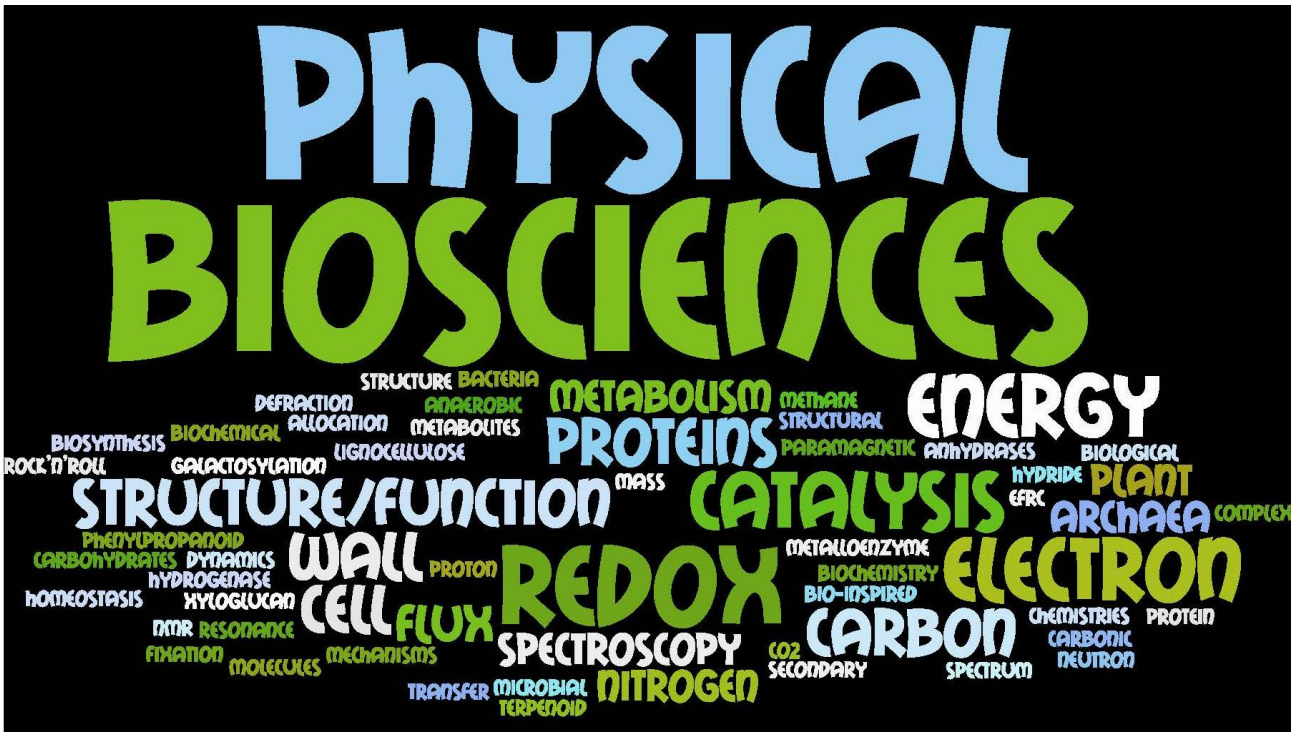
# 2012




# Research Meeting

**Bolger Conference Center**
**Potomac, MD**
**October 14-17, 2012**

U.S. DEPARTMENT OF ENERGY | Office of Science

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

# 2012 Physical Biosciences Research Meeting

## Program and Abstracts

Bolger Conference Center
Potomac, MD
October 14-17, 2012

Chemical Sciences, Geosciences, and Biosciences Division
Office of Basic Energy Sciences
Office of Science
U.S. Department of Energy

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# Foreword

This volume provides a record of the 3nd biennial meeting of the Principal Investigators (PIs) funded by the Physical Biosciences 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). Physical Biosciences and Photosynthetic Systems are the two complimentary programs within DOE-BES that fund basic research in energy-relevant biological sciences. These two programs, along with Solar Photochemistry, comprise the Photochemistry and Biochemistry Team within DOE-BES.

We believe there is significant benefit in building a vibrant community of some of the best and brightest minds out there engaged in generating the foundational knowledge upon which future bio-inspired and/or biomimetic energy systems can be developed. Consequently, it is our expectation that you attend and participate in these all-PI meetings on a biennial basis as they serve many critically important functions. The format of these meetings is specifically designed to: 1) promote sharing of information on your DOE-funded work with your colleagues; 2) facilitate development of new collaborations between individual research groups with complementary strengths; 3) stimulate creativity by exposing you to new ideas and methodologies; 4) challenge you with new ideas and paradigms; and, 5) provide opportunities for you to interact with DOE Program Managers and staff on a formal as well as informal basis. And last but not least, this meeting gives you an important opportunity to learn the latest in our thinking for the continuing evolution of the Physical Biosciences program.

As has become our custom, we have lined up two exciting invited guest speakers for you. We first introduced you to the promise of neutron beamlines back in 2008 when the technology and facilities were still in their infancy. Since then capabilities and applications have exploded in this area, and Paul Langan from Oak Ridge National Laboratory (ORNL) will be providing us with a timely update in his keynote talk on Monday morning. On Tuesday morning, James Evans from the Pacific Northwest National Laboratory (PNNL) will introduce us to dynamic transmission electron microscopy (DTEM) and ultrafast x-ray diffraction for pump-probe imaging of single particle and 2-D protein crystals that can enable unprecedented spatiotemporal resolution of biological processes.

This year's meeting will also feature oral presentations from three of DOE's Energy Frontier Research Centers (EFRCs) with relevance to the Physical Biosciences program, and, of course, on many of your amazing individual research programs. Two evening poster sessions will fill out our agenda – these really provide the best opportunity to have in-depth interactions/discussions with the presenter. In keeping with the theme of this year's meeting, whether you manage one of these larger centers or lead one of these smaller "bands", we view each and every one of you as a "rock star"...

In closing, we want to express our appreciation to all of you for your many contributions to this meeting. We also wish to thank Diane Marceau and Dawn Adin from DOE-BES and Connie Lansdon and Verda Adkins-Ferber from Oak Ridge Institute for Science and Education (ORISE) for their invaluable help in planning and executing the many logistical and other tasks associated with putting on a meeting of this type. OK now – are you ready to rock and roll?

**Robert J. Stack,** Program Manager, Physical Biosciences, DOE-BES
**B. Gail McLean,** Program Manager, Photosynthetic Systems, DOE-BES
**Richard V. Greene,** Lead, Photo- and Bio-Chemistry Team, DOE-BES

# *Agenda*

# AGENDA

3rd Biennial Physical Biosciences Research Meeting
Bolger Conference Center, Potomac, MD
October 14-17, 2012

## Sunday, October 14, 2012

3:00 – 6:00 p.m. Registration
5:30 – 6:30 Reception (No Host) at the Pony Express Bar & Grill
6:30 – 8:00 Dinner at Osgood's Restaurant

## Monday, October 15, 2012

7:15 – 8:00 a.m. Breakfast at Osgood's Restaurant

*Session I:* ***Welcome and Keynote Presentation*** Stained Glass Hall

8:00 – 8:30 a.m. Welcome, DOE Updates, and Physical Biosciences Program Notes
**Robert Stack,** Program Manager, Physical Biosciences, DOE-BES

8:30 – 9:30 Neutron technologies for understanding catalysis and proton transport in biological and biologically-inspired systems
**Paul Langan,** Oak Ridge National Laboratory

9:30 – 10:00 Break

*Session II:* *Carrie Harwood, Moderator*

10:00 – 10:30 Biochemistry of Gamma Class Carbonic Anhydrases from Strictly Anaerobic Species in the Domains Archaea and Bacteria
**James G. Ferry,** Pennsylvania State University

10:30 – 11:00 Structural and mechanistic studies on the enzymes involved in microbial propylene metabolism
**John W. Peters,** Montana State University

11:00 – 11:30 Role of HydF in Hydrogenase Maturation
**Joan B. Broderick,** Montana State University

11:30 – 12:00 Computational Design of Proteins, Molecules and Materials with Novel Chemistries
**David Baker,** University of Washington, Seattle

12:00 – 1:00 Lunch at Osgood's Restaurant

1:00 – 4:00 **Free/Discussion Time** (Put up odd-numbered posters in Franklin I)

*Session III:* *Tobias Baskin, Moderator*

4:00 – 4:30 The Role of CSLD Proteins During Polarized Cell Wall Deposition in Arabidopsis Root Hair Cells
**Erik Nielsen,** University of Michigan

4:30 – 5:00 Secondary Wall Formation in Fibers
**Zheng-Hua Ye,** University of Georgia

5:00 – 5:30 Genetic and Biochemical Analysis of Xyloglucan Galactosylation in Arabidopsis
**Wolf-Dieter Reiter,** University of Connecticut

5:30 – 6:00 Role of the Plant Cell Wall in Resistance to Pathogen Attack
**Jane Glazebrook,** University of Minnesota

6:00 – 7:30 Dinner at Osgood's Restaurant

*Poster Session I*

7:30 – 9:30 Poster Session, Odd Numbered Posters (No-Host), in Franklin I

## Tuesday, October 16, 2012

7:15 – 8:00 a.m. Breakfast at Osgood's Restaurant

*Session IV:* *Bob Stack, Moderator*

8:00 – 9:00 Dynamic TEM and LCLS: New Capabilities for Visualizing Protein Structural Dynamics
**James E. Evans,** Pacific Northwest National Laboratory

9:00 – 9:30 Photodynamics of Single Biomolecules in Solution by Suppression of Brownian Motion
**William E. (W.E.) Moerner,** Stanford University

9:30 – 10:00 Break **(Take down odd-numbered posters)**

*Session V:* *Paul King, Moderator*

10:00 – 10:30 Mass Spectrometric Imaging of Plant Metabolites
**Basil Nikolau,** The Ames Laboratory and Iowa State University

10:30 – 11:00 Two-Dimensional Electronic Spectroscopies for Probing Electronic Structure and Change Transfer: Applications to Photosystem II
**Jennifer Ogilvie,** University of Michigan

11:00 – 11:30 High resolution field cycling NMR – a new tool for weak binding (e.g. osmolytes and proteins)
**Mary F. Roberts,** Boston College

11:30 – 12:00 Conformational States of Cytochrome P450 in Catalytic Cycle by Advanced Electron Paramagnetic Resonance
**R. David Britt,** University of California – Davis

12:00 – 1:00 Lunch **(Reminder: Take down odd-numbered posters)**

1:00 – 4:00 Free/Discussion Time **(Put up even-numbered posters)**

*Session VI:* *Gail McLean, Moderator*

4:00 – 4:30 The DOE Center for Plant and Microbial Complex Carbohydrates at the University of Georgia
**Alan Darvill,** University of Georgia

4:30 – 5:00 Center for Lignocellulose Structure and Formation (CLSF): A DOE-funded Energy Frontiers Research Center **
**Daniel Cosgrove,** Pennsylvania State University

5:00 – 5:30 The Center for Direct Catalytic Conversion of Biomass to Biofuels (C3Bio): Our roadmap for selective deconstruction of lignocellulosic biomass to advanced biofuels and useful co-products **
**Maureen C. McCann,** Purdue University

5:30 – 6:00 Center for Advanced Biofuel Systems (CABS): Enhancing photosynthetic energy conversion efficiency **
**Richard Sayre,** New Mexico Consortium/Los Alamos National Laboratory/Donald Danforth Plant Science Center

6:00 – 7:30 Dinner at Osgood's Restaurant

*Poster Session II*

7:30 – 9:30 Poster Session, Even Numbered Posters (No-Host), in Franklin I

## Wednesday, October 17, 2012

7:15 – 8:00 a.m. Breakfast at Osgood's Restaurant

*Session VII:* *John Shanklin, Moderator*

8:00 – 8:30 Jasmonate Hormone: Regulating Synthesis of Reduced Carbon Compounds in Plants
**John Browse,** Washington State University

8:30 – 9:00 Suppression of Photosynthesis by the Plant Stress Hormone Jasmonate
**Gregg A. Howe,** Plant Research Laboratory, Michigan State University

9:00 – 9:30 The Rhizobial Nitrogen Stress Response and Effective Symbiotic Nitrogen Fixation
**Michael L. Kahn,** Washington State University

9:30 – 10:00 CHX Transporters at Dynamic Endomembranes: Roles in pH Homeostasis Critical for Vegetative and Reproductive Success of Land Plants
**Heven Sze,** University of Maryland – College Park

10:00 – 10:30 Break **(Take down odd-numbered posters)**

*Session VIII:* *Mike Sussman, Moderator*

10:30 – 11:00 Quantitative Analysis of Central Metabolism and Seed Storage Synthesis
**Jorg Schwender,** Brookhaven National Laboratory

11:00 – 11:30 Unraveling the Regulation of Terpenoid Oil and Oleoresin Biosynthesis for the Development of Biocrude Feedstocks
**Mark Lange,** Washington State University

11:30 – 12:00 Regulation of Carbon Allocation to Phenylpropanoid Metabolism: The Role of Components of the Mediator Complex
**Clint Chapple,** Purdue University

12:00 – 1:00 Lunch **(Reminder: Make sure all posters are down)**

*Session IX:* *Bob Stack, Moderator*

1:00 – 3:00
**Robert Stack,** Program Manager, Physical Biosciences, DOE-BES
**Gail McLean,** Program Manager, Photosynthetic Systems, DOE-BES
**Richard Greene,** Lead, Photochemistry and Biochemistry, DOE-BES

** Energy Frontier Research Center-supported project

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** Energy Frontier Research Center-supported project

# *Session I*

# Neutron technologies for understanding catalysis and proton transport in biological and biologically-inspired systems

Paul Langan

Director, Biology and Soft Matter Division
Oak Ridge National Laboratory, Oak Ridge, TN
Email: langanpa@ornl.gov

Hydrogen (H) is the most common element found in biological systems where it plays a variety of roles in chemical interactions and reactions that underlie life processes. Over the years neutron crystallography has been developed as an experimental method for directly determining the location of H atoms in and around biological macromolecules, and therefore how they participate in H bonds and electrostatic interactions, how they are transferred during the chemical reactions catalysed by enzymes, and how they move during charge transport. H atoms are often difficult or impossible to see using other techniques such as X-ray crystallography, and therefore the information provided by neutrons is unique and of critical importance for understanding the chemistry in biology. In this talk, I will review how neutron crystallography is being used to help provide a detailed understanding of the catalytic mechanism of enzymes, with specific examples including human carbonic anhydrase and xylose isomerase. This information is guiding the re-engineering of these proteins so that they have improved catalytic properties.

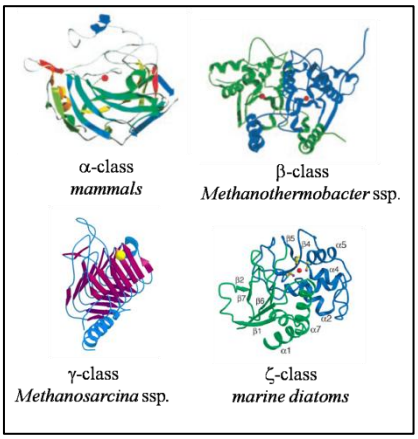
Despite the important advantages of neutrons, growth in the application of neutron crystallography to biology has been slow. A disadvantage of neutron crystallography has been the relatively low flux of available neutron beams, which requires either big crystals or very long exposure times for smaller crystals in order to have a measurable diffraction signal. Recently, the prospects for this field have changed dramatically and there has been great increase in the application of neutrons in biology. This is partly due to an increase in the number of available neutron beam lines. In 2010 there was 1 neutron beamline (the PCS at Los Alamos National Laboratory) compared to about 50 synchrotron X-ray macromolecular crystallography stations in North America. By the end of 2013 there will be 4 neutron macromolecular crystallography stations in North America including 3 new powerful beam lines at Oak Ridge National Laboratory (MaNDi, IMAGINE and TOPAZ) and 10 worldwide. However, it is also due to improvements in beamline instrumentation, neutron sources, data collection and sample preparation methods, and new approaches to and computational tools for structure determination. These advances are pushing practicable sample sizes down to fractions of a cubic mm, data collection times down to a few days or even hours, and are allowing increasingly complex biological systems to be studied.

# *Session II*

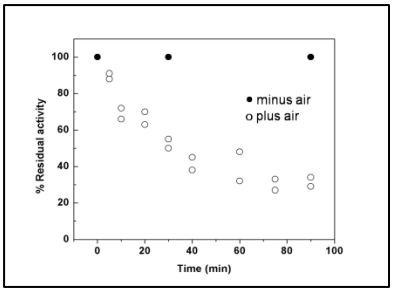
**Biochemistry of Gamma Class Carbonic Anhydrases from Strictly Anaerobic Species in the Domains *Archaea* and *Bacteria***

*James G. Ferry, P.I.*
*Department of Biochemistry and Molecular Biology*
*The Pennsylvania State University*
*University Park, Pa 16801*
jgf3@psu.edu

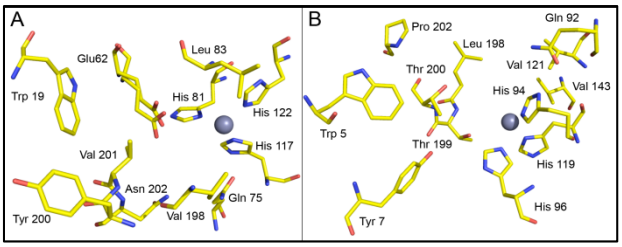
Carbonic anhydrase (CA) is a metalloenzyme catalyzing the interconversion of carbon dioxide and bicarbonate ($CO_2 + H_2O = HCO_3^- + H^+$). The great majority of studies have been conducted with CAs from mammals and plants with few studies pertaining to prokaryotic CAs, particularly for anaerobic microbes. The gamma class is one of five independently evolved classes of CA with no structural or sequence identity among them (see figure). The gamma class CA (Cam), from the strictly anaerobic methanogen *Methanosarcina thermophila*, is the founding member of the class and only one of three gamma class CA's characterized despite wide spread occurrence of homologs identified in sequenced genomes of diverse species from all three domains of life. No gamma class CA's have been characterized from the domains *Bacteria* or *Eukarya*. The prevalence of the gamma class in diverse species from the domains *Archaea* and *Bacteria* is indicative of important physiological functions and novel biochemical characteristics yet to be discovered. This study focuses on the biochemistry and physiology of gamma class CAs from strictly anaerobic microbes from the domains *Archaea* and *Bacteria*.




Cam from *M. thermophila* when over produced in the closely related methanogen *Methanosarcina acetivorans* contains ferrous iron, the first CA reported to contain iron in the active site Cam is the first CA isolated from a strictly anaerobic species raising the question of the occurrence of iron in CAs from anaerobes and if this characteristic extends into the domain *Bacteria*. Thus, the gamma class CA from anaerobe *Pelobacter carbinolicus* (domain *Bacteria*) was biochemically characterized. The enzyme produced in *Escherichia coli*, and purified anaerobically, contained an iron:zinc ratio of 23:1. The effective $k_{cat}$ (normalized to metal content) was 13.2 ± 5.2 $10^{-4}$ $s^{-1}$. The enzyme lost all activity when exposed to air (see figure) consistent with oxidation of ferrous iron to ferric that is lost from the active site. The results suggest iron is the preferred metal for catalysis by the gamma CA from *P. carbinolicus*.




The active site residues of Cam have no resemblance to any CA for which the catalytic mechanism is known except for two residues (Trp19 and Tyr200) positioned similar to Trp5 and Tyr7 important for catalysis in the human alpha class HCA II (see figure). Trp19 and Tyr200 were investigated by structural and kinetic analyses of replacement variants. Steady-state $k_{cat}/K_m$ and $k_{cat}$ values decreased 3- to 10-fold for the Trp19 variants whereas the Y200 variants showed a 5-fold increase in $k_{cat}$. Rate constants for proton transfer decreased nearly 10-fold for the Trp19 variants, and an




increase of ~2-fold for Y200F. The p$K_a$ values for the proton donor decreased ~2-fold for Trp19 and Y200 variants. The variant structures revealed a loop composed of residues 62-64 that occupies a different conformation than previously reported. The results show that, although Trp19 and Y200 are non-essential, they contribute to an extended active-site structure distant from the catalytic metal that fine tunes catalysis. Trp19 is important for both $CO_2$/bicarbonate interconversion, and the proton transfer step of catalysis. The results extend an understanding of the active site and catalytic mechanism of Cam which further contributes to a general understanding of the gamma class.

**Mechanistic insights into microbial mediated alkene and ketone metabolism**

John W. Peters, Principal Investigator
Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717
Email: john.peters@chemistry.montana.edu
Web: http://www.chemistry.montana.edu/~john.peters/

Overall research goals: The research objectives are to examine the catalytic mechanism of unique carboxylation enzymes involved in the metabolism of propylene and acetone. Studies of alkene and ketone metabolism have identified a central role for $CO_2$ and specifically $CO_2$ fixation reactions in these processes. The $CO_2$ fixing enzymes of the pathways are distinct carboxylases with unique molecular properties and cofactor requirements. We are utilizing a multidisciplinary approach involving kinetic studies, site specific amino acid substitution studies, and the determination of high resolution structures of enzymes in the presence of substrate, products, their analogs, and mechanism based inhibitors to ascertain information concerning the biochemical mechanisms of enzyme catalyzed reactions.

Significant achievements from prior support: Probably the most seminal accomplishment of the funding period is our structural characterization of 2-ketopropyl coenzyme M oxidoreductase / carboxylase (2-KPCC) with bound $CO_2$. Capturing target substrates or products can be challenging and capturing $CO_2$ at the active site of 2-KPCC has been a goal for some time but was extremely difficult. After screening numerous potential scenarios for capturing $CO_2$ at the active site, we found we were able to observe linear density consistent with $CO_2$ at the active site when crystals of 2-KPCC were soaked prior to data collection with the reaction products acetoacetate and CoM. Under these conditions it appears that we were able to catalyze the back reaction and effective decarboxylation of acetoacetate to form $CO_2$ that remained bound at the active site. Serendipitously, the additional electron density in the active site could be fit to 2-ktopropyl coenzyme M (2-KPC), also consistent with the products of the back reaction, such that the position of $CO_2$ relative to 2-KPC indicates $CO_2$ is bound in a position ideal for electrophilic attack of the proposed carbanion intermediate.

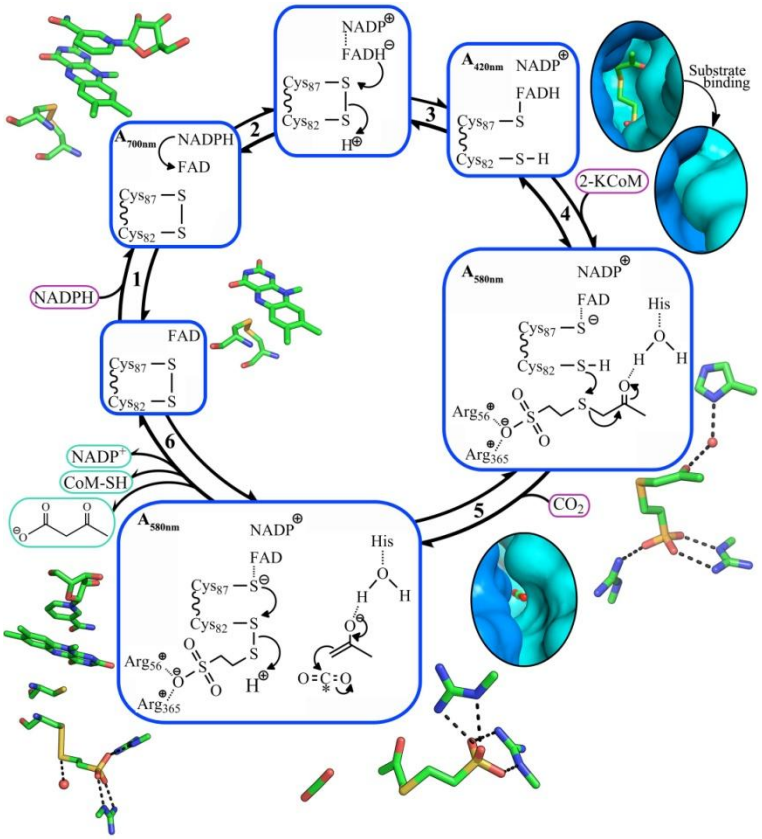



Figure 1. The reaction mechanism of 2-KPCC derived from the previously proposed mechanism and substrate-free (PDB ID 1MOK), substrate-bound (1MO9), mixed-disulfide substrate-bound (PDB ID 2C3C), and $CO_2$-bound crystal structures.

Additional accomplishments include defining the functional role of specific amino acid residues in catalysis for both 2-KPCC and the stereo selective R- and S- hydroxylpropylthioethane

sulfonate dehydrogenases and defining a new class of FAD binding proteins in the structure of AbpE.

Science objectives for 2012-2013:

- Mutagenesis studies will be conducted to examine the mechanism by which 2-KPCC discriminates between carboxylation and protonation and the production of acetoacetate versus acetone. Our working hypothesis is that encapsulation of substrate is the key determinant that enables the enzyme to preferentially discriminate between carbon dioxide and protons favoring the production of the desired product acetoacetate over the production of the unproductive protonation product acetone. We believe the key features to the encapsulation of the substrate are regions of the N- and C- termini and a *cis*-Pro flanked loop region. We will examine this hypothesis directly through mutagenesis studies selectively deleting N- and C- terminal regions as well as the unique *cis*-Pro flanked loop regions.
- XecB is a small protein (66 amino acids) of unknown function required for expression of active 2-KPCC. Our hypothesis is that XecB is involved in formation of the metal stabilized *cis*-Pro substrate binding site shielding loop is a unique feature in biology and it is our hypothesis that XecB is in some manner involved in the maturation of 2-KPCC by facilitating formation of the observed confirmation of the loop either by specific divalent metal insertion and/or Pro isomerization. XecB will be expressed and purified and the preliminary biochemical characterization will be initiated.
- A system for the controlled facile hyperexpression and purification of acetone carboxylase from *Azotobacter vinelandii* will be developed. We were very excited when it was revealed that the genome of *A. vinelandii* encoded an acetone carboxylase. Similar to 2-KPCC, it has been difficult to identify a platform by which we can express acetone carboxylase in a controlled manner such that genetic manipulations can be introduced into the protein to facilitate purification and/or probe aspects of the mechanism. We have been using *A. vinelandii* for many years as a model organism for studying structural and biochemical features of nitrogenase, the enzyme responsible for biological nitrogen fixation. The methods for efficient transformation and gene replacement are very well developed for this organism and have been used routinely in our laboratory.

References to work supported by this project in the previous funding cycle:


1. A. M. Krishnakumar, D. A. Sliwa, J. A. Endrizzi, E. S. Boyd, S. A. Ensign, J. W. Peters, "Getting a handle on the role of coenzyme M in alkene metabolism." Microbiol. Mol. Biol. Rev. **723**:445-456 (2008)
2. D. A. Sliwa, A. M. Krishnakumar, J. W. Peters, S. A. Ensign, "Molecular basis for enantioselectivity in the (*R*)- and (*S*)-hydroxypropylthioethanesulfonate dehydrogenases, a unique pair of stereoselective short-chain dehydrogenases/reductases involved in aliphatic epoxide carboxylation." Biochemistry **49**:3487-98 (2010)
3. J. M. Boyd, J. A. Endrizzi, T. L. Hamilton, M. R. Christopherson, D. W. Mulder, D. M. Downs, J. W. Peters, "FAD binding by ApbE protein from *Salmonella enterica*: a new class of FAD-binding proteins." J. Bacteriol. **193**:887-895 (2011)
4. A. S. Pandey, D. W. Mulder, S. A. Ensign, J. W. Peters, "Structural basis for carbon dioxide binding by 2-ketopropyl coenzyme M oxidoreductase/carboxylase." FEBS Lett. **585**:459-464 (2011)
5. M. A. Kofoed, D. A. Wampler, A. S. Pandey, J. W. Peters, S. A. Ensign, "Roles of the redox-active disulfide and histidine residues forming a catalytic dyad in reactions catalyzed by 2-ketopropyl coenzyme M oxidoreductase/carboxylase." J. Bacteriol. **193**:4904-4913 (2011)

**Role of HydF in Hydrogenase Maturation**

Joan B. Broderick, Principal Investigator
John W. Peters, Stephen P. Cramer, Co-PIs
Eric M. Shepard, Postdoctoral Research Associate
Department of Chemistry & Biochemistry, Montana State University, Bozeman, MT 59717
Email: jbroderick@chemistry.montana.edu, Web: http://www.chemistry.montana.edu/jbroderick/

Overall research goals: The main goal of this project is to elucidate the mechanism of maturation of the [FeFe]-hydrogenase, with a specific focus on biosynthesis of the active site H-cluster. The hydrogenase H-cluster is an unusual iron-sulfur cluster assembly that exists as a [4Fe-4S] cluster cubane bridged to a 2Fe cluster containing multiple inorganic (CO and $CN^-$) ligands as well as an exogenous bridging dithiolate ligand; the proper assembly of this H-cluster is necessary for hydrogenase activity, and is carried out by hydrogenase-specific accessory proteins. The potential for harnessing biological hydrogen production as an energy solution cannot be fully realized without a complete fundamental understanding of how the complex metal clusters at the active sites of hydrogenases function and are synthesized. The proposed studies are focused on examining the specific biochemical processes responsible for the synthesis of the H-cluster.

Significant achievements 2010-2012: Iron-sulfur cluster coordination in HydF has been probed by examining the biochemical and biophysical effects of amino acid substitution of potential ligands identified by sequence alignments and structural studies. All variants showed impaired ability to activate the hydrogenase HydA; the results of biochemical and biophysical studies implicate three cysteines and one histidine in FeS cluster binding in HydF. HydF binds both a [4Fe-4S] and a [2Fe-2S] cluster, and the four amino acid ligands identified by these studies are insufficient to bind both clusters simultaneously to a single protein monomer; thus these results suggest that HydF may function as a multimer with shared cluster coordination, a suggestion that is supported by recent gel filtration studies indicating that the dimeric form of HydF is most active in activating HydA.

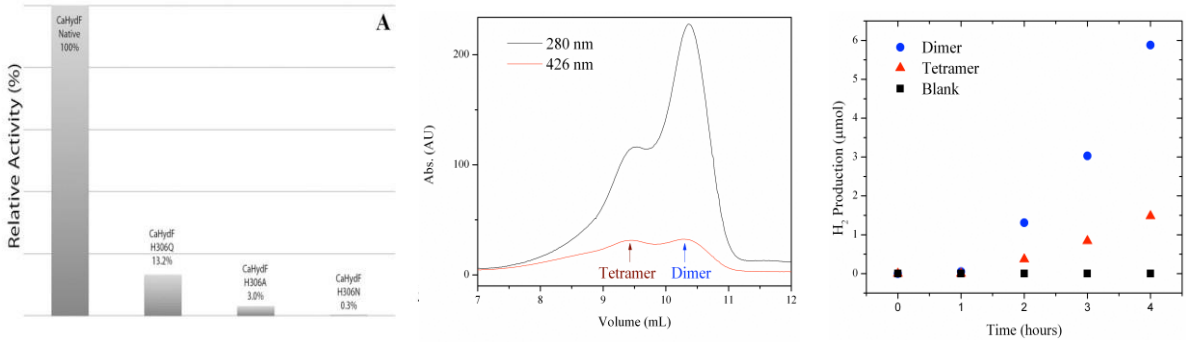



Figure 1. Left panel: Variants in which potential metal-binding amino acids were substitututed show decreased capacity to activate HydA. Most variants also showed decreased metal binding and/or altered spectroscopic properties (data not shown). Middle panel: HydF is typically found as a mixture of dimer and tetramer species, with the dimer species generally predominant. Similar distributions of dimer and tetramer were also found for the HydF variants described above. Right panel: Assays for activation of HydA by HydF demonstrate that the greatest activation ability is associated with the dimeric form of HydF. These observations, together with recent structural studies, provide insight into the most likely cluster binding scenarios in HydF.

Science objectives for 2012-2013:

- EPR and FTIR will be used to identify and characterize H-cluster assembly intermediates on HydF. Although we have intriguing EPR spectroscopic data providing clues to the order of modifications occurring to the 2Fe cluster on HydF, we have not yet been able to obtain definitive evidence for the structure of any assembly intermediates on HydF. These studies will be carried out on HydF produced in

a background of HydE alone or HydG alone, and then purified from this background and compared to the previously characterized HydF expressed the either the absence or presence of both accessory enzyme HydE and HydG.

- We will attempt to carry out *in vitro* modification of the 2Fe cluster on HydF using the purified radical SAM enzymes HydE and HydG. The substrate for HydG is known to be tyrosine, which is cleaved to form *p*-cresol and presumably either a glycine radical or dehydroglycine intermediate which is further converted to the CO and $CN^-$ ligands of the H-cluster. We will carry out the HydG reaction in the presence of HydF, and then use analytical and spectroscopic approaches to examine any modifications to HydF. We believe that we have identified the substrate of HydE as well, and so parallel experiments will be carried out with HydE. These experiments will be carried out in the presence and absence of GTP in order to better elucidate the role of GTP in H-cluster biosynthesis.
- We will pursue crystallization and structural characterization of cluster-bound forms of HydF. The recently-published structure of the metal-free form of HydF raises numerous questions regarding the nature of metal cluster binding as well as the relevance of different oligomeric states of the enzyme. HydF can be produced in different genetic backgrounds in an attempt to capture stable intermediates for structural characterization.
- ENDOR spectroscopy will be used to probe whether GTP binds to HydF in proximity to the iron-sulfur clusters. We have previously shown that GTP affects the EPR signals of the iron-sulfur clusters of HydF, yet the iron-sulfur clusters do not affect the rates of GTP hydrolysis. The question of whether there is any direct interaction between the clusters and GTP therefore remains. ENDOR spectroscopy will probe the interactions between NMR-active nuclei on GTP (including P, N, H/D, and C) with the EPR-active clusters found in reduced HydF.

