2011 Photosynthetic Systems Research Meeting

Baltimore Marriott Waterfront Hotel
Baltimore, MD
November 6-9, 2011

U.S. DEPARTMENT OF ENERGY | Office of Science

Office of Basic Energy Sciences
Chemical Sciences, Geosciences & Biosciences Division

2011 Photosynthetic Systems Research Meeting

Program and Abstracts

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Foreword

This volume provides a record of the second biennial meeting of the Principal Investigators (PIs) funded by the Photosynthetic Systems program and is sponsored by the Chemical Sciences, Geosciences, and Biosciences Division of the Office of Basic Energy Sciences (BES) in the U.S. Department of Energy (DOE). Within DOE BES, there are two programs that fund basic research in energy-relevant biological sciences, Photosynthetic Systems and Physical Biosciences. These two Biosciences programs, along with a strong program in Solar Photochemistry, comprise the current Photochemistry and Biochemistry Team.

This meeting specifically brings together under one roof all of the PIs funded by the Photosynthetic Systems program, along with Program Managers and staff not only from DOE BES but also other offices within DOE, the national labs, and other federal funding agencies. Our goal in holding these research meetings is to provide an environment that fosters exchange of information on your DOE-funded work; facilitates cooperation and collaboration among research groups; provides opportunities for discussions with DOE Program Managers and staff; and promotes sharing of new ideas and methodologies. Accordingly, "down time" is scheduled to encourage informal discussions and interactions.

This year's agenda features two invited speakers. We are pleased to have Dr. John Turner from the National Renewable Energy Laboratory who will talk about hydrogen production from photoelectrochemical cells (PECs), providing "food for thought" regarding the economic and theoretical considerations of PECs. We are also delighted to have Dr. W.E. Moerner from Stanford University who will tell you about exciting developments in the use of Anti-Brownian ELectrokinetic (ABEL) trap technology to understand behavior of individual photosynthetic antenna proteins and redox enzymes.

While we extend a warm welcome and our sincere appreciation to our invited speakers, the real star at this year's meeting is **you**. Your productivity, creativity, and commitment to world-class science come across in your abstracts and not only make a meeting like this possible but also gratifying and enjoyable. We hope that the meeting will further enhance the innovation and success of your research efforts and build a robust community of researchers in the study of photosynthesis and energy capture, conversion, and storage in plants, algae, and photosynthetic microbes. Whether you are delivering a talk or presenting a poster, we sincerely appreciate your contribution to this meeting.

It has been an extraordinary time in DOE's Office of Science, and we also thank you for your many contributions to the successful execution of our many funding opportunities over the last few years. Finally, we thank Diane Marceau and Dawn Adin from DOE BES and Connie Lansdon, Tim Ledford, and Verda Adkins-Ferber from Oak Ridge Institute for Science and Education (ORISE) for their invaluable help in planning and successfully executing the many logistical tasks associated with this meeting.

B. Gail McLean, Program Manager, Photosynthetic Systems, DOE BES
Robert J. Stack, Program Manager, Physical Biosciences, DOE BES
Richard V. Greene, Lead, Photochemistry and Biochemistry Team, DOE BES

Agenda

AGENDA

2011 Photosynthetic Systems Research Meeting
Baltimore Marriott Waterfront Hotel, Baltimore, MD
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Sunday, November 6, 2011

3:00 – 6:00 p.m.	Registration
5:30 – 6:30 p.m.	Reception (No Host)
6:30 – 7:30 p.m.	Dinner at Baltimore Marriott Waterfront Hotel
7:30 – 8:00 p.m.	Welcome and Opening Remarks **Gail McLean,** Program Manager, Photosynthetic Systems **Richard V. Greene,** Lead, Photochemistry & Biochemistry Team, DOE BES

Monday, November 7, 2011

7:30 – 8:30 a.m.	*****Continental Breakfast*****

Session I

8:30 – 9:00 a.m.	Welcome and Photosynthetic Systems Program Update **Gail McLean,** Program Manager, Photosynthetic Systems
9:00 – 10:00 a.m.	Hydrogen Production from Photoelectrochemical Cells: Economic and theoretical considerations and experimental results **John Turner,** National Renewable Energy Laboratory
10:00 – 10:30 a.m.	*****Break*****

Session II *Moderator: **Dave Tiede**, Argonne National Laboratory*

10:30 – 11:00 a.m.	Structure and Mechanism of the Mn_4Ca Cluster in Photosystem II Using X-ray Spectroscopy **Vittal Yachandra,** Lawrence Berkeley National Laboratory
11:00 – 11:30 a.m.	Studies of Photosynthetic Reaction Centers and Biomimetic Systems **Marilyn Gunner,** City College of New York
11:30 – 12:00 p.m.	Maximizing Photosystem II Water Oxidizing Efficiency through the Identification of Optimal Protein Coordination Environments **G. Charles Dismukes,** Rutgers University
12:00 – 1:00 p.m.	*****Lunch*****
1:00 – 1:45 p.m.	DOE Update and News **Eric Rohlfing,** Director, Chemical Sciences, Geosciences & Biosciences Division, DOE BES

Session III *Moderator: **Rob Burnap**, Oklahoma State University*

1:45 – 2:15 p.m.	Membrane-attached Electron Carriers in Photosynthesis and Respiration: Cytochrome c maturation (Ccm-System I) in a facultative photosynthetic bacterium **Fevzi Daldal,** University of Pennsylvania

2:15 – 2:45 p.m. Controlling Electron Transfer Pathways in Photosynthetic Proteins
Christine Kirmaier, Washington University and
Deborah Hanson, Argonne National Laboratory

2:45 – 3:15 p.m. Nature-Driven Photochemistry for Solar Fuels Production: Photosynthetic Interprotein and Biohybrid Electron Transfer
Lisa M. Utschig, Argonne National Laboratory

3:15 – 6:00 p.m. *****Free/Discussion Time (Put up your poster too!)*****

6:00 – 6:30 p.m. *****Reception (No-Host)*****

6:30 – 7:30 p.m. *****Dinner at Baltimore Marriott Waterfront Hotel*****

Poster Session I

7:30 – 9:30 p.m. *****Odd Numbered Posters (No-Host)*****

Tuesday, November 8, 2011

7:30 – 8:30 a.m. *****Continental Breakfast*****

Session IV: Physical Biosciences Program Guest Lecturer
*Moderator: **Bob Stack**, Program Manager, Physical Biosciences*

8:30 – 9:30 a.m. Photodynamics of Single Antenna Proteins and Redox Enzymes in Solution by Suppression of Brownian Motion
W.E. Moerner, Stanford University

9:30 – 10:00 a.m. *****Break*****

Session V *Moderator: **Sergei Savikhin**, Purdue University*

10:00 – 10:30 a.m. Photoreceptor Regulation And Optimization Of Energy Harvesting In *Nostoc punctiforme*
J. Clark Lagarias, University of California, Davis

10:30 – 11:00 a.m. Carotenoid Antennae in Retinal-based Light-driven Proton Pumps
Janos K. Lanyi, University of California, Irvine

11:00 – 11:30 a.m. Characterization of the Conformational Flexibility in Light Harvesting Complexes
Ann E. McDermott, Columbia University

11:30 – 12:00 p.m. Molecular Analysis of the FMO Antenna Complex from Green Sulfur Bacteria
Robert Blankenship, Washington University

12:00 – 1:00 p.m. *****Lunch*****

Session VI *Moderator: **Steve Theg**, University of California, Davis*

1:00 – 1:30 p.m. Antisense RNAs in the Chloroplast
David Stern, Boyce Thompson Institute for Plant Research

1:30 – 2:00 p.m. MSH1: a novel interorganellar environmental response mechanism in higher plants
Sally Mackenzie, University of Nebraska

2:00 – 2:30 p.m. Post-Transcriptional Regulation of Ethylene Perception and Signaling in Arabidopsis
Eric Schaller, Dartmouth College

2:30 – 3:00 p.m. Eukaryotic initiation factor 3 (eIF3) and mRNA leader sequences as agents of translational regulation in Arabidopsis
Albrecht G. von Arnim, University of Tennessee

3:00 – 7:30 p.m. *****Free/Discussion Time and Dinner on Your Own*****

Poster Session II

7:30 – 9:30 p.m. *****Even Numbered Posters (No-Host)*****

Wednesday, November 9, 2011

7:30 – 8:30 a.m. *****Continental Breakfast*****

Session VII *Moderator: **Mike Salvucci**, USDA – Agricultural Research Center*

8:30 – 9:00 a.m. Role of the Rubisco Small Subunit
Robert Spreitzer, University of Nebraska

9:00 – 9:30 a.m. Structure, Function, and Assembly of Rubisco Activase
Rebekka M. Wachter, Arizona State University

9:30 – 10:00 a.m. Disruption of C_4 photosynthesis by changes in light quantity and quality: Implications for CO_2 fixation and photosynthetic efficiency in C_4 crop and biofuel species
Asaph Cousins, Washington State University

10:00 – 10:30 a.m. *****Break*****

Session VIII *Moderator: **Ralph Henry**, University of Arkansas*

10:30 – 11:00 a.m. Tha4 topology and direct interaction with translocating precursor mature domain during transport on the cpTat pathway
Carole Dabney-Smith, Miami University

11:00 – 11:30 a.m. Thylakoid Biogenesis – Significance of Protein Maturation and Mechanism of Protein Targeting
Kentaro Inoue, University of California, Davis

11:30 – 12:00 p.m. Thylakoid membrane biogenesis in cyanobacteria
Willem F.J. Vermaas, Arizona State University

12:00 – 12:30 p.m. Chloroplast Dynamics and Photosynthetic Efficiency
Maureen Hanson, Cornell University

12:30 – 1:30 p.m. *****Lunch *****
(DON'T FORGET TO TAKE DOWN YOUR POSTER!)

***Session IX**:* *Moderator: **Gail McLean,** Program Manager, Photosynthetic Systems*

1:30 – 3:00 p.m. Discussion of photosynthesis research needs and Closing Comments

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Session I

Hydrogen Production from Photoelectrochemical Cells:
Economic and theoretical considerations and experimental results

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Hydrogen represents a possible energy carrier that could simultaneously offer a transportation fuel as well as large-scale energy storage for variable energy systems such as wind and solar. Producing hydrogen from water using electrolysis driven by the energy from solar cells or wind power provides a pathway for these intermittent renewable power sources to meet our continuous energy demand. While wind can supply a significant amount of our energy needs, only solar provides the resource to "do to all". The combination of solar and PV/electrolysis could supply both our electrical and energy carrier demands, however, using the sun's energy to directly split water into its component gases directly via an artificial photosynthesis route would be both more efficient and cost-effective than the two-step PV/electrolysis process. Photoelectrolysis represents one approach in this direction.

The photoelectrolysis of water is the splitting of water into hydrogen and oxygen using sunlight. Such water-splitting systems consist of three main components, a light-harvesting system (the semiconductor) and two gas-evolving electrodes, one for hydrogen and one for oxygen. The semiconductor material is placed into an aqueous solution and illuminated with sunlight, driving the water splitting reaction directly. One of the gases, either hydrogen or oxygen, is evolved off the surface of the semiconductor, the other gas is evolved on an electrode is spatially separated so that the two gases, hydrogen and oxygen do not mix. This configuration combines the solar cell and the electrolyzer into a single device. The result of this configuration is that sunlight is the only energy input and that hydrogen is evolved with no external electron flow. The major barrier to implementing this approach is identifying a semiconducting material that satisfies all the necessary criteria.

The simplest PEC based direct water splitting system would consist of an illuminated single gap semiconductor having a bandgap greater than 1.7 electron volts coupled to a surface catalyst immersed in an aqueous solution. One gas would be evolved on the illuminated side and the other gas would be evolved off of the back side.

To date, no semiconducting material has been discovered that simultaneously meets all the criteria required for economical hydrogen production via light-driven direct water splitting. Considerable work has been directed at metal oxides due to their expected stability, unfortunately most of the approaches are empirical in nature or seemingly random walks though the periodic chart, primarily focusing on the 3-d metal oxides. The result is that after 35 years of work, little progress has been made: efficiencies for these oxides remains abysmally low. This is not surprising, in general when it comes to solar photoconversion, oxides have very poor electronic properties, and thus there are no PV devices where an oxide is the active light absorbing material. In the PEC area little

thought has been given to the requirement that these PEC devices must have the same fundamental internal quantum efficiency as the commercial high efficiency PV devices. Still there are many good reasons to continue to study mixed metal oxides. One is the very large number of possible combinations; there are easily 50,000 combinations of ternary oxides and easily 2 million quaternary oxides not including stoichiometric variations. Since high temperature superconductors are 4 or 5 component mixed metal oxide, there exists the real possibility that there is an analogous material with the correct properties for a viable photoelectrochemical device. Additionally, the photo-electrodes must be stable in aqueous solutions under illumination for years, and clearly oxides are one of the better prospects to meet that requirement. Finally, the possibility of utilizing nanostructure to address one or more material limitations to enhance the photoresponse is viable in these system, since water forms a conformable junction with any structure.

Multi-component transition metal oxides are complex materials, making intuitive guesses impossible and a focused search very challenging. So to achieve suitable photo-electrode materials, the electronic properties of the materials and their response to defect formation must be understood. Strong electron-electron and electron-phonon interactions in oxides are responsible for the exotic quantum critical behaviors which are inherently difficult to study.

A computational approach may be the only approach that can give us the necessary insight into these mixed metal oxides and allow us to narrow the composition space leading us towards a successful material.

The III-V nitride materials have shown excellent stability as evidenced by corrosion analysis; however, they show a significant decrease in overall conversion efficiency as compared to other non-nitride III-Vs.

This talk will discuss issues relating to metal oxides and summarize our efforts III-V nitride materials and their application to tandem cells for photoelectrochemical water splitting.

Session II

Structure and Mechanism of the Mn_4Ca Cluster in Photosystem II Using X-ray Spectroscopy

Junko Yano, Kenneth Sauer, Vittal Yachandra, Principal Investigators
Jan Kern, Rosalie Tran, Benedikt Lassalle, Postdocs
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Overall research goals: The specific questions that are the focus of our studies are: 1) What is the geometric and electronic structure and the changes of the Mn_4Ca cluster of the photosynthetic oxygen-evolving complex (OEC) as it traverses the enzymatic cycle driven by four successive photons? 2) What is the mechanism of the photosynthetic water-oxidation reaction that is catalyzed by the Mn_4Ca cluster? We are using steady state and time-resolved X-ray spectroscopy and crystallography methodologies in pursuit of these goals.

Significant achievements 2009-2011: 1) X-ray emission spectra, particularly the so-called cross-over transitions ($K\beta_{2,5}$ and Kβ"), that result from ligand atom 2p and 2s to Mn 1s transitions contain unique information on ligand type, ligand protonation state, and Mn-ligand distances. Using model systems, we have demonstrated that the spectral shape is sensitive to protonation of ligands and allows ligands, which differ only slightly in atomic number (e.g. C, N, O), to be distinguished. A theoretical description of the main spectral features has been developed using DFT calculations in terms of molecular orbitals for a series of Mn model systems. We have collected the XES spectrum of PS S_1 state and demonstrated the feasibility of collecting the weak Kβ" transition peak from dilute protein samples like PS II, and to our knowledge this is the first example of such spectroscopy on a biological sample. We have compared the S_1 state spectrum to those from several well-characterized oxo-bridged Mn complexes. We plan to study all the S-states using the XES method during the present proposal period.

2) We have used RIXS spectroscopy to understand the electronic structure and d-orbital population of Mn in PS II by comparison with Mn oxides and coordination compounds. These data indicate the charge density changes per formal oxidation state changes and demonstrates how the charge density on the atom differs markedly from the formal oxidation state of the complexes. The comparison of PS II with ionic and coordination complexes shows that the change in charge density on Mn atoms during the S-state transition is smaller than that of the coordination complexes. This implies that during the S-state turnover the charge is delocalized on to the ligands thus the formal oxidation state is very different from the charge density of the metal.

3) We used range-extended EXAFS data to resolve the distance heterogeneity in the short Mn-Mn distances of the S_1 and S_2 states and thereby provided firm evidence for three Mn-Mn distances of 2.7-2.8 Å. We have completed the range-extended EXAFS study of the S_3 state and the higher-resolution data clearly shows that metal-metal distance changes in the S_2 to S_3 transition as seen in the changes in FT peak II. The two 2.7 and one 2.8 Å Mn-Mn distances in the S_2 state lengthen to 2.8 and 2.9-3.0 Å in the S_3 state. This result together with our work of Sr EXAFS on Sr-PS II shows that there is a major structural change in the S_2 to S_3 transition, and this is a critical step before the O-O bond formation occurs in the latter step(s).

4) We are using site-specific mutants to study the critical role of ligands in the OEC of PS II. The effect of replacing a histidine ligand on the properties of the OEC and the structure of the Mn_4Ca cluster in PS II was studied by XAS using PS II core complexes from the *Synechocystis sp*. PCC 6803 D1 polypeptide mutant H332E. The Mn XANES spectrum of D1-H332E shifts to a lower energy compared with that of the native WT samples, suggesting that the electronic structure of the Mn cluster is affected by the presence of the additional negative charge on the OEC of the mutant. The EXAFS spectrum shows that the geometric structure of the cluster is altered substantially from that of the native WT state, resulting in an elongation of Mn-ligand and Mn-Mn interactions in the

mutant. This substantial structural change provides an explanation not only for the altered properties of the D1-H332E mutant but also the importance of the histidine ligand for proper assembly of the Mn_4Ca cluster. In the current X-ray crystal structure Glu354 of the CP43 polypeptide is the only amino acid ligand of the Mn_4Ca cluster that is not provided by the D1 polypeptide. Mn XAS studies have shown that the CP43-E354Q mutation only subtly perturbs the structure of the Mn_4Ca cluster in the S_1 state, in contrast to that seen in the D1-H332E mutant, while it substantially alters the S_2 to S_1 state decay time.

Science objectives for 2011-2012:

- We are making good progress with the polarized XAS studies of single crystals of PS II from PS II monomers and also dimers in the S_2 and S_3 states.
- We have been given two beamtimes at the new X-ray laser facility at Stanford over the next year, where we plan to conduct simultaneous X-ray crystallography and X-ray emission spectroscopy studies of all the S-states, and particularly follow the fast steps between the S_3 to S_0 transition in a time-resolved manner. We have commissioned an energy-dispersive emission spectrometer that will enable us to collect 'snapshots' of the emission spectrum using the X-ray laser. These spectra will then be correlated to the diffraction and the structure of the Mn_4Ca cluster in the intermediate states and the transient states between S_3 and S_0.

References to work supported by this project 2009-2011:

Papers

1. Service, R. J., Yano, J., McConnell, I., Hwang, H. J., Niks, D., Hille, R., Wydrzynski, T., Burnap, R. L., Hillier, W., Debus, R. J. (2011) Participation of Glutamate-354 of the CP43 Polypeptide in the Ligation of Manganese and the Binding of Substrate Water in Photosystem II. *Biochemistry,* **50**, 63-81.
2. Yano, J., Walker, L. M., Strickler, M. A., Service, R. J., Yachandra, V. K., Debus, R. J. (2011) Altered Structure of the Mn_4Ca Cluster in the Oxygen Evolving Complex of Photosystem II by a Histidine Ligand Mutation. *J. Biol. Chem*. **286**, 9257-9267.
3. Lassalle-Kaiser, B., Hureau, C., Pantazis, D. A., Pushkar, Y., Guillot, R., Yachandra, V. K., Yano, J., Neese, F., Anxolabéhère-Mallart, E. (2010) Activation of a Water Molecule Using a Mononuclear Mn Complex: From Mn-aquo, to Mn-hydroxo, to Mn-oxyl via Charge Compensation. *Energy Environ. Sci.*, **3**, 924-938.
4. Pushkar, Y., Long, X., Glatzel, G., Brudvig, G. W., Dismukes, G. C., Collins, T. J., Yachandra, V. K., Yano, J., Bergmann, U. (2010) Direct Detection of Oxygen Ligation to the Mn_4Ca Cluster of Photosystem II by X-ray Emission Spectroscopy. *Angew. Chem. Int. ed*. **49**, 800-803.
5. Smolentsev, S., Soldatov, A. V., Messinger, J., Merz, K., Weyhermuller, T., Bergmann, U., Pushkar, Y., Yano, J., Yachandra, V. K., Glatzel, P. (2009) X-ray Emission Spectroscopy to Study Ligand Valence Orbitals in Mn Coordination Complexes. *J. Am. Chem. Soc.* **131**, 12451-12457.

Reviews

1. Yachandra, V. K., Yano, J. (2011) Calcium in the Oxygen-Evolving Complex: Structural and Mechanistic Role Determined by X-ray Spectroscopy. *J. Photochem. Photobiol. B.* **104**, 51-59.
2. Yano, J., Yachandra, V. K. (2009) X-ray Absorption Spectroscopy. Special Issue, 'Basics and Applications of Biophysical Techniques in Photosynthesis and Related Processes' *Photosynth. Res*. **102**, 241-254.

Studies of Photosynthetic Reaction Centers and Biomimetic Systems

Marilyn Gunner, Principal Investigator
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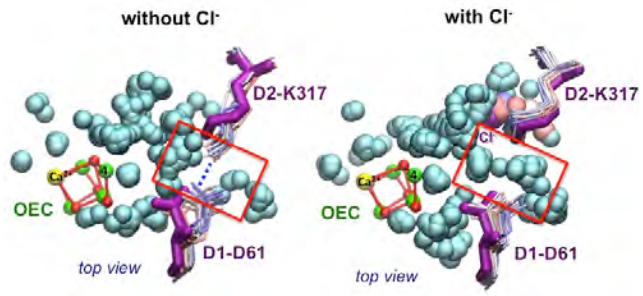
Overall research goals: The funded project brings together laboratories that are experts in complementary computational methods applied to Photosynthetic Reaction Centers. The aim is to understand the basic cofactor chemistry and how this is modified by the proteins in natural photosynthesis to achieve high efficiency charge separation and water splitting. Gunner (CCNY) contributes Monte Carlo techniques for calculations of pK_as and electrochemical midpoints (E_ms) in proteins; Batista (Yale) contributes expertise in quantum mechanics and QM/MM methods of PSII and biomimetic oxomanganese complexes. Collaborations with Bruce (Brock) provides expertise in large-scale molecular dynamics of photosynthetic reaction centers; while Brudvig (Yale), provide extensive experience in spectroscopic, structural and mechanistic studies of O_2 evolution in PSII and model system and photoprotection in PSII.

Significant achievements 2000-2011: The initial focus of our work has been on the OEC in PSII. A working computational model that integrates information from DFT, QM/MM, classical electrostatics and molecular dynamics has been built. All OEC models are developed using EXAFS, EPR, and redox titration analysis of small model Mn complexes and the best protein models. This is a unique multi-technique, dynamic method that lets us study the coupling between the redox and protonation states of the OCE with the protonation and ion binding states (Cl here) in the surrounding protein. The new model has been compared with the 1.9Å crystal structure and with EXAFS spectra.[2] In addition to a high level DFT model of the OEC we are developing a simple classical, valence model of the OEC. This has the advantage of being able to look at the electrostatic interactions between the protein and OEC as well as the changes in protein and OEC protonation states and Cl position and stoichiometry through the S state cycle.



Salt-bridge formed between K317 and D61 (left, without Cl^-), and interrupted by water (right, with Cl^-). X-ray configuration is shown in magenta. Molecular Dynamics and Monte Carlo sampling have shown the disruption of the salt bridge when Cl binds. Rivalta et al Biochemistry. 2011;50:6312.[3]

A novel Monte Carlo approach has been taken to investigate the order of oxidation of the four Mn in the Oxygen Evolving Cluster (OEC) of Photosystem II. Classical electrostatic and van der Waals interactions are considered between all atoms in the cluster, which have some latitude to rearrange on a fine lattice. This approach has been shown to provide surprisingly good agreement with Mn E_ms and μ-oxo and terminal water pK_as in a small diMn cluster. When applied to the OEC cluster the order of oxidation of Mn and atomic movements of the Mn agree with previous measurements. An order of deprotonation of the μ-oxo bridges and terminal waters are proposed. The coupling between the Mn oxidation state and the protonation states of amino acids and the number and positions of bound Cl are examined as a function of the OEC redox S state.

Science objectives for 2011-2012:

QM/MM, MD and MCCE analysis of PSII. The QM/MM approach provides the best view of the charge distribution and atomic positions in the OEC. MCCE is used to bring all protons in the protein into equilibrium with the OEC redox state and protein conformation. Cycles of QM/MM (for OEC charge distribution, atomic geometry and bridging and terminal oxygen pK_as), MCCE (for amino acid

protonation states and coupling of Cl occupancy and group protonation) and MD (for relaxation of the whole protein and sampling of Cl and water positions) will be carried out in each S state. Comparison of the optimized OEC will be made with available EXAFS data. Simulations will be carried out both in a 30Å sphere around the OEC as well as in the whole, membrane embedded protein. Teragrid time has been obtained to make the whole protein analysis possible.

The goals of the calculations for each S state are to determine the changes in protonation of surrounding residues (by MCCE) and of the OEC oxygens and waters (by DFT) to assess the relative importance of the protein and the OEC itself in proton release coupled to OEC oxidation; to determine the Cl- occupancy and position to establishe the role of this key ion; and obtain a detailed mechanism of the Tyr Z and His complex that oxidizes the OEC. New network theory methodologies for understanding correlations in biological systems currently under development will be applied to track the pathways for proton release given the positions of waters and potential hydrogen donors/acceptors in the MD and Monte Carlo simulations. We are also working on new, faster classical methods to analyze redox and protonation states of the OCE and their coupling to the protein. This will be used to look at how changes in protein conformation, pH or mutation change the behavior of the OEC.

References to publications supported by this project 2010-2011:

1. Science (2011) . Comparing photosynthetic and photovoltaic efficiencies and recognizing the potential for improvement, Blankenship, R. E., Tiede, D. M., Barber, J., **Brudvig, G. W**., Fleming, G., Ghirardi, M., **Gunner, M. R.**, Junge, W., Kramer, D. M., Melis, A., Moore, T. A., Moser, C. C., Nocera, D. G., Nozik, A. J., Ort, D. R., Parson, W. W., Prince, R. C., and Sayre, R. T. 332:805-9.
2. Biochemistry (2011) S1 State Model of the O2-Evolving Complex of Photosystem II **Sandra Luber, Ivan Rivalta**, Y.Umena, K. Kawakami, Jian-R. Shen, N. Kamiya, **Doug Bruce, Gary Brudvig, and Victor S. Batista**. 50:6308-11.
3. Biochemistry (2011) Structural/Functional Role of Chloride in Photosystem II **Ivan Rivalta, Muhamed Amin, Sandra Luber, Serguei Vassiliev, Ravi Pokhrel**, Y. Umena, K, Kawakami, J.-R. Shen, N. Kamiya, **Doug Bruce, Gary W. Brudvig, M. R. Gunner, and Victor S. Batista.** 50:6312-5
4. Biochem. Biophys. Acta (2011)Efficiency of photosynthesis in a Chl d-utilizing cyanobacterium is comparable to or higher than that in Chl a-utilizing oxygenic species. S. P. Mielke, N. Y. Kiang, R. E. Blankenship, **M. R. Gunner,** D. Mauzerall. 1807:1231-6.

Maximizing Photosystem II Water Oxidizing Efficiency through the Identification of Optimal Protein Coordination Environments

G. Charles Dismukes, Principal Investigator
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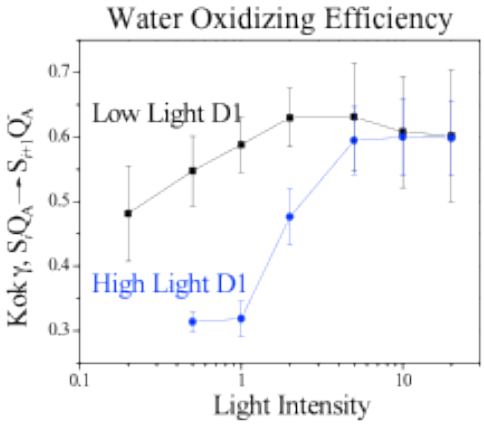
Overall Research Goals:
This research project seeks to characterize the diversity of catalytic performance by photosystem II water-oxidizing complexes (PSII-WOC) found naturally *in vivo*. The PSII-WOC is the primary photochemical engine for solar fuel production from water, being responsible for generation of intracellular hydrogen equivalents (electrochemical energy in the form of electrons, protons and a proton gradient), while releasing O_2 as byproduct. Remarkably, a single conserved protein scaffold (with amino acid variants) and a conserved inorganic core ($CaMn_4O_5$) have been identified in all PSII-WOC centers examined to date. The proposed experiments will survey a genetically diverse range of oxygenic phototrophs in search of atypical catalytic performance, utilizing a non-invasive fluorescence technique to investigate *whole cells* without having to isolate the enzyme. Subsequent experiments on selected strains will utilize inorganic substitutions and site-specific mutations to characterize the consequences on PSII-WOC catalytic efficiency, kinetic bottleneck(s) and adaptive regulation. The long-term goal will be to understand the various factors that throttle and control the primary photochemical engine of natural photosynthesis.

Significant achievements 9/2010-8/2011:
Photosystem II Water Oxidizing Efficiency as a Function of Natural and Unnatural Variations in the D1 Protein[1]. Done with graduate students David Vinyard and Javier Gimpel (UCSD); research Prof. Gennady Ananyev; external collaborators: Profs. Stephen Mayfield (UCSD) and Susan Golden (UCSD).

The D1 protein of PSII provides most of the ligating amino acid residues for the Mn_4CaO_5 core of the WOC. Most cyanobacteria have 3-5 copies of the *psbA* gene coding for at least two isoforms of D1 that are used to adapt to varying environmental conditions, notably light intensity. By expressing natural or unnatural *psbA* sequences in the model green alga *Chalmydomonas reinhardtii*, we can compare the efficiency of photosynthetic water oxidation as a function of D1 protein in a single background. *This project aims to identify the correlation between D1 sequence and WOC catalytic fitness.* A long-term goal of this work is to improve upon nature's existing D1 isoforms by engineering protein motifs that maximize the efficiency of photosynthesis in a given set of environmental conditions. Such custom D1 proteins could be used in biofuels and agronomic applications.

a) Eighteen D1 mutants in *Chlamydomonas* have been constructed by the Mayfield Laboratory (UCSD). These include strains containing heterologous D1 sequences from *Arabidopsis thaliana*, *Thalassiosira pseudonana*, and *Synechococcus* sp. PCC 7942 (both D1.1 "low light" and D1.2 "high light" isoforms), as well as point mutations designed to target specific amino acid differences between varying isoforms.



b) The Dismukes Laboratory (Rutgers University) is actively analyzing strains constructed by the Mayfield Laboratory (UCSD), as well as previously constructed *Synechococcus* mutants from the Golden Laboratory (UCSD). Most notably, a distinct phenotypic difference has been identified in *Chlamydomonas* mutants containing either the "low light" or "high light" D1 isoforms found in cyanobacteria (Figure 1).

Figure 1. Fast repetition rate Chl fluorometry of low light and high light D1 isoforms. When the amplitude of the period-four oscillations in variable Chl fluorescence intensity are fit to

Shinkarev's extended Kok model, to obtain the efficiency of WOC turnover (γ), it is significantly higher in the low light D1 isoform, especially at low light intensities.

We tentatively attribute this difference in water oxidizing efficiency (estimated both through Kok cycle modeling and model-independent Fourier Transform analysis) to secondary charge recombination within PSII, likely involving intrinsic electron donors other that water (including Q_A^-, $Y_D^{\cdot}$, cyt b_{559}) that are more active in PSII centers containing the high light D1 isoform.

Scientific Objectives for 2011-2012:

a) Measure QY directly; Identify and quantify secondary electron transport reactions in low light and high light D1 isoforms.
b) The specific amino acid residues that control the phenotypic differences between low light and high light D1 isoforms will be determined through targeted point mutations.
c) Survey of PSII QY of genetically diverse strains from culture collections and field sites.

2. **What Are the Oxidation States of Manganese Required to Catalyze Photosynthetic Water Oxidation?**[2] Done by former postdoc Derrick R. J. Kolling, visiting predoctoral student Nicholas Cox (ANU-Canberra), Research Prof. Gennady M. Ananyev, and collaborator Ron J. Pace (ANU-Canberra).

This project addresses a long-standing, unresolved, fundamental question about oxygenic photosynthesis (see title). Answering this question is an essential prerequisite for elucidation of the mechanism of photosynthetic water oxidation, which is hotly debated. It is equally important for advancing the extensive worldwide research to develop bioinspired (synthetic) water oxidation catalysts. This question was answered by counting the number of short light flashes required to produce the first burst of O_2 gas upon reconstitution of Mn^{2+} and Ca^{2+} to the cofactor-depleted apo-WOC PSII complex from spinach (photo-assembly. We find the number of photo-assembly intermediates required to reach the lowest oxidation state of the WOC, S_0, to be three, suggesting a net oxidation state 3 equivalents above that of four Mn^{II}, formally $(Mn^{III})_3Mn^{II}$, while the highest (O_2 releasing) oxidation state, S_4, has a net oxidation state corresponding to formally to $(Mn^{IV})_3Mn^{III}$. These net Mn oxidation states differ by two electrons from those proposed on the basis of Mn X-ray absorption spectroscopy and ^{55}Mn-electron nuclear double resonance spectroscopy. Consequences for the catalytic mechanism of water oxidation are significant and discussed in the paper[2].

3. **Identification of an oxygenic reaction center *psbADC* operon in the cyanobacterium *Gloeobacter violaceus* PCC 7421**[3]. Done with former postdoc T. A. Nguyen; undergraduate student J. Brescic; graduate student D. J. Vinyard; and high school student T. Chandrasekar.

Gloeobacter violaceus, the earliest diverging oxyphotobacterium (cyanobacterium) on the 16S rRNA tree, has five copies of the Photosystem II *psbA* gene encoding the D1 reaction center protein subunit. These copies are widely distributed throughout the 4.6 Mbp genome with only one copy co-localizing with other PSII subunits, in marked contrast to all other *psbA* genes in all publicly available sequenced genomes. A clustering of two other *psb* genes around *psbA3* (glr2322) is unique to *Gloeobacter*. We provide experimental proof for the transcription of a *psbA3DC* operon, encoding three of the five reaction center core subunits (D1, D2, and CP43). This is the first example of a transcribed gene cluster containing the D1/D2 or D1/D2/CP43 subunits of PSII in an oxygenic phototroph (prokaryotic or eukaryotic). Implications for the evolution of oxygenic photosynthesis are discussed.

References to Work Supported by this Project:

1. In preparation for submission.
2. Submitted, under review.
3. Molecular Biology and Evolution 2011; doi: 10.1093/molbev/msr224

Session III

Membrane-attached Electron Carriers in Photosynthesis and Respiration:
Cytochrome *c* maturation (Ccm-System I) in a facultative photosynthetic bacterium

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Overall research goals: are to define the nature, mechanism of function, and biogenesis of bacterial cytochromes (cyt) and cyt containing cellular energy transduction complexes. We seek a mechanistic understanding of this process, which is essential for photosynthesis and respiration, using the facultative photosynthetic bacteria of *Rhodobacter* species. Our current focus is on the attachment of a covalent heme cofactor to an apocyt *c* (cyt *c* maturation, Ccm) to yield a mature holocyt *c* that is competent as an electron carrier.

Significant achievements 2008-2010: We, and others, in this field have now defined the major component involved in the Ccm process. These components are grouped into two categories: **a**- those that are exclusively specific to the Ccm process (*e.g.*, the heme ligation core complex), and **b**- those that are required for Ccm but also used by other major cellular functions (*e.g.*, periplasmic thiol oxidation for protein folding). Studies of both sets of components are of significance. Currently we focus on the Ccm specific components, which we think are more important for our long-term aims and for energy-specific goals of the DOE program. We grouped these components into three distinct operational modules that are depicted in the figure, and described in its legend. The work is now at a stage that we seek mechanistic understandings of these modules, and the components constituting these modules.

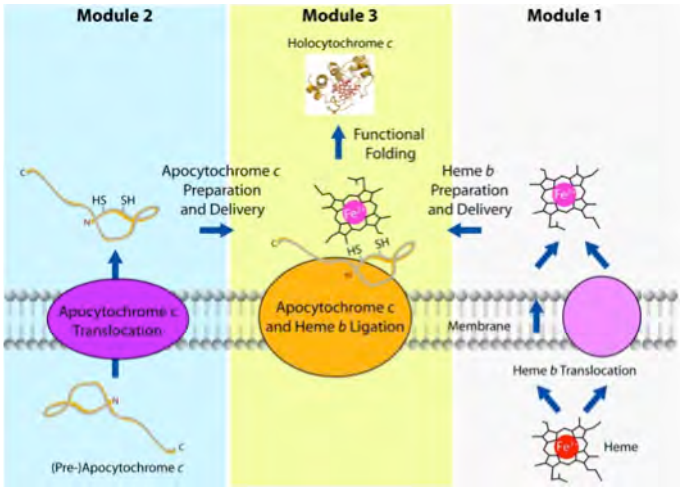

Figure. Modular organization of Ccm. Three coordinately operating membrane-integral modules carry out the Ccm process. Module 1 (M1, heme translocation and relay) transports heme across the membrane and makes it available to Module 3 (M3, apocyt *c*-heme ligation), to which Module 2 (M2, apocyt *c* thioredox and chaperoning) conveys apocyt *c* upon its translocation. M1 and M2 produce ligation-competent heme and apocyt *c* substrates, respectively, and M3 catalyzes thioether bond formation between the appropriate vinyl and Cys groups of these substrates.

Science objectives for 2011-2014: The following specific aims are being pursued:

- Characterize the physical interactions between soluble apocyt c_2 and CcmI of the CcmFHI heme ligation complex complex during the early stages of cyt *c* maturation.
- Define the interactions between the amino-terminally and carboxyl-terminally membrane-anchored different types of apocyts and the two domains of CcmI during their "capture" from the translocation apparatus by the CcmFHI core complex.

- Explore the interactions between the two critical Ccm substrates (apocyt *c* and heme) and the other essential components of Ccm besides CcmI, including CcmG, CcmH, CcmE components.
- Our ultimate aims are to develop *in vitro* an active Ccm system that is able to carry out apocyt *c*-heme ligation using not only natural protoporphyrin IX-iron (heme *b*) but also non natural synthetic variants of hemes and even chlorophylls to create novel cyts hopefully with novel energy-transduction related physicochemical properties.

References to work supported by this project 2008-2010:

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3. Sanders, C., Turkarslan, S., D-W. Lee and **F. Daldal**. (2010). Cytochrome *c* biogenesis: The Ccm system. *Trends Microbiol.* 18:266-74.
4.

Papers:

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9. Pawlik, G., Kulajta, C., Sachelaru, I., Schröder, S., Waidner, B., Hellwig, P., **Daldal, F**. and Koch' H-G. (2010). The putative assembly factor CcoH is stably associated with the cbb_3-type cytochrome oxidase. *J. Bacteriol.* **192**: 6378-89.

Controlling Electron Transfer Pathways in Photosynthetic Proteins

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Overall research goals: Photosynthetic reaction centers (RCs) convert light energy into chemical energy in a series of extremely efficient transmembrane electron transfer reactions. The X-ray structures of RCs reveal two pseudo-symmetrical branches of cofactors (Fig. 1) that are functionally asymmetric; bacterial RCs use the A pathway exclusively. The goal of the project is to identify a mutant RC that utilizes the B pathway exclusively for quinone (Q_B) reduction with the same high yield as that of the A pathway in the native protein, thereby gaining fundamental insight into the factors necessary for *de novo* design of an efficient electron transfer pathway. Site-specific mutagenesis has produced an RC that performs charge separation solely via B-branch activity, but the best overall yield of state $P^+Q_{B^-}$ is still very low. Previous attempts at rational design have provided neither the means nor understanding necessary to engineer an efficient B-branch electron-transfer pathway. Therefore, a directed molecular evolution approach is being used that implements streamlined mutagenesis and high-throughput spectroscopic screening steps to sample a large number of RC variants. Our experimental vehicle is the RC from *Rhodobacter capsulatus*.

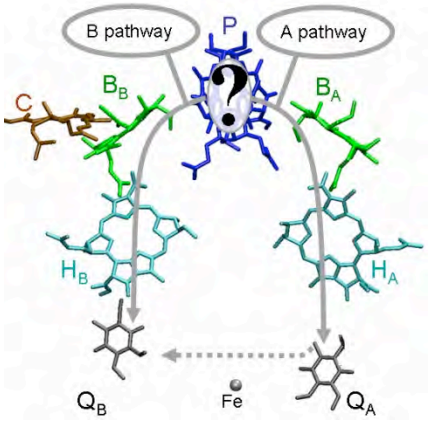

Figure 1. *Arrangement of cofactors in the bacterial RC.*

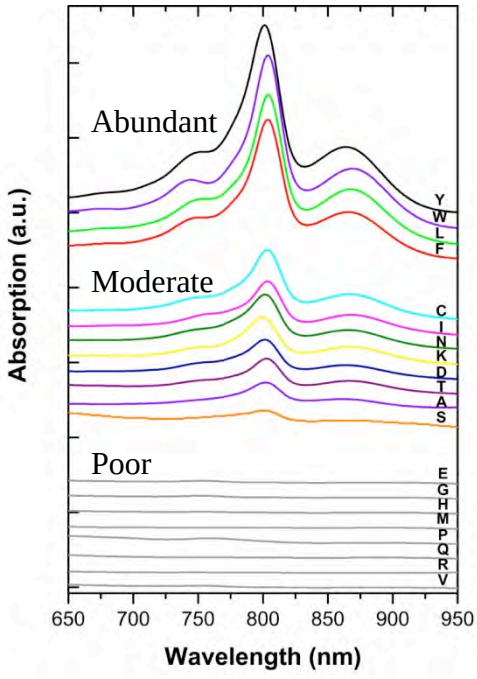

Fig. 2. *UV-vis spectra of RCs purified from F(L181)"X" set of mutants that express the complex abundantly (top), moderately (middle), or poorly (bottom).*

Significant achievements 9/2009 – 9/2011: Mutagenesis approaches employing synthetic biology and automated liquid handling systems have been implemented at ANL to enable hundreds of mutant RCs to be generated in the amount of time required previously for the construction of one mutant. Following multiplexed protein purification and determination of the expression yields of mutant RCs via UV-vis spectroscopy (*e.g.*, Fig. 2), they are screened for use of the B pathway at WU on a time-resolved (msec-sec) spectroscopic screening apparatus specifically built for this project and extensively optimized for performance with miniaturized sample volumes

(96-well plates). Representative data are shown in (Fig. 3). To date, we have constructed, purified and screened 135 mutant RCs. A summary chart comparing the yields of B-branch charge separation (producing state $P^+Q_{B^-}$) in a subset of these mutant RCs is shown in Fig. 4.

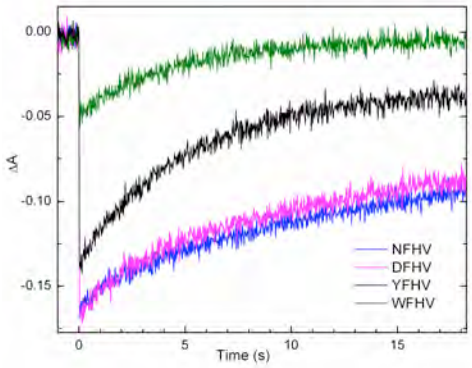

Fig. 3. *Data from the millisecond high-throughput assay showing increased P-bleaching amplitudes (850 nm) for state $P^+Q_{B^-}$ in two mutant RCs (blue, magenta), and one with lower amplitude (green), versus the YFHV control (black).*

These procedures have underscored the plasticity of the RC in accommodating substitutions that result in a range of B-branch activity. A significant fraction of mutant RCs display increased B-branch electron transfer activity compared to the YFHV control mutant RC – results confirmed by ultrafast spectroscopic measurements at WU revealing correspondingly higher yields of state $P^+H_{B^-}$. Many of these RCs carry unexpected amino acid substitutions that would not have been targeted by rational design.

The work to date thus validates the overall "directed evolution" strategy and rapid screening methodologies that are being newly developed and implemented in this project. These mutant RCs are providing new insights into how to engineer and control the directionality of electron transfer, knowledge that could not be obtained so readily via traditional approaches.

Science objectives for 2011-2012:

- Construct a large number of mutants targeting other regions surrounding the cofactors and purify screening-level amounts of RCs.
- Identify promising candidate RCs by using "ultraslow" time-resolved optical absorption spectroscopy. Purify selected mutant RCs in larger scale for characterization of their photochemistry in more detail to understand how the mutations affect the rate constants of the electron-transfer steps and the yields of the charge-separated states.
- Modify the RC complex further by building upon mutations found in the most promising candidates.
- For mutants that show significant, efficient B-side electron transfer, select photocompetent phenotypic revertants that utilize the B-branch cofactors exclusively for quinone reduction.

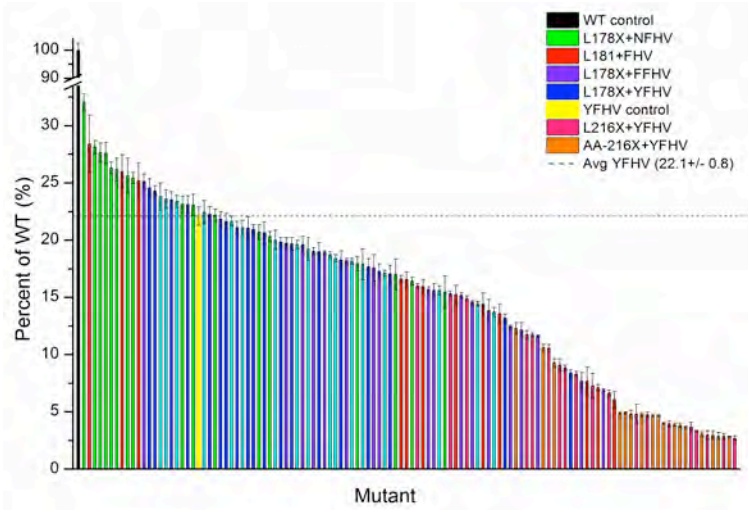

Fig. 4. *Yields of B-side $P^+Q_{B^-}$ formation from millisecond time-resolved assays of mutant RCs relative to wild-type.*

References to work supported by this project 2009-2011: Two manuscripts are in preparation.

