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Entitled:

Regulation of oil biosynthesis in algae

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Summary

The widely recognized need for the development of biomass-based domestic production of high energy liquid transportation fuels can potentially be addressed by exploring oil (triacylglycerol) biosynthesis in microalgae. Many microalgae, including *Chlamydomonas*, accumulate triacylglycerols when cultures encounter nutrient limitation. However, the regulatory factors and enzymes that govern triacylglycerol biosynthesis in microalgae have not been studied at the molecular level. *Chlamydomonas* is being used as a microalgal model to identify genes and regulatory mechanisms required for triacylglycerol biosynthesis following nutrient deprivation. Multiple global and focused approaches are being pursued towards this goal: 1. A gene disruption mutant screen of 32,000 lines initially yielded 80 putative mutants, some of which are disrupted in genes encoding enzymes such as lipases, and others encoding novel proteins that could be central to the regulation of triacylglycerol biosynthesis. Six of these mutants are currently under detailed investigation. 2. Deep sequencing using 454 and Illumina based technologies enabled a global expression analysis that covers nearly all genes of the *Chlamydomonas* genome. These expression data were corroborated using metabolic labeling analysis. This work provides a first picture of metabolic and physiological changes following nitrogen deprivation and oil accumulation. 3. Following proteomic analysis of isolated lipid droplets, the expression of a major lipid droplet associated protein was repressed by RNAi in *Chlamydomonas*, resulting in increased oil body size. A sensitive and specific polyclonal antibody against this protein was developed. Preliminary data indicate that it is an excellent diagnostic tool to study lipid droplet formation in *Chlamydomonas*. 4. Candidate genes involved in oil biosynthesis were studied as identified using the approaches described above. The focus was on diacylglycerol acyltransferases, lipases, betaine lipid synthase, a fatty acid desaturase and transcription factors. Their activities were studied in heterologous systems and their role was tested through overexpression and repression experiments. 5. A mass spectrometry based TAG profiling approach was developed, which was applied to the analysis of lipid mutants.

Major Accomplishments and Findings during the Reporting Period:

1. *Plasmid disruption mutagenesis*: Of 32,000 individual disruption lines initially 80 putative mutants were isolated. We completed rescreening leaving us currently with 6 lines with reproducible lipid phenotypes. One line is disrupted in a lipase encoding gene that is induced during N-deprivation. Detailed lipid profiling and pulse chase labeling using acetate suggest that this triacylglycerol (TAG) deficient mutant is affected in the

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transfer of fatty acids from membrane lipids to TAG during N-deprivation. It seems possible that this mutant will allow us to distinguish between *de novo* TAG biosynthesis and TAG biosynthesis as a result of lipid remodeling in response to N-deprivation. Overall, the analysis suggests that at least two processes contribute to the accumulation of TAGs. Aside from these findings on lipid metabolism, the mutant also exhibits loss of viability, loss of chlorophyll, and increased lipid peroxidation after 2 days of N-deprivation. These three phenotypes can be rescued by supplementing the medium with DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), an inhibitor of photosynthetic electron transport. Study of this phenomenon will shed light on the physiological significance of oil production in microalgae. A first paper describing this interesting mutant is in preparation.

Another mutant which our efforts are focused on, has increased amounts of TAG during N-deprivation. This mutant appears to be disrupted in a putative protein kinase. We are currently in the process of corroborating the identity of the gene using an independent miRNA inactivation approach. The respective gene is currently our best candidate encoding a regulatory protein involved in TAG biosynthesis. Four additional low oil mutants are currently at different stages of analysis towards the identification of the affected gene.

2. Deep sequencing of transcripts: We published this aspect of the project: Miller, R., Wu, G., Deshpande, R.R., Vieler, A., Gaertner, K., Li, X., Moellering, E.R., Zäuner, S., Cornish, A., Liu, B., Bullard, B., Sears, B.B., Kuo, M.H., Hegg, E.L., Shachar-Hill, Y., Shiu, S.H., and C. Benning. 2010. Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen-deprivation predict diversion of metabolism. *Plant Physiol.*, 154:1737-1752. I am citing from the Abstract as follows: "...high-throughput sequencing technology was employed to generate large numbers of expressed sequence tags of eight biologically independent libraries, four for each condition, N-replete and N-deprived, allowing a statistically sound comparison of expression levels under the two tested conditions. As expected, N-deprivation activated a subset of control genes involved in gametogenesis, while down-regulating protein biosynthesis. Genes for components of photosynthesis were also down-regulated, with the exception of the PSBS gene. N-deprivation led to a marked redirection of metabolism: the primary carbon source, acetate, was no longer converted to cell building blocks by the glyoxylate cycle and gluconeogenesis, but funneled directly into fatty acid biosynthesis. Additional fatty acids may be produced by membrane remodeling, a process that is suggested by the changes observed in transcript abundance of putative lipase genes. Inferences on metabolism based on transcriptional analysis are indirect, but biochemical experiments supported some of these deductions. The data provided here represent a rich source for the exploration of the mechanism of oil accumulation in microalgae."

