Los Alamos NATIONAL LABORATORY

IGET: Informatics-based genetic tools for rapid enhancement of production strains

Blake Hovde

March 9th, 2021

Project Overview

- **Challenge**: Limited genetic tools are available for algal engineering applications.
- **Goal:** Rapidly generate a validated library of distinct promoter sequences to be used for generating specific gene expression levels.
- **Relevance**: Augmenting the *variety* of promoter sequences available to researchers, advancements in genetic engineering of algae will be obtained.

Currently, algal genetic engineering tools are limited across algal systems:

Currently:

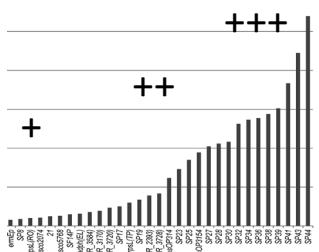
Strong constitutive Promoters available

+++

PSAD RBCS2
FCP CaMV35S

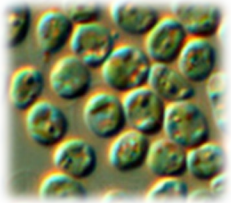
Goal:

Variety in promoter strengths



(Myronovski *Nat Prod Rep*. 2016)

Applications: Enabling fine-tuning of target product and co-product pathways.

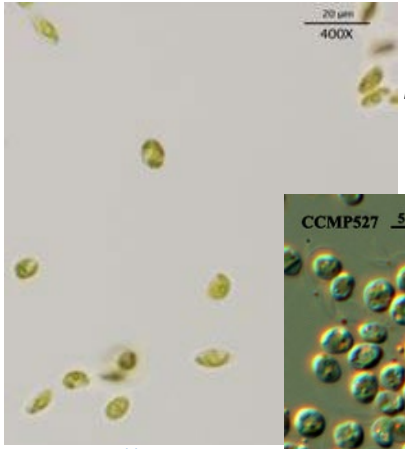


Biofuels

Co-products

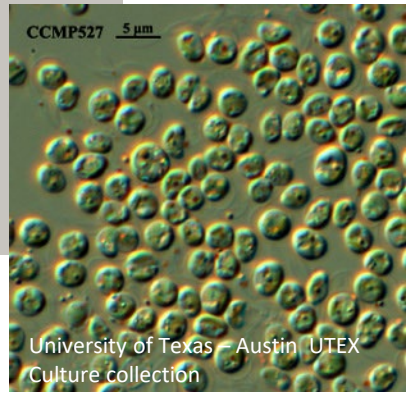
Project Overview

- Technical objectives:
  - Generate promoter libraries for industrially relevant algal strains *Nannochloropsis salina, Scenedesmus UTEX393 and Microactinium* sp. -
  - Utilization of transcriptomic data to identify promoter sequences analysis gene expression
  - Validation of promoter strength/inducibility in-vitro



Nannochloropsis

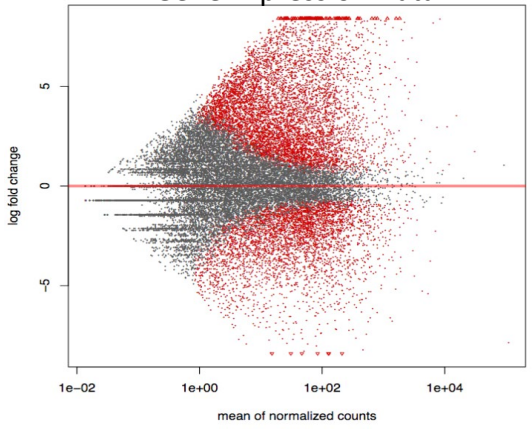
Scenedesmus



University of Texas – Austin UTEX Culture collection

NCMA: https://ncma.bigelow.org/ccmp527

Gene Expression Data



1 – Management

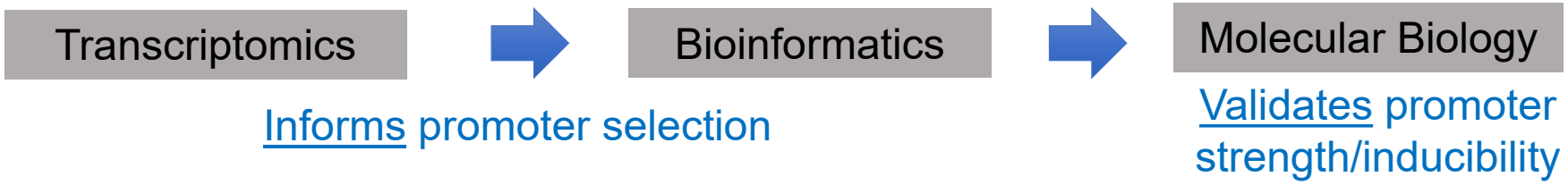
- *Blake Hovde* (PI – LANL) – Bioinformatics – gene expression analysis/promoter identification
- *Jackie Mettler* (Post Masters student – LANL/UNM) – Molecular Biology – transgene cloning and qPCR analyses
- *Raul Gonzales* (Scientist II – LANL) – Molecular Biology – cloning strategies and transgene design
- *Sangeeta Negi* (Scientist II – LANL) Algal cultivation and genetic engineering support

Project Structure:

Risks and mitigation: The main risk of this project is the contradiction of predicted promoter constructs and validation of gene expression during validation.