Nature-Driven Photochemistry for Solar Fuels Production: Photosynthetic Interprotein and Biohybrid Electron Transfer

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Overall research goals: Photosynthetic reaction center (RC) proteins are finely tuned molecular systems optimized for solar energy conversion. The primary reaction in RCs involves rapid, sequential electron transfer that results in stable charge separation. Following efficient charge separation, the energy captured is utilized in a series of reactions that ultimately drive the chemical conversion of CO_2 into carbohydrates. Our group has initiated efforts to mimic Nature and make use of the light-induced RC reactions to drive non-native chemical reactions for solar fuels production. To this end, recent work is focused on understanding structure-function relationships in natural photosynthetic systems with the intent of applying this knowledge to the design and optimization of novel biohybrid systems. Specifically, our goals include (1) development of experimental methods to interrogate native protein docking and electron transfer events that take place subsequent to photoinduced charge-separation in the Photosystem I (PSI) RC and (2) design and spectroscopic characterization of H_2-producing PSI-catalyst complexes.

Significant achievements 2009-2011:

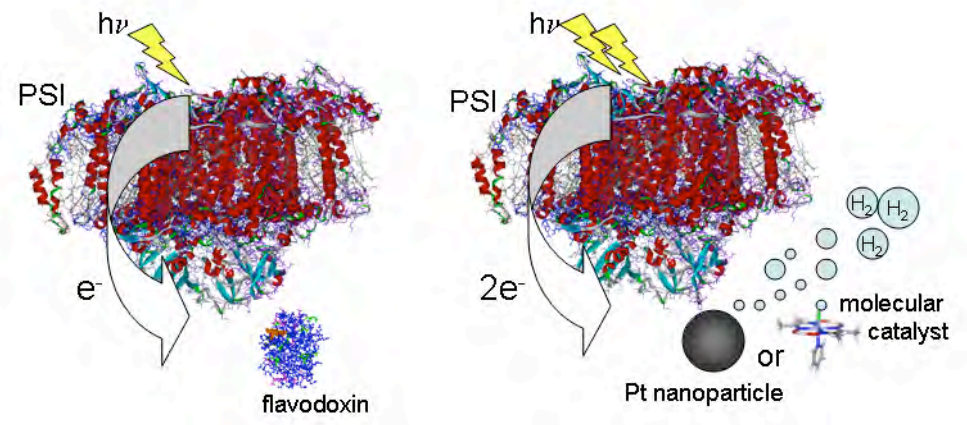

Figure 1 EPR spectroscopy has been used to interrogate the interprotein electron transfer between Photosystem I (PSI) and flavodoxin (Left). These fundamental photosynthetic reaction center studies have led to the development of new PSI-catalyst hybrid systems for hydrogen production (Right).

Photosystem I-Flavodoxin Interprotein Electron Transfer. Electron paramagnetic resonance (EPR) was used to directly examine the photoinitiated interprotein electron transfer reaction between PSI and flavodoxin. Deuteration of flavodoxin enables the signals of the reduced flavin acceptor and oxidized primary donor P_{700}^{+} to be well-resolved at X- and D-band EPR. A remarkable light-dependence on the photoreduction of oxidized flavodoxin by PSI at cryogenic temperatures was observed. These studies suggest light-induced "redox reorientation" of flavodoxin within the PSI docking site as a novel mechanism that allows a high quantum yield efficiency for the interprotein electron transfer reaction.

PSI-Pt Nanoparticle H_2 Production. A photocatalytic hydrogen-evolving system based on intermolecular electron transfer between native PSI and electrostatically associated Pt nanoparticles has been developed. Visible-light-induced H_2 production occurs for the PSI/Pt

nanoparticle biohybrid at a rate of >21,000 mol H_2 (mol PSI)$^{-1}$ h^{-1}. These results demonstrate that highly efficient photocatalysis of H_2 can be obtained for a self-assembled, noncovalent complex between PSI and Pt nanoparticles; a molecular wire between the terminal acceptor of PSI, the [4Fe-4S] cluster F_B, and the nanoparticle is not required.

PSI-Cobaloxime H_2 Production. Although a great catalyst, platinum is a rare and expensive noble metal. Thus, we are investigating new strategies for solar fuel production that involve the insertion of sustainable first-row transition metal *molecular* catalysts into PSI. As a first attempt at incorporation of molecular catalysts into PSI, we have targeted the well-known proton reduction cobaloxime electrocatalysts, pseudo-macrocyclic bis(dimethylglyoxamato) cobalt complexes. Cobaloxime self-assembles with PSI, and the resultant complex provides the first example of light-driven hydrogen production from a synthetic molecular catalyst linked to PSI. This system provides a benchmark for creation of future PSI-molecular catalyst hybrids, presenting new opportunities for solar fuel production that merge synthetic inorganic capabilities with classic bioinorganic approaches.

Science objectives for 2011-2012:

- We are designing experimental systems to look in detail at PSI interprotein interactions. We are interested in looking at the native PSI reduction of ferredoxin and flavodoxin in solution using freeze-trap methods and EPR spectroscopy, metal ion-induced positioning of these carrier proteins, as well as light-induced conformational changes in PSI.

- Current PSI-catalyst biohybrid systems are being optimized. New bioinorganic and acceptor protein-based strategies to form biohybid complexes are being developed. Fundamental ET mechanisms of PSI-catalyst hybrids will be investigated with optical and EPR spectroscopy.

References to work supported by this project 2009-2011:

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Poster Session I

Regulation of Thylakoid Lipid Biosynthesis in Plants

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Overall Research Goal: Galactoglycerolipids are the predominant lipids of photosynthetic membranes. Galactosyl transferases associated with the inner and outer chloroplast envelope membranes synthesize the bulk of galactoglycerolipids from UDP-galactose and diacylglycerol. The diacylglycerol moiety can be assembled at the endoplasmic reticulum (ER) or at the inner chloroplast envelope membrane. As a consequence, precursors for a substantial amount of galactolipids must be imported into the plastid. Arabidopsis mutants deficient in several aspects of this process are available. Mutants disrupted in the import of lipids from the ER accumulate unusual oligogalactolipids and triacylglycerols for reasons currently not understood. Oligogalactolipid synthesis has been observed in isolated chloroplasts as well, and was attributed to the activation of a processive galactolipid:galactolipid galactosyl transferase (GGGT) associated with the chloroplast outer envelope. The gene proposed to encode GGGT of Arabidopsis was identified as *SENSITIVE TO FREEZING*. The long term goal is to provide an understanding of GGGT function in chloroplast biogenesis and maintenance, its biochemical properties, and its regulation of activity. Specific objectives are (1) to study SFR2 function in its physiological context, (2) to understand the role of SFR2 activation in lipid trafficking mutants, (3) to determine the biochemical mechanism of SFR2 activation, and (4) to probe diacylglycerol (DAG) pools, as they are affected by the activity of SFR2.

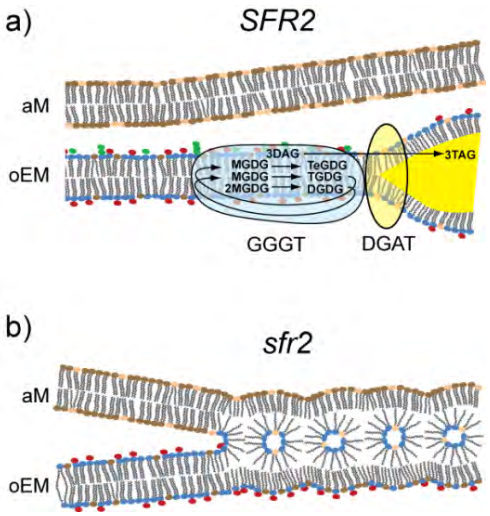

Figure 1. A proposed model for GGGT-dependent galactolipid remodeling during freezing. In wild-type plants (a) (SFR2), lamellar membrane structure is maintained between the chloroplast outer envelope (oEM) and various apposed extraplastidic membranes (aM) through SFR2-dependent galactolipid remodeling (GGGT) and as yet unidentified triacylglycerol biosynthetic enzyme(s) (DGAT). Following the loss of GGGT in the *sfr2* mutant (b), the outer envelope membrane and other apposed membranes have an increased tendency for inter bilayer hexagonal II phase formation and membrane fusion leading to freezing damage. Lipids: DAG, diacylglycerol, DGDG, digalactosyldiacylglycerol; MGDG, Monogalactosyldiacylglycerol; TAG, triacylglycerol; TGDG, trigalactosyldiacylglycerol (Moellering and Benning, 2011).

Significant Achievements 2010-2011:

- The initial characterization of SFR encoding GGGT activity was completed. The enzyme was expressed in yeast and its activity was reconstituted in yeast microsomes (Moellering et al., 2010).

- A novel hypothesis was proposed linking freezing tolerance in plants to the lipid remodeling activities of SFR2 (Figure 1; Moellering and Benning, 2011). However, the presence of SFR2 activity was also tested and observed in a variety of non-freeze tolerant tropical plants, implying that freeze tolerance is not the only biological rationale for SFR2 activation.
- The SFR2 protein structure was modeled providing guidance for a mutational structure-function analysis of SFR2. First truncations of SFR2 were generated and their activity tested in yeast microsomes.
- Crossing lipid trafficking and *sfr2* mutants determined that the accumulation of oligogalactolipid in the lipid trafficking mutants is indeed due to SFR2 activity.
- Transgenic plants were generated, targeting *E. coli* DAG kinase to different membranes to probe different DAG pools. Of all the constructs tested, targeting the *E. coli* enzyme to the chloroplast intermembrane space had severe effects on growth and led to a strong accumulation of phosphatidic acid and a reduction in galactolipid accumulation suggesting the proposed approach is working.

Science Objectives for 2011-2012:

- A major focus will be on the construction of deletion and point mutants of SFR2 and analysis of their activity in the yeast microsome assay. If the protein contains a specific regulatory domain, its removal or alteration could constitutively activate SFR2.
- To address the function of SFR2 in naturally freezing sensitive plants, we will isolate and begin to investigate an SFR2 ortholog from tomato. It seems possible that the activation of tomato SFR2 is different than the Arabidopsis ortholog.
- Particular focus will be on the analysis of transgenic Arabidopsis plants targeting *E. coli* DAG kinase to different membranes. A detailed lipid analysis of severely affected lines, in which the DAG kinase is targeted to the chloroplast intermembrane space versus the other faces of the envelope membranes, should allow us to gain first insights into the importance of specific DAG pools for thylakoid lipid biosynthesis.
- We will begin to cross mutants such as *sfr2* or lipid trafficking mutants with the *E. coli* DAG Kinase expressing lines to probe for changes in DAG pools in these mutants. In addition, we will subject the transgenic lines to freeze treatment, which activates SFR2 and creates an additional DAG pool at the outer envelope membrane. Different diagnostic phenotypes are expected depending on the targeting of the DAG kinase and the alteration of DAG pools through activation of SFR2 or the presence of mutations affecting lipid trafficking.

References to work supported by this project 2010-2011:

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Multifrequency Pulse EPR Studies of Ligation of the PSII Manganese Cluster

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Overall research goals: The Mn-Ca cluster of the Photosystem II reaction center splits water and produces molecular oxygen. New X-ray structures give more detailed pictures of the geometry of this 'oxygen evolving complex' in its more reduced states. Spectroscopies such as EPR can probe later oxidation states following flashes to advance the oxygen evolving S-state cycle. Specifically EPR can provide details of electronic structure by measuring the hyperfine interactions between the unpaired electron spin and magnetic nuclei of ligands to the cluster. This provides new insight into the mechanism of the reaction, particularly when combined with high level electronic structure calculations.

Significant achievements 2011: Our DOE Photosynthesis Program funding is just beginning. We are working with the Debus laboratory to expand our initial ^{13}C ENDOR study of carboxylate binding to the Mn cluster (1) with labeling strategies beyond only labeling the terminal alanine, and also with instrumental improvements. We are extending our 31-35 GHz high resolution ESEEM of the S_2 multiline signal nitrogen ligation (2) to other spectral forms and to the *Synechococcus sp*. used in the X-ray diffraction studies. We are continuing to develop ENDOR coupled to high frequency/field (130 GHz, 4.7 T) pulse EPR as a tool for studying the Mn-Ca centers.

References to work supported by this project 2011:

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Light Energy Transduction in Green Bacteria

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Overall research goals: The long-term objectives of this research program are to understand the photosynthetic apparatus of green bacteria, and in particular to understand the structure, function, and biogenesis of chlorosomes, their light-harvesting antennae. Chlorosomes occur in all phototrophic *Chlorobi* (GSB), in some filamentous anoxygenic phototrophs (FAPs) belonging to the phylum *Chloroflexi*, and in *Candidatus* Chloracidobacterium thermophilum, a newly discovered aerobic chlorophototrophic member of the phylum *Acidobacteria*. Genomic, bioinformatic, genetic and biochemical approaches are employed to define chlorophyll and carotenoid biosynthetic pathways, reaction centers, electron transport chains, and other metabolic properties of green bacteria that are critically important to their ability to transduce light energy efficiently into chemical energy. We have recently expanded our studies of carotenoid biosynthesis to include pathways in purple sulfur bacteria (*e. g.*, for okenone and rhodopinal).

Significant achievements 2009-2011: We completed the identification of enzymes in the biosynthetic pathways for bacteriochlorophyll (BChl) *c* and *d* as well as the carotenoid, okenone. We characterized FMO and chlorosomes of *Ca.* C. thermophilum isolated and additionally characterized the oxygen-stable, homodimeric type-1 reaction centers (RCs) of this organism. These RCs contain ~2 molecules of Zn-BChl *a′*, which may act as the primary electron donor. The *in situ* transcription patterns of all major chlorophototrophs in microbial mats of Mushroom Spring in Yellowstone National Park (YNP) were determined at 1-h time intervals over a full diel cycle. These data revealed that oxygenic and anoxygenic phototrophs differ dramatically in their patterns of photosynthetic gene transcription. The data also suggested that chlorosome-containing organisms may perform photo-phosphorylation at night. Metagenomic and meta-transcriptomic approaches led to the discovery and description of 3 novel chlorophototrophs belonging to the phyla *Chlorobi* and *Chloroflexi* in alkaline siliceous hot spring mats in YNP. Genetics, solid-state NMR, cryo-EM, and molecular modeling were used to determine the structure of BChl *c* in chlorosomes of a *bchQ bchR* mutant of *C. tepidum* (Fig. 1).

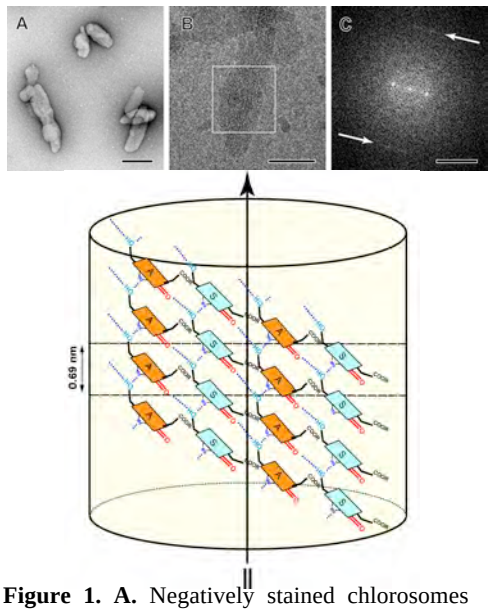

Figure 1. A. Negatively stained chlorosomes from the *bchQ bchR* mutant of *C. tepidum*. **B.** Cryo-EM image of chlorosomes from *bchQ bchR* mutant. Fourier transform of image in panel **B**. The model below shows the all-*syn* and all-*anti* parallel monomer stacks of BChl *c* and the origin of the 0.69-nm repeat visible as the layer lines in panel **C**.

Objectives for 2011-2013:

- Characterize key enzymes of the biosynthetic pathways for BChl *c/d/e* and carotenoids
- Continue characterizing the homodimeric reaction centers of *Ca.* C. thermophilum
- Isolate and verify inferred properties of *Candidatus* Thermochlorobacter aerophilum

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Investigations into the pfkB family of proteins affecting chloroplast function

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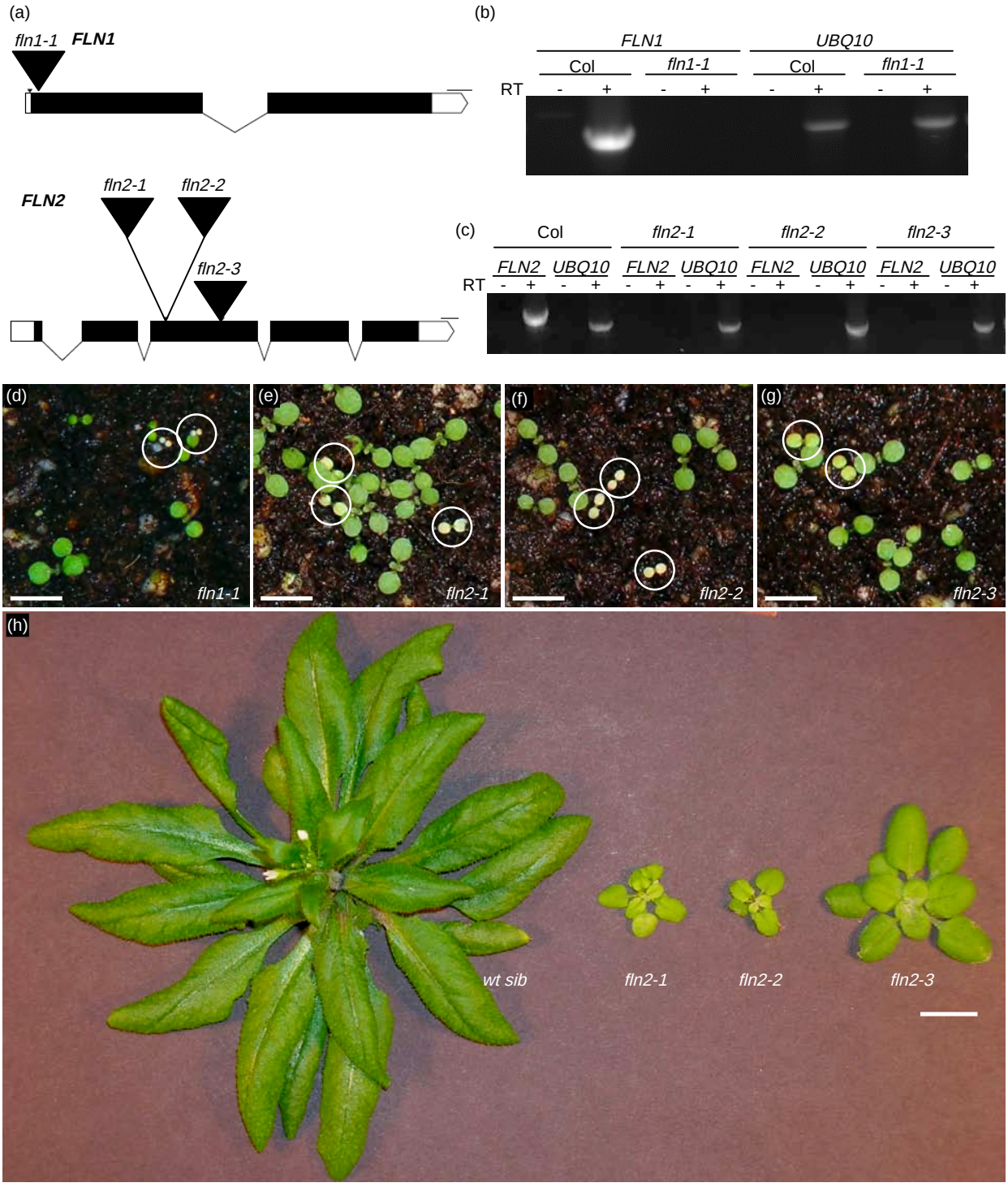
Overall Research Goals: The long-term research goals are to understand the biological function of the pkkB family of carbohydrate kinases by: 1) studying the effects of loss-of-function mutants in individual family members and in multiple mutants; 2) to develop an assay to test the biochemical function of two family members whose chloroplast localization has been determined; and to 3) use this assay to determine whether catalytic activity and/or substrate binding are essential to in vivo function.

Significant achievements 2009-2011. 1) We have characterized in detail the phenotype of loss of function mutants in *FLN1* (for *Fructokinase-like protein*) and *FLN2*, encoding two closely members of the pfkB family of carbohydrate kinases of which most have unknown function. Loss of function T-DNA insertional mutants in either At3g54090 (*FLN1*) or At1g69200 (*FLN2*), result in chlorotic plants that differ in severity. *fln2* mutants green slowly and remain small, but eventually can flower and set seed. On the other hand *fln1* plants never green and die on soil (Figure 1). Significant development of white *fln1* plants occurs on synthetic media with exogenous sugar. However, double homozygous *fln1 fln2* plants fail to develop past cotyledon opening, even on synthetic media. Normal plastid development in both light and dark requires at least one of the FLNs, but surprisingly skotomorphogenesis is unaffected in *fln* seedlings. These studies establish the significance of these proteins for choroplast function. 2) We developed an *in vivo* assay for function through introduction of a transgene into the mutant background. This was not trivial, as over-expression of FLN proteins either was toxic, or transgene-induced silencing of the endogenous genes occurred in this system. Finally, we were successful by inducing a low level of expression using reduced amounts of dexamethasone in achieving a dex-dependent wild-type phenotype in the hmz T-DNA mutant background; 3) We have made some progress in isolating T-DNA insertion alleles for other members of the pfkB family. There are a total of 20 pfkB carbohydrate kinase-encoding genes in *Arabidopsis thaliana*. Two phosphorylate adenine and 3 phosphorylate fructose *in vitro*. Another member, NARA5, plays an unknown developmental role, while the other 12 (not counting FLN1 and 2) are completely uncharacterized. Currently, no phenotypic effects of single loss of function mutants are seen, so creation of mutant in closely related genes is in progress.

Objectives for 2011-2012: 1) Complete the isolation of available T-DNA insertion lines in the pfkB family member and obtain mutants in multiple family members: 2) Create and test the in vivo function of site-directed mutants of FLN1 or FLN2- in either predicted kinase active site or predicted substrate binding to determine which is required and 3) Complete analysis of *fln1* and *fln2* mutant lines.

Figure 1 (next page). Identification and characterization of *fln* mutant lines.
(a) Structure of the *FLN1* (At3g54090) and *FLN2* (At1g69200) genes with positions of T-DNA insertions shown. (b) and (c) RT-PCR analysis of *fln1* and *fln2* alleles from 7-day old light grown seedlings. *UBQ10* reactions show equal RNA inputs, and minus (-)RT reactions demonstrate amplification from cDNA. (d) to (g) 7-day old phenotype of *fln* mutants. Seeds segregating for *fln* alleles were germinated and seedlings grown under a 16h photoperiod at 18^{o} C. Homozygous individuals are circled. Scale bars, 0.5 cm. (h) 40-day old phenotype of *fln2* alleles. Seedlings from (d)-(f) were transferred to individual pots for continued growth. Scale bar, 1 cm.


(a)
fln1-1
FLN1
FLN2
fln2-1
fln2-2
fln2-3
(b)
FLN1
UBQ10
Col
fln1-1
Col
fln1-1
RT
- + - + - + - +
(c)
Col
fln2-1
fln2-2
fln2-3
FLN2 UBQ10 FLN2 UBQ10 FLN2 UBQ10 FLN2 UBQ10
RT
- + - + - + - + - + - + - + - +
(d)
fln1-1
(e)
fln2-1
(f)
fln2-2
(g)
fln2-3
(h)
wt sib
fln2-1
fln2-2
fln2-3

FTIR Studies of Photosynthetic Oxygen Production

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Overall research goals: The oxidation of water to molecular oxygen is an extremely demanding chemical reaction, both thermodynamically and kinetically. The Mn_4Ca cluster in Photosystem II catalyzes this reaction far more efficiently than any synthetic catalyst. The reason for this efficiency is that the protein environment of the Mn_4Ca cluster controls the cluster's reactivity at each step in the catalytic cycle by carefully choreographing the proton and electron transfer reactions associated with water oxidation and by carefully managing both substrate (water) access and proton egress. Our overall goal is to identify the amino acid residues that are responsible for this control and to determine the role of each. Our specific goals are to identify and further delineate the dominant water access and proton egress pathways that link the Mn_4Ca cluster with the thylakoid lumen and to identify the amino acid residues that serve as the critical bases that facilitate the oxidation of the Mn_4Ca cluster in its higher oxidation states. Our approach is to characterize mutant Photosystem II core complexes representing residues identified crystallographically or computationally as potentially participating in networks of hydrogen bonds or serving as catalytic bases. Our primary investigative tool is FTIR difference spectroscopy. Infrared spectroscopy is particularly suited for analyzing protonation/deprotonation reactions, pK_a shifts, and hydrogen bonded structures in proteins. Our recent work has focused on residues thought to participate in a dominant proton egress pathway leading from the Mn_4Ca cluster to the thylakoid lumen involving the Chloride ion (Cl^- 1) that is ligated by side chain amino group of D2-Lys317 and the backbone nitrogen of D1-Glu333.

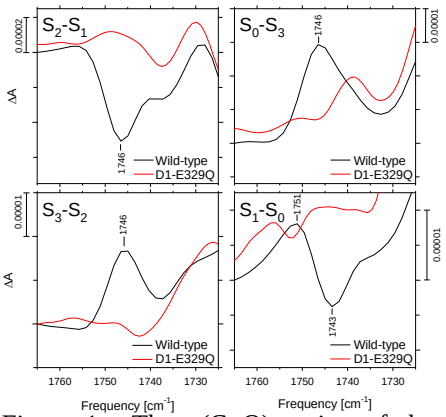

Figure 1. The ν(C=O) region of the S_{n+1}-*minus*-S_n FTIR difference spectra of wild-type (black) and D1-Glu329Gln (red) PSII core complexes.

Significant achievements 2010-2011:

- We obtained evidence that the carbonyl stretching [ν(C=O)] modes of protonated carboxylic acids serve as suitable probes of changes to networks of hydrogen bonds during all the S state transitions (our previous work had focused on the S_1 to S_2 transition). For example, the D1-Glu329Gln mutation eliminates the ν(C=O) features from all of the S_{n+1}-*minus*-S_n FTIR difference spectra (Figure 1), suggesting that this mutation alters the network of hydrogen bonds sufficiently that the carboxylate residues responsible for these features are no longer sensitive to the structural perturbations that accompany any of the S state transitions. In

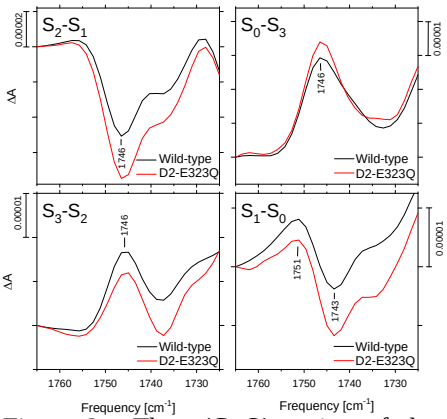

Figure 2. The ν(C=O) region of the S_{n+1}-*minus*-S_n FTIR difference spectra of wild-type (black) and D2-Glu323Gln (red) PSII core complexes.

contrast, the D2-Glu323Gln mutation eliminates none of these features (Figure 2) implying either that D2-Glu323 does not participate in the same network of hydrogen bonds or that this residue is located at the periphery of the network, so that its replacement with a non-protonatable residue does not prevent the carboxylate groups giving rise to the ν(C=O) features from sensing the structural changes associated with the S state transitions.

- We obtained evidence that D2-Lys317 binds a catalytically active Cl^- ion. Changing this residue to Ala, Glu, or Gln perturbs the S_2-*minus*-S_1 FTIR difference spectrum and prevents efficient advancement beyond the S_2 state (Figure 3), as would be expected if proton release is disrupted because the catalytically active Cl^- ion is no longer bound to the Cl^- 1 site. In contrast, the S_2-*minus*-S_1 FTIR difference spectrum is not altered and the S state transitions proceed normally when D2-Lys317 is changed to Arg.

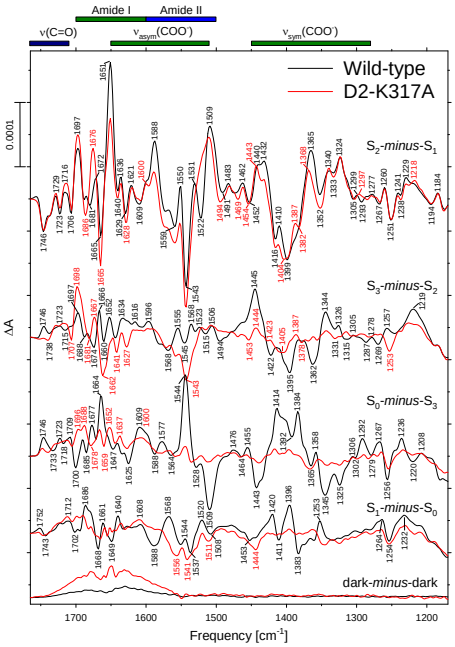

Figure 3. Comparison of the S_{n+1}-*minus*-S_n FTIR difference spectra of wild-type (black) and the corresponding spectra induced by four flashes given to D2-Lys317Ala PSII core complexes (red).

- We obtained evidence that D1-Glu333 influences the efficiency of proton release from the Mn_4Ca cluster. Changing this residue to Gln decreases the efficiency of the S_2 to S_3 and S_3 to S_0 transitions, as would be expected for the mutation of a residue required for efficient proton release. Because D1-Glu333 interacts directly with Cl^- 1 in addition to ligating two Mn ions, the apparently decreased efficiency of proton release may be caused by structural perturbations that alter the Cl^- 1 site.

<u>Science objectives for 2011-2012:</u>

- Additional mutations in the putative dominant proton egress pathway leading from the Mn_4Ca cluster to the lumen via Cl^- 1 will be examined to further characterize the network of hydrogen bonds that comprise this pathway.

- Measurements of proton release to the exterior of Photosystem II will attempted on the basis of S_{n+1}-*minus*-S_n FTIR difference spectra recorded in the presence of 200 mM MES (at this concentration, all protons released from the Mn_4Ca cluster are trapped by the exogenous MES, producing characteristic protonation-induced changes to the buffer's asymmetric SO_3^- and morpholino ring vibrations).

- Time resolved IR measurements will be performed in attempts to follow the progress of protonation changes of carboxylate residues and hydrogen-bonded water molecules as a function of S state.

Dynamic transcription factor binding reveals role of EIN3 in hormone crosstalk

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Overall research goals: Although many significant insights have been gained into the mechanisms of plant growth, major unanswered questions still remain about how plants integrate the various environmental and endogenous signals to control their growth. We aim to identify new signalling pathway components and the major transcriptional gene targets for plant growth regulators with a focus on ethylene gas. In particular, we will use a variety of types of experimental approaches to explore the hormone signalling pathway targets, their roles in ethylene signalling and cross-talk with other hormone response pathways. Our hypothesis is that through the identification and analysis of hormone pathway targets, we will be able to functionally dissect the various transcriptional output pathways of ethylene that control the numerous and important biological processes regulated by this simple gas (biomass production, biotic and abiotic stress, senescence, etc.) The research objectives are to understand the ethylene signalling network and how it communicates with other signalling networks by: 1) identifying and characterizing EIN3 dependent ethylene response output pathways, and 2) determining the roles of additional ethylene signalling components in the regulation of EIN3.

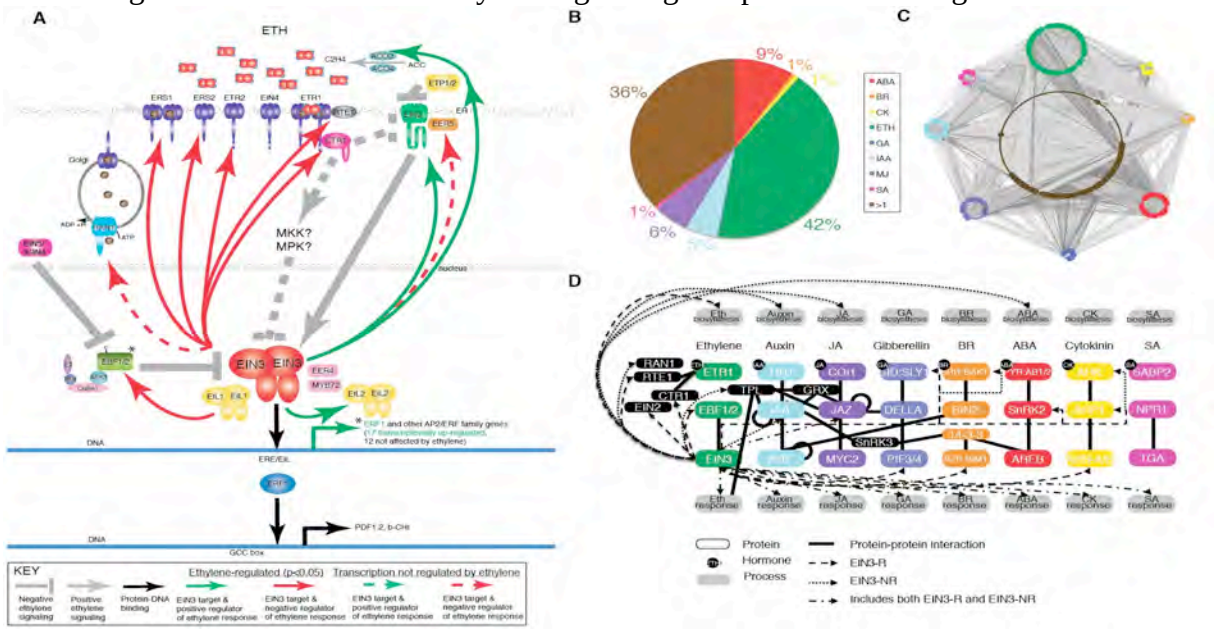

Figure 1. Functional classification of EIN3 targets reveals genes involved in hormone responses. (A) Feedback within the ethylene signalling pathway and feedforward to downstream effectors. **(B, C)** EIN3 targets are involved in hormone crosstalk. Node color represents hormone annotation, as indicated in B; large nodes are EIN3 targets. Blue edges represent protein-protein interactions (PPI) and black edges are protein-DNA interactions (PDI). **(D)** EIN3-mediated ethylene crosstalk occurs at many different levels. PPIs are from the Arabidopsis Interactome Mapping Consortium (2011), and EIN3 PDIs are from this study.

Significant achievements 2009-2011: The plant hormone ethylene regulates a multitude of growth and developmental processes, however the temporal control and organization of

ethylene signalling remains elusive. We characterized the dynamic ethylene transcriptional response by identifying targets of the master regulator of the ethylene signalling pathway, ETHYLENE INSENSITIVE3 (EIN3), using chromatin immunoprecipitation sequencing and transcript sequencing during a timecourse of ethylene treatment (Chang et al. submitted). We found ethylene-induced transcription occurs in waves, and EIN3 binding established downstream transcriptional cascades, feedback circuitry of the ethylene signalling pathway, and interconnections between hormone response pathways. EIN3 directly targeted *HLS1*, a signal integrator of ethylene, auxin, and light. We further characterized the role of the HLS1 gene family using genetics. Mutants in the *HLS1* gene family exhibited auxin response phenotypes in several growth and developmental stages, thus confirming the role of *HLS1* in hormone crosstalk as a signal integrator.

Science objectives for 2011-2012:

To elucidate how individual hormone signaling pathways are regulated by *cis*-regulatory circuitries, how these circuitries facilitate cross-communication with other hormone pathways and how they coordinate the establishment of effective defense mechanism, a comprehensive and genome-wide annotation of functional *cis*-elements is necessary. Since no genome-wide annotation maps of functiona *cis*-elements exist in Arabidopsis, we aims to generate genome-wide in vitro and *in vivo* maps of the *cis*-regulatory landscape. These experiments will uncover how the mutual crosstalk of ET and other signalling is manifested at the level of TF-binding. In summary, our temporally-resolved hormone-specific signatures will unravel the dynamics of TF appearances and disappearances on the network level and on the single gene level, providing a new view into the molecular architecture of plant growth processes.

References to work supported by this project :

Katherine N. Chang, Shan Zhong, Gary Hon, Mattia Pelizzola, Hai Li, Robert J. Schmitz, Mark Urich, Dwight Kuo, Joseph Nery, Hong Qiao, Trey Ideker, Ziv Bar-Joseph, Joseph R. Ecker, Dynamic transcription factor binding reveals rol;es of EIN3 in hormone crosstalk, submitted

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Identification of key parts of energy allocation systems in plants and their role in pathogen susceptibility

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Overall research goals: The research objectives are to study carbohydrate allocation in plants by (1) developing optical sensor technology to monitor carbohydrate flux in plants non-invasively; (2) to specifically study carbon sequestration using the optical sensor technology; (3) and to identify the transporters involved in carbon sequestration. Unexpectedly, we have succeeded in identifying key components of the machinery involved in carbohydrate translocation from photosynthetic leaves to roots, flowers and seeds using the optical sensor technology. Increased yield potential of today's crop plants can be attributed primarily to improvements in allocation efficiency (ε_p), also termed harvest index, which is defined as the amount of total biomass energy partitioned into harvestable biomass. We have refocused the project to characterize these novel transport mechanisms in more detail. Even more unexpectedly, we learned that pathogens hijack these important components of carbohydrate translocation and thus have begun to characterize the role of plant sugar transporters and the architecture of the sugar translocation machinery in pathogen defense.

Significant achievements: We have developed a suite of genetically encoded Förster Resonance Energy Transfer sensors for pentoses, hexoses and disaccharides. We have successfully implemented these sensors to monitor sugar flux in intact plants. We have implemented microfluidic RootChips for efficient analysis of FRET responses in roots of Arabidopsis. We have identified novel sugar transport activities in roots with properties of uniport systems that could play important roles in carbon sequestration. We have used the optical sensor technology to identify novel sugar transporters named SWEETs. Our data demonstrate that these transporters play key roles in carbohydrate allocation, that they are positioned in strategic locations in the plant to limit leakage of sugars into the cell wall space, most probably to limit nutrient supply to pathogens, and we can show that the transporters are directly activated by pathogens in order to tap into the plants sugar allocation system. Inactivation of the transport activity prevents pathogen infections.

Science objectives: We intend to fully develop the RootChips as a routine technology and explore ways to distribute the technology to the community. We intend to continue the studies and fully define the mechanisms involved in sucrose efflux in leaves as a key to biomass productivity, implement the biosensors in leaves and roots and study members of the family involved in carbon sequestration in roots.

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Regulation of H_2 and CO_2 Metabolism: Factors Involved in Partitioning of Photosynthetic Reductant in Green Algae

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Overall Research objectives**:** The overall objective of this project is to develop fundamental understanding about the regulation of the partitioning of photosynthetic reductants between the H_2-production and the CO_2-fixation pathways. Specific objectives are to: (a) identify additional protein factors whose expression may be required for optimal hydrogenase expression; (b) identify active promoter regions and transcriptional elements for the two algal hydrogenases; and (c) determine whether the recently identified FXL homologs in *C. reinhardtii* play a role in O_2-sensing mechanism and mediate components of the anoxic regulatory response that leads to hydrogenase expression.

Significant Achievements in 2011

Mutant library screening and genetic characterization of mutants: Dr Patrice Hamel's group at Ohio State University is analyzing eleven mutants attenuated for the production of hydrogen from a collection of transformants (~15,000) generated with the hygromycin B resistance insertional cassette. Molecular analysis of the mutants revealed that the affected loci correspond to novel genes that were not previously assigned to hydrogen production or to any other known biological process. We named these mutants *ahp* for (attenuated for *hyd*rogen production). Five *AHP* genes (*AHP1* to *AHP5*) were recovered from the genetic screen. Dr Matthew Posewitz's group at Colorado School of Mines is analyzing the progeny from genetic crosses of the mutants described above to determine whether the hydrogen phenotypes are linked to the antibiotic markers. The Posewitz laboratory has quantified H_2 production from the spores and will send the results to NREL for genetic and physiological characterization.

H_2 production in the trebouxiophyte *Chlorella* NC64A: *Chlorella* NC64A is the first alga known to encode both the H and F-clusters of [FeFe**]**-hydrogenases. We show for the first time that F-cluster-containing hydrogenases are coupled to both anoxic photosynthetic electron transport and dark fermentation in a green alga. H_2 photoproduction in *Chlorella* NC64A is as sensitive to O_2 inactivation as in *C. reinhardtii*. Phylogenetic analysis indicates that all known algal HYDA enzymes are monophyletic, suggesting that they emerged once within the algae. Furthermore, phylogenetic reconstruction indicates that the multiple *HYDA* copies in the algal taxa are the result of gene duplication events that occurred independently in each algal lineage, and that the ancestor of the Trebouxiophyceae and Chlorophyceae likely encoded a single, H and F-cluster-containing HYDA. F-cluster encoding HYDA transcripts have been verified.

Isolation of *hyda2hyda1* double mutant: To define the role of individual hydrogenases in green algae, we screened a Chlamydomonas *hydA2* mutant background for mutants lacking all hydrogenase activity. A double *hydA2hydaA1* mutant, as well as single *hydA1* and *hydA2* mutants have been isolated and are being further characterized. These mutants will have a major role not only in the identification of the specific roles of each hydrogenase in algal metabolism, but they will also serve as a clean background for expression of foreign hydrogenases in Chlamydomonas.

Analysis of hydrogenase promoter elements: To understand the mechanism that underlies the anoxic regulation of hydrogenase in *C. reinhardtii*, we have fused the truncated promoter of HYDA1 and HYDA2 to the SNAP reporter gene lacking its own promoter. Several deletions within the HYDA1 and

HYDA2 promoter regions -644 to -1 and -544 to -1 respectively were introduced into *C.reinhardtii*. Transformants expressing the SNAP protein were shown to transcribe under anaerobic condition. The expression of SNAP protein was high enough to allow quantitative measurements. The results indicate that region between position -144 and -1 for HYDA1 and -149 to –1 for HYDA2 with respect to the transcription start site is required for anaerobic specific gene expression.

Identification of transcription factor for HYDA1/HYDA2: To identify the transcription factors that are required for hydrogenase expression, promoter sequences of HYDA1 and HYDA2 were biotinylated and used to bind proteins from induced (anaerobic) nuclear extract from *C. reinhardtii.* The biotinylated promoter fragments from HYDA1 and HYDA2 were bound to streptavidin and then incubated with nuclear extract from *Chlamydomonas.* Proteins strongly bound to biotinylated HYDA1/HYDA2 promoter were separated and visualized using SDS-denaturing gel electrophoresis. Random DNA was biotinylated for use as a control. Proteins differentially bound to the promoter fragments were identified using LC-MS-MS. We have identified 6 proteins for HYDA1 and 9 proteins for HYDA2 from above experiments. These proteins were analyzed using a database of *cis*-acting regulatory elements.

Characterization of *Chlamydomonas* FXL homologs: To investigate the potential role of the FXL homologs in O_2-sensing in *C. reinhardtii*, we did several biochemical experiments. Spectrophotometric titration of FXL1 and FXL5 with hemin showed saturation at a ratio of 1:1. Under non-denaturing electrophoresis, heme-specific staining for FXL1 and FXL5 showed a single, diffuse band similar in specificity to that of hemoglobin. Both heme proteins are unstable and get oxidized slowly when exposed to air. Oxygen binding measurements on the reduced peptide–heme complexes showed that these compounds bind oxygen and CO_2 and give visible spectra that were typical of oxygenated heme-proteins. O_2 and CO_2 binding measurements were done under different partial pressures. The dissociation constants *(Kd)* of O_2 for FXL1 and FXL5 were ~140 and ~287 μM, respectively. However, CO_2 has much higher affinity for both proteins, with *Kd* values of ~31 and ~21 μM, respectively. Transcriptional analysis of FXL1 and FXL5 showed a slight increase under anaerobic conditions.

Science objectives for 2012

- *C. reinhardtii* mutants with aberrant H_2 production will be further analyzed. Performance of q-RT-PCR transcript levels of HYDA1 and HYDA2 hydrogenase expression will be carried out. Complementation of the the phenotype with the transformed gene to confirm correlation between the disrupted gene and the H_2 production phenotype will be performed.
- Active promter regions of HYDA1 and HYDA2, carrying a SNAP tag will be transformed into double hydrogenase mutants to confirm their assignment. As an additional approach to determining regulatory elements controlling hydrogenase gene expression, we will identify the transcription factor binding to the promoter regions of HYDA1 and HYDA2.
- The potential transcription factor identified in the screen will be used to fish out downstream proteins interacting with it under anaerobic conditions. Antibodies will be generated against the transcription factor protein and used to study its expression under various conditions such as aerobiosis, anaerobiosis, high light intensity, sulfur deprivation etc. Once the regulatory protein counterparts to the transcription factor are identified, they will be genetically modified in order to analyze their role in H_2 production.
- To understand the signal transduction pathway from FXL proteins, promising FXL candidates will be tagged (streptavidin or histidine tag) and over expressed in *C.reinhardtii*. Tagged FIXL proteins will be used to fish out hybridized cellular proteins in the search for interacting proteins involved in signal transduction. FXL6 is predicted to contain a putative ankyrin protein-protein interaction domain. Ankyrin has been shown to bind to a number of plasma membrane-associated proteins. We will also clone this domain to search for interacting proteins in Chlamydpmonas.
- To further determine whether O_2 sensing signal from FXL proteins are related to hydrogenase regulation, we will generate mutant lines for each of the FXL proteins and analyze HYDA1/HYDA2 expression under

aerobic and anaerobic condition. We will also generate RNAi silencincing lines for FXL (by identifying the common sequence among all nine FXL proteins). This method will potentially be able to silence mRNAs of other FXL genes that carry the same sequence of nucleotides.

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Research papers in preparation/submitted

Narayana Murthy U. M., Matt S. A. Wecker, Matthew C. Posewitz, Marie-Alda Gilles-Gonzalez and Maria L. Ghirardi (2010) Novel FixL homologues in *Chlamydomonas reinhardtii* bind heme and O_2 (submitted).

