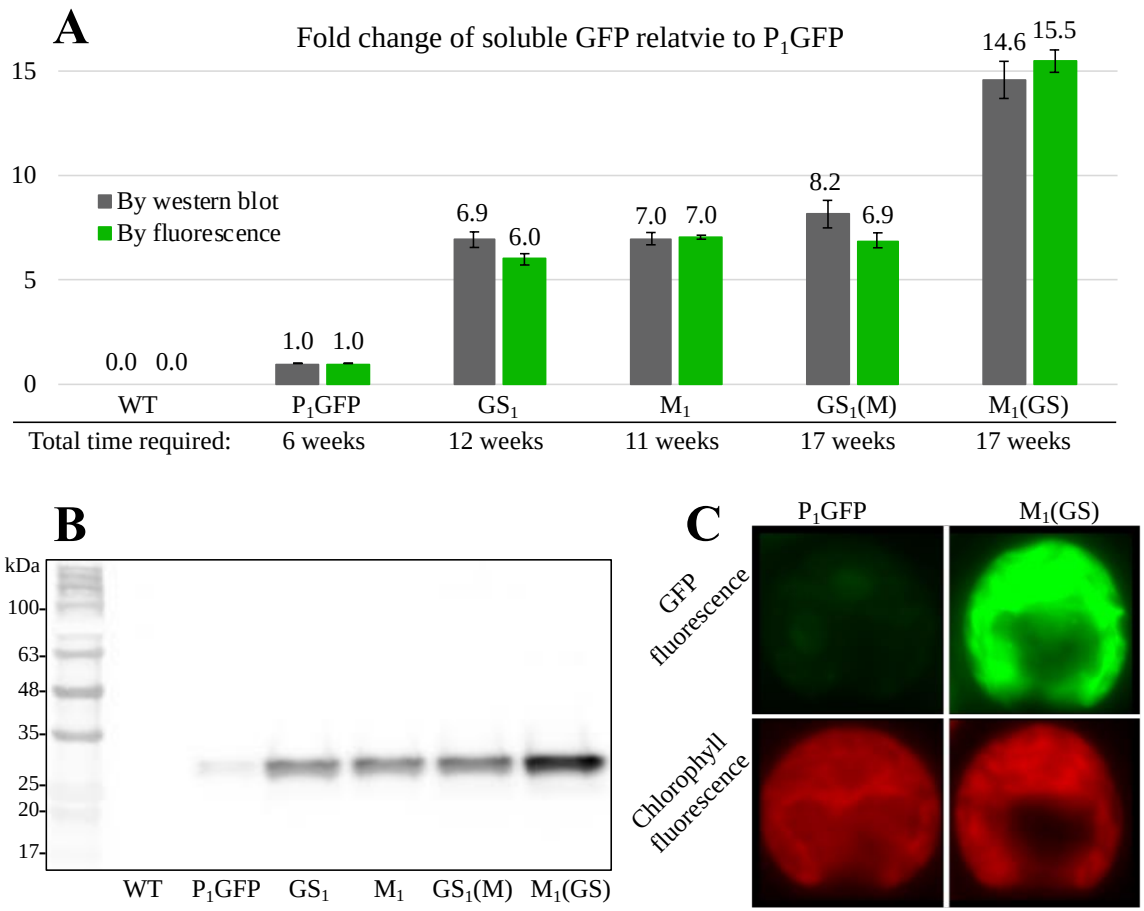




Mutagenesis to introduce genetic diversity Resulting in increased GFP expression



We can go from a wild type strain to an optimized strain in 17 weeks

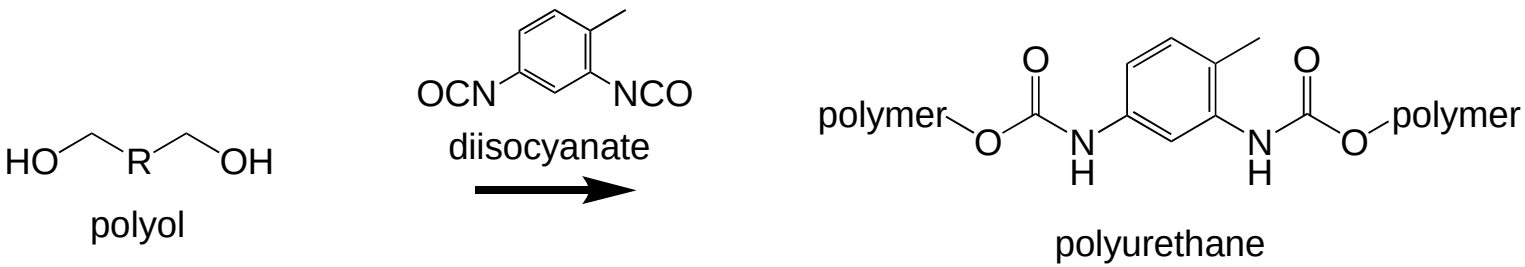


3 – Technical Progress - Algae oil extraction and conversion methods – M. Burkart