Regular progress updates:
-Weekly full team meetings for strategy/troubleshooting
-Quarterly reporting on deliverable to BETO

2 – Approach



- Utilization of transcriptomic analysis to identify genes that are differentially expressed in each algal species
- Identify a variety in a strength and inducible promoters to be validated using molecular biology techniques

Risks:

- Quantitation of gene expression is measurable/consistent?
- Promoter sequences identified provide a reliable level of gene expression in practice?

2 – Approach

Gene expression analysis (transcriptomics) to inform promoter selection

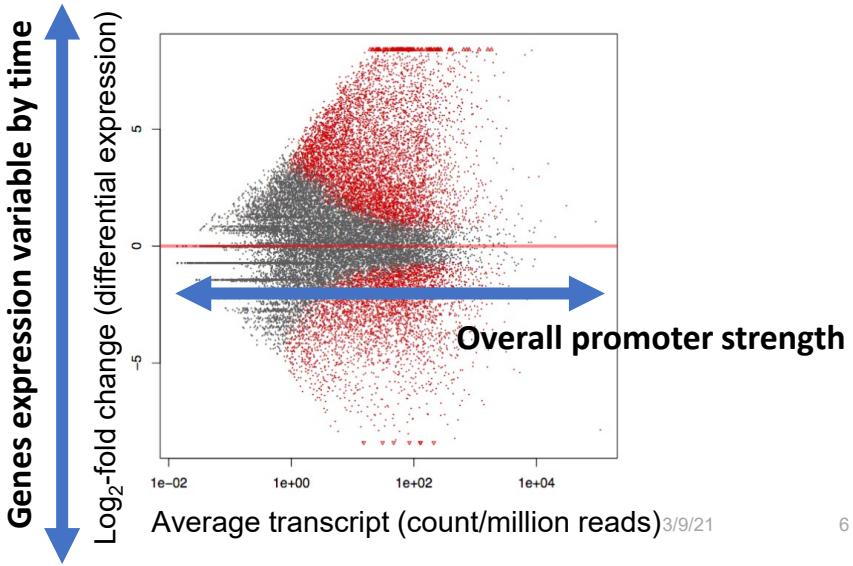
Transcriptomics → **Bioinformatics**

Informs promoter selection

General Concept:

- Select promoters with low variability over the experimental time-course
- Select a variety of promoters based on overall predicted strength

Gene expression data collected over 24 hours (light and dark periods)



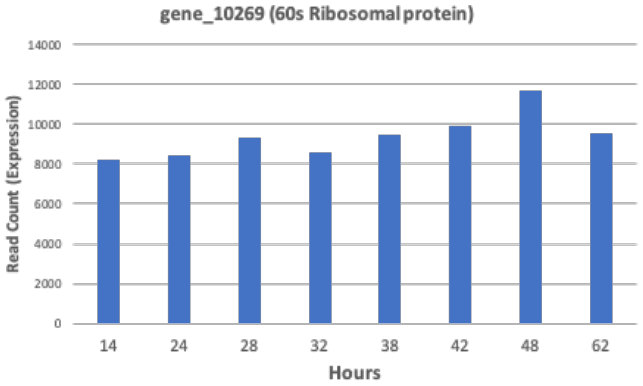
2 – Approach

Utilization of transcript data to identify promoters for the promoter library

gene_9489	13516	11196	12165	10787	14853	12790	17286	15680
gene_2709	10758	14298	14536	12861	12403	13547	21176	13865
gene_1581	11272	13903	13742	12082	11856	13335	20609	14770
gene_8128	10085	14379	14091	12002	9901	13129	20435	14741
gene_1996	9369	24247	26807	15070	7374	7604	23866	10779
gene_876	17682	9224	14510	17805	14965	5777	10060	11954
gene_9498	10145	12962	13614	11923	9927	11336	18835	15808
gene_5230	12220	11359	12429	10419	11097	11292	17284	14606
gene_13680	10003	11423	11989	10707	11130	11712	15237	14440
gene_12177	8926	11678	14098	12057	10511	10783	17014	13176
gene_9700	11081	10614	11909	10523	9887	11665	17521	17833
gene_6828	9579	11047	11537	9794	10312	11092	16345	13453
gene_8183	11625	8490	10199	9658	12055	10230	12911	15073
gene_954	10580	15259	17542	15201	11193	5264	15131	10458
gene_5455	10401	12895	18179	22419	10700	5736	10100	11324
gene_8286	14168	9501	13382	11760	9592	8575	13662	21653
gene_11425	10052	10941	10906	9575	10303	11004	16425	12556
gene_900	10842	9272	10520	8563	8919	9209	14770	18665
gene_145	11370	9544	11333	8894	10573	10540	12425	14082
gene_13539	8872	9299	10066	8422	9116	9810	15232	13202
gene_10365	8828	9716	10624	9795	9357	8784	14409	11377
gene_14125	12378	6457	11977	15697	13989	3127	5545	14032
gene_2287	7889	9558	9941	8423	9711	9006	14078	11890
gene_9668	9712	8143	8912	7992	8877	8602	12660	15423
gene_14792	9401	9576	13623	15796	10350	6471	10001	7722
gene_10269	8201	8407	9305	8589	9475	9906	11700	9569
gene_10135	12436	7162	10986	12185	11489	4444	7703	15199
gene_16837	8647	9309	10228	8565	6578	8473	13201	14928
gene_7613	8902	8449	8968	7731	8497	8980	12759	12130
gene_4317	7243	12183	23639	6155	6336	2097	10921	14768
gene_8428	8961	8574	11613	12226	12197	5877	8084	10228
gene_792	8836	7984	8762	7490	8069	8588	12419	13023
gene_7822	11617	5951	9420	11219	13541	4558	5335	9913
gene_1488	8305	8163	8733	7559	8101	8576	12201	11260
gene_6443	7744	8070	8327	7437	7785	8138	12553	11983
gene_5282	7487	8314	8559	7401	7691	9174	12483	10320
gene_8531	14458	3396	6547	8668	12609	5383	4031	10495

