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REGULATION OF OIL BIOSYNTHESIS IN ALGAE

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14. ABSTRACT

Microalgae produce triacylglycerols (TAGs) which can serve as feedstock for the production of bio-based aviation fuel. TAG accumulation is maximal following nutrient deprivation, when algae enter a state of quiescence accompanied by changes in metabolism and cessation of growth. The long term goal was to increase basic knowledge of lipid metabolism and its regulation in the green algae Chlamydomonas as a model to provide targets and strategies for the engineering of biotechnologically relevant microalgal strains for maximal production of TAGs. Specific accomplishments include the discovery of a galactolipase and of a lipid transport protein and their role in TAG accumulation leading to a revision of our understanding of lipid metabolism and lipid trafficking in microalgae. A diacylglycerol

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Introduction

The search for sustainable sources of biofuels has led to renewed interest in microalgae as potential feed stock for renewable fuels including high energy density aviation fuels. Microalgae accumulate large quantities of oils in the form of triacylglycerols (TAGs), albeit mostly under nutrient-deprivation when growth also ceases posing a conundrum hampering the efficient production of algal fuels. A thorough analysis of the underlying molecular mechanism was at its infancy at the onset of this work. At the time, the unicellular green alga *Chlamydomonas reinhardtii* was the premier microalgal molecular model for the analysis described here but new models such as *Nannochloropsis sp.* emerged during the course of this study as described below. Although Chlamydomonas is not under direct consideration for the production of biomass for biofuel feed stocks, the analysis of its metabolism and physiology is expected to provide basic insights into mechanisms of TAG accumulation relevant to other microalgae and perhaps dedicated biofuel crop plants. Knowledge and understanding of algal model systems as gathered during the course of this work coupled with recent advances in genetic and genomic technologies have opened a wealth of new possibilities for the engineering of plants and algae and for further research. These new advances promise rapid progress towards the development of algal systems that could potentially produce feedstocks for aviation fuels on a scale that might soon supplement and possibly one day replace fossil high energy density liquid fuels.

Long Term Objectives.

Under permissive conditions, i.e. nutrient deprivation, many microalgae accumulate oils (TAGs) which provide an excellent feed stock for high energy aviation fuel production. It was the long-term goal of the funded work to identify genes encoding the enzymes and regulatory factors that are necessary for oil biosynthesis in microalgae using the model green alga Chlamydomonas. The work was undertaken with the premise that understanding the basic mechanisms of TAG biosynthesis and its regulation, and identifying factors and genes essential to the process will enable the engineering of improved algal strains as feedstock for the sustainable production of high energy density aviation fuels. To accomplish this goal, a set of specific objectives was pursed as described in the following.

Specific Objectives.

1. Analysis of insertional mutants. During prior work, extensive screening of Chlamydomonas lines carrying random insertions into their genome yielded 7 lines with reproducibly altered triacylglycerol amount or composition. A detailed analysis of the mutant phenotypes and the disrupted genes was pursued. Ultimately, focusing on two of the most promising lines led to the discovery of two important proteins and processes involved in TAG accumulation as will be summarized below.

2. Analysis of differentially regulated genes following nutrient deprivation. Global transcript analysis under oil inducing and standard growth conditions yielded a large set of differentially regulated genes, providing candidates with a function in lipid accumulation. Two particular genes encoding a lipase and a diacylglycerol acyltransferase, respectively, were further explored and led to novel insights as summarized below. An initial focus on putative transcription factors

with high abundance under TAG producing conditions did not identify a specific regulator of TAG accumulation and this approach was abandoned.

3. Analysis of proteins associated with lipid droplets. The most abundant protein in the lipid droplet fraction of Chlamydomonas is the Major Lipid Droplet Associated Protein, MLDP. Determining the exact role of MLDP in lipid droplet formation and its possible role in recruitment of other proteins to the lipid droplet was pursued to gain sights into the formation and turnover of lipid droplets as summarized below.