The Homodimeric Type I Reaction Center in *Heliobacterium modesticaldum*

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Overall research goals: Our research objectives are to understand the structure, function and organization of homodimeric Type I reaction center found in *Heliobacterium modesticaldum*. Our goals include: (i) What are the 3-dimensional structures of the heliobacterial reaction center core and the F_A/F_B-containing polypeptides? (ii) Are the PshB1 and PshB2 polypeptides true subunits of the heliobacterial reaction center, or are they quasi-mobile acceptor proteins? (iii) Do other ferredoxin-like proteins function as electron acceptors from F_X? The overall idea is to 'jump start' the study of a simple Type I reaction center, so that with the acquisition of high-resolution X-ray crystal structures, mutagenesis techniques, biochemical protocols, and biophysical tools, structure/function studies can be carried out on a simplified photochemical reaction center that works at the reducing end of the biological redox scale.

Significant achievements 2010-2011:

(1) Purification of the Heliobacterial RC to homogeneity and crystallization. We have extended the purification originally published for the HbRC by adding a second chromatography step (cation exchange chromatography). The resulting preparation has no contamination by cyt c_{553} and several ABC transporters, which were a problem with the previous prep. We have performed an extensive characterization of this purified HbRC core ($PshA_2$) with a wide range of analytical, biochemical, and spectroscopic techniques. The stoichiometry of cofactors within the HbRC was determined to be 19±3 BChl *g* : 2 BChl *g'* : 2 8^1-OH-Chl a_F : 1 4,4'-diaponeurosporene : 1.6 menaquinone (7:1 ratio of MQ-9:MQ-8). Flavodoxin reduction kinetics were very similar to the original prep. In single photon counting fluorescence experiments, a 25-ps component comprising 99% of decay and exhibiting an emission maximum at ~815 nm was assigned to excitation trapping. Many of the experiments repeat what has been done on this prep previously or on preparations of the HbRC form other species, but they were essential to do, as we are engaged in crystallization of the HbRC from this prep. Preliminary crystallization and diffraction results will be presented.

(2) Identification of a second F_A/F_B-containing protein in *Heliobacterium modesticaldum*. The gene that codes for PshB, HM1_1462, is part of a predicted dicistronic operon that contains a second gene, named HM1_1461, which codes for a second ferredoxin-like protein with high sequence homology to PshB, including the two traditional [4Fe-4S] cluster binding motifs. RT-PCR results confirm that both genes are transcribed as a single transcript. We have cloned the HM1_1461 gene through PCR amplification of the *H. modesticaldum* chromosomal DNA and overexpressed the apoprotein in *Escherichia coli*. Reconstitution studies with inorganic reagents have shown that the holoprotein harbors ~8 iron and ~8 sulfide atoms in the form of two [4Fe-4S] clusters. Incubation of the reconstituted holoprotein with heliobacterial reaction center cores results in a charge-separated state characteristic of electron transfer past the F_X cluster to the terminal [4Fe-4S] clusters F_A and F_B. These results suggest that the HM1_1461 product, which we have named PshBII, is capable of functioning in lieu of PshB (renamed PshBI) as an alternative terminal electron transfer protein.

Thus, unlike PS I, to which PsaC is tightly bound, two loosely bound ferredoxins, PshBI and PshBII, interact with the heliobacterial reaction center.

(3) Soluble redox proteins can be reduced directly by F_X in heliobacterial reaction centers. We have proposed earlier that PshBI and PshBII are quasi-mobile electron transfer proteins that, upon reduction, dissociate from the HbRC and reduce downstream metabolic partners. Using P_{800}-F_X cores devoid of PshBI and PshBII, we show that the iron-sulfur cluster F_X directly reduces the souble redox protein flavodoxin without the involvement of PshBI and PshBII. The maximum rate of flavodoxin reduction occurs at pH 8.0 in the presence of 5 mM $MgCl_2$ and 20 mM NaCl with phenzine methosulfate as the electron donor to P_{800}^+. Increasing the ionic strength leads to a significant loss of activity, an effect reminiscent of the ionic strength-dependent loss of PshBI/PshBII from membranes and HbRCs. The reduction of flavodoxin is suppressed by the presence of PshBI or PshBII, an effect that can be explained on the basis of competition for the electron on F_X. This is the first report of the F_X cluster participating in forward electron transfer to a completely soluble redox protein in any Type I reaction center and implies that the membrane-embedded PshA homodimer should be capable of donating electrons directly to a variety of soluble redox partners in heliobacteria.

Science objectives for 2011-2012:

1. Produce first gene deletion mutants in *H. modesticaldum.*
2. Continue X-ray crystallography of the HbRC core and PshB protein, and NMR work on the latter.
3. Produce recombinant cyt c_{553} and FdxB to have native electron donors and acceptors.
4. Continue experiments to test double-reduction of menaquinone by the HbRC in presence of high-potential electron donors (*e.g.* cyt c_{553}), and test ability of QB-type inhibitors to bind to the HbRC in the MQ site and affect electron transfer within the RC.
5. Study the effect on primary charge separation when bacteriochlorophyll *g* is converted to a chlorophyll *a*-like molecule in the presence of molecular oxygen.

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Excitation energy transfer in photosynthetic complexes and chlorophyll trefoils: hole-burning (HB) and single complex/trefoil spectroscopic studies.

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Overall research goals: The research objectives are to better understand natural and artificial photosynthetic complexes. HB and modelling studies are used to provide more insight into the excitonic structure and excitation energy transfer (EET) processes. It is anticipated that experimentally determined parameters will allow for better modelling of excitonic structure and EET dynamics at a quantitative level.

Significant achievements 2010-2011:

- ***CP47 complex of PSII: simulation of optical spectra and revised structural assignments***. We simulated steady-state absorption, emission, and non-resonant HB spectra for the CP47. Excitonic calculations allowed for the assignment of the Chls contributing to the lowest excitonic states. The search for realistic site energies was guided by experimental constraints and aided by fitting algorithms [*J. Phys. Chem. B 114, 11884 (2010)*]. A new insight into the electronic structure of CP47 complex has been provided [*JACS, 132 (2010) 4214*].
- ***Accurate lineshape functions for modeling of ΔFLN.*** We have shown that previous analysis of ΔFLN spectra obtained for many photosynthetic complexes led to strong artificial lowering of apparent electron-phonon coupling strength. *J. Phys. Chem. Lett. 1, 2310 (2010).*
- ***Low-T frequency domain study of EET in ethynyl-linked Chl-trefoils and aggregates.*** We showed that EET in ethynyl-linked chlorophyll trefoil monomer is very fast (~2.5 ps) and nearly retained in various trefoil aggregates. *J. Phys. Chem. B 115, 1520 (2011).*
- ***On the primary electron donor and electron acceptor in the RC of PSII from Chlamydomonas reinhardtii.*** While most researchers over the last two decades assigned the Q_y-states of $Pheo_{D1}$ and $Pheo_{D2}$ bands near 678-684 nm and 668-672 nm, respectively, recent modeling of the electronic structure reversed the location of the active ($Pheo_{D1}$) and inactive ($Pheo_{D2}$) pheophytins, suggesting that the mean site energy of the $Pheo_{D1}$ is near 672 nm. To provide more insight into the site energy of electron acceptor, and to attest that the above assignment is incorrect, we studied isolated RCs from spinach and wild-type *Chlamydomonas reinhardtii* (at different level of intactness), as well as the D2-L210H mutant, in which the active branch $Pheo_{D1}$ is replaced with Chl *a*. We show that the Q_x-/Q_y-region site-energies of $Pheo_{D1}$ and $Pheo_{D2}$ are ~545/680 nm and ~542/670 nm, respectively, and demonstrate that the primary electron donor in *intact* algal RCs is P_{D1} (analogous to P_L of the special BChl pair of the bacterial RCs) and not Chl_{D1}, although the latter can also be a primary electron donor. We showed that the pathway of charge separation in *isolated* RCs depends on intactness of the so-called P680*. A highly dispersive charge-separation occurs on the picoseconds time scale. *(Two manuscripts are in preparation, 2011).*

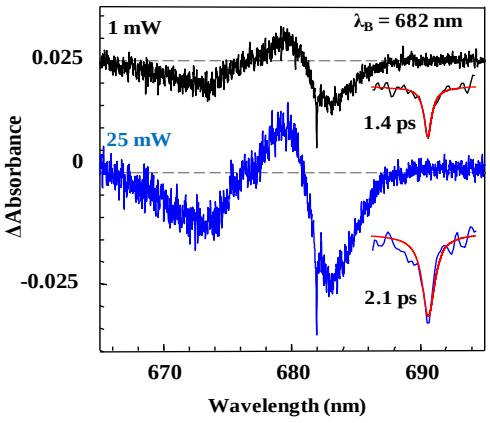

Figure 1. *Resonant transient HB spectra for Chlamydomonas reinhardtti revealing dispersion of CS rates (manifested as variations in homogeneous line widths). Note a negative signal at ~673 nm expected for oxidation of P_{D1}.*

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New DOE-BES project: *"Resonant and Non-Resonant Hole-Burning (HB) and Delta Fluorescence Line-Narrowing Study of BChls in Excitonically Coupled Photosynthetic Systems"*; (Sep. 2011-).

Science objectives: This project focuses on excitonically coupled BChls. HB, ΔFLNS, and modeling studies will be used to provide insight into the excitonic structure and vibrational frequencies, electron-phonon and vibronic couplings, ligation of pigments, and excitation energy transfer (EET) processes in: **1)** *De novo* designed proteins with *one* and *two* Zn-BChls will provide relatively simple functional models of BChls in the protein environment as well as serve as a complementary strategy for better understanding the roles of specific elements within the natural systems. **2)** The Zn-reaction center (RC) protein from *Rb. Sphaeroides* (Zn-RC with six Zn-BChls) and its mutants to evaluate how the coordination state of the Zn in the H_A Zn-BChl affects excitonic structure, HB spectra, and EET/ET rates. **3)** Various FMO antenna protein complexes. This study is warranted due to the discovery of the 8th BChl molecule in addition to the seven molecules that have been known and studied experimentally and theoretically for many years. The 8th pigment is sandwiched between two monomers and has BChl ligands from two different subunits. Our preliminary data suggest that pigment #8 may absorb in the 804-818 nm spectral region (see Figure 2) and not near 787 nm, as recently suggested by theoretical calculations [T. Renger *et al. J. Phys. Chem. Lett.* 2011, 2, 93]. Note broader zero-phonon holes near 804-808 nm region (research in progress). Studies of FMO complexes will be done in collaboration with Dr. R. Blankenship.

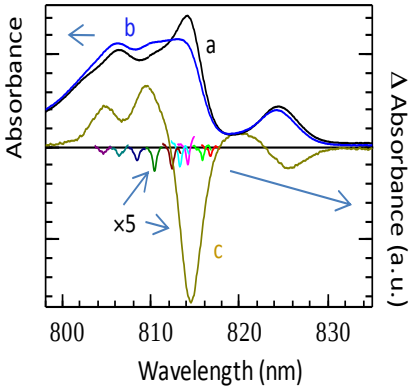

Figure 2. *5 K absorption spectra of PFMO before (curve a) and after HB at 496.5 nm (curve b). Curve c=b-a is a saturated nonresonant HB spectrum obtained with λ_B = 496.5 nm. The narrow resonant ZPHs (in the 804-818 nm region) were obtained at constant fluence (i.e. corresponding to zero-phonon action spectrum) obtained after saturation of the persistent hole (see curve c) with the laser at 496.5 nm turned on.*

Manuscripts in preparation supported by the DOE BES project (September 2011-present):

1. B. Neupane… and R. Jankowiak, *"Electron Transfer in the Rhodobacter Sphaeroides Reaction Center Containing Zn-Bacteriochlorophylls and its β-Zn-RC Mutant: Hole Burning Study", J. Phys. Chem. B (2011), to be submitted.*

2. K. Acharya, A. Kell, J. Wen, R. Blankenship, and R. Jankowiak, "Hole-Burning Study of Various FMO Complexes", *J. Phys. Chem. B (in preparation).* 3. M. Reppert and R. Jankowiak, *"Modeling of Resonant Hole-Burning Spectra in FMO Complexes: An Application of Redfield Theory" JACS (in preparation).*

The Energy Budget of Steady-State Photosynthesis: Mechanism and Regulation of Cyclic Electron Flow in Higher Plants Chloroplasts

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Overall Research Goals: The overall research goals focus on how photosynthesis balances its energy budget, matching the output of energy in NADPH and ATP to precisely meet biochemical demands. If this balancing does not occur, the system will fail, leading to photodamage. Efforts to improve the efficiency of photosynthesis by introducing CO_2 concentrating mechanisms, altering metabolism or biosynthetic pathways to shunt energy to alternative products will likely exacerbate these imbalances (*1*). The goal of the proposed research is thus to understand the mechanisms by which this balance is achieved and regulated to allow future improvements in photosynthesis. Cyclic electron flow around photosystem I (CEF1) balances the ATP/NADPH budget of higher plant photosynthesis. Despite its important role, there are many open questions about the mechanism and regulation of this process.

Significant achievements (2009-2011) (*1-10*): To address these questions, we isolated a series of "*hcef*" (high cyclic electron flow) mutants in Arabidopsis mutants with constitutively high CEF1. Elevated CEF1 in these mutants involves the chloroplast NADPH:PQ oxidoreductase-like complex, NDH, and not the proposed AA-sensitive PGR5 pathway. A novel set of *in vivo* assays of proton and electron fluxes suggest that CEF1 involves a proton pumping plastoquinone reductase in CEF1, consistent with the complex I-like NDH rather than PGR5 or the cytochrome b_6f complex. We also used the *hcef* mutants to test regulatory models, eliminating state transitions and Calvin-Benson cycle intermediates as potential regulators. Mutations that knock out chloroplast ferredoxin:$NADP^+$ oxidoreductase increased CEF1, indicating that events upstream of FNR (e.g. Fd redox state, reactive oxygen species) are likely triggers for CEF1. In line with this suggestion, our *hcef* mutants also produced high levels of H_2O_2, leading us to test whether H_2O_2 was a cause or an effect of high CEF1. H_2O_2 was introduced into leaves, either by infiltration or, in mutants expressing glycolate oxidase in the chloroplast, by exposure to ambient CO_2. Both methods resulted in strong activation of CEF1. We propose a regulatory model in which H_2O_2 is produced when PSI electron acceptors are strongly reduced, under conditions of ATP/NADPH imbalance. H_2O_2, in turn, may induce synthesis of NDH genes and activate the NDH complex, perhaps involving a phosphorylation cascade, as suggested by previous work.

Science Objectives for 2011-2013:

1) We will directly test proton-pumping capacity of NDH using a new luciferase assay.

2) We will test the H_2O_2 regulatory cascade, and delineate by mapping and characterizing (with in vivo spectroscopy, comparative proteomics) three newly isolated *hcef* mutants.

3) We will test for alternative ATP regulatory mechanisms by introducing a highly-active, type II (non-proton pumping), quinone reductase, thereby initiating constitutive CEF1, and altering ATP/NADPH energy balance.

4) We will explore the function of a putative chloroplast-targeted NDC complex with partial homology to type II quinone reductase, which could function in CEF1 under some conditions.

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Elucidating the Principles that Control Proton-Coupled Electron Transfer Reactions in the Photosynthetic Protein, Photosystem II.

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Overall Research Goals: The solar water-splitting protein complex, photosystem II (PSII), catalyzes one of the most energetically demanding reactions in Nature by using light energy to drive a catalyst capable of oxidizing water. Proton-coupled electron transfer (PCET) reactions, which are exquisitely tuned by smart protein matrix effects, are central to the water-splitting chemistry of PSII. Elucidating the water-splitting chemistry of PSII is of major importance in designing bio-inspired catalytic systems for solar fuels production. Proton motion coupled to electron transfer is the basic mechanism of biological energy conversion. However, the details of PCET processes are not yet understood because of the inability of conventional methods to directly probe PCET processes. A major challenge is to develop methods to directly probe PCET processes to understand the structural requirements for minimizing the energetic penalty for multiple charge transfers. The objective of our research is to elucidate the tuning and regulation of PCET reactions of PSII and to determine their role in the early charge-transfer steps of photosynthesis. We are studying the factors that control the coupling of proton and electron transfer pathways at the catalytic tetranuclear manganese-calcium-oxo (Mn_4Ca-oxo) cluster and the redox-active tyrosine and quinone cofactors of PSII by the development of new state-of-the-art multi-frequency multi-dimensional electron paramagnetic resonance (EPR) spectroscopy methods.

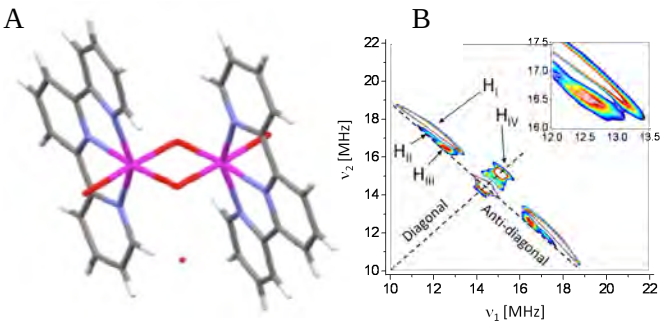

Figure 1. (A) The X-ray crystal structure of (A) $[H_2O(terpy)Mn^{III}(\mu\text{-}O)_2Mn^{IV}(terpy)OH_2](NO_3)_3$ (terpy = 2,2':6',2''-terpyridine) (**1**) and **(B)** The 2D ^{1}H HYSCORE (A) spectrum of **1** in protonated aqueous buffer at pH 4.3.

Significant Achievements 2009-2011:

(1). In a very exciting development, we have unambiguously identified the substrate water molecules that are bound to the catalytic Mn_4Ca-oxo cluster in the S_2 state of photosystem II. This provides, for the first time, a direct window into the activation and catalysis of bound substrate water molecules in the solar water oxidation reaction of photosystem II.

(2). We have demonstrated, for the first time, the presence of three nitrogen atoms that are magnetically interacting with the Mn_4Ca-oxo cluster in the S_2 state of photosystem II. We unambiguously assign the interactions to the imino and amino nitrogen atoms of a strongly ligated histidine residue and the terminal nitrogen atom of an arginine residue that is proximal to the catalytic cluster.

(3). We have characterized the electronic properties of the water molecules that are directly coordinated to the mixed-valence manganese ions in synthetic dimanganese di-μ-oxo complexes (Figure 1). These are excellent structural and functional mimics of the catalytic Mn_4Ca-oxo cluster of photosystem II and provide insight on the mechanism of the solar water oxidation reaction of photosystem II.

(4). We have detailed the individual steps of the highly efficient proton-coupled electron transfer reactions mediated by the redox-active tyrosine residues of photosystem II. This study provides a blueprint for biological proton-coupled electron transfer processes in solar energy conversion.
(5). We have conducted detailed multi-dimensional EPR spectroscopy investigations of the primary and secondary quinone and phylloquinone cofactors of photosystem II and photosystem I, respectively. This study provides insight on the factors that tune and control the function of quinones in solar energy conversion.
(6). We are making rapid progress on solids NMR spectroscopy investigations of the oxygen-evolving complex and the quinone acceptors of photosystem II.

Science Objectives for 2011-2012:

- Thus far, we have detailed the environment of the catalytic Mn_4Ca-oxo cluster and the redox-active tyrosine and primary quinone cofactors of PSII. We are expanding the initial studies to include site-directed mutagenesis to engineer PSII in which the environment of these key cofactors that are participating in the solar water oxidation has been modified to alter the efficiency of proton-coupled electron transfer reactions. Our goal is to better understand the role of the protein environment in determining the functional properties of the PCET cofactors of PSII. These studies will also investigate the tuning of the secondary quinone cofactor of PSII. ◄

- We are embarking on solids NMR investigations to map the structure and dynamics of the tyrosine and quinone cofactors of PSII. This will enable the direct determination of structural parameters, protonation states, hydrogen-bonding interactions and dynamics of these cofactors and provide valuable insight into the functional tuning of the PCET cofactors of PSII.

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Dissecting the function of immunophilins in the assembly of photosynthetic complexes

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Overall research goals: Originally defined as cellular receptors for immunosuppressants, immunophilins have been identified in a wide range of organisms from bacteria to fungi, plants, and animals. Almost all immunophilins function in protein folding processes as molecular chaperones and foldases. Arabidopsis, a flowering plant with the smallest genome, contains more than 50 genes encoding immunophilins. In particular, 16 immunophilin members are predicted to target chloroplast thylakoid lumen, implicating these proteins in the regulation of photosynthesis. Indeed, genetic and biochemical analyses have demonstrated critical functions for several immunophilins in the biogenesis and maintenance of photosynthetic complexes such as PSII. Studying immunophilins thus provides a unique opportunity to understand the molecular mechanism underlying assembly and maintenance of protein machines involved in photosynthetic light reactions. The long term goal of this project is to understand the regulation of photosynthesis by chloroplast immunophilins. The specific objectives for this funding period will be to dissect the mechanism of action of two thylakoid lumen immunophilins, FKBP20-2 and CYP38, that are involved in the assembly and maintenance of PSII. At the same time, we will expand our effort into the functional analysis of at least one other immunophilin that is required for the accumulation of Cyt b6f complex.

Significant achievements 2008-2010:

1. To identify the partner proteins for FKBP20-2 using yeast two hybrid and transgenic lines expressing HA-FKBP20-2: A key step towards understanding the mechanism of FKBP20-2 function is to identify the proteins that associate with FKBP20-2. Because FKBPs are protein foldases that often assist conformational changes of proteins and protein complexes, it is crucial to identify the "partner proteins" of FKBP20-2 in the PSII complex. In the previous year, we produced transgenic lines that express HA-tagged FKBP20-2. Several lines were initially characterized to express HA tagged protein of right size. However, the level of expression of these lines are too low to be used effectively to pull-down the fusion protein and its associated partner proteins. We are building new transgenic lines using different vector and tags. While this line of work is in progress, we used yeast two hybrid screen as a parallel approach to identifying potential partners. Along this line, we have constructed a cDNA library expressing all thylakoid lumen proteins (where FKBP20-2 is located). The screen so far has generated several positive clones that encode PSII subunits. This is a exciting progress because FKBP20-2 is involved in the assembly of PSII supercomplex. Identifying the PSII subunits provide a foundation for understanding the mechanism of action of this FKBP.

2. Structural analysis of CYP38: Cyclophilin 38 (CYP38) is one of the highly divergent cyclophilins from *Arabidopsis thaliana*. Here we report the crystal structure of the AtCYP38 protein (residues 83-437), at 2.39 Å resolution. The structure reveals two distinct domains: an N-terminal helical bundle and a C-terminal cyclophilin β-barrel, connected by an acidic loop. In this structure, N-terminal residues become part of the C-terminal β-barrel, thereby making it an inactive PPIase. Furthermore, the helical domain, although predicted to be similar to leucine zipper, contains several new elements for protein-protein interaction which may be of

functional significance. To this end, we show that the N-terminal helical domain is structurally closely packed together with the C-terminal PPIase domain, an indication of intramolecular interaction. This idea was confirmed by the protein-protein interaction assay using yeast two hybrid system. This study describes the first structure of a plant cyclophilin and presents a possible mechanism of auto-inhibition of PPIase function through an intramolecular interaction.

3. Studies on other FKBPs in the chloroplast: In the past year, we made significant progress on the understanding of the only stromal cyclophilin in the chloroplast. The immunophilin member CYP20-3 is required for the stress tolerance induced by reactive oxygen species (ROS). It interacts directly with a metabolic enzyme involved in cysteine biosynthesis and thereby regulates total thiol level in the chloroplast leading to changes in ROS responses (see publication below).

Science objectives for 2009-2010:

1. To dissect the mechanism of action for FKBP20-2 and CYP38 in the accumulation of PSII supercomplex. Our recent studies (Lima et al., 2006; Fu et al., 2007) demonstrated that FKBP20-2 and CYP38 are both required for the accumulation of PSII supercomplex, placing these immunophilin members as the first group of protein factors specifically involved in the regulation of supercomplex assembly and/or stability. We plan to further determine the mechanistic details for the functions of these two immunophilins in the assembly or maintenance of the complex. In doing so, we will identify the protein targets associated with these two immunophilins and solve their 3-D structures, providing a stepstone for understanding the atomic interaction between the foldases and their targets.

2. To determine the function of an immunophilin in the assembly and maintenance of Cyt b6f complex. We have systematically analyzed the function of a number of immunophilins and identified at least one member that functions in the accumulation of Cyt b6f complex. The main approach has been inducible RNAi because lack of Cyt b6f complex is lethal for plants. Upon induction of RNAi, the plants that lack expression of one immunophilin gene began to show severe necrosis and bleaching phenotype, accompanied by reduction in the electron transfer activity (at a step between PSII and PSI) and reduced level of Cyt b6f subunits. We will confirm the function of this immunophilin and further dissect the mechanism of action of this immunophilin in this aspect.

3. Functional analysis of other thylakoid lumen immunophilins. Although we will focus our effort on those members involved in assembly of PSII and Cyt b6f complexes, we will continue the functional analysis of other lumenal immunophilins using genetic and biochemical analysis, as an ongoing effort towards the long-term goal of connecting each thylakoid immunophilin to a specific process in photosynthesis.

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Imaging the Dynamics of Photosynthetic Membranes

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Overall research goals: In plants and algae, the photosynthetic membrane of chloroplasts responds to changing light levels and spectral composition, regulates the balance of activities of the two photosystems, and repairs and replaces critical components as they are damaged by photo-oxidation. By integrating programs in plant genetics, biophysics, advanced imaging, ultrafast spectroscopy, engineering, and computational modeling, our goal is to obtain a detailed molecular description of the organization of the photosynthetic membrane, the regulation of its composition, and the membrane dynamics occurring during photosynthetic regulation and repair. We follow protein rearrangements in photosynthetic membranes using a range of cutting-edge imaging techniques, which are ideally suited to reveal the complex structural dynamics of the membrane at different levels of spatio-temporal resolution. The imaging approaches are integrated with parallel modeling and theoretical efforts, which will provide new insights into the fundamental biophysical mechanisms governing protein interactions and the partitioning of protein complexes in the photosynthetic membrane.

Significant achievements 2009-2011: Isolated spinach grana membranes were observed by negative stain electron microscopy (EM). Disorganized and partially organized regions of the membrane were observed and further analyzed by data processing techniques. Three-dimensional reconstruction of the *Arabidopsis* thylakoid network was performed after high-pressure freezing and freeze substitution of leaf sections. Atomic force microscopy (AFM) was used to visualize photosynthetic supercomplexes from *Arabidopsis* and grana membranes from spinach. LHCII-PSII supercomplexes were observed in grana membranes in air and in liquid. We completed major instrumentation improvements that are necessary for measuring the mechanical properties of thylakoid membranes and associated changes in properties during state transitions in chloroplasts. These measurements are based on an AFM that has been modified to simultaneously quantify changes in force, dimension, and compliance of thylakoid membranes under dynamic lighting conditions. Differences in membrane mechanical properties were detected in stacked and destacked grana preparations. Construction progressed on a microscope capable of visualizing the PSII repair process in the thylakoid membrane. The microscope combines stimulated emission depletion (STED) to achieve sub-diffraction spatial resolution and fluorescence lifetime imaging (FLIM) to incorporate high temporal resolution. We developed a new photoactivatable fluorophore that can be used for multiple super-resolution microscopies. We developed a coarse-grained model of transmembrane alpha helices which has greater fidelity to the geometry of the alpha helix than our previous barrel-like model, while retaining the overall simplicity of representation.

Science objectives for 2011-2012:

- We will develop high-pressure freezing and freeze substitution methods for *Chlamydomonas* cells and will use EM and 3D reconstruction to investigate and compare changes in thylakoids and membrane protein complexes during NPQ and state transitions in *Arabidopsis* and *Chlamydomonas*.

- We will continue to refine the method for obtaining AFM images of grana membranes in liquid. We will image wild-type membranes illuminated under different conditions to determine changes due to NPQ, in comparison with membranes from NPQ-deficient mutant plants.
- We will perform dynamic AFM measurements of membrane mechanical properties during state transitions, induced by illuminating de-enveloped chloroplasts with state I and state II specific illumination (740 nm and 640 nm). An AFM probe in contact with a de-enveloped chloroplast will be used to measure the height change or force generated by the reorganization of the thylakoid membranes. Simultaneous fluorescence measurements will allow tracking of the chloroplast.
- We will finish construction of the STED-FLIM microscope, which consists of coupling and aligning both beams into the instrument. Initial experiments are planned using fluorescent beads (FluoSpheres® far red 0.02 μm) to characterize and optimize the microscope. These beads absorb and fluoresce at wavelengths similar to chlorophyll *a*, allowing us to fine-tune the STED-FLIM microscope and determine its resolution.
- We are currently validating our basic model of membrane-mediated interactions against experimental results in simplified protein-model bilayer systems. Once this validation is complete we shall progress towards implementing this model of lipid-mediated interactions in more realistic models of photosynthetic membranes. New directions that we will be pursuing include (1) a highly coarse-grained model of PsbS and PSII supercomplexes in order to study the inhibiting effect of PsbS on the formation of the semi-crystalline PSII arrays that are thought to affect the extent of qE and (2) a molecular-level model of the nonbilayer lipids in the thylakoid membrane in order to understand how the geometry of such lipids may impact the membrane-mediated interactions.

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Regulation of Photosynthetic Light Harvesting

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Overall research goals: Photosynthetic light-harvesting systems are regulated, protected against photo-oxidative damage, self-assembled into supercomplexes, and rely on a subtle balance of electronic interactions to produce a highly robust system. In this project, we apply a multidisciplinary approach that combines genetic and biochemical techniques with ultrafast spectroscopy to understand the mechanism(s) by which green plants and algae regulate the efficiency of light harvesting in Photosystem II. In response to fluctuations in light intensity, nonphotochemical quenching (NPQ) mechanisms that regulate photosynthetic light harvesting are induced. The qE type of NPQ is turned on and off rapidly by changes in thylakoid lumen pH, whereas the slower qI type downregulates PSII during long-term light stress. Our specific aims are (1) to investigate a novel type of violaxanthin de-epoxidase (VDE) enzyme in *Chlamydomonas*, (2) to identify the structural features that make LHCSR a qE-quenching protein instead of a light-harvesting protein, (3) to determine if LHCSR expression is sufficient for qE and to identify new factors that control LHCSR expression in *Chlamydomonas*, (4) to assess the roles of LHCSR (and PsbS) in other algae, and (5) to gain new insight into qI in *Arabidopsis*.

Significant achievements 2009-2011: The *npq1* mutant of *Chlamydomonas* is defective in VDE activity and is unable to convert violaxanthin into zeaxanthin in high light. The molecular basis for this mutation has been mysterious, because the *Chlamydomonas* genome lacks an obvious ortholog of the VDE found in plants and other algae. Therefore, we mapped the *npq1* mutation and identified the *NPQ1* gene as a homolog of a type of carotenoid cyclase. The *npq4* mutant of *Chlamydomonas* affects two genes encoding LHCSR3, an ancient member of the LHC protein family. In collaboration with Prof. Roberto Bassi's group, we expressed LHCSR3 in *E. coli* and reconstituted a pigment-protein complex *in vitro*. The protein binds 6 or 7 chlorophylls and 2 or 3 xanthophylls, and it exhibits a very fast, pH-sensitive fluorescence decay, with lifetimes below 100 ps. Using transient absorption spectroscopy, we found that LHCSR3 is highly active in the transient formation of a carotenoid radical cation, a species proposed to act as a quencher in the heat dissipation process. LHCSR1, a second isoform of LHCSR, also appears to contribute to qE. We isolated a partial suppressor of *npq4* that overexpresses LHCSR1 and has more qE. We identified a loss-of-function *lhcsr1* mutant by TILLING. The *lhcsr1* mutant does not have a major impairment of qE compared to wild type, but an *lhcsr1 npq4* double mutant lacking both LHCSR1 and LHCSR3 has essentially no qE, suggesting that all qE in *Chlamydomonas* is LHCSR-dependent. To explore the necessity of PsbS in *Arabidopsis*, we isolated mutants that no longer require PsbS for rapid induction of NPQ. The *eqi1* mutant exhibits rapid NPQ that does not depend on a low lumen pH, zeaxanthin, or the STN7 kinase, so it is a type of NPQ other than qE, qZ, or qT. The relaxation of this NPQ in the dark is very slow, so it is a type of qI. By map-based cloning, we showed that two independent point mutants affect the same gene, which encodes a chloroplast protein with a thioredoxin (Trx)-like domain. T-DNA insertion alleles have the same phenotype as the point mutants.

Science objectives for 2011-2012:

- By trying to complement the *npq1* mutation in *Arabidopsis*, we will test if the protein encoded by the *NPQ1* gene of *Chlamydomonas* has VDE activity. We will also determine its subcellular

location in *Chlamydomonas* cells, which might differ from the plant-type VDE that is located in the thylakoid lumen.

- We will investigate structure-function relationships in LHCSR3 by site-directed mutagenesis of acidic residues and putative chlorophyll ligands and by domain swapping with a light-harvesting LHC protein.
- We will determine whether expression of LHCSR1 or LHCSR3 is sufficient to allow for qE in low-light-grown cells and will begin to map a suppressor mutation that allows for overexpression of LHCSR1.
- To determine whether our results with LHCSR proteins in *Chlamydomonas* can be extrapolated to other algae, we will isolate and characterize *npq* mutants of a divergent prasinophyte green alga (*Ostreococcus*) and a chromalveolate (*Nannochloropsis*).
- Targets of the Trx-like domain of the EQI1 protein will be identified using a covalent affinity trap approach in which the buried cysteine of the redox-active disulfide has been replaced by serine. Interacting proteins will be trapped *in planta* or *in vitro* and then identified by mass spectrometry. To determine the timescales of qI in the mutant, we will measure fluorescence lifetimes in the mutant and wild type by time-correlated single photon counting.

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Functional Analysis of the Chloroplast Division Proteins ARC6 and PARC6

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Overall research goals: The proliferation of chloroplasts by division during leaf development is essential for maximizing the efficiency of light harvesting and CO_2 fixation in plants. Further, by regulating chloroplast size and shape, chloroplast division plays an important role in the ability of plant cells to fine-tune chloroplast distribution to optimize photocollection and photoprotection in fluctuating light environments. The overall goal of the funded research is to define key functional aspects of two critical components of the chloroplast division complex, ARC6 and its paralog PARC6 using Arabidopsis as a model system. These proteins both reside in the inner envelope membrane and function partly to regulate stromal FtsZ ring (Z-ring) assembly and partly to coordinate the internal (stromal) and external (cytosolic) contractile machineries across the two envelope membranes (Fig. 1). Our long-term goal is to understand the mechanisms by which chloroplast division is achieved.

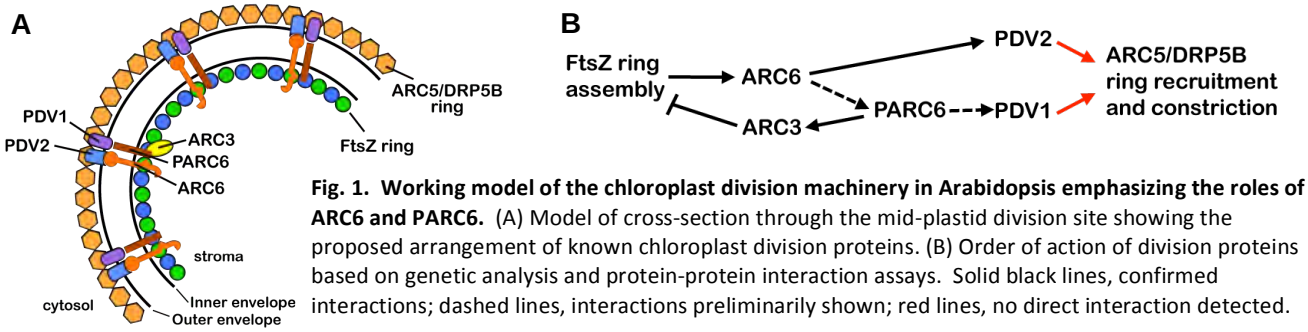

Fig. 1. Working model of the chloroplast division machinery in Arabidopsis emphasizing the roles of ARC6 and PARC6. (A) Model of cross-section through the mid-plastid division site showing the proposed arrangement of known chloroplast division proteins. (B) Order of action of division proteins based on genetic analysis and protein-protein interaction assays. Solid black lines, confirmed interactions; dashed lines, interactions preliminarily shown; red lines, no direct interaction detected.

Significant achievements 2009-2011: ARC6 localizes to the mid-plastid division site and functions in part as positive regulator of FtsZ assembly *in vivo*. The stromal region of ARC6, which interacts with FtsZ (Fig. 1), bears a conserved, predicted DnaJ-like domain (JLD). As *E. coli* DnaJ functions as an HSP70 cochaperone, we tested whether ARC6 might have a related activity in chloroplasts. We found that the ARC6 JLD partially restored the heat-sensitive phenotype of an *E.coli dnaJ* mutant and interacted with cpHSP70 proteins in yeast-two hybrid and pull-down assays, but that the J-domain from *E. coli* DnaJ could not fully replace the ARC6 JLD in an *arc6* mutant. We further showed that the ARC6 JLD could not reproducibly stimulate the ATPase activity of chloroplast HSP70, a hallmark function of bona-fide J-domain proteins. We conclude that while ARC6 has retained some DnaJ-like properties, its JLD has evolved a function distinct from that of typical J-domain-containing co-chaperones. Our recent genetic and cytological analyses in Arabidopsis have shown the ARC6 JLD is required for Z-ring assembly and for localization of the Z ring and ARC6 to the chloroplast division site. It is also required for ARC6 interaction with PARC6 (see below).

While ARC6 functions as a positive regulator of Z-ring assembly, we have established that its paralog PARC6 negatively regulates Z-ring assembly. However, unlike ARC6, PARC6 does not interact with FtsZ. Instead, it interacts with another negative regulator of FtsZ assembly, ARC3 (Glynn et al., 2009). PARC6 and ARC3 localize similarly in chloroplasts, and some *parc6* mutant phenotypes resemble those of *arc3*. These data suggest that the inhibitory effect of

PARC6 on FtsZ assembly is a consequence of its interaction with ARC3. However, analyses of chloroplast and FtsZ filament morphologies in the *parc6 arc3* double mutant indicate that PARC6 also regulates FtsZ assembly independently of ARC3. Further, we have detected a direct and specific interaction between PARC6 and the ARC6 JLD motif, suggesting that PARC6 may also influence FtsZ assembly through ARC6. Together, our results show that vascular plants have evolved complex mechanisms to regulate FtsZ dynamics, one of which involves the functional divergence of ARC6 and PARC6.

Science objectives for 2011-2012:

- We and others have detected a potentially complex set of interactions between PARC6, ARC3, ARC6 and FtsZ that probably regulate FtsZ assembly, disassembly and dynamics *in vivo*. We hypothesize that a conserved motif in the stromal region of PARC6, the MORN motif, as well as the JLD of ARC6, are important regulators of these interactions. We will continue to investigate the roles of these regions in specific protein-protein interactions and the significance of such interactions *in vivo*. These studies will be important for elaborating the mechanisms regulating the activity of the chloroplast contractile machinery.
- We showed that PARC6 is required for localization of PDV1 to the division site (Glynn et al., 2009). We have recently detected a direct interaction between PARC6 and PDV1 through their intermembrane space (IMS) regions by yeast two-hybrid assay. We plan to verify this interaction using pull-down assays and use genetic and biochemical analysis to determine whether this interaction is required for PDV1 localization.
- We showed previously that the IMS regions of ARC6 and PDV2 interact, and that this interaction positions PDV2 at the mid-plastid, enabling PDV2 to subsequently recruit the dynamin-related protein ARC5/DRP5B to the division site to pinch apart constricted chloroplasts (Glynn et al, 2008, Plant Cell) (Fig. 1). Using yeast two-hybrid analysis, we have now mapped the PDV2-binding domain of ARC6 and determined it contains three predicted serine/threonine phosphorylation sites. We have established that two of these sites can be phophorylated *in vitro* and that phophomimic mutations at any of these sites phenocopy *pdv2* mutants *in vivo*. These findings are consistent with our hypothesis that ARC6-PDV2 interaction is regulated by phosphorylation of ARC6 in the IMS. A goal of the coming year will be to determine whether the PDV2-binding domain of ARC6 is phosphorylated *in vivo* and if so whether phosphorylation may be regulatory for chloroplast division.

Published work supported by this project 2009-2011:

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Electronic Structure of Cobaloxime Catalyst for Biomimetic Hydrogen Production: Multi-Frequency EPR & DFT Study

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Overall research goals: The ultimate goal of our natural photosynthetic energy research is to resolve fundamental mechanisms of photochemical energy conversion in photosynthetic proteins and to use this information in the design of bio-inspired materials for solar fuels production. Currently, our group is designing both synthetic supramolecular photocatalytic systems as well as Photosystem I (PS I)-catalyst biohybrids that photochemically produce hydrogen (Fig.1). Further development and improvement of these systems relies on understanding the inherent, fundamental mechanisms for coupling captured photons to fuel generation. To this end, we are applying advanced spectroscopic techniques such as multifrequency, pulsed electron paramagnetic resonance (EPR) to elucidate important structure-function relationships in our artificial and biochemical complexes.

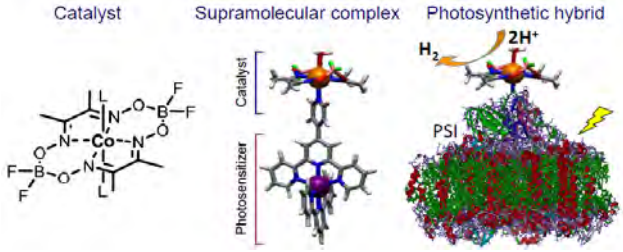

Figure 1. Structure of BF_2 "capped" cobaloxime (left) and schematic representation of the supramolecular, and photosynthetic hybrid complexes.

Significant achievements 2009-2011: As the catalysts for our biohybrid research we are using cobaloxime derivatives. BF_2-capped cobaloxime $Co(dmgBF_2)_2$ rank among the most promising first row transition metal complexes for the reduction of protons to molecular hydrogen. The catalytic properties of cobaloximes depend on the local surrounding and in particular on the direct ligands to the central metal ion. The knowledge of the electronic properties is essential for understanding the catalytic properties of the molecule. EPR is an excellent tool to achieve this goal. In case of PS I, the other essential component of our biohybrid system, EPR spectroscopy has contributed tremendously to the current understanding of its electronic structure and function. In the work reported here the multi-frequency approach along with simultaneous fitting of the EPR spectra at X-, Q-, and D-band microwave frequencies allows us to determine g-tensor anisotropy and hyperfine splitting due to the central metal (^{59}Co) and coordinating ligands that have magnetic nuclei (Fig.2). A variety of different

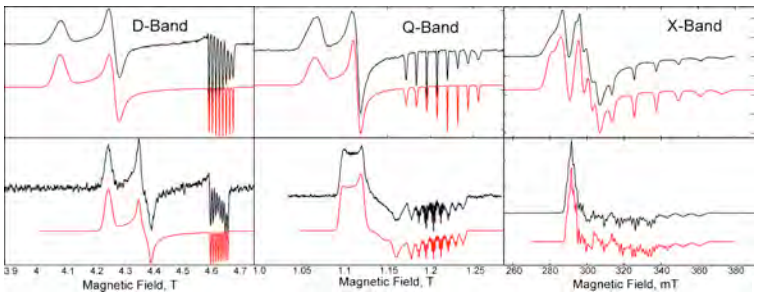

Figure 2. Experimental (black) and theoretically simulated (red) multi-frequency ERP spectra of cobaloxime in pure methanol (upper row) and methanol with pyridine (lower row).

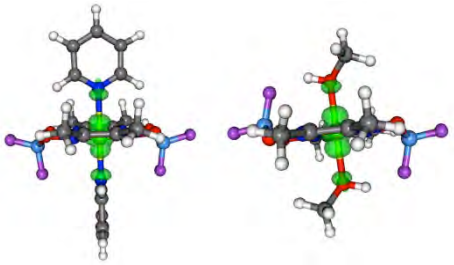

Figure 3. DFT geometry optimization and spin density distribution for $Co(dmgBF_2)_2$ complex with pyridine (left) and methanol (right).

solvents with a range of polarities and stochiometric amounts of potential ligands to the cobalt ion were studied. These experimental results were compared to the first comprehensive set of DFT calculations on $Co(dmgBF_2)_2$ model systems with various axial ligands. Comparison with experimental values for the "key" magnetic parameters like *g*-tensor and ^{59}Co hyperfine coupling tensor allows identification of the stable conformers and validate the electronic structure of the axial ligand(s)-$Co(dmgBF_2)_2$ complexes.

Science objectives for 2011-2012: Recently, we have prepared a Photosystem I-cobaloxime biohybrid complex. PSI and the cobalt-containing catalyst self-assembles and the resultant complex rapidly produces hydrogen in aqueous solution upon exposure to visible light. We are going to apply our knowledge from EPR research of PS I and cobaloximes to determine where and how cobaloxime is binding to the protein, as well as identify important intermediates in the photo-catalytic cycle. This research will help us optimize our biohybrid system and provide a benchmark for designing future RC-based biohybrids for solar fuel production.

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Regulation of Chloroplast Biogenesis: the *immutans* Variegation Mutant of Arabidopsis

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Overall research goals: The Arabidopsis *immutans* (*im*) variegation mutant has green- and white-sectored leaves due to the absence of IMMUTANS (IM), a terminal oxidase in plastid membranes (also called PTOX) that bears homology to the mitochondrial inner membrane alternative oxidase (AOX). The project has three research aims: a) to elucidate the mechanism of *im* variegation; b) to examine structure/function relationships of PTOX and AOX; and c) to determine whether PTOX functions as a "safety valve" in photosynthetic electron transport. These studies should provide insight into the regulation of PTOX activity, and the role of PTOX in photosynthesis, chloroplast biogenesis, and plant growth/development. In addition, because the green and white sectors of *im* have a uniform genotype (*im/im*), research directed toward understanding the mechanism of variegation should lead to the identity of factors or processes that are able to compensate for a lack of PTOX in the green sectors of the mutant.

Significant achievements 2009-2011

Aluru et al., 2009*:* Morphological, biochemical, molecular and microarray analyses have been carried out on the green and white sectors of *im.* The green leaf tissues have significantly higher rates of photosynthesis and sucrose production than wild type leaves, and these increases appear to be due to sink demand from the white tissues. The green tissues also have alterations in gene expression and morphology that are consistent with adaptations to photooxidative stress early in leaf development, when chloroplasts develop from proplastids. Retrograde (chloroplast –to-nucleus) signaling likely plays a role in development of the two types of leaf tissues and in optimizing interactions between them.