Expression data for ~12000 genes

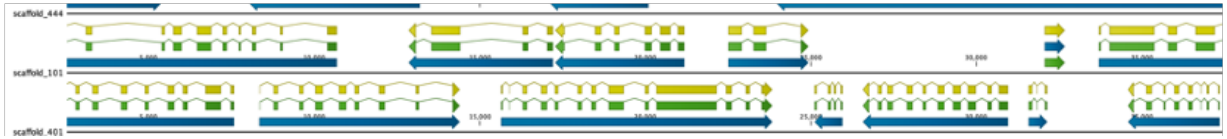
…



Identification genes with consistent expression over 48 hours

Annotated Scenedesmus genome

Find gene in the annotated genome

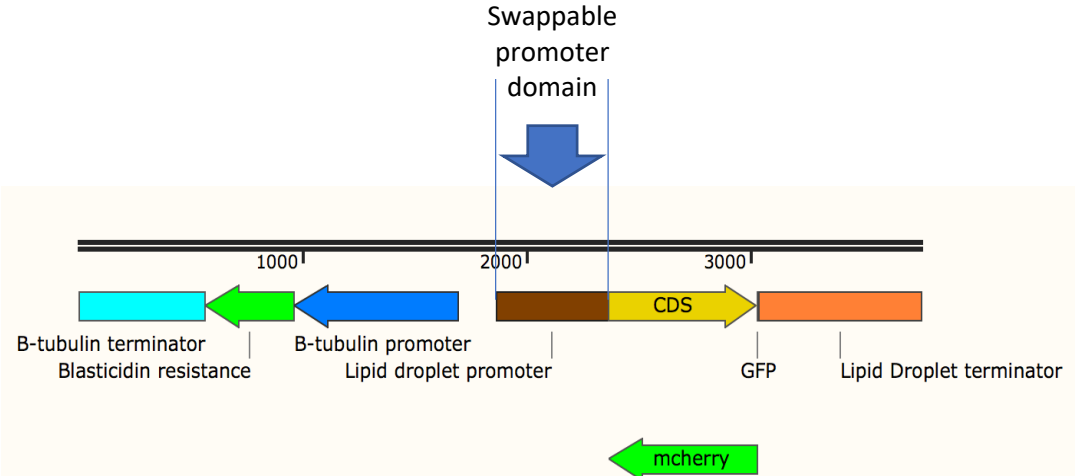


Capture upstream promoter sequence for cloning

2 – Approach
Cloning of candidate promoters into algal strains

Transcriptomics → Bioinformatics → **Molecular Biology**

- Originally, we planned to use random integration of transgene constructs
- Transgene Cassettes contain a swappable promoter domain for rapid cloning of new promoter sequences



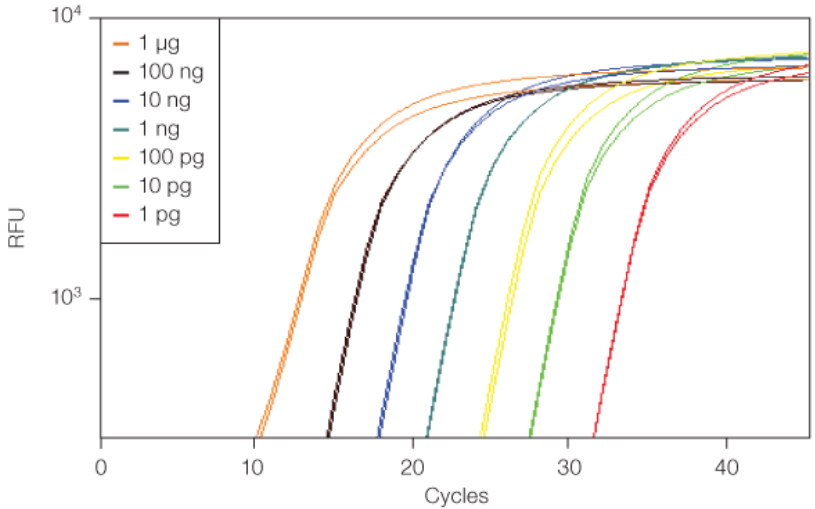
2 – Approach
Validation of promoter sequences using qPCR

Transcriptomics → Bioinformatics → **Molecular Biology**

To validate promoter strength:

- Quantitative PCR (qPCR) will be used to enumerate a more precise transcript number as a measure of promoter strength.

- qPCR has a much higher dynamic range than fluorescent signal



*Placeholder for real data

3 – Impact

- Goal: improve genetic engineering tools for algae by developing variable strength promoter libraries in a stable Cas9 algal cell line.
- If successful in completing these objectives:
  - Release of promoter library sequences in three industrially relevant algae.
  - Follow on work would include publication of a publicly available tool “ExpressTrain” that would allow any user to rapidly identify candidate promoter libraries for any organism with transcriptomic (RNAseq) data available including any strain passed through the BETO Blueprint project.
  - Each algal species is unique and requires a promoter library – this process democratizes this process.
- Industry impact
  - The promoter library is immediately useful to the academic and industrial algal research community utilizing *Nannochloropsis, Scenedesmus* and *Microactinium* species.