4. Identifying proteins involved in the regulation of quiescence. As the analysis of the original mutant population neared completion, a new screen for mutants that could not degrade TAG following nutrient refeeding was conducted with the goal to identify factors controlling the exit of algal cells out of quiescence. Quiescence induced by nutrient deprivation is a reversible state in the life cycle of unicellular algae and conceptually encompasses the adjustment of cellular energy metabolism, such as the induction of TAG biosynthesis following nutrient deprivation, in the context of the cell life cycle. Understanding of the factors and mechanism regulating quiescence will enable engineers to address a major conundrum hampering the efficient production of fuels from microalgae, i.e. the accumulation of TAGs at the cost of growth and biomass production. As will be described below, work under this objective has led to a very important breakthrough.

5. Development of Nannochloropsis oceanica CCMP1779 as a new microalgal model. The microalga *Nannochloropsis oceanica* CCMP1779 is unlike Chlamydomonas a biotechnologically relevant marine alga with a metabolism geared toward TAG production. To enable the development of a molecular tool box for Nannochloropsis, the genome was sequenced and basic technology such as stable nuclear transformation was developed for this organism. Students supported by the grant participated in the annotation of the genome and the development of the tools. It is anticipated that basic knowledge gained during the overall project can be applied towards the engineering of high TAG-yielding production strains of Nannochloropsis in the near future.

6. Lipidomics and quantitative lipid analysis. During the course of this work, it was necessary to define the TAG profile of the different algae under investigation. An improved lipidomics protocol was established and quantitative lipid analyses were conducted in collaboration with other AFSOR funded groups as summarized below.

Specific Accomplishments

Accomplishments are summarized in order of the objectives listed above and the basic experimental approaches are mentioned. As most of the results have been published, a brief synopsis of the respective papers will be provided and the reader is referred to the actual papers for further details.

1. Analysis of insertional mutants.

1.1 Identification of a novel galactolipase required for TAG accumulation in Chlamydomonas. One of the mutants showing decreased TAG accumulation following nutrient deprivation was *pgd1* (*plastid galactoglycerolipid degradation1*). The *pgd1* mutant shows a 50% reduction in TAG content following N deprivation. *In vivo* pulse chase labeling indicated that the galactoglycerolipid pool of the chloroplast is involved in providing acyl groups for triacylglycerol synthesis. *In vitro* enzyme assays showed that the PGD1 protein is a galactolipase that digests newly synthesized monogalactosyldiacylglycerol. Based on the analysis of the

mutant phenotype and the respective PGD1 protein a new hypothesis proposing the routing of de novo synthesized fatty acids through the galactoglycerolipid pool into TAGs was formulated. This new hypothesis supports suggestions by others that TAG biosynthesis in algae involves the chloroplast envelopes. The *pgd1* mutant also provides evidence for a physiological role of TAGs, namely relieving a detrimental overreduction of the photosynthetic electron transport chain. The mutant bleaches following N-deprivation, a phenotype that can be rescued by blocking the photosynthetic electron transport chain suggesting that TAG biosynthesis serves as a sink for reductant and carbon under conditions of nutrient deprivation. A mutant suppressor screen was conducted in the *pgd1* mutant background. It led to the identification of a reductase-encoding gene disrupted in the suppressor line providing further corroboration for TAG biosynthesis as sink for cellular reductant. The discovery of the *pdg1* mutant, the PGD1 protein, and its physiological function has been published in an original research article (Li et al., 2012b) and the hypothesis has been explained in detail in a published review (Liu and Benning, 2013). Work on the suppressor is ongoing.

1.2 Identification of a component of a lipid transporter in the chloroplast envelope of Chlamydomonas. One of the insertional mutants of Chlamydomonas which showed increased TAG content under nutrient replete conditions carries a deletion in the gene encoding an ortholog, *TGD*2, of a substrate binding protein component of a well known lipid transporter in plants. The ABC transporter in plants is involved in the transfer of lipids from the endoplasmic reticulum to the chloroplast, a process previously not thought to occur in Chlamydomonas. However, pulse-chase labeling experiments with radioactive acetate as the precursor of fatty acid biosynthesis clearly indicated that the mutant is impaired in lipid trafficking from the ER to the plastid. This represents an important finding, revising some of the current assumptions about lipid metabolism in Chlamydomonas. Additional labeling experiments with isolated chloroplasts using radioactive UDP-galactose demonstrated an increase in galactolipid biosynthesis. Given the above mentioned role of galactolipid turnover in the synthesis of TAGs (see *1.1*), this change in galactolipid synthesis rates in this mutant could possible explain the observed increase in TAG accumulation. In support of this hypothesis, fatty acids in TAG accumulating in the *tgd2* mutant resemble those of the major galactolipid suggesting that TAG is derived from galactolipid turnover in this mutant. However, we do not yet have a full understanding of the function of the TGD2 protein in TAG biosynthesis and the mechanistic studies to determine its function are ongoing. It should also be noted that cultures of this Chlamydomonas *tgd2* mutant age much faster than the wild-type demonstrating that the protein and the respective process it is involved in are essential for biomass production in Chlamydomonas. The work is ongoing and a publication of these findings is in preparation.