References to work supported by this project 2010-2012:


1. Eric M. Shepard, Eric S. Boyd, Joan B. Broderick, and John W. Peters, "Biosynthesis of complex iron-sulfur enzymes," *Curr. Op. Chem. Biol.* **2011**, 319-327.

2. David W. Mulder, Eric M. Shepard, Jonathan E. Meuser, Neelambari Joshi, Paul W. King, Matthew C. Posewitz, Joan B. Broderick, and John W. Peters, "Insights into [FeFe]-Hydrogenase Structure, Mechanism, and Maturation," *Structure* **2011**, *19(8)*, 1038-1052.

3. Kevin D. Swanson, Benjamin R. Duffus, Trevor E. Beard, John W. Peters, Joan B. Broderick, "Cyanide and Carbon Monoxide Ligand Formation in Hydrogenase Biosynthesis," *Eur. J. Inorg. Chem.* **2011**, 935-947.

4. John W. Peters and Joan B. Broderick, "The Maturation of [FeFe]-Hydrogenases: A Paradigm for Complex Iron-Sulfur Cofactor Assembly and Insertion," *Annu. Rev. Biochem.* **2012**, *81*, 32.1 – 32.22.

5. Benjamin R. Duffus, Trinity L. Hamilton, Eric M. Shepard, Eric S. Boyd, John W. Peters, and Joan B. Broderick, "Radical AdoMet enzymes in complex inorganic metallocluster biosynthesis," *BBA – Proteins and Proteomics*, **2012**, *in press*.

6. Neelambari Joshi, Eric M. Shepard, Amanda S. Byer, Kevin D. Swanson, Joan B. Broderick, and John W. Peters, "Iron-sulfur cluster coordination in the [FeFe]-hydrogenase H Cluster biosynthetic factor HydF," *FEBS Lett.*, in revision.

7. Eric M. Shepard, Amanda S. Byer, Kevin D. Swanson, Eric S. Boyd, John W. Peters, and Joan B. Broderick, "[FeFe]-Hydrogenase H-Cluster Assembly," in *Metals in Cells, Encyclopedia of Inorganic and Biological Chemistry*, submitted.

## Computational Design of Proteins, Molecules and Materials with Novel Chemistries

David Baker, Principal Investigator
Dan DuBois, Wendy Shaw PNNL, Collaborators.
Box 357350 University of Washington, Seattle, WA 98195
Email: dabaker@u.washington.edu, Web: http://www.bakerlab.org/

Overall research goals: Computational design of hybrid protein-organometallic molecules and materials, combining the specificity, affinity and modularity of proteins with the potent chemical reactivities of molecular catalysts. We apply our methodology to the design of a wide range of peptides, proteins and small molecules, as well as interfaces between these components.

Significant achievements 2011-2012: The widely used Rosetta and RosettaDesign software has been extended to model novel protein / small molecule catalysts in which one or many small molecule active centers are supported and coordinated by protein scaffolding. Symmetric modeling and design of scaffolding has been developed and applied. Peptide sequences for organometallic coordination and material assembly have been designed, synthesized and characterized experimentally. Figures 1 and 2, display both models and experimentally determined structures of several protein and peptide designs with novel chemical topologies, enhanced functionalities, and self-assembly properties. Symmetrical outer coordination scaffolds for the DuBois nickel phosphine hydrogenase have been designed (figure 1A) and techniques for synthesis and characterization of these constructs are in development with collaborators DuBois and Shaw.

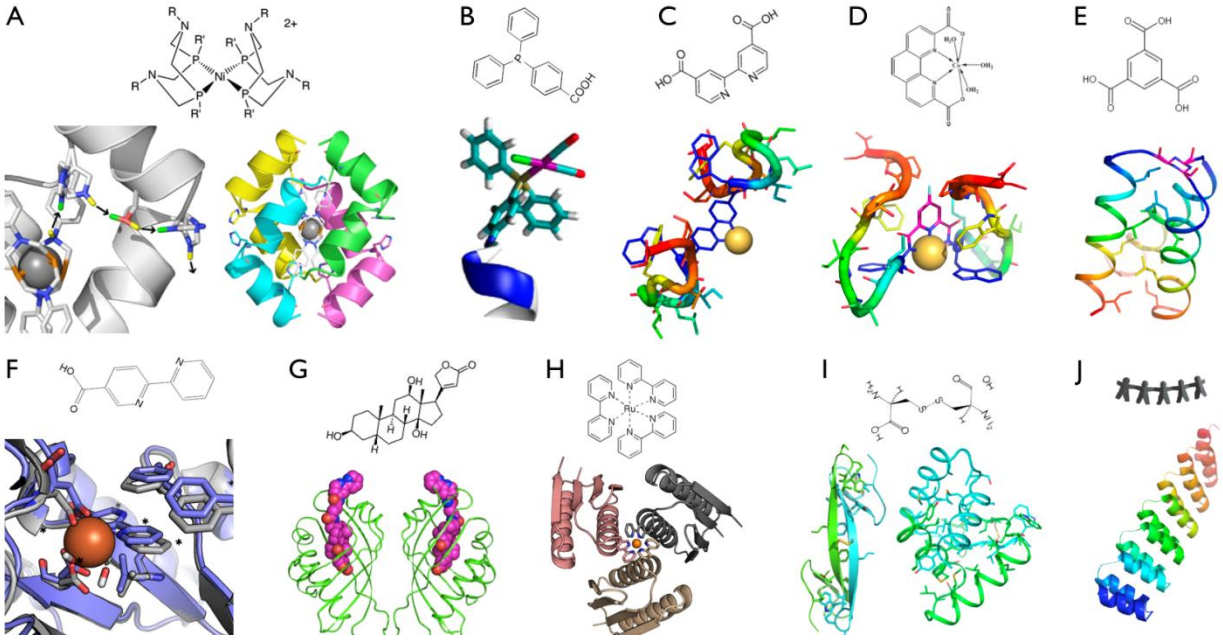



Figure 1: (A) Model of designed proton relay scaffolding P2N2 Hydrogenase Catalyst, synthesis in development. (B) rhodium-based hydrogenation catalyst attached to peptide, modeling in development. (C) NMR structure of designed dimeric bipyridine hybrid construct, NMR. (D) Structure of designed dimeric dipicolinic hybrid construct, NMR. (E) Structure of designed timeric trimesic hybrid, NMR. (F) Crystal structure of designed iron binding protein with bipyridine amino acid. (G) NMR structure of designed dimeric disulfide peptides. (H) Light Scattering model of designed tris-bipyridine trimer (I) NMR structure of denovo symmetric disulfide peptides designed with Foldit. (J) Crystal structure of peptide repeat protein.

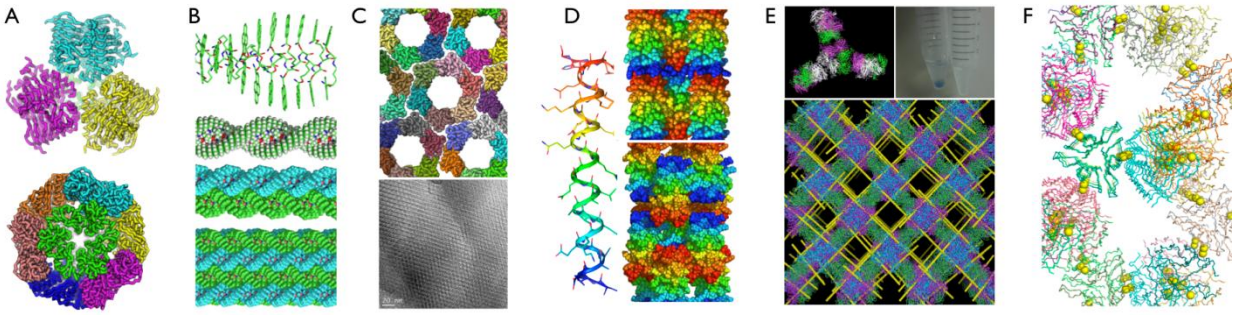



Figure 2: (A) Crystal structures of *de novo* designed tetrahedral and octahedral protein cages. (B) Model of bio-inspired pyrene wire and photo-voltaic material. (C) Model and image of Denovo designed protein surface, EM. (D) Model and image of designed catalytic protein crystal, characterization in progress. (E) Model of difulfide linked peptide crystal.

Science objectives for 2012-2013:

Integrate recently developed methodology for modeling non-natural chemistries into Foldit, allowing community driven design of peptides, chemical hybrids and symmetric complexes.

Design and characterization of more diverse metal binding peptides and proteins, most significantly peptide scaffolding to enhance and modulate the rhodium-phosphine hydrogenation catalyst of Shaw and DuBois.

Further develop and design new peptide and protein-based materials: cages, wires, surfaces and crystals. A heterogeneous self-assembly design protocol will be developed, allowing multiple components to be incorporated into integrated materials, as well as a novel method for inverse kinematic assembly of symmetric materials that is built around covalently attached organic molecules or metal binding sites.

References to work supported by this project 2011-2012:

King NP, Sheffler W, Sawaya MR, Vollmar BS, Sumida JP, André I, Gonen T, Yeates TO, Baker D., "Computational design of self-assembling protein nanomaterials with atomic level accuracy.." Science. 2012 Jun 1;336(6085):1171-4.

# *Session III*

# The role of CSLD proteins during polarized cell wall deposition In *Arabidopsis* root hair cells

**Erik Nielsen, Principal Investigator**
Department of Molecular, Cellular, and Developmental Biology
University of Michigan, Ann Arbor, MI 48109
Email: nielsene@umich.edu

**Overall research goals:** Our long term goal is to characterize the molecular machinery responsible for polarized secretion of cell wall components in *Arabidopsis thaliana*. Specific research objectives for this funding period are: (1) examine how CSLD3 function is integrated with regard to other members of the CSLD family and other cell wall synthetic enzymes (e.g. CESA cellulose synthase components), (2) determine if CSLD proteins assemble into multi-subunit complexes, and (3) characterize CSLD synthetic activity and the examine the nature of the polysaccharides synthesized by these enzymes. The results obtained from this research proposal are expected to provide fundamental insights into cellular mechanisms that are crucially important during plant development and growth, and elucidate processes that underpin the synthesis and deposition of the plant cell wall, which is an important source of plant biomass.

**Significant achievements 2009-2011:** To identify potential cargo proteins involved in cell wall synthesis in root hairs cells we had proposed to examine and compare the subcellular trafficking of CESA and CSLD proteins through the RabA4b compartment. We showed that cell walls in the tips of actively growing root hairs accumulate significant levels of cellulose-like polysaccharides, and that inhibition of cellulose synthase activity resulted in cell rupture during tip growth. Intriguingly, EYFP-CSLD3, but not fluorescently tagged CESA proteins, selectively accumulated in this tip-growing root hair cells.

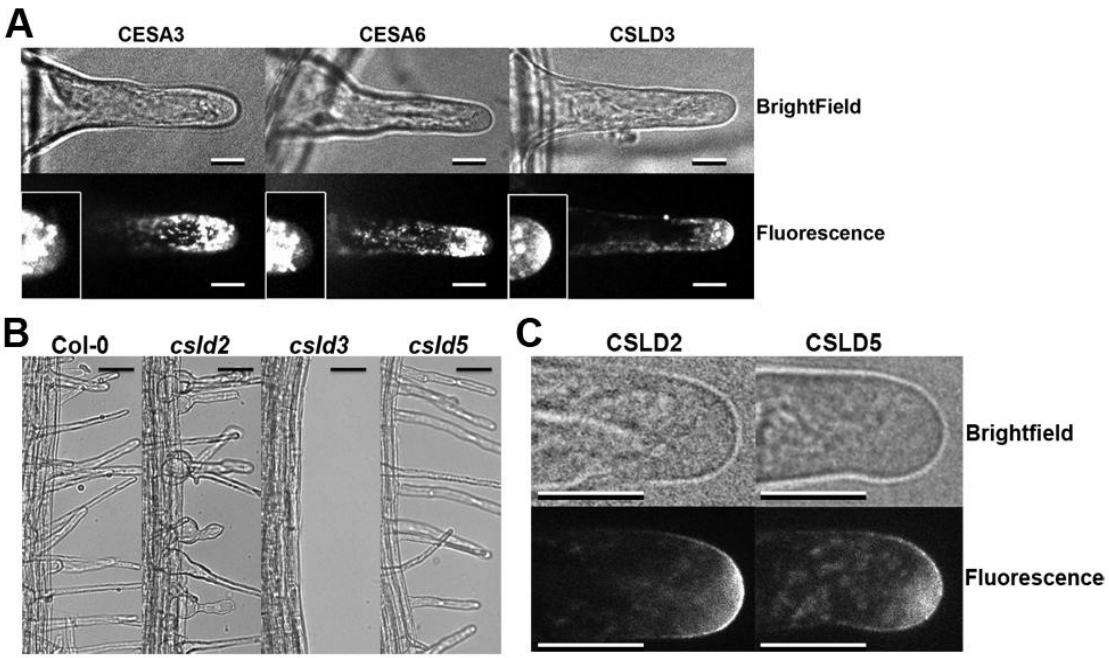



**Figure 1. Localization of fluorescent CESA and CSLD fusion proteins in growing root hair cells** (A). Medial confocal sections were collected from root hair cells of *A. thaliana* stably expressing EYFP-CESA3 (left), EGFP-CESA6 (middle), and EYFP-CSLD3 (right) using brightfield (upper panels) or spinning-disk confocal microscopy and the appropriate excitation and emission filter sets (lower panels; bars = 10 um). Inset boxes represent expanded images to show details of subcellular localization in root hair tips. (B) Brightfield images of wild type (Col-0), *csld2*, *csld3*, and *csld5* mutant root hairs (bars = 50 um). (C) Apical membrane localization of EYFP-CSLD2 and EYFP-CSLD5 fusions in rescued *csld2*, and *csld5* mutant plants (bars = 10 um).

**Science objectives for 2011-2012:**

**Examine the functional relationship between CSLD family members in tip-growing cells.** We will use functional fluorescent fusions of CSLD proteins, T-DNA insertional mutants of CSLD genes, and transgenic tools to examine the functional redundancy within this gene family.

**Determine if CSLD proteins organize into multi-subunit complexes.** We will use doubly marked fluorescent CSLD2, CSLD3, and CSLD5 fusions along with photoactivation, and FRAP methods to determine if different CSLDs organize into discrete fluorescent structures in apical root hair membranes. In addition, we will use co-immunoprecipitation methods to characterize CSLD protein complexes form detergent-solubilized membranes.

**Determine CSLD functions in tissues and cell types other than tip-growing cells.** CSLD genes are required for normal tip-growth in root hairs and pollen tubes, but whether these plants have defects in other tissues is not known. We will use genetic analysis and biochemical cell wall fractionation methods to examine whether CSLD proteins play roles during cell wall deposition in cells and tissues other than previously characterized roles in tip-growing root hairs and pollen tubes.

**Identification of the cell wall polysaccharide class that CSLD proteins synthesize.** We will use photo-affinity labeling methods to determine whether UDP-glucose or other sugar nucleotides are substrates for CSLD proteins. Cell wall fractionation and immuno-electron microscopy will be used to examine nature and type(s) of cell wall polysaccharides CSLD proteins synthesize in tip-growing root hair cells.

**References to work supported by this project 2009-2011:**


1).Park, S., Szumlanski, A.L., Gu, F., Guo, F., and **Nielsen, E.** (2011) A role for CSLD3 during cell wall synthesis in apical plasma membranes of tip-growing root hair cells. *Nature Cell Biol.,* ***13,*** 973-80.

2).Munnik, T., and **Nielsen, E.** Green light for polyphosphoinositide signals in plants. (2011) *Curr. Opin. Plant Biol.,* ***14,*** 489-97.

3).Kang, B-Y[†], **Nielsen, E**.[†], Preuss, M., Mastronarde, D., and Staehelin, L.A. (2011) Electron tomography of RabA4b and PI-4Kb1 labeled trans-Golgi network compartments in Arabidopsis. *Traffic,* ***12***, 313-29. ([†]co-first and co-corresponding author)

4).Szumlanski, A., and **Nielsen, E.** (2009) The *Arabidopsis thaliana* Rab GTPase, RabA4d, is required for the regulation of pollen tube tip growth. *Plant Cell*, **21**, 526-544.

5).Vermeer, J.E.M., Thole, J.M., Goedhart, J., **Nielsen, E.**, Munnik, T., and Gadella Jr., T.W.J. (2009) Imaging phosphatidylinositol 4-phosphate dynamics in living plant cells. *Plant J.*, **57**, 356-372.

6).Szumlanski, A., and **Nielsen, E.** (2009) Phosphatidylinositol 4-phosphate is required for tip growth in Arabidopsis thaliana. *In* Munnik, T., (ed.) Lipid Signaling in Plants, Heidelberg, Germany: Springer-Verlag, pp. 65-77.

7).Preuss, M.L., Wiedman, P., and **Nielsen, E**. (2009) *In* Nava Segev (ed.), Protein Trafficking: Mechanisms and Regulation, Austin, Texas, USA: Landes Bioscience, 15-41.

## Secondary Wall Formation in Fibers

Zheng-Hua Ye, Principal Investigator
Department of Plant Biology, University of Georgia, Athens, GA 30602
Email: zhye@plantbio.uga.edu; Web: www.plantbio.uga.edu/~zhye/zhye.html

Overall research goals: The goal of the DOE-funded project is to study the functional roles of glycosyltransferases in xylan biosynthesis during secondary wall formation. Xylan in dicots (typically called glucuronoxylan) is composed of a linear backbone of β-1,4-linked xylosyl residues, about 10% of which are substituted with α-1,2-linked glucuronic acid and/or 4-*O*-methylglucuronic acid residues. Although a number of genes have been implicated in xylan biosynthesis in genetic studies, biochemical assignment of their enzymatic activities is largely lacking. Because the presence of xylan in cellulosic biomass has been shown to hinder the efficiency of conversion of biomass into bioethanol, further understanding of how xylan is made will not only contribute to our knowledge of cell wall biosynthesis in general but also have important economic and agronomic implications, such as providing genetic tools for custom-designing cell wall composition tailored for biofuel production.

Significant achievements for 2009-2012: We have found that three Arabidopsis glycosyltransferases belonging to family GT8 are glucuronyltransferases involved in the addition of glucuronic acid side chains onto the xylan backbone. Simultaneous mutations of these three GT8 glycosyltransferase genes result in a complete loss of glucuronic acid side chains on xylan (Fig. 1). Enzymatic assay demonstrated that these three GT8 members exhibit xylan glucuronyltransferase activity. We have found that GT43 glycosyltransferases from both Arabidopsis and poplar possess xylan xylosyltransferase activity when expressed in tobacco BY2 cells, indicating that they are part of the long- sought xylan synthase. Co-expression of two functionally non-redundant GT43 members confers a high level of xylosyltransferase activity that is capable of transferring multiple xylosyl residues onto xylooligomer acceptors, thus generating β-(1,4)-linked xyloligosaccharides (Fig. 3). This represents an important advance in our understanding of the biochemical mechanism underlying xylan biosynthesis. We performed comprehensive genetic and functional studies of four *Arabidopsis REDUCED WALL ACETYLATION* (*RWA*) genes and demonstrated their involvement in the acetylation of xylan during secondary wall biosynthesis. We have extended our findings on xylan biosynthesis in Arabidopsis to unravel the biosynthesis of xylan during wood formation in a tree species. We have demonstrated that the xylan content could be manipulated by alteration of the expression of a GT47 gene and that xylan modification could improve the digestibility of wood cellulose by cellulase. Because wood is the most important raw material for traditional forest products and can potentially be used for biofuel production, our findings have significant biotechnological implications.

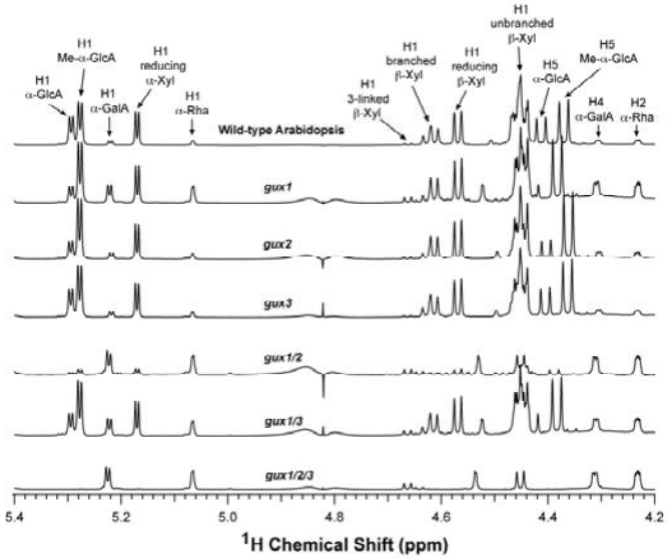


Figure 1. Structural analysis of xylan from the wild type and the *gux* mutants by NMR spectroscopy. Xylooligosaccharides generated by B-endoxylanase digestion of alkaline-extracted xylan were subjected to $^{1}$H-NMR analysis. Resonances are labeled with the position of the assigned proton and the identity of the residue containing that proton. The resonances of H1 of α-D-GalA, H1 of α-L-Rha, H1 of 3-linked B-D-Xyl, H4 of α-D-GalA, and H2 of α-L-Rha are from the xylan reducing end tetrasaccharide sequence. Note the complete loss of resonances of α-GlcA and Me-α-GlcA in the *gux1/2/3* triple mutant compared with the wild type.

Science objectives for 2012-2013:

In addition to our continuous efforts in investigating biochemical functions of xylan-associated GT43 and GT8 members, we are also investigating other groups of enzymes that are involved in the biosynthesis of xylan structure. We are in the process of generating mutants for these genes of interest for their functional characterization. We expect that our work will lead to a better understanding of the biosynthesis of xylan, one of the major wood components.

References to work supported by this project 2009-2012:


1. Lee, C., Teng, Q., Huang, W., Zhong, R., and Ye, Z.-H. (2009) Downregulation of PoGT47C expression in poplar results in a reduced glucuronoxylan content and an increased wood digestibility by cellulase. **Plant Cell Physiol.** 50, 1075-1089.
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6. Lee, C., Teng, Q., Zhong, R., and Ye, Z.-H. (2011). Molecular dissection of xylan biosynthesis during wood formation in poplar. **Mol. Plant** 4, 730-747.
7. Lee, C., Teng, Q., Zhong, R., and Ye, Z.-H. (2011). Four Arabidopsis RWA genes are specifically expressed in secondary wall-containing cells and required for the acetylation of xylan. **Plant Cell Physiol.** 52, 1289-1301.
8. **8.** Zhong, R., Lee, C., McCarthy, R.L., and Ye, Z.-H. (2011). Transcriptional activation of secondary wall biosynthesis by rice and maize NAC and MYB transcription factors. **Plant Cell Physiol.** 52, 1856-1871.
9. Lee, C., Zhong, R., and Ye, Z.-H. (2012). Arabidopsis family GT43 members are xylan xylosyltransferases required for the elongation of the xylan backbone. **Plant Cell Physiol.** 53, 135-143.
10. **10.** Lee, C., Zhong, R., and Ye, Z.-H. (2012). Biochemical characterization of xylan xylosyltransferases involved in wood formation in poplar. **Plant Signal. Behav.** 7, 332-337.
11. Lee, C., Teng, Q., Zhong, R., and Ye, Z.-H. (2012). Arabidopsis GUX proteins are glucuronyltransferases responsible for the addition of glucuronic acid side chains onto xylan. **Plant Cell Physiol.** 53, 1204-1216.

## Genetic and Biochemical Analysis of Xyloglucan Galactosylation in Arabidopsis

Wolf-Dieter Reiter, Principal Investigator
Department of Molecular and Cell Biology, University of Connecticut,
75 North Eagleville Road, Storrs, CT 06269
Email: wdreiter@uconn.edu; Web: http://reiter.uconn.edu

Overall research goals: Our research objectives are to determine the rate-limiting steps in galactose attachment to the backbone of the hemicellulose xyloglucan. The main experimental approaches are as follows: (1) increase the availability of the predicted donor substrate UDP-galactose by overexpressing UDP-glucose 4-epimerase in the cytosol or the endomembrane system of Arabidopsis plants, (2) characterize the biochemical properties of known or predicted xyloglucan galactosyltransferases from Arabidopsis by recombinant expression of the respective proteins in *Pichia pastoris* followed by enzyme assays with a variety of acceptor substrates, and (3) overexpress these proteins in Arabidopsis either individually or in combination under the control of a strong constitutive promoter. Xyloglucan from the transgenic lines will then be analyzed for changes in the degree of galactosylation by mass spectrometry and NMR of enzymatically generated oligosaccharides. We hope that this approach will provide insights into the mechanisms by which monosaccharides from nucleotide sugars are allocated to the synthesis of specific cell wall components. Furthermore, the transgenic plants may display interesting changes in cell wall properties since structural changes in xyloglucan are expected to affect the mechanical strength of xyloglucan-cellulose interactions.

Significant achievements 2011-2012: (1) The putative galactosyltransferase MUR12 (a.k.a. AtGT18) was shown to transfer radiolabeled galactose from UDP-galactose to specific xylose residues in the seed storage xyloglucan from *Hymenaea courbaril*. Considering that the recombinant protein was obtained in a yeast expression system, the enzyme clearly acted as a *bona fide* galactosyltransferase without the need for plant-derived cofactors. Somewhat surprisingly, the MUR12 protein converted XXLG subunits to XLLG but did not produce XLXG from XXXG. In enzyme assays conducted in parallel to those described above, recombinant MUR3 protein converted XXXG to XXLG but did not convert XLXG to XLLG. Both MUR3 and MUR12 used the *Hymenaea*-specific building block XXXXG as an acceptor substrate with the formation of XXXLG and XLXXG, respectively. (2) Subcellular localization studies demonstrated targeting of both MUR3 and MUR12 to the Golgi; however, co-localization of the two proteins was partial rather than complete. (3) Homozygous Arabidopsis plants overexpressing both MUR3 and MUR12 were generated from previously characterized lines, and are now ready for analysis of their xyloglucan structure.

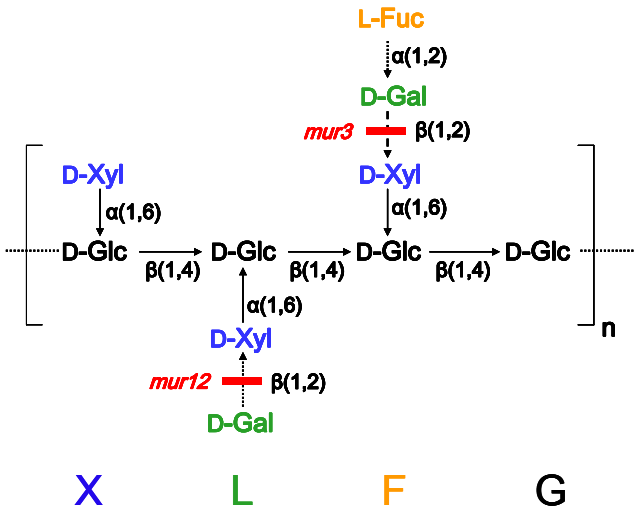


**Figure 1.** Structure of a xyloglucan building block with complete decoration of the XXXG core subunit. In naturally occurring xyloglucans, the galactosylation is partial rather than complete. In *mur3* knockout plants, the α-L-Fuc-(1→2)-β-D-Gal side chain is undetectable whereas galactosylation of the central xylose residue is absent in *mur12* plants. *mur3 mur12* double mutants contain only the unsubstituted xyloglucan core structure.

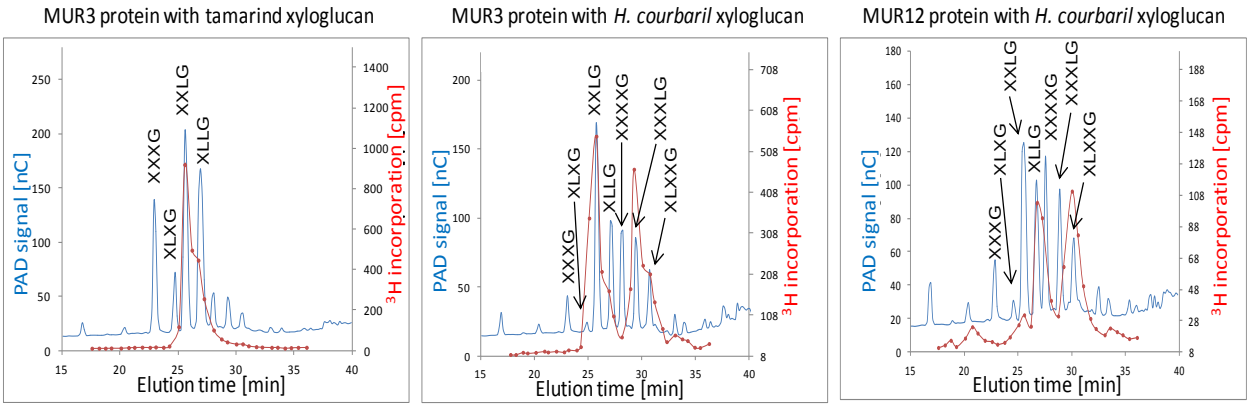


**Figure 2.** Results from *in vitro* enzyme assays using UDP-α-D-[6-$^{3}$H]galactose as the donor substrate, and intact seed storage xyloglucan from *T. indica* or *H. courbaril* as the acceptor substrate. After digestion with an endoglucanase, the xyloglucan-derived oligosaccharides were separated by HPLC, and the amount of radiolabel in the individual fractions was quantified via liquid scintillation counting. The HPLC trace is shown in blue, and the abundance of $^{3}$H is shown in red.

Science objectives for 2013:

- With the availability of Arabidopsis plants overexpressing both MUR3 and MUR12, we plan to determine the structure of their xyloglucan via MALDI-TOF MS and NMR. The transgenic plants will also be evaluated for visible phenotypes.
- Yeast and Arabidopsis lines that overexpress cytosolic and Golgi-targeted versions of UDP-glucose 4-epimerase will be evaluated for changes in the abundance of this enzymatic activity. To achieve an adequate degree of sensitivity, we have developed a novel assay system in which UDP-α-D-[UL-$^{13}C_6$]galactose serves as the substrate, and the formation of $^{13}$C-labeled UDP-glucose is quantified by GC-MS of the corresponding alditol acetate. Sub-microgram quantities of labeled sugars can easily be detected by this method.
- If the above enzyme assays lead to promising results, the transgenic Arabidopsis lines will be analyzed for the galactose content of total cell wall material and xyloglucan-derived oligosaccharides. If no meaningful increase in UDP-glucose 4-epimerase activity is observed, we may have to modify the tag attached to the recombinant proteins. In this case, we will use a codon-optimized synthetic gene rather than the wild-type version in an effort to maximize the efficiency of protein synthesis.

## Role of the Plant Cell Wall in Resistance to Pathogen Attack

Jane Glazebrook, Principal Investigator
Gerit Bethke, Research Associate
Department of Plant Biology, University of Minnesota,
1445 Gortner Ave. Rm 250 BioSciences Center, Saint Paul, MN 55108
Email: jglazebr@umn.edu

Overall research goals: Plant cell walls are thought to present a physical barrier to attack by microbial pathogens. In addition to this structural effect, plants may sense changes in cell wall structure as indicators of pathogen attack. Yet, relatively little is known about the roles of plant cell walls in disease resistance. Previously, we have screened *Arabidopsis* thaliana mutants with altered cell walls for changes in disease resistance. Presently, we are working to understand how mutations impacting cell wall structure affect resistance. Specific goals are (1) Create multiply-mutant plant genotypes with more severe cell wall structure and disease phenotypes, (2) Determine the nature of perturbations in the plant defense system responsible for altered disease phenotypes in cell wall mutants and (3) Test the hypothesis that Wall Associated Kinases (WAKs) link certain cell wall changes to activation of defense responses.

Significant achievements 2010-2012: We found that mutations in several different pectin methylesterase genes (PMEs) caused small but statistically significant increases in susceptibility to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* strain ES4326 (*Pma* ES4326). Plants with mutations in two or more *PME* genes allow approximately three-fold more bacterial growth than wild-type plants, indicating a positive role for *PME* genes in resistance. Using a gel-diffusion assay for PME activity, we have found that activity increases greatly in plants infected with *Pma* ES4326 or a necrotrophic fungal pathogen, *Alternaria brassicicola*. This increased activity does not occur in plants with defects in jasmonic acid signaling (*dde2*), a major sector of the plant defense signaling network. Defects in other major defense signaling sectors, including salicylic acid (*sid2*, *pad4*) and ethylene (*ein2*), do not affect PME activity. Using antibodies specific for methylesterified or demethylesterified pectin, we found that the degree of methylesterification declines over the course of infection by either *Pma* ES4326 or *A. brassicicola.*

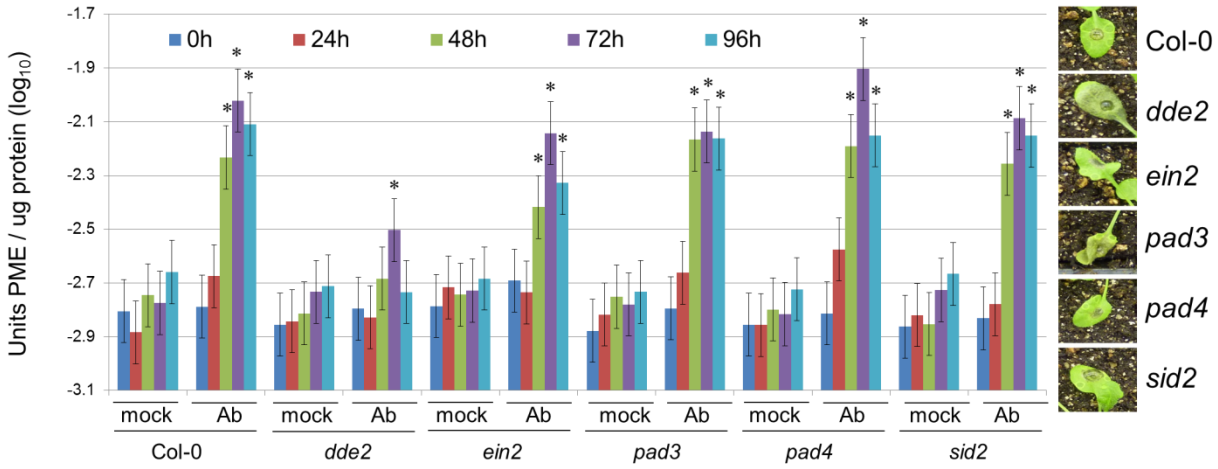


Figure 1. Total PME activity was determined in the indicated genotypes at various times after infection with *A. brassicicola.* Each bar represents the mean and standard error of 2 independent replicates, each with three technical replicates, calculated using a mixed linear model. Asterisks indicate significant differences from mock ($q<0.01$). Images at the right show plants 96 h after infection.