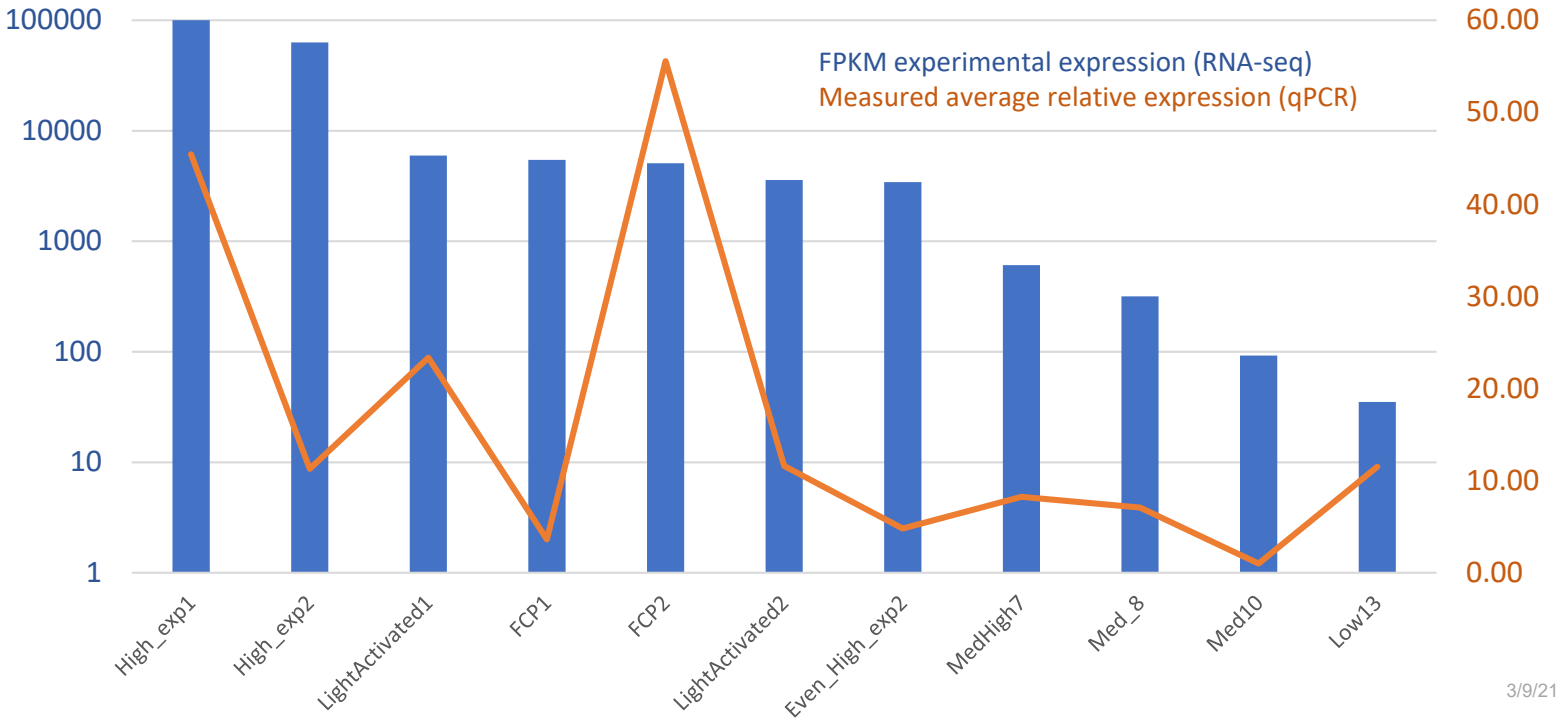
3 – Impact
Why Native promoters?

- Native promoter libraries are a complementary approach to the development of synthetic promoter libraries, but include a number of advantages
  - Rapid mobilization
  - Reduction of the potential for gene silencing
  - Condition specific expression