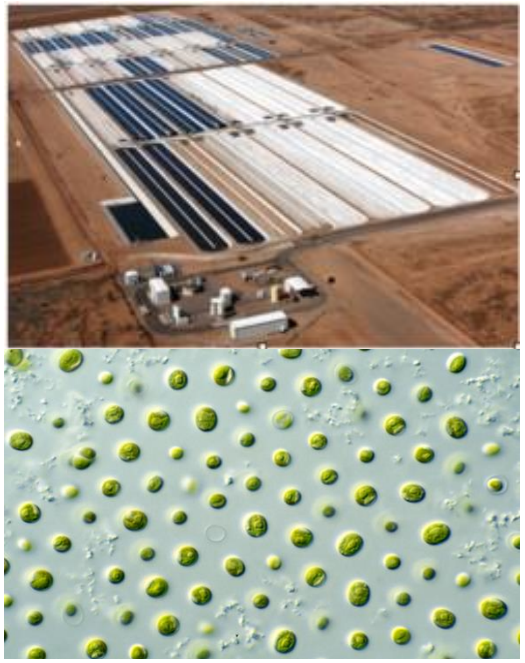
- *Isolation and conversion of lipids from algae and cynobacteria will prove critical to development of products for real-world applications*
- *Initial focus has been placed on polyols for flexible polyurethanes*