Fu et al., 2009*:* IM (PTOX) and AOX are members of the diiron carboxylate quinol oxidase (DOX) class of proteins, and they contain 20 highly-conserved amino acids, six of which are Fe-binding ligands. In previous structure/function studies, *in vitro* and *in planta* activity assays were used to examine the functional importance of the Fe-binding sites (Fu et al., 2005; *J. Biol. Chem*. 280: 42489-42496). These assays have been extended to the 14 other conserved sites, and it was observed that they fall into three classes: sites that are dispensable for activity; essential for activity; and important but not essential for activity. The data are consistent with the proposed role of some of these residues in active site conformation, substrate binding and/or catalysis.

Fu et al. 2011*:* We have found that mitochondrial AOX1a, when re-targeted to chloroplasts of *im,* is incorporated into thylakoids and is able to rescue the *im* defect. We also found that activation tagged AOX2 is similarly targeted to chloroplasts and able to rescue *im*. Because AOX2 is targeted to plastids using its own sequences, we concluded that it is normally dual-targeted to mitochondria and chloroplasts, where it functions to supplement PTOX activity during the early events of chloroplast biogenesis. Further research has demonstrated that AOX1a and AOX2 form monomers in thylakoids, and that AOX2, but not AOX1a, also forms dimers, reminiscent of AOX regulation in mitochondria. Both proteins are also present in higher molecular weight complexes in thylakoids, but their presence there does not markedly disturb steady-state photosynthesis. We have concluded that the ability of AOX1a and AOX2 to substitute for PTOX in the correct physiological and developmental contexts is a striking example of the capacity of a mitochondrial protein to replace the function of a chloroplast protein, and illustrates the plasticity of the photosynthetic electron transport chain.

Science objectives for 2011-2013:

• To gain insight into the mechanism of *im* variegation, suppressor screens have been carried out using EMS and T-DNA tagging mutagenesis. Five suppressor lines have been isolated, and three suppressor genes have been cloned and identified. Efforts over the coming year will focus on characterizing the suppressor lines, and how the suppressor genes influence the activity of PTOX during electron transport.

• Work will continue to test whether IM acts as a "safety valve" in photosynthesis. Prior experiments have shown that IM does not act as a safety valve in Arabidopsis during steady state photosynthesis (Rosso et al., 2006; *Plant Physiol.* 142: 574-585); the current experiments focus on early chloroplast biogenesis using Arabidopsis de-etiolation as a model system (Rosso et al., 2009; *Plant Cell* 21: 3473–3492). We are exploring the idea that PTOX poises the PQ pool in the dark to optimize photosynthesis when light is provided.

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Intrinsic photoprotection in strongly coupled (bacterio)chlorophyll complexes via triplet exciton formation

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Overall research goals: The research objective is to investigate novel photoprotection mechanism in strongly coupled (bacterio)chlorophyll complexes by: (1) time resolved and steady state spectroscopy, (2) infrared phosphorescence detection and (3) EPR techniques. The goals is to fully understand this exceptionally efficient intrinsic photoprotection discovered recently by our group and develop framework that would allow to predict this photoprotection in natural system from their structure and design robust artificial systems that incorporate this novel mechanism.

Significant achievements 2009-2011:

EPR study was performed on chlorophylls and their aggregated states as well as on natural chlorosomal light harvesting antenna complexes that incorporate up to a 200,000 bacteriochlorophyll molecules in a strongly coupled quasi periodic array. The EPR signals in dimeric chlorophyll samples exposed to light flashes exhibited typical deviations expected for triplet excited state that is delocalized over both pigments, supporting out hypothesis that triplet states have excitonic character. The EPR signals in highly aggregated samples as well as in chlorosomes revealed the formation of a radical in response to a flash which is most probably associated with charge separation or triplet excimer molecule formation that does not recover into the ground state under liquid helium temperatures. These states readily disappear upon warming up the sample to room temperature and thus don't play physiological role. Since EPR in these samples cannot be performed at room temperature the stress in research was shifted to infrared phosphorescence study as was proposed in the original timeline of the proposal. At that time the first version of ultrasensitive IR phosphorescence spectrometer is built and first spectra of model porphirin phosphorescence and chloropphills performed. The preliminary data indicates that the phosphorescence in aggregated samples is shifted to red as expected from the proposed triplet exciton model, however, the spectrometer needs to be (and currently is) modified to obtain clear data.

Science objectives for 2011-2012:

- The phosphorescence spectrometer will be modified to increase its sensitivity by about 10 times via the use of new detector (will come in September 2011) and the use of faster shutter to cutoff significant fraction of fast fluorescence background. The new laser was also received in 2011 and will be updated with OPO that would allow to tune excitation wavelength over absorption bands of monomeric, dimeric, and highly aggregated forms of Chlorophylls. The latter proved to be critical in interpreting the phosphorescence data.
- The phosphorescence spectra will be measured for 10 different chlorophylls and triplet energies will be determined for several of them for the first time. We expect this data to be published promptly afterwards as a separate paper.
- The phosphorescence spectra for natural aggregates of bacteriochlorophylls will be measured. We will characterize the chlorosomes and also attempt to study Fenna Matthews Olson complex which is known to be exceptionally photostable in spite the fact that it contains no carotenoids and thus we propose that it is protected through triplet exciton formation only.
- The procedures for formation of aggregates of chlorophylls of controlled size will be developed.

Molecular Regulation of Photosynthetic Carbon Dioxide Fixation in Nonsulfur Purple Bacteria

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Overall Objective: Determine the mechanism by which a transcriptional activator protein affects CO_2 fixation (*cbb*) gene expression in nonsulfur purple photosynthetic bacteria, with special emphasis to *Rhodobacter sphaeroides.*

Significant achievements (2009-2011): In *Rhodobacter sphaeroides*, CbbR and RegA (PrrA) are transcriptional regulators of the cbb_I and cbb_{II} (Calvin-Benson-Bassham CO_2 fixation pathway) operons. Both proteins interact specifically with promoter sequences of the *cbb* operons. RegA has four DNA binding sites within the cbb_I promoter region, with the CbbR binding site and RegA binding site 1 overlapping each other. We recently demonstrated that CbbR and RegA interact and form a discrete complex in vitro, as illustrated by gel mobility shift experiments, direct isolation of the proteins from DNA complexes, and chemical cross-linking analyses. For CbbR/RegA interactions to occur, CbbR must be bound to the DNA, with the ability of CbbR to bind the cbb_I promoter enhanced by RegA. Conversely, RegA interactions with CbbR did not require RegA to bind the cbb_I promoter. RegA itself formed incrementally larger multimeric complexes with DNA as the concentration of RegA increased. The presence of RegA binding sites 1, 2 and 3 promoted interactions at significantly lower concentrations of RegA than when RegA binding site 3 is not present in the cbb_I promoter**.** These studies support the premise that both CbbR and RegA are necessary for optimal transcription of the cbb_I operon genes of *R. sphaeroides*. A model depicting how the CbbR-RegA complex might interact with the transcription machinery of the cell is shown (Fig. 1).

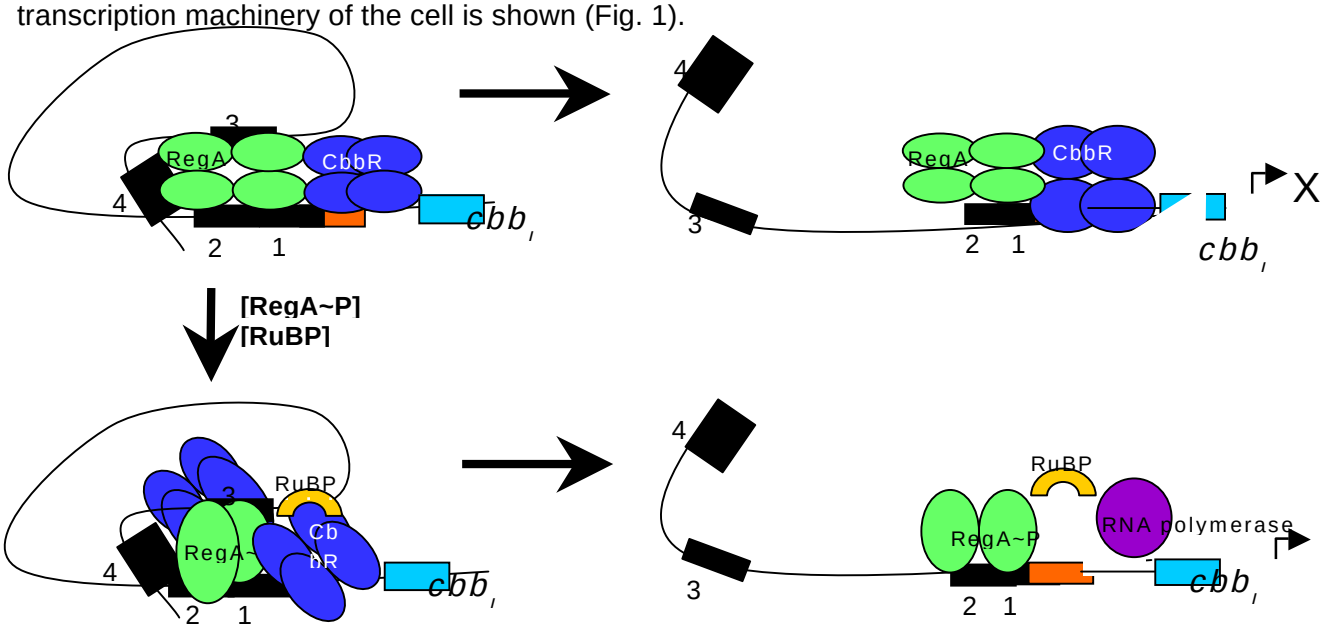

Fig. 1. Model of cbb_I transcriptional regulation in *Rb. sphaeroides*

A somewhat different situation occurs in *Rhodopseudomonas palustris* CGA010, as CbbR interacts with two unusual response regulators (from a different two-component system) that cannot by themselves bind to DNA. Here CbbR specifically controls transcription of the *cbbLS* genes encoding form I RubisCO. Previous genetic and physiological studies had indicated that this unique two-component (CbbRRS) system influences CbbR-mediated *cbbLS* transcription

under conditions where CO_2 is the sole carbon source. We have recently established direct protein-protein interactions between the response regulators of the CbbRRS system and CbbR, using a variety of techniques. The bacterial two hybrid system established a specific interaction between CbbR and CbbRR1 (response regulator 1 of the CbbRRS system), confirmed in vitro by chemical crosslinking. In addition, both response regulators (CbbRR1 and CbbRR2) played distinct roles in influencing the CbbR-*cbbLS* promoter interactions in gel mobility shift assays. CbbRR1 increased the binding affinity of CbbR at the cbb_I promoter 3-5 fold while CbbRR2 appeared to stabilize CbbR binding. Specific interactions were further supported by surface plasmon resonance (SPR) analyses. In total, the results suggested that both response regulators, with no discernible DNA binding domains, must interact with CbbR to influence *cbbLS* expression. Thus the CbbRRS system provides an additional level of transcriptional control beyond CbbR alone, and appears to be significant for potentially fine tuning *cbbLS* expression in *Rps. palustris*.

Objectives for 2011-14: There will be several approaches taken under the rubric of this major goal to discern the mechanism and consequences of transcription factor complexes in controlling CO_2 fixation gene expression, including: (1) Mutational analysis to determine residues required for specific interactions between the transcriptional apparatus and the major and ancillary regulator proteins; (2) Cross-linking approaches to delineate specific protein interactions; (3) Bacterial two-hybrid analyses to probe specific protein interactions; (4) DNA bending analyses; (5 Employ In vitro assays to determine and quantify the influence of RegA and effectors on CbbR-RNAP interactions; (6) Determine how and whether CbbR impacts regulation and metabolism in unexpected ways; (7) Determine the role of CbbX; (8) Compare and contrast to other systems where CbbR plays an important role in CO_2

Recent publications supported by this project (2009-2011):

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3. Joshi, G. S., Romagnoli, S., VerBerkmoes, N. C., Hettich, R. L., Pelletier, D., and Tabita, F. R. Differential accumulation of form I RubisCO in *Rhodopseudomonas palustris* CGA010 under photoheterotrophic growth conditions with reduced carbon sources. J. Bacteriol. **191** (2009) 4243-4250.
4. Tabita, F. R. The hydroxypropionate pathway of CO_2 fixation: fait accompli. Proc. Natl. Acad. Sci. USA. **106** (2009) 21015-21016.
5. Laguna, R., Joshi, J.S., Dangel, A. W., Luther, A. K., and Tabita. F. R. Integrative control of carbon, nitrogen, hydrogen, and sulfur metabolism: the central role of the Calvin-Benson-Bassham cycle. Adv. Exp. Med. Biol. **675** (2010) 265-271.
6. Rizk, M. L., Laguna, R., Smith, K. M., Tabita, F. R., and Liao, J. C. Hydrogen production and redox homeostasis phenotypes in RubisCO-deficient *Rhodobacter sphaeroides* via ensemble modeling. Biotech. Prog. **27** (2011) 15-22.
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Interdisciplinary Research and Training Program in the Plant Sciences

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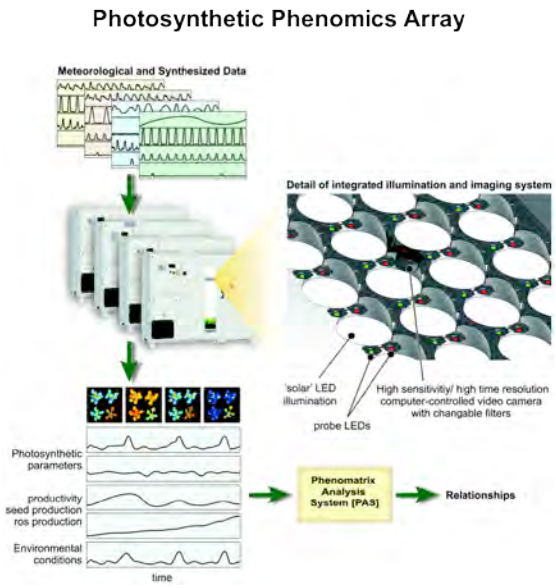
Overall research goals: The MSU-DOE Plant Research Laboratory (PRL) was established in 1965 as a joint venture between the U.S. Department of Energy (then the Atomic Energy Commission) and Michigan State University to conduct basic interdisciplinary research on fundamental questions in plant biology and to train graduate students and postdoctoral researchers in this area of science. Over the years, the specific topics of research in the PRL have changed, but all have contributed to developing a greater understanding of the flow of energy from photon capture to its deposition in energy-rich molecules, and how the associated processes are affected by plant interactions with the environment. Current research has two general themes: interactions of photosynthetic organisms with the environment (He, Howe, Kramer, Montgomery, Thomashow, Walton); and the biogenesis, biochemistry and biophysics of cellular energy systems (Brandizzi, Hu, Keegstra, Kramer, Montgomery, Wolk). In addition, the Plant Bioenergy Network—"PlaNet"—project, is a recently initiated collaborative research effort directed at developing an integrated understanding of the regulatory, biochemical, and metabolic networks that govern energy flow in photosynthetic organisms.

Examples of recent achievements: During the past three year funding period, the PRL faculty has published more than 110 articles. A complete listing of these publications can be found at the PRL website (http://www.prl.msu.edu/). All of these findings cannot be summarized here, but a few recent highlights provide examples of current research efforts. In the area of photosynthetic organisms interacting with the environment, our recent research: established a role for alternative splicing in modulating responses to the plant stress hormone jasmonic acid (JA)-isoleucine (Ile) (Chung et al., 2010); led to the discovery of the first enzyme involved in deactivation of JA-Ile (Koo et al., 2011); elucidated a mechanism by which the plant defense enzyme deplete essential amino acids in the gut of insect herbivores (Gonzales-Vigil et al., 2011); provided evidence that during gene-for-gene resistance, Arabidopsis blocks pathogen-mediated degradation of a host target as a novel mechanism to counter pathogen virulence (Nomura et al., 2011); uncovered a broad role of the bacterial JA-mimicking toxin coronatine in overcoming both stomate- and mesophyll-based defenses in Arabidopsis (Zheng et al., 2011); determined key genes that integrate circadian and low temperature regulatory pathways that are required for maximum freezing tolerance (Dong et al., 2011); and demonstrated that root-localized phytochromes impact light-dependent root elongation and root sensitivity to JA (Costigan et al., 2011).

In the area of cellular energy systems, our research: established key roles for regulation of the chloroplast ATP synthase (Rotta et al., 2011) and cyclic electron transfer (Strand et al., 2011) in governing the balance between photosynthetic efficiency and photoprotection; identified a gene that plays a major role in determining the relative frequency of H_2-producing heterocysts and the vegetative cells that provide them with reductant (Liu and Wolk, 2011); and established that the endoplasmic reticulum has a significant role in controlling root hair development (Slabaugh et

al., 2011; Chen et al., 2011) and managing secretory protein overload (Chen and Brandizzi, 2011).



Finally, research conducted under the PlaNet project has shown that the expression of photosynthesis associated genes (PAGs) is globally down-regulated in response to both abiotic and biotic stresses and has identified more than 90 transcription factors that potentially have roles in PAG regulation. The roles of these regulatory proteins in this regulation and the significance of PAG down-regulation in energy capture and conversion is now under study. These and additional lines of study will be facilitated through the development of novel high-throughput "phenomics" technology, including the Photosynthetic Phenomics Array, that will be used to study the regulation of photosynthesis in response to fluctuating environmental conditions

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Structure and Mechanism of the Mn_4Ca Cluster in Photosystem II Using X-ray Spectroscopy

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Overall research goals: The specific questions that are the focus of our studies are: 1) What is the geometric and electronic structure and the changes of the Mn_4Ca cluster of the photosynthetic oxygen-evolving complex (OEC) as it traverses the enzymatic cycle driven by four successive photons? 2) What is the mechanism of the photosynthetic water-oxidation reaction that is catalyzed by the Mn_4Ca cluster? We are using steady state and time-resolved X-ray spectroscopy and crystallography methodologies in pursuit of these goals.

Significant achievements 2009-2011: 1) X-ray emission spectra, particularly the so-called cross-over transitions ($K\beta_{2,5}$ and Kβ"), that result from ligand atom 2p and 2s to Mn 1s transitions contain unique information on ligand type, ligand protonation state, and Mn-ligand distances. Using model systems, we have demonstrated that the spectral shape is sensitive to protonation of ligands and allows ligands, which differ only slightly in atomic number (e.g. C, N, O), to be distinguished. A theoretical description of the main spectral features has been developed using DFT calculations in terms of molecular orbitals for a series of Mn model systems. We have collected the XES spectrum of PS S_1 state and demonstrated the feasibility of collecting the weak Kβ" transition peak from dilute protein samples like PS II, and to our knowledge this is the first example of such spectroscopy on a biological sample. We have compared the S_1 state spectrum to those from several well-characterized oxo-bridged Mn complexes. We plan to study all the S-states using the XES method during the present proposal period.

2) We have used RIXS spectroscopy to understand the electronic structure and d-orbital population of Mn in PS II by comparison with Mn oxides and coordination compounds. These data indicate the charge density changes per formal oxidation state changes and demonstrates how the charge density on the atom differs markedly from the formal oxidation state of the complexes. The comparison of PS II with ionic and coordination complexes shows that the change in charge density on Mn atoms during the S-state transition is smaller than that of the coordination complexes. This implies that during the S-state turnover the charge is delocalized on to the ligands thus the formal oxidation state is very different from the charge density of the metal.

3) We used range-extended EXAFS data to resolve the distance heterogeneity in the short Mn-Mn distances of the S_1 and S_2 states and thereby provided firm evidence for three Mn-Mn distances of 2.7-2.8 Å. We have completed the range-extended EXAFS study of the S_3 state and the higher-resolution data clearly shows that metal-metal distance changes in the S_2 to S_3 transition as seen in the changes in FT peak II. The two 2.7 and one 2.8 Å Mn-Mn distances in the S_2 state lengthen to 2.8 and 2.9-3.0 Å in the S_3 state. This result together with our work of Sr EXAFS on Sr-PS II shows that there is a major structural change in the S_2 to S_3 transition, and this is a critical step before the O-O bond formation occurs in the latter step(s).

4) We are using site-specific mutants to study the critical role of ligands in the OEC of PS II. The effect of replacing a histidine ligand on the properties of the OEC and the structure of the Mn_4Ca cluster in PS II was studied by XAS using PS II core complexes from the *Synechocystis sp*. PCC 6803 D1 polypeptide mutant H332E. The Mn XANES spectrum of D1-H332E shifts to a lower energy compared with that of the native WT samples, suggesting that the electronic structure of the Mn cluster is affected by the presence of the additional negative charge on the OEC of the mutant. The EXAFS spectrum shows that the geometric structure of the cluster is altered substantially from that of the native WT state, resulting in an elongation of Mn-ligand and Mn-Mn interactions in the

mutant. This substantial structural change provides an explanation not only for the altered properties of the D1-H332E mutant but also the importance of the histidine ligand for proper assembly of the Mn_4Ca cluster. In the current X-ray crystal structure Glu354 of the CP43 polypeptide is the only amino acid ligand of the Mn_4Ca cluster that is not provided by the D1 polypeptide. Mn XAS studies have shown that the CP43-E354Q mutation only subtly perturbs the structure of the Mn_4Ca cluster in the S_1 state, in contrast to that seen in the D1-H332E mutant, while it substantially alters the S_2 to S_1 state decay time.

Science objectives for 2011-2012:

- We are making good progress with the polarized XAS studies of single crystals of PS II from PS II monomers and also dimers in the S_2 and S_3 states.
- We have been given two beamtimes at the new X-ray laser facility at Stanford over the next year, where we plan to conduct simultaneous X-ray crystallography and X-ray emission spectroscopy studies of all the S-states, and particularly follow the fast steps between the S_3 to S_0 transition in a time-resolved manner. We have commissioned an energy-dispersive emission spectrometer that will enable us to collect 'snapshots' of the emission spectrum using the X-ray laser. These spectra will then be correlated to the diffraction and the structure of the Mn_4Ca cluster in the intermediate states and the transient states between S_3 and S_0.

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Session IV

Photodynamics of Single Antenna Proteins and Redox Enzymes in Solution by Suppression of Brownian Motion

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Overall research goals: The objective of this research is to study and understand the behavior of individual photosynthetic antenna proteins and redox enzymes in their native solution environment by optical measurements over extended time periods. This objective relies on (a) development of advanced Anti-Brownian ELectrokinetic (ABEL) trap designs which suppress Brownian motion and enables extended-time single-molecule measurements without surface attachment or encapsulation, (b) detailed exploration of the photodynamics of specific antenna proteins such as Allophycocyanin (APC) with the ABEL trap, and (c) direct observation of electron transfer events in single redox enzymes such as nitrite reductase. Our approach enables extraction of information on molecule–to-molecule photophysical heterogeneity as well as details of intrinsic time-dependent state changes without the use of a surface or host matrix that can significantly affect the photophysics of the antenna or the enzyme. Greater understanding of the optically-driven dynamics of antenna proteins should be useful for the design of photosynthetic mimics with improved light-harvesting properties, and a microscopic understanding of the enzymatic cycle of redox enzymes can lead to improved electron transfer behavior.

Significant achievements 2008-2010: Our work concentrates in deep method development to achieve optical performance of the ABEL trap as a trapping device with minimum wasted photons as well as specific applications of the trap to study of biomolecules of interest to energy transduction. In the area of trap development, we have redesigned the trapping concept to include a "knight's tour" motion of the laser beam for the extraction of position information, and we have implemented a Kalman filter for optimal feedback (Figure 1). Moreover, we have directly used the error signal from the filter (the "innovation") to convert the system into an *adaptive* trap, which senses the diffusion coefficient and the mobility of the object in the trap in *real time*.

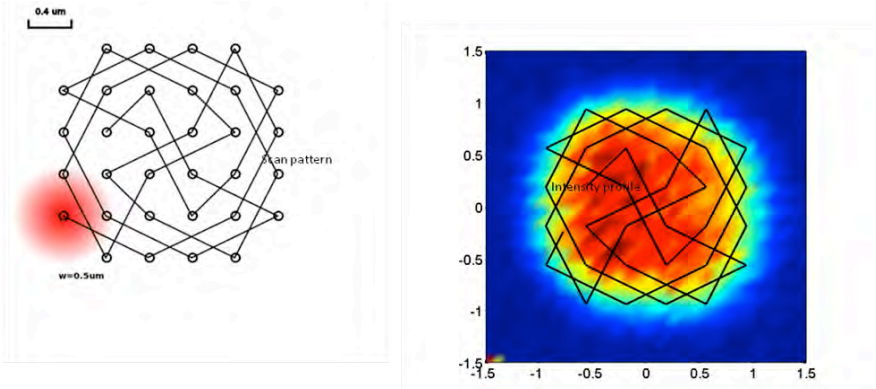

Figure 1. Left Panel: Schematic of the Knight's tour beam scanning pattern for our new advanced ABEL trap design (3). The laser beam spot (red) traverses the points shown in the plane of the microfluidic cell. Whenever a photon is detected, the position of the laser spot at that moment represents a probability distribution for the location of the particle. By analysis of the photon stream using Kalman filtering, optimal feedback forces can be generated. Right Panel: Illustration of the time-averaged pumping laser intensity showing that the molecule is trapped in a region of uniform excitation, in strong contrast to single focal spot methods like single-burst spectroscopy or FCS.

To utilize the trap directly for biomolecular studies, we explored the conformational and photo-dynamics of single APC antenna proteins via simultaneous intensity and lifetime measurements (Figure 2) and completed a statistical extraction of state changes and kinetic pathways. In addition, we recently used the trap for measurements of the conformational and chemical dynamics of the redox enzyme Nitrite Reductase containing a fluorescent reporter near one copper site, in collaboration with G. Canters. This approach allowed us to observe and fully characterize single electron transfer events and the dwell time distributions as a function of nitrite in solution. The kinetic analysis allowed a detailed extraction of the rate constants for the observed behavior.

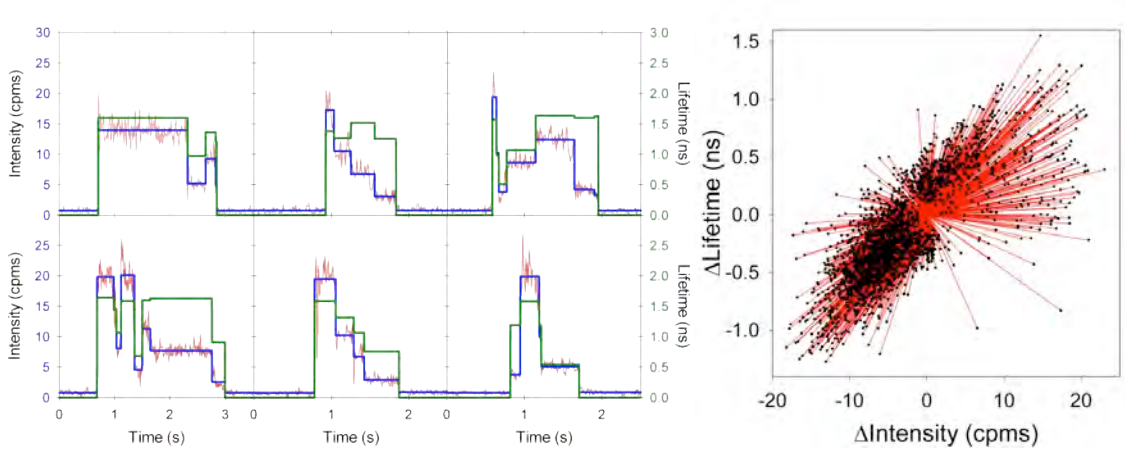

Figure 2. Left Panel: Examples of photodynamics of six APC antenna proteins in the ABEL trap (4). Red: Intensity trajectory (left ordinate). Blue: Brightness states extracted by a change-point finding algorithm. Green: Excited state lifetime during each state (right ordinate). Correlated changes in brightness and lifetime suggest creation of quenching centers at one or more of the phycocyanobilin chromophores, but anticorrelated changes also occur. Lifetime changes without brightness change and other novel behaviors are also observed. Right Panel: Vector sunburst plot showing all intensity-lifetime changes observed for 1,048 single molecules of APC.

Science objectives for 2011-2012:

- Recognizing that our Kalman filter design is most optimal in the presence of Gaussian noise, and that background photons can seriously limit the trap performance, we will mathematically and experimentally explore advanced statistical control strategies such as expectation maximization.
- Because the fascinating photodynamics we observed with single APC antennas suggest a range of different quenching states of the complex, we will measure time-dependent *spectral* dynamics for each molecule in the trap.
- We will study single copies of the important Fenna-Mathews-Olson (FMO) antenna complex in the ABEL trap in order to study the excitonic interactions between the chromophoric units and the effect of added reductant, in collaboration with R. Blankenship.

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Session V

SISGR: PHOTORECEPTOR REGULATION AND OPTIMIZATION OF ENERGY HARVESTING IN NOSTOC PUNCTIFORME

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Overall Research Goals. The long-term goal of this project is to create the knowledge base necessary to engineer cyanobacteria for light-regulated energy production. We are examining photosensory proteins of the phytochrome (Cph1 and Cph2) and cyanobacteriochrome (CBCR) families (1) in the model cyanobacterium, *Nostoc punctiforme* ATCC 29133. *N. punctiforme* is capable of both oxygenic photosynthesis and nitrogen fixation, is amenable to molecular genetic techniques, has a sequenced genome completed under DOE sponsorship, and exhibits diverse metabolism and physiology. This system lets us approach both the *in vitro* photochemical properties and the *in vivo* function of these photoreceptors in a single organism, establishing paradigms for designing artificial light-regulated systems for gene expression (optogenetics approaches) which can be combined with other organisms and with other target genes for production of hydrogen, oxygen, and/or biomass.

Significant Achievements 2009-2011. In two years, the Lagarias lab has successfully cloned, purified and characterized 41 photosensory proteins from *N. punctiforme,* along with six other proteins that do not function as photosensors. Only one target protein remains to be characterized. The results from this survey reveal unexpected diversity in the CBCRs: six previously unknown photocycles and a new CBCR subfamily, the insert-Cys (i-Cys) CBCRs, have been discovered. This proves that CBCRs can sense the entire visible spectrum and even extend into the near-UV (Fig. 1). *N. punctiforme* also contains a trichromatic phytochrome, unexpectedly extending the R/FR photosensory response of phytochromes into the violet. In work published this year (2), the Lagarias lab has shown that both the trichromatic phytochrome and i-Cys CBCRs employ dual-Cys photocycles to convert a red-absorbing linear tetrapyrrole chromophore into a violet or near-UV sensor (Fig. 1).

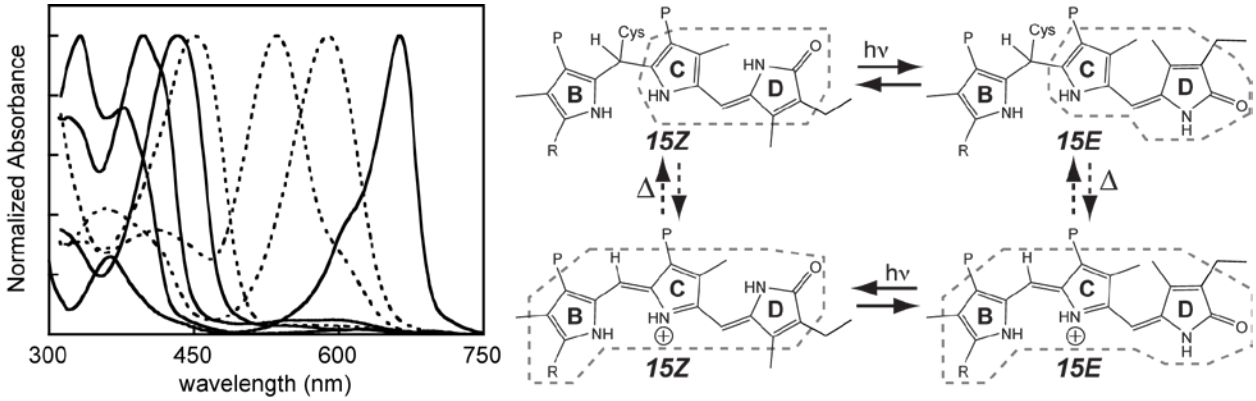

Figure 1. Dual-cysteine CBCRs. *(left)* Spectral coverage of representative dual-Cys photosensors. Absorbance spectra are shown for three dual-Cys CBCRs, demonstrating the spectral coverage of these photosensors. Wild-type *15Z* Cph1 (peak absorbance 660 nm) is shown for comparison. *(right)* A general dual-Cys photocycle. In the dark-adapted *15Z* ground state (top left), there is a covalent linkage between C10 and the second Cys. Light induces 15,16 photoisomerization to produce a *15E* species with an intact second linkage (top right). In some examples, the 15E species is stable while in others, the second linkage is labile, red-shifting the photoproduct absorbance (bottom right).

Previously, the Lagarias lab and others described such dual-Cys photocycles in another CBCR subfamily, the DXCF or blue/green CBCRs. We and others have also shown that DXCF CBCRs play important roles in phototaxis in *Synechocystis* (3). The DXCF subfamily in *N. punctiforme* exhibits surprising diversity as well: in work submitted to *Biochemistry* (4), the Lagarias lab has found novel blue/teal, blue/orange, and green/blue photocycles, along with a DXCF CBCR that mimics the well-known green/teal photocycle of the photoswitching phycobiliprotein α-phycoerythrocyanin. The Lagarias lab has also shown that the thermal relaxation of the photoproduct to the dark-adapted state can vary over six orders of magnitude in CBCRs, permitting these proteins to be sensors of both light intensity and color. Based on these studies, a unified mechanism for dual-Cys photocycles is shown in Figure 1. Spectral tuning is accomplished by several mechanisms, including variable second linkage stability (1, 2), isomerization of the phycocyanobilin chromophore to phycoviolobilin (4), and bilin protonation-deprotonation (work performed on the CBCR RcaE in collaboration with Dr. Yuu Hirose and Prof. Masahiko Ikeuchi, University of Tokyo).

Work performed in the Meeks lab under this project focuses on characterizing the phototaxis sensor of *N. punctiforme* hormogonia and the biological function of cyanobacterial phytochromes. The Meeks lab has established that the *NpF2161-2168* locus is required for the phototactic response and has recently established that hormogonia are most sensitive to red light. Based on the Lagarias lab survey of photosensors, this implicates two specific CBCR domains in NpF2164 as candidate sensors for this response, with specific deletions of those two domains now under construction. The Meeks lab has also constructed a deletion mutant in the conventional red/far-red phytochrome NpCph1, an apparent ortholog of the well-studied *Synechocystis* Cph1. Preliminary results suggest a role for NpCph1 in adaptation to high light, in contrast to the unknown function of Cph1.

The Huser lab is in the process of moving to Germany; work performed to date has used three-dimensional high- and super-resolution imaging to characterize cell morphology and phycobiliprotein distribution in *N. punctiforme*. Additionally, proof-of-concept imaging of a GFP fusion to a CheY-like protein has revealed distinct double ring structures at the junctions between cells in vegetative filaments. Single molecule analysis of fluorescent GAF domains from NpR2164 is in progress using two-focus FCS, photon antibunching spectroscopy, and pulsed interleaved FRET to probe the quaternary structures of these proteins.

Owing to the departure of Dr. Huser, Dr. Jim Ames has recently joined the project. A protein NMR spectroscopist, Ames has successfully obtained high resolution ^{15}N-^{1}H HSQC and one-dimensional ^{13}C NMR spectra of both photostates of the i-Cys CBCR NpR2164g3, which exhibit a violet/orange photocycle (2). These studies reveal that photoisomerization triggers thermal cleavage of a second covalent linkage to the bilin chromophore at C10, providing direct confirmation of the dual-Cys photocycle. In addition, all four bilin nitrogens are rigorously assigned in the orange state and are protonated, providing important information about the chemical structure of this state. The NMR peak widths in the ^{15}N-^{1}H HSQC spectra reveal a possible light-dependent protein dimerization. The ^{15}N NMR relaxation parameters (R_1/R_2) correspond to an average rotational correlation time of 20 ± 0.2 ns (dark state) and 12 ± 0.2 ns (light state), consistent with a molar mass of 42 kDa (dimeric dark-state) and 24 kDa (monomeric light state) in solution under NMR conditions. Gel filtration chromatography performed in the Lagarias lab provides further confirmation that this protein exhibits light-

dependent dimerization. This exciting discovery raises the potential of this protein for light-driven optogenetic approaches.

Profs. Britt and Larsen are scheduled to attend and will be presenting their work separately. Work in the Britt lab has focused on establishing site-directed spin-labeling systems in phytochromes, which has proved difficult to date. Work in the Larsen lab has used pump-probe and pump-dump-probe spectroscopy to analyze the photocycles of four CBCRs on an ultrafast timescale. This work has shown a very high quantum yield in some CBCR photocycles, along with considerable ground-state heterogeneity.

Science objectives for the next year: The Lagarias lab will complete the survey of *N. punctiforme* photosensors and test ten candidate CBCR domains for structural studies (both supplying samples for preliminary ^{15}N-^{1}H HSQC spectra and carrying out crystal trials ourselves). Cases such as the phototaxis sensor NpF2164, in which domains with distinct photocycles are arranged in tandem, will also be examined to determine whether photoperception by CBCRs in such tandem sensors is independent or interacting.

The Meeks lab will complete deletion mutants in the *NpF2164* phototaxis sensor and complement them. In combination with an improved action spectrum for phototaxis, this work will identify the individual CBCR domain(s) within NpF2164 that trigger phototaxis. This will provide insight into both the signal transduction event in phototaxis and the function of tandem CBCRs. The Meeks lab will also complete phenotypic characterization of the NpCph1 mutant.

The Britt lab will attempt to complete their study of spin-lableled Cph1 using DEER to measure inter-label distances in the two photostates and will evaluate two to three other CBCR systems in such assays.

The Ames lab will complete its characterization of the violet/orange CBCR NpF2164g3 (1) by NMR spectroscopy and will evaluate candidates for determination of solution structures.

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Carotenoid Antennae in Retinal-based Light-driven Proton Pumps

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Overall research goals: We study the light-harvesting antenna in dual-chromophore carotenoid/retinal proteins that function as light-driven transmembrane proton pumps. The first of such proteins we had described, is xanthorhodopsin, a transmembrane proton pump related to bacteriorhodopsin. Understanding energy migration in such simple one-donor/one-acceptor systems should yield insights to how light-receptors are optimized for capturing photons.

The research objectives are to describe the structural requirements for carotenoid binding, and the geometry of the two chromophores that allows efficient excited-state energy transfer. This goal is aided by the fact that we found at least one such system additional to xanthorhodopsin, gloeobacter rhodopsin, and sequence information from the Protein Data Base predicts that there may be many more. The carotenoid serves also as reporter molecule for studying electrical and structural changes during the light-initiated retinal reaction cycle.

Significant achievements 2009-2011:

We identified a second retinal/carotenoid-based proton pump, in the cyanobacterium, *Gloeobacter violaceous*. The *E. coli*-expressed protein binds salinixanthin (from xanthorhodopsin) and echinenone (a native *Gloeobacter* carotenoid). Once bound, these carotenoids function as antenna, in the same way as in xanthorhodopsin. A requirement for binding near the retinal is the replacement of a residue that is a conserved tryptophan near the β-ionone-ring in many rhodopsins with a glycine. Genes with high homology to xanthorhodopsin contain such a replacement, making them candidates for antenna binding. Another requirement is the keto group of the carotenoid ring, as the corresponding alcohol will not bind.

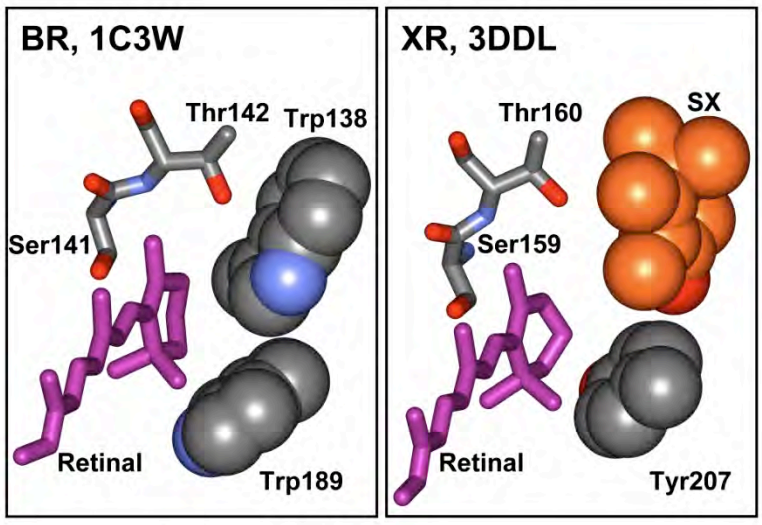

Figure 1. Region of the ionone ring of the retinal and the keto ring of the salinixanthine. Trp138 in bacteriorhodopsin (left panel) is replaced by a glycine, which makes room for the keto ring of the carotenoid in xanthorhodopsin (right panel).

Femtosecond absorption spectroscopy described the rate of excited-state energy-transfer as comparable to the rate of the S2 to S1 internal conversion. This confirmed that the efficiency of energy transfer is ca. 50%. Carotenoid spectral shifts suggested development of a very high electric field during the excited state of the retinal.

Procedures were developed to remove the carotenoid without affecting the retinal, and this made it possible to study these proteins with retinal alone or carotenoid alone.

Science objectives for 2011-2013:

- We will define the carotenoid binding site in gloeobacter rhodopsin by mapping with single mutations, as detailed in the last grant proposal.
- We will describe the spectral changes of the carotenoid in the photocycle. Preliminary results indicate that they reveal the existence of an intense local electrostatic field in the excited state and the initial steps of the photocycle, and what may be a large-scale conformational change that affects carotenoid binding.
- We will explore the ability of the carotenoid to quench potentially harmful excited states using quantum dots. The model system is the light-harvesting cascade QD → salinixanthin → retinal that can collect energy in a broad range from UV to the red region. Preliminary results indicate that the carotenoid can serve as a sink for the electronic excitation near the cell membrane.

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Characterization of the Conformational Flexibility in Light Harvesting Complexes

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Overall research goals: We propose to characterize the structure and conformational dynamics of light harvesting complex I of *Rb. sphaeroides* using solid-state NMR studies of the complex. Disorder and dynamics are expected to be important in the efficiency of function of the apparatus. Our signal assignments for the complex indicate enable molecular level structure and dynamics studies of this system by SSNMR. Such a study will allow us to test specific hypothesis about functionally significant structure and disorder associated with specific conformational degrees of freedom in the naturally occurring complexes.

Significant achievements 2009-2011:
One major objective of the proposal was the characterization of the fast conformational dynamics of the chromophores and nearly protein functional groups. During this year we established and tested methods for dynamic characterization of the protein. Ms. Kuo-yin Huang has written a manuscript regarding a new method using of ^{2}H or deuterium NMR of extensively isotopically triply enriched (^{15}N, ^{13}C, ^{2}H) proteins to obtain order parameters describing the extent of fast limit motions. The deuterium dimension in a multidimensional experiment can yield an order parameter and motional asymmetry, and additionally the ^{2}H – ^{13}C optimal magnetization transfer conditions can be independently used to determine the order parameter. The methods have been applied to model systems and to a small protein, and cross-validated against other strategies to obtain order parameters.

In order apply this approach to light harvesting complexes, we established a collaboration with Richard Cogdell of the University of Glascow, with whom we are preparing and characterizing the LH1 complexes from Rhodobacter Paulstris Acidophila and Spheroides. We optimized expression in appropriate isotopic media, scaled up the purification preparation, and established conditions for reconstitution of the purified materials into artificial membranes. Comparative studies of the preparations from the three organisms is underway. In preliminary results on complexes from Sphaeroides we characterized a potentially important conformational flexibility in LH1, namely the torsional angle in the exocyclic acetyl group of the BChl pigments. This conformational degree of freedom has been indicated previously as potentially powerful for tuning the optical properties of the key chromophores of the light harvesting system, but its plasticity has not been studied experimentally previously, and NMR offers a wonderful opportunity to do so.

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Molecular Analysis of the FMO Antenna Complex from Green Sulfur Bacteria

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Overall research goals**:**

All photosynthetic organisms contain a light-harvesting antenna system, which absorbs solar photons and transfers energy to the reaction center complexes where photochemistry takes place and the energy is stored ultimately in high-energy compounds such as biomass. Photosynthetic antenna complexes are extremely diverse both in terms of structural organization and type of pigment utilized, yet all have a similar function of light collection and must operate using the same set of physical principles. The research described here centers on the Fenna-Matthews-Olson or FMO antenna protein that is found in anoxygenic green sulfur photosynthetic bacteria. The FMO protein is a model system for photosynthetic antennas. It has been studied in great detail using numerous types of structural, spectroscopic and theoretical methods.

Significant achievements 2009-2011**:**

- The FMO protein is probably the most thoroughly studied photosynthetic antenna complex, and was the first chlorophyll-containing protein to have its X-ray structure determined more than 30 years ago. It has continued to be an extremely fruitful system to deepen our understanding even further. Previous work in collaboration with the group of Graham Fleming from UC Berkeley on the use of 2-dimensional spectroscopy of the FMO protein revealed quantum coherence effects. This technique has revealed new energy transfer pathways and quantum coherence effects, the first time that such effects have ever been observed in any biological system. This work has been continued in collaboration with the group of Greg Engel from the Univ. of Chicago (10, 17).
- We have collaborated with the group of Don Bryant from Penn. State Univ. to characterize the FMO from the very distantly related organism *Candidatus* Chloracidobacterium thermophilum (8, 16). This gives insights into the attachment points of the FMO to the chlorosome and reaction center.
- A collaboration with Dale Tronrud from the Univ. of Oregon produced an ultrahigh resolution X-ray structure of the FMO protein at 1.3 Å resolution (1). This study reveals a previously undetected eighth pigment present in substoichiometric quantities (approximately one per FMO trimer) and that the Mg in this pigment is hexacoordinate. We have also collaborated with Jim Allen from Arizona State University on an X-ray structure of the FMO protein from *Pelodictyum phaeum* (14).
- High performance mass spectrometry has been used to probe the FMO protein in collaboration with Michael Gross (2, 12, 15, 16, 18, 19). The main focus in this work has been the eighth pigment, which we have now concluded is present in stoichiometric amounts in the complex in vivo (15).
- We have carried out a detailed analysis of the protein composition and spectroscopy of the photosynthetic membrane in *Roseiflexus castenholzii*, a close relative of *Chloroflexus aurantiacus*, but without chlorosome antennas (3, 13). This is an excellent system with which to investigate the integral membrane antenna complexes in this group of organisms, as there is no interference from the optically dense chlorosome. We have also written a review chapter on antenna systems (20).

