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1. REPORT DATE (DD-MM-YYYY) 10-09-2012		2. REPORT TYPE Final			3. DATES COVERED (From - To) 04/01/2010-01/31/2012							
4. TITLE AND SUBTITLE Developing Molecular Genetic Tools to Facilitate Economic Production in Green Algae					5a. CONTRACT NUMBER 5b. GRANT NUMBER FA9550-10-1-0052 5c. PROGRAM ELEMENT NUMBER 5d. PROJECT NUMBER 5e. TASK NUMBER 5f. WORK UNIT NUMBER							
6. AUTHOR(S) Georgianna, David, R Gimpel, Javier Hannon, Michael, J Mayfield, Stephen, P					8. PERFORMING ORGANIZATION REPORT NUMBER 							
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12. DISTRIBUTION/AVAILABILITY STATEMENT DISTRIBUTION STATEMENT A: Approved for public release. Distribution is unlimited only.												
13. SUPPLEMENTARY NOTES N/A												
14. ABSTRACT It is now accepted that algae have enormous potential to generate economically viable and environmentally sustainable liquid fuels that can help mitigate the effects of a diminishing supply of fossil fuel. The achievement of economic biofuel production from any algal species will almost certainly require advanced genetic techniques. Although these tools are available for model algal species they are not readily available for algae that are being identified as potential biofuel production strains. Our work was focused on developing the genetic tools required to enable green algae to become efficient biofuel production strains. Being able to efficiently apply genetic transformation techniques to green algae species will allow us to generate strains that contain ideal traits for maximally efficient fuel production, and will also enable crop protection, improved nutrient utilization, and increased harvesting efficiency, all of which will help drive the economic viability of algal fuel production. Algae have two genomes that are typically transformed: the nuclear genome and the chloroplast genome, and each of these genomes require distinct sets of transform												
15. SUBJECT TERMS Algae, Biofuels, Algal Genetic Tools, Marine Algae												
16. SECURITY CLASSIFICATION OF: <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <td style="width: 33%; padding: 2px;">a. REPORT</td> <td style="width: 33%; padding: 2px;">b. ABSTRACT</td> <td style="width: 33%; padding: 2px;">c. THIS PAGE</td> </tr> <tr> <td style="text-align: center; padding: 2px;">U</td> <td style="text-align: center; padding: 2px;">U</td> <td style="text-align: center; padding: 2px;">U</td> </tr> </table>			a. REPORT	b. ABSTRACT	c. THIS PAGE	U	U	U	17. LIMITATION OF ABSTRACT UU		18. NUMBER OF PAGES 5	
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			19a. NAME OF RESPONSIBLE PERSON Stephen P. Mayfield 19b. TELEPHONE NUMBER (Include area code) 858-822-7743									

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AFOSR Final Performance Report

Project Title: Developing Molecular Genetic Tools to Facilitate Economic Production in Green Algae

Award Number: FA9550-10-1-0052

Start Date: 04/01/2010

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DEVELOPING MOLECULAR GENETIC TOOLS TO FACILITATE ECONOMIC PRODUCTION IN GREEN ALGAE

ABSTRACT

It is now accepted that algae have enormous potential to generate economically viable and environmentally sustainable liquid fuels that can help mitigate the effects of a diminishing supply of fossil fuel. The achievement of economic biofuel production from any algal species will almost certainly require advanced genetic techniques. Although these tools are available for model algal species they are not readily available for algae that are being identified as potential biofuel production strains.

Our work was focused on developing the genetic tools required to enable green algae to become efficient biofuel production strains. Being able to efficiently apply genetic transformation techniques to green algae species will allow us to generate strains that contain ideal traits for maximally efficient fuel production, and will also enable crop protection, improved nutrient utilization, and increased harvesting efficiency, all of which will help drive the economic viability of algal fuel production. Algae have two genomes that are typically transformed: the nuclear genome and the chloroplast genome, and each of these genomes require distinct sets of transformation vectors and protocols. In this proposal we aimed to improve the potential of marine algae by developing genetic tools for chloroplast through the following aims.

COMPLETED WORK

We have successfully met the three aims outlined in our AFOSR funded project (FA9550-09-1-0336). These aims were:

- 1) Identify regulatory elements of chloroplast genes from algae by functional analysis in the *C. reinhardtii* chloroplast**
- 2) Develop transformation technologies for the chloroplast of the marine algae *Dunaliella tertiolecta***
- 3) Express a recombinant protein at economically viable levels in a candidate production species**

In an effort to design what we hoped could be a set of universal algal transformation vectors, we identified highly conserved regions of plastid genomes and explored a number of regulatory regions from both characterized species as well as several marine uncharacterized species, including *D. tertiolecta*. Genes that were examined included: *psbA*, *tufA*, *atpA*, and *psbD*. From this work we identified a number of promoter and regulatory regions required for chloroplast gene expression, and designed a transformation strategy for new algal species based on this analysis. We have submitted an article describing this work, entitled “Analysis of Heterologous Regulatory and Coding Regions in Algal Chloroplasts” to the journal *Applied Microbiology and Biotechnology*.