Science objectives for 2012-2014:

- Further explore the differences in pectin methylesterification in PME mutant lines vs. wild-type plants, before and during infection. This could be done using specific antibodies and/or by FTIR.
- T-DNA insertion lines in 4CL, UXS and GAE genes showed small yet significant changes in susceptibility towards *Pma* ES4326. We have created double and multiply-mutant lines for these gene families. We will test these for altered pathogen susceptibility. Any genotypes with strong phenotypes will be studied further by expression profiling. We will also attempt to discover how the cell wall is altered in the mutant lines.

References to work supported by this project 2010-2012:


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# *Poster Session I*

## Hyperthermophilic Multiprotein Complexes and Pathways for Energy Conservation and Catalysis

Michael W. W. Adams, Principal Investigator
Department of Biochemistry & Molecular Biology, University of Georgia, Athens, GA 30602
Email: adams@bmb.uga.edu; Web: http://adams.bmb.uga.edu/

Overall research goals: The focus of this research is non-covalent multi-protein complexes that are involved in novel mechanisms of energy conservation and catalysis. The protein complexes under study have the remarkable property of being synthesized (self-assembling) at temperatures near 100°C in so-called hyperthermophilic microorganisms. Moreover, they are involved in the conversion of low potential reducing equivalents into gaseous end products with concomitant energy conservation in the form of ion gradients. Conversion of low potential reductant to a useable form of energy is a fundamental issue in all reaction systems that utilize light to produce biofuels.

Significant achievements in 2010-2012: The model organism for these studies is *Pyrococcus furiosus* (Pf), an archaeon that grows optimally at 100°C. Pf obtains carbon and energy for growth by fermenting carbohydrates and producing $H_2$ and by reducing elemental sulfur (S°) to $H_2S$. It has a respiratory metabolism in which it couples $H_2$ production by a ferredoxin-dependent, membrane-bound hydrogenase (MBH) to ion translocation and formation of a membrane potential that Pf utilizes to synthesize ATP (Figure 1). Pf also contains a cytoplasmic hydrogenase (SHI) that has the rare property of evolving $H_2$ from NADPH, a reaction of utility in $H_2$ production systems (Figure 1). Addition of S° to Pf prevents the synthesis of MBH and SHI, and induces the synthesis of a highly homologous membrane complex which we term MBX. MBX is proposed to oxidize ferredoxin, reduce S° and conserve energy by an as yet unknown mechanism. The specific aims of the proposed research are to characterize the novel energy-conserving complexes MBH and MBX, and to structurally characterize native SHI and minimal forms of four-subunit SHI and fourteen-subunit MBH. Over the past two years we have taken advantage of the recently developed genetic system in Pf and have obtained the genome sequence of the competent strain. We have engineered Pf to over-produce an affinity-tagged holoenzyme form of SHI and a tagged two-subunit form that directly interacts with a pyruvate-oxidizing enzyme without an intermediate electron carrier. An affinity-tagged version of MBH has also been constructed but yields of the detergent-solubilized protein, which apparently contained all 14 subunits, was low. Deletion strains of Pf lacking SHI, MBH and MBX have been constructed and characterized, along with strains lacking NSR and SipA, two additional enzymes involved in elemental sulfur metabolism. An extensive phylogenetic and bioinformatic analysis of MBH and MBX revealed that they are modular in nature and represent ancestral respiratory complexes. The results of this research will provide a fundamental understanding of how the metabolism of S° and $H_2$ leads to energy conservation (MBH and MBX) in Pf using novel catalytic mechanisms, and the structure and function of $H_2$-activating enzymes.

Science objectives for 2012-2013:

- To optimize production of detergent-solubilized affinty-tagged forms of fourteen-subunit MBH and thirteen-subunit MBX for structural and biochemical characterization.
- To obtain a five-subunit form of MBH and a single-subunit form of SHI for structural and biochemical characterization.

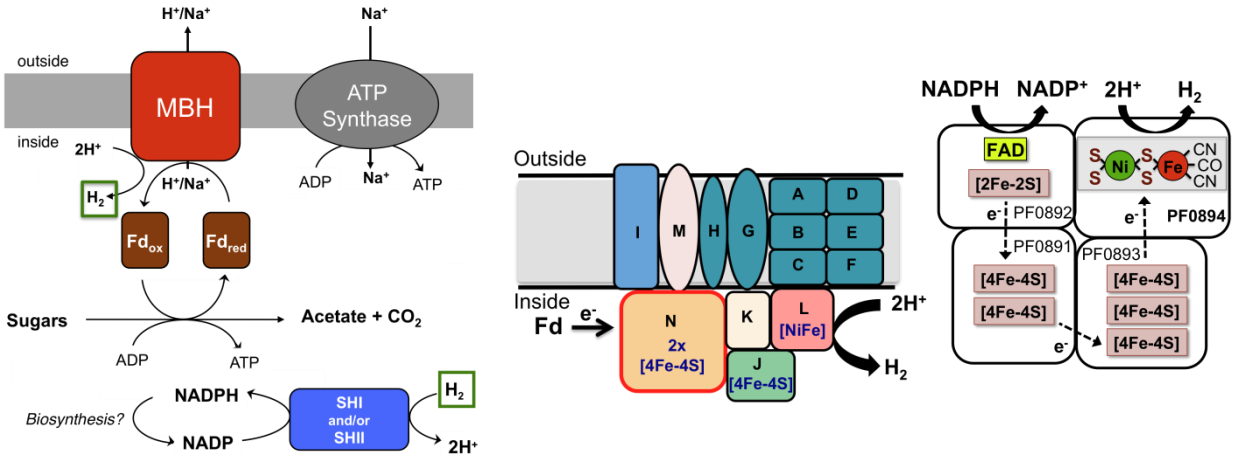


**Figure 1.** Roles of hydrogenases in *P. furiosus* (*left*) and proposed subunit organization, cofactor content and electron flow pathway in fourteen-subunit MBH (*center*) and four-subunit SHI (*right*).


References to work supported by this project 2011-2012:

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## Cellulose Synthesis and the Control of Growth Anisotropy

Tobias I. Baskin, Principal Investigator

Karen Osmont, Senior Research Fellow
Biology Department, 611 N. Pleasant St., University of Massachusetts, Amherst, MA, 01003
E-mail: baskin@bio.umass.edu; Web: http://www.bio.umass.edu/biology/baskin/

Overall research goals: The major research goal is to understand the way in which cellulose controls the anisotropic expansion of plant organs. To reach this goal, the project takes advantage of the new model grass species, *Brachypodium distachyon*. The project objectives for 2012-2015 are to accomplish the following specific aims:

**1.** Characterize root morphology mutants in *B. distachyon*.

**2.** Characterize variability in *B. distachyon* accessions for root morphology and cellulose synthesis rate.

**3.** Use reverse-genetic approaches to study the function of *B. distachyon* genes suspected to be important in cellulose synthesis.

**4**. Develop immuno-gold methods for SEM to characterize cell wall architecture.

Significant achievements 2010-2012: The main thrust over the first three years of this project has been establishing techniques for *B. distachyon*, a new system for the PI, and to accomplish objectives similar to the above. We have screened nearly 5,000 M2 families and obtained putative root morphology mutants. Additionally, we have mapped highly significant quantitative trait loci for root elongation rate.

**Forward genetics (aim 1):**

We developed a forward-genetic screen to identify conditional alleles in loci affecting root morphology. Because insertion knockouts are often lethal, we used chemical mutagenesis (EMS). We mutagenized the Bd21 line, bulked up nearly 5,000 M2 families and have screened them all, using ~12 seeds per family to check for segregation. For screening, we have developed conditions to find temperature-dependent root swelling because constitutive loss-of-function could be lethal. About 200 families are screened at one time, and great care is needed to ensure sterility and uniform germination. To increase mutant yield, we have subjected most of these families to a secondary screen for resistance to the cellulose synthesis inhibitor, isoxaben. We prioritize those in which the phenotype is segregating in the screened F2 family. So far, we have bulked up numerous mutants with swelling, isoxaben resistance, and other phenotypes such as root-hair-less and bifurcation.

**Natural variability (aim 2):**

In screening various *B. distachyon* lines held at UMass, we noticed that two well known lines, Bd21 and Bd3-1, differ in root elongation rate by a factor of three. Fortuitously, these are the parents of a set of ~160 genotyped recombinant inbred lines. We obtained these lines from the Garvin lab and screened them for root elongation rate. By repeating this experiment three times, we have identified four highly significant quantitative trait loci, accounting for considerable variation in root elongation rate. Now we are screening for cellulose synthesis rate, using a streamlined assay based on uptake of radio-labeled glucose into acid-insoluble cell wall.

**Reverse genetics (aim 3):**

For reverse genetics, we began with CESA genes. We determined which ones are actively expressed in roots and designed artificial microRNA (amiRNA) constructs to silence them, singly and in combination. Unfortunately, despite many attempts, we have not been able to obtain plants with the desired gene suppression. It appears that the amiRNA approach has

likewise proven to be difficult for other labs. Instead, we have started to build silencing constructs based on inverted repeats. Furthermore, because transformation involves selection on callus, where silencing might impose an undue burden, we are driving constructs with an inducible promoter. Additionally, in working with the *B. distachyon* CESA gene family, we have set up a screen for functional complementation, where we transform *Arabidopsis thaliana* CESA mutants with putative orthologs from *B. distachyon*, driven by the native *A. thaliana* promoter. These experiments are on-going, but so far we have evidence that two gene pairs can be considered true orthologs based on at least partial complementation. Finally, we are also developing single-cell methods to allow assays that bypass transforming and regenerating plants.

Science objectives for 2013-2014: For forward genetics, as true breeding lines are in hand, we will analyze them genetically and physiologically, focusing on microtubules and microfibrils.
For reverse genetics, we will document the inducible promoter system and use it to drive the loss of function of specific genes, such as CESAs, cobra, or korrigan, known to be essential for controlling growth anisotropy in *A. thaliana*. Parallel assays in single cells should also be accomplished. For natural variability, we will determine to what extent cellulose synthesis rate varies and possibly map the trait. Finally, we will develop procedures for high resolution scanning electron microscopy where specific structures are labeled with gold-conjugated probes.

**Amidase Mediated Modulation of N-Acylethanolamine (NAE) Signaling**

Kent Chapman, Principal Investigator
Elison Blancaflor, Co-Principal Investigator
University of North Texas, Department of Biological Sciences, Denton, TX 76203
Samuel Roberts Noble Foundation, Plant Biology Division, Ardmore OK 73401
Email: chapman@unt.edu; eblancaflor@noble.org
Web: http://www.biol.unt.edu/~chapman http://www.noble.org/staff/blancaflor-elison/

Collaborators: Dr. Barney J. Venables, University of North Texas, Denton, TX
Dr. Ruth Welti, Kansas State University, Manhattan, KS
Dr. Peter Koulen, University of Missouri, Kansas City, Kansas City, MO
Dr. Ivo Feussner, Georg August University, Gottingen, Germany

Overall research goals: Research in our labs, supported since 2005 by Basic Energy Sciences, has led to the discovery of a new lipid mediator pathway that influences phytohormone regulation of plant growth and development—the so-called *N*-acylethanolamine (NAE) regulatory pathway. This pathway in plants shares conserved metabolic machinery with the endocannabinoid signaling system of vertebrates that regulates a plethora of physiological and behavioral processes in mammals, suggesting that the metabolism of NAEs is an important regulatory feature of eukaryotic biology. The current evidence in plants points to interaction between NAE metabolism and ABA signaling, and here we focus our efforts mainly on the transition from embryonic development to seedling establishment and the acquisition of photoautotrophic growth. Our main hypothesis is that fatty acid amide hydrolase (FAAH), a member of the amidase superfamily of proteins, influences plant growth by both its catalytic hydrolysis of endogenous bioactive NAEs and through its interaction with proteins involved in RNA metabolism. Evidence suggests that these bifurcating actions of FAAH are central to modulating ABI3 transcript levels, a key transcription factor regulating the embryo-to- seedling transition, and thereby provides a connection between NAE metabolism and ABA signaling in the regulation of seedling growth. Three specific aims are proposed:

- (1) Delineate the mechanisms by which FAAH1 interacts with proteins to modulate ABI3 transcript levels independent of its catalytic activity toward NAEs.
- (2) Attribute specific effects of polyunsaturated NAE species on aspects of seedling growth—Ca2+ signaling in root cells, root elongation, and chloroplast development in cotyledons.
- (3) Examine the impact of FAAH2 on the interaction between ABA signaling and NAE metabolism.

Significant achievements in 2010-2012: Pharmacological approaches by application of NAEs to plants have generally supported a role for these compounds as negative regulators of growth, especially in seedlings. Evidence indicates that ectopic overexpression of this FAAH1 in *Arabidopsis* leads to enhancement of overall plant size, resulting in part from increased cell size/expansion. Interestingly, the enhanced growth phenotype also is associated with marked hypersensitivity to abscisic acid (ABA) and to a number of abiotic and biotic stresses. Probing a mechanistic explanation for these different activities of FAAH1 using site-directed mutants, we discovered that growth regulation is attributed to its enzymatic activity whereas hypersensitivity to stress and phytohormones (ABA) is independent of catalytic activity, with FAAH1 perhaps via protein-protein interactions. Two candidate interactors with FAAH1were identified through a yeast-two hybrid screen and interaction in vitro occurs specifically through an arginine rich motif near the C-terminus. This motif is conserved in other plant FAAH proteins.

Separately we have identified a second candidate NAE amidase, designated FAAH2. Disruptions in this gene locus results in seedlings with increased NAE sensitivity (like faah1 knockouts) although not as severe as with disruptions in *FAAH1*, suggesting some partial redundancy of these two NAE amidases. In addition to the hydrolysis of NAEs by FAAH enzymes, polyunsaturated NAEs can be oxidized by the lipoxygenase (LOX) pathway and we have recently developed methods to document the formation of novel NAE oxylipins derived from NAE18:2 and NAE18:3 in Arabidopsis seedlings. Our evidence suggests that FAAH and LOX cooperate to metabolize endogenous NAEs and we are investigating the potential function of NAE18:3 oxidation metabolities in chloroplast development. This regulation may be more complex than appreciated because we also discovered that NAE12.0 is a potent competitive inhibitor of plant

LOX enzymes and may modulate plant growth and development through the regulation of oxylipin formation.

In other work, we are using targeted and untargeted approaches to identify the functional role(s) of NAE metabolism in plants, and these include screens for allosteric regulators of FAAH1 activity, chemical genomics and forward genetics. Overall, our work continues to point to a complex regulatory interaction between NAE metabolism and plant growth regulation and responses to environmental stress. The molecular and biochemical tools that we have developed through support of DOE should help enable us to unravel this lipid regulatory pathway in the coming years.

Science objectives for 2012-2013:

- We will continue to examine the mechanisms by which FAAH proteins participate in the regulation of plant growth, development and responses to environmental stresses. We are following up with FAAH1 domain-specific protein interactions and its relevance in planta. In other experiments, synthetic analogues of NAEs are being evaluated for their direct effects on FAAH1 catalytic activity. New compounds were identified that prevent product inhibition by free fatty acids, and these compounds are being evaluated for their ability to influence plant growth and development.

- Additional enzymes/pathways for NAE metabolism continue to be investigated. The LOX pathway is intriguing in that this may represent a source of novel oxylipins with biological activity and we are evaluating NAE18:3 and its metabolites for their role in chloroplast development in young seedlings. Further we have generated various knockouts and overexpressors of FAAH2 in Arabidopsis to assess the action(s) of this protein in vivo.

- We are employing unbiased approaches to study the functional role of NAE metabolism in plants. A chemical genetic screen (library of 10,000 compounds) is being conducted to identify potential, novel targets of NAEs in plants. Also, an activation-tagged population was screened for mutants tolerant to NAE, and one promising line is in the process of genetic and molecular characterization.

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**Asparagine synthetase gene regulatory networks and plant nitrogen metabolism**

Gloria M. Coruzzi, Principal Investigator
Center for Genomics & Systems Biology, Department of Biology, New York University
100 Washington Square East, 1009 Silver Center, New York, NY 10003
Email: gloria.coruzzi@nyu.edu; Web: http://coruzzilab.bio.nyu.edu/

Overall Research Goals: The goal of this DOE project is to model and alter gene regulatory networks affecting N-assimilation into asparagine (Asn), a C- and N-efficient amino acid used to transport and store nitrogen in seeds. Altering transcription of ASN1, the major gene controlling Asn synthesis in Arabidopsis, effects increases in seed-N, and this technology is in field trials of corn and other crops. Using a combined genetic, genomic and systems biology approach, we have uncovered components regulating Asn synthesis and metabolism in response to carbon (C), light (L) and nitrogen (N) signals. In a positive genetic selection, a complete deletion of EFS/SDG8, a histone methyltransferase, was shown to impair the C and L repression of ASN1. Our goal is to explore the epigenetic and transcriptional control of ASN1 and N-assimilation in response to nutritional and environmental stimuli, with a combination of epigenomic (Aim1), transcriptomic (Aim2) and metabolic approaches (Aim3).

Significant achievements from 2010-2012:

**AIM 1. CHROMATIN: Role of histone methylation in the ASN1 metabolic regulatory network.** We used a combined genetic, genomic and systems approach to uncover components involved in the regulation of ASN1 gene expression and Asn synthesis in response to environmental and nutrient signals including Light (L), Carbon (C) and Nitrogen (N) status (Thum et al., 2004). The ASN1 promoter was used in a positive genetic selection to identify Arabidopsis mutants (*cli*) impaired in ASN1 repression by C and L signals (Thum et al 2008). We showed that one such mutant, *cli186*, is in a gene encoding a histone lysine methyltransferase (H3K4/K36), also known as *EFS/SDG8*. The genome-wide CLI186-mediated epigenetic and transcriptional control in response to light and carbon was profiled by ChIP-SEQ and Affymetrix microarrays. EFS/CLI186 has a unique zf-CW domain predicted to read H3K4me3 to guide the "writing" of H3K36me3. In concert with this hypothesis, we observed a dramatic decrease in H3K36me3 levels for ~4,000 genes, while their H3K4me3 levels remain unchanged. Site-directed mutation lines are being generated to further characterize the function of the zf-CW domain of EFS/CL186 in coordinating different histone codes. Moreover, the H3K36me3 pattern in the cli186 mutant shifted from the gene body towards the 5' of the targeted genes, suggesting a role of CLI186 in transcription elongation in plants, a parallel of the function of its yeast homolog SET2. Combining the epigenomic and transcriptomic data, we identified ~1,200 genes as potential direct targets of histone modification by CLI186. The functional annotation of those genes reveals CLI186 as a master regulator in integrating biotic and abiotic stimuli to regulate expression of genes involved in development and metabolism, including epigenetically modifying transcription factors critical for nitrogen metabolism, which are our focus in Aim2. **Aim 2. TRANSCRIPTION: Role of Transcription Factors (TFs) in the ASN1-metabolic regulatory network.** Four TFs predicted to regulated ASN1 expression based on network analysis [At2g20570 (GLK1), At5g39610 (NAC2), At1g01060 (LHY1), and At1g68840 (RAV2)] are predicted to be targeted by epigenetic regulation by CLI186, as evidenced by decreased H3K36me3 and reduced expression level in the *cli186* mutant. In addition, our gene regulatory networks also predict that RAV2 (At1g68840), ANAC047 (At3g04070), and ANAC102 (At5g63790) reciprocally regulate Asn levels via synthesis (via ASN1) and degradation (via ANS1). Currently these TFs predicted to regulate Asn synthesis and/or degradation are being tested with knockout mutants and overexpressors, as well as using a transient inducible overexpression system to verify their role in ASN1-metabolic regulatory network. **Aim 3. METABOLITES: Role of metabolic control of transcription in the ASN1-metabolic regulatory network.** We aim to integrate our studies on transcriptional control of N-assimilation into Asn into a regulatory network model that encompasses changes in N-metabolites. We have begun measuring changes in temporal levels of the primary N-assimilation products (Glu, Gln, Asp, Asn) using $^{15}N$ labeling, in WT and in mutants in Asn metabolism genes (both synthesis and degradation) and in putative regulatory TFs (from Aim2). We have developed a strategy to determine the ratios of unlabeled, single and double labeled N species, in which a portion of a sample is taken for transcriptional analysis and the other portion is split for derivatization by N-methyl-N-

(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) and N(O,S)-heptafluorobutyryl isobutyl (HFBI). The combined transcriptional and metabolic analyses will let us determine whether/how perturbations in N-assimilation into endogenous Asn affects gene regulation, and to better define the role of Asn in N-signaling.

Science objectives for 2012-2013: Our scientific aims for 2012-13 encompass further elucidating the regulatory mechanisms mediating N-assimilation into asparagine operating at the level of chromatin regulation (Aim 1), transcriptional regulation (Aim 2), and changes in N-metabolites (Aim 3). This integrated approach should enable us to identify regulatory factors that enable plants to coordinate N assimilation and storage with related processes including photosynthesis, energy and carbon metabolism. Modifying the regulatory factors that mediate this integration should have implications for modifying N-use efficiency in crop plants at a systems wide level as opposed to a single enzyme level.

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**Patents :** The technology covered by these patents has been commercially licensed by two major US agricultural companies for crops including trees and corn, both major biofuel crops.

- Patent #60/919,818 "Methods of affecting nitrogen assimilation in plants". Inventors: Gloria Coruzzi, Damion Nero and Rodrigo Gutierrez. Filed: March 23, 2007.
- Patent #5,955,651 "Transgenic plants that exhibit enhanced nitrogen assimilation". Inventors: Coruzzi and Brears. Issued: September 21, 1999.
- Patent #5,256,558 "Genes encoding plant asparagine synthetase". Inventors: Coruzzi and Tsai. Issued: Oct 1993.

## Molecular Mechanisms of Plant Cell Wall Loosening: Expansin Structure & Function

Daniel J. Cosgrove, Principal Investigator
Nicholas Georgelis, postdoc
Dept. of Biology, Penn State University, University Park, PA 16802
Email: dcosgrove@psu.edu; Web: http://bio.psu.edu/directory/fsl

**Overall research goals:** To elucidate the mechanisms underlying growth (irreversible enlargement) of plant cell walls. Our focus is on the structure of the cell wall loosening proten expansin and its interactions with cell wall polymers, with the aim of understanding how expansins modify wall polymer interactions and structures, resulting in physical effects such as wall stress relaxation, polymer creep, and increases in surface area.

**Significant achievements 2010-12:**

- **Matrix weakening and solubilization by plant beta-expansin.** We discovered that treatment of maize cell walls with purified b-expansin from maize pollen led to solubilization of wall matrix polysaccharides, dominated by highly-substituted feruloylated glucuronoarabinoxylan (60%) and homogalacturonan (35%). Several tests for lytic activities by b-expansin proved negative and polysaccharide solubilization had weak temperature dependence, suggestive of a non-enzymatic process. Concomitant with matrix solubilization, b-expansin treatment induced creep, reduced the breaking force and increased the plastic compliance of wall specimens. From comparisons of the pH dependencies of these processes, we conclude that matrix solubilization was linked closely to changes in wall plasticity and breaking force, but not so closely coupled to cell wall creep. Because matrix solubilization and increased wall plasticity have not been found with other expansins, we infer that these novel activities are linked to the specialized role of grass pollen b-expansins in promotion of penetration of the pollen tube through the stigma and style, most likely by weakening the middle lamella.
- We carried out detailed structural and functional analysis of expansin proteins from bacterial sources because they were readily expressed in active form in E. coli, whereas plant expansins have proved to be recalcitrant to heterologous expression.
- **Structure-function analysis of a bacterial expansin**. We made use of EXLX1, an expansin from *Bacillus subtilis*, to investigate protein features essential for its plant cell wall binding and wall loosening activities. The two expansin domains, D1 and D2, need to be linked for wall extension activity. D2 mediates EXLX1 binding to whole cell walls and to cellulose via distinct residues on the D2 surface. Binding to cellulose is mediated by three aromatic residues arranged linearly on the putative binding surface that spans D1 and D2. Mutation of these three residues to alanine eliminated cellulose binding and concomitantly eliminated wall loosening activity measured either by cell wall extension or by weakening of filter paper but hardly affected binding to whole cell walls, which is mediated by basic residues located on other D2 surfaces. Mutation of these basic residues to glutamine reduced cell wall binding but not wall loosening activities. Based on these results, domain D2 became the founding member of a new carbohydrate binding module family, CBM63, but its function in expansin activity apparently goes beyond simply anchoring D1 to the wall. Several polar residues on the putative binding surface of domain D1 are also important for activity, most notably Asp82, whose mutation to Ala or Asn completely eliminated wall loosening activity. The functional insights based on this bacterial expansin may be extrapolated to the interactions of plant expansins with cell walls.
- **Structural basis for entropy-driven cellulose binding by expansin / type-A CBM**. Type-A cellulose-binding modules (CBMs) bind to crystalline cellulose and enhance enzyme effectiveness, but structural details of the interaction are uncertain. We analyzed cellulose binding by EXLX1, whose domain D2 has type-A CBM characteristics. EXLX1 strongly binds to crystalline cellulose via D2, whereas its affinity for soluble cello-oligosaccharides is weak. Calorimetry indicated cellulose binding was largely entropically driven. We solved the crystal structures of EXLX1

complexed with cellulose-like oligosaccharides to find that EXLX1 binds the ligands through hydrophobic interactions of three linearly arranged aromatic residues in D2 (Fig 1). The crystal structures revealed a unique form of ligand-mediated dimerization, with the oligosaccharide sandwiched between two D2 domains in opposite polarity. These results clarify the molecular target and the specific molecular interactions of an expansin/type-A CBM.

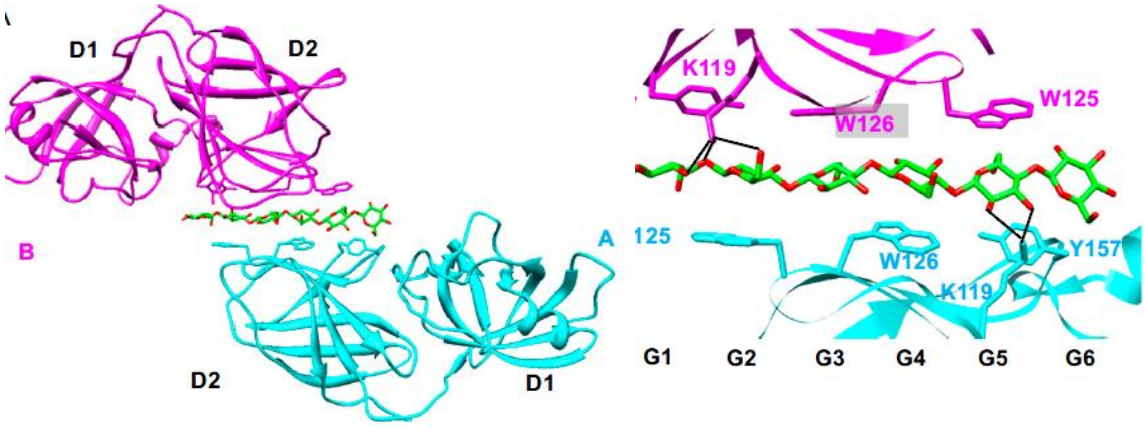


Figure 1. Structure of BsEXLX1 with cellohexaose. (Left) Sandwich structure consisting of two molecules of EXLX1 (A and B) and one molecule of cellohexaose (green). (Right) Direct hydrogen bonds and CH–π interactions between each EXLX1 molecule and cellohexaose. Glucose numbering starts from the reducing end of cellohexaose.

Science objectives for 2012-2013: We will try to:

- develop heterologous expression systems for plant expansins
- make crystal complexes of beta-expansin with oligosaccharides
- crystallize apo- and ligand-bound expansin from 1-2 other microbial sources
- elucidate the mechanism of solubilization of matrix polymers by beta-expansin
- use AFM and FESEM to image the site of expansin binding to cell walls

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## Energetics and Structure of the ZIP Metal Transporter

Dax Fu, Principal Investigator
Optional Postdoc, Postdoctoral Research Associate, Wei Lin (2008-2010), Zhening Zhang (2012- )
Biology Department, Brookhaven National Laboratory, Upton, NY 11973
Email: dax@bnl.gov; Web: http://www.bnl.gov/biology/People/fu.asp

Overall research goals: The Zrt-, Irt-like Proteins (ZIPs) are ubiquitous and conserved metal uptake transporters responsible for cellular metal acquisition. In their roles of transporting zinc, ZIPs selectively bind zinc ions, and then move the bound zinc ions down an energy path across the membrane barrier. The transient zinc binding challenges the commonly held concept of zinc sites as permanent fixtures in metalloproteins. The research objective is to understand the structural basis for selective binding and energized movement of metal ions in ZIP proteins that control the zinc loading in chloroplasts. A critical technical barrier to structure analysis of plant ZIP proteins is the lack of a eukaryotic expression system for the production of plant membrane proteins in a crystallographic quantity. We have overcome this barrier using a bacterial homolog of plant ZIP proteins, termed ZIPB. ZIPB is the first member of the ZIP family that is accessible to X-ray crystallographic analysis. This provides a unique opportunity to explore how metal binding affinity and selectivity are built into a protein structure, and how protein dynamics may reshape metal binding sites to render mobility. We will determine the crystal structure of ZIPB by X-crystallography. At present, there is no structural knowledge for any zinc uptake transport protein. The ZIPB structure will fill in a critical knowledge gap. The structural information may transform the static view of metallochemistry into a dynamic and quantitative model that describes physicochemical principles of metal coordination and energized movement in atomic detail. Metals are essential co-factors of photosynthetic supercomplexes in chloroplasts. The knowledge gained in the proposed research will facilitate engineering metal transport systems to overcome a critical bottleneck in biogenesis of the photosynthetic apparatus.

Significant achievements 2008-2012: X-ray crystallographic analysis of integral membrane proteins in general is hindered by the difficulty of obtaining an adequate amount of pure, stable protein samples. The over-expression of zinc uptake transporter ZIPs appears to be particularly difficult. We have developed the ZIPB project from ortholog selection and high throughput expression screening through purification and crystallization to initial x-ray analysis and crystal optimization. ZIPB was identified by high throughput expression screening of a collection of 53 prokaryotic ZIP orthologs. A library of detergents, metal ions, and buffers in various combinations was screened for an optimal stabilizing condition. The resulting ZIPB in a stabilized form was concentrated to ~20 mg/ml. Measurement of the absolute protein mass in the ZIPB-detergent-lipid complex indicated that ZIPB was purified as a homodimer. Functional characterization of purified ZIPB in reconstituted proteoliposomes indicated that ZIPB is a zinc-selective electrodiffusional channel that exploits *in vivo* zinc concentration gradients to move zinc ions into the cytoplasm. An initial crystallization condition was obtained by biased sparse matrix screens. We iteratively pursued two avenues to improve ZIPB crystals: modifying the protein construct and optimizing the

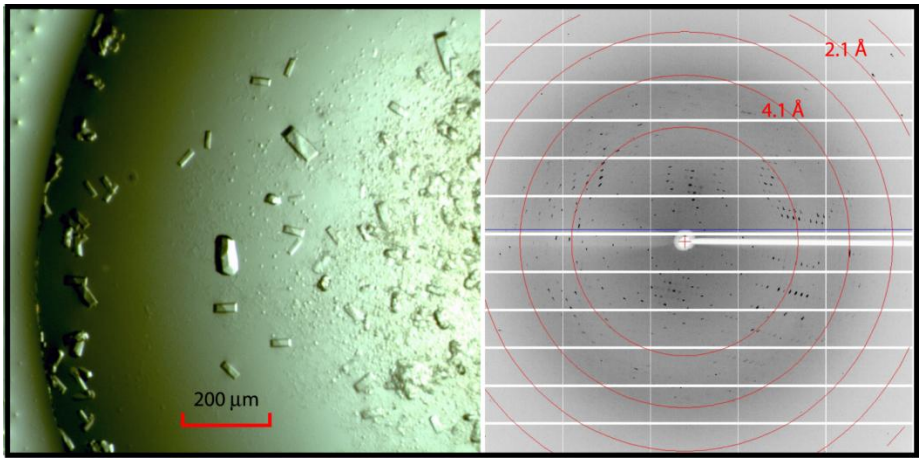


**Fig. 1** Crystal optimization and improved X-ray diffraction. Note: 2D- and 3D-crytsals co-exist in the same drop.

crystallization condition. Under an optimal condition, ZIPB grew into thin, plate-like crystals in about two weeks, and then slowly transformed into 3-D like crystals over a course of six months (Fig. 1, left panel). The chunky 3-D crystal (P2 space group, a=71.3, b=65.6, c=82.9 Å, β=92.6$^{o}$) diffracted beyond 3-angstrom brag spacings (Fig. 1, right panel).