2. Analysis of differentially regulated genes following nutrient deprivation.

2.1 Identification of a TAG lipase with broad substrate specificity. One of the most drastically affected classes of genes following N-deprivation encodes lipases. Of those the focus was on a highly induced gene encoding LIP1. Using a yeast lipase null mutant strain, it was shown that the LIP1 gene from Chlamydomonas suppresses the lipase deficiency-related phenotypes of the yeast mutant. In Chlamydomonas LIP1 transcript levels and TAG abundance were inversely correlated under conditions of TAG accumulation and degradation. In vitro assays of the recombinant LIP1 protein demonstrated its lipolytic activity against diacylglycerol (DAG) and polar lipids. Reducing the abundance of LIP1 in Chlamydomonas using an artificial microRNA approach resulted in an apparent delay in TAG lipolysis when nitrogen was resupplied. These

data suggest that LIP1 is involved in TAG turnover in Chlamydomonas. The findings on LIP1 and its role in Chlamydomonas have been published (Li et al., 2012a).

2.2 Characterization of a type 2 DGAT from Chlamydomonas and its introduction into plants. Diacylglycerol acyltransferases (DGATs) catalyze the final step of TAG biosynthesis. Chlamydomonas has 5 Type 2 DGATs that were characterized. Some of the genes encoding these enzymes are induced following nutrient deprivation. Expression of the respective genes in yeast allowed the characterization of DGAT activity in this heterologous system. It was noted that DGTT2 had particularly high activity in this system and in a collaborative effort the respective gene was expressed in the plant Arabidopsis to determine its utility for the production of TAG in plant vegetative tissues. As such this is an example of how findings made in Chlamydomonas and tools generated under this project are used for translational research. TAG synthesis in vegetative tissues of plants to enhance the energy density of biofuel crops is an alternative approach to the production of biofuel feed stocks from algae. Expression of DGTT2 altered the acyl carbon partitioning in the vegetative tissues of the transgenic plant. It was demonstrated that DGTT2 can accept a broad range of acyl-CoA substrates. Expression of *DGTT2* in Arabidopsis increased leaf TAG content. Notably some TAG molecular species contained very-long-chain fatty acids, likely due to the substrate specificity of DGTT2. Caterpillars of a generalist herbivore reared on transgenic plants gained more weight. Thus, the nutritional value and/or energy density of the transgenic lines was increased by ectopic expression of DGTT2 from Chlamydomonas. This work has been published (Sanjaya et al., 2013).

3. Analysis of proteins associated with lipid droplets. During a lipid droplet proteomics study leading up to this project a major lipid droplet associated protein, MLDP, was discovered in Chlamydomonas. A specific antibody against MLDP was generated and its versatility for tracking the abundance of lipid droplets during changing nutritional conditions was demonstrated. Thus MLDP can serve as a marker for the abundance of lipid droplets and indirectly TAGs. Based on this finding the antibody can be potentially used in detection kits to monitor TAG abundance in algal cultures. Moreover, the MLDP antibody was used to screen novel mutants of Chlamydomonas unable to degrade TAGs following nutrient resupply (see below, *4.*). It was hypothesized that MLDP recruits other proteins to the lipid droplet involved in the synthesis or turnover of TAGs. Using immunoprecipitation, five high molecular complexes containing MLDP and other proteins were identified. Using mass spectrometry, different proteins were found associated with the MLDP complexes, among them BTA1, an enzyme responsible for betaine lipid biosynthesis, TGD2, the protein discussed above under *1.2*, and every microtubule component (i.e. alpha- and beta-tubulin, and cytoplasmic dynein) suggesting association of lipid droplets with the cytoskeleton. Follow up studies to corroborate the functionality of these protein-protein interactions in vivo are still under way. For this purpose, a new in situ immunolocalization protocol for Chlamydomonas was developed that allows the detection of co-localization of specific proteins with lipid droplets and other structures.