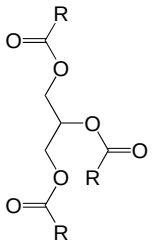
Photosynthetic -Sourced Polyols

Polyurethane Chemistry

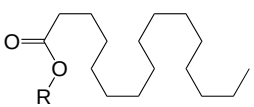


TAGs from Autotrophic Algae Cultivation

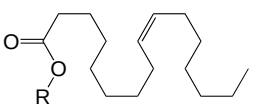




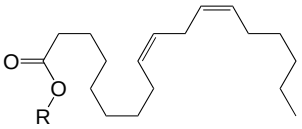
R = fatty acids



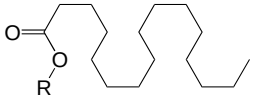
14:0 6%



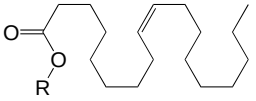
16:1 41%



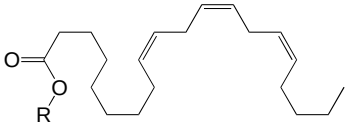
18:2 2%



16:0 20%



18:1 3%

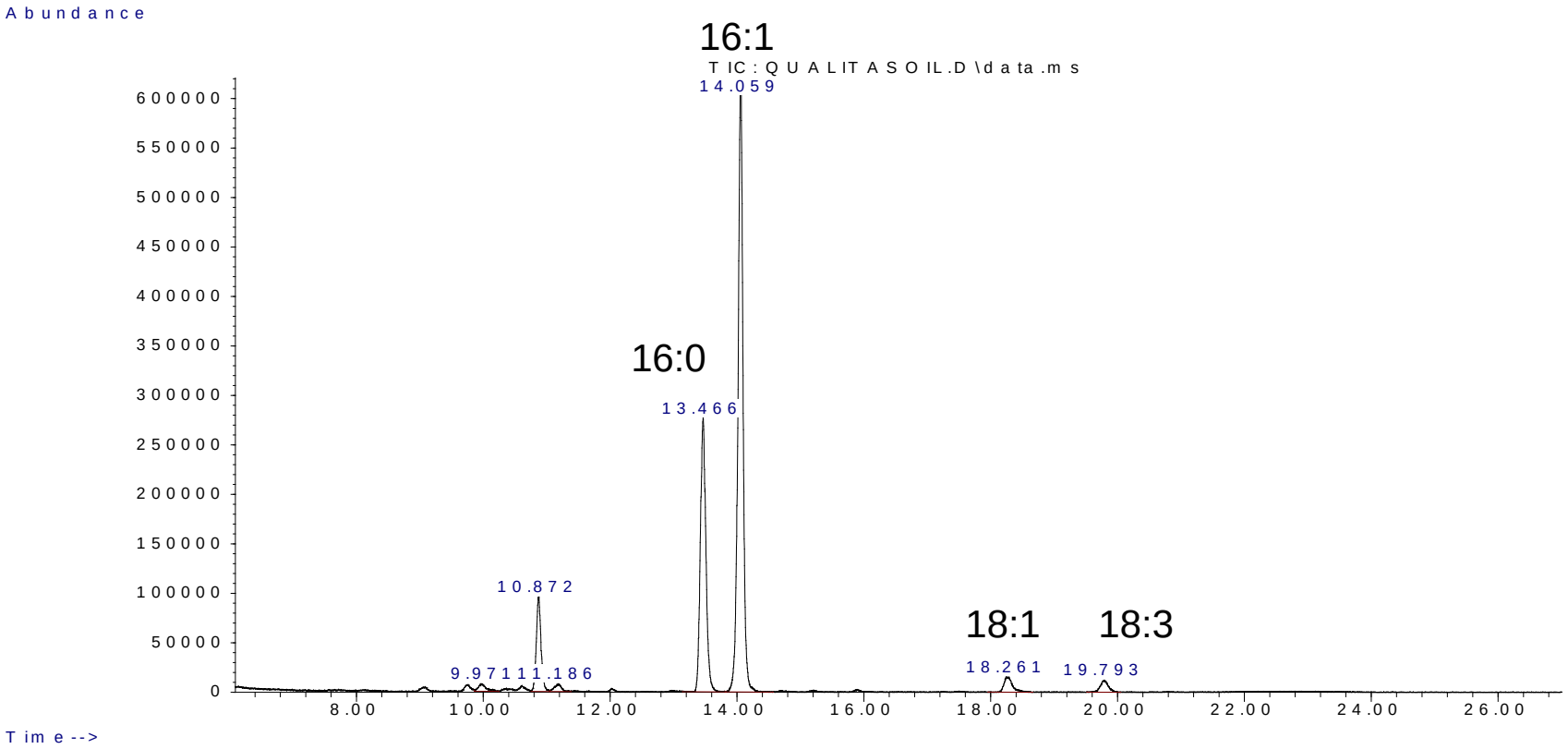


C20:3 4%

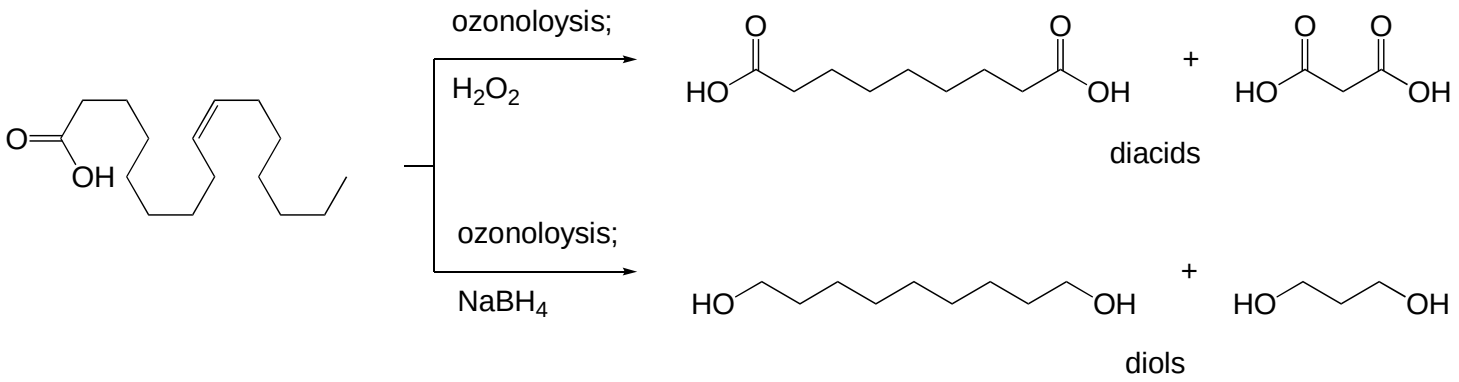
Nannochloropsis salina

Qualitas Inc.
Columbus, NM

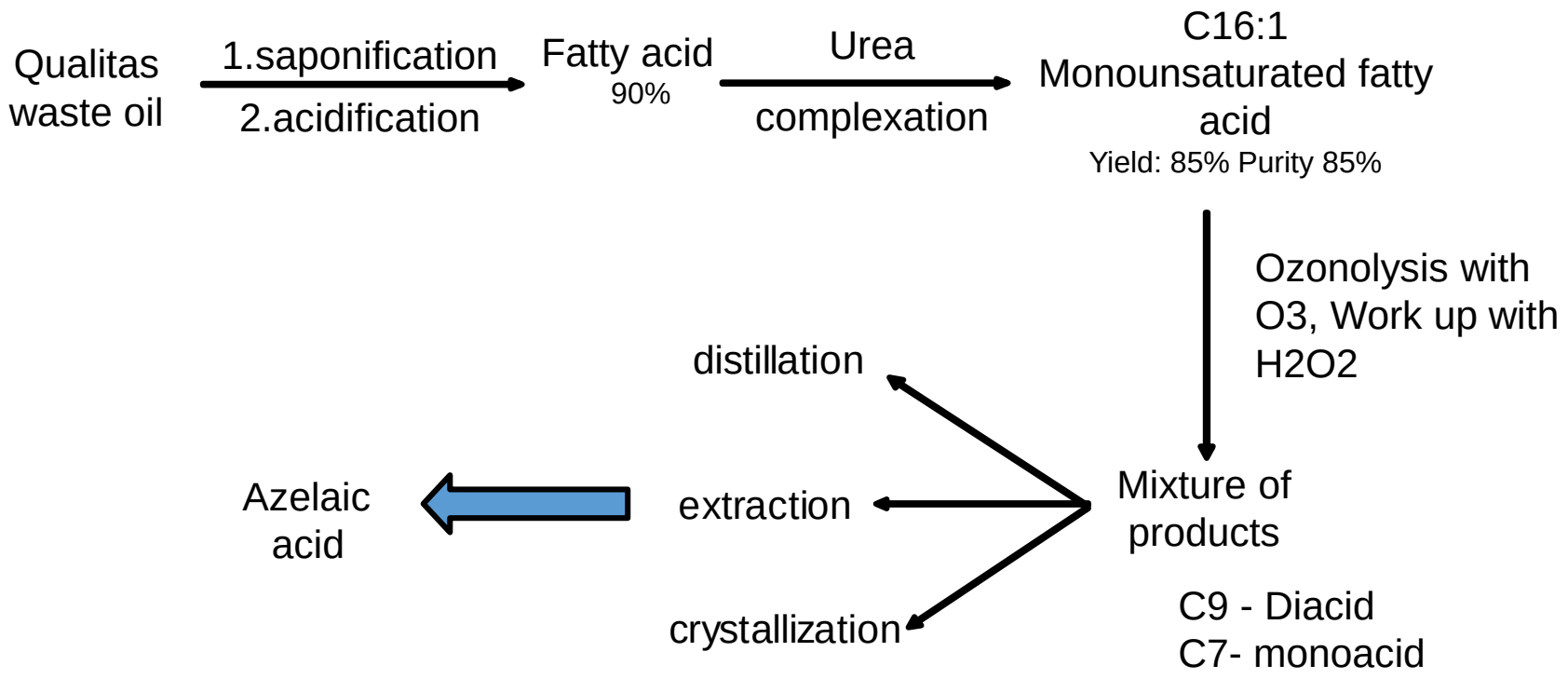
GCMS of FAMEs



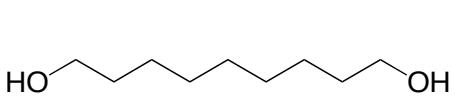
Diacid and Diol Preparation



Chemistry Flow
Original Azelaic Acid Pathway

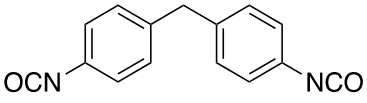


Polyurethanes produced with algae oil diols and Methylene diphenyl diisocyanate



1, 9 nonanediol

+



MDI

4 – Relevance

Developing advanced genetic tools, high-throughput screening methods, and breeding technologies for microalgae

- This project will enable algae biofuels to be more cost competitive with fossil fuels by improving yields and developing high value co-products
- Directly supports BETO's mission "to enable the cost-effective and sustainable production of drop-in biofuels"