Science objectives for 2012-2013:

- Reproduce ZIPB crystals in large quantity for multi-crystal data collection. Our crystals are ultrasensitive to temperature fluctuation. We do not have a temperature-controlled facility in the Biology Department. This gives tremendous difficulty to reproduce the existing crystallization condition. If we are not able to reproduce the existing ZIPB crystals, we will explore detergents and additives to identify additional crystallization conditions that may be less sensitive to temperature fluctuation.
- Determine the initial protein phases by single wavelength anomalous dispersion (SAD)/multiple wavelength anomalous dispersion phasing. Alternatively, we will derivatize crystals with heavy atoms for multiple isomorphous replacement/SAD phasing.
- Carry out phase refinement to obtain protein electron density maps.
- Build an atomic model into the electron density maps
- Refine the model to obtain the first atomic resolution structure of a ZIP protein
- Interpret the ZIP crystal structure and propose the mechanism of zinc selectivity and transport.

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## Dissecting *Arabidopsis* Gβ signal transduction on the protein surface

Alan M. Jones, PI
Departments of Biology) and Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-3280, USA
Donald R. Ort, subcontractor
Global Change and Photosynthesis Research Unit, Agricultural Research Unit, United States Department of Agriculture, Institute for Genomic Biology, 1206 West Gregory Drive, Urbana, IL, 61801, USA

Overall Research Goals. Understanding how plants sense sugars is critical for us to engineer crops that have greater energy yields. My lab has shown that the signaling module called "G protein"-coupled signal transduction is a major means by which plants sense sugar and control the efficiencies of key plant physiologies such as photosynthesis and disease resistance, both of great interest to the DOE Bioenergy Science Program.

Significant Achievement of the 2011-2012 Period. The heterotrimeric G protein complex provides signal amplification and target specificity. The *Arabidopsis* Gβ subunit of this complex (AGB1) interacts with and modulates the activity of target cytoplasmic proteins. This specificity resides in the structure of the interface between AGB1 and its targets. Important surface residues of AGB1, which were deduced from a comparative evolutionary approach, were mutated to dissect AGB1-dependent physiological functions (see figure 1). Analysis of the capacity of these mutants to complement well-established phenotypes of Gβ-null mutants revealed AGB1 residues critical for specific AGB1-mediated biological processes, including growth architecture (see Figure 2), pathogen resistance, stomatal mediated leaf-air gas exchange, and photosynthesis. These findings provide promising new avenues to direct finely-tuned engineering of crop yield and traits.

Science Objectives for 2012-2013. The effects of directed changes on the surface of the Gβ subunit caused changes in photosynthesis but these effects varied. The objective of the next cycle is to determine what parameters are causing this variability so that we can control them in an engineered plant with increased photosynthesis efficiency.

DOE Publications Since 2011.


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## Figure 1

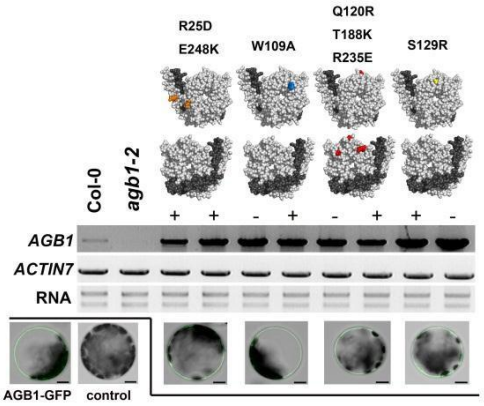


Figure 1.Site-directed mutagenesis of AGB1.
The upper panel shows the top and bottom views of AGB1 (light grey) and the closely associated Gγ subunit (dark grey). Colored residues indicate point mutations generated. From left to right: R25D E248K, W109A, Q120R T188K R235E, and S129R. The middle panel shows results of RT-PCR analysis of Myc-tagged (+) and non-tagged (-) mutated *AGB1* expression in the transgenic lines used in this study. Total RNA was extracted from 10-d-old seedlings. PCR was repeated twice for each biological sample and a total of two biological replicates were tested. *ACTIN7* was used as a loading control. Ethidium bromide (EB) stained rRNAs (28S rRNA and 18S rRNA) are shown as a quality control for the RNA samples. The lower panel shows plasma membrane localization of mutant AGB1 proteins. Mesophyll protoplasts were isolated from 5-week-old Col-0 plants and subsequently transfected with 20 µg of plasmids that allow transient expression of N-terminally GFP-tagged WT or mutated *AGB1*. Each image shows a single optical section in the focus at the plasma membrane. No GFP signal was detected in untransfected (ctrl) protoplasts. Bar = 10 µm

## Figure 2

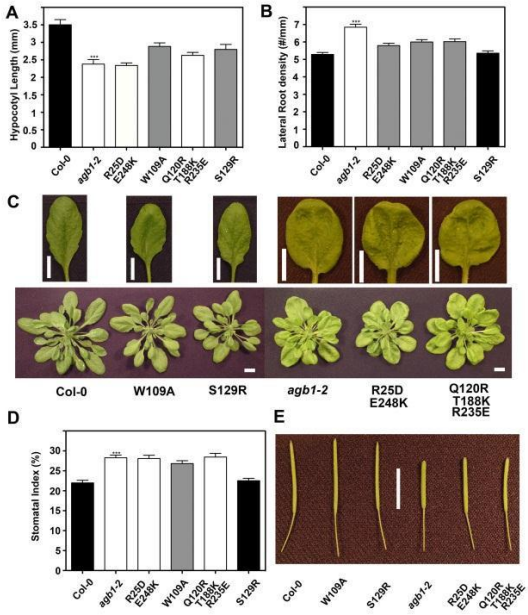


Figure 2. Analyses of developmental phenotypes in wild type, *agb1-2*, and transgenic plants.
A, Hypocotyl Length of fifteen 50-hour-old etiolated seedlings. B, Density of lateral root (visible lateral roots + lateral root primordia) of thirty 10-day-old seedlings. C, Morphological comparison of fully expanded rosette leaves and aerial part of plants. D, Stomatal index on abaxial epidermis of cotyledons from 9-day-old seedlings. E, Morphological comparison of mature siliques. The grey scale of bars indicates the extent to which a phenotype was rescued (black = totally rescued, grey = partially rescued, white = not rescued). Data for wild-type plants are plotted in black and those for *agb1-2* mutants are plotted in white. All values are mean ±SE and assays were repeated 2-4 times with similar results. *Significant difference from wild-type plants (*** $p<0.001$; Student's *t*-test). Bars in C and E = 1 cm.

## Dynamic Regulation of the Photosynthetic Energy Budget

David M. Kramer, P.I.
Leticia Ruby Carrillo, Jeffrey A. Cruz, Atsuko Kanazawa, Kaori Kohzuma, William Kovac
MSU-DOE Plant Research Lab, Michigan State University, East Lansing, MI 48823
Email: kramerd8@msu.edu; Web: http://www.prl.msu.edu/

Overall research goals: Our work focuses on how photosynthesis balances its energy budget, matching the output of energy in NADPH and ATP to precisely meet biochemical demands while controlling the dissipation of energy to prevent photodamage. Understanding this balancing is critical for improving the efficiency of photosynthesis by introducing $CO_2$ concentrating mechanisms, and altering metabolism or biosynthetic pathways to shunt energy to alternative products (1, 2). Key developments in our current work lead us propose testable hypotheses as to how this balancing is achieved through cyclic electron flow around photosystem I, regulation of the chloroplast ATP synthase, partitioning of the proton motive force into electric field, and ΔpH components and exchange of ATP/ADP+$P_i$ across the chloroplast envelope.

Significant achievements 2010-2012:

***1) In vivo probes of steady-state photosynthetic processes (3-5).*** The work has led to the introduction of new spectroscopic tools and methods to explore the photosynthetic energy budget *in vivo* that enabled us to address key questions posed in the current work. In this poster, we will highlight new tools for high throughput applications, including non-invasive imaging of photosynthetic phenotypes.

***2) New classes of mutants with modified proton circuits (6-10).*** We isolated and characterized new classes of mutants with altered cyclic electron flow, regulation of the chloroplast ATP synthase and partitioning of the thylakoid proton motive force (pmf) into ($\Delta\psi$) and ΔpH components. Analysis of these and a series of reverse-genetics mutants, led to the following conclusions: 1) CEF involves the NDH complex, but not PGR5; 2) State transitions are not required for activation of CEF; 3) CEF is likely activated by redox status or reactive oxygen species. We now have evidence that long-term regulation of CEF involves $H_2O_2$ and the *hcef3/ocp3* proteins; 4) The partitioning of pmf is regulated in vivo by endogenous mobile buffers. Differential synthesis of these mobile buffers can modulate the feedback regulation of photosynthesis to changing environmental conditions.

***4) Multiple roles for the chloroplast ATP synthase (8, 11-13).*** We have demonstrated that the activity of the chloroplast ATP synthase is controlled by metabolic factors and constitutes a key regulator of the photosynthetic energy budget. Through a series of genetic modifications, we showed that regulatory behavior the ATP synthase is altered by differential expression of different homologues of the γ-subunit, allowing this complex to serve different functions in photosynthesis and plant development.

***5) A new component required for CEF activity.*** We have shown that CEF activity is irreversibly lost during standard isolation of thylakoids, but that this activity can be retained in the presence of certain antioxidants. CEF activity in vitro did not correlate with the rate of "fluorescence rise" upon addition of NADPH and ferredoxin, an effect previously attributed to the CEF-related reduction of $Q_A$ upon reduction of the plastoquinone pool by PGR5. In fact, we demonstrate that the fluorescence rise is unrelated to photosystem II. We therefore conclude that CEF activity does not involve PGR5 or plastoquinone accessible to the majority of photosystem II, consistent with a highly organized CEF supercomplex.

***6) Observation of rapid ATP export from the chloroplast.*** Our experiments to measure CEF in intact chloroplasts led us to the striking, and serendipitous finding that intact chloroplast preparations were capable of rapid export of ATP from stroma to the external phase. We are currently testing which transporters are involved in this process.

Science objectives for 2012-2013: 1) Characterize our recently isolated mutants using our high throughput techniques with altered capacity for *pmf* regulatory responses; 2) Develop "inverse genetics" approaches to uncovering functions of genes of unknown function using high throughput photosynthetic phenotyping under dynamically fluctuating environmental conditions; 3) Identify new components required for regulation of the chloroplast ATP synthase and CEF; 4) Identify the transporters involved in rapid ATP exchange

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**Transmethylation reactions during methylotrophic methanogenesis in methanogenic Archaea**

Joseph A. Krzycki, Principal Investigator
Department of Microbiology, Ohio State University, 484 West 12th, Columbus OH 43210
Email: krzycki.1@osu.edu; Web: http://microbiology.osu.edu/faculty/krzycki-joseph

Overall research goals: Most methane produced from biological sources comes from methanogenic Archaea. Of these organisms, *Methanosarcina* spp. and their relatives have the most diversified substrate range. Our overall goal is to understand the enzymes and molecular biology underlying these methanogenic pathways. We have primarily focused on methanogenesis from monomethylamine, dimethylamine, and trimethylamine. Methanogenesis from these substrates is began by three methyltransferases that methylate cognate corrinoid proteins, which are then used to methylate coenzyme M, forming the direct precursor of methane. The methylamine methyltransferases are non-homologous, yet each of their encoding genes contains an in-frame UAG codon. Metabolism of methylamines obligately depends on translation of an amber codon as pyrrolysine. We have recently made inroads into understanding how pyrrolysine is biosynthesized and are currently examining the function of pyrrolysine in the methylamine methyltransferases by site directed mutagenesis of the amino acid.

Significant achievements in 2010-2012: During this time, DOE solely funded the completion of a longstanding project: how pyrrolysine is synthesized by methanogens. We used a recombinant system in which *E. coli* transformed with *pylTSBCD* and *mtmB1* carries out the synthesis of pyrrolysine that is then incorporated into MtmB (the MMA methyltransferase). We fed isotopically labelled lysine to cells in minimal medium, and established by tandem mass spectroscopy that pyrrolysine is entirely derived from this amino acid. We showed that during pyrroline ring formation the epsilon nitrogen of lysine is eliminated from the ring, with the alpha nitrogen retained. We further showed that D-ornithine added to the medium is incorporated into a new pyrrolysine derivative that lacks a methyl group, i.e. desmethylpyrrolysine. Unlike pyrrolysine biosynthesis, formation of desmethylpyrrolysine does not require PylB, but only PylC and PylD. This allowed us to surmise that PylB, a Radical SAM family enzyme, carries out the conversion of lysine to a methylated ornithine derivative, which is then ligated to lysine by PylC, before oxidation of this methylornithine-lysine intermediate by PylD results in

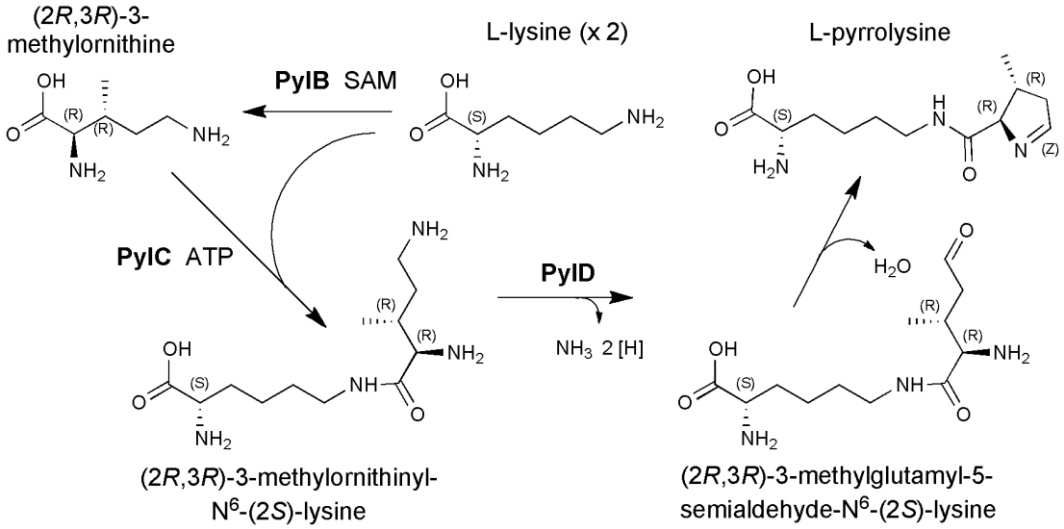


Fig. 1. The complete biosynthesis of pyrrolysine from lysine.

pyrrolysine formation. We showed the ligation product of D-ornithine and L-lysine was made in cells bearing PylC. The pathway is presented in Fig 1. We have also used this same general method to provide evidence that the first step of the proposed pathway requires a intramolecular rearrangement of lysine with accompanying loss of otherwise stable protons—indicative that PylB employs a radical mechanism similar to that of adenosylcobalamin-dependent glutamate mutase. This is surprising, as radical SAM proteins like PylB were not previously thought to carry out such reactions.

Science objectives for 2011-2012:

- Theories of how pyrrolysine functions in the methylamine methyltransferases remain essentially untested. In this next year, we are continuing to focus on establishing the consequences of replacement of the pyrrolysyl-residue of the TMA methyltransferase using expression of *Methanosarcina barkeri* enzymes in *Methanosarcina acetivorans* host. These studies will be buoyed by our recent discovery that a non-pyrrolysine homolog of TMA methyltransferase lacks methyltransferase activity with TMA, but is instead a betaine methyltransferase.

- We had previously explored the effects of mutagenesis of the MMA methyltransferase pyrrolysyl-residue in the same system, but results were complicated by the presence of host enzyme in recombinant methyltransferase preparations. We are currently exploring alternative solutions to this problem employing both cultural and genetic approaches.

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**Lignin biopolymer assembly and primary structure: A (bio)chemical characterization**

Norman G. Lewis, Principal Investigator
Laurence B. Davin, Kim K. Hixson, Chanyoung Ki, Syed G.A. Moinuddin, Team Members
Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340
Email: lewisn@wsu.edu

Lignins are the major contributors in vascular plant cell walls to so-called lignocellulosic recalcitrance. A considerable challenge is, however, not only in the study of lignin primary structures and how these are controlled, but also in how to overcome the ability of these primary chains to self-associate via strong non-covalent bond interactions. This self-association thus represents one of the major challenges and drawbacks in the study of lignin structure(s) and their differential deposition in distinct-cell wall types, as well as the biophysical properties associated with same; yet these are the main underlying reasons for lignocellulosic recalcitrance.
Consequently, the study of lignin biopolymer structure and assembly has necessitated the development of new incisive approaches that can overcome such difficulties, including analysis at the single cell wall level.

Overall research goals: There are two specific objectives in this proposal: (i) To further define and establish lignin primary sequence(s), and their biophysical/biomechanical properties from specific cell-wall types and tissues and (ii) to obtain lignin dehydropolymerizates *in vitro* that are accurate facsimiles of native lignin macromolecular configuration.

Significant achievements 2010-2012: Summarized below are some of the recent major advances made in probing/defining lignin primary structure and lignin analyses.

Molecular Analysis at the Single Cell Level. Laser microdissection technologies, as well as (metabolite) tissue imaging of cross-sections of plant stem material, have brought the study of lignins and other cell-wall forming processes to the single-cell type level. These approaches permit various analyses including proteomics (Cho *et al.*, manuscript in finalization; Hixson *et al.*, manuscript in preparation), transcriptome profiling/bioinformatics (work in progress) and chemical analysis [via pyrolysis/GC/MS (Patten *et al.*, 2010, Corea *et al.*, 2012a,b)], and more recently of the entire cell wall type matrix (e.g., interfascicular fibers vs. vascular bundles) via development of methods to intactly solubilize and analyze the entire cell wall matrix (work in progress).

Dissecting Plant Cell Wall Biosynthetic Processes and Physiological Consequences: Crossing the Rubicon. The ability to modulate segments of the lignin biosynthetic pathway and related polymeric carbohydrate cell wall processes has provided much insight into the nature of the defects introduced by such manipulations. In addition, various manipulations [e.g., of the Phe-forming arogenate dehydratase gene family (Corea *et al.*, 2012a,b,c), 4-coumarate CoA ligase (Voelker *et al.*, 2010), NAC transcription factors, etc.] has provided new insights into: differential carbon allocation into lignifying cell wall types, vascular bundles vs. interfascicular fibers (Corea *et al.*, 2012a,b,c); as well as providing the means to determine quantitatively the physiological and biophysical consequences of modulating cell wall development/lignin deposition thereby preventing normal secondary wall thickening processes, using, for example, nano-indentation technologies [Cardenas *et al.*, manuscript in finalization]; the physiological consequences of manipulating various cell-wall forming processes (lignin deposition) in various transgenics in field trials to evaluate growth and development.

Lignin Primary Structures and Lignin Association: The Devil is in the Details: There is an ever-growing need to be able to develop new approaches that permit the incisive analysis of lignins both *in situ* and in solubilized form, including without the added complications of self-association of lignin chains. Our research group has thus begun to develop new approaches to overcome these long-standing difficulties and challenges.

In this context, lignins can be removed and/or chemically degraded/derivatized by various techniques such as sulfonation, ball-milling of plant tissue and polar solvent extraction, ionic liquid treatment, as well as via inter-unit linkage cleavage (e.g., thioacidolysis). Some of these approaches giving various lignin preparations now offer the opportunity to study not only lignin primary structure, but also how to begin to overcome the strong non-covalent bond interactions due to self-association.

For examples, we have, therefore, developed methods to probe/quantify lignin inter-unit bond frequencies, partial primary structures, effects of sulfonation protocols on lignin composition, properties, and structures (Moinuddin *et al.*, 2010, work in progress). A significant effort is now underway to develop approaches to further overcome lignin association phenomenon, using a variety of new analytical separation technologies coupled to HRMS analysis (work in progress) and via enzymatic means to degrade the lignin polymers via cleavage of specific inter-unit bonds (work in progress). These approaches are now beginning to form the basis of rational evaluation and elucidation of the lignin biopolymeric matrix.

Science objectives for 2012-2014:

(i) Continue Molecular Analysis of Lignin Structure at the Single Cell Level
(ii) Continue Establishing Biophysical Consequences Lignin/Cell Wall Modifications
(iii) Continue Lignin Primary Chain Structure and Association Determinations, and Analysis

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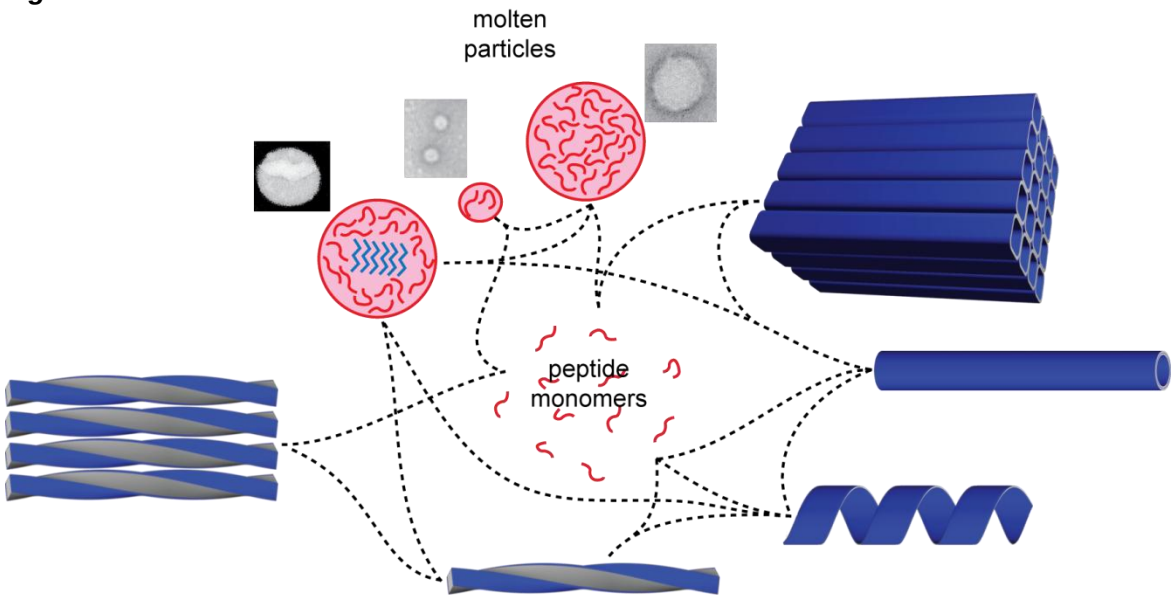
# Engineering Intelligent Scaffolds by Supramolecular Self-Assembly

*PD/PI:* David G Lynn and Co-PI Vincent P Conticello
Emory University, Atlanta, GA 30322
1515 Dickey Drive, Departments of Chemistry and Biology
404 727-9348; david.lynn@emory.edu
Web: http://www.chemistry.emory.edu/faculty/lynn/index.html

### *Overall Goals*

We proposed that a new generation of self-assembled intelligent materials able to respond, adapt, and learn from their environment, could be created from self-assembling peptide systems. This goal required that we realize such self-organizing behaviors through the assembly of diverse structural forms, demonstration of their self-propagation and selection, and the emergence of new function. We have made significant progress in assembling diverse forms and understanding the energetic surfaces that dictate their folding landscape, propagating and selecting specific forms, and demonstrating the emergence of new chemical functions.

### *Significant Achievements*




We have demonstrated [1-5] that the seven-residue nucleating core of the Alzheimer's disease peptide, Ac-KLVFFAE-$NH_2$, can access a variety of self-assembled forms as summarized above [6]. Except the molten particles, all the assemblies have a cross-β architecture with peptides H-bonded into anti-parallel β-sheets (**Figure 1**) The protonation state of the glutamate side chain acts as a switch, directing peptide assembly into nanotubes at acidic pH and fibers at neutral pH [1,5]. Replacing the glutamate residue with the uncharged leucine results in tube assembly at all pH (**Fig 1A**). The peptides within the tubes assemble into anti-parallel out-of-register β-sheets (**Fig 1E**) that create a positively-charged solvent-exposed faces (blue surface in **Fig 1E**). The positive charge results in tube-tube repulsion in solution. However the addition of a divalent counter-ion (sulfate or phosphate) results in surpamacromolecular ordering and bundled tubes (**Fig 1D**).

Recently, we have expanded the diversity of side chains that can be exposed as potentially catalytic functional groups and assembled histidine (Ac-HLVFFAL-$NH_2$) and arginine (Ac- RLVFFAL-$NH_2$) tubes. These tubes have an identical morphology to the lysine tubes and remain suspended as long as the histidine or arginine side chains remain positively charged.

In contrast to the tube morphology, fibers have four major solvent exposed surfaces. Two surfaces are composed of β-sheet faces and two are composed of peptide termini. At neutral pH, both the lysine and glutamate side chains of Ac-KLVFFAE-$NH_2$ are oppositely charged and the cooperative intermolecular electrostatic attraction directs the peptides to form anti- parallel β-sheets. The anti-parallel nature of the β-sheets results in the two fiber surfaces, composed of the peptide termini, being identical, (purple surfaces in **Fig. 1E**). Fibers assembled with parallel β-sheets would break the symmetry of peptide termini faces, and we predicted that H-bonding between side chains on adjacent peptides would overcome the cooperative K-E electrostatic interaction. Both Ac-KQVFFAQ-$NH_2$ and Ac-KLVFFAQ-$NH_2$ assembled into fibers (**Fig 1C**) and isotopic $^{13}C$ enrichment allowed us to determine that the resulting fibers formed parallel β-sheets via isotope-edited and solid-state NMR. With these structural tools, we have demonstrated that glutamine incorporation at any position along the peptide sequence results in parallel β-sheet assemblies (7). Further, we have demonstrated that peptide/lipid chimeras allow us to access an entirely new class of self-assembled material and allows for fine-tuning of the tube and fiber surfaces (8, 9). Now, with precise control of the functional groups of the tube/fiber surfaces, we are positioned to design systems that have small molecule binding specificity and catalytic function (10, 11).

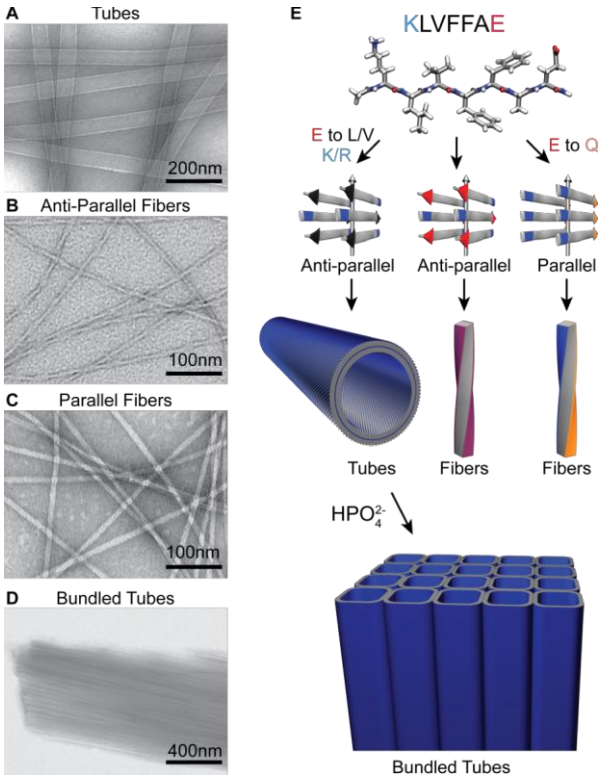



**Figure 1.** *TEM images of (**A**) Ac-KLVFFAL-$NH_2$, (**B**) Ac-KLVFFAE-$NH_2$, (**C**) Ac-KLVFFAQ-$NH_2$, and (**D**) $Na_2HPO_4$ bundled Ac-KLVFFAL-$NH_2$ nanotubes. (**E**) Summary of structurally characterized amyloid polymorphism observed within KLVFFAE variants.[1-3] The anti-parallel strand nanotubes have a single surface, anti-parallel fibers have three distinct surfaces, and parallel fibers display four distinct surfaces.*

***Future Science Objectives***

- As the rules for peptide assembly are clarified, it becomes possible to investigate more complex mixed assemblies. The chimeric assemblies represent the first step and indeed have taught us much about the plasticity of the cross-β structures. Already we have seen great morphological diversity accessed with the presence of small molecules and simple peptides.
- With the structural details of these assemblies, predictions of chemical function have now been possible. Indeed, the nanotubes as effective aldol catalysts, and while their catalytic efficiencies do not achieve those of highly evolved enzymes, they do offer enantiomer-enriching catalytic networks. The demonstration that these highly ordered peptide phases can selectively control reactions of C-C bonds, the central reaction in the construction of the metabolism of today, provides a clear first step toward the catalytic networks necessary for intelligent materials that learn from their environments.
- Finally, these materials also offer unique insights into functions that can be integrated into cells so as to compliment and extend their existing functions.

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## Bioenergetic aspects of syntrophic fatty and aromatic acid metabolism

Michael J. McInerney, Principal Investigator
University of Oklahoma, 770 Van Vleet Oval, Norman, OK 73019
Email: mcinerney@ou.edu ; Web: http://mpbio.ou.edu/michael-j-mcinerney

Collaborators: Dr. Robert P. Gunsalus, UCLA, Los Angeles, CA
Dr. Rachel R. Ogorzalek Loo, UCLA, Los Angeles, CA
Prof. Dr. Matthais Boll, University of Freiburg, Germany
Dr. Lee Krumholz, University of Oklahoma, Norman, OK
Dr. Gregory B. Hurst, Oak Ridge Natl. Lab., Oak Ridge, TN
Dr. Caroline Plugge, Wageningen Universiteit, Wageningen, the Netherlands
Prof. Dr. Alfons J. M. Fons, Wageningen Universiteit, Wageningen, the Netherlands

Overall research goals: Syntrophic metabolism is essential in the global cycling of organic matter to methane and carbon dioxide. A distinctive feature of syntrophic metabolism is the need for reverse electron transfer. The objectives of our project are: (1) to detect the membrane complexes involved in reverse electron transfer, (2) to conduct gene expression and operon analyses to determine if key gene systems are induced under growth conditions that require reverse electron transfer, (3) to determine the functions of a butyrate-induced, membrane complex in *Syntrophomonas wolfei* and an NADH:ferricyanide oxidoreductase activity in *Syntrophus aciditrophicus*, and (4) to determine if *S. wolfei* and *S. aciditrophicus* have electron-bifurcating hydrogenases and formate dehydrogenases that could be used for reverse electron transfer of electrons from NADH to $H_2$ or formate.

Significant achievements in 2011-2012: We previously identified a membrane complex in *S. wolfei* that was present only in cells growth with butyrate in co-culture with *Methanospirillum hungatei*. Mass spectral analyses revealed that the complex contained electron transfer flavoprotein (Etf), FeS oxidoreductase (Fso) and cytochrome *b*-linked hydrogenase subunits (Fig. 1). The complex was not detected when *S. wolfei* grew syntrophically, or axenically, with crotonate, a compound that does not require reverse electron transfer for its metabolism. Activity staining showed that the complex has hydrogenase activity. The *fso* and *hydIIA* (gene for the alpha subunit of the hydrogenase) were induced when *S. wolfei* was shifted from axenic growth on crotonate to syntrophic growth on butyrate. These data indicate that the complex generates hydrogen from electrons generated during beta-oxidation (Fig. 1).

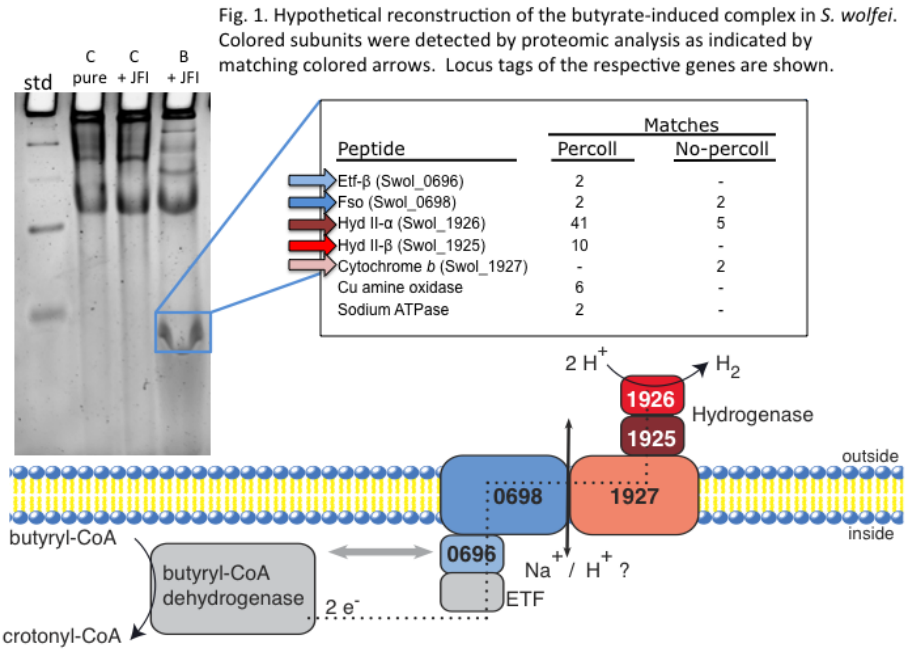


Fig. 1. Hypothetical reconstruction of the butyrate-induced complex in *S. wolfei*. Colored subunits were detected by proteomic analysis as indicated by matching colored arrows. Locus tags of the respective genes are shown.

Syntrophic co-cultures grow slowly and very little biomass is made, making enzyme purification difficult. To circumvent this problem, we developed a new method of cultivation where the syntrophic metabolizer is grown axenically with crotonate and then once a large amount of cells are obtained, syntrophic metabolism is induced by adding the syntrophic substrate and the methanogenic partner. This approach allows for a rapid turnaround time of large bioreactors and we were able to obtain 365 g of syntrophically grown *S. wolfei*.