4 – Progress and Outcomes

Example – Nannochloropsis promoters

Very poor correlation between predicted and measured expression of clones

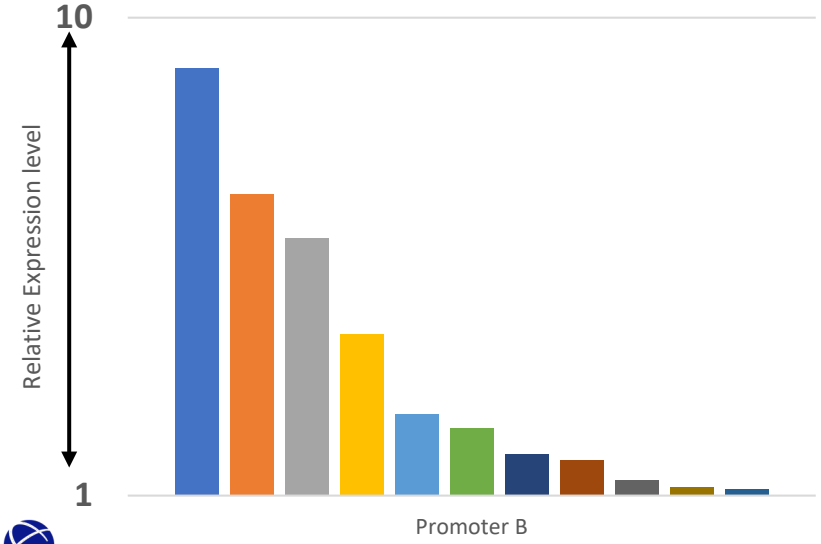


4 – Progress and Outcomes

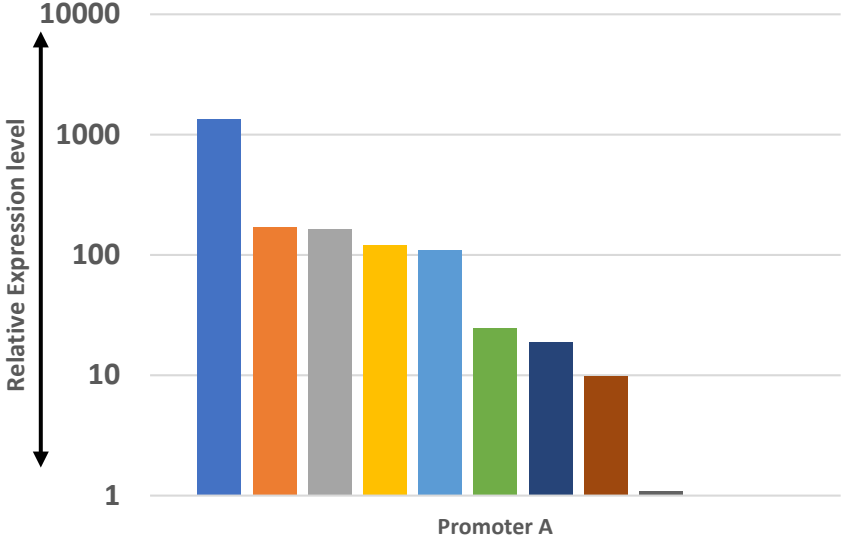
Example – Nannochloropsis promoters

High variability of expression between individual clones of the same promoter sequence likely due to random integration

Lower variability (still 10 fold difference) “Promoter B”



Extreme variability “Promoter B”



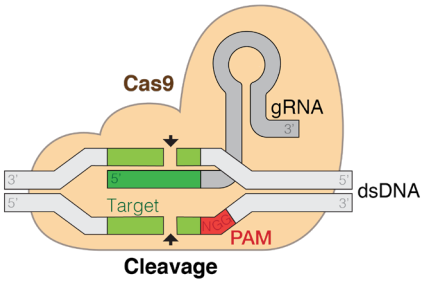
4 – Progress and Outcomes

Promoter libraries have been developed for *Nannochloropsis* and *Scenedesmus*

However, variable expression levels of these libraries – likely due to random integration effects - have made these libraries ineffective to date.

To remedy this:

We have been developing Cas9 (CRISPR) safe harbor cloning methods to consistently insert the test expression constructs into the same genomic location to improve replication and accurate promoter strength measurement



Summary

Overview: This project will provide researchers verified genetic tools for algal engineering and will enhance the genetic engineering toolbox greatly.

Approach:

Accomplishments:

- Development of use of two stable Cas9 expressing algal cell lines
- Determination of promoter variance based on consistent integration of promoter sequences

Relevance:

- Rapid development of promoter libraries
- Generation of stable Cas9 cell lines utilizing the developed promoter libraries
- Stable Cas9 cell lines lead to rapid genome engineering applications

Timeline

- Start date: 10/1/18
- End date: 9/30/21

	FY20	Active Project
DOE Funding	(10/01/2019 – 9/30/2020) $200,000	$600,000

Project Partners*

- No current partners

Project Goal:

To develop a standard method to rapidly generate a validated library of distinct promoter sequences to be used for generating specific gene expression levels.

End of Project Milestone:

Publication of a library of promoter sequences for three organisms as a public resource for the algal biotech community. Organisms include *Nannochloropsis salina, Scenedesmus obliquus* UTEX393, and *Microactinium* sp.

Barriers addressed

19Ft-C Feedstock Genetics and Variety Improvement:

Development of new tools for modern genetic engineering

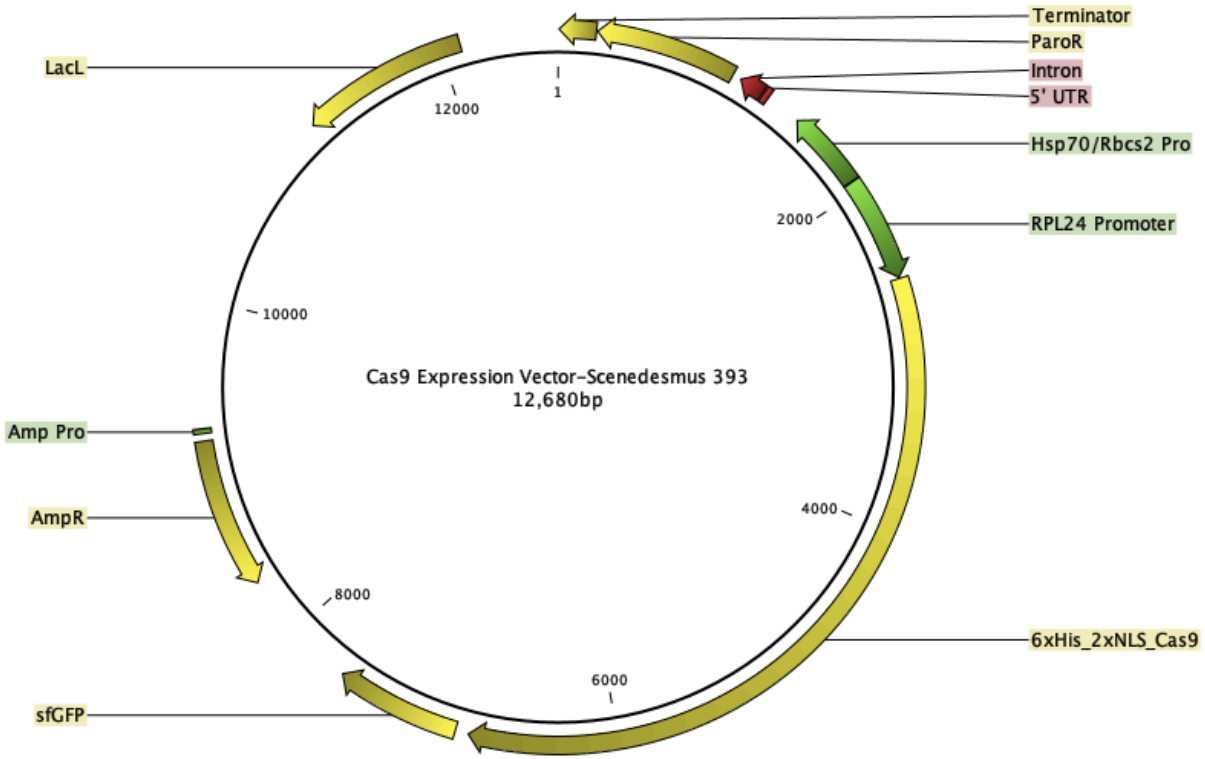
Funding Mechanism

N/A

AOP project

Additional Slides

Scenedesmus stable Cas9 vector



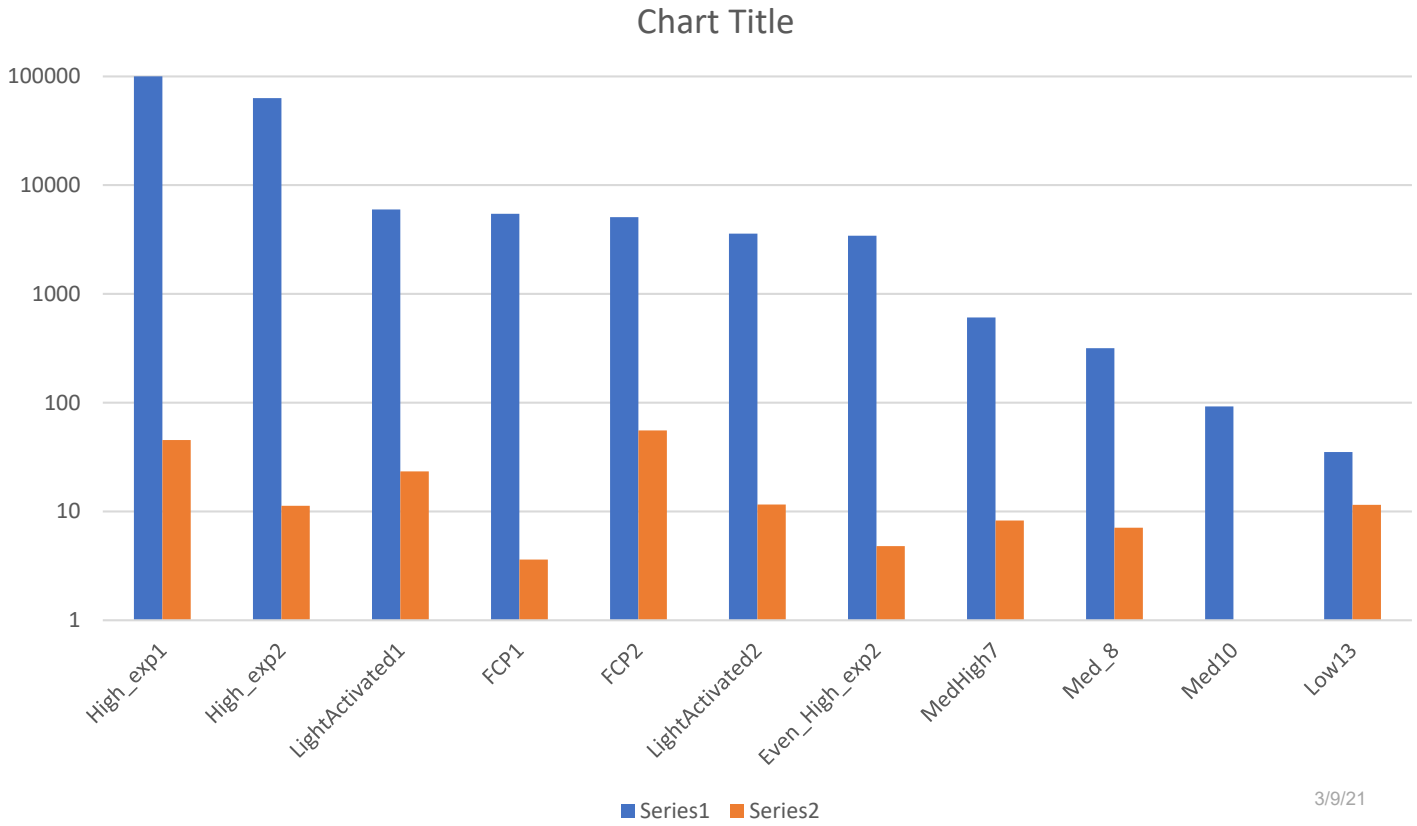


Chart Title
100000
10000
1000
100
10
1
High_exp1
High_exp2
LightActivated1
FCP1
FCP2
LightActivated2
Even_High_exp2
MedHigh7
Med_8
Med10
Low13
Series1
Series2