4. Identifying proteins involved in the regulation of quiescence. To identify factors involved in the regulation of quiescence, a state of the cell that encompasses the cessation of growth and accumulation of TAG in Chlamydomonas following nutrient deprivation, random gene disruption mutants of Chlamydomonas were isolated that were impaired in degrading TAGs after nitrogen (N) deprivation followed by N resupply. The abundance of MLDP of Chlamydomonas,

which tracks with TAG abundance (see above *3.*), was used as proxy for lipid droplet turnover in an immunoblot screen. Of several candidates isolated, *cht7* (*compromised in hydrolysis of TAG 7*) showed strongly delayed degradation of MLDP and TAG. While growth of *cht7* in liquid N-replete medium was not obviously impaired, N-deprived cultures were slow to regrow following N-refeeding. This effect was not N-specific because phosphate starvation followed by refeeding or Rapamycin treatment, which causes a quiescence-like cessation of growth, followed by its removal showed a similar delay of *cht7* growth. Moreover, the viability of *cht7* during the first five days in liquid culture following N-deprivation was not compromised. Taken together, these data suggest that the *cht7* mutant is generally compromised in exiting nutrient deprivation induced quiescence.

The genome of *cht7* mutant harbors an ~18 kb deletion next to the tagging sequence affecting four predicted genes. Complementation analysis identified a single gene (Cre11.g481800, *C. reinhardtii* genome v5.3.1; *CHT7*) as responsible for the phenotypes described above. The predicted CHT7 protein contains two cysteine-rich motifs comprising CXC domains (Pfam 03638). CXC proteins have been shown to bind zinc and specific DNA sequences through their CXC domains. Green fluorescent fusion (GFP) protein based imaging and cell fractionation followed by immunoblotting localized the CHT7 protein to the nucleus. Global transcript profiles of *cht7* and the parental line were compared by RNA-Seq during mid-log phase of an N-replete culture and after 48 h of N deprivation. In line with previous observations, ~2600 genes were up-regulated and ~3300 down-regulated in wild-type N-deprived cells compared to wild-type N-replete cells. Comparing *cht7* N-replete and wild-type N-replete cells, ~1500 genes were found up-regulated and ~1500 down-regulated in *cht7*. Most strikingly, there was a substantial overlap in genes up-regulated (~500) and down-regulated (~900) between the two comparisons. Thus, a subset (~49%) of all genes that were misregulated in *cht7* during N-replete conditions, were expressed as if the cells had already entered quiescence. Based on this result it was hypothesized that CHT7 acts as a repressor in safeguarding against the premature activation of global transcriptional changes associated with quiescence during N-replete growth, and also to fully revert quiescence after N resupply. Activators of quiescence are postulated to fully turn on quiescence following N deprivation, but their inactivation after N resupply is insufficient in the absence of CHT7 to restore growth. To exert its effects on gene expression related to quiescence, one may hypothesize that abundance of CHT7 changes in response to the N supply. However, immunoblotting indicated that CHT7 protein abundance was relatively constant during the conditions tested. Using Blue Native (BN) gel electrophoresis, it was also determined that CHT7 is part of a larger protein complex that does not change in apparent size or abundance following N deprivation. The discovery of CHT7 and its possible role in regulating quiescence was recently accepted for publication in Proc. Natl. Acad. USA (Tsai et al., 2014), which is accompanied by a perspectives article, and an invention disclosure covering the use of this gene for the engineering of TAG accumulating algal strains has been filed.

The discovery of this protein is not only of great significance in addressing the current conundrum of the inverse relationship between TAG production and cell growth but also has potential implication for human health. How cells sense their metabolic status and translate this into decisions whether to divide or not is still poorly understood but of critical importance to the development of cancer. CXC domain proteins similar to CHT7 have been found in association with the retinoblastoma tumor suppressor protein RB in mammals, which represses cell division and is ablated in many types of tumors. Unlike yeast, Chlamydomonas has an RB protein as well

as CXC proteins like CHT7 and, hence, this green alga may serve as a unicellular model to study the regulatory relationship of cell metabolic status and cell division.