We determined the membrane protein complexes in two phylogenetically related syntrophic microorganisms: the aromatic and fatty acid oxidizer, *Syntrophus aciditrophicus*, and the propionate-oxidizing bacterium, *Syntrophobacter fumaroxidans*. We detected peptides for ATP synthase, NADH:ferredoxin oxidoreductase, pyrophosphatase, and others. Interestingly, contrary to our prediction, the NADH:ferredoxin oxidoreductase peptides were only detected in *S. aciditrophicus* cultures grown under conditions not requiring reverse electron transfer. NADH:ferredoxin oxidoreductase peptides were not detected in *S. fumaroxidans*.

Science objectives for 2013:

- We will purify the butyrate-induced complex in *S. wolfei* and determine its activity and subunit composition.
- We will purify and characterize hydrogenases and formate dehydrogenases in *S. wolfei* and *S. aciditrophicus* to determine if they couple the unfavorable production of $H_2$ or formate from NADH with the favorable production of $H_2$ or formate from reduced ferredoxin.
- We will purify and characterize the NADH:ferricyanide oxidoreductase activity in *Syntrophus aciditrophicus.*

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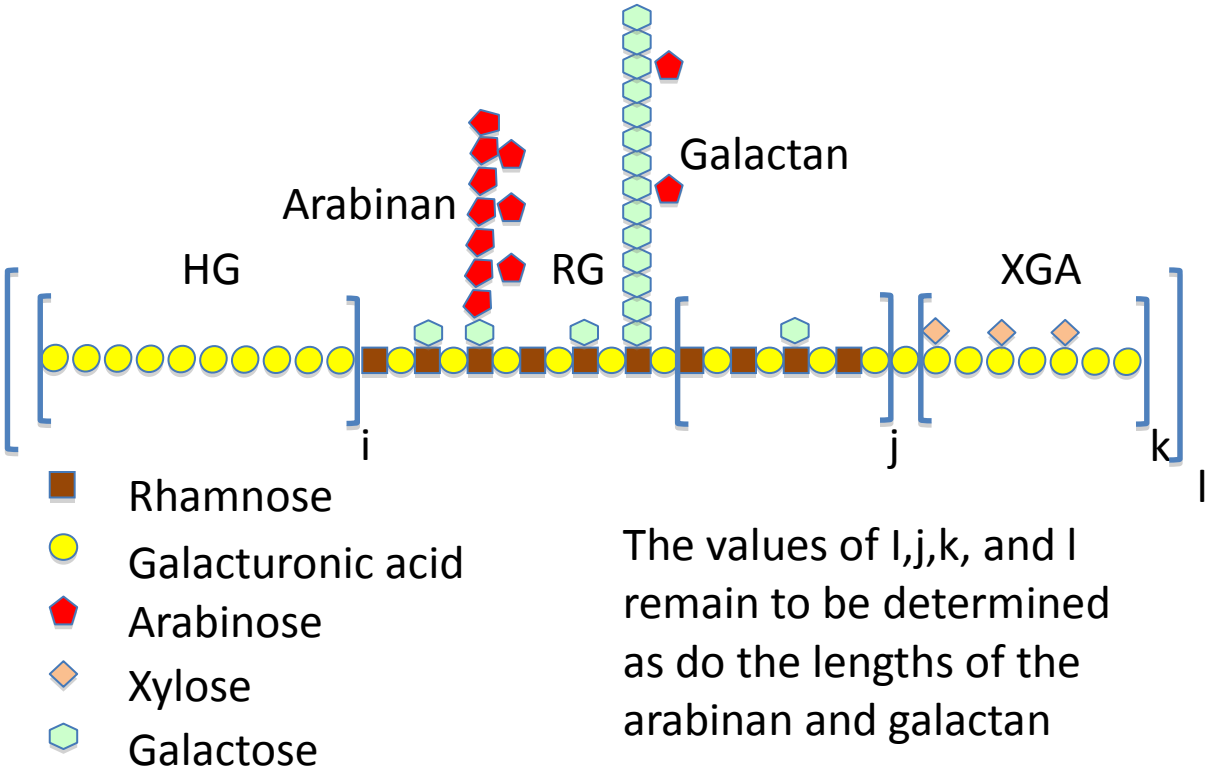
## The Structure of Pectins

Andrew Mort, Principal Investigator
Xiangmei Wu, Postdoctoral Research Associate
Songyue Chen, Graduate Student
Soreiyu Umezu, Research Assistant
246 NRC, Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, Oklahoma 74078.
Email: andrew.mort@okstate.edu; Web: http://biochemistry.okstate.edu/faculty-research-1/andrew-mort

Overall research goals: The research objectives are: 1) To determine how, and if, the various structurally distinct regions of pectins are linked together, what range of sizes each region has, to determine the arrangement of the regions, and 2) To characterize the linkage between pectin and xyloglucan.

Significant achievements in 2010-20012: Figure 1 shows our current model of the structure of apple pectin. In this model pectin starts at its non reducing end with a stretch of homogalacturonan (HG) followed by a stretch of rhamnogalacturonan (RG) with galactan, arabinan, and single galactose sidechains followed finally by a stretch of xylogalacturonan (XGA).




The main approach leading to this model is to selectively digest the pectin with cloned enzymes to allow isolation of fragments containing the junctions between two different regions of pectin, or to obtain intact regions with only small fragments of what used to be the adjacent region, for their characterization by mass spectrometry, NMR spectroscopy, and static light scattering. Isolation of individual fragments requires various forms of HPLC separations. With much help from Chris Somerville's lab we have collected together a large number of *Pichia pastoris* clones each expressing a different enzyme sequence derived from Aspergillus nidulans or in a few cases other fungi. From Rolf Prade's lab we have obtained additional enzymes cloned from a thermophylic fungus and some from hyperthermophylic bacteria. We have determined the mode of action of many of these enzymes on model substrates and have tested various sequences of application of the enzymes for generation of the desired small fragments containing two or more regions of pectin.

Using a combination of the cloned enzymes mentioned above, in combination with semi-preparative ion exchange and size exclusion chromatography, we have isolated several additional oligosaccharides reflecting junctions between HG and RG and between RG and XGA. These include RG oligomers with two GalA residues at their non-reducing ends, giving more credence to the linear relationship between HG and RG, and RG oligomers with XGA fragments at their reducing ends.

All of the RG oligomers containing arabinose appear to have a Gal residue linked to a Rha in the RG with the Ara linked to the Gal.

We have repeated the isolation of an XG/RG complex from cotton suspension cultures by collecting the alkali-extracted fraction of XG that binds strongly to an anion exchange column. After digesting this fraction with an arabinosidase to de-branch the arabinan and then digestion with the pure endoarabinanase the majority of the XG could be separated from the RG on the anion exchange column. Thus, it does appear that XG and RG can be linked together via a branched arabinan. Unfortunately we have not succeeded yet in isolating enough of the fragment of the XG linked to the arabinan to characterize it. We have repeated the experiments with Arabidopsis culture cell walls with similar, but not identical results.

Science objectives for 2012-2013:

- Characterize more oligomers from apple pectin showing the linkage between arabinans and RG.
- Characterize oligomers from sugar beet showing the linkage between arabinans and RG.
- Continue efforts to obtain small characterizable fragments containing both RG and XGA regions.
- Characterize the arabinan fragment left on the XG after the arabinanase treatment.
- Determine the molecular weights of the individual pectin regions
- Develop an assay for transglycosylases using BlotGlyco beads.

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**Translational Repressor Mutants Reveal Novel Functions for Auxin Responsive Factors in Plant Development**

Natasha V. Raikhel, Principal Investigator
Postdoctoral Research Associates: Abel Rosado, Ruixi Li Wilhemina Van de Ven
Undergraduate student: Emily Hsu
Center for Plant Cell Biology and Department of Botany and Plant Sciences, University of California-Riverside, CA 92521, USA
Email: nraikhel@ucr.edu; Web: http://cepceb.ucr.edu/people/raikhel.html

Overall research goals: The long-terms goals of this project are to: 1) Characterize the biochemical reactions and cellular mechanisms involved in the uptake, compartmentalization and mobilization of energy-relevant nutrients and storage proteins; 2) Define the genetic elements required for the accumulation and mobilization of reserves into the vacuole(s) of both vegetative and seed tissues in Arabidopsis; 3) Design experimental approaches aimed at improving biomass, production yield, and fitness through the enhancement of various reserves in vacuoles; and 4) Translate this knowledge to crops of biotechnological interest.

Significant achievements 2011-2012:

Earlier we identified the ribosomal protein RPL4A as an important element for the sorting of vacuolar cargoes in a process regulated by auxins (Rosado et al., 2010). We then analyzed how RPL4A, as well as other ribosomal components such as RPL4D and RPL5A, modulated the auxin responses through the translational regulation of multiple ARF containing 5'-UTRs leader sequences in Arabidopsis. Upstream open reading frames (uORFs) are elements found in the 5' leader sequences of specific mRNAs that modulate the translation of downstream ORFs encoding major gene products. In Arabidopsis, the translational control of Auxin Responsive Factors (ARFs) by uORFs has been proposed as a regulatory mechanism required to properly respond to complex auxin signaling inputs. We identified and characterized the aberrant auxin responses of mutants defective in the ribosomal complex in which multiple ARF transcription factors are simultaneously repressed at the translational level. This characteristic lends itself to use these mutants as genetic tools to bypass the genetic redundancy among members of the ARF family in Arabidopsis. Using this approach, we were able to assign novel functions for ARF2, ARF3 and ARF6 in plant development.

Science objectives for 2012-2013:

We will continue our careful examination of the regulatory mechanisms mediated by the RPL4 family and their implication in vacuolar trafficking. Our preliminary *in silico* identification of uORFs in Arabidopsis indicates that multiple vacuolar trafficking elements, including transcription factors, machinery elements and cargoes, might potentially be translationally regulated by uORFs. We will be studying the role of uORFs in the regulation of vacuolar trafficking pathways using biochemical and genetic approaches.

References to work supported by this project 2011-2012:

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## Functions of Plant Sodium and Potassium Transporters in Salinity Tolerance and in Chloroplast Function.

Julian Schroeder, Principal Investigator
Division of Biological Sciences, Cell and Developmental Biology Section,
University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116, USA

**Overall Research Goals:**

Salinity stress is progressively causing reduced plant growth in irrigated lands and in natural saline soils, causes dramatic reductions in plant biomass production and is detrimental to photosynthetic activity. Salinization of soils has become a major and increasing concern. Our DOE-supported research has shown that the AtHKT1;1 transporter mediates a key mechanism for protection of plants from salinity stress by excluding toxic $Na^+$ ions from leaves (Horie et al., 2009). Recent mapping of salinity resistance quantitative trait loci (QTL) in grasses in several labs, and analyzing candidate HKT genes in rough mapping domains suggest that the same HKT mechanisms that the P.I.'s lab has identified in *Arabidopsis* are key salinity resistance mechanisms in grasses. Our DOE research focuses on the basic biophysical, physiological, and regulatory mechanisms through which HKT sodium cation transporters and $K^+$ transporters play central roles in mediating salt tolerance in plants.

The molecular $K^+$ transport mechanisms across chloroplast membranes remain largely unknown. Our recent research is further characterizing chloroplast-targeted $K^+$ transporters and their roles in plant growth, chloroplast function and salinity responses.

**Significant Achievements 2010-2012:**

***In vivo* Electrophysiological Characterization of AtHKT1;1 Transporter and its Role in Protection of Leaves from Salinity Stress**

Salt over-accumulation in leaves causes major damage to plants by inhibiting photosynthesis and metabolic enzymes. Our DOE-supported research has led to the model that AtHKT1;1 unloads sodium directly from xylem vessels to xylem parenchyma cells. However, direct comparative *in vivo* electrophysiological analyses of a plant HKT transporter in wild-type and *hkt* loss-of-function mutants had not yet been reported. Enhancer trap *Arabidopsis* plants with GFP-labeled root stelar cells were used to investigate AtHKT1;1-dependent ion transport properties using patch clamp electrophysiology (Xue et al., 2011). We reported key functions of AtHKT1;1-mediated ion currents in their native root stelar cells, including $Na^+$ and $K^+$ conductances, AtHKT1;1-mediated outward currents, and shifts in reversal potentials in the presence of defined intracellular and extracellular salt concentrations (Xue et al., 2011).

**$K^+$ transport by the OsHKT2;4 transporter from rice (*Oryza sativa*) with atypical $Na^+$ transport properties and competition in permeation of $K^+$ over $Mg^{2+}$ and $Ca^{2+}$ ions**

Members of the class II of HKT transporters have thus far been identified uniquely in grasses. But the physiological functions of this $K^+$ transporting class II of HKT transporters remained unknown in plants, with exception of the distinct class II $Na^+$ transporter, OsHKT2;1 (Horie et al., 2007). The genetically tractable rice (background Nipponbare) possesses two predicted $K^+$ transporting class II HKT transporter genes, *OsHKT2;3* and *OsHKT2;4*. We characterized the ion selectivity of the class II rice (*Oryza sativa*) HKT transporter, OsHKT2;4, (Horie et al., 2011). GFP-OsHKT2;4 is targeted to the plasma membrane in transgenic plant cells. Comparative analyses of cation permeabilities in several HKT transporters, including AtHKT1;1, TaHKT2;1, OsHKT2;1, OsHKT2;2 and OsHKT2;4 revealed that only OsHKT2;4 and to a lesser degree TaHKT2;1 mediate $Mg^{2+}$ transport. Interestingly, cation competition analyses demonstrate that the selectivity of both of these class II HKT transporters for $K^+$ is dominant over divalent cations (Horie et al., 2011).

**Differential sodium and potassium transport selectivities of the rice OsHKT2;1 and OsHKT2;2 transporters in plant cells**

$Na^+$ and $K^+$ homeostasis are crucial for plant growth and development. The $Na^+/K^+$ selectivities of the $K^+$ permeable HKT transporters had not yet been studied in plant cells. We therefore analyzed two highly homologous HKT transporters in plant cells, OsHKT2;1 and OsHKT2;2, that show differential $K^+$ permeabilities in heterologous systems. At millimolar $Na^+$ concentrations OsHKT2;2 mediated $Na^+$ influx into plant cells. In addition, the presence of external K $K^+$ and $Ca^{2+}$ down-regulated OsHKT2;1-mediated $Na^+$ influx in two plant systems, BY2 cells and intact rice roots (Yao et al., 2010).

**$K^+$ transporters in *Arabidopsis*: AtHAK5 and AKT1 are vital under low $K^+$ conditions** Potassium ($K^+$) is a major plant nutrient required and potassium uptake is an important contributor to salinity ($Na^+$) resistance. Epstein and colleagues showed that plant roots absorb $K^+$ through high-affinity and low-affinity transport kinetics. We previously characterized AtHAK5 as the major high-affinity $K^+$ uptake transporter in $K^+$-starved roots (Gierth et al., 2005). New results demonstrated that AtHAK5 and AKT1 are the major, physiologically relevant molecular entities mediating potassium uptake into roots (Pyo et al., 2010). In addition, we published reports describing transporters and their functions and regulation mechanisms (Mendoza-Cozatl et al., 2010; Brandt et al., 2012) and have collaborated on additional transporter characterizations (Li et al., 2010; Song et al., 2010) with DOE support.

**Science objectives for 2012-2013:**

Our present research sets out to answer the following questions:

1. Identify the regulation mechanisms that function in controlling *AHKT1;1* expression and $Na^+$ tolerance.
2. Functional characterization of plastid-localized putative $K^+/Na^+$ transporters crucial for chloroplast function and salinity responses.

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## Nanotube-Supported Phospholipid Bilayers

Alex I. Smirnov, Principal Investigator
Antonin Marek and Maxim A. Voynov, Postdoctoral Research Associates
2620 Yarbrough Dr., Department of Chemistry, North Carolina State University, Raleigh, NC 27695
Email: Alex_Smirnov@ncsu.edu; Web: http://www.ncsu.edu/chemistry/people/ais.html

Overall research goals: The overall long term objective of our BES DOE project is to develop a new class of nanoscale biotechnological objects - substrate-supported lipid nanotubes - and to utilize these nanoscale structural elements for building robust hybrid biological nanodevices that are based on functionally active membrane proteins. This will be accomplished by:

- Developing efficient experimental protocols for loading and self-assembling bilayer membranes of various lipid compositions inside the nanochannels formed in anodic aluminum oxide (AAO) substrates.
- Further improving technology for fabricating homogeneous nanoporous substrates with desired pore dimensions and low light absorbance to enable biophysical studies of light-harvesting biomolecular systems. Collaborating with DOE BES investigators on further dissemination of the lipid nanotube technology and nanoporous substrates as being developed in the course of this project.
- Investigating effects of surface chemistry through surface modification of nanoporous substrates on the lipid self-assembly and the properties of the lipid bilayers formed. Specifically, we propose to compare i) bilayer self-assembly on alumina vs. silica surfaces formed by sol-gel methods over the AAO nanopores and ii) effects of a series of chemical modifications on the properties of the lipid bilayers of various compositions including surface electrostatic potential.
- Developing His-grab technology in application to nanoporous substrates for one-step purification of membrane proteins and directional incorporation of various functional membrane proteins within the nanopores including a photosynthetic reaction center (RC) protein from *Rhodobacter Sphaeroides*.
- Demonstrating initial feasibility of employing lipid nanotube technology for building hybrid nanostructures based on membrane proteins. Specifically, carry out measurements of the lipid bilayer surface potential upon illuminating RC proteins incorporated into lipid nanotube arrays.

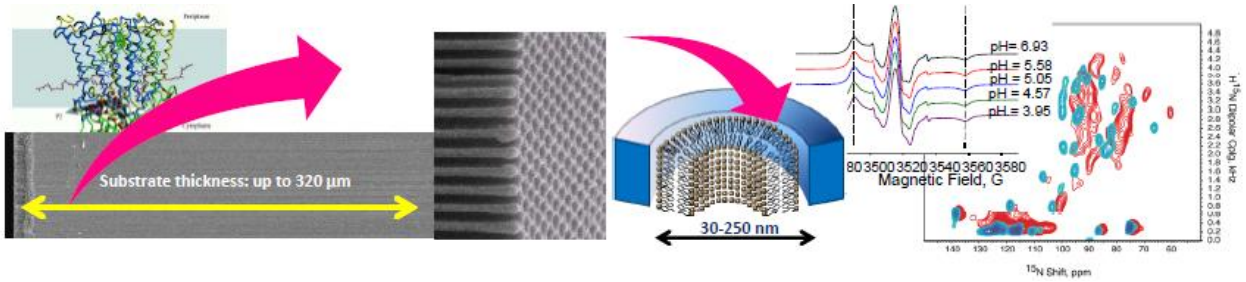


Figure 1. Left Panel: (Top) – a ribbon diagram of a photosynthetic reaction center (RC) protein from purple bacterium *Rhodobacter Sphaeroides;* (Bottom) – a scanning electron microscopy (SEM) image of the entire 320 µm cross-section of the AAO substrate fabricated at NCSU. Center Panel: a close-up SEM of the AAO surface shows highly ordered hexagonally packed pores with an average diameter of 48 nm. Right Panel: a cartoon of a lipid nanotube formed inside a nanopore and a series of EPR spectra of spin-labelled RC protein as a function of pH (top, multiple colours). The panel at the far right compares solid state 500 MHz NMR spectra of uniformly labelled pf1 coat protein in DMPC/DHPC bicelles (blue) and DMPC lipid nanotubes (red) aligned in 60 nm AAO.

Significant achievements 2011-2012:

- We have developed a number of high throughput procedures for fabricating high quality nanoporous substrates with pore diameters ranging from 25 to 90 nm and exceptionally narrow (5 to 6 nm) pore diameter distributions. Examples include substrates with a gradient of pore diameters and nanoporous matrices framed in metallic aluminum to improve mechanical stability.
- A series of substrates of various diameters have been prepared and tested for incorporation of membrane proteins and subsequent structure-function studies of membrane proteins by ssNMR (reported at ENC 2012 in Miami, FL and ICMRBS in Lyon, France: the manuscript in final stages of preparation).
- We have successfully labeled a photosynthetic reaction center (RC) from purple bacteria with a new electrostatic molecular label developed in the PI's lab. A new pH-sensitive spin-labeled lipid has been synthesized, characterized, and tested to assess surface electrostatics of lipid bilayer by EPR (manuscript under review by Biophysical J., minor revisions have been requested).
- Experimental protocols have been developed for assessing the free radical generating activity of a variety of nanomaterials that could be related to their biotoxicity (Ref. [1]).

Science objectives for 2012-2013:

- Develop experimental protocols and demonstrate fabrication of nanoporous substrates having continuous variable pore size along one dimension.
- Investigate surface electrostatics of lipid nanotubular bialyers as a function of bilayer curvature using EPR of pH-sensitive phospholipids we described recently.
- Demonstrate initial feasibility of employing lipid nanotube technology for building hybrid nanostructures based on membrane proteins. Specifically, carry out measurements of the lipid bilayer surface potential upon illuminating RC proteins incorporated into lipid nanotube arrays.
- Collaborate with other DOE BES grantees on investigation of self-assembly of the amyloidgenic peptide Aβ in nanopores of different diameters.

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   Note: this is collaborative work between PIs supported by different DOE grants.

## Regulation of Actin Filament Ends: The Role of Capping Protein in Stochastic Dynamics

**Christopher J. Staiger,** Principal Investigator
Dept. of Biological Sciences, Purdue University, W. Lafayette, IN 47907-2064
Email:staiger@purdue.edu; Web: www.bio.purdue.edu/development_disease/directory.php?refID=107

Overall research goals: Our goal is to understand the molecular mechanisms that underpin actin filament turnover in plant cells. Specifically, we will investigate the properties and function of the heterodimeric actin capping protein from *Arabidopsis* (CP). The specific aims of this project are: 1) characterizing the role of CP in actin stochastic dynamics with reverse-genetics and advanced imaging methods; 2) dissecting synergies between CP and other cappers within cells and using a biomimetic model of cytoskeletal dynamics; and 3) understanding where CP is located in living cells and how it contributes to organelle function.

To understand how actin filaments are organized and turn over *in vivo*, we applied variable-angle epifluorescence microscopy (VAEM) to living epidermal cells expressing an actin reporter. In the first quantitative description of single actin filament dynamics in plant cells [1], we found that filaments grow extremely rapidly but are rather short-lived. Filament disassembly is mediated by prolific severing activity rather than depolymerization from ends. A new model, based on the biochemical/biophysical properties of plant actin and actin-binding proteins was developed to describe this stochastic dynamic behavior [2,3,7]. Here, we will test the hypothesis that loss of CP leads to increased actin polymer levels and changes the availability of filament ends in living cells.

Significant Accomplishments, 2009–2012:

- We completed a phenotypic characterization for three capping protein (*cp*) mutant alleles (*cpa-1*, *cpb-1* and *cpb-3*). All three T-DNA insertion lines are knock-down mutants. Homozygous seedlings grown in the dark are characterized by significantly longer hypocotyls with measurably longer epidermal cells in all regions of the hypocotyl.
- We developed and validated metrics for quantifying global actin cytoskeleton architecture in live plant cells [4]. This provides us with powerful tools for measuring the extent of actin filament bundling (*skewness*) and the percent occupancy of filament arrays (*density*). Both metrics were applied to the developmental gradient of elongating cells in dark-grown hyocotyls allowing us to correlate axial cell expansion with actin hypocotyls allowing us to correlate axial cell expansion with actin organization. Specifically, rapidly elongating cells at the apex of the hypocotyl have actin cytoskeletal arrays that are more dense and less bundled than cells that have completed axial expansion near the base of the hypocotyl. In the *cp* mutants, all cells have a significantly higher percent occupancy compared to wild-type cells (Fig. 1; [9]). The increase in density is consistent with elevated actin filament levels in *cp* mutants and supports our hypothesis about CP function in plant cells.

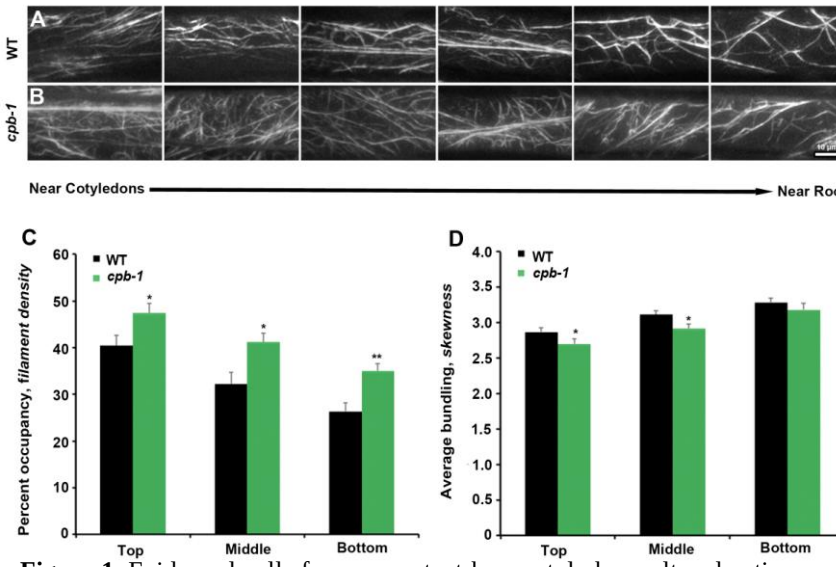


**Figure 1.** Epidermal cells from *cp* mutant hypocotyls have altered actin organization. (A,B) Actin architecture in epidermal cells along the axial gradient of cell expansion in representative hypocotyls from WT and *cpb-1* seedlings. (C,D) Quantitative analysis of actin filament architecture, bundling (D) and percent occupancy (C), show that *cp* mutants have increased filament density.

- Single actin filament dynamics in all three *cp* mutant lines have been measured with time-lapse VAEM imaging [9]. In addition to the previously described parameters [1], we developed new metrics that focus on properties of filament ends. First, we quantified filament origin/nucleation (*de novo*; from the side of another filament; and from pre-existing ends). The *cp* mutants have significantly more filaments originating from pre-existing ends and fewer that are nucleated *de novo* or off the side of a mother filament. We also measured filament-filament annealing, and *cp* mutants had 3- to 6-fold more annealing events compared to wild type. As a consequence of these changes in filaments end properties, maximum filament lengths and lifetimes were both significantly elevated in *cp* mutants. Thus, we have compelling evidence that CP plays a role in the turnover of actin filaments through its regulation of filament barbed ends *in vivo*.
- Previously we demonstrated that recombinant CP binds to and is regulated by the signaling lipid, phosphatidic acid (PA). Coarse-grained molecular dynamics (MD) simulations and molecular docking experiments indicate that the C-terminus of CPα forms an amphipathic helix involved in phospholipid binding. Moreover, by soaking wild-type seedlings with exogenous PA, we demonstrate that filament density increases in a dose-dependent fashion and mimicking the *cp* mutant phenotype. Consistent with a model whereby exogenous PA uncaps filament barbed ends, the *cpb-1* mutant is less responsive to lipid treatment compared to wild-type epidermal cells [9].

Scientific Objectives for 2012–2013:

- CP subcellular localization will be explored by cell fractionation, immunocytochemistry and with functional YFP-CP fusion proteins. Preliminary data indicates that a sub-population of CP interacts with an endomembrane compartment, perhaps the Golgi.
- Over-expression lines with elevated levels of both CP subunits have been recovered; these will be examined for differences in filament array architecture and stochastic dynamics by live-cell imaging. Moreover, double mutants between *cp* and other barbed-end capping factors (e.g. *vln*, *aip1*) will be recovered and examined for growth phenotypes and perturbation of actin dynamics.

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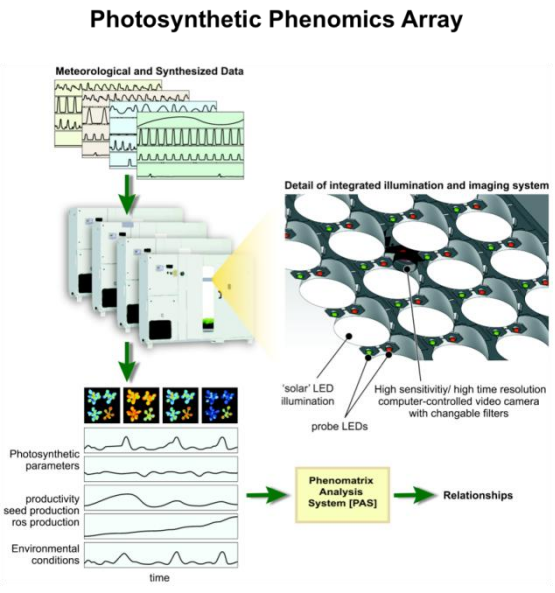
**Interdisciplinary Research and Training Program in the Plant Sciences**

Michael Thomashow, Principal Investigator
Federica Brandizzi, Jin Chen, Sheng Yang He, Gregg Howe, Jianping Hu, Ken Keegstra, David Kramer, Beronda Montgomery, Jonathan Walton, and Peter Wolk, Co-PIs
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48823
Email: thomash6@msu.edu; Web: http://www.prl.msu.edu/

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 conducted in the PRL have changed, but all have contributed to a basic understanding of plant growth and how it is affected by environmental factors. Current research conducted in the labs of individual faculty focuses on the biogenesis, biochemistry, and biophysics of cellular energy systems (Brandizzi, Hu, Keegstra, Kramer, Montgomery, and Wolk); the biosynthesis of plant cell walls (Brandizzi, Keegstra and Walton); and the interactions of photosynthetic organisms with the environment (He, Howe, Kramer, Montgomery, and Thomashow). In addition, there is the Plant BioEnergy Network—PlaNet—project, a collaborative effort devoted to understanding the regulatory, biochemical, and metabolic networks that govern energy flow within photosynthetic organisms. The current goals are to determine the regulatory networks that impart down-regulation of photosynthesis-associated genes in response to biotic and abiotic stresses, and photosynthetic acclimation to rapidly fluctuating environmental conditions. Addressing these questions includes the use of novel high-throughput "phenometrics" technology—the Photosynthetic Phenometrics Array (PPA)—under development in the laboratory of David Kramer (Kramer et al., 2012, 2012). The PPA facility will not only provide a powerful resource for use in PRL research, but will also provide a major new resource for those in the scientific community studying photosynthesis.




Examples of recent achievements 2011-2012: During the past three year funding period, the PRL faculty published more than 100 articles. A complete listing of these publications can be found at the PRL website (http://www.prl.msu.edu/). Recent highlights on the topic of photosynthetic organisms interacting with the environment include: discovery of the first enzyme involved in deactivation of the plant stress hormone jasmonic acid (JA) (Koo et al., 2011); elucidation of a

mechanism that increases the stability of proteins that repress expression of JA-responsive genes (Shyu et al., 2012); description of a signaling cascade by which the bacterial JA-mimicking toxin coronatine overcomes both stomatal- and mesophyll-based plant defenses (Zheng et al., 2012); discovery of a conserved mechanism by which cross-talk between the gibberellin and JA hormone signaling pathways regulate the balance between growth and defense in flowering plants (Yang et al., 2012); identification of key genes that integrate circadian and low temperature regulatory pathways required for maximum freezing tolerance (Dong et al., 2011); and the finding that light-dependent regulation of cellular morphology in the cyanobacterium *Fremyella diplosiphon* is likely to be linked to cellular mechanisms that protect against light-induced damage, in addition to regulating photosynthetic efficiency and pigmentation production (Pattanaik and Montgomery, 2012; Singh and Montgomery, 2012). In the areas of cell wall synthesis and cellular energy systems, our research: identified a membrane-anchored GTPase that controls the structural integrity of the endoplasmic reticulum, the cellular site where most of the enzymes responsible for cell wall biosynthesis are synthesized (Chen et al., 2011; Stefano et al., 2012); established that an ER-anchored kinase and ribonuclease responsible for managing secretory protein overload has a key role in plant immune responses (Moreno et al., 2012); established key roles for the thylakoid proton motive force in regulation of photosynthesis governing the balance between photosynthetic efficiency and photoprotection (Kohzuma et al., 2012; Ioannidis et al., 2012); discovered a novel regulatory pathway that controls the morphogenesis and proliferation of both peroxisomes and mitochondria (Aung and Hu, 2011); and identified a likely glycoside transporter with a specific physiological role in production of heterocyst envelope polysaccharide (López-Igual et al., 2012).

Recent results from the PlaNet project are presented in two additional abstracts, one authored by Gregg Howe and colleagues and the other by David Kramer and colleagues.

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## DEFINING THE ASSEMBLY, ORGANIZATION, AND TURNOVER OF THE *ARABIDOPSIS* 26S PROTEASOME

***Vierstra, R.D.[a], R.M. Marshall[a], A.J. Book[a], N.P. Gladman[a], D.C. Gemperline[a], K.-H. Lee[a], M. Scalf[b], and L.M. Smith[b]***

Departments of [a]Genetics and [b]Chemistry, University of Wisconsin-Madison, Madison, WI 53706, USA

Central to the ubiquitin (Ub) proteolytic system is the 26S proteasome, a 2.5-MDa proteolytic complex that specifically degrades Ub-protein conjugates. The 26S proteasome is composed of two subparticles, the 20S core protease (CP) that compartmentalizes the protease active sites, and the 19S regulatory particle (RP) that recognizes and translocates appropriate substrates into the CP lumen for breakdown. Our studies with *Arabidopsis thaliana* during the current grant period have revealed a remarkably large number of events that affect the activity, substrate specificity, and/or abundance of the plant particle. These include: (i) synthesis by a integrated transcriptional regulon that measures proteasome capacity, (ii) an intricate assembly cascade involving a suite of dedicated chaperons, (iii) incorporation of alternative subunit isoforms that may have divergent functions, (iv) capping with different regulatory complexes, (v) association with accessory proteins and target shuttle factors, (vi) various post-translational modifications, and (vii) a route to remove excess or aberrant proteasome complexes. Key to our success in defining the organization of plant 26S proteasomes was a recently developed affinity method to rapidly purify the particle intact from *Arabidopsis thaliana* that involved genetic replacement of the $\alpha$7 subunit of the CP with a Flag epitope-tagged version. In-depth mass spectrometric (MS) analyses of the resulting preparations confirmed that the CP-RP complex is actually a heterogeneous set of particles assembled with paralogous pairs for most of the 33 core subunits. A number of these subunits were found to be modified post-translationally by proteolytic processing, acetylation, and/or ubiquitylation. RPN1 and RPN2 in particular appear to be prevalent Ub targets. Measurement of 26S proteasome levels in various mutants defective in autophagy, strongly suggests that this ubiquitylation helps direct the complex to the vacuole for breakdown via an autophagy-type transport system. Presumably, this transport is encouraged by the NBR1 autophagy receptor that tethers the Ub moieties to the lipidated ATG8 protein that decorates the autophagic compartment as it assembles.