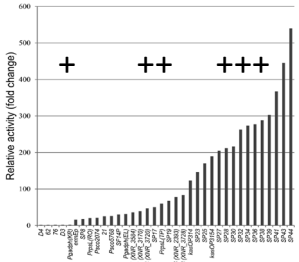
Rationale: Limited genetic tools are available for algal engineering.

Current:
Strong constitutive promoters

+++

PSAD RBCS2
FCP CaMV35S

Goal:
Variety in promoter strength



(Myronovski *Nat Prod Rep*. 2016)

Inducible promoters

+++

NIT1

"On" with nitrate

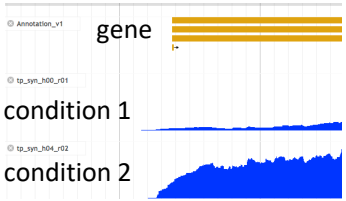
Additional conditions for gene activation

Light
Temperature
Nitrogen starvation

Approach:

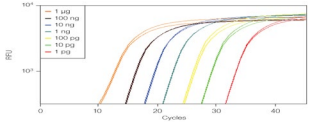
Transcriptomics

Informs promoter selection



Molecular Biology

Verifies promoter strength/inducibility



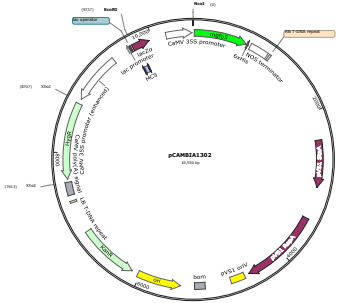
qPCR and fluorescent detection of actual promoter strength

Outcomes: a library of ten native promoters representing a variety of gene expression strengths and three additional inducible promoters.

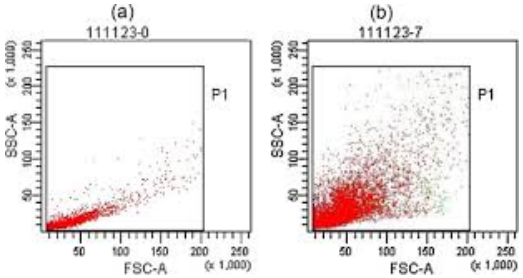
3-year AOP concept proposed from successful completion of seed: "IGET: Informatics-based genetic tools for rapid enhancement of production strains" – Application of these tools to three BETO algal production strains

PCAMBIA1302 as the backbone vector for promoter testing in *Scenedesmus*

- The pCAMBIA1302 plasmid has been used for *Scenedesmus obliquus* transformation by electroporation



- *Some Scenedesmus* cells transformed with pCAMBIA1302 show increased fluorescence on GFP channel on flow cytometer

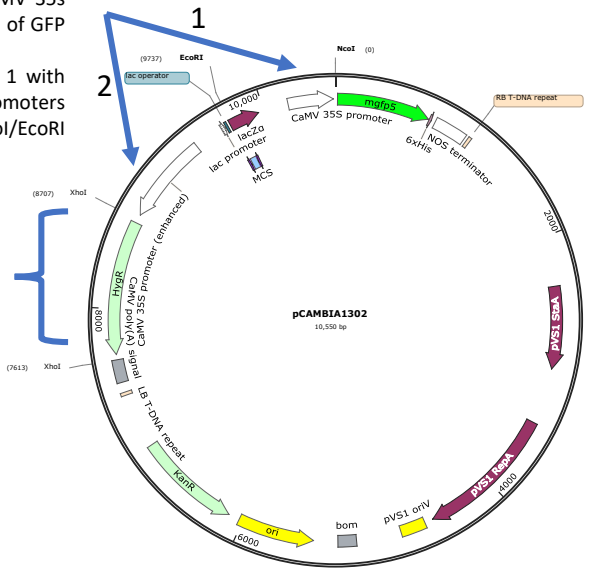


Guo et al. (2012). *J Biotechnol* 163: 61-68)

- Original vector uses CAMV 35s promoter for expression of GFP and antibiotic cassette.

- We will replace promoter 1 with *Scenedesmus* native promoters using NcoI/EcoRI restriction/ligation.