a

Strain	Rel. mRNA Accumulation	Doubling Time [hour]
Duna	9.04 ±0.72	15.6 ±0.4
Haema	0.742 ±0.048	0
Scene	29.1 ±1.4	16.8 ±0.1
Oedo	1.54 ±0.10	0
Stige	2.57 ±0.22	0
Pseud	1.66 ±0.13	0
Chlor	1.60 ±0.12	0
Lepto	0.810 ±0.028	0
Micro	0.158 ±0.003	0
Pedi	0.739 ±0.068	0
Syne3	1.06 ±0.12	0
Chlamy	100 ±7	12.7 ±0.3

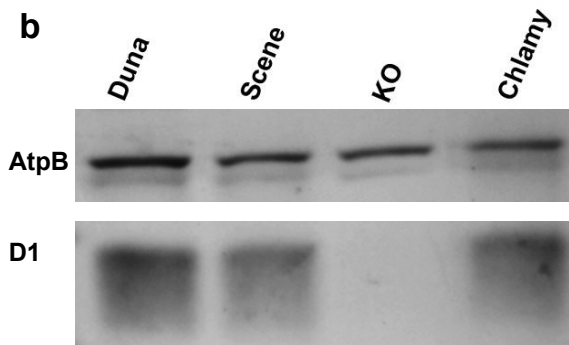
b

Fig. 1 *psbA* mRNA accumulation, photosynthetic growth rates and D1 protein accumulation of strains carrying heterologous *psbA* promoters and 5' UTRs. **a** Relative *psbA* mRNA accumulation quantified by qPCR. Photosynthetic growth rates in HSM media. **b** Anti-D1 Western blot. 15 µg of total protein were loaded in each well. The AtpB band is shown as a loading control.

integrates between the *psbH* and *psbB* genes and contains a *psbD* promoter/UTR driving expression of any gene of interest and an *rbcL* promoter/UTR driving erythromycin resistance for selection. Using this strategy we engineered expression of several recombinant enzymes in *D. tertiolecta* chloroplasts, including a xylanase gene from *Trichoderma reesei*; two homologues of α -galactosidase, one from *Lactobacillus acidophilus* and one from *T. reesei*; a phytase from *E. coli*, and a phosphatase from *C. crescentus* (**Figure 2**). These enzymes were chosen because

By examining heterologous promoters and terminators we were able to determine that while high levels of recombinant mRNA could be observed, recombinant protein production was lacking. As a result heterologous regulatory regions appear not to be a viable option for engineering the chloroplast of algae, due mainly to the high degree of species-specificity for utilization of 5' untranslated regions (UTRs) from plastid mRNAs (**Figure 1**). However, our data do show that heterologous proteins can be produced very well in *C. reinhardtii* chloroplasts, as long as codon bias is maintained within the coding sequence. Thus we determined that the best approach to achieve reliable expression of transgenes in chloroplasts of new algal species is to use endogenous regulatory regions coupled to coding sequences modified to reflect the codon bias of that species. For transforming the chloroplast of non-sequenced algae species it would be prudent to retrieve the host regulatory sequences along with homologous flanking regions for integration into the plastid genome, and this can be easily done with PCR using conserved primers.

Using the aforementioned endogenous promoter/UTR strategy we have successfully transformed the chloroplast of the marine algae *D. tertiolecta*. In collaboration with Sapphire Energy we have developed a complete homologous recombination vector for the chloroplast of *D. tertiolecta*. This vector