During current studies, we also developed a purification strategy that focuses on the RP subcomplex specifically by replacing the RPT4 isoform with a Flag-tagged version. MS analyses of these preparations identified several new proteasome-interacting proteins, with the complete list now including the SEM2 and RPN13 core subunits, the maturation factor UMP1/2, the assembly chaperons PBAC2 and ECM29, several Ub ligases, and the deubiquitylating protease UBP16. Two novel interactors (PIP1 and PIP2) of unknown function were discovered that interact specifically with PAG1 and potentially other $\alpha$-ring subunits of the CP. Both PIP1 and PIP2 appear to be unique to land plants and each contains a C-terminal HbYX motif (hydrophobic-Tyr-any amino acid) first discovered in yeast as being essential for $\alpha$-ring interactions. The presence of this HbYX sequence suggests that PIP1/2 act as chaperons that aid $\alpha$ ring assembly. In addition, we detected a particle consisting of the CP capped by the single subunit PA200/Blm10 activator, which also bears a C-terminal HbYX motif. Even though null *pa200* mutant are developmentally normal, a role for the PA200-CP complex in proteolysis is indirectly supported by a dramatic increase in the PA200 protein upon treating plants with the proteasome inhibitor MG132.

We have continued our reverse genetic analyses of several *Arabidopsis* CP and RP subunits, which has further reinforced that notion that individual core components have diverse roles in plant growth and development. For example, whereas null mutants eliminating the RPT4a isoform has little phenotypic impact, null mutants eliminating RPT4b display a striking hyposensitivity to the stress hormone abscisic acid (ABA). At least part of this insensitivity is driven by dampened accumulation of the ABI5 transcription factor that is central to ABA responsiveness. This effect is in striking contrast to a knock-down mutation affecting another RP subunit RPN10, where causes *Arabidopsis* seedlings to be hypersensitive to ABA by accumulating high levels of ABI5. Taken together, our studies indicate that the 26S proteasome is a highly dynamic protease complex that is regulated by numerous mechanisms and controls a number of critical processes within plant cells by removing proteins upon ubiquitylation.

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## Functional Analysis of Plant Sucrose Transporters

John M, Ward, Principal Investigator
Anke Reinders, Postdoctoral Research Associate
Plant Biology Department, University of Minnesota
250 Biological Sciences Center, 1445 Gortner Ave. St. Paul, MN 55108
Email: jward@umn.edu; Web: http://www.cbs.umn.edu/lab/ward

Overall research goals: The main research goal is to understand the transport activity and structure/function relation of plant sucrose transporters (SUTs, also called SUCs). These are proton coupled sucrose uptake transporters in the plasma and vacuole membranes in plants. SUTs are essential for the long distance transport of carbohydrate in vascular tissue and for uptake into sink tissues such as seeds. We are studying the transport activity (substrate affinity, specificity, regulation) using mutagenesis, heterologous expression and electrophysiology. To study the physiological function of SUTs, we are mainly using complementation of Arabidopsis mutants as a model. The ability to modify SUT activity is important for engineering changes in carbon partitioning.

Significant achievements 2010-2012:

- The function of cDNAs for all five sucrose transporter genes from rice was tested by expression in yeast and Xenoppus oocytes. OsSUT1 and OsSUT5 were functional in both expression systems and were analyzed in detail using two-electrode voltage clamping (TEVC) in oocytes and $^{14}$C-sucrose uptake in yeast [1]. We found that OsSUT2, the vacuolar sucrose transporter from rice, was functional when expressed in yeast but not in oocytes [1]. We collaborated with Dr. Jong-Seong Jeon (Kyung Hee University, Korea) to study the activity of OsSUT2 and the phenotype of *ossut2* mutants [2]. This led to the hypothesis that vacuolar storage of sucrose contributes to phloem loading capacity in rice [3].
- The OsSUT1 structure was modeled using the known structures of LacY, GlpT, and EmrD, distantly related prokaryotic transporters, as templates. This provided a revised transmembrane model and allowed us to select six conserved charged amino acid positions within transmembrane spans for muatagenesis. We found three positions that appear to be essential for transport activity [4]. The OsSUT1 R188K mutant did not transport $^{14}$C-sucrose but encodes a $H^+$ leak that is blocked by sucrose. Some non-transported glucosides also block $H^+$ transport through the R188K mutant.
- We developed a novel fluorescent assay for sucrose transporters. Yeast expressing type I SUTs accumulate the fluorescent coumarin glucoside esculin and can be detected using several methods including fluorescence-activated cell sorting (FACS) [5]. We used this transport assay in conjuntion with a novel gene shuffling method that we call Synthetic Template Shuffling (STS) to identify positions in sucrose transporter proteins that contribute to substrate specificity [6]. Briefly, type I SUTs can transport esculin while type II SUTs can not. We identified 63 differentially conserved positions in type I vs. type II SUTs and used gene shuffling between a synthetic template (OsSUT1m63, a non-functional type II SUT with 63 mutations) and wild-type OsSUT1 to generate a shuffled library. Yeast transformed with this library were selected for esculin uptake using FACs. We identified 5 amino acid changes that convert a type II SUT to type I SUT substrate specificity [6].
- Recently completed genome sequence for the lycophyte *Selaginella*, the bryophyte *Physcomitrella*, several species of red algae and RNAseq data for several green algae enabled our analysis of the origins of higher plant SUTs. We conclude that type I SUTs, present only in eudicots, evolved from type III SUTs that are localized to the vacuole [7]. It is of particular interest for this project that monocots use a type II SUT for phloem loading while eudicots use a type I SUT and that these two types of SUTs differ strongly in substrate specificity. We demonstrated that the type II SUT from barley, HvSUT1, reverses the growth phenotype of an Arabidopsis *atsuc2* mutant [7].
- In collaboration with Doris Rentsch (University of Bern) we analyzed the activity of two peptide transporters AtPTR1 and AtPTR5 from Arabidopsis. Both showed coupling ion ($H^+$) leak in the absence of substrate, block of this leak by non-transported dipeptides, and voltage-dependent transport activity[8].

Science objectives for 2012-2013:

- The R188K mutant of OsSUT1 revealed a $H^+$ leak that could be blocked by transported as well as non-transported glucosides [4]. We are analyzing this mutant by electrophysiology with a variety of glucosides to differentiate substrate binding involved in initial recognition of substrate and binding involved in substrate translocation.
- To confirm the structural model of OsSUT1 [4], the membrane topology of the protein needs to be determined experimentally. We are testing the function of a Cys-free SUT that would allow us to use cysteine scanning accessibility mutagenesis to investigate topology. This strategy also requires development of a protein purification protocol and that will facilitate further structural analysis.
- In order to make significant progress in understanding the structure/function relation of sucrose transporter, a protein crystal structure is necessary and we need to take the first steps towards this long-term goal. Therefore we are testing different protein tags to identify positions that allow purification of an active protein.
- We are using the esculin uptake assay and FACS [5] with gene shuffling strategies [6] to identify mutations that modify transport activity. The goal is to develop SUT variants with modified substrate specificity and affinity that can be used to engineer plants with differences in carbohydrate partitioning.

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7. Reinders A, Sivitz AB, Ward JM (2012) Evolution of plant sucrose uptake transporters (SUTs). Front Plant Sci 3: 00022.
8. Hammes UZ, Meier S, Dietrich D, Ward JM, Rentsch D (2010) Functional properties of the Arabidopsis peptide transporters AtPTR1 and AtPTR5. J Biol Chem 285: 39710-39717.

# *Session IV*

## Dynamic TEM and LCLS: New Capabilities for Visualizing Protein Structural Dynamics

James E. Evans[1]

Co-PIs: Nigel D. Browning[1] (DTEM) & Matthias Frank [2] (LCLS)

[1] Pacific Northwest National Laboratory, Richland, WA 99352
[2] Lawrence Livermore National Laboratory, Livermore, CA 94550
Email: James.Evans@PNNL.gov

In 1953, the structure of DNA was solved, and five years later, the first three-dimensional model of a protein was determined at atomic resolution (1, 2). Since that time, biologists have strived to link the structure of biological systems to their physiological function and dynamics. Yet, countless details still remain obscure for most research foci because biological systems are highly complex and their processes occur over a wide range of spatial and temporal scales (from femtoseconds to hours and angstroms to meters). Ultrafast x-ray diffraction using x-ray free electron lasers (3) and dynamic transmission electron microscopy (DTEM) (4) are two burgeoning technologies that are being developed for pump-probe biological applications.

Currently, the best preservation of biological samples for structural analysis with x-rays or electrons is achieved by rapidly freezing the samples to liquid nitrogen temperature. This vitrification enhances high-resolution analysis of the sample by affording more tolerance to irradiation during imaging and by immobilizing the sample in a specific conformation to prevent motion blur. Unfortunately, such cryogenic preparation completely negates the posssiblity of dynamic observations that occur on shorter timescales than the freezing process (<1 ms). Therefore, in order to expand the pump-probe experimental regime to more natural environmental conditions and permit physiologically relevant dynamic observations, *in situ* liquid chambers or diffract-and-destroy imaging at room temperature are critical.

The DTEM combines the high spatial resolution of electron microscopy with the high temporal resolution of pulsed lasers and the incorporation of these two components into a single instrument provides a perfect platform for *in situ* pump-probe observations of dynamic processes. Ultrafast x-ray diffraction at the Linac Coherent Light Source is also capable of pump-probe experiments and takes advantage of femtosecond x-ray pulses of much higher peak brightness than previous synchrotron sources to outrun x-ray induced damage and enable single-shot analysis at room temperature.

This talk will describe the current and future applications of *in situ* liquid microscopy, DTEM and ultrafast x-ray diffraction for pump-probe imaging of single particle and 2-D protein crystals with unprecedented spatiotemporal resolution. The synergy between both advanced approaches should permit time-resolved multimodal observations of macromolecular reactions and conformational switching with unprecedented spatiotemporal resolution to help better link protein structure and function.

References:

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## Photodynamics of Single Biomolecules in Solution by Suppression of Brownian Motion

William E. (W. E.) Moerner, Principal Investigator
Gabriela S. Schlau-Cohen, Postdoctoral Research Associate
Department of Chemistry, Mail Code 5080, Stanford University, Stanford, CA 94305-5080
Email:wmoerner@stanford.edu; Web: http://www.stanford.edu/group/moerner/sms_trapping.html

Overall research goals: The objective of this research is to explore and understand the dynamics of single enzymes and single photosynthetic proteins in a solution environment for an extended observation window (tens of seconds) using optical spectroscopy and electrokinetic trapping. Achievement of this objective requires continued development of our advanced Anti-Brownian ELectrokinetic (ABEL) trap, which suppresses Brownian motion for single nanoscale objects, to provide extended-time, single-molecule fluorescence information without perturbative surface attachment or encapsulation. One goal of this research is to understand single-molecule enzymatic dynamics by a detailed exploration of the catalytic cycle of electron-transfer redox enzymes, such as bNIR (bacterial Nitrite Reductase), which will reveal the kinetics of intramolecular electron transfer and sense the intrinsic heterogeneity of the enzyme. A second primary goal involves investigation of the photodynamics of light-harvesting antenna proteins such as light-harvesting protein 2 (LH2) from purple bacteria and allophycocyanin (APC) from cyanobacteria in order to characterize molecule-to-molecule photophysical heterogeneity and time-dependent state changes. A deeper understanding of energy and electron transfer processes can provide insight into the function of natural systems for the development of efficient artificial light-harvesting devices.

Significant achievements 2010-2012: We have achieved major strides forward in ABEL trap development, in characterizing intramolecular energy transfer in redox enzymes, and in exploring the photophysics of light-harvesting proteins.

Following our development of a stochastic differential equation model, we have implemented an ABEL trap with knight's tour beam scanning and a Kalman filter-based real-time tracking algorithm, extending the trap's capability of confining dim and fast-diffusing nano-objects down to the level of single-dye labeled 30nt DNA (1).

Considering the importance of redox electron transfer events in the energy conversion and storage process, we have completed a detailed study of single electron transfer events in blue nitrite reductase (Fig. 1) (2). In this system, a fluorophore covalently attached to the protein near the type I Cu site experiences quenching when the Cu is oxidized, yet becomes brightly fluorescent when the Cu site is reduced. In this way, the fluorophore emission reports directly on the redox state of the type I copper atom as nitrite is converted to NO. When a single enzyme is trapped, we observe high and low emission periods and can thus extract dwell times in the individual states. With a detailed rate equation analysis, specific rate constants of various internal processes have been extracted.

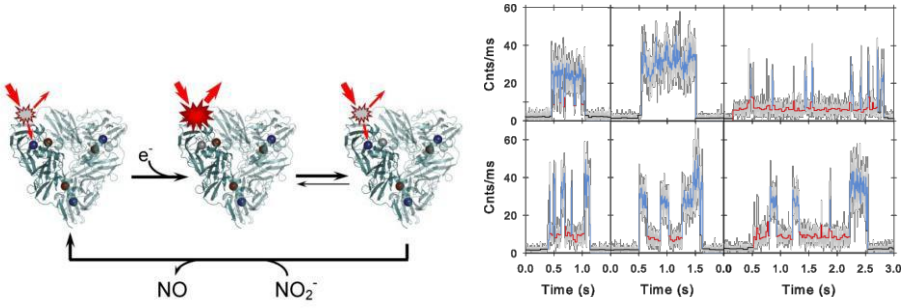


**Figure 1.** Left Panel: Scheme of assay showing fluorophore sensing of the redox sgtate of the type I Cu. Right Panel: Example emission traces of 6 single enzymes, showing high (reduced) and low (oxidized) levels. These unprecedented data allow detailed kinetic analysis.

We have also had success employing the ABEL trap for simultaneous intensity and lifetime measurements of photosynthetic antenna proteins (3, 4). In the case of the primary light-harvesting protein in purple bacteria, LH2, statistical extraction of state changes shows reversible, intensity-dependent switching between more and less quenched states (Fig. 2).

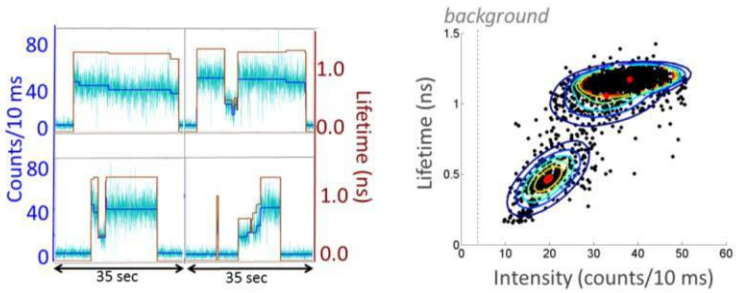


**Figure 2.** Left Panel: Examples of photodynamics of single LH2s. Cyan: Intensity trajectory (left ordinate). Blue: Brightness states extracted by a change-point finding algorithm. Brown: Excited state lifetime of each state (right ordinate). Correlated changes in brightness and lifetime suggest switching between a more and less quenched state of LH2. Right Panel: Scatter plot of intensity-lifetime points for each intensity level, with clustering by a Gaussian mixture model.

By extending the capability of the ABEL trap to *simultaneously* record intensity, lifetime, and spectrally-resolved fluorescence emission spectra, different states of APC monomers along the photobleaching pathway are resolved. Based on spectrally shifted emission and different excited state lifetime, a physical model is proposed for the photodynamics of the APC monomer. (Fig. 3).

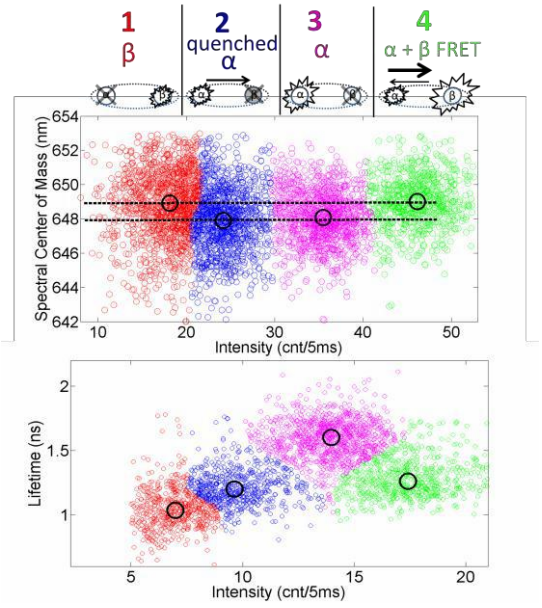


**Figure 3** Lifetime, brightness, and spectrally-resolved spectroscopy of single APC monomers in solution using the ABEL trap. a) Spectra-intensity (upper, dotted lines highlight the difference in emission spectrum between clusters) and lifetime-intensity (lower) mapping of the observed states in single molecules of APC monomers. Black circles denote the average values of each cluster. Different populations are color-coded in the scatter plots and a physical model is illustrated at the top.

<u>Science objectives for 2012-2013:</u>

- Measure time-dependent spectral dynamics for single LH2 to fully characterize the states.
- Begin study of the intramolecular electron transfer dynamics of the oxygen-reducing multi-copper enzyme, Fet3p, in order to study the four-electron catalytic cycle, in collaboration with E.I. Solomon.
- Further extend the capability of the trap to simultaneously monitor fluorescence spectrum, lifetime and intensity dynamics at the single-fluorophore level.
- Begin investigation of the conformational dynamics of the crucial enzyme, F0F1 ATP synthase, via single-molecule FRET in the ABEL trap in collaboration with M. Boersch.

<u>References to work supported by this grant in 2010-2012:</u>

1. Quan Wang and W. E. Moerner, "An Adaptive Anti-Brownian ELectrokinetic Trap with Real-time Information on Single-Molecule Diffusivity and Mobility," ACS Nano 5, 5792-5799 (2011).
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4. Samuel Bockenhauer, Quan Wang, and W. E. Moerner, "Spectrally Resolved Anti-Brownian ELectrokinetic (ABEL) Trapping of Single Peridinin-Chlorophyll-Proteins in Solution," *Proc. SPIE* **8427**, 84274C(1-9) (2012).

# *Session V*

## Mass Spectrometric Imaging of Plant Metabolites

Basil Nikolau, Principal Investigator
Young-Jin Lee, Robert S Houk, Co-Principal Investigators
The Ames Laboratory, Iowa State University, 3254 Molecular Biology Building, Ames, IA 50011
Email: dimmas@iastate.edu; Web: http://www.ameslab.gov/cbs/fwp/mass-spectrometric-imaging

Overall research goals: The research objectives are to develop mass spectrometric imaging (MSI) technologies to spatially map metabolite distributions within plant tissues. We are using these techniques to address specific biological questions concerning the differential distribution of metabolites among diverse cell-types, and dissect metabolic regulatory processes. Such imaging technology is of particular importance in multicellular organisms, such as plants, in order to identify and characterize cooperative and antagonistic genetic interactions, and environmental modifiers that asymmetrically regulate the complexity of plant metabolism. This imaging analytical capability is being developed in the context of understanding plant metabolic processes that impact the collection and storage of solar-energy in the form of energy-dense biochemicals.

Significant achievements 2010-2012: Significant achievements where accomplished in three different categories: 1) improvements to enhance the spatial resolution of the MSI technology, achieving single cell level spatial resolution (down to 12 μm); 2) improvements in the mass resolution to enhance the chemical identification of analytes; 3) multiplexed MSI scheme to reduce the time-required for data acquisition; 4) integrating MSI technology into functional genomics analysis of genes required for the biosynthesis of energy-dense biochemicals (see Figure 1, below).

Science objectives for 2012-2014:
The project will use the currently available MSI technology to address biological questions associated with specialized metabolism that is asymmetrically distributed among discreet cellular-sized structures. The choice of systems is guided by three criteria: (1) focus on metabolism leading to the biosynthesis of energy-dense molecules (i.e., molecules composed primarily of carbon and hydrogen, lacking oxygen), (2) use the current resolution power of our imaging technology (~10 μm resolution) to visualize metabolites and metabolism to the level of about a single plant cell and (3) take advantage of our team's expertise in the biochemistry and genetics of acetyl-CoA metabolism.

References to work supported by this project 2010-2012:

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**Perdian, D.C., Cha, S., Oh, J., Sakaguchi, D., Yeung, E.S., and Lee, Y.J.** (2010) In Situ Probing of Cholesterol in Astrocytes at the Single Cell Level using Laser Desorption Ionization Mass Spectrometric Imaging with Colloidal Silver. *Rapid Communications in Mass Spectrometry* **24**, 1147-1154.

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**Korte, A.R., Song, Z., Nikolau, B.J., and Lee, Y.J.** (2012) Mass Spectrometric Imaging as a High-Spatial Resolution Tool for Functional Genomics: Tissue-Specific Gene Expression of TT7 Inferred from Heterogeneous Distribution of Metabolites in Arabidopsis Flowers. *Analytical Methods*, **4**, 474-481.

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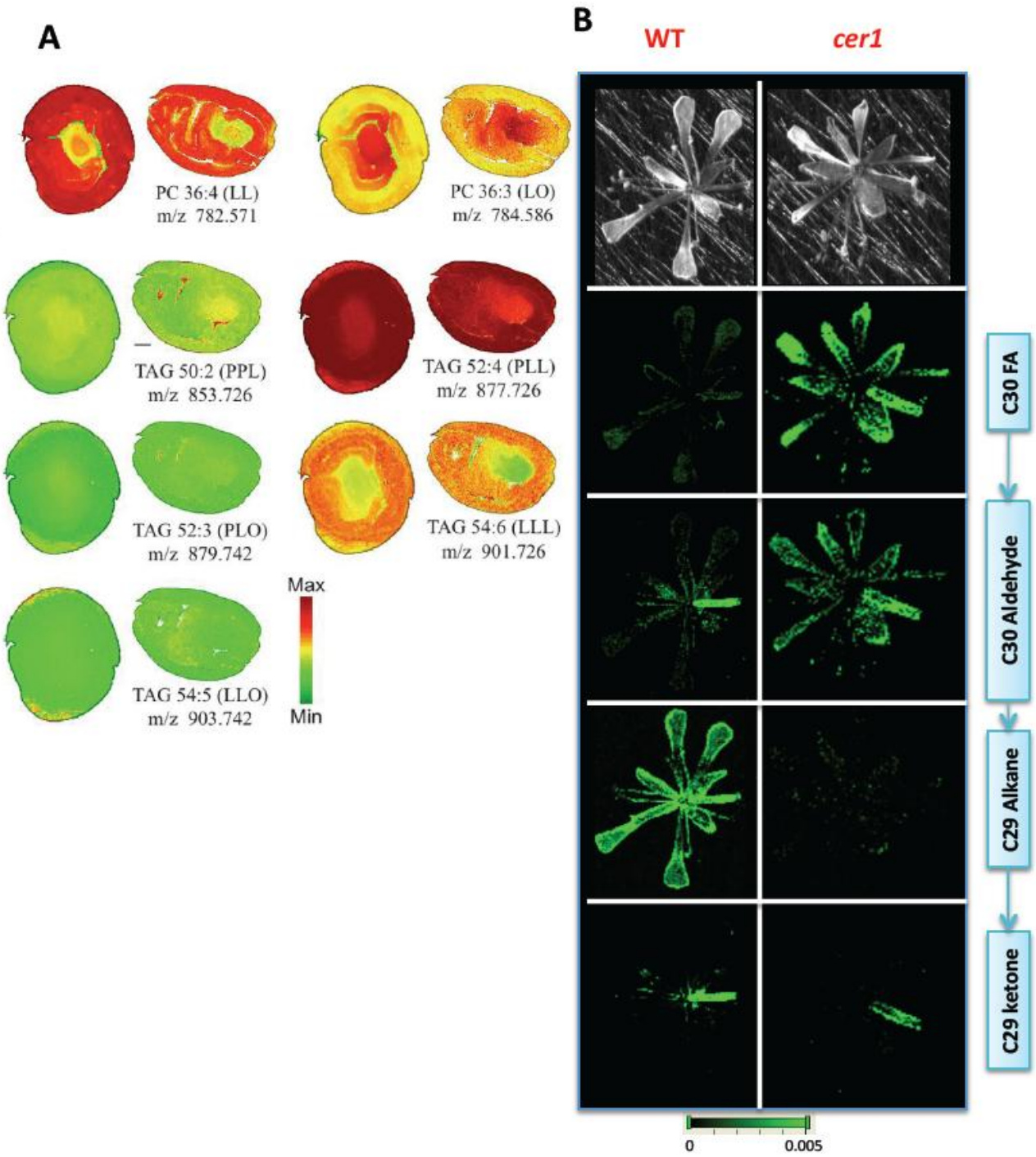


**Figure 1.** Examples of MSI technology applied to solving functional genomics analysis of plant metabolism. A) Asymmetric distribution of phosphatidylcholine (PC) and triacylglycerol (TAG) molecular species with cotton embryos (Horn et al., 2012). B) Asymmetric distribution of cuticular lipids on the surface of Arabidopsis flowers of wildtype (WT) and *cer1* mutant plants. The *cer1* mutation blocks the conversion of the C30-aldehyde to the C29-alkane (Song et al., in preparation).

## Two-Dimensional Electronic Spectroscopies for Probing Electronic Structure and Charge Transfer: Applications to Photosystem II

Jennifer Ogilvie, Principal Investigator
University of Michigan, Department of Physics and Biophysics, 450 Church St., Ann Arbor, MI 48109
Email: jogilvie@umich.edu; Web: http://www-personal.umich.edu/~jogilvie/Ogilvie_Group/Welcome.html

Overall research goals:

This project focuses on addressing key deficits in our current understanding of the PSII reaction center (PSII RC). Understanding the PSII RC's design principles has importance for both fundamental and applied sciences wishing to mimic its remarkable properties. The project aims to address the following open questions: 1) What is the electronic structure of the PSII RC? 2) What is the charge transfer mechanism in the PSII RC? 3) Does electronic coherence facilitate energy transfer in the PSII RC? To address these questions we extend our previous work employing two- dimensional electronic spectroscopy (2DES), a method that enables a direct view of electronic couplings and energy and charge transfer processes over a broad range of frequencies with ultrafast time resolution. In addition we are developing 2DE-Stark spectroscopy which utilizes an applied electric field to aid in separating exciton and charge transfer states and allow control of the charge separation rate.

Significant achievements 2010-2012:

*Testing exciton models of the PSII RC:* We have made progress in using our 2DES data [1] to test current excitonic models of the PSII RC. Figure 1 shows the predicted 2D spectra based on an existing exciton model, alongside our data. We also show a modified model where we have changed several parameters, most notably the system-bath coupling and disorder, to achieve better agreement with our 2DES data. This work is currently under review [2].

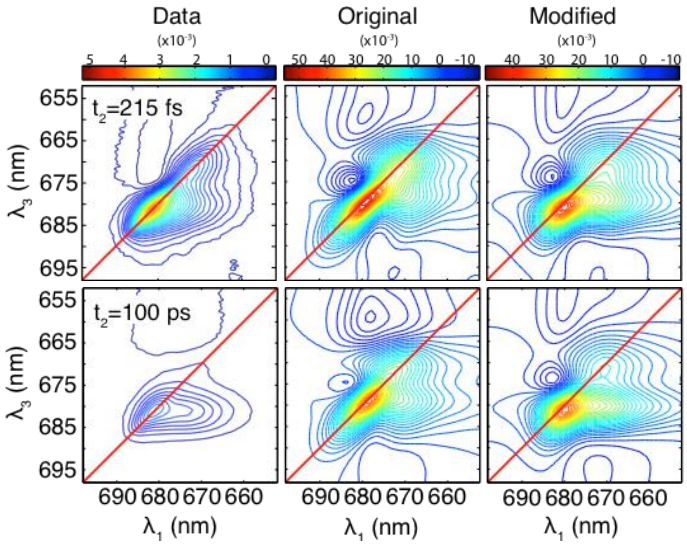


Figure 1: 2D electronic spectra for different waiting times $t_2$=215 fs, 600 fs, 1.5 ps and 100 ps. Experimental (left), and simulations based on an existing PSII RC exciton model (center) and modified model (right). Simulations are each averaged over 1500 instances of disorder.

*2DES with a continuum probe:* In the previous grant period we developed an experimentally simplified 2DES method that employs a broadband probe, extending the available detection wavelength range to span the full visible spectrum. We recently demonstrated a straightforward method for correcting spectral distortions in 2DES data that can arise from the use of a chirped continuum probe [3]. We are currently using the continuum probe method to aid in the assignment of charge transfer events by probing the pheophytin ion bands at 460 nm. The goal here is to correlate changes in the Qy region with a clear spectroscopic marker of charge transfer. In the process we have observed signatures of electronic coupling between carotenoids and chlorophyll A pigments that could be of significance for understanding the excitonic structure of the PSII RC.

*2D electronic Stark spectroscopy:* The goal of this new spectroscopy is to identify charge transfer states and their involvement in charge separation in the PSII RC; the large electric field applied in a Stark spectroscopy experiment preferentially shifts charge transfer states, facilitating their identification. Both the zero waiting time 2D spectra, and time-resolved measurement will be used to gain insight into what states and kinetic processes involve charge transfer. We have received all of the necessary parts for the experiment, have constructed a Stark cell design for room temperature and 77 K experiments and are currently debugging the experiment using charge transfer dyes embedded in a polymer matrix at room temperature.

Science objectives for 2012-2014:

- In collaboration with Shaul Mukamel and Darius Abramavicius we are simulating 2DES spectra based on recently-developed charge transfer model. This model is already showing improved agreement with our experimental 2DES data, allowing us to test different models of charge separation.
- We have recently made improvements to the sensitivity of our 2DES continuum probe experiment. Improved broadband measurements on D1-D2 PSII RC samples, and on reduced D1-D2 PSII RC samples will provide new insight into the charge separation process and the excitonic structure of the PSII RC.
- 2DES experiments on PSII core samples will enable testing of the validity of the D1-D2 PSII RC samples as a model system for understanding the primary processes of energy transfer and charge separation in the PSII RC.
- Upon performing 2D electronic Stark spectroscopy on model systems we will then apply the method to the PSII RC.

References to work supported by this project 2010-2012:


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 b559 photosystem II reaction center complex," Journal of Physical Chemistry Letters **1**, 2774-2780 (2010).
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4. K. L. M. Lewis and J. P. Ogilvie, "Probing photosynthetic energy and charge transfer with two-dimensional electronic spectroscopy," Journal of Physical Chemistry Letters **3**, 503-510 (2012).

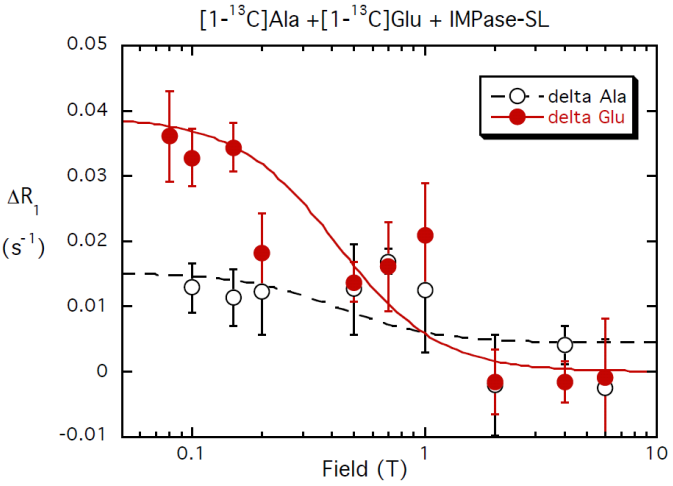
## Osmoregulation in Methanogens

Mary F. Roberts, Principal Investigator
Merkert Chemistry Center, Boston College
2609 Beacon Street. Chestnut Hill m A02467
Email: mary.roberts@bc.edu

Overall research goals: This DOE sponsored project aims to categorize the solutes synthesized in archaea and related bacterial hyperthermophiles in response to osmotic and thermal stress and then to explore any specific interactions of these solutes with macromolecules.

Significant achievements 2010-2012: For several archaeal proteins we have seen that certain anionic solutes are much more effective than zwitterionic or nonionic solutes – possibly indicative of a specific interaction. Since solute concentrations needed for protection are > 50 mM, solute binding is likely to be weak. Capturing such weak binding and determining if a specific site is targeted is difficult with conventional methods. We have developed the use of high resolution field cycling NMR relaxation studies as a way to not only monitor binding but to determine where sites exist. Our test archaeal protein is the dimeric inositol monophsphatase from *Archaeoglobus fulgidus*. The mid-point of its denaturation is is 87$^{o}$C and it is protected from thermal denaturation by the presence of 50-300 mM α- or β-glutamate (solutes accumulated by that organism), as well as aspartate, and a wide range of dicarboxylic acids (but not tricarboxylic acids). Structures (obtained under cryo-conditions) of protein crystals formed in the presence of high concentrations of glutamate and aspartate show four bound amino acids per monomer. However, whether or not these sites are occupied in solution and at higher temperatures is unclear.