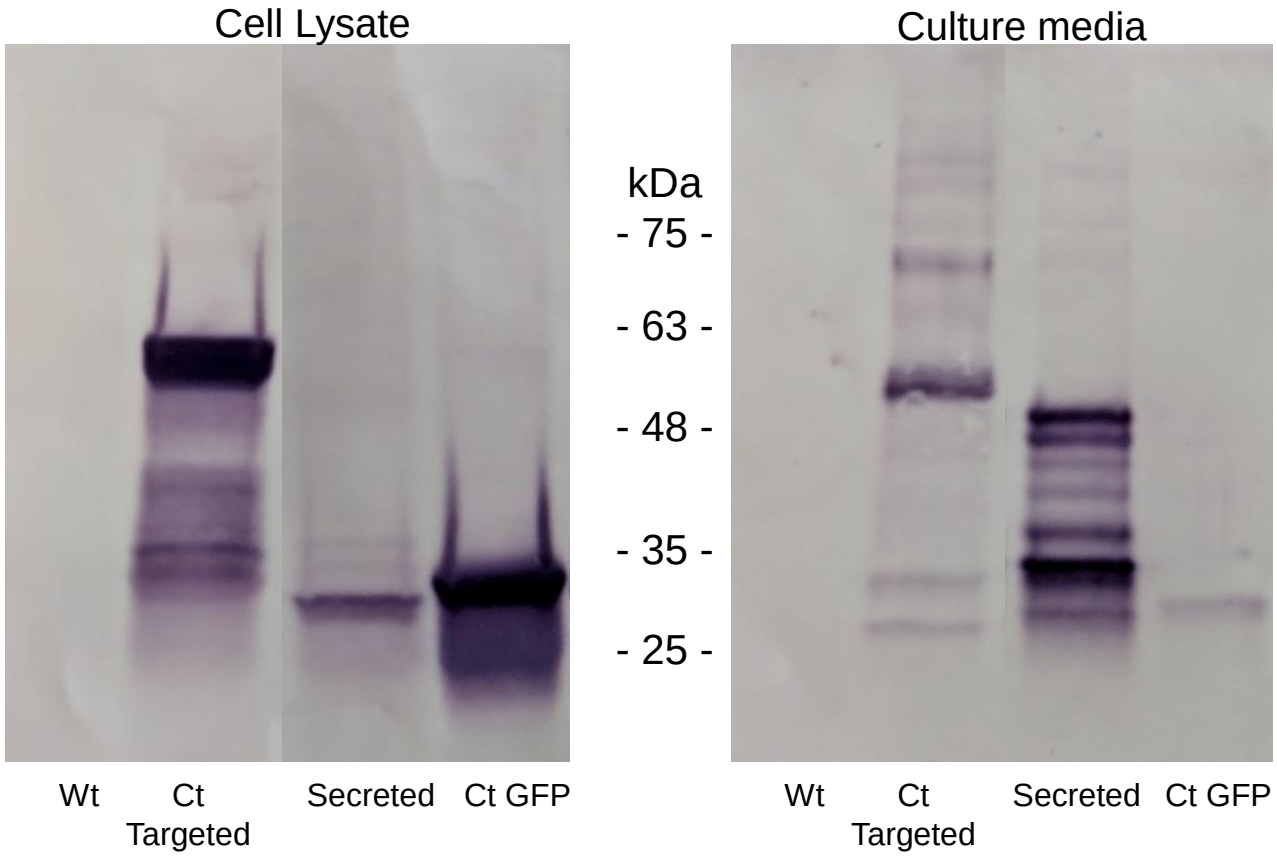
Relevance to Bioenergy Industry

- Metabolic engineering and co-products will both be required to enable economic fuel production from algae
- We are developing a *process* that can be applied to any algae species, especially those that are commercially relevant
- We are working directly with cost share partners to develop algae based co-products including recombinant proteins and renewable polymers