5. Development of Nannochloropsis oceanica CCMP1779 as a new microalgal model. Because none of the algae currently in use for biofuel production have a history of domestication, and engineering of algae is still difficult, there is a need to develop algal strains that can be readily optimized for industrial large-scale production of aviation fuels and other desirable compounds. To contribute to the development of such an algal model system and to enable a move from Chlamydomonas to a more biotechnological relevant alga, we sequenced the genome and two sets of gene transcripts in *Nannochloropsis oceanica* CCMP1779, assembled the genomic sequence, identified putative genes and began to interpret the function of selected genes. This species was chosen because it is readily transformable with foreign DNA and grows well in culture. Moreover, it has been reported by others that applying reverse genetics by targeted gene replacement in *Nannochloropsis oceanica* has been feasible. The assembly of the 28.7 Mb genome of *N. oceanica* CCMP1779 was completed and RNA sequencing data from N-replete and N-depleted growth conditions supported a total of 11,973 genes, which were manually inspected by students funded under this grant to predict the biochemical repertoire for this organism. Importantly, more than 100 genes putatively related to lipid metabolism were identified and it became obvious that, while many gene families were reduced in size compared to other organisms, gene families encoding proteins involved in TAG biosynthesis such as DGATs were expanded in Nannochloropsis. As this organism also does not synthesize starch but accumulates TAGs transiently during the day, it appears that its primary metabolism is optimized for the production of TAGs. Further studies in this organism have been initiated to determine the factors that allow this alga to accumulate TAG up to 70% of dry weight. For this purpose, protocols for the transformation and insertional mutagenesis of *N. oceanica* CCMP1779 have been developed. The genomic and transcriptomic data along with the tools developed provide the basis for future detailed gene functional analysis and genetic engineering of Nannochloropsis species. This work has been published (Vieler et al., 2012).

6. Lipidomics and quantitative lipid analysis. During the course of this project it was necessary to routinely analyze TAG profiles for phenotyping of mutants and transgenic strains. For this purpose a facile liquid chromatography–mass spectrometry protocol was developed for TAG profiling, achieving identification and quantification of intact TAG molecular species in *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica* grown in nitrogen (N)-replete or N-depleted medium. The quantification of algal TAGs and fatty acids was accomplished by employing two internal standards taking advantage of the presence of pheophytin and specific fatty acids in the respective algal samples. This allowed a simplification of the comparative analysis of TAG levels in both algae, paving the way for TAG-based, high-throughput mutant screening. The results of this work were published (Liu et al., 2013).

Using this expertise in lipid profiling, contributions were made to several AFOSR funded studies initiated by collaborator Sabeeha Merchant at UCLA. These studies included the identification of additional Chlamydomonas acyltransferases and a nitrogen responsive regulator (Boyle et al., 2012), the analysis of the changes in transcriptome and lipidome of Chlamydomonas under dark anoxia conditions (Hemschemeier et al., 2013), a study of the remodeling of membrane lipids in iron-starved Chlamydomonas (Urzica et al., 2013), and a

system-wide analysis of N-deprivation induced modifications in a starch-less mutant of Chlamydomonas (Blaby et al., 2013).