$^{13}$C-high resolution field cycling NMR with $^{13}$C-carbonyl labeled Glu, Asp, succinate and Ala is used to assess if and where solutes bind to the protein, which is spin-labeled on Cys to enhance sensitivity. We can easily detect for 25-100 mM solute with 50-100 μM protein by the increase in spin lattice relaxation rate ($\Delta R_1$) of the $^{13}$C comparing samples with spin-labeled and non-labeled protein. The figure at right shows that with a mixture of Glu and Ala, the Glu is preferentially binding to the protein (and detection requires fields below 1 T). The proximity of the spin-label on the protein dominates the dipolar relaxation of the bound small molecule, which is rapidly exchanging on and off the protein. Analysis of the field dependence profile allows one to estimate a correlation time for the complex, and by examing several concentrations on can estimate a $K_d$. For the IMPase, mutagenesis can then be used to sequentially remove different Glu sites and determine which sites are sensed by the spin-labels on two modifiable Cys of the protein. We have acquired data up to 50˚C and can suggest which of the four sites seen in the crystal are occupied in solution at higher temperatures. The results of this particular part of our work should help to provide a molecular picture for osmolyte specificity, when it is observed. *The methodology is robust and useful for a wide variety of systems where one wants to explore weak binding of ligands to macromolecules.*



**Conformational States of Cytochrome P450 in Catalytic Cycle by Advanced Electron Paramagnetic Resonance**

R. David Britt, Principal Investigator
David B. Goodin, Co-PI
William K. Myers, Young-Tae Lee, Postdoctoral Research Associates
Chemistry Department, Univ. of California Davis, One Shields Ave., Davis, CA 95616
Email: rdbritt@ucdavis.edu, Web: http://brittepr.ucdavis.edu

Overall research goals: The research objectives are to study conformational states of cytochrome P450 in its entire catalytic cycle by: (1) perform dipolar electron-electron resonance (DEER) measurements on intermediate states and the interaction of the putidaredoxin (Pdx) redox partner; (2) investigate the dipolar interaction of the heme iron with site directed spin labels by study temperature-dependent relaxation dynamics; (3) apply innovative pulse forming technology for increasing the dipolar information available by pulsed EPR. Cytochrome P450 enzymes have essential roles as monooxygenases, peroxidases and peroxygenases in archaea, bacteria and eukarya, and they are a source of inspiration for synthetic catalysts. While extensive biophysical studies utilizing x-Ray crystallography have provided great detail in P450 substrate interactions, information on global protein conformational variations such as due to the influence of Pdx have been largely absent. This important system was the rationale for early work focused on model heme-spin label distance measurements. We are now measuring the influence of the heme spin state on the DEER signal and relaxation properties of spin labels attached to P450. With one commercial and three laboratory-built pulsed spectrometers spanning 8-130 GHz, we are in an excellent position to contribute to the development of next-generation pulsed EPR instrument designs by applying AWG-driven pulse shaping to control the orientation selection in DEER.

Significant achievements 2011-2012: We have used the DEER method to show that the channel closes upon substrate addition, amounting to a shift of 6-8 Å of the spin label attached to the loop connecting F and G helices. Subsequently, the influence of putidaredoxin has been shown to promote the open substrate channel under aerobic conditions but it has little influence in a reduced, CO-bound state. Finally, in the product-bound state we find that the protein adopts an open conformation, at odds with an x-Ray structure of product-soaked crystals.

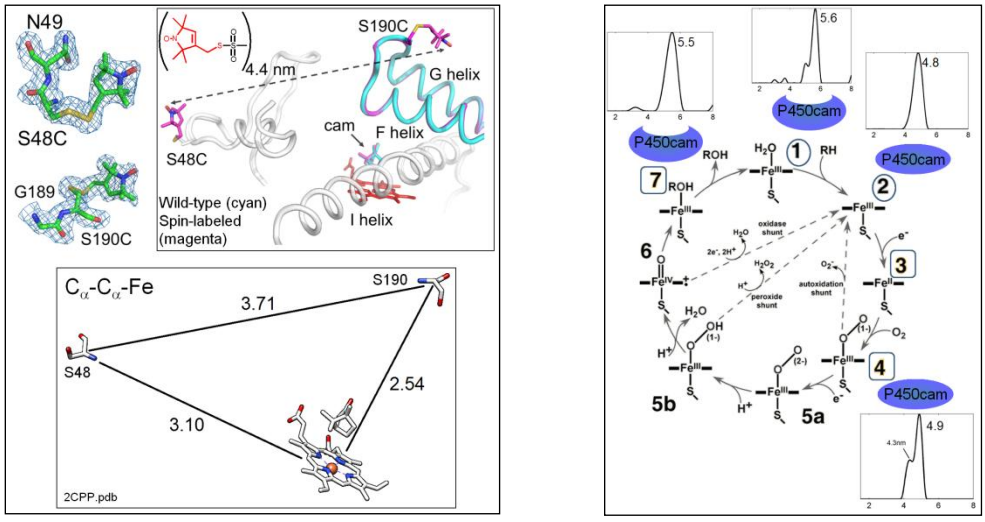



Figure. Left Panel: Distances derived from crystallization of P450 with MTSL attached to S48C and S190C side chains and the alpha-carbon distances with respect to the heme. Right Panel: Scheme of P450 catalytic cycle and extent of current data corresponding to individual states.

Science objectives for 2013-2014:

- We will obtain DEER measurements on the remaining four states of the P450 catalytic cycle. Clearly, some of these states are very difficult to trap and will require methods such as rapid freeze-quench. Addition of a nitrosyl group to the reduced form would also provide a better analog to the $O_2$-bound state 4, as opposed to the present Fe(II)-CO analog. The Pdx interaction could be studied by using DEER with single spin labels attached to Pdx and P450 and also by use of the $[2Fe\text{-}2S]^+$ cluster itself, following a recent paper that details the g-tensor orientation in a Pdx analog, PuxB.
- At a distance comparable to that between spin labels, the heme interaction with the spin labels differs in its effect, not as creating dipolar electron modulations, but rather damping those of the spin labels. The catalytic cycle of P450 offers three oxidation states of iron. How these heme iron oxidation states interact with the spin-lattice and phase memory time of spin labels can be probed at several positions. At present, P450 S190C and S48C mutants are single labels attached at axial and equatorial positions to the heme plane, respectively.
- Following installation of an ELDOR synthesizer channel at 130 GHz, a 10W upgrade at 34 GHz and construction of large bandwidth resonators at 31 and 34 GHz, we will develop pulse shaping capability with an AWG and feedback loop to optimize pulses with respect to the instrument transfer function. Pulse shaping is common in NMR and microwave communications but has only recently become practical for EPR laboratories.

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# *Session VI*

**The DOE Center for Plant and Microbial Complex Carbohydrates at the University of Georgia**

Alan Darvill, Principal Investigator
Parastoo Azadi, Russell W. Carlson, Co-PIs
315 Riverbend Road, Complex Carbohydrate Research Center
University of Georgia, Athens, GA 30602-4712
Email: adarvill@ccrc.uga.edu; Web: www.ccrc.uga.edu

Project Goals: The Complex Carbohydrate Research Center (CCRC) of the University of Georgia is a national resource for the study of complex carbohydrates. The DOE Center enables the CCRC to provide services and training to academic, government, and industrial researchers who study the complex carbohydrates of plants and microbes by providing support of experts who operate, maintain, and assist in interpreting the data obtained from a variety of scientific instrumentation. The expanding need for expertise in studies of the structure/function of complex carbohydrates is rapidly growing as the importance of carbohydrate research in areas such as biomedical glycobiology, vaccine development, and biomass conversion to biofuels is being recognized [1].

Analytical Services: The DOE Center enables several types of service to be offered to researchers. Scientists who request analytical services (see below) receive a written report containing a description of (i) the analytical procedures used, (ii) publishable quality results (data) of the analyses of their samples, and (iii) an authoritative interpretation of the results. CCRC personnel also provide collaborative service by becoming involved in "in depth" scientific research projects with individuals from other laboratories. One hallmark of the service offered is the continued addition of new technologies originally developed in the CCRC research laboratories. For example, in the fall of 2012 several new services will be offered, including glycome profiling and lignin and/or lignin/carbohydrate analysis. Over the past four years, the CCRC has provided analytical service or collaborative service to over 370 scientists. CCRC personnel consult with external scientists via e-mail and telephone, helping the scientists address specific analytical problems or interpretation of data. The CCRC's website (www.ccrc.uga.edu) provides freely accessible, internet-searchable databases in aspects of carbohydrate science, a scheduler for shared use of instrumentation, and descriptions of the CCRC's various research projects.

The CCRC provides several "high demand" analytical services to the scientific community as a result of the funding by the DOE Center. The major analytical services that we currently provide include:

1. Purification and analysis of plant and microbial polysaccharides
2. Purification and characterization of plant and microbial glycoproteins
3. Molecular weight determination by SEC, MALDI-MS, or ESI-MS
4. Glycosyl composition analysis: GC-MS and HPAEC
5. Glycosyl linkage analysis
6. Determination of absolute configuration
7. Structural characterization by mass spectrometry
    a. ESI-MS and ESI-MS/MS
    b. MALDI-MS and MALDI-MS/MS
    c. Online liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS/MS)
8. 1-D and 2-D NMR spectroscopy
9. Lignin analysis using Py-MBMS and Py-GC-MS

10. CarboSource Services: production and distribution of rare nucleotide-sugars, acceptors required for polysaccharide and glycoconjugate biosynthesis, and of monoclonal antibodies reactive against plant cell wall epitopes

In the fall 2012, the CCRC will incorporate several new services, which include:

1. Development of new improved reduction steps for the linkage analysis of uronic acid-containing oligo- and polysaccharides
2. Expanding lignin and tannin analysis by Py-MBMS and Py-GC-MS
3. Reducing end analysis of polysaccharides
4. Glycome profile analysis

Training: Training students and scientists in various fields of carbohydrate science is a very important part of the CCRC's mission. Training occurs when undergraduate students, graduate students, postdoctoral fellows, and visiting scientists undertake research projects with or take formal courses from CCRC faculty and staff. In addition, several annual one-week training courses and one- and/or two-day specialized courses are offered for individuals from academic institutions, government laboratories, and private industry.

Training courses currently offered at the CCRC include:

Course 1. *Techniques for Characterization of Carbohydrate Structure of Polysaccharides*

Course 2. *Separation and Characterization of Glycoprotein and Glycolipid Oligosaccharides*

Course 3. *Analytical Techniques for Structural Analysis of Glycosaminoglycans (GAGs)*

Course 4. *Mass Spectrometry of Glycoproteins*

References

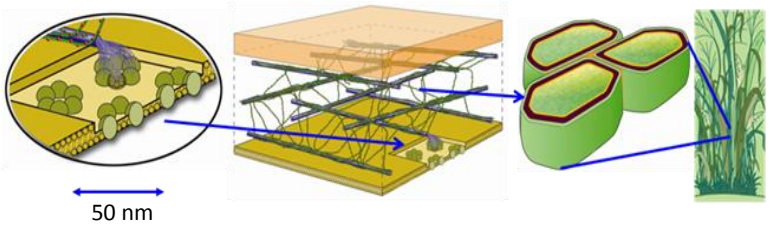
*Transforming Glycoscience: A roadmap for the future*. National Academy of Sciences (2012). A pdf of this report is available at The National Academies Press.
http://www.nap.edu/catalog.php?record_id=13446.

**Center for Lignocellulose Structure and Formation (CLSF)**
**A DOE-funded Energy Frontiers Research Center**

Daniel Cosgrove, Principal Investigator & CLSF Director
Dept. of Biology, Penn State University, University Park, PA 16802
Email: dcosgrove@psu.edu, Web: www.lignocellulose.org

*Mission Statement: To dramatically increase our fundamental knowledge of the formation and physical interactions of bio-polymer networks in plant cell walls to provide a basis for improved methods for converting biomass into fuels.*

CLSF is a multi-institution center whose research activities are organized into three interacting themes.




Center theme 1: Understand Cellulose synthesis

Center theme 2: Understand Lignocellulose assembly

Center theme 3: Understand relationship between nano scale structure and macro scale

**Theme 1** deals with the mechanism of cellulose synthesis and the nano-scale structure and properties of cellulose. Specific objectives include:

**Computational modeling:** Predict secondary and three-dimensional structure of an individual CesA protein. Build a computational model of protein packing within the CSC. Explore the packing of predicted transmembrane helices in a membrane using multiscale molecular dynamics modeling. Predict the structure of the "rosette" using molecular mechanics simulations. Model the structure of crystalline cellulose and how crystallization occurs.

**CSC (Cellulose Synthase Complex) function & control:** Crystallize the catalytic core of cellulose synthases and develop an X-ray based structural model; Identify novel components of the CSC in Arabidopsis and other organisms by biochemical and genetic approaches. Label components of the CSC with fluorescent tags and assess potential interactions of CSC components using fluorescence energy transfer methods. Work with modelers to incorporate all proven aspects of CSC structure and operation into their emerging models; Apply freeze fracture transmission electron microscopy to visualize the CSC and, possibly, sites of microfibril extrusion in protoplasts; carry out parallel work for the bacterial CSC. Develop *Physcomitrella* as a model system for studying CSC assembly and functions of specific molecular components.

**Cellulose microfibril structure:** Refine models of cellulose microfibril structure by combining experimental spectroscopic data with structural predictions of the computational modeling. Develop sum frequency generation spectroscopy, combined with computational modeling, to refine structural models of cellulose crystalline forms. Experimentally modify cellulose structure through genetic modifications and through the introduction of hemicelluloses and other interacting molecules, to assess the mechanism of microfibril assembly and its impact on cellulose digestibility by defined cellulases. Reconstitute partial or complete cellulose synthase complexes in artificial membranes or nanotubes to assess requirements for glucan and microfibril synthesis and control of microfibril structure.

**Theme 2** focuses on how cellulose microfibrils, matrix polysaccharides, structural proteins and lignin assemble to make multifunctional cell walls with unique and diverse properties. Objectives include:

**Binding studies:** Characterize the dynamics and energetics of specific cellulose-polysaccharide-protein-lignin binding interactions using isothermal titration calorimetry, surface plasmon resonance techniques and other methods. Explore the dependence of binding parameters on the form of cellulose and the

details of xyloglucan, arabinoxylan and lignin structure. Use defined Arabidopsis mutants and specific enzymes to test models of cell wall structure and its control of cell wall rheology. Combine data with molecular modeling results to understand the key molecular elements of cellulose-matrix binding interactions. Correlate self-assembly and binding with structure, enabling further correlations with results from ITC, vibrational spectroscopy, and computational modeling. Perform computational modeling of interactions and assembly of binary wall components.

**Cell wall assembly and structural properties:** Analyze cell wall mechanical properties and their dependence on cell wall composition/structure and their modification by endoglucanases, xylanases and other cell wall-loosening enzymes, using atomic force microscopy, extensometry and spectroscopic methods. Identify novel proteins that interact with glycosidases and lignin pathway enzymes upregulated in poplar wood-forming tissue. Develop model 3D synthetic plant cell wall systems implementing aligned cellulose fibrils, and use them to assess the impact of matrix polymers on wall assembly and physical characteristics of cell walls.

**Spectroscopy and scanning probe microscopy studies:** Elucidate cellulose-matrix interactions with experimental and computational IR using models generated by the center to address issues such as the site of binding to cellulose (crystalline or amorphous domains). Develop and test novel nonlinear spectroscopic methods to analyze cellulose structure *in situ*. Experimentally study the effect of lignin type (degree of branching, molecule size, etc.) and reactant sequence on the solubility of xylan in cellulose-xylan-lignin complexes.

**Theme 3** is directed at understanding how the macroscopic properties of cell wall materials emerge from their nano-scale properties and interactions focused on in themes 1 and 2. The following are specific objectives:

**Nanoscale characterization and modeling of cell wall structure:** Perform computational multiscale modeling of cell walls with application to structural, mechanical, and other physical properties. Extend atomistic modeling to larger length and time scales by use of a coarse-grained model which incorporates atomistic detail; Develop a coarse grained simulation model for cellulose structure and crystallization. Identify 4-5 model structures for study using small angle neutron scattering. Develop improved method to visualize lignin in the cell wall and study its interaction with wall components. Correlate cellulose crystallinity and fibril structure with material properties. Characterize interdiffusion of water and deuterated polysaccharides in interfacial regions using neutron reflectivity.

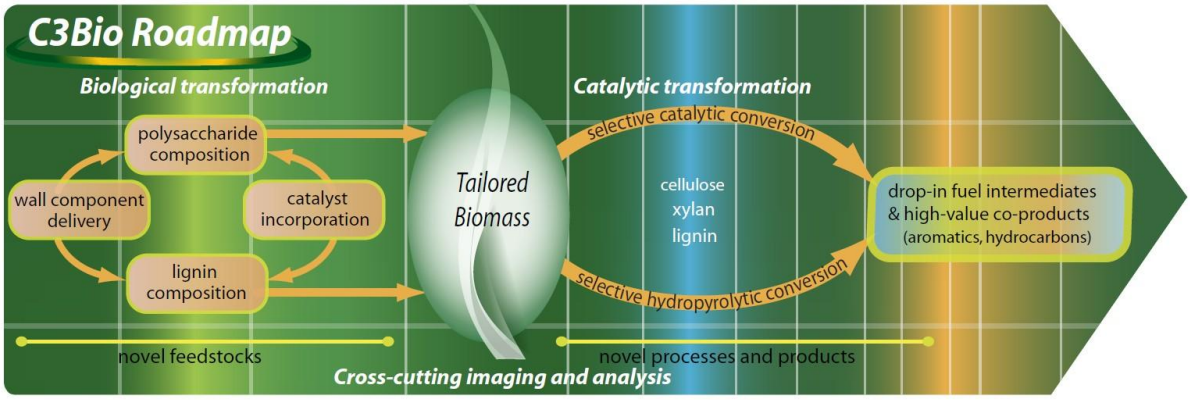
| **Center for Lignocellulose Structure and Formation** | |
|---|---|
| Pennsylvania State University | Daniel Cosgrove (Director), Charles Anderson, Nicole Brown, Jeffrey Catchmark, Vincent Crespi, Ying Gu, James Kubicki, Teh-hui Kao, Seong Kim, Janna Maranas, Virendra Puri, Tom Richard, Ming Tien, Bernhard Tittmann, Linghao Zhong |
| North Carolina State University | Candace Haigler (Associate Director), Alex Smirnov, Yaroslava Yingling |
| University of Rhode Island | Alison Roberts |
| Virginia Tech University | Alan Esker |

**A roadmap for selective deconstruction of lignocellulosic biomass to advanced biofuels and useful co-products**

Maureen C McCann, Principal Investigator
Mahdi Abu-Omar[1], Rakesh Agrawal[1], Craig Barnes[2], Joe Bozell[2], Alison Buchan[2], Nicholas Carpita[1], Clint Chapple[1], Kari Clase[1], Michael Crowley[3], Nicholas Delgass[1], Shi-You Ding[3], Bryon Donohoe[3], Michael Himmel[3], Hilkka Kenttamaa[1], Lee Makowski[4], Ian McNulty[5], Nathan Mosier[1], Angus Murphy[1], Wendy Peer[1], Fabio Ribeiro[1], Garth Simpson[1], Christopher Staiger[1], Daniel Szymanski[1], Kendall Thomson[1], Melvin Tucker[3], Co-PIs
[1]Purdue University, [2]University of Tennessee, [3]National Renewable Energy Laboratory, [4]Northeastern University, [5]Argonne National Laboratory
Email: mmccann@purdue.edu; Web: http://c3bio.org/

Overall research goals: Our Center mission is *to integrate fundamental knowledge and enable technologies for catalytic conversion of engineered biomass to advanced biofuels and value-added products.* We have 4 major research objectives: (1) Adding value to lignin by enabling its catalytic conversion; (2) Synthesis, assembly and deconstruction of cellulose microfibrils; (3) Engineer tailored biomass for highly efficient, direct catalytic conversion to liquid fuels and value-added products; (4) Fast-hydropyrolysis to maximize carbon efficiency.

Second-generation biofuels will be derived from lignocellulosic biomass using biological catalysis to use the carbon in plant cell wall polysaccharides for ethanol or other biofuels. However, this scenario is both carbon- and energy-inefficient. The major components of biomass are cellulose, hemicellulose and lignin. Biological conversion routes utilize only the polysaccharide moiety of the wall, and the presence of lignin interferes with the access of hydrolytic enzymes to the polysaccharides. Living micro-organisms, required to ferment released sugars to biofuels, utilize some sugars in their own growth and co-produce carbon dioxide. In contrast, chemical catalysis has the potential to transform biomass components directly to alkanes, aromatics, and other useful molecules with improved efficiencies. The Center for Direct Catalytic Conversion of Biomass to Biofuels (C3Bio) is a DOE-funded Energy Frontier Research Center, comprising an interdisciplinary team of plant biologists, chemists and chemical engineers. We are developing catalytic processes to enable the extraction, fractionation, and depolymerization of cellulose and hemicellulose coupled to catalytic transformation of hexoses and pentoses into hydrocarbons. Additional catalysts may cleave the ether bonds of lignin to release useful aromatic co-products or that may oxidize lignols to quinones. In a parallel approach, fast-hydropyrolysis is a relatively simple and scalable thermal conversion process. Our understanding of biomass-catalyst interactions require novel imaging and analysis platforms, such as mass spectrometry to analyze potentially complex mixtures of reaction products and transmission electron tomography to image the effects of applying catalysts to biomass and to provide data for computational modeling. By integrating biology, chemistry and chemical engineering, our data indicate how we might modify cell wall composition, or incorporate Trojan horse catalysts, to tailor biomass to tailor biomass for physical and chemical conversion processes. We envision a road forward for directed construction and selective deconstruction of plant biomass feedstock.

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**The Center for Advanced Biofuel Systems (CABS): A DOE-BES Energy Frontier Research Center**

*Richard T. Sayre*
*New Mexico Consortium/Los Alamos National Laboratory*
*and the*
*Donald Danforth Plant Science Center*

The overall objective of the **Center for Advanced Biofuel Systems** (CABS) is to increase the thermodynamic and kinetic efficiency of select plant- and algal-based fuel production systems using rational metabolic engineering approaches grounded in modern systems biology. Our strategy is to increase the efficiency of solar energy conversion into oils and other specialty biofuel components by channeling metabolic flux toward products using advanced catalysts and rationale design. This will be achieved by: 1) employing novel protein catalysts that increase the thermodynamic and kinetic efficiencies of photosynthesis and oil biosynthesis, 2) engineering metabolic networks to enhance acetyl-CoA production and its channeling towards lipid synthesis, and 3) engineering new metabolic networks for the production of hydrocarbons required to meet commercial fuel standards. These strategies will be informed by comprehensive metabolic flux analyses and computational modeling of metabolic pathways in both the oil seed crop *Camelina* and a model microalga, Chlamydomonas. Information obtained from these studies will direct engineering strategies for enhanced biofuel production systems. A unique feature of our program is the integration of all aspects of metabolism, from the earliest events in photosynthetic energy conversion to the synthesis and accumulation of oils and novel biofuel products. Using a "systems" approach, we will continuously improve our strategy and inputs for biofuel production and product development. I will provide a brief overview of CABS and then present some of the research efforts of our (Sayre) lab in more detail.

# *Poster Session II*

**Analysis of Cellulose Synthesis, Organization, and Dynamics as a Function of Pectic Cell Wall Composition in *Arabidopsis thaliana* and *Physcomitrella patens***

Charles T. Anderson, Principal Investigator
Daniel J. Cosgrove, Director, Center for Lignocellulose Structure and Formation
Peter Dowd, Melissa Ishler, and Pooja Shah, Research Personnel
201 Life Sciences Building, Pennsylvania State University, University Park, PA 16802
Email: cta3@psu.edu

Overall research goals: As part of the Center for Lignocellulose Structure and Formation, a DOE-funded Energy Frontiers Research Center, this project seeks to characterize functional interactions between pectins and cellulose in plant cell walls, using the model organisms *Arabidopsis thaliana* and *Physcomitrella patens*. Cellulose is a primary target for bioenergy production, is a major load-bearing component of the cell wall, and is synthesized at the plasma membrane by the cellulose synthase complex. Pectins are complex wall matrix polymers that constitute a large fraction of primary cell walls, where they are thought to function in wall hydration and cell adhesion. They have also recently been implicated in the production of secondary cell walls. Pectin-cellulose interactions have been detected *in vitro* and using NMR-based structural analyses of cell walls, and this project seeks to determine how these interactions influence cell wall structure and plant growth. Funding for this project started in January 2012.

Achievements in 2012: We have screened a large collection of *Arabidopsis* mutants that possess altered pectin biosynthesis and modifying activities with respect to cellulose organization in root epidermal cells. Preliminarily, we have found that the *gaut12-2* and *qua2* mutants, which lack the GAUT12 (a putative galacturonosyltransferase) and TSD2 (a putative pectin methyltransferase) gene products, respectively, show altered cellulose organization as compared to wild-type controls (Figure 1). These results indicate that the amount and methylesterificiation status of pectin in the cell wall can affect the organization of cellulose.

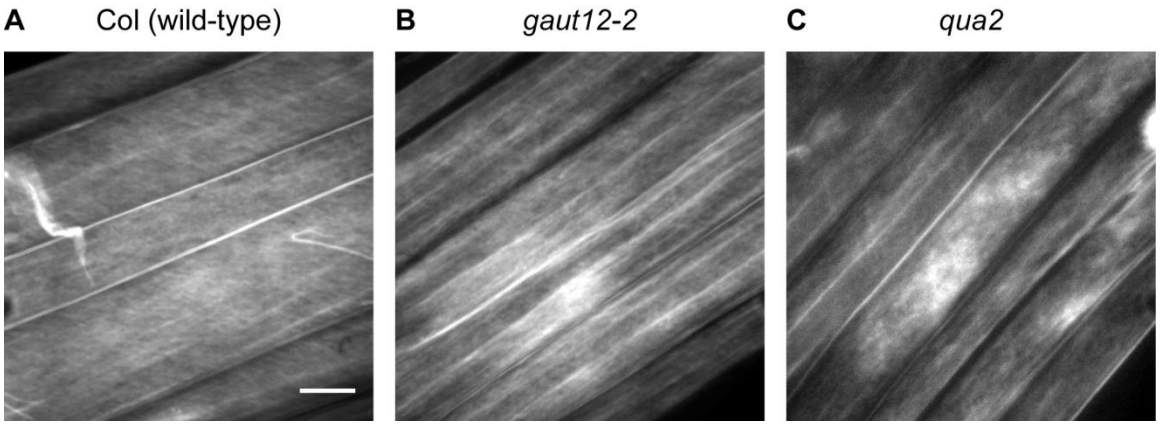


Figure 1. Cellulose organization in differentiated root epidermal cells of 5-day-old *Arabidopsis* seedlings of the indicated genotypes, as detected using Pontamine Fast Scarlet 4B staining for cellulose and spinning disk confocal microscopy. In wild-type seedlings (**A**) cellulose fibers are arranged in a regular diagonal pattern; in *gaut12-2* mutants (**B**), cellulose is arranged in a more longitudinal pattern; in *qua2* mutants (**C**), cellulose fibers are less clearly detectable. Scale bar in **A** = 10 µm; images are maximum projections of z-series.

Science objectives for 2012-2013:

- Transform *gaut12-2* and *qua2* mutants with a GFP-tagged form of CELLULOSE SYNTHASE 3 to enable measurements of the rates and patterns of cellulose synthesis in the context of altered pectin matrices.

- Analyze cell wall composition, cellulose crystallinity, and cell wall ultrastructure in *gaut12-2* and *qua2* mutants, using experimental capabilities contained within the Center for Lignocellulose Structure and Formation.

- Generate mutant *Physcomitrella* plants that lack specific pectin biosynthetic and modifying genes using a targeted gene knockout approach, and characterize the growth, morphology, cell wall organization, and cell wall composition of these mutants using kinetic morphometry, confocal microscopy, and biochemical approaches.

Publications supported by this project:

Wallace, I.S., Anderson, C.T. 2012. Small molecule probes for plant cell wall polysaccharide imaging. *Frontiers in Plant Science* doi: 10.3389/fpls.2012.00089

## Using the *Corngrass1* microRNA gene to enhance the biofuel properties of crop plants

Sarah Hake, Principle Investigator
George Chuck, Co-PI
Plant Gene Expression Center /U.C. Berkeley, Albany California 94707
Email: georgechuck@berkeley.edu; Web: http://www.pgec.usda.gov

Overall research goals: Our project seeks to enhance the biofuel properties of crop plants by modifying the activity of the *Corngrass1* (*Cg1*) microRNA gene and its targets by: (1) controlling the tissue and temporal expression of *Cg1* by using different promoters; (2) elucidating the functions of the *SPL* targets of *Cg1* by generating gain and loss of function alleles; (3) isolating downstream targets of select *SPL* genes using deep sequencing and chromatin immunoprecipitation. By achieving these goals, it will be possible to control biomass accumulation, cell wall properties, and sugar levels of different crop plants through manipulation of either the *Cg1* gene and/or its *SPL* target genes. The information derived from these studies will be applied to other potential biofuel crop plants that are not as genetically tractable as maize.

Significant achievements 2010-2012. The maize *Cg1* gene was transferred into several different crop plants and analyzed for enhanced biofuel traits. A complete composition analysis was completed for field trial samples of *Cg1* overexpressing switchgrass driven by the *ubiquitin* (*UBI*) promoter (Fig. 1A) and recently published. In summary, we discovered that weakly expressing *Cg1* transformants are likely to be the most effective as biofuels, as they produce nearly normal amounts of biomass (Fig. 1B) but have higher levels of fermentable substrates based on 1-year field trial data. In addition, we found that the transformants do not flower, even after three years of growth in the greenhouse and two years in the field. This result could serve as an important mechanism to minimize the potential for transgene escape in crop plants. The most significant finding was that starch levels were increased in our transformants up to 2.5 fold allowing the recovery of more glucose per weight from our transformants compared to wild type (Fig. 1C). This was achieved by simply modifying our saccharification protocol to include startch-degrading enzymes such as alpha amylase to our enzyme mix to break down starch in addition to the cell walls (Fig. 1D).

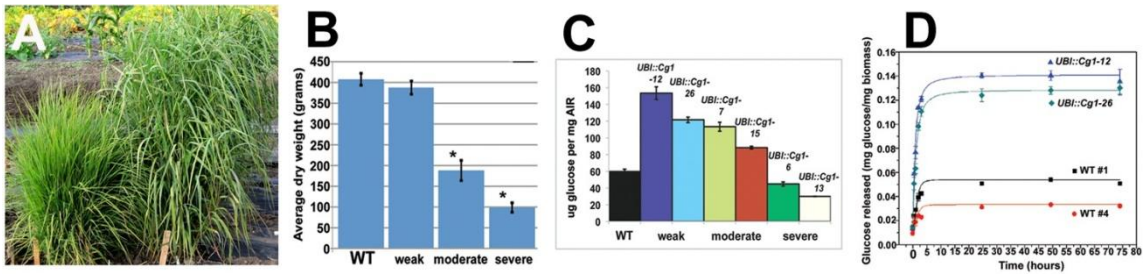


Figure 1. Analysis of biofuel properties of *UBI::Cg1* switchgrass plants
A) *UBI::Cg1* plants (left) compared to normal (right). *Cg1* plants have not flowered after three years of growth. B) Dry weights of field grown plants. Weakly expressing transformants produce almost as much biomass as normal plants. C) Starch levels of transformants. The stems of weakly expressing transformants have up to 2.5 times more starch. D) Saccharification assay using starch-degrading enzymes of stems of weakly expressing transformants versus wildtype. *Cg1* plants produce 3-4 times more glucose than normal plants.

We have extended our analysis to include *Cg1* overexpression in poplar, wheat, and sorghum, and observed similar phenotypes.

Science objectives for 2012 and beyond:

• In light of the fact that *Cg1* has been shown to enhance switchgrass as a biofuel, we will assess the biofuel properties of the new *Cg1* transformants in different crop plants. The biofuel traits are expected to vary greatly between crop plants.

• New switchgrass transformants driving *Cg1* behind different promoters have been generated and are undergoing field trials. We will determine whether their biofuel properties are comparable to the previous transformants ubiquitously expressing *Cg1*.

• High throughput cDNA sequencing has recently been completed on *Cg1* plants in maize, *Brachypodium* and switchgrass. We will compare the list of differentially expressed genes and determine a common list of targets that are either up or down- regulated in all *Cg1* grasses. This will allow us to identify conserved downstream genes shared amongst the different crop plants and provide new targets for functional analysis.

• Chromatin immunoprecipitation was completed for the *Cg1* target gene *tasselsheath4*. Putative targets of *tasselsheath4* are currently being validated.

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**Resolving protein-semiquinone interactions by advanced EPR spectroscopy**

Sergei Dikanov, Principal Investigator
Antony R. Crofts, Robert B. Gennis, Colin A. Wraight, Co-PIs
University of Illinois at Urbana-Champaign (UIUC), Urbana, IL 61801
Email: dikanov@illinois.edu; Web: http://ierc.scs.uiuc.edu/

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 $Q_i$-site of the $bc_1$ complex, and the $Q_H$-site of the $bo_3$ quinol oxidase, - 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 2010-2012:

***Semiquinone in the $Q_H$ site of cytochrome $bo_3$ ubiquinol oxidase.*** Selective $^{15}N$ isotope labeling of the cytochrome $bo_3$ ubiquinol oxidase from *E. coli* with auxotrophs was used to characterize the hyperfine (hf) couplings with the side-chain nitrogens from R71, H98, and Q101 residues and peptide nitrogens from R71 and H98 residues around the semiquinone (SQ) in the high-affinity $Q_H$ site. The 2D ESEEM (HYSCORE) data have directly identified the $N_\varepsilon$ of R71 as an H-bond donor carrying the largest amount of the unpaired spin density. In addition, weaker hf couplings with the side-chain nitrogens from all residues around the SQ were determined. These hf couplings reflect a distribution of the unpaired spin density over the protein in the SQ state of the $Q_H$ site and strength of interaction with different residues. The approach was extended to the virtually inactive D75H mutant, where the intermediate SQ is also stabilized. We found that the $N_\varepsilon$ from a histidine residue, presumably H75, carries most of the unpaired spin density instead of the $N_\varepsilon$ of R71, as in the wild-type $bo_3$. However, the detailed characterization of the weakly coupled $^{15}N$s from selective labeling of R71 and Q101 in D75H was precluded by overlap of the $^{15}N$ lines with the much stronger ~1.6 MHz line from quadrupole triplet of the strongly coupled $^{14}N_\varepsilon$ from H75. Therefore, a reverse labeling approach, in which the enzyme was uniformly labeled except for selected amino acid types, was applied in order to probe the contribution of R71 and Q101 to the $^{15}N$ signals. Such labeling has shown only weak coupling with all nitrogens of R71 and Q101. We utilize density functional theory based calculations to model the available information about $^{1}H$, $^{15}N$ and $^{13}C$ hf couplings for the $Q_H$ site and to describe the protein-substrate interactions in both enzymes. In particular, we identify the factors responsible for the asymmetric distribution of the unpaired spin density and ponder the significance of this asymmetry to the quinone's electron transfer function.