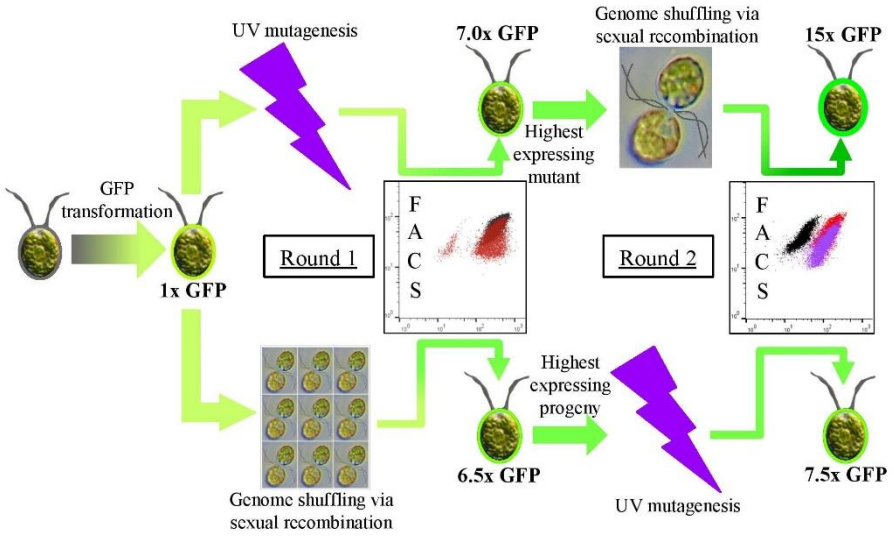
5 – Future Work

- *Identify improved synthetic promoters for recombinant protein expression*
  - *Screening of promoter library*
  - *Condition-specific inducible promoters*
  - *Develop site-specific integrase tools*
- *Strain improvement and commercial production*
  - *Mutagenesis and breeding for strain improvement*
  - *Production of commercial strains at Cal-CAB field station*
- *Develop genetic tools for commercial strains*
  - *Transcriptomics under different growth conditions*
  - *Promoter library and regulated expression systems*
- *Optimization of metabolite production*
- *100% algae polyurethane*

Accumulation of OPN in chloroplast or secreted by nuclear expression and protein targeting



Overlay Synthetic Biology and classical strain improvement for commercial production strains



Strain improvement process developed for Task 5.

GM and non-GM strains will be grown in greenhouse using paddle wheel ponds allowing characterization of transgenic strains without any EPA permitting

Summary

1. Overview – We are developing advanced genetic tools and high throughput screening processes that will enable increased productivity and value added co-products for commercially relevant strains
2. Approach – We are using a design – build – test – learn approach to develop tools that will enable the algae industry, not just a few select labs or companies
3. Technical Accomplishments – We have demonstrated the utility of the approach to increase recombinant protein production up to 15 fold for one reporter proteins, and have begun development of cyanobacteria vectors for commercial strains. We have also been able to advance a key product (renewable polyurethanes) that are already on their way to becoming a commercial product
4. Relevance – The bioeconomy is rapidly appraoching and these technologies will allow algae to play a significant role in this new era
5. Future work – The potential for algae as a bioproducts platform is clear, we need to develop the enabling tools that will allow this potential to be realized

Additional Slides

Responses to Previous Reviewers' Comments

- This is a new project that was not previously reviewed
- Also provide highlights from any Go/No-Go Reviews

Publications, Patents, Presentations, Awards, and Commercialization

Publications

- Hussam, N., Specht, E., Ostrand, J., Hoang, K., Karunanithi, P., Mayfield, S. (2018) High-throughput system for quantifying and characterizing homologous recombination in *Chlamydomonas reinhardtii*. Algal Research 31, 167-172
- Molino J., de Carvalho, J., Mayfield, S. (2018) Comparison of secretory signal peptides for heterologous protein expression in microalgae: Expanding the secretion portfolio for *Chlamydomonas reinhardtii*. PloS one 13
- Molino, J.D.V., de Carvalho, J.C.M., Mayfield, S. (2018) Evaluation of secretion reporters to microalgae biotechnology: Blue to red fluorescent proteins. Algal Research 31, 252-261
- Roulet, J., Taton, A., Golden, J.W., Arabolaza, A., Burkart, M.D., Gramajo, H. (2018) Development of a cyanobacterial heterologous polyketide production platform. Metabolic Engineering 49, 94-104.
- Fields, F.J., Ostrand, J.T., Tran, M., Mayfield, S.P. (Under Review) Nuclear genome shuffling increases recombinant protein expression in the chloroplast of *Chlamydomonas reinhardtii*. Submitted to Algal Research February 20, 2019.

Presentations

- Mayfield, Stephen (2019, January). “Use of Breeding, Mutagenesis and High Throughput Screening for Enhanced Recombinant Protein Production in Algae.” Presented at The 2019 Gordon Research Conference on Chloroplast Biotechnology, Ventura, CA.

Patents

- Roulet, J., Taton, A., Golden, J.W., Arabolaza, A., Burkart, M.D., Gramajo, H. Engineering polyketide synthase machinery in cyanobacteria. Patent Application No. 62643370, filed March 15, 2018.