Publications summarized above and citing AFOSR Support

1. **Blaby, I.K., Glaesener, A.G., Mettler, T., Fitz-Gibbon, S.T., Gallaher, S.D., Liu, B., Boyle, N.R., Kropat, J., Stitt, M., Johnson, S., Benning, C., Pellegrini, M., Casero, D., and Merchant, S.S.** (2013). Systems-level analysis of nitrogen starvation-induced modifications of carbon metabolism in a *Chlamydomonas reinhardtii* starchless mutant. Plant Cell **25,** 4305-4323.
2. **Boyle, N.R., Page, M.D., Liu, B., Blaby, I.K., Casero, D., Kropat, J., Cokus, S.J., Hong-Hermesdorf, A., Shaw, J., Karpowicz, S.J., Gallaher, S.D., Johnson, S., Benning, C., Pellegrini, M., Grossman, A., and Merchant, S.S.** (2012). Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in Chlamydomonas. J Biol Chem **287,** 15811-15825.
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4. **Li, X., Benning, C., and Kuo, M.H.** (2012a). Rapid triacylglycerol turnover in Chlamydomonas reinhardtii requires a lipase with broad substrate specificity. Eukaryot Cell **11,** 1451-1462.
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6. **Liu, B., and Benning, C.** (2013). Lipid metabolism in microalgae distinguishes itself. Curr Opin Biotechnol **24,** 300-309.
7. **Liu, B., Vieler, A., Li, C., Jones, A.D., and Benning, C.** (2013). Triacylglycerol profiling of microalgae Chlamydomonas reinhardtii and Nannochloropsis oceanica. Bioresour Technol **146,** 310-316.
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9. **Tsai, C.H., Warakanont, J., Takeuchi, T., Sears, B.B., Moellering, E.R., and Benning, C.** (2014). The protein COMPROMISED HYDROLYSIS OF TRIACYLGLYCEROLS 7 (CHT7) acts as a repressor of cellular quiescence in Chlamydomonas. Proc Natl Acad Sci U S A **in press**.
10. **Urzica, E.I., Vieler, A., Hong-Hermesdorf, A., Page, M.D., Casero, D., Gallaher, S.D., Kropat, J., Pellegrini, M., Benning, C., and Merchant, S.S.** (2013). Remodeling of membrane lipids in iron-starved Chlamydomonas. J Biol Chem **288,** 30246-30258.
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Hegg, E.L., Jiang, N., Kuo, M.H., Lu, Y., Niyogi, K.K., Ohlrogge, J., Osteryoung, K.W., Shachar-Hill, Y., Sears, B.B., Sun, Y., Takahashi, H., Yandell, M., Shiu, S.H., and Benning, C. (2012). Genome, functional gene annotation, and nuclear transformation of the heterokont oleaginous alga Nannochloropsis oceanica CCMP1779. PLoS Genet **8,** e1003064.

Public presentations of the findings from AFSOR support by the PI, Christoph Benning

1. 01/30/12 Genetics Program MSU. Towards the engineering of lipid metabolism in microalgae for the production of biofuels.
2. 02/10/12 Microalgal Biotechnology: Principles and Applications IBt-UNAM, Cuernavaca, Mexico. Towards the engineering of lipid metabolism in microalgae for the production of biofuels.
3. 02/17/12 Carnegie Institute, Stanford University, Menlo Park, CA. Lipid biosynthesis and lipid droplet dynamics in microalgae.
4. 03/27/12 GIAVAP, University of Le Mans, France. Lipid biosynthesis and lipid droplet dynamics in microalgae.
5. 05/01/12 Univ. of Kentucky, Lexington, KY, Regulation of lipid biosynthesis in microalgae.
6. 07/12/12 International Symposium on Plant Lipids, Seville Spain. Triacylglycerol metabolism in the microalga *Chlamydomonas reinhardtii*
7. 08/06/12 AFSOR Annual Meeting, Arlington VA. Triacylglycerol metabolism in the microalga *Chlamydomonas reinhardtii*
8. 08/27/12 Biomolecular Science (BMS) Retreat, East Lansing Hannah Community Center. Lipid metabolism in microalgae
9. 10/06/12 Inaugural Symposium on Plant Biotechnology for Health and Sustainability, Michigan State University. Engineering of Microalgal Lipid Metabolism for Biofuel Feedstocks.
10. 10/23/12 IPMB 2012 Jeju, Rep. of South Korea. Exploring Microalgal Triacylglycerol Metabolism for the Production of Biofuels.
11. 11/16/12 USDA NC1200. Reno, Nevada. Redirecting primary metabolism to enhance biofuel crops and algae.
12. 01/28/13 GRC Plant Lipids, Galveston, TX. Biogenesis and turnover of lipid droplets in algal models.
13. 02/05/13 University of British Columbia, Botany Dept. Regulation of lipid metabolism in microalgae.
14. 02/11/13 MSU Plant Biology Club. Regulation of lipid metabolism in microalgae.
15. 03/25/13 Lipid metabolic Networks and Switching. Tokyo tech, Yokohama, Japan. Regulation of Lipid Metabolism in Microalgae.
16. 04/04/13 The Plant Journals Editor's Symposium, London UK, Regulation of Oil Biosynthesis in Microalgae
17. 05/30/13 Michigan State University, Genomics Summer Program. Understanding plant and algal lipid metabolism for crop protection and biofuel production
18. 06/17/13 3rd International Conference on Algal Biomass, Biofuels & Bioproducts, Toronto Canada. Regulation of triacylglycerol biosynthesis and turnover in microalgae
19. 08/12/13 16th International Congress on Photosynthesis Research, St. Louis, MO.