Science objectives for 2011-2012**:**

- Our major objectives for 2012 include the production and detailed analysis of site-directed mutants of the FMO protein. Mutants have been prepared by Dr. Yisheng Kang in our group and the characterization is under way. We also are working to establish experimentally the site energy of the eighth bacteriochlorophyll in the FMO protein and how the FMO protein is coupled both structurally and energetically to the chlorosome energy donor and the reaction center energy acceptor.

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Session VI

Antisense RNAs in the Chloroplast

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Overall research goals: Our project aims to characterize the diversity and functions of non-coding RNAs (ncRNAs) in chloroplasts, an RNA class which is abundant but poorly understood in organelles, with a particular focus on antisense RNAs (asRNAs). Our experimental goals are: (1) to identify the full complement of chloroplast-encoded asRNAs, and address their biological functions; (2) to understand how certain asRNAs are selectively accumulated, given that the entire chloroplast genome is fully transcribed on both strands; and (3) to explore roles of two chloroplast ribonuclease III (RNC) homologs in their possible metabolism of double-stranded sense-antisense RNA duplexes.

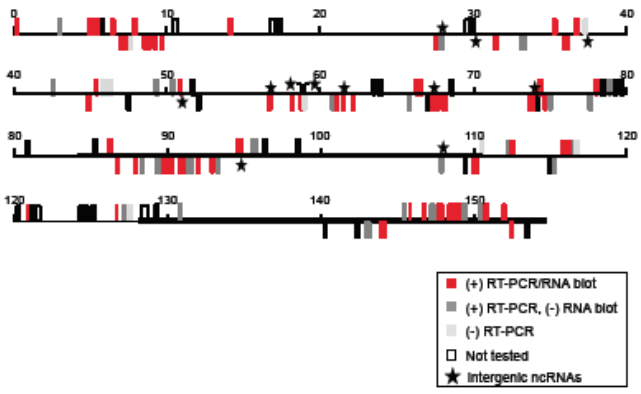

Figure 7. Summary of ncRNA analysis. The chloroplast genome is depicted in linear form, with the inverted repeats shown as heavy lines. The zero position corresponds to the beginning of the large single copy region. Each ncRNA tentatively identified by RNA-Seq is shown; the legend at bottom right indicates the verification status.

Significant achievements 2009-2011: We established both *in vivo* and *in vitro* systems for testing chloroplast asRNAs functionally, by studying an asRNA called AS5. Overexpression of AS5 in transformed chloroplasts was deleterious to plant growth, and inhibited accumulation of the cognate sense RNA (5S rRNA). Using the *in vitro* system, we found that AS5 prevents correct maturatio nof 5S rRNA. To determine the suite of ncRNAs that occurs in chloroplasts, we collaborated with the Ecker laboratory at the Salk Institute, and examined strand-specific RNA-seq data. From these data we identified over 100 candidate ncRNAs, and verified over 90% by subsequent molecular analysis. As the Figure shows, ncRNAs are encode throughout the chloroplast genome, giving them many possible roles in gene regulation. We also studied the phenotype of plants deficient for RNase J, which is present in chloroplasts and some bacteria. To our surprise, RNase J deficiency resulted in rampant accumulation of asRNA, as shown in the lower figure for the gene *atpBE*. The same figure shows that the asRNA is resistant to the single strand RNA-specific mung bean nuclease, showing that it is in duplex form. We further found that the sense RNAs in these duplexes cannot be translated, causing plant mortality. Thus, asRNA is widely produced in chloroplasts but mostly removed by RNase J, leaving behind the specific ncRNAs we identified by RNA-seq.

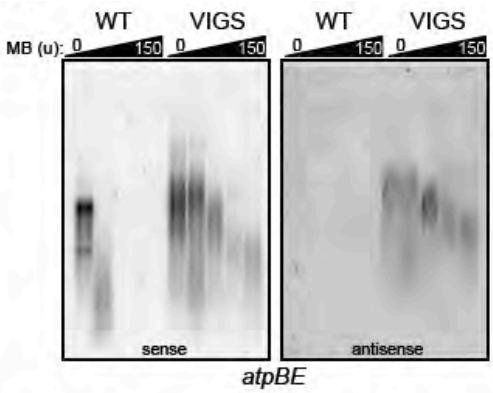

Figure 7 Duplexed antisense and sense RNAs accumulate in the RNase J-deficient leaves. Total RNA isolated from WT or RNase J-deficient material was treated with increasing amounts of mung bean nuclease (0, 0.15, 1.5, 15 or 150 u) for 15 min prior to gel blot analysis. Blots were hybridized with strand-specific *rbc*L **(A)** or *atp*B **(B)** RNA probes to detect transcripts from the indicated strands.

Science objectives for 2011-2012:

- Explore possible functions of ncRNAs. We are also exploring their conservation among different species, determining if any encode small proteins, and ascertaining if they are present in dsRNA form *in vivo*. We are also exaiming possible RNA editing of ncRNAs.
- We have obtained a double mutant lacking the two RNase III-like proteins RNC3 and RNC4. This plant appears to grow fairly normally, but we have started to identify some molecular defects in chloroplast RNA that will be further studied.
- Continue to study the specificity and roles of RNase J in assuring correct gene expression in the chloroplast.

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MSH1: a novel interorganellar environmental response mechanism in higher plants

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Overall research goals: The proposed research aims to understand the functions of a plant-specific protein, MSH1, in altering plastid properties and influencing the plant developmental program. The specific aims of this research are (1) to confirm MSH1 as a component of the PSII oxygen evolving complex, (2) To define both protein and DNA binding sites within MSH1, and (3) to assess environmental responsiveness of *MSH1* expression. From the proposed studies, we expect to confirm and define the nature of MSH1 nucleoid/thylakoid association within the plastid, to gain insight into when and how MSH1 expression is influenced by environmental effects like high light stress, and to incorporate this information into a model of environmental sensing and organelle response that may account for observed plastid, mitochondrial, and nuclear epigenetic effects.

Significant achievements 2010-2011: Over the past year, several important advancements have been made in our understanding of MSH1 in plant development. The gene encodes a DNA binding protein that serves to stabilize both the mitochondrial and plastid genomes. Disruption of *MSH1* results in a variegation phenotype and low frequency chloroplast genome rearrangement. Yeast-2-hybrid screening for potential MSH1 protein interactors suggest possible interaction of MSH1 with components of the PSII-oxygen evolving complex. MSH1 co-fractionates with the thylakoid membrane. Loss of MSH1 function results in altered chloroplast redox status, reduced levels of both plastoquinone and phylloquinone, and the electron chain in more highly reduced state under dark conditions. Plants with this altered plastid state show evidence of enhanced high light tolerance.

Science objectives for 2011-2012: We plan to address three primary questions in the coming year. It will be necessary to confirm MSH1 interaction with the thylakoid membrane by conducting protein topology studies. Our working hypothesis is that MSH1 spans the thylakoid membrane, permitting interaction with the chloroplast genome as well as with PS-II. We expect to carry out directed mutagenesis studies to identify DNA and protein interaction domains within the MSH1 protein. We also expect to confirm MSH1 protein interactors within the chloroplast.

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Post-Transcriptional Regulation of Ethylene Perception and Signaling in Arabidopsis

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Overall research goals: The simple gas ethylene functions as an endogenous regulator of plant growth and development. Ethylene is perceived in the plant Arabidopsis by a five-member family of receptors related to bacterial histidine kinases. Our data support a general model in which the receptors exist as parts of larger protein complexes. Our goals are (1) to identify components of these receptor complexes, (2) to determine the roles that physical interactions among components of the complex play in signalling, and (3) to determine the role of histidine kinase activity in signaling by the receptors, including how it may affect interactions among components of the receptor complex. These studies will provide fundamental insights into how physical interactions function in coordinating receptor signal output. These studies will also shed light on ethylene signal transduction, which regulates multiple aspects of plant growth and development including plant bio-mass, cell wall composition, and photosynthetic capacity.

Significant achievements 2009-2011:

- Demonstration that receptors function as parts of larger protein complexes (published as Chen et al., 2010, PLoS ONE). Our results confirm that the receptors function as components within high-molecular-mass protein complexes, that differences exist among the protein complexes formed by different members of the receptor family, and that disulfide linkages play a role in stabilizing the receptor complexes. Significantly, our results also indicate that novel components within the complexes still remain undiscovered.
- Demonstration that dynamic changes occur in ethylene receptor complexes in response to ligand binding, with both receptors and the down-stream signaling component CTR1 being degraded. Interestingly, ETR1 does not exhibit pronounced ligand-induced degradation, except in mutant backgrounds that lack CTR1 protein; this result indicates that the interaction of CTR1 with ETR1 may serve to protect ETR1 from degradation.
- Identification and characterization of the *Ethylene Feedback Mediator* (*EFM*) Gene Family. Our characterization of the four-member *EFM* family is consistent with their acting to negatively regulate signaling through the ethylene pathway. They are rapidly induced by ethylene, localized to the ER, and overexpression results in decreased sensitivity to ethylene signaling. Initial data suggests that they interact with ethylene receptors.
- Characterization of a role for histidine autophosphorylation in signaling by the receptors. We have been taking a genetic approach looking at lines of plants in which kinase-inactive versions of the receptor ETR1 have been introduced. Analysis of known ethylene-responsive genes by microarray and qRT-PCR indicates that they have reduced expression, suggesting that kinase activity serves a stimulatory role in regulating their expression.

Science objectives for 2011-2012:

- Conclude analysis of ethylene-induced turnover of components within the ethylene receptor/CTR1 complex. In particular, we will determine how mutations within other components of the primary signaling pathway affect turnover of CTR1.
- Characterization of the *Ethylene Feedback Mediator* (*EFM*) family of genes in modulation of signal output from the receptors. New over-expression and loss-of-function lines will be analyzed for effects on physiological and molecular responses to ethylene. We will use BiFC and co-IP approaches to analyze interactions with the ethylene receptors.

- Further characterization of the role for histidine kinase activity of the receptors in modulating signal output. These studies will take advantage of receptor null backgrounds into which various mutant versions of the receptors are expressed. We will test models to determine if phosphorylation affects (1) activation of CTR1 and/or (2) operates through the two-component signaling pathway.

References to work supported by this project 2009-2011:

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Eukaryotic initiation factor 3 (eIF3) and mRNA leader sequences as agents of translational regulation in Arabidopsis

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Overall research goals: Protein synthesis is an energy-intensive component of cellular metabolism. Protein synthesis (translation) is also a target for regulatory controls during gene expression in response to external environmental factors and internal/developmental cues. However, questions abound concerning (i) the limitations imposed by translation on the solar-to-bioenergy conversion, (ii) the RNA sequence motifs and the molecular machinery that support translational regulation, and (iii) the corresponding cellular signaling pathways. This project approaches these questions with an emphasis on the molecular machinery, taking a genetic approach in Arabidopsis thaliana. Specific aims are (1) to assign molecular functions to subunits of the largest translation initiation factor, eIF3, starting with the 38kDa eIF3h subunit, using mutant analysis as well as single-gene and genome-scale assays of translational efficiency; (2) to examine the role of translational regulation in development, in particular stem cell function in the shoot apical meristem; (3) computer modeling of translation initiation as constrained by mRNA sequence motifs; (4) comparative genomic approaches to define the phylogenetic plasticity of mRNA sequence elements that drive translational control.

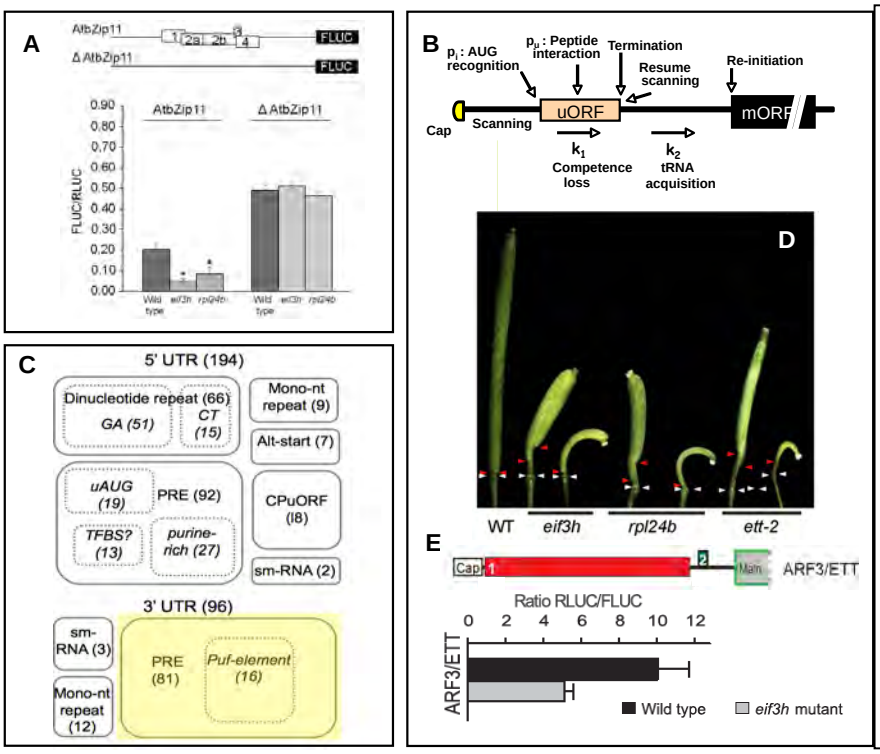

Fig. 1. Translation factors and mRNA sequence elements in translational control of gene expression **(A)** eIF3h and RPL24 cooperate in reinitiation on the uORF containing mRNA AtbZip11. (Zhou et al., 2007). **(B)** Events during translation initiation on a uORF-containing mRNA; k_1, k_2, p_i, and p_u refer to parameters implemented in our computational model (Roy et al., 2010). **(C)** Classification of 290 conserved mRNA sequence elements found in 5' and 3' UTRs of dicot plants (Vaughn et al., submitted). **(D)** Mutant phenotypes and **(E)** in planta gene expression data support the notion that eIF3h and RPL24 contribute to translation reinitiation on the uORF containing ARF3 mRNA (Zhou et al., 2010).

Significant achievements 2009-2011: We continued our molecular analysis of the translation reinitiation machinery, which must engage when ribosomes encounter and translate upstream open reading frames (uORFs) in the 5' leader sequence of the mRNA [Fig. 1B]. Using extensive mutational dissection of the 5' leader sequence of the AtbZip11 mRNA as a model system, we discovered that the h subunit of eIF3 supports translation reinitiation, most likely by supporting a form of molecular memory, such that the ribosome maintains, rather than loses, its competence to

reinitiate translation during translation elongation on a uORF (Roy et al., 2010). eIF3h cooperates with a ribosomal protein, RPL24B, in bringing about translation reinitiation [Fig. 1A] (Zhou et al., 2010). A computational model of the translation initiation process was constructed to test and refine hypotheses concerning translation reinitiation by eIF3h (Roy et al., 2010).

At the developmental level, the eIF3-modulated translational control by uORFs plays a role in controlling stem cell activity and organ initiation in the control center of plant growth, the shoot apex (F. Zhou and AG von Arnim, in preparation). In cooperation with RPL24B, eIF3h supports translation of uORF containing mRNAs for auxin response transcription factors such as ARF3 [Fig. 1D, E] (Zhou et al., 2010). eIF3h and other eIF3 subunits also support pollen development, as was demonstrated using a novel GFP tagging approach (Roy et al., 2011).

A comparative genomics pipeline was constructed and tapped to identify nearly three hundred mRNA sequence elements in 5' and 3' UTRs, which have been conserved over 70 million years of plant evolution [Fig. 1C]. Conservation of these elements suggests a wealth of gene regulatory processes at the posttranscriptional level (Vaughn et al., submitted).

Finally, two early genetic screens for mutants with defects in dark-responsive gene expression both yielded genes involved in RNA-biology. The *FRY1* gene codes for a phosphatase that detoxifies PAP, a ribonuclease inhibitor, which is a byproduct of sulfur assimilation in the chloroplast (Kim et al., 2009). Second, the *BPG2* gene codes for a small GTP-binding protein that specifically binds to chloroplast ribosomal RNA and is required for its proper processing (Kim et al., in preparation).

Science objectives for 2011-2012:

- Continued identification and characterization of mutants in eIF3 subunits other than eIF3h.
- Assembly of a panel of translational reporter genes that allows us to probe the translational machinery for defects in many if not all events critical for successful initiation.
- Taking steps to measure translational efficiency by next-generation sequencing of ribosome-protected fragments, to complement ongoing polysome microarray analysis.
- Identification of patterns of evolution in 5' and 3' UTRs of plant mRNAs.

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Poster Session II

Structure, Function and Assembly of Photosystem II

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Overall research goals: Our overall research goals are: (1) the identification of factors that contribute to the assembly/stability of cyanobacterial PS II and elucidation of the functional mechanism of these factors, (2) the identification of the binding domains for the PsbP and PsbQ proteins in higher plants and CyanoQ and CyanoP in cyanobacteria, and, (3) identification of novel components required for optimal photoautotrophy.

Principal achievements 2008-2011: Our most recent achievements have centered on three areas: (1) Numerous protein components are required for the assembly/stability of PS II. We have identified a novel, cyanobacterial-specific assembly factor for PS II in *Synechocystis*. The protein Sll0606 is absolutely required for PS II assembly beyond the CP43-less reaction center core complex. (2) In higher plants, the PsbP protein of PS II is the founding member of a large lumenally localized family of proteins. A subset of these components, the PPD-like proteins, has not been well studied. We have examined Arabidopsis mutants (T-DNA and RNAi) lacking the PPD5 and PPD1 components. Mutants lacking the PPD5 protein exhibit a range of developmental defects consistent with a lesion in strigolactone biosynthesis and/or sensing. Mutants producing varying amounts of the PPD1 protein exhibit a variegated phenotype, the severity of which appears to correlate with the amount of accumulated PPD1 protein. (3) Numerous studies have used computational methods in an attempt to identify putative oxygen and water channels in PS II. We hypothesized that amino acid residues in contact with oxygen channels would be particularly prone to oxidative modification. Using Fourier-Transform mass spectrometry coupled with collision-induced dissociation, we have recently mapped the presence of oxidatively modified residues in spinach PS II and found that the only residues oxidatively modified and located within 20 Å of the Mn_4CaO_5 cluster are the CP43 residues ^{354}E, ^{355}T, ^{356}M, ^{357}R and ^{370}R.

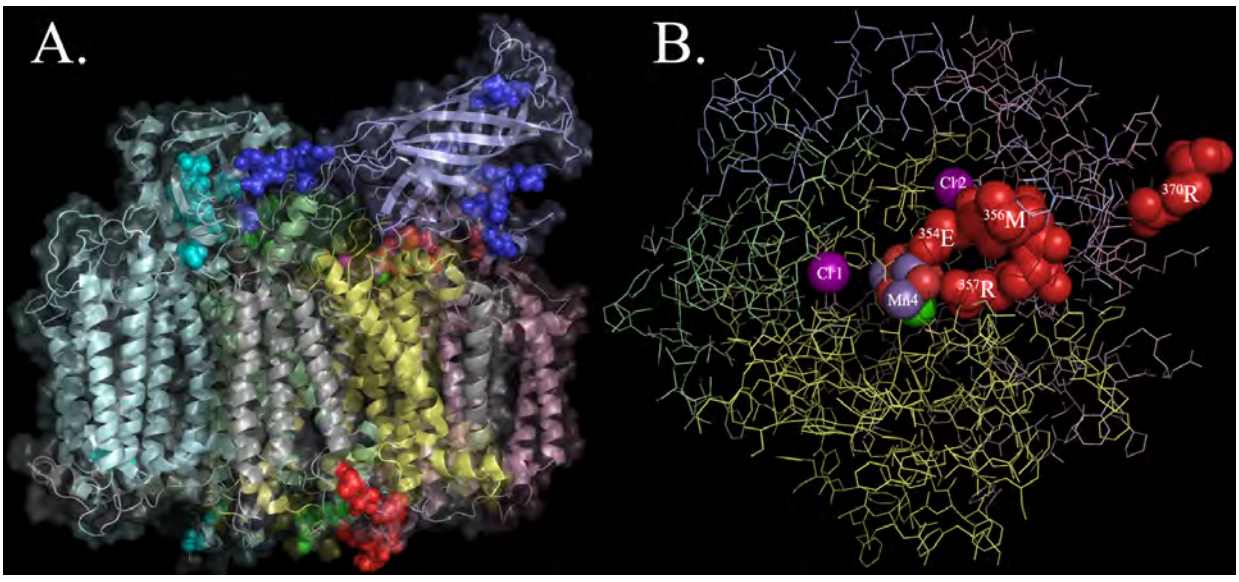

Figure 1. Oxidatively Modified Residues in PS II. A. Overview of modified residues mapped onto the 1.9 Å crystal structure of *Thermosynechococcus vulcanus* PS II. Eighty-seven residues were identified and those resolved in the crystal structure are shown as colored spheres. The majority of these (80%) are surface-exposed to the bulk oxygenated solvent. B. Oxidatively modified residues within 20 Å of the Mn_4CaO_5 metal cluster. This is shown in approximately the same orientation as presented in (A.); note that some unmodified residues were removed for clarity. Key: The D1 protein is shown in pale yellow, D2 is shown in pale green, CP43 is shown in pink, PsbB is shown in pale cyan and PsbO is shown in pale blue. Oxidatively modified residues are shown in darker shades on their respective proteins. The Mn_4CaO_5 cluster is also illustrated, as are the two associated chlorides.

Science objectives for 2011-2012:

- Our previous work has indicated that the Sll0606 protein in *Synechocystis* is a critical assembly factor for PS II. With an antibody now in hand, we will determine if this component is a membrane or a soluble protein, its subcellular location, and identify proteins which interact with the Sll0606 component.
- Examination of the PPD-like proteins in Arabidopsis will continue. The hypothesis that the PPD5 protein is involved with strigolactone biosynthetic and/or sensing pathways will be tested. The mechanism by which loss of the PPD1 protein leads to plant variegation will be investigated.
- We have performed radiolytic mapping at the LSU CAMD synchrotron on spinach PS II in an attempt to determine (1) the location of putative water/oxygen channels in PS II, and (2) the organization of the extrinsic proteins PsbP and PsbQ within the higher plant photosystem. Analysis of these radiolytic mapping experiments is ongoing. Additionally, protein crosslinking experiments are ongoing which will complement and extend the radiolytic studies.
- We continue to analyze additional *Synechocystis* mutants produced by *in vitro* transposon mutagenesis, identifying genes required for optimal photoautotrophy. It should be noted that the *sll*0606 gene was identified in this manner.

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Redirecting Electron Transfer in Photosystem II
From Water to Redox-Active Metal Complexes

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Photosystem II (PSII) functions to couple the light-induced oxidation of water to the reduction of quinones. The steady-state turnover of PSII is limited by the slow reduction and exchange of quinones at the Q_B-binding site, which is 20 times slower than the slowest step in the oxidation of water, and slow turnover of the electron-acceptor side of PSII is results in photodamage associated with the production of reactive oxygen species. In order to bypass the rate-limiting quinone electron acceptors, we have redirected electron transfer from Q_A^- to water-soluble electron acceptors. A negatively charged region on the surface of PSII near Q_A has been identified as a docking site for cationic exogenous electron acceptors. Oxygen evolution activity, which is inhibited in the presence of the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), is recovered by adding Co(III) complexes. Thus, a new electron-transfer pathway is created with Co(III) as the new terminal electron acceptor from Q_A^-. This binding site is saturated at ~2.5 mM [Co(III) complex], which is consistent with the existence of low-affinity interactions with a solvent-exposed surface. This is the first example of a higher plant PSII in which the electron-transfer pathway has been redirected from the normal membrane-associated quinone electron acceptors to water-soluble electron acceptors. The newly identified binding site for cationic electron acceptors may enable faster turnover of PSII with minimal formation of reactive oxygen species and may enable efficient collection of electrons generated from photochemical water oxidation by PSII immobilized on an electrode surface.

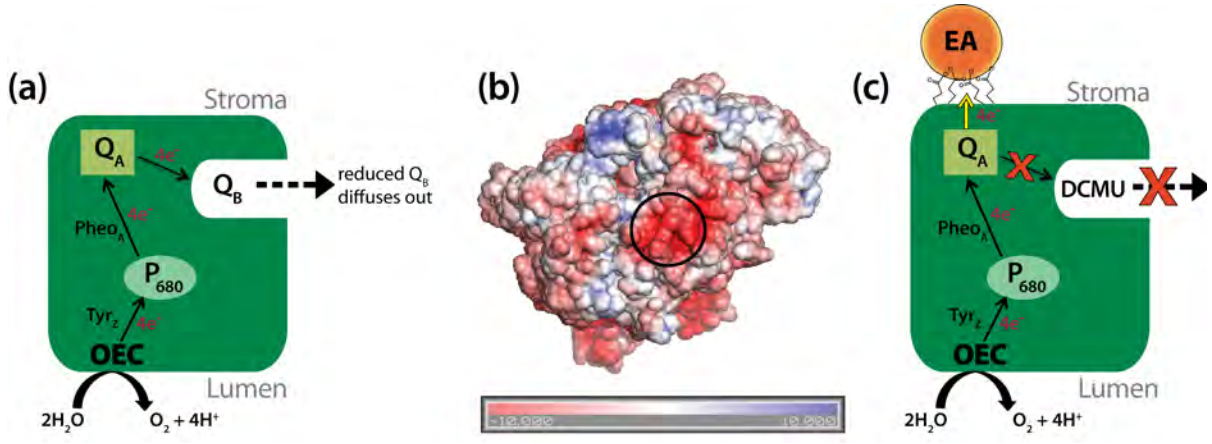

Figure 1. (a) A simplified scheme of photosystem II showing a view along the membrane plane of the electron-transfer pathway from the oxygen-evolving complex (OEC) to the membrane-diffusing quinone, Q_B. (b) Electrostatic potentials of the solvent-exposed stromal surface of PSII shown at -10 kT/e (in red) and +10 kT/e (in blue). The patch outlined within the black circle was identified as a putative binding site for cationic small molecules. (c) Redirection of the electron-transfer pathway within PSII. DCMU, a potent herbicide, binds to the Q_B site and blocks electron transfer. Catalytic activity can be restored by electron transfer to an exogenous electron acceptor (EA) that binds to a surface site near Q_A (as shown bound by the black circle in Fig. 1b).

Overall research goals: PSII is the only natural photosynthetic reaction center that generates an oxidant sufficiently strong to oxidize its own light-harvesting pigments. As a result of this propensity for oxidative damage, PSII is distinct from other photosynthetic complexes in having secondary electron donors that include carotenoid (Car) and chlorophyll (Chl) molecules. Thus, carotenoids in PSII have a unique redox function in addition to their roles also found in other photosynthetic complexes as light-harvesting pigments, as photoprotective molecules in triplet energy-transfer processes, as singlet O_2 scavengers and as components that stabilize pigment-protein structures. This makes PSII an ideal system in which to determine the factors that control carotenoid function in natural photosynthetic systems. The long-term objective of this project is to determine the primary factors that influence carotenoid function in PSII.

Science objectives for 2008-2011:

- characterize the secondary electron-transfer reactions of PSII.
- characterize the photoprotective functions of the secondary electron-transfer reactions in PSII.
- characterize the role of carotenoids in PSII assembly.

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Integration of the light and dark reactions of oxygenic photosynthesis

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Overall Research Goals:

The inducible high affinity carbon concentrating mechanism (CCM) is studied to understand how it is integrated with the light and dark reactions of photosynthesis. The basic hypothesis was that a tight integration is achieved through specific regulatory interactions between photosynthetic metabolites and the transcriptional regulatory proteins that control the expression of the structural genes for the inducible CCM. While such interactions had been hypothesized, the actual mechanisms had remained unresolved. *The DOE-supported analysis revealed that metabolic intermediates of the light and dark reactions, notably NADP$^+$ and α-ketoglutarate (α-KG), of cyanobacterial photosynthetic metabolism act as allosteric effectors of the DNA-binding proteins which modulate the expression of the CCM genes.* The current research objectives involve:

1. Study the interactions between effector metabolites, transcriptional regulator, and the regulatory DNA sequences of the genes in the inducible CCM. Define the basic mechanics of the regulatory circuits of the CCM, which will benefit by a new collaboration with an expert in the structural and functional analysis, of the LysR-type transcriptional regulators involved (Prof. Cory Momany, U Georgia).
2. Connect transcriptional regulation findings with physiological analysis that probes how the deduced regulatory circuitry explains certain cellular responses to changing light and inorganic carbon availability conditions. Here we have made very good progress tracking the redox state of the cell, notably, the NAD(P)H/NAD(P)$^+$ ratio during the onset of inorganic carbon limitation.
3. Construction and analysis of experimental systems that will test the function of the CCM and test the feasibility of assembling genetically chimeric forms of the CCM. This will allow teasing apart some of the unique regulatory and enzymatic features of the CCM and will provide insight into the feasibility of one day transplanting the cyanobacterial CCM into eukaryotes.

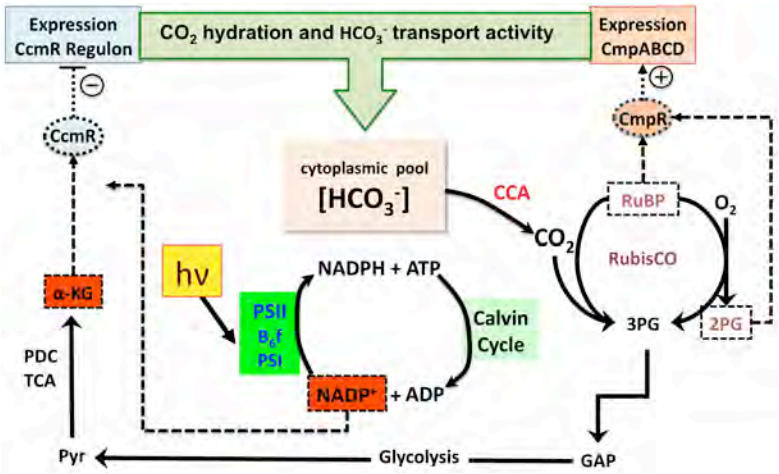

Figure 1. Working model for the CCM regulatory network within *Synechocystis* showing CcmR and CmpR. Regulatory molecules and interactions are indicated with dashed lines, metabolic fluxes indicated with solid arrows. Ligand molecules for CcmR and CmpR are enclosed in dashed boxes. Using Surface Plasmon Resonance (SPR) Shawn Daley showed that NADP$^+$ and α-KG (red dashed boxes) function as co-repressors for CcmR. The depletion of these metabolites results in the de-repression CCM transcriptional expression to alleviate the scarcity of inorganic carbon (Ci).

Major Accomplishments 2010-11

1. Progress towards real-time tracking of physiological cues for gene expression: NADPH/NADP$^+$ ratio dynamics in vivo: The NADPH/NADP$^+$ ratio now appears key in the coordination of the expression of the CCM, carbon status, and the light reactions. Continuous measurements of chlorophyll *a* and NAD(P)H fluorescence during limitation of inorganic carbon shows a dramatic increase prior to a significant decrease of NAD(P)H levels in *Synechocystis* while a strain bearing the deletion of the regulator *ccmR* (constitutively expressing the high affinity CCM) showed little changes in NADPH fluorescence traces during carbon limitation. The data suggest that *Synechocystis* detects changes in the energy state of the cell prior to increased oxygenase activity of RuBisCO and

that the full activation of the CcmR regulon causes increased consumption of NAD(P)H through the specialized NDH-I complexes while decreasing the severity of the response to carbon deprivation. Experiments with cells adapted to high inorganic carbon (HCi) and low inorganic carbon (LCi) subjected to increasing light intensity while measuring Chl and NADPH fluorescence showed an increase in NADPH levels until growth light intensity and a decrease in levels at higher light intensities in HCi adapted cells but no decrease in LCi adapted cells. This shows that the cells adapt to maximize $NADP^+$ reduction at growth light conditions and that an abundant inorganic carbon source causes a decrease in the level of NADPH at high light intensities presumably through increased consumption of NADPH and increased cyclic electron transport to balance increased consumption of ATP within carbon metabolism. The measurement of the dynamic changes of NADPH during carbon deprivation and changing light intensities has led to the development of further understanding of the flow of reductant within cyanobacteria.

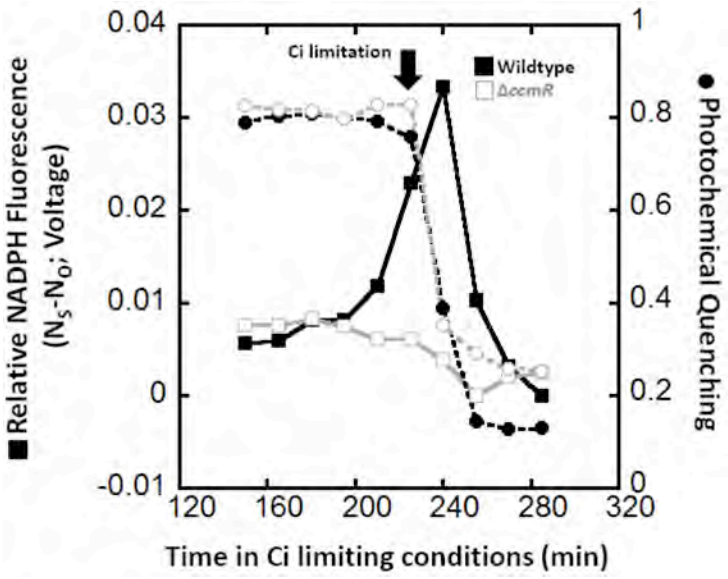

Figure 2. Carbon limitation affects the redox state of the PQ and the NAD(P)H pools with kinetics that depend upon induction of the CCM. Wild-type cells (closed symbols) show a dramatic decline in photochemical quenching (squares) upon Ci limitation reflecting an over-reduction of of the PQ pool due to loss of electron sink capacity. Increased reduction of $NAD(P)H/NAD(P)^+$ pool (circles) also occurs, but declines after about an hour, possibly due to synthesis of the high afinity CCM proteins such as CupA. Consitutive synthesis of the CCM in repressor knockout strain, Δ*ccmR* (open symbols), prevents the heightened reduction of the $NAD(P)H/NAD(P)^+$ pool. The continued overreduction of the PQ pool in both strains likely refelcts the fact that electrons routed through the CCM are returned to the PQ pool.

2. Progress towards the construction 'markerless' strain lacking CCM activity to facilitate engineering the CCM. The NAD(P)H-Dehydrogenase Type 1 complex (NDH-1) protein family is diverse group of protein complexes which use reductant (NADH or NADPH) to power proton translocation and reduce quinone substrates. Cyanobacteria use these complexes to capture CO_2 escaping from the carboxysome and convert it to bicarbonate. This is accomplished through the use of accessory proteins associating with the NDH-1 complex, namely CupA and CupB. In order to study NDH-1, CupA, and CupB, a mutant strain of *Synechocystis* sp. PCC 6803 lacking the entire carbon concentrating mechanism is being created. This knockout will allow the addition and deletion of genes in order to manipulate carbon transporters. The knockout of the *ndhF3/ndhD3/cupA* operon, induced during carbon limitation, has been developed. Characterization of its carbon uptake ability is currently underway and the knockout of further genes is in development.

Manuscripts:

Daley, S. M. E., Kappell, A., Holland, S., Carrick, M., and Burnap, R. (in preparation) $NADPH/NADP^+$ ratio controls the expression of the high affinity inorganic carbon concentrating mechanism in cyanobacteria.

The effects of inorganic carbon limitation on the dynamic changes in NADPH levels of *Synechocystis* sp. PCC 6803 Kappell, A., Holland, S., Carrick, M., and Burnap, R. (in preparation)

Objectives 2011-2012:

The finding that $NADP^+$ is a key regulator of inorganic carbon acquisition provides important insight regarding the integration of the light and dark reactions of oxygenic photosynthesis. However, what additional biochemical details can be obtained regarding the regulation mechanism and how do these detailed features play out in terms of physiological dynamics in the face of different nutrient and light regimes?

1. The key objective this year must be to convert the considerable experimental progress to manuscripts.
2. Continue to connect global gene expression profiles with changes in physiological state. A prime objective will be to assay changes in $NADPH/NADP^+$ as a function of light and C_i and using different mutant strains.
3. Complete the construction of the markerless ΔCCM strain, evaluuate the properties of the ΔCupA mutant in terms of photosynthetic metabolism.

Intracellular Signaling from Chloroplasts to the Nucleus

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Overall research goals: The major goal of this project is to define the signal(s) and signaling pathways from chloroplasts that regulate nuclear gene transcription. In previous years of this award, we isolated a large number of mutants that defined six *GUN* genes. Five of the six genes (*GUN2-6*) encode enzymes of the tetrapyrrole pathway that cluster around the chlorophyll and heme branchpoint, while the precise function of GUN1, a pentatricopeptide repeat protein (PPR), has been mysterious. We also initiated a genetic screen to identify new genes involved in the high light stress response. The proposed studies will integrate genetic, genomic, molecular, and biochemical approaches in *Arabidopsis* in an effort to: (1) Test the hypothesis that GUN1, a chloroplast PPR protein, is a regulator of tetrapyrrole synthesis; (2) Further explore our recent finding that a specific pool of heme is the source of a positive signal that chloroplasts are functional; (3) Continue genetic and biochemical dissection of the signaling pathway(s) initiated from changes in photosynthetic electron transport redox state in response to excess light. Our screens have already identified 7 new genes involved in acclimation to excess light; we will assign these genes to a genetic pathway, and determine the molecular mechanisms by which plants acclimate to excess light.

Significant achievements 2009-2011:

- GUN1: GUN1 contains 10 PPRs and a second domain called the SMR (small MutS-related) domain. In bacteria, the SMR domain has endonuclease activity. We generated recombinant GUN1 protein and showed that GUN1 contains nuclease activity. Deletions showed that the 10 PPR motifs are required for the interaction between GUN1 and its target. GUN1 has a role in chloroplast metabolism, most likely as an RNA processing enzyme for which the PPRs conferred substrated specificity, whereas the SMR domain is involved in catalysis.
- PGCN: We explored the role of plastid genome copy number (PGCN) in retrograde signaling with genotoxic agents that target DNA gyrase, inhibiting the replication of the genome, reducing PGCN. Our data uncovered a light-independent, retrograde response for expression of genes encoding nuclear-localized, chloroplast-destined proteins. Opposite to other known pathways of retrograde signaling, changes in plastid DNA copy number affect accumulation of plastid-encoded transcripts, but not protein synthesis. *Gun1* mutants uniquely failed to suppress the expression of chloroplast-destined proteins when DNA content is reduced. Thus, the signal that triggers PGCN-mediated response may depend on the accumulation of a chloroplast RNA processed by GUN1.
- Heme as a Retrograde Signal: We discovered a new gain-of-function mutant of *Arabidopsis*, called *gun1-6D*, which has a constitutive retrograde response. This mutant overexpresses plastid ferrochelatase 1 (FC1), which encodes an enzyme involved in heme biosynthesis. Surprisingly, overexpression of the paralog, FC2, is not a *gun* mutant, suggesting a model in which heme, specifically produced by FC1, may be used as a retrograde signal to coordinate gene expression. These data are published (Woodson et al., 2011, *Curr Biol.*).
- High light stress screen: Light is required for photosynthesis, but too much light (excess light or EL) can create a situation that is harmful to plants. If the amount of light exceeds a plant's ability to fix carbon dioxide, the plant may become photo-inhibited or go into oxidative stress. A third signaling pathway mediates signals derived from the reduction/oxidation (redox) state of the photosynthetic electron transfer chain (PET) and affects both photosynthesis-related and stress-related genes. Several studies have provided evidence for a role of the redox state the PQ pool as a sensor of EL and a regulator of nuclear gene expression. We used a reporter gene

fusion to identify mutants defective in the response to high light stress, and found genes involved in both early and late phases of the response. Early on, 3 heat shock factors (HSFs) of greater than 20 found in the genome play an important role in this process. For later responses, we are currently working through our mutant collection to clone genes. So far, we have identified mutations in 4 genes involved in later phases of light stress; these include: cryptochrome, two RNA processing enzymes, and a co-activator. We are trying to order the genes into a network.

Science objectives for 2011-2012:

- We are analyzing transgenic plants that stably overexpress the 3 HSFs that we have shown are involved in the rapid response to high light. Preliminary results indicate that their overexpression can protect *Arabidopsis* from high light-induced stress (bleaching, growth arrest, etc). We are very excited about this result as plant stress responses are a major contribution to loss of yield.
- We continue to work on the precise function of GUN1, and are following up on several leads to identify the specific RNA bound by the PPR domain of GUN1.
- We are attempting to identify the 2 proposed pools of heme, by purifying FC1 and FC2 complexes from plants. We have some candidates for possible components of the complexes.
- The successful completion of the proposed projects should allow us to begin to engineer these crucial pathways. Since heat, cold, and high light stress share at least some common signaling elements, generation of new genotypes will ultimately influence our abilities to manipulate plant growth and development, and will aid in the understanding of the developmental control of photosynthesis.

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Resolving protein-semiquinone interactions by advanced EPR spectroscopy

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Overall research goals: Our focus is on use of modern, high-resolution EPR spectroscopy to explore the catalytic domains trapped in states with semiquinone (SQ) as an intermediate. The catalytic sites we propose to study, - the Q_A and Q_B-sites of the reaction center, the Q_H-site of the bo_3 quinol oxidase, and the Q_i-site of the bc_1 complex, - all operate using ubiquinone, but have different electron transfer partners, and different operating potentials. EPR probes interactions between the electron spin of SQ and local magnetic nuclei, which provide direct information about *spatial* and *electronic* structure of the SQ and the immediate protein and solvent environment. The main question to be addressed is that of how the protein environment modifies the spatial and electronic structure of the SQ in different sites to fit the physiological function.

Significant achievements 2010-2011:

Q_A and Q_B site of bacterial reaction center. Photosynthetic reaction centers from *Rhodobacter sphaeroides* have identical ubiquinone-10 molecules functioning as primary (Q_A) and secondary (Q_B) electron acceptors. The SQ_A forms H-bonds with nitrogens of H-M219 and A-M260. The hyperfine (*hfi*) tensors of two H-bond protons were previously characterized by orientation-selective Q-band ENDOR. Powder X-band 2D ESEEM was validated against the ENDOR with good agreement for two H-bond protons. 2D ^{1}H ESEEM was then applied to the SQ_B where we found lines from four H-bonded protons (ref. 4). In addition, 2D ^{14,15}N ESEEM has unambiguously provided *hfi* and nuclear quadrupole (*nqi*) couplings for H-bond nitrogen donors carrying transferred unpaired spin density (USD) for both sites (refs. 2,4). These data define the H-bond network around the SQ_B. Critical analysis of USD distribution to the SQ_B, previously frustrated by lack of information about the number of H-bonds and the strength of coupling, is now feasible. We used QM/MM calculations on a Q_B site model to assign the *hfi* tensors to specific H-bond interactions, and we compared this with the Q_A site (ref. 4). The calculated ^{1}H and ^{14}N *hfi* tensors have shown very good agreement with experimental values for both sites. The QM/MM simulations also reproduce a difference in the asymmetry of USD distribution between SQ_A and SQ_B. This work has been performed in collaboration with Dr. P. O'Malley (University of Manchester, UK). The computational approach tested on the Q_A and Q_B has now been applied to the Q_H site of the cyt bo_3 and Q_i site of cyt bc_1.

Selective isotope labeling of cyt bo_3 for pulsed EPR characterization of the SQ_H. The most unambiguous way to identify the nitrogen H-bond donors around SQ is by selective ^{15}N labeling of particular residues. Efficient selective labeling of proteins necessitates auxotrophic hosts. For proteins expressed in *E. coli* this is straightforward, and the laboratory of Prof. Gennis has constructed a set of auxotrophs in a commonly used *E. coli* expression strain C43(DE3), a derivative of *E. coli* BL21(DE3), which can be used for isotopic labeling of individual amino acids or sets of amino acids (ref. 6). These strains have general applicability to either soluble or membrane proteins that can be expressed in *E. coli*. Selective ^{15}N isotope labeling of different nitrogens in Arg, His and Gln with *E. coli* C43(DE3) auxotrophs was used to identify the N_ε of R71 as the H-bond donor carrying the most transferred USD and to measure weak *hfi* couplings with the side-chain and peptide nitrogens from R71, H98, and Q101 residues. The approach was extended to the D75H mutant, where the intermediate SQ_H is also stabilized, but the protein is virtually

inactive. We found that the N_ε from a histidine residue, presumably H75, carries most of the USD instead of N_ε of R71, as in wild-type *bo*$_3$. All nitrogens of R71 are weakly coupled suggesting much weaker H-bond with R71 than in wild-type *bo*$_3$. These data form a comprehensive basis for QM/MM modeling of the SQ_H state in wild-type and D75H cyt *bo*$_3$.

Determination of SQ electronic structure, and of the geometry of substituents *in vivo* and *in vitro*, requires selective ^{13}C labeling in ring positions and in CH_3 and OCH_3 substituents. Selective ^{13}C labeling of methyl groups was achieved by using L-methionine (methyl-^{13}C) as the only source of ^{13}C in the *bo*$_3$ proteins (ref. 5). The ^{13}C labeled UQ_8 isolated from *E. coli* was used for model experiments with the anion-radical. In the model system we observed 2D ESEEM spectra with significantly different ^{13}C *hfi* couplings when compared with the SQ_H in cyt *bo*$_3$, or with D75E and D75H mutants. The ^{13}C *hfi* couplings in proteins reflect not only the USD redistribution induced by the new environment but also the conformation of the methoxy groups.