3. Isolation and characterization of lipid droplets and analysis of specific lipid droplet associated proteins: The isolation and characterization of lipid droplets was published (Moellering, E.R., and C. Benning. 2010. RNAi silencing of a major lipid droplet associated protein affects lipid droplet size in *Chlamydomonas reinhardtii* Euk. Cell 9:97-106). During this study we identified a Major Lipid Droplet Protein, tentatively designated with the acronym MLDP. This protein is specific to the green algal lineage of photosynthetic organisms. We developed an excellent antibody against this protein that is

highly specific and sensitive. Western analysis shows that the presence of this protein correlates directly with the accumulation and turnover of TAG and therefore can be used as a marker for lipid droplet formation. This is critical information for the development of a diagnostic kit to monitor oil formation in culture as proposed in a recent invention disclosure. We are also currently using this antibody in a new round of insertional mutant screening to identify mutants deficient in the turnover of lipid droplets following re-feeding of nitrogen. This antibody will also be tested to determine whether MLDP can recruit other proteins to the lipid droplet or is part of a larger protein complex. These proteins may be involved in lipid turnover or lipid droplet formation and will provide potential new targets for engineering of production strains.

4. Analysis of specific genes required for oil biosynthesis. The investigation of five potential DGAT candidates, DGTT1-5, is close to being completed. One of the genes has been successfully expressed in *Arabidopsis* leading to oil accumulation in seed and vegetative tissues and demonstrating its utility for the engineering of oil biosynthesis in plants. We are currently preparing a manuscript describing these genes and their applicability to biofuel crop engineering.

Our analysis of transcription factors identified as being induced or repressed under oil producing conditions is ongoing. We are focusing on overexpression of the respective cDNAs to test oil accumulation under N-replete or N-deprived conditions. At this time we have examined 10 candidate factors. Overexpression of the respective genes has not led to altered oil phenotypes. It seems possible that multiple factors will be required to induce oil biosynthesis and that the concept of a single gene, i.e. the "lipid trigger", is far too simplistic. This finding is also corroborated by the fact that at this time none of our mutants is carrying an insertion in a transcription factor gene. However, the available gene space has not yet been fully explored by any of our approaches. We are also testing an N-responsive transcription factor known to regulate genes involved in N-metabolism. We are testing the hypothesis that this factor could also regulate lipid metabolism genes.

Because betaine lipid synthase was identified as major lipid droplet associated protein, we constructed RNAi and artificial miRNA lines to study its role in oil accumulation and lipid droplet formation. We have obtained two Tilling lines carrying point mutations in this gene, which we are currently studying. Preliminary studies show that TILLING mutant strains accumulate less oil under nitrogen depletion.

We have targeted several lipase encoding genes that were identified as possible candidates in the gene expression and proteomics approaches described above. Five (3 down-regulated, 2 up-regulated following N-deprivation) were expressed in a yeast lipase mutant with one of them complementing the TAG and growth phenotypes. This gene is named *CrLIP1*. In *Chlamydomonas* the expression of *CrLIP1* is decreasing during nitrogen deprivation and gradually increasing during nitrogen re-supply. Knock down lines were created with artificial microRNA. The lines with lower mRNA level did not show increased TAG accumulation during N deprivation, but exhibited delayed TAG degradation after N was re-supplied. Blast search with the *Arabidopsis* TAG lipase SDPI sequence leads to one hit in *Chlamydomonas* genome (named *CrLIP4*). Knock down of *CrLIP4* also slows down the TAG degradation, similar to that of *CrLIP1*. A double mutant with two independent amiRNA constructs is underway and will be tested for TAG turnover.

A visiting scientist, Simone Zauner, has identified an unusual plastid-targeted desaturase with an N-terminal cytochrome b5 fusion domain. She has shown in repression and overexpression *Chlamydomonas* lines and transgenic *Arabidopsis* lines that this enzyme is responsible for the 16:4 fatty acids present in chloroplast galactolipids. A paper describing this finding has been positively reviewed and is awaiting revision.