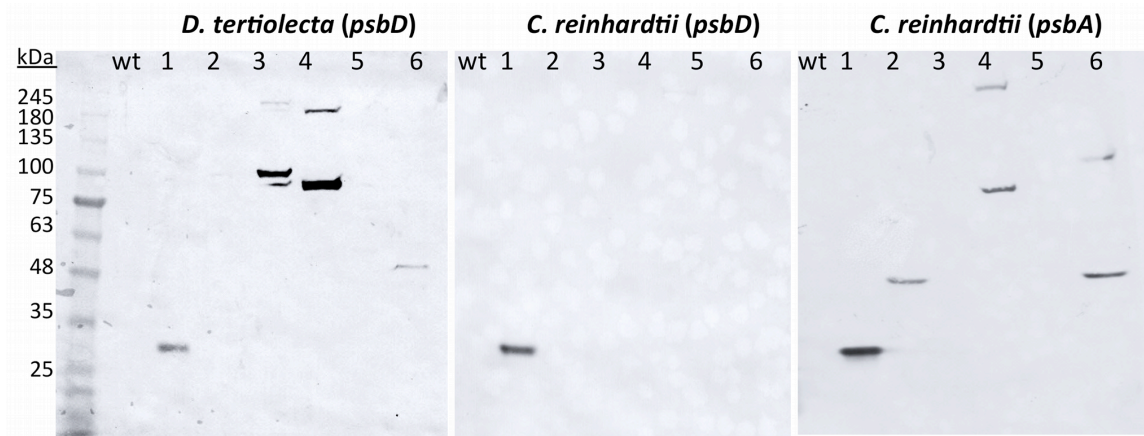


Figure 2: Western blot detection of recombinant proteins. Six out of 14 proteins were observable by western blot analysis. **wt** = wild-type; **1** = Xylanase from *T. reesei*; **2** = manannase from *A. sulphureus*; **3** = α -galctosidase from *L. acidophilus*; **4** = α -galctosidase from *T. reesei*; **5** = phytase from *E. coli* (a very faint band is visible for *D. tertiolecta*); **6** = a phosphatase from *C. crescentus*.

Table 1: Recombinant Enzyme Activity			
	<i>D. Tertiolecta</i> (<i>psbD</i> promoter)	<i>C. reinhardtii</i> (<i>psbD</i> promoter)	<i>C. reinhardtii</i> (<i>psbA</i> promoter)
Xylanase (<i>Trichoderma reesi</i>)	57.79 $\pm$ 10.04 U/mg	49.19 $\pm$ 9.28 U/mg	276.58 $\pm$ 34.92 U/mg
b-Mannanase (<i>Aspergillus sulphureus</i>)	0	0	0
α-Galactosidase (<i>Lactobacillus acidophilus</i>)	480 $\pm$ 340 mU/mg	0	1.7 $\pm$ 1 mU/mg
α-Galactosidase (<i>Trichoderma reesei</i>)	980 $\pm$ 200 mU/mg	10 $\pm$ 2 mU/mg	580 $\pm$ 260 mU/mg
Phytase (<i>Eschericia coli</i>)	180 $\pm$ 10 mU/mg	0	170 $\pm$ 8 mU/mg
Phosphatase (<i>Caulobacter crescentus</i>)	2.1 $\pm$ 0.2 mU/mg	0	4.4 $\pm$ 0.7 mU/mg

these are valuable industrial enzymes already marketed from bacterial production systems, and thus are potential co-products from algae grown for biofuel production. Both xylanase and α -galactosidase are essential components for the breakdown of complex carbohydrates from cellulosic material, thus these enzymes can potentially be used to produce sugars from cellulose for ethanol fermentation. In addition, we have shown improved production of xylanase by modifying the enzyme to increase expression levels, and we have expressed a thermo-stable variant of the enzyme. A manuscript entitled “Production of recombinant enzymes in the marine alga *Dunaliella tertiolecta*” has been submitted to the journal *Algal Research*. This manuscript describes the basic *D. tertiolecta* transformation system, and the production of industrial enzymes at levels potentially viable for commercial production in this marine biofuel production strain. **Figure 2** shows a comparison of production in *D. tertiolecta* relative to the model algae, *C. reinhardtii*. We observed that recombinant protein accumulation and enzymatic activity levels are similar and in some cases better for *D. tertiolecta* produced proteins (**Figure 2** and **Table 1**).

CONCLUSIONS

From this work we have learned that mRNA accumulation is less tightly regulated than protein accumulation and thus, endogenous 5'-UTRs appear to be crucial for the production of proteins. Using this knowledge we successfully engineered the biofuel production candidate marine green algae *Dunaliella tertiolecta* and produced several protein at levels equal to or exceeding levels achieved in *C. reinhardtii*.

We aim to later expand the repertoire of proteins expressed in *D. tertiolecta*, particularly with regard to modification of lipid production, and additionally work towards developing efficient transformation and recombinant protein production in other algae species. The work outlined here also sets the basis for future work on improving protein production and developing a universal heterologous or synthetic set of promoters/UTRs to allow facile and robust genetic modification of production algae strains.

Publications and manuscripts resulting from AFOSR funding

1. S.P. Mayfield (2011) Fuel for debate. *Nature* 476, 402-403
2. C.S. Jones and S.P. Mayfield (2011) Algae Biofuels: Versatility for the Future of Bioenergy. *Current Opinion in Biotechnology*. 23 1-6
3. J. Gimpel, and S.P. Mayfield (2012) Analysis of Heterologous Regulatory and Coding Regions in Algal Chloroplasts. *Plant Physiology*. *submitted*
4. D.R. Georgianna, M.J. Hannon, M.J. Hannon, M. Marcuschi, S. Wu, K. Botsh, A.J. Lewis, J. Hyun, M. Mendez, S.P. Mayfield (2012) Production of recombinant enzymes in the marine alga *Dunaliella tertiolecta*. *Algal Research*. *submitted*.