Science objectives for 2011-2012:

- Construction of a comparative structural model describing the H-bonding around SQ_H in wild-type and D75H *bo*$_3$, based on the ^1H and ^{15}N *hfi* couplings and QM/MM calculations.
- ^{13}C couplings provide insight into the SQ binding to the protein. We will apply a biochemical approach for selective ^{13}C labeling of ring carbons in the SQ_H. The quinones biochemically labeled in *bo*$_3$ enzyme will also be used in studies of bacterial reaction center and *bc*$_1$ complex.
- We will exploit Q-band ^1H and ^2H ENDOR to address specific questions about the *orientation* of the H-bonds around SQ in Q_B and Q_H sites based on the *hfi* (and *nqi*) tensors of exchangeable protons (deuterons).

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Mimicking Photosynthetic Systems Using Chemically Modified Viral Capsids

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Overall research goals: The protein capsids of viruses provide a convenient source of rigid, nanoscale scaffolds for the construction of complex multifunctional materials. These proteins can be produced in large quantities through expression in *E. coli*, and can be genetically tailored to possess reactive groups for the positioning of synthetic molecules on their surfaces. In this project, we have explored the use of two capsid-forming proteins to build artificial light harvesting systems and connect them to photocatalytic groups. In one example, we have developed chemical strategies to attach the rod-like protein shell of the tobacco mosaic virus to polymers,[1] carbon nanotubes,[2] light harvesting chromophores,[3] and porphyrins. We have also developed methods to transfer the modified viral capsids into organic media for the purposes of embedding them into polymeric films.[4] As a second target, methods have been developed to append new functionality to both the external and internal surfaces of MS2 viral capsids.[5] These spherical assemblies have been used to house chromophores that collect light and transfer the energy to catalysts located on the exterior surface. Taken together, these new scaffolds provide many new avenues for the integration of multiple functional components with defined spatial relationships. Equally important for these studies is the set of chemical strategies that has been developed to modify biomolecules in multiple locations with high site selectivity and yield. As a result of these studies, we are in an excellent position to answer basic questions about the mechanisms of energy transfer, the effects of geometry on light harvesting efficiency, the roles of photoprotectants (such as carotenoids) in increasing system longevity, and types of spatial relationships that are ideal for electron transfer.

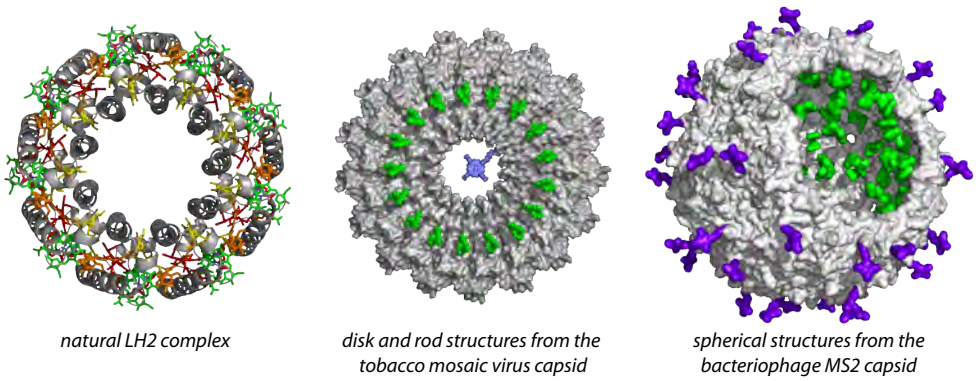

Figure 1. The integrated chromophore networks found in natural light harvesting systems can be efficiently mimicked using the self-assembling proteins that comprise viral capsids. Complex systems with varied geometries and multiple functional components can be accessed through the use of tandem bioconjugation strategies.

Recent Program Achievements:

- We have developed efficient methods for the covalent attachment of chromophores to TMV coat protein monomers (TMVP) and the subsequent self assembly of these proteins to access highly efficient light harvesting systems.[3] These systems have been characterized using steady-state and time-resolved spectroscopic methods.[6]

- One unique aspect of TMVP-templated light harvesting arrays is the fact that they are inherently three-dimensional, and thus could possess redundant energy transfer pathways that could

circumvent defect sites better than two-dimensional systems. To test this possibility, we have recently developed a chemical strategy to introduce well-defined numbers of "bleached" chromophores into the arrays.[7] These studies have shown that the three-dimensional structures do indeed have an improved tolerance for defects, showing a linear decrease in efficiency as photoinactive sites are introduced.

- From our first studies, the assembly state of TMV-based light harvesting systems has been observed to exert a dramatic effect of light harvesting performance, with rod assemblies consistently significantly outperforming disks. Using computer modeling, we first determined that the principal transition dipole of the chromophores is oriented along the long axis of assembled rods, suggesting that energy transfer is much more efficient in this direction. Disks lack this transfer orientation. Secondly, we have developed a computational simulation of these systems. This model tracks the fate of large numbers of absorbed photons as they transfer from donor to donor, and finally to the acceptor groups. These studies have revealed an important difference in the energy dissipation rates of the donors in the two systems, and they have shown that the small number of chromophores in disk systems is more subject to statistical variations during the assembly process.[7] We are now using these simulations to guide the choice of appropriate photocatalysts for use in these systems.

- We have developed several new routes for the synthesis of porphyrins[5] and phthalocyanines that possess the proper chemical functional groups for protein bioconjugation. In particular, we have synthesized a number of new structures that possess alkoxyamine groups, which can be attached to reactive carbonyls. Several methods are now available for the introduction of ketone and aldehyde groups on proteins,[8] including our oxidative coupling strategy that targets artificial amino acids.[9] With these new molecules and strategies, we can now dictate the position of photocatalytic groups on the genetic level.

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Studies on the cytochrome bo_3 ubiquinol oxidase from *E. coli*

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Overall research goals:

Cytochrome bo_3 is a member of the superfamily of heme-copper oxidoreductases, which include the proton-pumping respiratory oxygen reductases that are present in most aerobic bacteria as well as in the eukaryotic mitochondria. The major aim of this grant is to investigate the structure and catalytic mechanism of the cytochrome bo_3 ubiquinol oxidase from *E. coli*. This enzyme catalyzes the two-electron oxidation of ubiquinol-8 within the cytoplasmic membrane of *E. coli* and the four-electron reduction of O_2 to water. The considerable free energy available from this reaction is used to generate a proton motive force across the bilayer. For each turnover of the enzyme, 8 charges are translocated across the membrane, resulting in a transmembrane electrical potential. The reaction catalyzed by the enzyme is as follows.

$$2\ UQH_2 + O_2 + 8\ H^+_{in} \qquad 2\ UQ + 2\ H_2O + 8\ H^+_{out}$$

The catalytic chemistry takes place at an active site containing a high-spin heme (heme o_3) and a nearby copper (Cu_B). Our major biophysical tools are pulsed EPR, done in collaboration with Dr. Sergei Dikanov, and solid state NMR, done with Dr. Chad Rienstra.

Our goals include a) To determine the detailed nature of the hydrogen bonding between the protein and semiquinone radical, and the functional significance of the manner in which the semiquinone is stabilized by the protein. b) To determine the location of the tyrosyl radical that is generated during the cleavage of the O-O bond during the catalytic cycle. c) To determine the detailed structural information on critical residues at the active site and within proton-input channels by using solid state NMR.

Radicals are generated in different steps of the mechanism.

1. The oxidation of each ubiquinol molecule occurs in two steps, with the one-electron reduced semiquinone species generated as an intermediate. The semiquinone species can be stabilized and provides a probe to examine the protein environment around the semiquinone by pulsed EPR methods.
2. The mechanism of the heme-copper oxygen reductases is proposed to require electron transfer from an active-site tyrosine (Y288 in cyt bo_3) during the cleavage of the O-O bond. This tyrosine is always cross-linked to a histidine which is one of the ligands to Cu_B. Reduction of the active site heme iron and copper can yield only 3 of the four electrons needed for O-O bond cleavage to give the known products.

$$Fe^{2+}_{o_3} + Cu^{+}_{B} + \text{Y-OH} + O_2 \qquad Fe^{4+}_{o_3} = O^{2-} + Cu^{2+}_{B}(^{-}OH) + \text{Y-O}^{\bullet}$$

Efforts to trap the neutral tyrosine radical ($YO^{\bullet}$) and directly observe it have not been successful, possibly due to electronic coupling to the paramagnetic metals and/or due to a low yield during the transient reaction. We are placing non-natural amino acids in place of the active-site tyrosine to attempt to stabilize and observe the tyrosyl free radical.

Significant achievements (2009-2011):

To assist in both pulsed EPR and solid state NMR experiments, we increased the yield of recombinant enzyme to allow us to incorporate selectively labeled amino acids containing ^{13}C, ^{15}N or ^{2}H. To facilitate this we generated a set of amino acid auxotrophs in the frequently used C43 expression strain, which is a derivative of *E. coli* BL21. These strains allow us to selectively label amino acids in the enzyme with high yield. We have also placed ^{13}C both uniformly and in the methyl/methoxy groups of ubiquinone-8. These preparations have allowed us to obtain considerable information about the interaction of the protein amino acid residues with the unpaired electron spin of the semiquinone bound to cytochrome bo_3. We have also demonstrated that selectively labeled cytochrome bo_3 substantially simplifies the solid state NMR spectra and should allow us to make assignments of critical residues in the near future. In addition to work on cytochrome bo_3, we have extended our studies to include the much smaller DsbB protein from *E. coli*, which is also a ubiquinol-binding protein, and the aa_3-600 menaquinol oxidase from *B. subtilis*, which is a homologue of cytochrome bo_3 which utilizes menaquinol in place of ubiquinol as a substrate.

Science objectives (for the next 2 years):

During the next two years we plan to

1) Extend the pulsed EPR with cytochrome bo_3 to include determination of the ^{13}C hyperfine interactions using labeled ubiquinone, and to work with a computational chemist, Dr. Patrick O'Malley at the University of Manchester (UK) to model the quinone/protein interactions. We will also extend the studies to analogs of ubiquinol which bind to the same site on the enzyme and form a radical.
2) Identify the tyrosyl free radical which we have recently observed upon interaction of cytochrome bo_3 with hydrogen peroxide. This project includes the use on non-natural amino acid mutations, particularly 3-aminotyrosine, which has a lower midpoint potential than does tyrosine, and may be useful to trap the radical.
3) Identify the crosslinked histidine/tyrosine cofactor at the active site of the enzyme in the solid state NMR spectrum and use this to determine how this cofactor is interacting with other species at the active site.

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Structural, Functional, and Integration Studies of Solar-Driven, Bio-Hybrid, H_2-Producing Systems

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Overall research objectives: The overall objective of the project is to conduct fundamental studies of the molecules, molecular interactions and molecular assemblies that generate, transfer and catalyze conversion of solar energy into electrons and energy carriers. The aims include elucidating parameters that control energy transduction processes in model bio-hybrid and photobiological systems for hydrogen production that utilize [FeFe]-hydrogenases (H_2ase) as model hydrogen (H_2) activation catalysts. The research objectives include: (i) developing theoretical models of H_2ase to understand electron/proton-transfer (ET and PT) and catalysis; (ii) characterizing the structure of H_2ase using a combination of biochemical and spectroscopic techniques; (iii) optical and electronic studies of H_2ase bio-hybrids to characterize assembly, photoinduced charge-transfer and catalytic conversion processes; and (iv) electrochemical characterization of native and structurally minimized H_2ases using electrochemical scanning tunneling microscopy (STM) and macroscopic techniques. The efforts made towards achieving these objectives will help to resolve how solar energy is allocated among competing processes in molecular systems, provide fundamental knowledge on how metalloenzymes catalyze H_2 activation and a basis for structural control of catalytic efficiencies, and how molecules interact and form complexes that achieve efficient transduction of light energy into chemical energy.

Significant achievements 2008-2011: (i) Computational chemistry studies of H-cluster models and configurations of the diatomic ligands revealed a potential role of the bridging ligand in buffering charge upon reduction. (ii) A quantum chemical H_2ase model encompassing the H-cluster, accessory [4Fe-4S]-clusters and surrounding protein was constructed. Custom computational methods allow for breaking the spin symmetry within accessory [4Fe-4S]-clusters, and integration of the initial gas-phase calculation into a QM/MM model of the complete H_2ase. (iii) The free energies along PT pathways were investigated using QM/MM and umbrella sampling techniques, and several important residues were identified along with pK_a estimations using a thermodynamics integration method. (iv) Brownian dynamics and molecular dynamics simulation techniques predicted the binding structures between H_2ase and SWNTs or bulk carbon surfaces, and ET rates. ET appears to be at least a 1000-fold faster for SWNTs than for a bulk carbon surface, and independent of SWNT diameter. (v) The native Clostridial H_2ase has been successfully adsorbed to Au electrodes bearing self-assembled thiol-based monolayers (SAMs) and retains activity. Binding is via interactions between positively charged patches on the enzyme and carboxylate groups on the SAM. Single-molecule images have been obtained in an electrochemical STM showing tunneling currents increase under an applied bias, which allowed for estimation of a lower limit k_{cat} of 20,000 s^{-1} in combination with macroscopic voltammetry. (vi) Assemblies of H_2ase and mercaptopropionic acid (MPA) capped CdS nanorods have been studied in solution. Kinetic studies demonstrated that the CdS nanorod was bound to the H_2ase at the Ferredoxin binding

surface, consistent with an electrostatically controlled binding complex where the CdS lies adjacent to the H_2ase distal [4Fe-4S]-cluster. Hydrogen was evolved under prolonged illumination for up to several hours, but declined due to the photolability of the MPA ligand, and subsequent inhibition by MPA of the H_2ase.

Science objectives for 2010-2011:

- Tunneling currents will be examined between accessory [4Fe-4S]-clusters and the H-cluster in the H_2ase computational model. Calculations of H-cluster IR spectra with anharmonicity included. Accounting for mode coupling may permit higher confidence band assignments, and in turn clearer and more accurate interpretation of the enzymatic states being observed in FTIR spectroscopy. The free energies along the PT pathways will be further refined based on the *pKa* calculation results.

- We will continue to investigate the binding free energies for H_2ase with SWNTs and bulk carbon surfaces using MD simulation techniques, and develop these models to predict binding modes, orientations and ET processes.

- We will characterize native algal [FeFe]-H_2ase prepared under different redox states using IR and EPR spectroscopy to correlate the vibrational and spin properties of the H-cluster under inhibited and catalytically relevant states.

- Single-molecule electrochemistry of immobilized H_2ase on Au-electrodes will continue to be developed to characterize H_2ases lacking accessory iron-sulfur clusters functioning in electron-transfer in order to learn more about the conductive path through the protein.

- We will continue to characterize the assembly, charge-transfer, catalytic and inhibition processes in both H_2ase-CdS and H_2ase-CdTe bio-hybrid complexes under photoexcitation using steady-state and time-resolved techniques. These efforts will address the effects of ligands and nanoparticle photophysics on charge-transfer and catalytic rates.

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Membrane Events in Post-translational Targeting of Light Harvesting Chlorophyll-binding Proteins by Chloroplast Signal Recognition Particle

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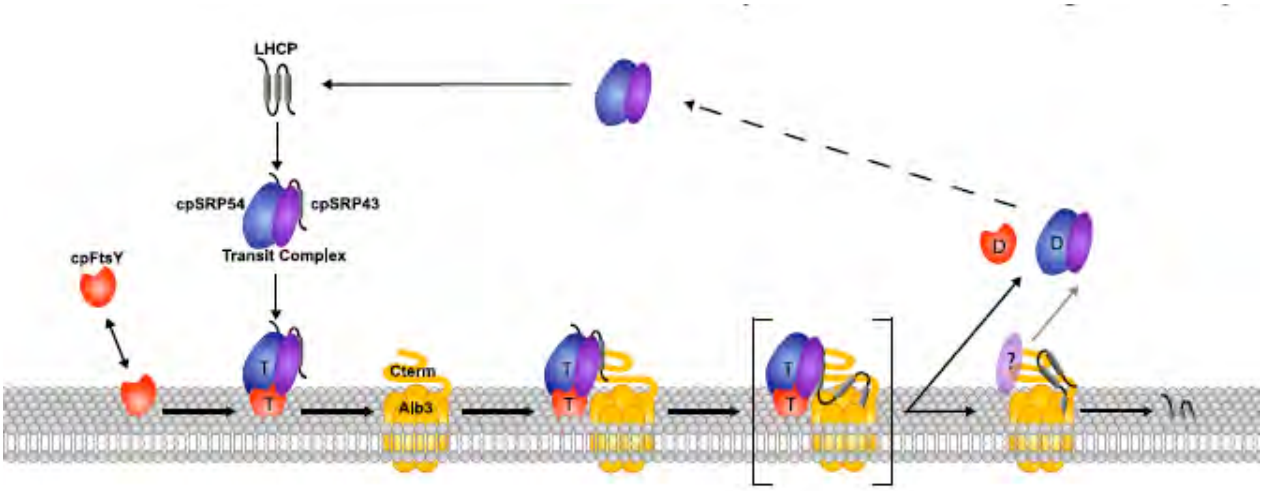
Overall research goals: The research objectives are to understand the structure based mechanism by which the chloroplast signal recognition particle (cpSRP) and its receptor (cpFtsY) direct light harvesting chlorophyll binding proteins (the LHCs) to Albino3 (Alb3), an insertase required for LHC insertion into chloroplast thylakoid membranes. **We hypothesize that binding of cpSRP43 subunit to the C-terminus of Alb3 is the primary interaction responsible for directing a cpSRP/LHC/cpFtsY membrane-associated targeting complex to Alb3. We also hypothesize that cpSRP-Alb3 binding serves to trigger LHC release from cpSRP and initiate disassembly of the targeting complex for cpSRP/cpFtsY recycling.** We are testing this hypothesis by (1) Identifying loss-of-function mutations in Alb3 C-terminal peptide that eliminate its ability to (a) bind cpSRP43 and (b) stimulate GTP hydrolysis by cpSRP/cpFtsY; (2) Using NMR to determine the structure of Alb3 Cterm alone and bound to the Ank-CD2 domain of cpSRP43; and (3) examining how interaction between cpSRP43 and the C-terminus of the Alb3 insertase promotes unidirectional transfer of LHC targeting substrates from cpSRP to Alb3.

Completion of the proposed studies will provide structural and functional details that are missing in our understanding of membrane events required for LHC insertion. Additionally, our results will set the stage for future studies aimed at addressing the potential for post- and co-translational targeting mechanisms to converge at Alb3 through binding of cpSRP43 (post-translational) or chloroplast ribosomes (co-translational) to the Alb3 C-terminus. It is also expected that the proposed work will have a general impact on understanding insertase activity of Alb3 homologues in bacteria, which are required for biogenesis of energy generating complexes in bacteria, which are being developed for biofuel production.

Significant achievements 2010-2011: Our data show that efficient association of Alb3 with cpSRP/cpFtsY relies on cpSRP43, a novel cpSRP subunit thought previously to function primarily in the soluble phase of protein targeting through its ability to bind LHCP and the GTPase subunit cpSRP54. The role of cpSRP43 in promoting association between Alb3 and soluble targeting components relies on high affinity of cpSRP43 for Alb3. The Ankyrin-repeat region of cpSRP43 interacts directly with the stroma-exposed Alb3 C-terminus. Alb3-Cterm peptide, which binds cpSRP43 (or the Ankyrin domain of cpSRP43) with nM affinity, promotes GTP hydrolysis by cpSRP54/cpFtsY *only* in the presence of cpSRP43. Importantly, mutational analysis of cpSRP43 demonstrates that its cpSRP54-binding domain (chromodomain 2) is needed to observe GTP hydrolysis in the presence of Alb3-Cterm peptide. In addition, Alb3-Cterm peptide appears to promote LHCP release from cpSRP. Taken together, our data supports a model (see below) in which cpSRP43/Alb3-Cterm interaction at the membrane is directly involved in release of LHC from cpSRP and initiates GTP hydrolysis by cpSRP/cpFtsY, an event that is required to recycle cpSRP and its receptor for subsequent rounds of targeting.

An unanticipated finding from our studies was the discovery that protein-protein interactions measured by Isothemal titration calorimetry (ITC) are sensitive to glycerol, a common additive in recombinant protein preparations. The sensitivity could be seen at glycerol concentrations as low as 5%. Even when present in both ligand preparations at equal concentrations, glycerol produces a heat of dilution sufficient to mask high affinity interactions. This is consistent with the mis-

identification by Falk et al. (Falk, S., Ravaud, S., Koch, J., and Sinning, I. (2010) J. Biol. Chem. 285, 5954–5962) of cpSRP43's chromodomain 2 as the Alb3 C-term binding domain within cpSRP43, an interaction that takes place with low (mM) affinity. We also showed the same buffers used by Falk et al. were not appropriate to support LHCP integration into isolated thylakoids. The affect of glycerol should be of general importance to all investigators using ITC to characterize protein-protein interactions in their studies and is illustrated in each of the two publications published in the Journal of Biological Chemistry.



Model for the role of cpSRP43 in targeting LHC polypeptides to the Alb3 insertase.

Science objectives for 2011-2012:

1. With the HSQC spectrum of cpSRP43 Ankyrin domains nearly complete, we will be able to use peak shift perturbation assays to identify amino acids in the cpSRP43 Ankyrin-repeat domain that interact with residues of the Alb3 C-terminus. cpSRP43 mutants designed from these studies are anticipated to lack Alb3 binding and lack the ability to release LHCs from cpSRP.

2. Using ITC and HSQC data from NMR studies, we will examine Alb3-Cterm mutants for loss of binding to cpSRP43 and loss of GTPase stimulation. These Alb3-Cterm mutants, as recombinant proteins, are also expected to lack the ability to stimulate LHC release from cpSRP.

References to work supported by this project 2010-2011:

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Magnetic Resonance, DFT and Electrochemical Study of Carotenoid Radicals

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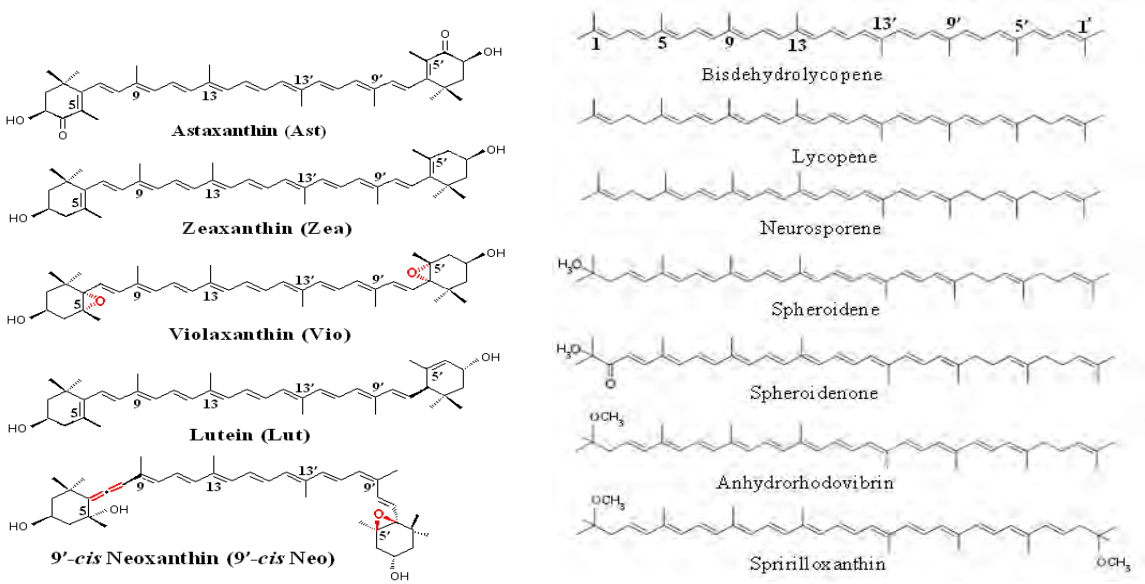
Overall research goals: The research objectives are to study how the host lattice affects the electron donor and charge transfer properties of carotenoids using electron paramagnetic resonance (EPR) spectroscopy, coupled with Density Functional Theory (DFT), optical and electrochemical measurements of the radical species formed. Such information is needed before optimum use of them as antennas, photoprotectors, antioxidants and radical scavengers can be made in artificial photosynthetic systems.

Significant achievements 2009 – 2011: Water soluble complexes of carotenoids were formed by complexing carotenoids with the natural polysaccharide arabinogalactan (PA). These complexes showed enhanced photostability by a factor of 10 in water solution, a decrease by a factor of 20 in the reactivity toward metal ions (Fe^{3+}) and reactive oxygen species in solution. Canthaxanthin radical cations formed in these complexes are stable for ~10 days at room temperature.

Photoirradiation of TiO_2 nanoparticles by visible light in the presence of the water-soluble polysaccharide (PA) complexes of carotenoid β-carotene leads to enhanced yield of the reactive hydroxyl (OH) radicals. The observed enhancement of the photocatalytic efficiency for carotenoid complexes, as measured by the quantum yield of the desired spin adducts, arises from the decrease in the rate constant for the back electron transfer to the carotenoid radical cation.

The carotenoid astaxanthin forms novel metal ion complexes with Ca^{2+}, Zn^{2+} and Fe^{2+}. MS and NMR measurements indicate that the two oxygen atoms on the terminal cyclohexene rings of astaxanthin adjacent to substituted hydroxyl group chelate the metal to form 1:1 complexes with Ca^{2+} and Zn^{2+} at low salt concentrations <0.2 mM. Pulsed EPR measurements were carried out on UV-produced radicals of astaxanthin supported on silica-alumina, MCM-41 or Ti-MCM-41.



Neutral radical intermediates formed upon catalytic or photoxidation of carotenoids inside MCM-41 molecular sieves showed that allene bond at the unprimed end and the epoxy group at the

prime end prevent proton loss at the C5 and C5' methyl groups by reducing the conjugation length so crucial for neutral radical stability. This is in agreement with the non-quenching ability of violaxanthin and 9 '-cis-neoxanthin. The proton loss from linear open chain carotenoids is dependent on the resulting conjugation length – the longer the length, the more likely the formation of a neutral radical.

Science objectives for 2011 – 2012: Electrochemical measurements of mono- and di-esters of astaxanthin and its salts will be carried out to establish the antioxidant activity and its relation to scavenging properties of peroxyl radicals as well as EPR studies of electron transfer reactions of carotenoids in silica alumina and mesoporous sieves. Resonance Raman spectra will be measured of carotenoid radical cations and neutral radicals produced electrochemically; a task that has proven difficult to achieve in past attempts.

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Primary Isomerization Dynamics of Phytochromes and Cyanobacteriochromes

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Overall Research Goals. Cyanobacteria are oxygenic, nitrogen-fixing photosynthetic organisms that hold great potential for biofuel production. Since they rely on light as an energy source, these organisms exploit biliprotein-based photosensory proteins to optimize light capture and photosynthesis. Hence, understanding how these photoreceptors operate should facilitate engineering of cyanobacteria as sustainable energy sources. Our studies were undertaken to address the primary photodynamics of several recombinant biliprotein-based systems: (1) Cph1, a red/far-red phytochrome from the cyanobacterium *Synechocystis* sp. PCC 6803; (2) the red/green photoswitchable cyanobacteriochrome (CBCR) GAF4 from the *NpR6012* locus of *Nostoc punctiforme ATCC 21933* (NpR6012g4); (3) the green/red photoswitchable CBCR of the complementary chromatic acclimation sensor RcaE from the cyanobacterium *Fremyella diplosiphon;* (4) the blue/green photoswitchable CBCR Tlr0924 from the thermophilic cyanobacterium *Thermosynechococcus elongatus;* and (5) the violet/orange CBCR NpF2164g3 from *Nostoc punctiforme ATCC 21933.*

Significant Achievements 2009-2011. Femtosecond dynamics of Cph1 and NpR6012g4 were resolved with incoherent multi-pulse pump-dump-probe spectroscopy. For the forward photoswitching of Cph1 (red to far-red), two photoisomerization models have been proposed: one in which isomerization is completed on the excited-state surface with subsequent de-excitation, and another in which isomerization and de-excitation occur simultaneously via a conical intersection. We observed dump-induced reduction of the Lumi-R photoproduct yield at early time points. This demonstrates that photoproduct formation is not occurring on the excited state; rather, excited-state quenching occurs before full isomerization is completed. Moreover, no signatures of equilibrium between the observed excited-state intermediates were resolved. These results support the conical intersection model for Cph1, with a quantum yield of 10-15% for Lumi-R formation. We also characterized the forward (red to green) photodynamics of NpR6012g4 using pump-dump-probe methods. Although the kinetic timescales for the NpR6012g4 photodynamics are significantly slower than those of Cph1, we demonstrate that forward isomerization to the Lumi-R photoproduct has a much higher yield (≥30%). This high yield reflects both ground- and excited-state evolution.

Reverse kinetics for NpR6012g4 (green to red) and both directions for RcaE, Tlr0924, and NpF2164g3 were examined using pump-probe spectroscopy with broadband detection. The

reverse reaction for NpR6012g4 exhibited even higher quantum yield. Two non-equilibrating ground state species were resolved with dual-wavelength interleaved pump pulses. These species rapidly equilibrate after excitation, indicating that the barrier to equilibration between these species is lowered on the excited-state surface. These results have important implications for the red/green photocycle.

Similar heterogeneity was observed with the forward (green to red) reaction for RcaE, although the RcaE quantum yields are much lower than those of NpR6012g4. In RcaE, through work performed by the Lagarias lab and their collaborators, we are able to interpret the heterogeneity of the green state as arising from two tautomers of a deprotonated phycocyanobilin chromophore, which becomes protonated in the red-absorbing photoproduct. RcaE exhibits much slower evolution than Cph1 or NpR6012g4, with the reverse reaction exhibiting excited-state signals at times in excess of 500 psec.

Tlr0924 also exhibits heterogeneity, which we are able to identify as arising from partial isomerization of phycocyanobilin to phycoviolobilin. The two bilin populations in Tlr0924 exhibit distinct rates for excited-state decay and photoproduct formation. In the forward direction, the stimulated emission band of Tlr0924 exhibits an exceptionally large Stokes shift of > 3000 cm^{-1}, consistent with a rubinoid species such as a C10 Cys adduct (the dual-Cys model). Unambiguous stimulated emission signals could not be recorded in NpF2164g3 to date, with strong excited-state absorbance overlapping the relevant region of the spectra. The primary photoproducts identified in Tlr0924 and NpF2164g3 are in good agreement with those proposed by the Lagarias lab in their published descriptions of those proteins. These analyses provide new insight into the rich dynamics of photo-induced bilin isomerization in the extended phytochrome photosensor family.

Science objectives for the next year: We will seek to complete and publish the analyses listed above. We will also use NpR6012g4 as a test-bed for two new systems constructed in the lab: a longtime system permitting us to examine spectral evolution on a timescale of nsec to msec with ultrafast pump pulses, and a pump-probe setup allowing us to visualize mid-IR signals with resolution better than 0.1 cm^{-1}. We will continue to examine other CBCRs of interest by pump-probe spectroscopy, using other techniques at need.

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Unraveling the Regulation of Terpenoid Oil and Oleoresin Biosynthesis for the Development of Biocrude Feedstocks

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Overall research goals: Terpenoid oils and oleoresins are characterized by a high volumetric energy density and high degree of reduction, and are thus viable biocrude feedstocks for liquid transportation fuels comparable to diesel and kerosene. A long-term goal of my laboratory is to develop quantitative conceptual models of terpenoid oil/oleoresin biosynthesis in plants that can guide researchers in developing superior terpenoid biofuel/biomaterial feedstocks. However, because these pathways are confined to specialized epithelial gland cells, which are scarce and not readily accessible to experimentation, pathway regulation is thus far poorly understood. We have made considerable advances in developing protocols for the isolation and biochemical characterization of metabolism in various types of epithelial gland cells, progress that has put us in a unique position to experimentally address gaps in our understanding of the regulation of terpenoid oil and oleoresin biosynthesis. We are currently using three experimental model systems to evaluate terpenoid pathway regulation: (1) peppermint essential oil glandular trichomes, (2) Citrus peel essential oil cavities, and (3) pine resin ducts. The proposed activities are designed to address critical gaps in our knowledge of how plants store energy in the form of terpenoid oils and oleoresins. This research will directly impact DoE's interests in enhanced biofuel/biomaterial production and in biomimetic strategies for assembly and storage of specialized materials.

Significant achievements 2009-2011:

- We developed a second-generation kinetic mathematical model of peppermint oil gland monoterpene biosynthesis. We are now able to accurate describe biochemical, developmental, environmental and genotypic determinants of essential oil composition and yield.

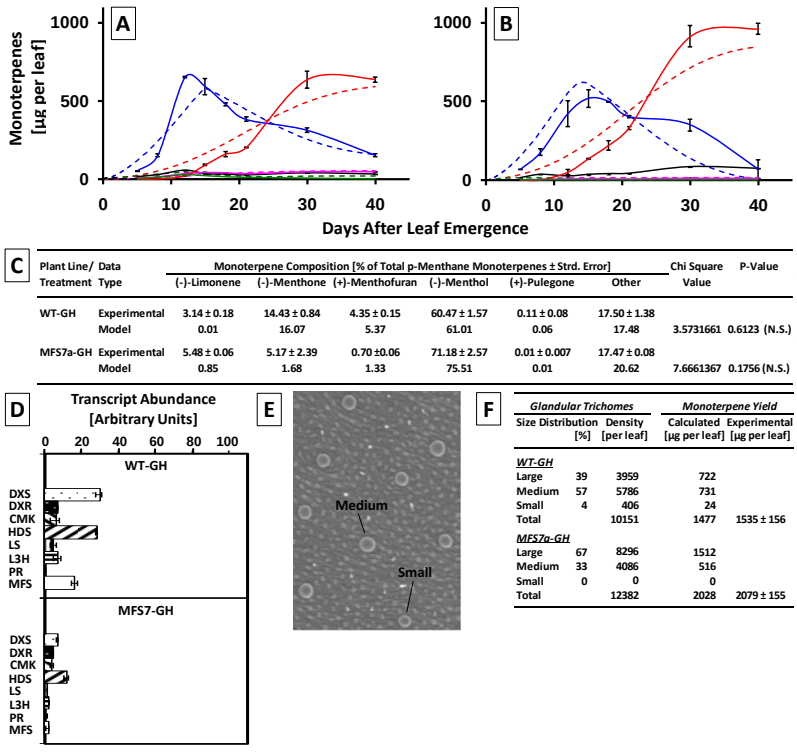
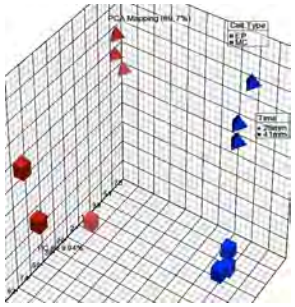


Fig 1. Comparisons of experimentally determined and simulated monoterpene profiles of (**A**) greenhouse-grown wild-type (WT-GH) and (**B**) a transgenic antisense line (MFS7a-GH), and Chi Square statistical analysis of goodnesss of fit at 40 d after leaf emergence (**C**). The different colors represent different mono-terpenes. Panel **D** depicts expression patterns of genes involved in peppermint monoterpene biosynthesis at 15 d after leaf emergence, as determined by qPCR. Panel **E** shows a microscopic image of the surface of a peppermint leaf. Panel **F** summarizes data on glandular trichome density and size distribution, as well total essential oil yield at 30 d after leaf emergence.

- Building on our mathematical modeling data we developed metabolic engineering approaches, which led to the successful generation of various transgenic peppermint lines with favourable oil composition and dramatically increased oil yields.
- We determined the chemical composition of Citrus essential oil obtained using a novel micropipetting technique, thus allowing us to evaluate artefacts introduced by the cold pressing process.
- We determined the regulatory characteristics of the monoterpene biosynthetic pathway at the transcriptional (Fig. 2), posttranslational and metabolite accumulation level and were able to correlate these data sets with microscopic measurements of oil cavity volumes. This now enables us to develop quantitative mathematical models of the oil filling process.

A



PCA analysis:

Microarray analysis using Partek:

***rma* for background correction**
(quantile normalization, median polish, summarization, log2 transformation)

↓

ANOVA
for differential expression analysis

↓

Multiple testings correction
(Fold change>2, p-value<0.01)

After filtering:
- 5788 in "28EC vs 28PC" list;
  2547 up-regulated in 28EC.
- 4505 in "41EC vs 41PC" list;
  2091 up-regulated in 41EC.

B

Column ID	Annotations	Fold change	
		28EC vs 28PC	41EC vs 41PC
Cit.10053.1.S1_at	1-Deoxy-D-xylulose 5-phosphate synthase	49.532	12.021
Cit.4968.1.S1_s_at	1-Deoxy-D-xylulose-5-phosphate reductoisomerase	5.384	3.434
Cit.14478.1.S1_at	2-C-Methyl-D-erythritol 4-phosphate cytidylyltransferase	5.452	4.375
Cit.3446.1.S1_s_at	4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase	7.803	5.097
Cit.1430.1.S1_s_at	2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase	5.645	4.046
Cit.985.1.S1_at	4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	8.931	4.878
Cit.17714.1.S1_at	4-Hydroxy-3-methylbut-2-en-1-yl diphosphate reductase	29.980	8.382
Cit.9842.1.S1_s_at	Isopentenyl pyrophosphate isomerase	3.430	2.800
Cit.9964.1.S1_s_at	(+)-Limonene synthase	9.540	3.194
Cit.2694.1.S1_at	beta-Pinene synthase	29.767	17.103
Cit.2936.1.S1_at	Terpene synthase/cyclase family protein	53.196	15.623
Cit.17284.1.S1_at	Terpene synthase/cyclase family protein	49.647	14.095
Cit.29748.1.S1_s_at	Terpene synthase/cyclase family protein	27.134	17.274
Cit.18744.1.S1_at	Terpene synthase/cyclase family protein	5.549	1.335

Fig. 2. Grapefruit peel cell-type specific microarray data. **A**, principal component analysis comparing gene expression patterns of ECs (epithelial gland cell synthesizing essential oil; red) and PCs (mesophyll parenchyma cells not involved in oil biosynthesis; blue). Squares, 28 mm fruit diameter; triangles, 42 mm fruit diameter. **B**, upregulation of genes putatively involved in essential oil biosynthesis in ECs.

- Using microscopic images and 3-dimensional reconstruction models, we determined the distribution and storage capacity of the resin duct system of entire loblolly pines. This now allows us to evaluate the determinants of resin biosynthesis in pine.

Science objectives for 2011-2012:

- Develop a genome-scale model of peppermint essential oil biosynthesis. These modeling efforts will help us to unravel the preferred precursor pathways generating oil precursors. We will also attempt to include active transport (sugar uptake and intermediate exchange) and cofactor status in models.
- Develop first generation quantitative model of essential oil biosynthesis in Citrus based on an integrative analysis of pathway regulation and oil accumulation capacity.
- Develop methods for the isolation of epithelial cells from loblolly pine resin ducts and subsequent mRNA extraction for RNAseq experiments. This will allow us to assess the biosynthetic capabilities of these specialized cell types.

References to work supported by this project 2009-2011:

1. Lange B.M., Mahmoud S.S., Wildung M.R., Turner G.W. Davis E.M., Lange I., Baker R.C., Boydston R. Croteau R.B. (2011) Improving peppermint essential oil yield and composition by metabolic engineering. *Proc. Natl. Acad. Sci. USA*, in press.
2. Rios-Estepa R., Lange I., Lee J.M., Lange B.M.* (2010) Mathematical modeling-guided evaluation of biochemical, developmental, environmental and genotypic determinants of essential oil composition and yield in peppermint leaves. *Plant Physiol.* 152, 2105-2119 (COVER STORY).

Ironing Out Metabolic Wrinkles in Nutrient Deficient Chlamydomonas

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Overall research goals

Iron limits life because of poor bioavailability. Photosynthesis on earth occurs in an iron-deficient environment, yet our understanding of photosynthetic metabolism is based in a nutrient-supplemented laboratory situation. We have established 4 "stages" of iron nutrition – operationally defined on the basis of phenotype with respect to expression of iron assimilation pathways and light energy utilization. The long term goal of this project is the discovery of mechanisms governing the iron-nutrition dependent programming of chloroplast metabolism in this reference organism. Previously, we identified iron-nutrition responsive genes involved in iron uptake and homeostasis. For the present project period, we have proposed to 1) distinguish the functions of various iron transporters (IRT1, IRT2 and NRAMP4) in assimilation vs. distribution, 2) undertake reverse genetic analysis of a) *CGLD27*, which is conserved in both plants and diatoms and whose expression is impacted by iron nutrition in multiple plants, and b) *TEF22*, whose iron-responsive expression is dependent on demand for respiratory vs. photosynthetic metabolism, and 3) identify cis-regulatory elements on key iron-responsive genes.

Significant achievements 2009 - 2011

In the previous two years, we have discovered that multiple oxidative stress pathways are up-regulated in iron-deficient and iron-limited cells. This includes mechanisms for increasing plastid superoxide dismutase activity, ascorbate biosynthesis and recycling, and Vitamin E accumulation. Two mechanisms contribute to increased plastid SOD activity: transcriptional regulation of the *MSD3* gene encoding a plastid-localized enzyme that uses manganese as a redox cofactor and prioritized allocation of recycled iron (resulting from degradation of abundant plastid proteins like ferredoxin) to the product of the *FSD1* gene encoding plastid FeSOD. In the case of ascorbate metabolism, we identified a pathway for Vitamin C synthesis in algae and found that *VTC2* (encoding the committed step in that pathway) is regulated by multiple stress situations. We have completed a survey of the transcriptome and soluble proteome of iron- deficient and –limited cells, and have identified genes whose expression is impacted in multiple plant species.

Science objectives for the next 2 years

Functional analysis of genes with a conserved pattern of expression (e.g. *CGLD27*) is a priority for the next project period. We will assess the phenotype of loss of function mutants in both Chlamydomonas and Arabidopsis and deduce the biochemical role of the protein. Promoter analysis is in progress; the identification of iron response elements will be critical for establishing the mechanism of regulatory factors when they are identified in future project periods. Finally, although not proposed in the original application, we are initiating metabolite flux analyses in collaboration with Professor Ana Alonso at Ohio State University.

References to work supported by this project 2009 – 2011:

1. Terauchi, A.M., Lu, S.F., Zaffagno, M., Tappa, S., Hirasawa, M., Tripathy, J.N., Knaff, D.B., Farmer, P.J., Lemaire, S., Hase, T., Merchant, S.S. (2009) Pattern of Expression and Substrate Specificity of Chlamydomonas Chloroplast Ferredoxins. J. Biol. Chem. 284:25867-25878.
2. Petroutsos, D., Terauchi, A.M., Busch, A., Hirschmann, I., Merchant, S.S., Finazzi, G., Hippler, M. (2009) PGRL1 participates in iron-induced remodeling of the photosynthetic apparatus and in energy metabolism in *Chlamydomonas reinhardtii*. J. Biol. Chem. 284:32770-32781.
3. Hirasawa, M., Tripathy, J.N., Sommer, F., Somasundaram, R., Chung, J.-S., Nestander, M., Kruthiventi, M., Zabet-Moghaddam, M., Johnson, M.K., Merchant, S.S., Allen, J.P., Knaff, D.B. (2010) Expression and Enzymatic Properties of a Ferredoxin-Dependent Nitrite Reductase from *Chlamydomonas reinhardtii*. Photosyn. Res. 103: 67-77.
4. Terauchi, A., Peers, G., Kobayashi, M., Niyogi, K.K., Merchant, S.S. (2010) Trophic status of *Chlamydomonas* influences the impact of Fe-deficiency on photosynthesis. Photosyn. Res.105:39-49.
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6. Kropat, J., Hong-Hermesdorf, A., Casero, D., Ent, P., Pellegrini, M., Merchant, S.S., Malasarn, D. (2011) A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. Plant J. 66:770-80.
7. Page, M.D., Allen, M.D., Urzica, E., Kropat, J., Karpowicz, S., Merchant, S.S. Two mechanisms for maintenance of superoxide dismutase capacity in iron deficient Chlamydomonas, in revision.
8. Lee, Y.-J., Huang, X., Kropat, J., Henras, A., Merchant, S.S., Dickson, R.C., Chanfreau, G.F. (submitted) Sphingolipid signaling mediates iron toxicity.

Two-dimensional Electronic Spectroscopy of the Photosystem II Reaction Center

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Overall research goals: The research objectives are to use two dimensional electronic spectroscopy (2DES) to elucidate the electronic structure of the photosystem II reaction center (PSII RC) and to map the energy and charge transfer pathways that produce the primary charge separation in this system. Towards this broad goal, we are developing new spectroscopic methods aimed at distinguishing the spectral signatures of energy and charge transfer. We are applying these methods to the PSII RC in several different preparations and from different organisms. In the previous funding period we developed a simple experimental method for performing 2DES based on pulse-shaping and we studied the D1-D2-cytb559 PSII RC from spinach [1]. We are currently testing existing excitonic models of the PSII RC against our 2DES data. We also demonstrated broadband 2DES, enabling the study of the ion band signatures during charge separation [4]. We are currently applying this method to the D1-D2-cytb559 PSII RC. Our future experimental methods development efforts include (1) combining 2DES with a coherent Raman probe to provide better assignment of spectral changes due to charge separation; (2) combining 2DES with Stark spectroscopy to better reveal charge transfer states and their participation in the energy and charge transfer processes in the PSII RC. We plan to apply these new methods to the isolated D1-D2-cytb559 PSII RC from spinach, as well as intact PSII core samples. We will also study preparations from Acaryochloris marina, a cyanobacterial system that employs primarily chlorophyll D, reducing the spectral overlap with other PSII RC pigments and facilitating the assignment of time-dependent spectral changes to specific energy and charge transfer processes.