Regulation of triacylglycerol biosynthesis and turnover in microalgae

20. 10/28/13 AFSOR Human Performance and Biosystems Review, Arlington, VA. Cellular mechanisms governing a nutrient stress-induced quiescence state in the model Chlamydomonas reinhardtii
21. 10/29/13 AFSOR Human Performance and Biosystems Review, Arlington, VA. Lipid profiling of algae grown under different stress conditions.
22. 05/11/14 The Plant Journal Editorial Symposium, Oxford, UK . Control of transcriptional and metabolic changes during quiescence in microalgae.
23. 05/30/14 3rd International Symposium on Microbial Lipids. Hamburg, Germany. Microalgal metabolism of triacylglycerols in response to changes in the metabolic status of the cell.
24. 06/08/14 16th International Conference on the Cell and Molecular Biology of Chlamydomonas. Pacific Grove, CA Microalgal glycerolipid metabolism in the context of cell development and cell division.
25. 06/16/14 4th International Conference on Algal Biomass, Biofuels & Bioproducts. Santa Fe, NM. Regulation of lipid metabolism in microalgae.
26. 06/27/14 Huazhong Agricultural University, Wuhan, China. Mechanisms of TAG biosynthesis and regulation in algae.
27. 07/10/14 21stISPL, Guelph, Canada. Breaking the link between TAG accumulation and cessation of growth in algae.

MSU Invention Disclosures from AFOSR support and pursued for US Patent Application

1. Method to increase algal biomass and enhance is quality for the production of fuel. Benning, C, Li, X, Liu, B, Kuo, MH, Sears, B MSU TEC2013-0041, 10/19/2012
2. Method to enhance calorific content and nutritional value of plant biomass for the production of fuel and feed. Benning, C, Sanjaya, Miller R. MSU TEC2013-0049, 10/26/2012
3. A novel regulator of algal lipid metabolism and cellular quiescence and its applications, Benning, C., and Tsai C.-H. 4/3/2014 TEC2014-0097

Human Resource Development under AFOSR funding.

The supported work was primarily carried out by graduate students and undergraduate students in the lab. The participating graduate students either partially or fully supported by AFOSR were:

1. Rachel Miller (Ph.D., PRL/Cell and Molecular Biol., graduated Spring 2013). She carried out the analysis of DGATs mentioned under 2.2.
2. Xiaobo Li (Ph.D., PRL, Plant Biology, co-mentored with Min-Hao Kuo, graduated Fall 2012, currently postdoctoral researcher at The Carnegie Institute at the University of Stanford, CA). He carried out the analysis of *pgd1* and LIP1 described under 1.1 and 2.1 and participated in the annotation of the Nannochloropsis genome described under 5.
3. Bensheng Liu (Ph.D., Biochemistry and Mol. Biol., graduated Spring 2014, since May 2014 Scientist at Mascoma Corporation, Lebanon NH). He conducted the lipidomics experiments described under 6 and participated in the annotation of the Nannochloropsis genome described under 5.
4. Chia-Hong Tsai (Ph.D., PRL, Plant Biology, scheduled to graduate in the Fall of 2014 and plans to join a biotech company). He conducted the experiments on MLDP described under

3. He also developed the concept of quiescence for Chlamydomonas and discovered the CHT7 protein described under 4. He also participated in the annotation of the Nannochloropsis genome described under 5.

5. Jaruswan Warakanont (Ph.D., Plant Biology, scheduled to graduate in the Spring of 2015 and plans to assume a lecturer position in her home country). She discovered and characterized the *tgd2* mutant described under 1.2.

The graduate students co-mentored the following undergraduate students who participated in the AFOSR supported studies described above:

1. M. Akeem Williams (Plant Genomics Program, Summer 2012)
2. Chrissy Iserman (German exchange student, Spring 2012)
3. Fabian Brandenburg (German exchange student, Spring 2012)
4. Cindy Amstutz (September 2012-May2014)
5. Benedikt Brink (German exchange student October 2012- April 2013)
6. Ryan Lee Wessendorf (Plant Genomics Program, Summer 2013)
7. Elena Justine Stickley Michel (since August 2013)