- Antibiotic marker can be swapped by XhoI restriction/ligation as needed for gene stacking

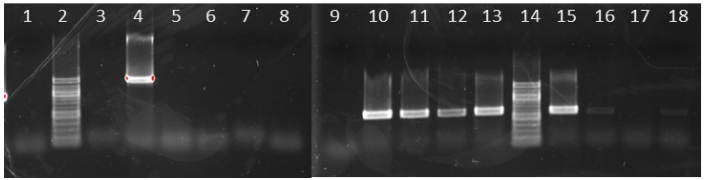


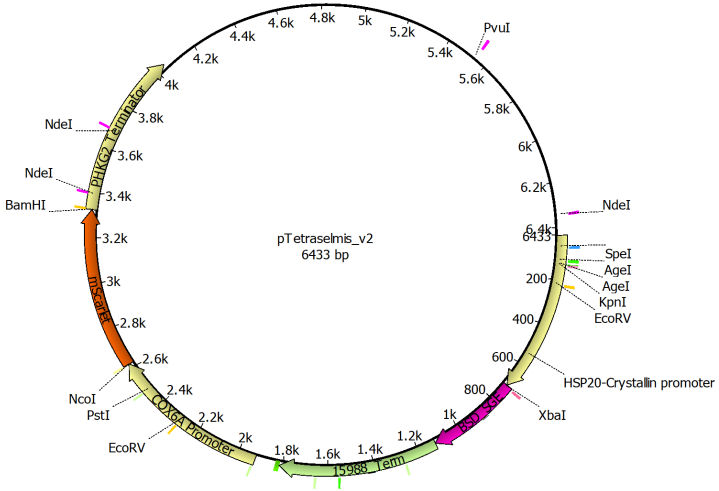
Strategy:

- Optimization of transformation conditions by flow cytometry/cell sorting.
- Preliminary testing of promoter strength by flow, with further confirmation by RT-PCR.

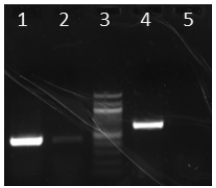
Current Status: Stable *Nannochloropsis* Cas9 lines

- Established stable Cas9 expressing strains:
  - N. salina (LANL)





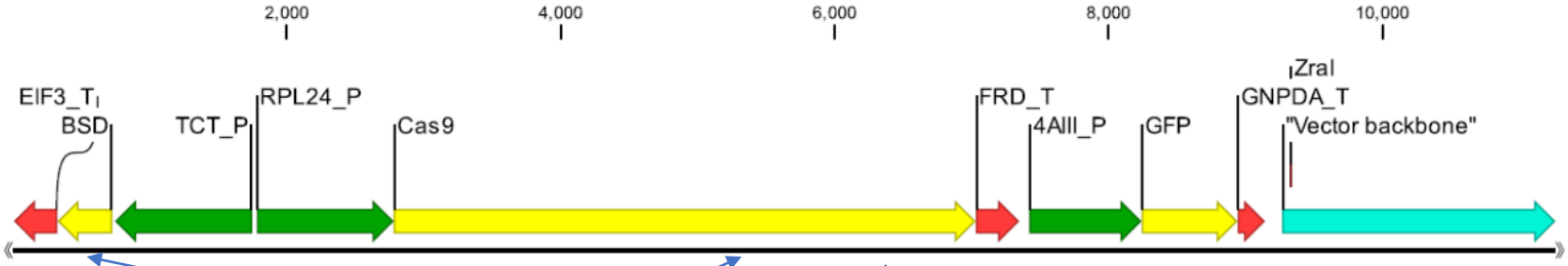
  - N. gaditana (Posewitz)



Shounak Banerjee, Scott Twary

Generation of CRISPR stable editing line
1)CRISPR expression

Randomly integrated Cas9 expression construct:



Codon optimized

Electroporation

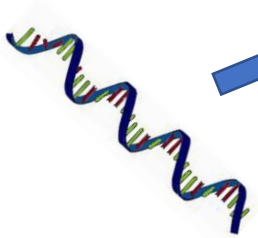
Nannochloropsis

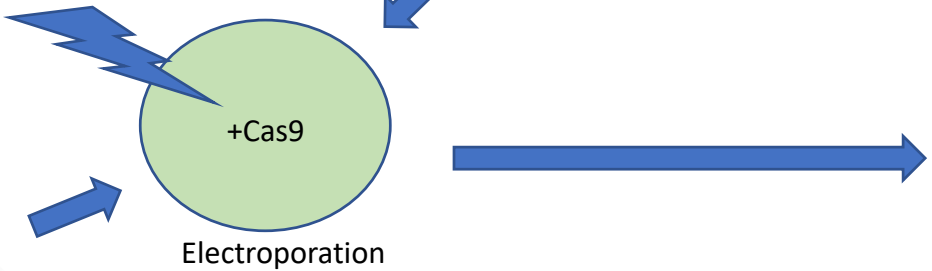
Screen for Blasticidin mutants and GFP

Ajjawi 2017, *Nat. Biotech*

Hygromycin expression construct:

gRNA:





Electroporation

Screen for Hygromycin mutants

Responses to Previous Reviewers' Comments

- If your project has been peer reviewed previously, address 1-3 significant questions/criticisms from the previous reviewers' comments which you have since addressed
- Also provide highlights from any Go/No-Go Reviews

Note: This slide is for the use of the Peer Reviewers only – it is not to be presented as part of your oral presentation. These Additional Slides will be included in the copy of your presentation that will be made available to the Reviewers.

Publications, Patents, Presentations, Awards, and Commercialization

- List any publications, patents, awards, and presentations that have resulted from work on this project
- Use at least 12 point font
- Describe the status of any technology transfer or commercialization efforts

Note: This slide is for the use of the Peer Reviewers only – it is not to be presented as part of your oral presentation. These Additional Slides will be included in the copy of your presentation that will be made available to the Reviewers.