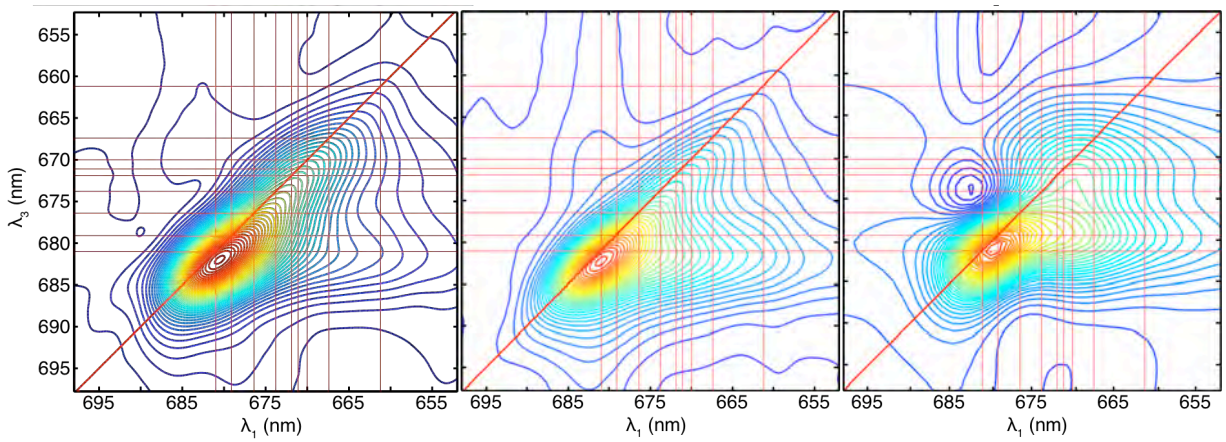

Figure 1. Left Panel: 2D spectrum of the D1-D2-cytb559 PSII RC at 77K at a waiting time of T=28 fs. Cross-peaks indicate excitonic coupling in the system. Middle: 2D spectrum at T=215 fs. The larger cross-peak below the diagonal indicates that considerable energy transfer has occurred by this time. Right: simulated 2D spectrum at T=215 fs, based on a current excitonic model of the D1D2 RC. The model matches some aspects of the 2D lineshapes but underestimates disorder and the strength of the system-bath coupling. We also find that using modified Redfield theory produces a poor match to the observed kinetics of energy and charge transfer.

Significant achievements 2009-2011:

- The highest time and frequency resolution measurements to date of the spectroscopic changes associated with energy and charge transfer in the PSII RC [1]
- The first direct observation of excitonic coupling in the PSII RC [1]
- A novel analysis, termed "2D decay associated spectra" (2D DAS) to extract 2D spectral features associated with the different kinetic processes in 2DES data [1]
- Investigation of the effect of chirp on 2D spectra [2]
- Demonstration of 2DES with a continuum probe [4] and chirp correction [3]
- Simulations of 2D spectra of the D1D2 RC based on current exciton models

Science objectives for 2011-2012:

- Isolation of PSII RC complexes from Acaryochloris marina
- 2DES studies of the D1D2 RC with continuum and coherent Raman probes to monitor charge separation kinetics
- Development of 2D electronic Stark spectroscopy for elucidation of charge transfer processes

References to work supported by this project 2009 - present:

1. J. A. Myers, K. L. M. Lewis, F. D. Fuller, P. F. Tekavec, C. F. Yocum and J. P. Ogilvie, Two-dimensional electronic spectroscopy of the D1-D2-cyt 559 photosystem II reaction center Journal of Physical Chemistry Letters, (2010) 1, 19 2774-2780.
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Enhancement of Photoassimilate Utilization by Manipulation of ADPglucose Pyrophosphorylase and Phosphorylase

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Overall research goals: ADPglucose pyrophosphorylase (AGPase) and the plastidial phosphorylase (Pho1) are two regulatory enzymes whose catalytic activities are essential for starch granule synthesis. Pho1 is essential for optimal formation of the pre-starch granule while conversion of the pre-starch granule to the mature form is dependent on AGPase, which produces ADPglucose, the substrate used by starch synthases. Although considerable information is available on the *in vitro* catalytic and regulatory properties of the AGPase enzyme activity from a wide variety of plant sources, much less is known about the structure-function relationships of this enzyme at the molecular level and the role and importance of this enzyme in governing plant productivity. Specific questions that remain unanswered are: why is the higher plant AGPase enzyme composed of a pair of large subunits (LSs) and a pair of small subunits (SSs) and what are the exact roles of each of these subunit types in enzyme catalysis and allosteric regulation? What is the exact contribution of this enzyme in controlling starch metabolism in photosynthetic and sink organs tissues and does starch metabolism influence photosynthetic capacity and, in turn, plant productivity? During the past funding period, this project was extended to starch phosphorylase Pho1 as genetic studies provided direct support for a biosynthetic role for this enzyme during the initial phase of pre-starch formation. Although Pho1 catalyzes a reversible reaction enabling it to extend or degrade starch, studies supported by our present DoE grant showed that the kinetic properties of this enzyme strongly favor glucan synthesis. Our research goals for this funding period are to (1) Determine the substrate and effector binding properties of AGPase homotetrameric LS and SS forms and heterotetrameric LS-SS forms; (2) Determine the X-ray crystal structures of potato AGPase heterotetramer and rice type L α–glucan phosphorylase Pho1; (3) Manipulate carbon flux and increase plant productivity in Poplar (*Populus trichocarpa* by the introduction and expression of up-regulatory AGPases; (4) Elucidate the control of Pho1 by post-translational modifications and by interacting proteins and (5) Determine the role of the unique L80 peptide of Pho1 in starch synthesis. Results from these biochemical and physiological studies will increase our fundamental understanding on how these important starch regulatory enzymes operate at the molecular level and *in planta*, which will aid in efforts to increase the utilization of plants as a renewable source of energy.

Significant achievements 2009-2011: Co-assembly of the LS activated forms, UpReg1 and UpReg2, with the SSs from potato, *Perilla*, and lettuce generated AGPase enzymes with enhanced sensitivity to 3-PGA activation and enhanced resistance to Pi inhibition. Transgenic lettuce expressing UpReg1 showed larger aerial biomass, suggesting that increased leaf starch metabolism resulted in higher photosynthetic capacity and, in turn, increased vegetative growth. This relationship between leaf starch, CO_2 assimilation and plant growth was confirmed in Arabidopsis and rice.

Studies were completed on assessing the major metabolites in transgenic developing rice seeds expressing the AGPase coded by the *Escherichia coli glgC*. As expected, ADPglc levels were elevated and seed weights increased although the % increases were substantially higher for AGPase levels than for seed weights. In addition to ADPglc, the levels of Glc 1-P, Glc 6-P, and UDPglc were also elevated in the transgenic plants. These results indicate that ADPglc formation is no longer a limiting step in starch synthesis and that a downstream process (ADPglc transport or utilization of ADPglc by starch synthases) constrains maximum carbon flow into starch. Present efforts are directed at elevating ADPglucose transport rates in these transgenic rice lines.

Rice mutants displaying severely shrunken seeds due to lack of starch synthesis have been found to contain missense mutations (T139I or A171V) in the AGPase large L2 subunit. Kinetic analysis of the purified recombinant AGPase mutants showed substantial decreases in sensitivity to 3-PGA and catalytic efficiencies. These results confirm that the rice seed AGPase activity is subjected to allosteric regulation and that the LS is required for this property.

Rice mutants for α-glucan phosphorylase (Pho1) were identified which showed severe starch deficiency at low temperature. Analysis of the kinetic properties of the recombinant rice phosphorylase Pho1 indicated that it favored the synthetic reaction as evidenced by the net incorporation of ^{14}C-Glc 1-P into α-glucan. This result

provides evidence that Pho1 may contribute to one or more early events involved in starch synthesis. The recombinant enzyme showed significantly high catalytic activity even at low temperatures (18°C), suggesting active involvement of this enzyme in starch metabolism at low temperature. Kinetic analysis of the recombinant Pho1 lacking the L80 peptide revealed that this highly charged domain is not required for catalysis.

Pho1 proteins were detected immunochemically at similar levels in all stages of seed maturation and in germinating seed, but not in root or leaf tissue, supporting the role of the enzyme for starch metabolism in rice seed. In order to facilitate the identification of the Pho-interacting proteins in rice endosperm, a HaloTag was fused to the N-termini of Pho1. The HaloTag::Pho1 protein was effectively soluble and purified to near homogeneity and will be used to identify Pho1-interacting protein(s).

In silico analysis of the poplar tree genome revealed that gene duplication events generated six LS and three SS genes. With the exception of one LS gene, the cDNAs for these AGPase subunits have been successfully cloned and expressed in bacteria. Very little, if any, glycogen accumulation was detected when the poplar AGPase subunits were co-expressed in bacteria, although a moderate level of glycogen was detected when the potato LS was co-expressed with the poplar AGPase S1. Further biochemical analysis of the poplar recombinant AGPase enzymes is currently underway.

Science objectives for 2011-2012:

- Characterize the effector binding properties of homotetrameric AGPase LS and SS forms as well as various AGPase heterotetrameric forms.
- Express and characterize the kinetic properties of plastid-localized phosphorylase and identify the ADPglucose effector binding site.
- Identify Pho1-interacting protein(s) by utilizing HaloTag technology
- Generate the homozygous transgenic rice plants over-expressing the BT1, the ADPglucose transporter, and assess its effect on starch synthesis.
- Generate transgenic poplar trees expressing up-regulatory AGPase large or small subunit variants in a tissue-specific manner.

References to work supported by this project 2009-2011:

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Role of Glutathione in Protection of the Photosynthetic Apparatus in Cyanobacteria

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Overall Research Goals: The objective of this project is to determine the mechanisms of newly identified thylakoid lumen localized redox proteins (redoxins) as well as other redox components in photoprotection and repair of the photosynthetic apparatus in cyanobacteria. There are two specific aims in this proposal: (1) To determine the mechanism for photoprotection by lumen localized redoxin proteins, and (2) To define other lumenal factors involved in such a protection and repair process.

Significant achievements 2009-2011: Our initial studies showed that redoxin proteins localized in the thylakoids of the cyanobacterium *Synechocystis* sp. PCC 6803 serve as important determinants in the protection of the photosynthetic apparatus under redox stress conditions. However, the lumenal redox partners of such redox partners are not known. An obvious candidate is glutathione, a non-ribosomal thiol tripeptide that has been shown to be critical for many processes in plants. Much less is known about the roles of glutathione in cyanobacteria. An understanding of glutathione metabolism in cyanobacteria is expected to provide novel insight into the evolution of the elaborate and extensive pathways that utilize glutathione in photosynthetic organisms. To investigate the function of glutathione in cyanobacteria, we generated deletion mutants of glutamate-cysteine ligase (*gshA*) and glutathione synthetase (*gshB*) in *Synechocystis* 6803. Complete segregation of the Δ*gshA* mutation was not achieved, suggesting that GshA activity is essential for growth. In contrast, fully segregated Δ*gshB* mutants were isolated and characterized. The Δ*gshB* strain lacks glutathione (GSH), but instead accumulates the precursor compound, γ-glutamylcysteine (γ-EC). The Δ*gshB* strain grows slower than the wild type (WT) strain under favorable conditions, and exhibits extremely reduced growth or death when subjected to conditions promoting oxidative stress. Furthermore, we analyzed thiol contents in WT and the Δ*gshB* mutant after subjecting the strains to multiple environmental and redox perturbations. We found that conditions promoting growth stimulate glutathione biosynthesis. We also determined that cellular GSH and γ-EC content decline following exposure to dark, blue light and during photoheterotrophic growth. Moreover, a rapid depletion of GSH and γ-EC is observed in WT and the Δ*gshB* strain, respectively, when cells are starved for nitrate or sulfate. Finally, compared to the WT strain, the photosynthetic apparatus of the Δ*gshB* mutant was more vulnerable to redox stress.

Our recent studies have shown that a significant amount of glutathione is present the thylakoid lumen of *Synechocystis* 6803. The role of this pool of glutathione in the protection of the photosynthetic apparatus during high redox stress will be discussed.

Science objectives for 2011-2012:

- We will investigate the role of lumenal glutathione in the protection of Photosystem I (PSI). Initial studies have indicated that in the Δ*gshB* mutant, PSI is highly susceptible to redox stress.
- We will examine the mechanism of transport of glutathione from the cytoplasm to the thylakoid lumen. Glutathione is synthesized in the cytoplasm, and little is known about

how it is transported across the thylakoid membrane. In particular, we will identify the transporter protein(s) involved in glutathione transport to the lumen.

References to work supported by this project 2009-2011:

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2. Singh, A. K., Elvitigala, T., Cameron, J. C., Ghosh, B. K., Bhattacharyya-Pakrasi, M. and Pakrasi, H. B. (2010) Integrative Analysis of Large Scale Expression Profiles Reveals Core Transcriptional Response and Coordination between Multiple Cellular Processes in a Cyanobacterium. BMC Systems Biol., 4:105
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Dynamic Antagonism Between Phytochromes and PIF-family bHLH Factors Generates Selective Reciprocal Responses During Deetiolation and Shade-avoidance in a Rapidly Light-responsive Transcriptional Network

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Overall research goals:
The long-term goal of this program is to define the molecular mechanisms by which the phytochrome (phy) family of sensory photoreceptors perceive and transduce informational light signals to photoresponsive genes in regulating plant growth and development. The central goal of this project is to define the mechanisms by which the phys induce and modulate the Shade-Avoidance Response (SAR) in green plants. Toward this goal, the specific aims for this funding period are: (1) To delineate the biological functions of the multiple known Phy-Interacting bHLH transcription Factor (PIF)-family members in controlling the SAR, including definition of the scope of functional overlap or redundancy between family members in this process. (2) To identify potential target genes of the PIF transcription factors under shade avoidance conditions, using genome-wide expression profiling to identify PIF-regulated early-response genes and chromatin immunoprecipitation (ChIP) procedures to identify promoters that are potential direct targets of these phy signaling partners. We are using two primary general strategies to approach these objectives: (1) Genetic approaches, including structured mutational analysis of the potential roles and interactions among the known PIF proteins in the SAR; and (2) Genomic approaches, including integrated genome-wide expression profiling and ChIP analyses aimed at identifying direct, primary targets of the PIF-bHLH transcription factors in the phy-regulated transcriptional network.

Significant achievements 2010-2011:
Plants respond to light signals informing them of imposed or impending vegetative shade, via the phytochrome (phy) photoreceptor system, by adaptive changes in growth and development, collectively termed the Shade Avoidance Response (SAR). To examine the roles of the PIF1, 3, 4 and 5 bHLH factors in transducing this perceived information to the transcriptional network, we compared the genome-wide transcription profiles of wild-type and quadruple *pif* (*pifq*) mutants in response to shade. The data identify a subset of genes, enriched in transcription-factor-encoding loci, that respond rapidly (within 1 h), in a PIF-dependent manner, to the shade signal, and that contain promoter-located G-box-sequence motifs (CACGTG), known to be preferred PIF binding sites. These genes are thus potential direct targets of phy-PIF signaling that function in the primary transcriptional circuitry that controls downstream response elaboration. A second subset of PIF-dependent, early-response genes, lacking G-box motifs, are enriched for auxin-responsive loci, suggestive of being indirect targets of phy-PIF signaling involved in the rapid cell-expansion responses known to be induced by shade. A meta-analysis comparing deetiolation- and shade-responsive transcriptomes identifies a further subset of G-box-containing genes that reciprocally display rapid repression and induction in response to light and shade signals at the inception of deetiolation and shade-avoidance, respectively. Collectively, these data define a core set of transcriptional and hormonal (auxin, cytokinin)

processes that appear to be dynamically poised to react rapidly to changes in the light environment in response to perturbations in the mutually antagonistic regulatory activities of the phys and PIFs.

Science objectives for 2011-2012:

We will continue to pursue: (1) Genetic approaches toward defining the potential roles and interactions among the known PIF proteins in the SAR, using comparisons of shade-responsiveness among the higher-order *pif*-mutant combinations that we have generated; and (2) Genomic approaches, including integrated genome-wide expression profiling and ChIP analyses (given sufficient resources) aimed at identifying direct, primary targets of the PIF-bHLH transcription factors in the phy-regulated transcriptional network, and at defining any differential targeting of sectors or pathways within the shade-responsive network by the individual PIF-family members.

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Quail, P. H. Phytochromes. 2010. Current Biology, 20: R504-R507. PMCID: PMC2954054 [Available on 2011/6/1].

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Optimizing Rubisco Activase to Increase Photosynthesis at Warmer Temperatures

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Overall research goals: The objectives focus on improving Rubisco performance, particularly under heat stress by: introducing a more heat-stable activase behind constitutive (1) and heat-inducible (2) promoters; and (3) using both redox- (α-) and non-redox-regulated (β-) forms of activases. Objective (4) is to identify the mechanism for regulation of activase in species that express only the non-redox-regulated β-form of activase. Camelina, a bioenergy crop, will be used to evaluate the effectiveness of introducing an improved activase on photosynthetic performance (1-3). For (4), a redox-regulated form of activase will be introduced into tobacco to evaluate its effect on photosynthesis under control and elevated temperatures.

Significant achievements 2010-2011: To determine the potential for improving photosynthetic performance under heat stress, we measured the temperature response of photosynthesis, Rubisco activation (in vivo) and Rubisco activase activity. The results established a temperature optimum of ≤ 30°C for these parameters in camelina (Fig. 1). In growth experiments, a moderate heat stress of 35°C for 4 h per day reduced seed yield by more than 50%. Thus, photosynthetic performance and yield in camelina are acutely sensitive to inhibition by heat stress, and should show considerable improvement from the introduction of a more thermostable activase.

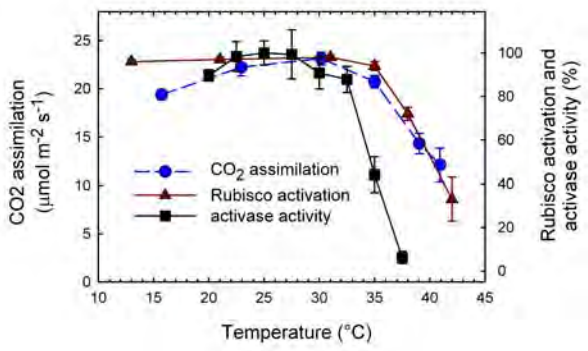

Fig. 1. *Effect of temperature on CO_2 assimilation, Rubisco activation and Rubisco activase activity in camelina*

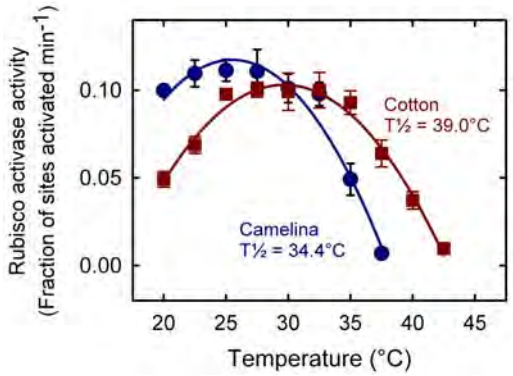

Fig. 2. *Temperature response of Rubisco activase activity in camelina and cotton extracts*

Assays with recombinant enzyme showed that cotton activase is capable of activating camelina Rubisco. Consequently, we are inserting the α- and β-forms of the activase gene from cotton into camelina to improve the thermotolerance of photosynthesis. Based on the temperature response of activity (Fig. 2), activase from cotton could provide a 5°C increase in thermal stability.

To understand activase regulation and the consequences for thermal stability, we have introduced a modified activase containing a redox-regulated element into tobacco, a species with only the non-redox regulated β-activase. T1 plantlets, developed in culture, are currently in soil and will soon be screened. In related experiments, photosynthetic induction was measured in tobacco (Nt), as well as in Arabidopsis (At) plants including At wild type (At wt) containing both α- and β-activase and

transgenics with only β-activase (At rwt43) or a chimeric β-activase composed of elements from both At and Nt (At_Nt). Unlike the other plants, photosynthetic induction in rwt43 was rapid regardless of the length of time in low light (Fig. 3). Also, activase activity in extracts of rwt43 was not inhibited by physiological ratios of ADP/ATP in contrast to At wt, chimeric At_Nt or tobacco activase (Fig. 4). Thus, although both lack redox-regulation, the β-activases in tobacco and Arabidopsis differ; tobacco β-activase is inhibited by ADP, whereas Arabidopsis β-activase is insensitive to ADP except when the α-form is present. The rwt43 plants that contain an activase that is insensitive to inhibition by ADP exhibited no lag in the induction of CO_2 fixation upon transition from low to high light, probably because Rubisco in these plants does not deactivate under low irradiance.

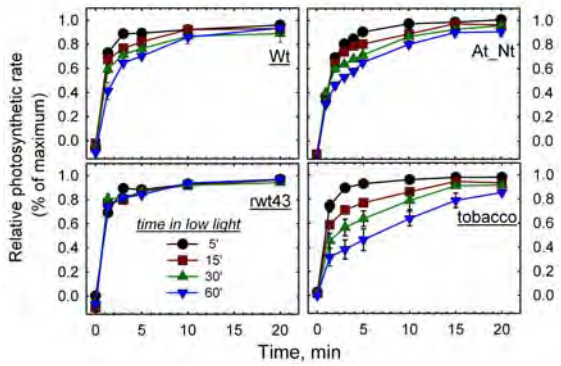

Fig. 3. *Induction of photosynthesis upon transition from low to high light after the indicated times at low light in plants containing different types of activase*

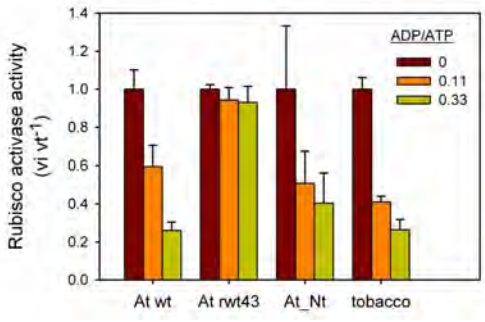

Fig. 4. *Effect of ADP/ATP ratios on Rubisco activase activity in plants containing various activases.*

Science objectives for 2011-2012:

- Construct transgenic camelina plants that express cotton activase (α- and β-forms) behind constitutive and heat-inducible promoters. Analyze the temperature response of photosynthesis and yield in the transformed plants.
- Analyze photosynthesis and Rubisco activation in transgenic tobacco plants containing a redox-regulated activase. Also, analyze the regulatory properties of the recombinant enzyme, particularly in response to redox modification by thioredoxin. Determine the structural basis for ADP inhibition of tobacco β-activase using directed mutagenesis of the recombinant enzyme.
- Replace the sensor-2 region of creosote activase with cotton to produce a more thermostable cotton activase for eventual introduction into camelina

References to work supported by this project 2010-2011:

1. C. Barta, A.M. Dunkle, R. M. Wachter, M.E. Salvucci**, "**Structural changes associated with the acute thermal instability of Rubisco activase," Arch. Biochem. Biophys. 499: 17-25 (2010)
2. A.E. Carmo-Silva, L. Marri, F. Sparla, M.E. Salvucci, "Isolation and compositional analysis of a CP12-associated complex of Calvin Cycle enzymes from *Nicotiana tabacum,"* Prot Pept Letts. 18, 618-624 (2011)
3. A.E. Carmo-Silva, and M.E. Salvucci**, "**The activity of Rubisco's molecular chaperone, Rubisco activase, in leaf extracts," Photosyn. Res., In press (2011)
4. J. N. Henderson, A. Kuriata, R. Fromme, M. E. Salvucci**,** R.M.Wachter, "Atomic resolution X-ray structure of the substrate recognition domain of higher plant Rubisco activase," J. Biol. Chem., In press (2011)

Amino Acid-Sensing Ion Channels in Plants

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Overall research goals: The goals of this project are to determine the molecular-level functions of glutamate receptor-like (GLR) proteins, presumed to be ligand-gated ion channels, and to elucidate their physiological or developmental roles in plants. Whether the GLR molecules function as amino acid-gated Ca^{2+} channels is the foremost molecular-level question to answer. We are approaching it with electrophysiological studies of heterologously expressed GLR proteins. To learn about developmental roles of GLRs in plants, we are studying phenotypes displayed by *glr* mutant plants.

Significant achievements 2009-2011: We have definitively shown that *GLR3.4*, one of the twenty *GLR* genes in Arabidopsis, functions as an amino acid-gated Ca^{2+}-permeable ion channel. This statement is based on successful expression of *GLR3.4* cDNA in human embryonic kidney (HEK) cells followed by patch-clamp experiments that show the transfected cells display amino acid-gated inward Ca^{2+} currents (Fig. 1A,B) and a rise in cytoplasmic Ca^{2+} concentration (Fig. 1C,D). Non-transfected cells displayed neither. The previous mutant plant studies had indicated that GLR3.4 was activated principally by Asn and Ser, less so by Gly, and not at all by the three other amino acids found to be effective in wild-type seedlings (Ala, Cys, and Glu). In exact agreement with this genetics-based prediction, patch-clamp studies showed GLR3.4 expressed in HEK cells was activated by Asn and Ser approximately equally. Gly was found to be 50% as effective, and the other three amino acids were ineffective. Our current hypothesis is that the amino acid-specificity of the GLR channels *in planta*, which are presumed to be tetramers, is determined by the specificity of the subunits comprising heteromeric channels. The HEK cell expression system we have now established will enable direct tests of this hypothesis.

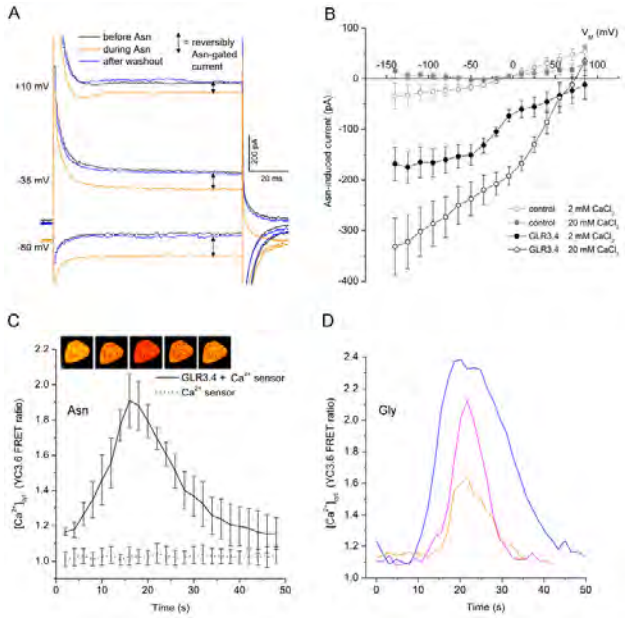

Figure 1. Ca^{2+} permeability of GLR3.4 channels expressed in HEK cells shown by whole-cell patch clamping and live-cell Ca^{2+} imaging. (*A*) Whole-cell current traces recorded from a HEK cell expressing GLR3.4 subjected to a voltage-step protocol before (*black*), during 2 mM Asn treatment (*orange*), and after agonist washout (*blue*). The voltage across the membrane was clamped at values ranging from -140 to +85mV in 15-mV increments for 100 ms each, with a 0.8-s recovery period at -70 mV between steps but only three steps were selected for display. The magnitude of the current reversibly gated by agonist (difference current) is indicated by arrows. (*B*) Difference current-voltage relationship of GLR3.4-expressing or control cells bathed in a NaCl-based buffer containing 2 mM or 20 mM $CaCl_2$. Control cells were transfected with the YC3.6 Ca^{2+} indicator only. The plotted data are means ± SEM, n=5 for control, n=6 for GLR3.4 transfected cells. (*C*) Cytoplasmic Ca^{2+} rise triggered by Asn in HEK cells expressing *GLR3.4* and the optical Ca^{2+} sensor YC3.6 or only YC3.6. The 535:480 nm fluorescence ratio, a direct measure of cytoplasmic Ca^{2+} concentration, rose transiently upon application of 500 µM Asn only in expressing GLR3.4. The values are means ± SEM ($n \geq 3$). Above the plots are confocal microscope images false colored to represent the magnitude of the FRET signal at selected points in a representative time series. (*D*) Cytoplasmic Ca^{2+} rise triggered by Gly in HEK cells expressing GLR3.4 and the optical Ca^{2+} sensor YC3.6. Three independent trials are shown.

We determined the expression pattern of GLR3.4 protein by adding GFP to the carboxy terminus of GLR3.4 and expressed the fusion protein under the control of its own promoter in a *glr3.4*

knockout. Phloem localization predicted by root transcript atlasing projects was confirmed (Fig 2A-F). Unexpected was the finding of discrete accumulations of the protein at the sieve plate junctions between sieve tube members (Fig. 2E). In 2010, the Benfey lab published a paper that identified patterns of oscillating gene expression causally related to the initiation of lateral root primordia. GLR3.4 and GLR3.2, but not GLR3.3, were identified on the basis of their expression behaviors as potentially important to the process of lateral root initiation. Our mutant analysis completely agreed with that prediction. Both *glr3.2* and *glr3.4*, but not *glr3.3*¸ were found to produce twice as many lateral root primordia as the wild type, though the number of emerged lateral roots and therefore the visible root system architecture was not affected (Fig 2G-I).

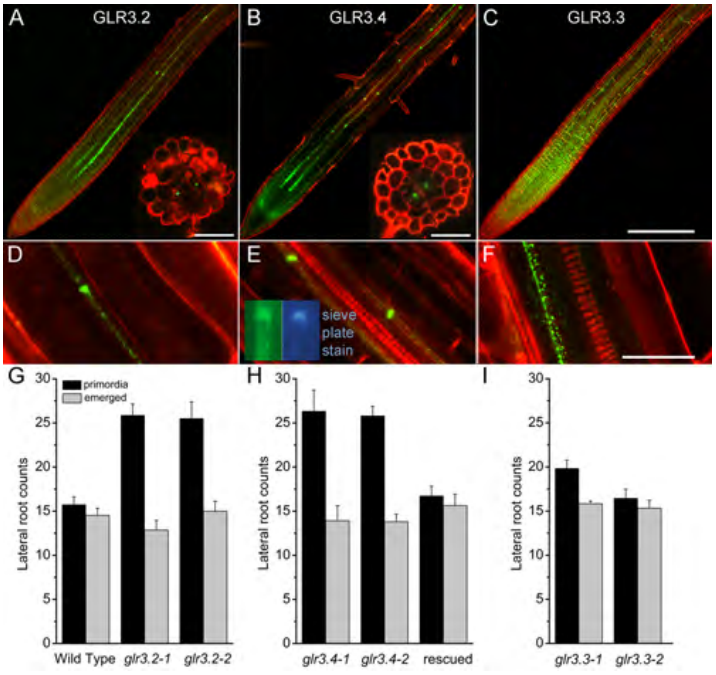

Figure 2. Localization of GLR3.2, GLR3.3, and GLR3.4 in roots and their effects on lateral root production. (A-C) Confocal microscope images of GFP-tagged GLR3.2, GLR3.4, or GLR3.3 (green) in primary root apices stained with propidium iodide (red) to mark cell boundaries. Insets in A and B show phloem-localized signal in hand-cut cross sections. (D-F) Higher magnification images of GFP-tagged GLR3.2, GLR3.4, or GLR3.3 signal in mature phloem. Inset in E shows that aniline blue, a sieve-plate indicator, stains a region of strong GLR3.4-GFP accumulation in the phloem. Scale bar in C = 200 µm and applies to the main images in A-C, inset scale bars = 10 µm. Scale bar in F is 20 µm and applies to D-F. (G-I) Counts of lateral root primordia or emerged lateral roots obtained with 12-d old Arabidopsis plants of the indicated genotype. The data labeled "rescued" were obtained with *glr3.4* mutant plants expressing the GLR3.4-GFP construct shown in B. The values are means ± SEM ($n \geq 6$).

Science objectives for 2011-2013:

- At the molecular function level, we will exploit our heterologous expression system to determine if co-expression of co-localized proteins (e.g. GLR3.2 and GLR3.4) produces a channel with novel transport properties or agonist specificities.
- At the genetic and organismal level, we will investigate the effects of combining mutations in pairs of genes encoding co-localized proteins (e.g. *glr3.2* and *glr3.4*). Will double mutant combinations exacerbate the phenotype? If the double mutant resembles the single mutants, we will think that the two GLR proteins function together in a multimeric channel (losing two is no worse than losing one if the functional channel is a heterotetramer).
- We want to determine by x-ray crystallography if the unique agonist specificity of GLR3.4 (asn, ser, gly) can be explained by structural features of a putative amino-acid-binding domain located extracellularly at the amino-terminus of the protein.

References to work supported by this project 2009-2011:

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2. Spalding EP, Harper JF (2011) The ins and outs of cellular Ca^{2+} transport. *Current Opinion in Plant Biology*. *in press*
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Energetics and mechanisms of protein transport across chloroplast membranes

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Overall research goals: The research objectives are to study the energetics of the protein transport reactions in chloroplasts by: (1) developing two fluorescent procedures, one (a) that will allow us to monitor the transport of fluorescent proteins across membranes in real time, and the other (b) which will allow us to measure the ΔpH across the thylakoid membrane using a pH-indicating fluorescent protein targeted to the thylakoid lumen; (2) determine the energetic cost of ATP-dependent protein import across the chloroplast envelope membranes; (3) measure the energetic cost of ATP-dependent and protonmotive force-assisted protein transport across and into the thylakoid membrane.

Significant achievements 2009-2010: Significant progress has been made on the first two research goals over the past 15 months. We have made numerous transgenic plants, both Arabidopsis and tobacco, containing lumen-targeted, pH-responsive GFP constructs, and have observed chloroplast-localized GFP signals from them (Fig. 1). We are presently growing these transgenic plants in order to prepare thylakoids and test them for the ability to report light-dependent lumen pH changes via these targeted GFP constructs. We have also successfully expressed precursors in bacteria and attached fluorescent reporters to them, allowing their detection with high sensitivity (Fig. 2a). Separately we have worked out a binding and chase import reaction that allows almost all of the precursors in our reactions to be transported (Fig. 2b). This is an important improvement that, unlike the usual protein import protocols, will allow most of the signal-carrying molecules to participate in the import reaction. Finally, we have completed our analysis of the translocation ATPase that powers protein import across the envelope membranes (research goal 2). Using two different precursors synthesized by three different methods, we have determined that the import of a single protein is accompanied by the hydrolysis of 586 ± 252 ATP molecules, placing the cost in Gibbs Free Energy (the $\Delta G_{protein\ transport}$) at some 29,000 kJ per mol protein imported.

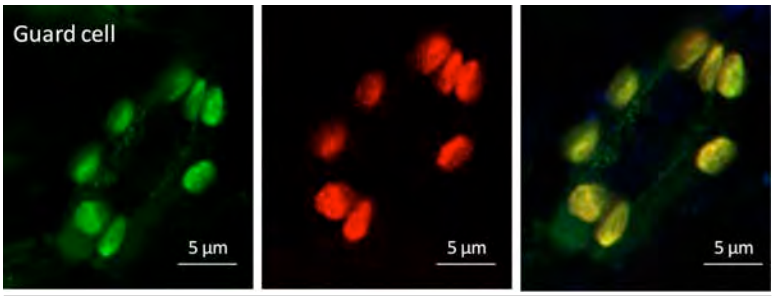

Figure 1. Ratiometric pHluorin behind the prOE17 transit peptide is localized to chloroplasts in Arabidopsis. Left and middle panels show the same confocal image of a transgenic guard cell through a GFP and autofluorescence filter set, respectively. Right panel shows the two images merged.

Science objectives for 2010-2011: In the year remaining before the project's termination we will have the following objectives:

- We will analyze the transgenic plants we have constructed, both Arabidopsis and tobacco, for the ability to use the targeted pH-sensitive GFPs as a monitor of light-induce pH changes in the thylakoid lumen. The low

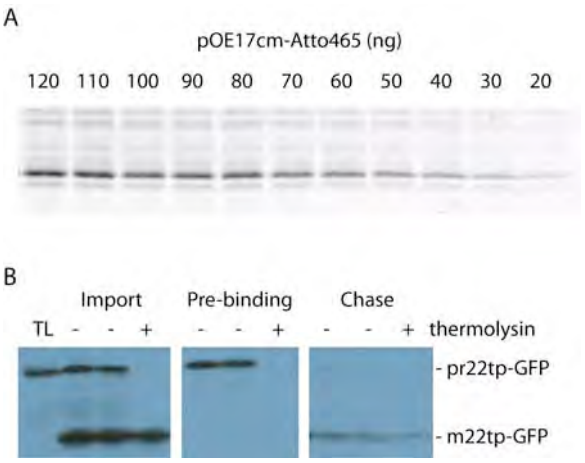

Figure 2. Properties of protein import reactions required for the development of a real time fluorescence-based import assay. A. Bacterially expressed precursor that is labelled with Atto465 can be detected with high sensitivity. The gel shows a dilution series of the Atto465-labelled protein, detected via fluorescence developed in a STORM phosphorimager. The detection limit is below 20 ng/reaction. B. Almost all precursor pre-bound to the surface of chloroplasts can be chased into the stroma. Left, middle and right panels show a set of three import, binding and subsequent chase reactions, respectively. Samples in the third lane of each set were treated with thermolysin to remove externally bound protein. TL, translation product; pr and m, precursor and mature protein, respectively.

amount of plant material presently on hand is the only impediment to performing this analysis at this time.

- We must combine the different aspects of the experiments seeking a real time fluorescence assay for protein transport across chloroplast membranes. We have successfully worked out each piece in isolation – fluorescent labeling and detection of precursor proteins, sequential binding, washing and chase of precursors into chloroplasts, and import of bacterially expressed proteins. Now we must combine all these elements into a single experiment to monitor the import of the labeled precursor via its fluorescent tag.

- In earlier work supported by this DOE program we measured the $\Delta G_{protein\ ransport}$ in a transporter that utilized only the thylakoid protonmotive force (Alder, NN and Theg, SM (1993) Cell 112:231-242). Here we have worked out the measurement of ATP hydrolysis accompanying protein import through an ATP-dependent transporter. It remains to combine these techniques to determine the energetic cost of protein transport via the chloroplast Sec pathway and protein integration via the cpSRP pathway, both of which derive energy from both NTP hydrolysis and the protonmotive force.

<u>References to work supported by this project 2009-2010:</u>

Shi, L-X and Theg, SM (2010) Measurement of the energetic cost of protein import across the envelope membranes of chloroplasts. Proc. Natl. Acad. Sci. USA (submitted).

Building Photochemical Electron Transfer Pathways in Multi-Heme Cytochrome Architectures

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Oleksandr Kokhan[1], Jens Niklas[1], Steven Karlen[3], Postdoctoral Research Associates. Kristen Brown[3], Graduate Student. Nina Ponomarenko[1], Research Consultant.
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Overall research goals: This program is targeted at the synthesis of biomimetic hybrid assemblies for solar fuels applications by combining biological and chemical synthetic approaches. The program investigates opportunities to exploit multi-cofactor redox proteins as building-blocks in supramolecular designs for solar chemical energy conversion, i.e., by using these redox proteins as host frameworks for abiotic light-harvesting pigments and transition metal catalysts. The multi-electron transfer capabilities of the proteins allow them to serve as functional components promoting multiple sequential photo-initiated electron transfer to transition metal catalysts. Program tasks include: (1) searching genomic databases as a source of electron transfer building-block architectures for hybrid supramolecular design; (2) design of synthetic light-harvesting and catalytic functionalities for insertion into biological frameworks; (3) application of time-resolved synchrotron x-ray, optical, magnetic resonance techniques and molecular modelling approaches to resolve active-site and ensemble structures on functional timescales. In particular, this program investigates opportunities to develop a solar hydrogen catalytic function within the multi-heme cytochrome architectures found in the dissimilatory metal-reducing bacteria.

Significant achievements 2009-2011: A variety of multi-heme c-cytochromes retrieved from the *Geobacter sulfurreducens* genome were efficiently produced in an *E. coli* expression system. Initial work focus centered on expression of PpcA as a model to investigate electrostatically-directed photosensitizer surface binding and to develop abiotic chromophore binding through genetic modification of a protein loop region (fig, 1 left). In addition, a PpcA heme-deleted mutant was developed providing a di-heme protein motif retaining two histidines for synthetic cofactor binding. In parallel, a series of water-soluble, ionic porphyrin, chlorophyll, perylene, and ruthenium complex chromophores were synthesized and investigated for photo-initiated electron transfer.

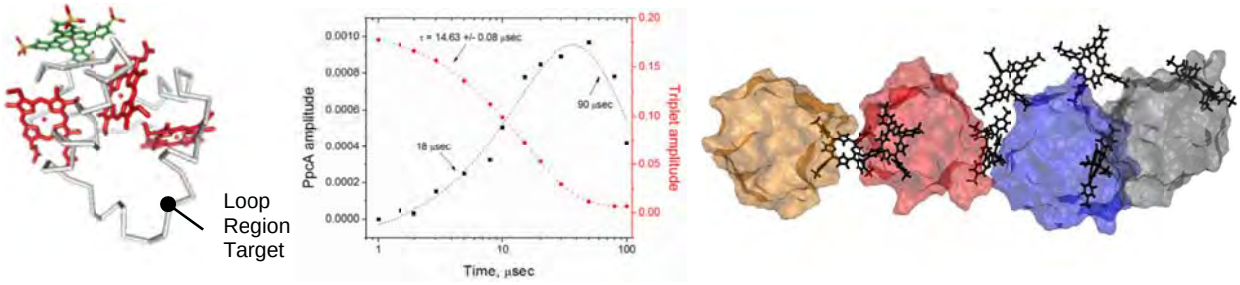

Figure 1. Left: Structure of the wild-type, three heme (red cofactors) cytochrome c_7 PpcA with conceptual view of electrostatically docked tetrakis(4-sulfonatophenyl) porphyrin (TPPS, green). Center: Transient absorption measurements showing simultaneous TPPS triplet quenching and heme reduction in the PpcA-TPPS assembly. Right: All atom MD simulation showing TPPS-induced cytochrome multimerization.

Transient optical absorption studies of the complexes formed between PpcA and anionic porphyrin and chlorophyll derivatives suggested heterogeneous photochemical pathways. A minor fraction exhibited reversible photo-induced heme reduction, as indicated by the matching of porphyrin triplet state quenching and heme reduction, followed by slower charge recombination. A major fraction showed porphyrin singlet and triplet quenching that is possibly indicative of charge separation and fast recombination on the sub-nanosecond scale. In addition, x-ray scattering studies showed that stoichiometric addition of anionic chromophore induced multimerization of the cytochrome, forming assemblies up to a tetramer dimension. Experimentally, he multimerization was found to be controlled significantly by the porphyrin metal and its axial ligand occupancy. Molecular modeling studies showed that the multimerization could be driven by porphyrin charge compensation and porphyrin bridging interactions between cytochromes.

The PpcA heme-deleted mutant showed analogous multiple modes for metal porphyrin interaction, including surface and free histidine coordinating sites. The relative occupancy of these sites was found to vary as a function of the metal porphyrin and its axial ligand. X-ray scattering studies showed the heme-deleted mutant to exist in largely unfolded conformation. Metal porphyrin coordination induced a conversion to a more compact structure, with partial recovery of tertiary structure. This work has provided strategies to developed multi-heme protein-chromophore assemblies with defined structure and potential for directed long-range electron transfer and photocatalytic function.

Science objectives for 2011-2012:

- Develop both electrostatic and metal coordination-directed assembly strategies for coupling multi-heme redox chemistry to supramolecular photosensitizer-acceptor and photosensitizer-catalyst dyad assemblies, with particular application for light-driven hydrogen generation.
- Evaluate abilities of multi-heme cytochromes architecture, ranging from 3 to 12 heme cofactors, to function as either secondary electron donors or acceptors in abiotic photosensitizer-driven photochemistry (depending on redox state), including evaluating opportunities to use redox gradients to trap long-range electron transfer.
- Carry-out structure-function analyses of the multi-heme cytochrome-photosensitizer assemblies using time-resolved synchrotron x-ray, optical, magnetic resonance techniques combined with molecular modelling.

References to work supported by this project 2009-2011:

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2. L. Morgado[1], V. B. Paixão[2], M. Schiffer[3], P. R. Pokkuluri[3], M. Bruix[4*], C. A. Salgueiro, "Solution structure of triheme cytochrome PpcA from *Geobacter sulfurreducens* reveals the structural origin of the redox-Bohr effet", Biochem. J. (2011) *in press.*
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Session VII

Role of the Rubisco Small Subunit

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Overall research goals: The goal of this project is to understand the structure-function relationships of the eukaryotic small subunit as a potential means for ultimately improving ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The nuclear gene family (*rbcS1* and *rbcS2*) that encodes the small subunit has been eliminated in the green alga *Chlamydomonas reinhardtii*, which is presently the only eukaryotic system that allows small-subunit genetic engineering. There are four specific aims in the project. (1) Random mutagenesis and mutant screening will be used to identify other regions of the Rubisco small subunit that play a role in Rubisco structure and function. (2) Genetic selection will be employed as a possible means for elucidating the structural basis for the influence of small-subunit substitutions on large-subunit active-site catalysis. (3) Directed mutagenesis will be used to further define interactions between the small subunit and large-subunit α-helix 8. (4) More-divergent small subunits will be assembled into hybrid Rubisco enzymes as a means for identifying other regions of the small subunit that determine Rubisco catalytic diversity and holoenzyme structure.

Significant achievements 2009-2011: Research has focused on the loop between β-strands A and B, which is the most variable structural feature of the Rubisco small subunit. The βA-βB loops from each of four small subunits at the top and bottom of the holoenzyme define the opening of the central solvent channel that passes through the large-subunit core (Fig. 1). Recently completed work has shown that a small-subunit L66F substitution at the interface between large and small subunits mimics the conformational dynamics of a large-subunit L290F substitution, and both mutant enzymes have decreases in CO_2/O_2 specificity. An L66G substitution, which introduces the smaller residue found in land-plant Rubisco, suppresses large-subunit L290F, and improves the CO_2/O_2 specificity and carboxylation catalytic efficiency of the wild-type enzyme. In an attempt to determine whether the central solvent channel may play a role in holoenzyme conformational changes that occur during catalysis, substitutions were made at Ile-58, which defines the narrowest diameter of the channel opening. Single substitutions had little or no effect. However, when three Trp residues were introduced in place of Ile-58 (introducing 12 Trp residues from four small subunits), CO_2/O_2 specificity and holoenzyme thermal stability were decreased. Similar results were obtained when chimeric small subunits were created with the short βA-βB loop and long carboxyl-terminal extension of the red-algal *Galdieria* small subunit. In *Galdieria*, carboxyl-terminal residues provide structural interactions at the opening of the solvent channel that are similar to those of the longer βA-βB loop in the *Chlamydomonas* small subunit. Altogether, these results indicate that the small-subunit βA-βB loop must influence large-subunit catalysis by a global effect on Rubisco conformation or conformational dynamics, and may serve as a future target for engineering improvements in the enzyme.