5. Analysis of triacylglycerols. We developed a robust protocol for the qualitative and quantitative analysis of triacylglycerol from *Chlamydomonas* as part of an amendment to the original project. Using this approach we identified 140 molecular species of TAG in *Chlamydomonas*. We applied this approach to one of the lipase mutants and detected losses of specific molecular species of TAG in this mutant. We assisted S. Merchant with lipid profiling and coauthored a paper describing these results: Castruita, M., Casero, D., Karpowicz, S.J., Kropat, J., Vieler, A., Hsieh, S.I., Yan, W., Cokus, S., Loo, J.A., Benning, C., Pellegrini, M., and S.S. Merchant. 2011. Systems biology approach in *Chlamydomonas* reveals connections between copper nutrition and multiple metabolic steps. *Plant Cell*, online.

Deviation from Original Proposal

No deviations are reported. All objectives are as proposed.

Publications of Findings and Patents

1. Moellering E.R., Miller, R., and C. Benning. 2009. Molecular genetics of lipid metabolism in the model green alga *Chlamydomonas reinhardtii*. In: *Advances in Photosynthesis and Respiration*. Govindjee (series ed). Vol 30. Lipids in Photosynthesis: Essential and Regulatory Functions. H. Wada and N. Murata (eds). Springer (Netherlands), pp. 139-155.
2. Moellering, E.R., and C. Benning. 2010. RNAi silencing of a major lipid droplet associated protein affects lipid droplet size in *Chlamydomonas reinhardtii* *Euk. Cell* 9:97-106.
3. Miller, R., Wu, G., Deshpande, R.R., Vieler, A., Gaertner, K., Li, X., Moellering, E.R., Zäuner, S., Cornish, A., Liu, B., Bullard, B., Sears, B.B., Kuo, M.H., Hegg, E.L., Shachar-Hill, Y., Shiu, S.H., and C. Benning. 2010. Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen-deprivation predict diversion of metabolism. *Plant Physiol.*, 154:1737-1752.
4. Castruita, M., Casero, D., Karpowicz, S.J., Kropat, J., Vieler, A., Hsieh, S.I., Yan, W., Cokus, S., Loo, J.A., Benning, C., Pellegrini, M., and S.S. Merchant. 2011. Systems biology approach in *Chlamydomonas* reveals connections between copper nutrition and multiple metabolic steps. *Plant Cell*, online
5. Zäuner, S., Jochum, W., Bigorowski, T. and C. Benning. 2011. A cytochrome b₅-containing plastid-located fatty acid Δ^4 -desaturase from *Chlamydomonas reinhardtii*. *Plant J.*, provisionally accepted.

Actively pursued disclosures:

Diacylglycerol acyltransferases involved in oil biosynthesis in microalgae and their use to enhance oil yield. Christoph Benning, Eric R. Moellering, Rachel Miller. MSU

TEC2008-0056, invention disclosure filed April, 2008; patent application filed in 2009

Regulatory factors controlling oil biosynthesis in microalgae and their use. Christoph Benning, Eric R. Moellering, Rachel Miller. MSU TEC2008-0057, invention disclosure filed April, 2008; patent filed in 2009.

Lipid drop protein markers for oil accumulation in green algae, Christoph Benning and Eric R. Moellering. MSU TEC2010-0029. Invention disclosure filed 10/5/2009.

Outreach and Education

Six graduate students (Eric Moellering, Rachel Miller, Xiaobo Li, Bensheng Liu, Chia-Hong Tsai and Jaruswan Warakanont), one postdoc (Simone Zauner) and four undergraduate students (Marie Fedewa, Cassandra Johnny, Tara Bigorowski, and Wibke Jochum) were involved in the project. Rachel Miller focuses on the transcription factors and DGTT enzymes. Eric Moellering, graduated last year and left to take a Scientist position at Synthetic Genomics in San Diego. Xiaobo Li works on the lipase mutants. He is conducting pulse chase label experiments to determine the role of those lipases in TAG metabolism. Jaruswan Warakanont is working on a different set of lipases. Bensheng Liu is conducting lipid profiling experiment and is characterizing a protein kinase mutant of *Chlamydomonas* with increased TAG content. He is also investigating the role of betaine lipid in TAG formation. Chia-Hong Tsai developed the MLDP antibody and is using it to study lipid droplet formation. Simone Zauner is studying fatty acid desaturases encoding candidate genes. She recently left for a new postdoctoral position at the University of Bonn, Germany. Other MSU faculty members participating in this effort are Barb B. Sears, who helps with *Chlamydomonas* genetics in particular in conducting *Chlamydomonas* matings and tetrad analyses, Shinhan Shiu, who is instrumental in analyzing the expression data, and Min-Hao Kuo, a yeast expert who is co-supervising Xiaobo Li in his studies of lipases.

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