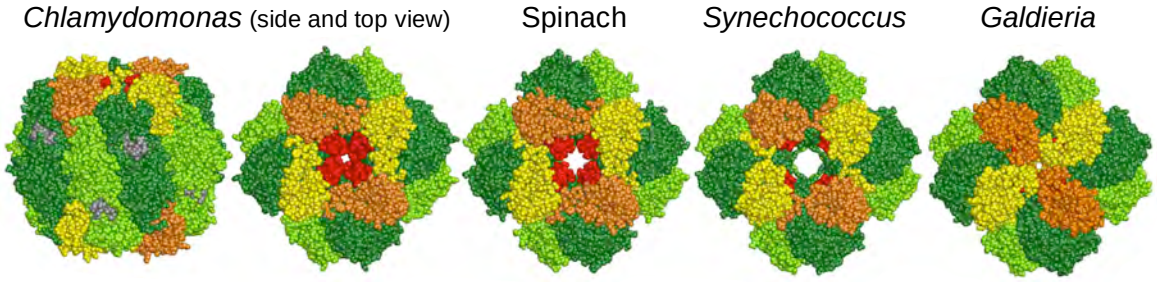

Figure 1. Rubisco x-ray crystal structures. Four nuclear-encoded small subunits (*yellow and orange*) cap the top and bottom of the core of eight chloroplast-encoded large subunits (*dark and light green*). One large-subunit dimer forms two active sites (active-site loop 6 in *gray*). Loops between β-strands A and B of the small subunit are colored *red*.

Ala-scanning mutagenesis of the most conserved small-subunit residues identified other interactions between large and small subunits that influence catalysis (Fig. 2). Recent work has focused on Tyr-32, which shields the carboxyl-terminal end of large-subunit α-helix 8 from solvent. Y32A is the only mutant substitution outside of the βA-βB loop that affects CO_2/O_2 specificity. Y32A, Y32D and Y32E substitutions decrease CO_2/O_2 specificity and holoenzyme thermal stability *in vivo* and *in vitro*, but Y32F and Y32R substitutions do not. It seems likely that either a phenolic ring or positive charge complements the partial negative charge at the carboxyl-terminal end of α-helix 8. This interaction may influence the position or amino-terminal charge of α-helix 8, which, in turn, may influence interactions between amino-terminal α-helix-8 residues and the carboxylation transition state. These results can explain how changes far from the active site affect catalytic efficiency.

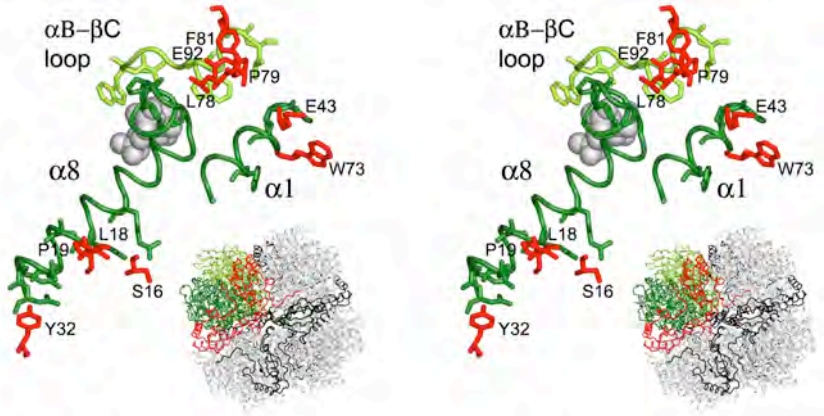

Figure 2. Conserved small-subunit residues in the x-ray crystal structure of *Chlamydomonas* Rubisco Looking down from the top of the Rubisco holoenzyme (*lower right with eight large subunits colored green or gray and four small subunits colored red or black)*, the large-subunit α/β-barrel is viewed from the side. The conserved small-subunit residues (*red*) cluster near large-subunit α-helix 8 (*dark-green* residues 413-432), its preceding loop (*dark-green* residues 403-412), α-helix 1 (*dark-green* residues 182-194), or part of the loop between α-helix B and β-strand C from a neighboring large subunit (*light-green* residues 65-74). The CABP transition-state analog is shown as *gray spheres*.

Science objectives for 2011-2012:

- To further elucidate the mechanisms by which small subunits influence large-subunit catalysis, genetic selection will be used to identify suppressor substitutions that complement mutant substitutions in the βA-βB loop and elsewhere. Because the mutant strains have temperature-conditional photosynthesis-deficient phenotypes, it has been difficult to recover "revertants." A "bioreactor" system is being developed for recurrent batch selection at elevated temperature.
- Random mutagenesis and site-directed saturation mutagenesis are being developed, coupled with genetic selection, to further assess the structural interactions between large and small subunits at the amino-terminal end of large-subunit α-helix 8 and at the small-subunit βA-βB loop. These experiments are being designed to select for improved Rubisco enzymes.
- Previous attempts have been successful at generating hybrid Rubisco enzymes comprised of plant small subunits and *Chlamydomonas* large subunits. More-divergent small subunits will be used to create hybrid enzymes as a means for identifying essential structural interactions between large and small subunits. A codon-optimized cyanobacterial (*Synechococcus*) *rbcS* gene has been synthesized for this purpose. Various gene constructs are being generated.

References to work supported by this project 2009-2011:

1. T. Genkov and R. J. Spreitzer, "Highly Conserved Small Subunit Residues Influence Rubisco Large Subunit Catalysis." J. Biol. Chem. **284**: 30105-30112 (2009).
2. T. Genkov, M. Meyer, H. Griffiths, and R. J. Spreitzer, "Functional Hybrid Rubisco Enzymes with Plant Small Subunits and Algal Large Subunits: Engineered *rbcS* cDNA for Expression in *Chlamydomonas*." J. Biol. Chem. **285**: 19833-19841 (2010) (*Paper of the Week*).

Structure, Function and Assembly of Rubisco Activase

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Overall research goals: To better understand the ATP-driven regulation of carbon assimilation, we are using biophysical methods to study the structure and function of higher plant Rubisco activase. The rapid release of tight-binding inhibitors from dead-end Rubisco complexes requires the activity of activase, an AAA+ ATPase that utilizes chemo-mechanical energy to open closed Rubisco sites. Activase is thought to play a central role in coordinating the rate of CO_2 fixation with the light reactions of photosynthesis, and has been proposed to modulate the thermotolerance of photosynthesis. The objectives of the DOE funded project are (1) to determine the denaturation temperatures of higher plant activases by means of circular dichroism spectroscopy, (2) to solve X-ray structures of full-length activases or structural domains, and (3) to elucidate the activase self-association mechanism by single-molecule spectroscopic methods and analytical ultracentrifugation.

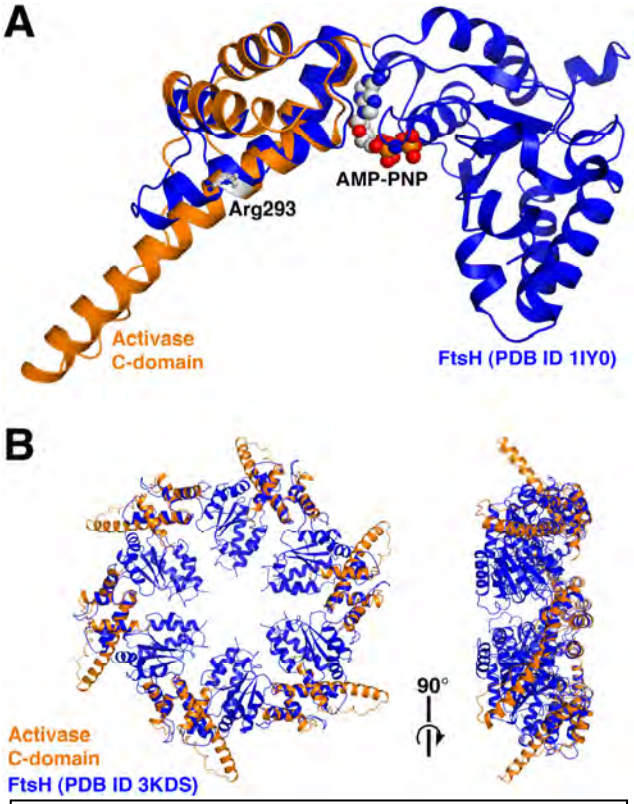

FIGURE 1. Superposition of the activase C-domain with FtsH. *A,* structural overlay of the C-domain (orange) with a monomer of the FtsH AAA+ module (blue). Shown in space-filling and ball-and-stick representations are the nucleotide of FtsH and activase Arg293, respectively. *B,* left: top view of the FtsH hexamer (blue) with six superimposed C-domains (orange) looking down the 6-fold axis; right: side view.

Significant achievements 2009-2011:

1. Thermostability of higher plant activases. Using circular dichroism spectroscopy, the mid-point temperatures (apparent T_m) for irreversible protein denaturation were determined for spinach, Arabidopsis, cotton, and tobacco activases. The data indicate that *in vitro*, the activase fold is highly thermolabile, with the onset of denaturation ranging from 30 to 40 °C. Overall, plants from cooler environments have activases with about 10 °C lower T_m values than plants from warmer environments.

2. 1.9 Å crystal structure of the activase recognition domain. All Rubisco activases examined to-date display a complex spread of oligomeric assembly states, a feature that has rendered crystallization of the holoenzyme difficult. To identify constructs with reduced size polydispersity, we have cloned, expressed and screened over 50 different activase fragments derived from a variety of plant species. Using MAD phasing of data collected on a selenomethionyl-derivatized crystal, we were able to solve the X-ray structure of a 97-residue fragment derived from the desert shrub creosote. The crystallographic model spans the core of the C-domain bearing several elements important for recognition, specificity and catalysis (Figure 1). The fold consists of a canonical four-helix bundle, from which a paddle-like extension protrudes that entails a 9-

turn helix lined by an irregularly structured peptide strand. The residues Lys313 and Val316 involved in the species-specific recognition of Rubisco are located near the tip of the paddle. Structural superpositions onto the distant homolog FtsH imply that the paddles extend away from the hexameric toroid in a fan-like fashion, such that the hydrophobic sides of each blade are facing inward, and the polar sides bearing Lys313 and Val316 are facing outward (Figure 1). Therefore, we speculate that upon binding, the activase protrusions embrace the Rubisco cylinder by placing their hydrophobic patches near the partner protein. This model suggests that conformational adjustments at the remote end of the paddle may relate to selectivity in recognition. We hope that the new structural information will aid in the development of appropriate models for Rubisco reactivation.

3. Activase self-assembly monitored by single-molecule spectroscopic methods. Cotton β activase was covalently labelled with an Alexa fluorophore, and a home-built optical setup housed in the Levitus lab was utilized to monitor fluctuations in the fluorescence signal. The data were analyzed in terms of their amplitude fluctuations (photon histogram counting, PCH) and their temporal behavior (fluorescence correlation spectroscopy, FCS). Irrespective of crowding agents, the results were consistent with a monomeric species at low to mid-nanomolar concentrations. To examine subunit self-association, experiments were carried out with mixtures of fluorescently labelled and unlabelled activase. The radius of gyration of the diffusing species was determined from the FCS decay curves (Figure 2). The results suggest that activase oligomerization occurs in the low μM range, and that a hexameric species is generated. Notably, the data imply that under physiologically relevant protein concentrations, the activase is fully assembled even in the absence of Rubisco.

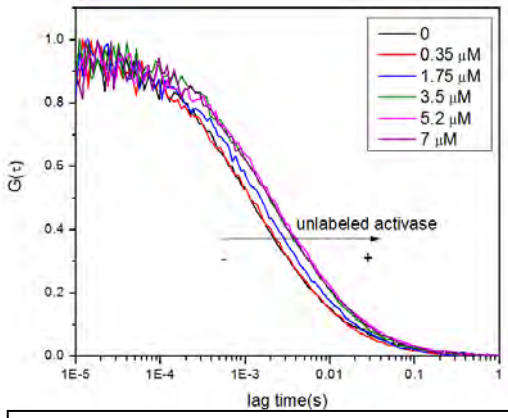

FIGURE 2: FCS decays measured with 100 nM fluorescently labeled activase in the presence of unlabeled activase at the concentrations indicated in the legend. All solutions contain 5% Ficoll as a crowding agent.

Science objectives for 2011-2012:

- We are planning to continue our crystallization efforts of full-length activases and activase fragments of varying sizes. To this end, we will first characterize the polydispersity of protein pools by dynamic light scattering, then carry out crystallization trials by taking advantage of a newly installed crystallization robotics system.
- To further examine the self-association behavior in bulk solution at micromolar concentrations, we are using equilibrium sedimentation methods. As the necessary nucleotide concentrations interfere with UV detection, we are planning experiments with Alexa-labeled activase preparations to be able to monitor sedimentation at 550 nm.
- To elucidate the equilibria responsible for assembly from a monomeric to a hexameric species, we will continue to use fluorescence correlation spectroscopy with mixtures of labeled and unlabeled protein. The experimentally determined change in the radius of gyration as a function of concentration will be compared to computer simulations based on different thermodynamic self-association models.

References to work supported by this project 2009-2011:

1. Henderson, J. N., Kuriata, A. M., Fromme, R., Salvucci, M. E., Wachter, R. M. (2011) Atomic Resolution X-ray Structure of the Substrate Recognition Domain of Higher Plant Rubisco Activase. *J. Biol. Chem. Report (in press)*.
2. Barta, C., Dunkle, A. M., Wachter, R. M., Salvucci, M. E. (2010) Structural Changes Associated with the Acute Thermal Instability of Rubisco Activase. *Archives of Biochemistry and Biophysics 499, 17-25.*

Disruption of C_4 photosynthesis by changes in light quantity and quality: Implications for CO_2 fixation and photosynthetic efficiency in C_4 crop and biofuel species.

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Overall research goals: The overall objective of this proposal is to determine the mechanisms controlling C_4 photosynthetic efficiency in response to changing light quantity and quality. Fluctuations in light availability are predicted to have a strong impact on C_4 photosynthesis, which requires coordination of the C_4 and C_3 cycle between two cell types. Our central hypothesis is that changes in light quantity and quality will alter the distribution of photosynthetic energy to C_4 photosynthesis and decrease the energy use efficiency of the CO_2 concentrating mechanism. The rationale for this research is that it will provide critical information on the efficiency of the CO_2 concentrating mechanism in C_4 plants in response to changes in light energy availability. Our research focuses on the C_4 crops *Zea mays* and *Sorghum bicolor*, and the biofuel species *Miscanthus x giganteus* and *Panicum virgatum*. By combining instrumentation and techniques not currently available elsewhere in the world, outcomes from this novel approach will provide a quantitative measure of the efficiency of C_4 photosynthesis under different light environments.

Significant achievements 2009-2010: Our first publication characterized the kinetic parameters of maize Rubisco to accurately model rates of photosynthesis (Cousins et al., 2010). We also demonstrated that applying the theoretical models for C_4 photosynthesis and isotope exchange assuming negligible fractionation during dark respiration and that bundle-sheath CO_2 concentration is large under low light leads to erroneous under estimates of the efficiency of C_4 photosynthesis (Ubierna et al., 2011). Subsequently, we determined the efficiency of C_4 photosynthesis under limited light in *Z. mays* and *M. giganteus* and *Flaveria bidentis* by combining measurements of gas exchange, photosynthetic discrimination ($\Delta^{13}C$), metabolite pools, spectroscopic assays, and models of C_4 photosynthesis and discrimination (Ubierna et al., under review). Spectroscopic and metabolite assays suggested that the energy partitioning between the C_4 and C_3 cycles was similar across light intensities. Additionally, calculations of leakiness (ϕ), the proportion of carbon fixed by PEP carboxylation that leaks out of the bundle-sheath cells, via the isotope method suggest little change in response to light availability (Fig. 1). However, neglecting respiratory fractionations or assuming large CO_2 concentration in the bundle-sheath cells led to large overestimation of ϕ under low light (Fig. 1). Our results suggest that C_4 species are able to coordinate the C_4 and C_3 activity at a given light to optimize carbon gain.

We also measured steady state and transition state leaf gas exchange, $\Delta^{13}C$, and pools of photosynthetic

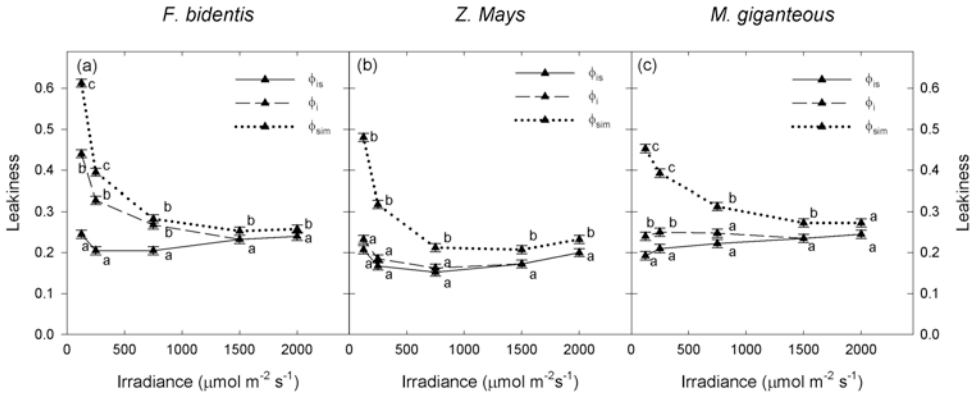

Figure 1. Estimations of leakiness (ϕ) at different light for *F. bidentis* (a), *Z. mays* (b), and *M. giganteous* (c). ϕ_{is}: solid line, (no assumptions); ϕ_i: dashed line, (assumes large bundle sheath CO_2 concentration, C_s); ϕ_{sim}: dotted line (assumes large C_s and negligible respiratory and photorespiratory fractionations). Mean ± LS-SE (n = 4).

intermediates in *Z. mays* and *M. giganteus* under white, red, green, and blue light. At a light intensity of 900 µmol m^{-2} s^{-1}, steady state net assimilation rates (*A*) under blue light was significantly lower than under white, red, and green light (Sun et al., Under review). However, no significant light quality effects were detected in steady state $\Delta^{13}C$ and ϕ. Metabolite analysis indicated little disruption of the C_3 and C_4 cycles. However, rapidly switching illumination from blue to red light increased $\Delta^{13}C$ and ϕ in *M. giganteus* but less so in *Z. mays* (Fig. 2; Sun et al., in prep). These data suggest that the mesophyll C_4 activity is initially reduced under blue light compared to red light; however, with time the rates of the C_4 and C_3 cycle adjust to minimize ϕ.

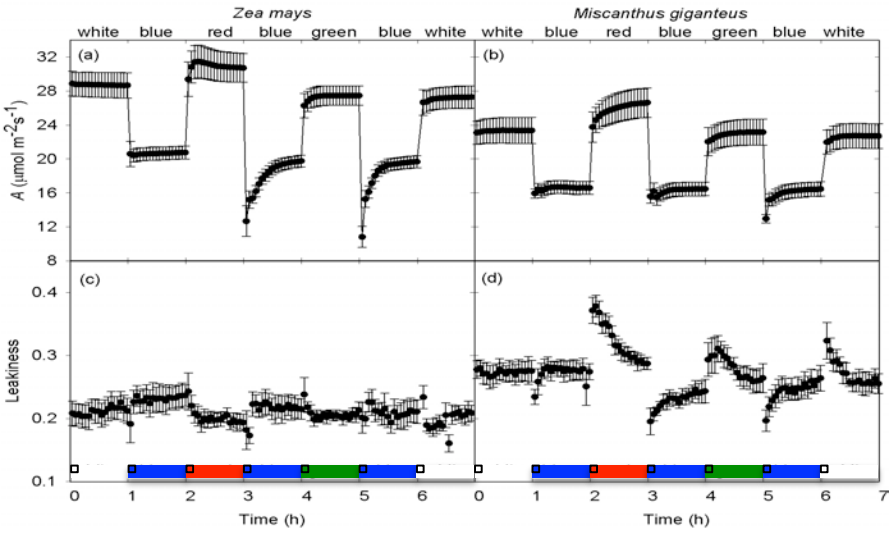

Figure 2. Variation in net CO_2 assimilation (a & c) and leakiness (ϕ; b & d) in response to changes in light quality *Z. mays* (a & c), and *M. giganteous* (b & d). Mean ± LS-SE (n = 6).

Science objectives for 2011-2012: We are currently measuring the effect of light availability on the photosynthetic efficiency of *Sorghum bicolor* (NADP-ME) and the NAD-ME type C_4 grass *P. virgatum* (Switch grass). This is an interesting comparison of the NADP-ME (*S. bicolor*, *M. giganteus* and *Z. mays*) and NAD-ME to determine if the different biochemical pathways respond similarly to light availability. We are also conducting a growth analysis of Miscanthus under two light treatments and three nitrogen levels to determine how these growth conditions influence leaf development and photosynthetic efficiency. Additionally, we are using a membrane inlet mass spectrometer to measure rates of photorespiration, pseudo-cyclic electron transport to O_2 and changes in the CO_2 compensation point in these C_4 grasses in response to different light qualities. These will be conducted simultaneous with measurements in rates of cyclic and linear electron flux using absorbance spectroscopy and chlorophyll *a* fluorescence.

Publications from research conducted in 2009-2011

1) Cousins A.B., Ghannoum, O., Badger M.R., von Caemmerer S. (2010) Measuring simultaneous determination of Rubisco carboxylase and oxygenase kinetic parameters in *T. aestivum* and *Z. mays* using membrane inlet mass spectrometer *Plant Cell and Environment* 33, 444-452

2) King J., Edwards, G.E., Cousins A.B. Efficiency of the CO_2 Concentrating Mechanism in Single-Cell C_4 Metabolism *Plant Cell and Environment* in press

3) Ubierna N., Sun W., Cousins A.B. (2011) The efficiency of C_4 photosynthesis under low light conditions: assumptions and calculations with CO_2 isotope discrimination. *Journal of Experimental Botany* 62, 3119-3134

4) Ubierna N., Sun W., Cousins A.B. Balancing the flux through the C_3 and C_4 cycle during C_4-photosynthesis under low light. *Plant Cell and Environment* under Review

5) Sun W., Ubierna N., Ma J-Y., Cousins A.B. The effect of light quality on steady state C_4 photosynthetic efficiency in *Z. mays* and *M. giganteus*. *Plant Cell and Environment* under review

Session VIII

Tha4 topology and direct interaction with translocating precursor mature domain during transport on the cpTat pathway

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Overall research goals: The research objectives are to study the mechanisms of protein transport into the lumen of thylakoids by the chloroplast Twin Arginine Transport (cpTat) pathway by: (1) identifying the cpTat component(s) that interact with the mature domain of the precursor during transport; (2) determine the organization of the cpTat translocon; and (3) compare topology of cpTat component, Tha4 in thylakoids during active transport and at rest. This particular protein transport pathway is predicted to translocate ~50% of the lumen proteins. Understanding cpTat system mechanism in chloroplasts will lead to a better understanding of the biogenesis of photosynthetic membranes potentially providing a means to engineer photosynthetic complexes into synthetic membranes for energy production.

Significant achievements 2010-2011: Proteins destined for the thylakoid lumen of chloroplasts are nuclear-encoded, synthesized in the cytoplasm, and must cross three membranes *en route* to their final destination. The chloroplast Twin Arginine Translocation (cpTat) system facilitates transport of about half of all proteins that cross the thylakoid membrane in chloroplasts. Known mechanistic features of the cpTat system are drastically different from other known translocation systems, notably in its formation of a transient complex to transport fully folded proteins utilizing only the protonmotive force for energy. However, key details such as the structure and composition of the translocation pore are still unknown. One of the three transmembrane cpTat components, Tha4, is thought to function as the pore by forming an oligomer. Yet, little is known about the basic topology of Tha4 in thylakoid, and little work has been done to detect precursor-Tha4 interactions, which are expected if Tha4 is the pore. First, we present topology studies of Tha4 via cysteine accessibility to membrane permeant and impermeant labels. N-terminal cysteine is not accessible to membrane impermeant labels but is accessible to membrane permeant labels. In addition, cysteine placed throughout the predicted amphipathic helix and carboxy tail region is susceptible to labelling with both kinds of labels. Taken together these data support a N_{out}-C_{in} topology for Tha4 in the thylakoid membranes. Second, we have the first evidence of interaction of the mature domain of precursor with Tha4. Using disulfide exchange we demonstrate that the mature domain of the precursor interacts specifically with the amphipathic helix of Tha4, suggesting a role for Tha4 as the precursor conduit. These data provide the first evidence that Tha4 participates directly in transport of precursor on the cpTat pathway.

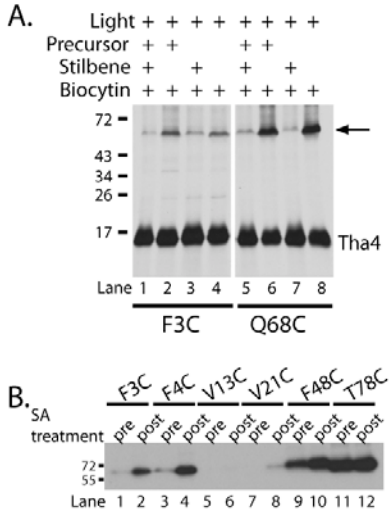

Figure 1. cpTat component, Tha4, exhibits a N_{out}-C_{in} topology in the absence of transport. Membrane impermeant (stilbene or streptavidin (SA)) cannot access cysteines near the N terminus of Tha4 (F3C, F4C), but have access to cysteines placed in the stromal domain (F48C, Q68C, T78C) as evidence by the decrease in biocytin labelling (lanes 5, 7). Neither membrane impermeant nor permeant (biocytin) molecules can bind cysteines in the apolar transmembrane region. (A) +/- stilbene pretreatment of thylakoids containing [^{3}H]Tha4 followed by a biocytin and SA post-treatment. The arrow indicates the presence of SA labelling of Tha4 via bound biocytin. (B) Thylakoids containing [^{3}H]Tha4 treated with biocytin were subsequently treated with SA before or after membrane solubilization with the detergent digitonin. SA could not label N-terminal cysteine (F3C, F4C) prior to membrane solubilisation, whereas APH or C-terminal cysteine were labelled even prior to solulbilization.

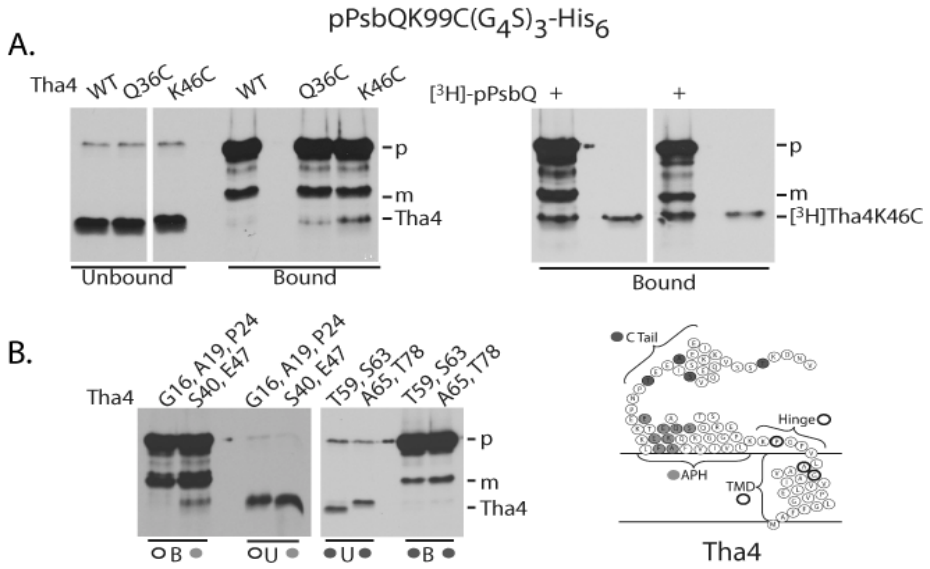

Figure 2. CpTat precursor, pPsbQ, interacts with cpTat component Tha4 in a cysteine-dependent manner. Ni^{2+}-affinity purifications of PsbQ precursor (p) containing a cys-substitution at position K99 and a His-Tag linked through a $(GGGGS)_3$ polylinker was transported into isolated thylakoids and processed to the mature (m) form. (A, *left panel*) Post transport, thylakoids were subject to affinity precipitation of the His-tagged protein and disulfide bound partners. WT [^{3}H]Tha4 (lacking Cys) was not affinity precipitated by precursor (Unbound vs Bound), whereas [^{3}H]Tha4 containing cys-subtitutions in the predicted amphipathic helix (APH; Q36C, K46C) were (Bound). (*right panel*) Cold precursor affinity precipitates [^{3}H]ThaK46C or [^{3}H]Tha4K53C. (B, *left panel*) [^{3}H]Tha4 containing cys-substitutions in the transmembrane domain (TMD), Hinge, or C Tail (G16C, A19C, P24C, T59C, S63C, A65C, T78C) were not precipitated by His-tagged pPsbQ (U=Unbound vs B=Bound), whereas [3H]Tha4 cys-substitutions in the predicted amphipathic helix (APH; Q36C, S40C, K46C, E47C, T53C were precipitated by his-tagged pPsbQ (B). (*right panel*) Cartoon diagram of the predicted topology of Tha4 showing the cys-sustituted amino acids. Open circles represent Cys placed in the TMD or Hinge; light gray circles the APH; and dark gray circles the C tail.

Science objectives for 2011-2013:

- Tha4 topology studies were performed on Tha4 in the absence of transport and did not look at changes in topology during the transport process. We will continue the topology studies to include investigation of topology changes during transport.
- While we can detect direct interactions between Tha4 and mature domain of protein, we do not know when the interaction occurs. Is the interaction prior to transport, during transport, or post transport? Further investigations with modified assays will allow us to investigate the nature and timing of the interaction.
- We are beginning investigations of the cpTat translocon by identifying the points of interaction between cpTatC and Hcf106 using a crosslinking and mass spectrometry approach.
- Structural studies on bacterial homologs of Tha4 have yielded limited functional information. Preliminary evidence in the lab suggests Tha4 responds, by changing conformation in some way, to the presence of a protonmotive force. We are currently using EPR spectrometry to probe the environment and topology of the cpTat components, Tha4 and Hcf106, in native thylakoid membrane. We hope to combine the two and undertake spin label EPR spectrometry experiments of Tha4 in thylakoids in the presence or absence of actinic light.

Manuscripts in preparation supported by this project:

1. Storm, A. and Dabney-Smith, C. *cpTat component, Tha4, exhibits an $N_{out}C_{in}$ topology*
2. Pal, D. and Dabney-Smith, C. *Direct interactions between precursor and Tha4 during transport.*

Thylakoid Biogenesis
– Significance of Protein Maturation and Mechanism of Protein Targeting -

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Joshua Endow (graduate student)
Takafumi Midorikawa (postdoctoral scholar – current)
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Overall research goals: There are three thylakoidal processing peptidase (TPP) homologs in the model plant *Arabidopsis thaliana*. Our previous study demonstrated that one of them called plastidic type I signal peptidase 1 (Plsp1) is necessary for photoautotrophic growth of the plant. The research objectives are to elucidate the molecular mechanism underlying the development of photosynthetic membranes by: (1) examining the significance of lumenal protein maturation for chloroplast development; (2) defining targeting mechanisms of Plsp1 to the chloroplast membranes; (3) examining interactions of Plsp1 with other proteins. Understanding the significance and mechanisms of processes vial for thylakoid development should serve as a foundation for future establishment of technologies that enable efficient capture and utilization of solar energy. The outcome of the research should also advance our understanding of basic biological processes, namely protein translocation and membrane biogenesis.

Significant achievements 2009-2011:
Significance of gene duplication that gave rise to multiple TPPs: Phylogenetic analyses revealed the presence of two distinct groups of TPPs in higher plants. One includes Plsp1 and another comprises the other two TPP homologs termed Plsp2A and Plsp2B in *A. thaliana*. Results of expression and genetic complementation assays suggest that the two groups evolved to play distinct roles although the exact function of the Plsp2 homologs remains elusive.
Functions and properties of Plsp1: Comprehensive analysis of the *plsp1*-null plastids revealed that Plsp1 was involved in maturation of the protein translocation channel in the chloroplast outer envelope (Toc75) and several proteins targeted to the thylakoid lumen (PsbO, PsbP and plastocyanin). Plastids of the *plsp1*-null mutant accumulated vesicle-like structures instead of properly flattened and stacked thylakoids. Results of biochemical and immunolocalization assays showed that unprocessed PsbO accumulated in the peripheral area of the vesicle-like structures in the *plsp1*-null plastids. By a genetic study, it was shown that complete maturation of Toc75 was dispensable for proper chloroplast development. Cytological and biochemical data suggest that Plsp1 is physically involved in maturation of proteins in both the envelope and thylakoids. The localization of Plsp1 in the two membranes correlated with the development of thylakoids from the envelope (Fig. 1). Thylakoid-localized Plsp1 was found to form oligomeric complexes by blue-native PAGE analysis. Genetic complementation assay was used to show that Plsp1 with a Citrine-tag in its N terminus (between the transit peptide and the mature portion) was functional *in vivo*. The tagged protein was used to isolate potential interacting partners including a protein involved in cyclic electron flow.

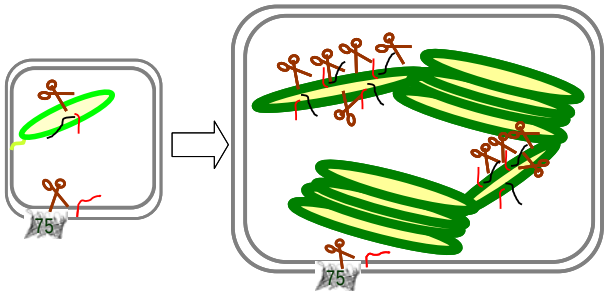

Figure 1. Schematic showing the developmental change of Plsp1 localization in chloroplasts. In developing chloroplasts (left), which are often found near meristematic tissues, Plsp1 (indicated as a scissor) is evenly distributed to the envelope and thylakoids to process Toc75 and lumenal substrates, respectively. In developed chloroplasts in mesophyll cells (right), Plsp1 is mainly located in thylakoids to process lumenal substrates.

Science objectives for 2011-2012:

- The results of our previous work suggest that lumenal protein maturation is necessary for proper thylakoid flattening. We will test this hypothesis by generating plants that accumulate immature forms of PsbO with the presence of Plsp1. Growth phenotypes and chloroplast development of the resultant plants will be analyzed.
- Association of Plsp1 and identified potential interacting partners, as well as its biological significance will be examined.
- We will use *in vitro* import assay to test whether Plsp1 utilizes known pathways to be targeted to thylakoids.

References to work supported by this project 2009-2011:

1. R. L. Shipman, and K. Inoue, "Suborganellar localization of plastidic type I signal peptidase 1 depends on chloroplast development." FEBS Lett **583**: 938-942 (2009).
2. R. L. Shipman-Roston, N. J. Ruppel, C. Damoc, B. S. Phinney, and K. Inoue K, "The significance of protein maturation by plastidic type I signal peptidase 1 for thylakoid development in Arabidopsis chloroplasts." Plant Physiol **152**: 1297-1308 (2010).
3. J. K. Endow, N. J. Ruppel, and K. Inoue, "Keep the balloon deflated: The significance of protein maturation for thylakoid flattening." Plant Sign Behav **5**: 721-723 (2010).
4. K. Inoue, "Emerging roles of the chloroplast outer envelope membrane." Trends in Plant Science doi:10.1016/j.tplants.2011.06.005 (2011).
5. S. C. Hsu, J. K. Endow, N. J. Ruppel, A. J. Baldwin, R. L. Roston, and K. Inoue, "Functional diversification of thylakoidal processing peptidases in Arabidopsis thaliana." Submitted to PLoS ONE (2011).

Thylakoid membrane biogenesis in cyanobacteria

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Overall research goals:

In the grant period that was recently completed a main objective of the project was to understand the interrelationships between chlorophyll (and its synthesis), chlorophyll-binding proteins and thylakoid membrane formation. Using stable-isotope labeling followed by mass spectrometry we monitored the rate of disappearance of "old" chlorophyll and chlorophyll-binding proteins, and measured the rate of biosynthesis of "new" pigment and proteins associated with the two photosystems. For these and other studies we used the cyanobacterium *Synechocystis* sp. PCC 6803, which easily takes up relevant stable isotope-labeled compounds and for which we have generated mutants that lack specific photosystem components.

Now that we have set up a system to distinguish between old and newly synthesized membrane components, we are extending these studies to understand thylakoid membrane biogenesis. Very little is known about how thylakoid membranes are formed, but it is clear that chlorophyll is required in both plants and cyanobacteria, for reasons as yet unknown. Another question is how thylakoids are formed and how new proteins are inserted, as we do not see a large number of ribosomes associated with either thylakoid or cytoplasmic membranes in cyanobacteria. One hypothesis is that membranes are formed from vesicles that in turn are synthesized de novo or bud off from existing membranes. One protein that may be relevant in this respect is VIPP-1 (vesicle-inducing plastid protein-1), which upon overexpression causes an increase the number of thylakoid membranes per cell. However, we are also focusing on several other proteins that may be involved with induction of vesicles or with protein integration into membranes. We are using a combination of genetic, biochemical and ultrastructural approaches to determine how membranes are formed. This is a long-standing problem that has proven to be very difficult to solve over the years, but we think that we have the right combination of expertise and approaches to contribute significantly to a solution.

Significant achievements 2010-2011:

- We have developed a stable-isotope/mass spectrometry method for monitoring protein lifetimes in the two photosynthetic complexes.
- Polypeptides in photosystem II dimers and photosystem I trimers as resolved on a native gel have the same lifetime as those found in monomers, suggesting that oligomeric forms of photosystems either exchange rapidly (within an hour) with monomeric forms or are preparational artifacts.
- Even though the Sll1906 protein is similar to PucC and other members of a group of putative chlorophyll delivery proteins, deletion of the corresponding gene did not lead to changes in chlorophyll delivery, suggesting that either there is another, parallel pathway for chlorophyll delivery, or Sll1906 is not a chlorophyll delivery protein.

- In a mutant that is unable to synthesize chlorophyll in darkness, thylakoid membranes are rapidly synthesized upon exposure to light and upon formation of chlorophyll. We are trying to fractionate cells and identify fractions that are involved in membrane biogenesis.

Science objectives for 2011-2012:

- Identify the thylakoid biogenesis pathway (proteins involved, and the sequence of events) in the mutant where chlorophyll biogenesis is light-dependent.
- Determine the role of a number of proteins that are potentially involved in thylakoid membrane biogenesis.
- Follow the lifetime and kinetics of synthesis of proteins that are important for membrane biosynthesis.

References to work supported by this project 2010-2011:

1. Yao, C.I.D, and Vermaas, W.F.J. (2011) Function of *sll1906*, a member of the bacteriochlorophyll delivery family, in the cyanobacterium *Synechocystis* sp. PCC 6803. In: 15th International Photosynthesis Congress Proceedings. Springer, in press.
2. Yao, C.I.D., Brune, D.C., Vavilin, D., and Vermaas, W.F.J. (2011) Photosystem II component lifetimes in the cyanobacterium *Synechocystis* sp. PCC 6803: Small Cab-like proteins stabilize biosynthesis intermediates and affect early steps in chlorophyll synthesis. In revision.
3. Yao, C.I.D., Brune, D.C.., and Vermaas, W.F.J. (2011) Lifetimes of photosystem I and II proteins in the cyanobacterium *Synechocystis* sp. PCC 6803. Submitted.

Chloroplast Dynamics and Photosynthetic Efficiency

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Overall research goals: Our project explores the roles of the myosin and actin cytoskeleton in positioning of chloroplasts to maximize solar energy utilization and to promote efficient exchange of metabolites with other compartments within the plant cell. Chloroplasts move within leaf cells to optimize light levels, maximizing their interception of light under limiting light conditions (accumulation) and minimizing interception under excess light conditions (avoidance). We are investigating whether members of the plant myosin XI family may play a role in chloroplast movement. We proposed to (1) Identify chloroplast-associated myosins by YFP fusions and use fluorescent labeling to discover which other organelles might be moved by the same myosins that interact with chloroplasts (2) Determine whether chloroplast-associated myosins undergo alternative splicing (3) Analyze Arabidopsis single and higher-order myosin mutants to observe whether chloroplast movement is affected (4) Assess the role of myosin function in photosynthesis and biomass accumulation and (5) Identify chloroplast-associated proteins that interact with myosins to mediate movement.

Significant achievements 2009-2011: Structural modeling was carried out on sequences of the predicted tail domains 12 of the 13 Arabidopsis myosin XIs observed to carry regions homologous to two different domains in yeast known to be important in organelle movement (Fig. 1).

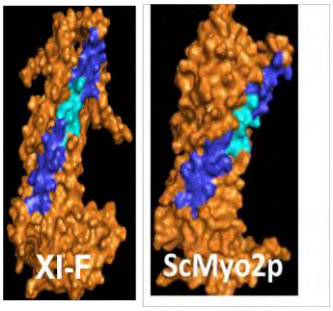


Fig. 1. Structural modeling of Arabidopsis myosin XI-F "PAL" domain homologous to yeast ScMyo2p vacuole-binding domain

YFP fusions were made to the 24 different Arabidopsis domains (termed "PAL" and "DIL" in plants) and examined by transient expression in Nicotiana leaves. One PAL domain YFP fusion was observed to label chloroplasts and stromules (Fig. 2) *(1, 2)*. DIL domain fusions labeled the endoplasmic reticulum, peroxisomes, Golgi, plasma membrane, nuclear envelope, and unidentified vesicles *(4)*. PAL domain fusions labeled mitochondria, peroxisomes, Golgi, plastids, and unidentified vesicles (manuscript in preparation).

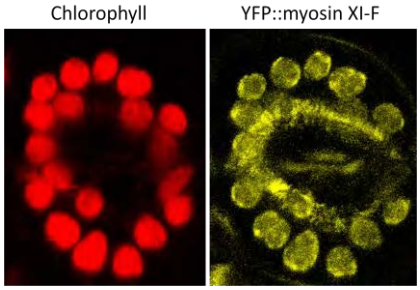


Fig. 2. Myosin XI-F YFP PAL fusion co-localizes with chlorophyll signal in transgenic tobacco guard cell pair.

Examination of transcripts of the tail domain of Arabidopsis myosin XI-F has not revealed any alternative splicing. Stable tobacco transgenic plants expressing YFP fusions with some of the myosin XIs have been produced. A second myosin XI localizing to chloroplasts was detected when the entire tail region was expressed.

Co-PI Owens assembled a dual source system (variable intensity actinic blue light to induce chloroplast movements, low intensity modulated red light) to measure changes in %T as small as 0.01%) (Fig. 3).

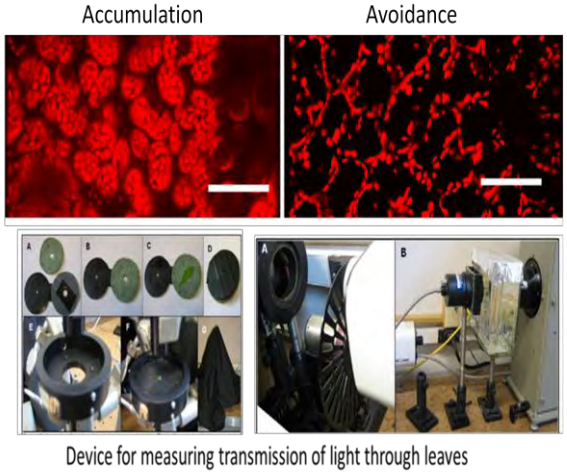
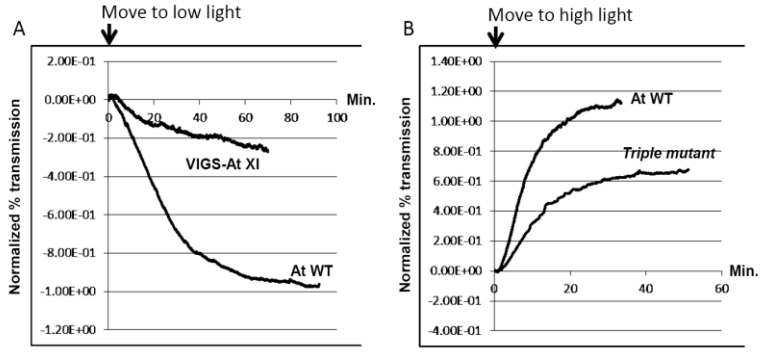


Fig. 3. Transmission through leaves with accumulated chloroplasts is lower than in leaves whose cells have moved to avoid light.

We have used this system to observe both the accumulation and avoidance movements in a variety of genotypes. Transgenic plants expressing a defective myosin comprised of the XI-F tail domain without a motor domain exhibited a dominant-negative effect on chloroplast movement. Arabidopsis plants undergoing virus-induced silencing of certain combinations of myosin XI genes also exhibit altered chloroplast movement (example in Fig. 4). Double and triple Arabidopsis myosin mutants exhibited altered accumulation and/or avoidance movement (example in Fig. 4). Our data support a previously neglected role of myosin XIs in chloroplast responses to light.



Science objectives 2011-2012

Construction of double and triple mutants will continue and such mutants will be assayed for chloroplast movement. The effect of altered myosin gene expression on photosynthesis and biomass accumulation will be examined. Existing plants carrying epitope tagged myoxin XI-F and an anti-myosin XI-F antibody will be used in immunoprecipitation experiments to identify associated chloroplast envelope proteins. Protein-protein interaction experiments will be carried out with the chloroplast-associated myosins and proteins such as CHUP1 and THRUMIN1 that are known to affect chloroplast movement.

References to work supported by this project 2009-2011:

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(2) Hanson MR and A Sattarzadeh. 2011. Stromules: recent insights into a long neglected feature of plastid morphology and function. *Plant Physiology* 155:1486-1492.

(3) Hanson MR and A Sattarzadeh. 2012. Fluorescent labeling and confocal microscopic imaging of chloroplasts and non-green plastids. *Methods in Molecular Biology*. Ed. P. Maliga. In press. Humana Press.

(4) Hanson, MR, E Schmelzer, and A Sattarzadeh. Analysis of organelle targeting by DIL domains of the Arabidopsis myosin XI family. *Frontiers in Plant Genetics & Genomics*. Minor revisions requested.

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