***Semiquinone in $Q_A$ and $Q_B$ site of bacterial reaction center.*** In the photosynthetic reaction center from *Rhodobacter sphaeroides*, the primary ($Q_A$) and secondary ($Q_B$) electron acceptors are both ubiquinone-10, but with very different properties and functions. $Q_B$ is always seen to occupy the proximal location when the RC was frozen under illumination - indicating that it is this conformation which traps the semiquinone ($SQ_B$). Structures with $Q_B$ in the proximal position show $HN_\delta$ of His-L190 as a potential H-bond donor to the carbonyl oxygen O4, and backbone NH groups from Ile-L224 and/or Gly-L225 plus the OH group of Ser-L223 as potential H-bond donors to the O1 carbonyl oxygen. The presence of a H-bond donation from the OH group of Ser-L223 is debatable with reports appearing for and against the presence of such a bond to the O1 of the quinone or the $SQ_B$. To study interactions of the $SQ_B$ with a protein environment that imparts functional differences, we have applied X-band 1D and 2D ESEEM. $^{14}N$ and $^{15}N$ 2D ESEEM spectra clearly show two nitrogens interacting with the $SQ_B$, each carrying

transferred unpaired spin density. Quadrupole coupling constants indicate them to be a protonated nitrogen of a histidine residue and the amide nitrogen of a peptide group. Lines from exchangeable protons with three different anisotropic couplings were found in $^{1}$H 2D ESEEM spectra. We also found negligible spectroscopic differences between the mutant with Ser-L223 changed to Ala and the wild type protein (WT), indicating only minor perturbations in the $SQ_B$ spin density for the mutant. Qualitatively this suggests that a strong H-bond does not exist in the WT between the Ser-L223 OH group and the $SQ_B$ O1 atom in the $Q_AQ_B^-$ state. We used QM/MM calculations on a $Q_B$ site model to assign the $^{1}$H and $^{14}$N tensors to specific H-bond interactions with $SQ_B$, and we compared this with the $SQ_A$ site. WT model in which the Ser-L223 OH group is rotated to prevent H-bond formation with the O1 atom of the $SQ_B$ predicts negligible change for the mutant. This, together with the better agreement between key QM/MM calculated and experimental $^{1}$H, $^{14}$N, $^{13}$C and $^{17}$O hf couplings for the non-hydrogen bonded model, leads us to conclude that no H-bond is formed between the Ser-L223 OH group and the $SQ_B$ O1 atom after the first flash. The calculations also reproduce a difference in the asymmetry of spin density distribution between $SQ_A$ and $SQ_B$, in agreement with $^{17}$O and $^{13}$C couplings of carbonyl groups.

Science objectives for 2012-2013:

- $^{13}$C couplings provide insight into the SQ binding to the protein. We will apply a biochemical approach for selective $^{13}$C labeling of methyl and ring carbons in the $SQ_H$. The quinones labeled in *bo*$_3$ enzyme will also be used in studies of reaction center and *bc*$_1$ complex.
- We will exploit Q-band $^{1}$H and $^{2}$H 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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**Composition and Regulation of Cellulose Synthase Complexes: What Have We Learned from *Arabidopsis thaliana***

Ying Gu, Principal Investigator
Daniel J. Cosgrove, Director, Center for Lignocellulose Structure and Formation
Shundai Li and Lei Lei, Research Personnel
262 North Frear, Pennsylvania State University, University Park, PA 16802
Email: yug13@psu.edu; Web: http://bmb.psu.edu/directory/yug13

Overall research goals: Research in my lab is focused on dissecting the molecular mechanism by which plant cells make cellulose. The lab is part of the "Center for Lignocellulose Structure and Formation", an Energy Frontier Research Center (http://www.lignocellulose.org/) led by Penn State. We use the cutting-edge confocal microscopy in conjunction with genetic and biochemical analysis to probe the composition and function of the cellulose synthesis complex. This work will substantially increase our knowledge of how plant cells make cellulose and provide unprecedented perspective that aids to increase the efficiency of biomass-based energy production. Funding for our projects started November 2010.

Significant achievements so far:
*Project I*: *How do Cellulose Synthase (CESA) complexes associate with microtubules?*
The above question has stood for many years. Here we present our recent work establishing that the link is mediated by a protein named CESA-interactive protein 1 (CSI1). CSI1 binds both microtubules and CESA. In the absence of CSI1, CESAs are uncoupled from microtubules. The characterization of CSI1 significantly enhances knowledge of how cellulose is aligned.

*Project II*: *Complexes with mixed primary and secondary CESAs are functional in planta*
The membrane-based yeast two hybrid (MbYTH) was used to assess the interactions between 3 primary (CESA1, CESA3, CESA6) and 3 secondary (CESA4, CESA7, CESA8) *Arabidopsis thaliana* CESA's. The results showed cross-interaction between primary CESAs and secondary CESAs. Analysis of transgenic lines showed that CESA1 could partially rescue CESA8 in the secondary cell wall whereas CESA7 can partially rescue CESA3 in the primary cell wall. These results demonstrate that additional selectivity exists within the plant cell, either through directed assembly or competition for interacting partners.

Science objectives for 2012-2014:
*Project I*: *Regulation of cellulose biosynthesis through CESA endocytosis*
Through conventional yeast two-hybrid (Y2H) screen using the central domain of primary CESA (CESA1, CESA3, and CESA6), we identified a protein with significant homology to the medium subunit (µ2) of the clathrin adaptor complex (AP2) in mammal. The interaction between At-µ2 and CESA6 has led to the hypothesis that At-µ2 might mediate the internalization of CESA proteins by recognizing and recruiting CESA to clathrin coated pits. The aims of this research are (i) to establish the role of At-µ2 as a player in Clathrin-mediated endocytosis (CME) in *Arabidopsis*, (ii) to investigate the interaction between At-µ2 and CESA, and (iii) to determine whether CESA is internalized through a CME mechanism.

*Project II*: *Characterization of CESA interactive proteins identified from membrane-based yeast two-hybrid system*
To identify additional CESA interactive proteins, we conducted split-ubiquitin membrane based yeast two-hybrid using full-length CESA3 and CESA6 as baits. Sequencing of putative interactive clones revealed that a total of 27 proteins interacted with both CESA3 and CESA6. We adopted a systematic approach to characterize these putative CESA interacting proteins: 1) Examine the expression pattern of candidate genes on the publically available microarray database and confirm the expression pattern by

promoter-GUS analysis; 2) Generate transgenic lines of *Arabidopsis* in which the candidate gene is fused with a fluorescent protein to examine whether it has a similar subcellular localization as CESAs; 3) Identify mutations in the candidate gene using the publically available T-DNA insertion lines or obtain RNAi mutants and examine the mutant for alterations in cell expansion, cellulose content, and CESA dynamics. Identification and characterization of these putative CESA interactive proteins will be critical to understand the composition and regulation of CESA complexes.

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**Seeing the light: Understanding how bacteriophytochromes function under anaerobic conditions**

Caroline (Carrie) S. Harwood, Principal Investigator
Kathryn Fixen, Postdoctoral Research Fellow
Department of Microbiology, University of Washington, Seattle, WA 98195-7735
Email: csh5@u.washington.edu; Web: http://depts.washington.edu/cshlab/index.html

Overall research goals: To generate foundational knowledge that will improve our ability to use dense suspensions of photosynthetic bacteria as biocatalysts to convert inexpensive feedstock compounds to hydrogen gas or other biofuels. Towards this end we are unraveling signal transduction cascades involved in the regulation of photosynthesis at low light.

Significant achievements in 2010- 2012. The anoxygenic photosynthetic bacterium *Rhodopseudomonas palustris* generates energy from light by cyclic photophosphorylation under anaerobic growth conditions. The synthesis of its photosynthetic apparatus is controlled in part by light-sensing bacteriophytochromes (BphPs). Two BphPs, designated BphP2 and BphP3, are configured as light- regulated histidine kinases that control expression of the operon encoding the LH4 antenna complex, which is one of the most highly expressed operons in *R. palustris* and is important for capturing light at low intensities. Structural and biophysical studies have shown that BphP2 and BphP3 respond to light quality by reversible conversion between a red absorbing form and a far red or a near red absorbing form for BphP2 and BphP3, respectively. Photoconversion requires incorporation of the light-absorbing chromophore biliverdin into the protein.

1. We determined that BphP2 and BphP3 are both required for the expression of the low light LH4 antenna complex under anaerobic conditions. However, since biliverdin requires oxygen for its synthesis by heme oxygenase, it was unclear to us how BphPs in anoxygenic bacteria are able to function.

2. We showed that BphP2 is bound to biliverdin *in vivo* in anaerobically-grown cells, and synthesis of biliverdin requires only one of four heme oxygenases encoded in *R. palustris*. This suggests that this heme oxygenase has a high affinity for oxygen, which allows it to synthesize biliverdin even under anoxic conditions.

3. We were surprised to find that deletion of the *R. palustris* heme oxygenase or mutations in the BphPs that prevent biliverdin binding did not disrupt BphP activity under anaerobic conditions. This indicates that BphP2 and P3 do not require biliverdin to function under anaerobic conditions, and the apophytochrome form of the BphP is sufficient for its activity. Instead, the main driver of LH4 gene expression under these conditions was light intensity not light quality.

4. We obtained evidence that light intensity is manifested as a redox signal sensed by a protein named RPA3018 in a BphP2/BphP3-controlled regulatory cascade.

Science objectives for 2012-2013.

- To test key aspects of the model for control of the low light response in *R. palustris* shown in Fig 1.
- Top priority will be to examine the redox state of RPA3018 *in vivo* to establish a direct link between light intensity, intracellular redox and transcription of the *pucAB* operon for LH4.
- To characterize and verify phosphotransfer reactions shown in Fig. 1 *in vitro*.

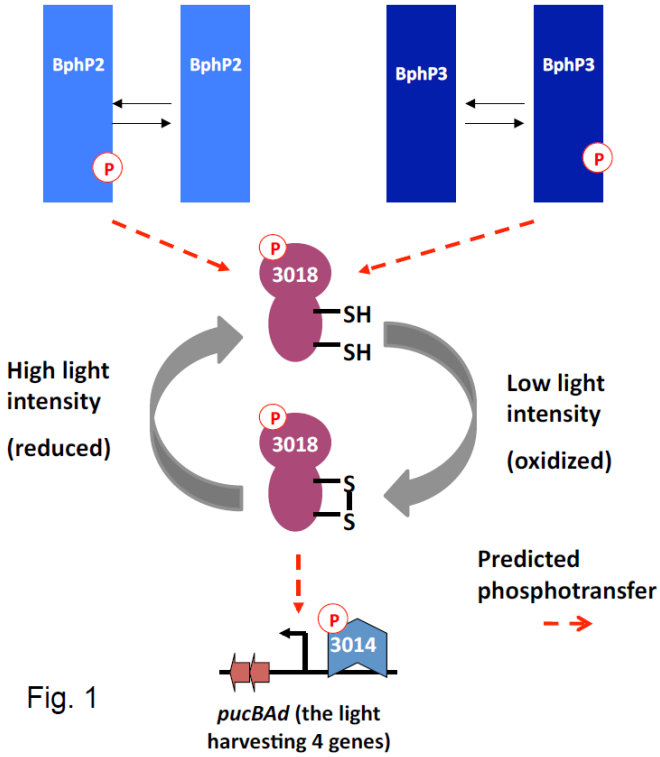


Fig. 1

**Fig. 1**. Model for signal transduction cascade leading to LH4 low light response under anaerobic conditions. Bph2 and Bph3 do not require bound chromophores but rather are in an equilibrium between forms that can autophosphorylate or not. Phosphotransfer to RPA3018 occurs. Under low light conditions, cells are relatively more oxidized because cyclic photophosphorylation occurs at a lower rate. This causes a disulfide bond to form in RPA3018 rendering it proficient to transphosphorylate and activate the transcription factor, RPA3014.

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## Understanding Thermal Chemical Conversion of Biomass to Fuels using a Multi-Modal Analytical Platform

B. Donohoe, P. Ciesielski, J. Matthews, M. Crowley, H. Wei, M. Tucker, M. Himmel, M. McCann

In support of C3Bio's mission to uncover routes for direct conversion of biomass into fuels and chemicals, we utilize NREL's multi-scale, multi-mode imaging facility, the Biomass Surface Characterization Laboratory (BSCL), to characterize novel sets of tailored biomass materials. One such set of materials, generated in the Chapple lab at Purdue, have genetically modified lignin composition and we show these mutants display enhance susceptibility to thermochemical pretreatment. In other biomass, cellulose binding modules (CBM), iron binding peptides (IBP), or ferritin have been engineered into the plants to precisely deliver metal ion co-catalysts into cell walls.

We find that corn stover impregnated by a 2 mM solution of iron sulfate significantly enhances both glucose and xylose monomer release by 14% and 29%, respectively in dilute acid pretreatment. We further show that iron delivered via ferritin-$Fe^{3+}$ is released similarly during pretreatment and enhances sugar release.

We have characterized the impact of Fe co-catalyzed dilute acid pretreatments on cell wall architecture by using 3D electron tomography to visualize and quantify cellulose microfibril geometry within pretreated cell walls. These measured geometries have been used to construct the first atomistic macromolecular models of cellulose microfibrils as they exist in pretreated cell walls, and molecular dynamics studies are beginning to elucidate the relationship between the energretics and nanotstructure of cellulose. In parallel, we have used molecular simulations to determine the structure and dynamics of cellulose at high temperature, a system that is difficult to study experimentally. Our simulations provide new molecular insight, consistent with experimental data, that indicate the formation of new hydrogen bonding schemes that form between layers cause cellulose microfibrils to form regions of local straightening offset by highly-strained regions of increased twisting. The goal of this work is to better understand structure-property relationships that effect the energetics and reaction mechanisms of cell wall deconstruction into liquid fuels.

## Photobiohybrid Solar Fuels

Paul W. King, Principal Investigator
C. Chang and K. Kim, NREL; D. Gust, A. L. Moore and T. A. Moore, Arizona State University, Tempe, AZ; Co-Principal Investigators
National Renewable Energy Laboratory, 15013 Denver West Parkway, Golden, CO 80401
Email: paul.king@nrel.gov

Collaborators: Prof. G. Dukovic, University of Colorado, Boulder, CO USA
Prof. T. Furtak, Colorado School of Mines, Golden, CO USA
Prof. A. Stuchebrukhov, University of California, Davis, CA USA

Overall research objectives: Photosynthesis provides a template for solar energy conversion into fuels to inspire the design of artificial light-harvesting and energy-transduction systems. Enzymes that catalyze the fuel production reactions also provide insight into structure-function for developing more efficient synthetic catalysts. The overall objectives of this project are to elucidate the fundamental photochemical processes that control energy conversion and catalytic $H_2$ production in natural and photobiohybrid systems that utilize [FeFe]-hydrogenases. The research areas being investigated include: (i) developing theoretical models to understand mechanisms of interfacial electron-transfer (ET), and enzymatic $H_2$ activation; (ii) characterizing [FeFe]-hydrogenases by a combination of biochemical, biophysical and structural techniques; (iii) optical and transient techniques for characterizing molecular self-assembly, charge-transfer and catalytic conversion; and (iv) electrochemical characterization of native and structurally minimized enzymes using electrochemical-scanning tunneling microscopy (STM). Integration of these research themes 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 the molecular interfaces for achieving efficient transduction of light energy into chemical energy.

Significant achievements 2010-2012: (i) Computational 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 [FeFe]-hydrogenase 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 H-cluster. (iii) The free energies along PT pathways were investigated using QM/MM and umbrella sampling techniques, and several important residues were identified along with p$K_a$ estimations using a thermodynamics integration method. (iv) Clostridial [FeFe]-hydrogenase was adsorbed to Au electrodes bearing self-assembled thiol-based monolayers (SAMs). Binding was mediated between positively charged patches on the hydrogenase and carboxylate groups on the SAM. Single-molecule images were obtained in an electrochemical STM and showed tunneling currents increased under an applied bias, which allowed for estimation of a lower limit $k_{cat}$ of 20,000 $s^{-1}$ in combination with macroscopic voltammetry. (v) Assemblies of [FeFe]-hydrogenase and mercaptopropionic acid (MPA) capped CdS nanorods were successfully formed with the CdS nanorod adsorbed to the ferredoxin binding surface of hydrogenase adjacent to the distal [4Fe-4S]-cluster. Rates of $H_2$ evolution were proportional to light intensity and donor concentration, with quantum yields (450 nm) of 20%. Long-term instability of the CdS-MPA ligand led to inhibition of hydrogenase. (vi) Time-resolved vibrational spectra of photoreduced [FeFe]-hydrogenase were observed by rapid scan FTIR of CdS-hydrogenase complexes under illumination. The technique will provide a window into the Fe-ligand transitions of the catalytic H-cluster following photoinduced ET from the CdS nanorod to hydrogenase.

Science objectives for 2011-2012:

- We have initiated a theoretical exploration of H-cluster diiron center conformations and potential intermediate structures in order to deconvolute experimental infrared (IR) absorption and electron paramagnetic resonance (EPR) spectra of [FeFe]-hydrogenases under turnover. The focus is on characterization of discrete candidate states, and includes basic vibrational spectrum and EPR g-tensor calculations. The free energies along the PT pathways in bacterial [FeFe]-hydrogenase will be further refined based on the p*Ka* calculation results.
- We will also investigate the interfacial environment one the hydrogenase-nanoparticle interface. Based on the MD simulation trajectory saved from previous study, the interfacial dielectric constant, $\varepsilon$, can be calculated. Reorganization energy, $\lambda$, is dependent on $\varepsilon$, and will help develop an understanding of how the interfacial environment may influence the ET process.
- We will continue to characterize native and PT mutants of bacterial and algal [FeFe]-hydrogenases prepared under different redox states using FTIR and EPR spectroscopy. Correlation of the results will help to unify dependence of Fe-ligand interactions with spin states in catalytic intermediates. We will continue to develop FTIR rapid scan as a dynamic technique for characterizing ET-induced transitions in the H-cluster.
- Single-molecule electrochemistry of immobilized [FeFe]-hydrogenase on Au-electrodes will continue to characterize native and accessory iron-sulfur cluster mutants in order to learn more about the conductive path through the protein.
- We will continue to characterize the self-assembly, charge-transfer, and photocatalysis of [FeFe]-hydrogenases with CdS or CdTe nanoparticles under photoexcitation using steady-state and time-resolved techniques. These efforts will address the effects of changes in nanoparticle ligand and diameter on charge-transfer and catalytic rates.

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## Ferredoxin-Dependent Plant Metabolic Pathways

David B. Knaff, Principal Investigator
Masakazu Hirasawa, Senior Research Scientist
Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX 79409-1061
Email: david.knaff@ttu.edu

Overall research goals: (1) To elucidate the detailed mechanisms of the complex, multi-electron transfer reactions catalyzed by several enzymes involved in nitrogen, carbon and sulfur assimilation, all of which use reduced ferredoxin as the physiological electron donor. (2) To characterize the complexes formed between ferredoxin and its target enzymes, by identifying the domains on the proteins that are involved in ferredoxin/enzyme complex formation and by measuring the thermodynamic parameters for complex formation. (3) To utilize the information gained from progress made on goals (1) and (2) to better understand how the distribution of electrons from reduced ferredoxin to its different targets is regulated and controlled.

Significant achievements 2011-2012:

(1) Nitrate Reductase: The roles of four absolutely conserved, basic amino acids in the ferredoxin-dependent nitrate reductase from the cyanobacterium *Synechococcus* sp. PCC 7942 have been investigated by site-directed mutagenic replacement. Replacement of either Lys58 or Arg70 by glutamine results in a complete loss of activity, when either reduced ferredoxin or a non-physiological substitute, reduced methyl viologen, serves as the electron donor. The fact that K58R and R70K variants are also inactive indicates that the requirement for these two amino acids is quite specific and that simply having a positively-charged side chain does not suffice. Replacement of Lys130 by glutamine causes substantial, but not total loss of activity, while replacement by arginine at this position has less effect. Replacement of Arg146 has no significant effect on activity. Replacement of Lys58 causes large losses of both the Mo-pterin and iron-sulfur cluster prosthetic groups and makes the cluster much more susceptible to oxidative damage. With the exception of the large decrease in the affinity of ferredoxin binding exhibited by the K58Q variant, these mutagenic replacements have relatively small effects on substrate-binding affinities. An *in silico* three-dimensional model of the enzyme has been used to provide a rationalization for these results.

(2) Arsenate Reductase: Cyanobacteria contain an arsenate detoxification system that involves an initial 2-electron reduction of arsenate to arsenite, followed by an ATP-dependent pumping of the arsenite out the cell. The arsenate reductase from the cyanobacterium *Synechocystis* sp. PCC 6803 uses one of three glutaredoxins present in cells, glutaredoxin A, as its preferred electron donor and we have used X-ray crystallography to obtain a three-dimensional structure of the glutaredoxin A protein at 1.8 Å resolution. We have used site-directed mutagenesis to demonstrate that only one of the two cysteines at the active site of glutaredoxin A, Cys15, is required for its activity as an electron donor to arsenate reductase. We have also used site-directed mutagenesis to demonstrate that only three of the five cysteine residues present in the reductase (Cys8, Cys80 and Cys82) are essential for activity, while the other two (Cys13 and Cys35) are not. We have combined these mutagenic studies with *in silico* structural modeling and kinetic studies to provide support for a proposed mechanism and to identify a key enzyme intermediate.

(3) Nitrite Reductase: Site-directed mutagenesis has been used to examine the roles of five conserved basic residues (four arginines and one lysine), all of which are located at or near the active site of the enzyme and are conserved in all ferredoxin-dependent nitrite reductases, in binding the spinach enzyme's siroheme and [4Fe-4S] cluster prosthetic groups and its electron-accepting substrate, nitrite. One of these conserved residues (Arg149 in the spinach enzyme) is replaced by a histidine in the closely-related and mechanistically similar sulfite reductases and an asparagine, conserved in nitrite reductases (Asn226 in spinach nitrite reductase) is replaced by a lysine in sulfite reductases. Site-directed mutagenesis is being used to investigate the roles of these two amino acids in determining the specificity of the enzyme for sulfite *vs*. nitrite.

(4) Glutamate Synthase: A loop, 27 amino acids in length, which is in close proximity to the enzyme's FMN and [3Fe-4S] cluster prosthetic groups, is present only in ferredoxin-dependent forms of the enzyme. We have prepared a series of deletion and replacement variants in the loop of the ferredoxin-dependent enzyme from the cyanobacterium *Synechocystis* sp. PCC 6803 and are in the process of characterizing their kinetic properties, their prosthetic group contents and their substrate-binding affinities, in order to test the hypothesis that this loop is part of the enzyme's ferredoxin-binding site.

(5) Disulfide/Dithiol Protein Redox Chemistry: The attachment of heme to apo-cytochromes *c* requires two reductive steps: reducing a disulfide at the CXXCH heme-binding site of the protein; and reducing the $Fe^{3+}$ of the heme to $Fe^{2+}$. We have provided evidence that the role of the yeast flavoprotein, Cyc2p, in this process is to catalyze the reduction of the heme iron, using NADPH as the electron donor. We have also characterized the redox properties of Cyc2p's flavin and of the disulfide/dithiol couple of yeast mitochondrial apo-cytochrome *c*. A fusion protein, combining peroxiredoxin (Prx) and nitroreductase (Ntr) domains, occurs naturally in the marine extreme thermophile *Thermatoga maritima*. We have characterized the redox properties of the two domains and shown that the Prx domain can reductively detoxify hydroperoxides using reduced glutaredoxin as an electron donor. The Ntr domain is also able to reduce hydroperoxides, using either NADH or NADPH as the electron donor. The two domains appear to function independently.

Science objectives for 2012-2013:

- To use site-directed mutagenesis, combined with kinetic, binding and spectroscopic measurements, to identify additional amino acids involved in substrate binding and catalysis in a ferredoxin-dependent cyanobacterial nitrate reductase.
- To use flash photolysis kinetic techniques to characterize electron transfer from reduced ferredoxin to nitrate reductase and to elucidate the kinetics of electron transfer between the iron-sulfur cluster and molybdopterin prosthetic groups of the enzyme.
- To obtain diffraction-quality crystals of the nitrate reductase and of its complexes with ferredoxin and with nitrate and to use these to determine three-dimensional structures for the enzyme and its substrate complexes.
- To use site-directed mutagenesis to explore the role of a unique 27 amino acid-long loop in a cyanobacterial ferredoxin-dependent glutamate synthase in binding ferredoxin to the enzyme.
- To use isothermal titration calorimetry to determine the enthalpies and entropies of substrate binding to four ferredoxin-dependent target enzymes and to examine cooperativity in substrate binding by these enzymes.

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# Genes Needed For Syntrophy in Sulfate Reducing Bacteria.

Lee R. Krumholz[1*], (Principal Investigator), Peter Bradstock[1], Luyao Wang[1], Yiwei Diao[1], Ozcan Gazioglu[1], Michael McInerney[1], Judy Wall[2]

[1]The University of Oklahoma, Norman, OK and [2]University of Missouri, Columbia, MO.

Email: krumholz@ou.edu

## Overall Research Goals

Research objectives are to study genes and proteins within *Desulfovibrio alaskensis* G20 that are involved in syntrophic interactions in coculture with a Syntrophic butyrate degrading bacterium and during lactate oxidation in coculture with a hydrogenotrophic methanogen. Specifically, this has involved screening mutants for loss of ability to grow in coculture in order to identify genes in *Desulfovibrio* needed for syntrophic growth. Mutants, mutated genes and the proteins encoded by these genes are being characterized to determine their specific function during syntrophic growth.

## Significant Acheivements for 2010-2012.

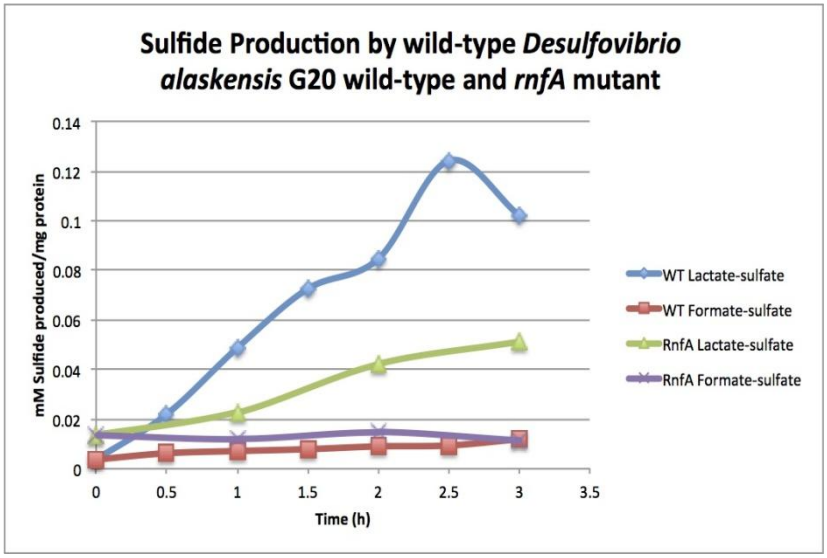
***Genes involved in syntrophic growth.*** A number of genes have been identified which appear to be required for syntrophic growth of *Desulfovibrio* as the $H_2$ user with *Syntrophomonas wolfei*. Some characterization experiments have been done and $H_2$ dependent growth and biofilm formation data is presented below (Table 1). Results show that flagella biosynthesis, Rnf protein and a chemotaxis sensory transducer play a role in $H_2$ uptake and syntrophy. Rnf is a large membrane spanning protein with homology to energy conserving oxidoreductases in many other bacteria. The results shown in figure 1 show that Rnf appears to play a key role in respiration in *Desulfovibrio*. Ongoing experiments explore the enzymatic function of the Rnf complex.

Table 1. Growth and biofilm formation by pure cultures of some syntrophy mutants. Results shown that mutations in flagellar function and pilus function affect both biofilm formation and growth on $H_2$. As well, rnf mutants do not grow on $H_2$. ND-not determined.

| Gene number | Gene Name | Proposed Function | Growth | | | Biofilm Formation |
|---|---|---|---|---|---|---|
| | | | H2 | Formate | Pyruvate | |
| Dde_0380 | flhA | Flagella Biosynthesis | ++ | +++ | ++++ | + |
| Dde_0353 | fliF | Flagellar Ring Protein | ++ | +++ | ++++ | + |
| Dde_2365 | tadC | Pilus assembly prot. | ++ | +++ | ++++ | ++ |
| Dde_0585 | rnfA | Electron Flow | - | - | ++++ | ND |
| Dde_0582 | rnfD | Electron Flow | - | - | ++++ | ND |
| Dde_3074 | | Methyl Accepting Chemotaxis Sensor | - | ++ | ND | ++ |
| Dde_2933 | qrc | Hydrogen metabolism | - | - | ++ | ND |
| Parent | | | +++ | +++ | ++++ | +++ |

***Membrane Protein Complexes in Desulfovibrio.*** Several mutants were grown in culture and their membranes were isolated and run on Blue Native gels to determine whether these proteins form large complexes. Gels run with a mutant in the QMO complex had three absent bands in comparison to the parental strain. Bands were excised and proteins were identified by mass spectroscopy. Several bands appear to be a complex containing both QMO and APS reductase. This complex has been speculated to operate together for the reduction of sulfate (APS) to sulfite as both QMO genes and APS reductase genes are located adjacent to each other on the chromosome. These results provide strong evidence for an association of QMO with APS reductase in *Desulfovibrio*.

**Figure 1**. Sulfide production by *rnf* mutant during incubations with formate and lactate.




**Science objectives for 2012-2013.**

***Determination of the role of syntrophy genes.*** . Rnf appears to play a role in the syntrophic interaction. It is a large membrane spanning protein complex that couples electron transfer to energy transduction (1) and will be further studied to determine its role in respiration of $H_2$ in *Desulfovibrio*. A group of three flagella and pilus genes have also been shown to be involved in syntrophy. The specific role that these genes play in Chemotaxis and in syntrophy will be investigated.

***Membrane Protein Complexes in Desulfovibrio.*** This work will build on the above results showing the presence of respiratory membrane complexes in strain G20. Our recent work has demonstrated a role for a protein now referred to as Qrc (Mop) in the uptake of $H_2$ by *Desulfovibrio* G20. Qrc has recently also been purified and shown to interact with both tetraheme cytochrome C3 in the periplasm and menaquinone (most likely in the membrane) (4). Rnf also appears to play a key role in $H_2$ uptake as shown above. It is therefore possible that Qrc or Rnf interacts directly with QMO for the oxidation of $H_2$ coupled to the reduction of adenosine phosphosulfate (APS). We will test this hypothesis by looking for protein complexes using affinity tagged proteins and if they occur, reconstituting the complex to characterize function.

***Transcriptomic Analysis of Syntrophic Cocultures.*** A number of genes have been identified that are involved in syntrophic interactions, based on growth assays. We are in the process of sequencing cocultures of *Syntrophomonas wolfei*, *Syntrophus aciditrophicus* and *Desulfovibrio* G20 grown with *Methanospirillum hungatei* using RNA seq. This will determine expression profiles and identify genes whose expression is influenced by syntrophy.

**Publication list acknowledging the DOE grant or contract**


1. Li , X., M.J. McInerney, D.A. Stahl and L.R. Krumholz. 2011. Metabolism of ***$H_2$*** **by** *Desulfovibrio alaskensis G20* **during syntrophic growth on lactate. Microbiology-SGM. 157:2912-2921.**
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**Electron Flow and Energy Conservation in a Hydrogenotrophic Methanogen**

John A. Leigh, Principal Investigator
Murray Hackett, Co-PI
Department of Microbiology, University of Washington, Seattle, WA 98195-7735
Email: leighj@uw.edu; Web: http://faculty.washington.edu/leighj/

Overall research goals: The biochemical steps of methanogenesis have been known for decades but a full understanding of electron flow and energy conservation has come only recently (Figure 1). The suggestion by R. Thauer that in hydrogenotrophic methanogenesis electron bifurcation at the heterodisulfide reductase (Hdr) step couples exergonic and endergonic reactions in the pathway appeared to solve the problem. However, evidence for the importance of electron bifurcation in vivo was lacking. In addition, in the electron bifurcation model there was no role for the energy- converting hydrogenase Eha which is thought to drive the endergonic reaction. Two key traits enabled us to address these and related questions in *Methanococcus maripaludis*: methods for the generation of mutations, and the ability to grow on formate alone as well as $H_2$ as the provided electron donor. Our objectives were to (1) take a genetic approach to understand the importance of three different types of hydrogenases in hydrogenotrophic methanogens, (2) determine the role of $H_2$, if any, when formate is provided as the electron donor for growth, (3) determine the role of Eha, and (4) test the electron bifurcation model in vivo.

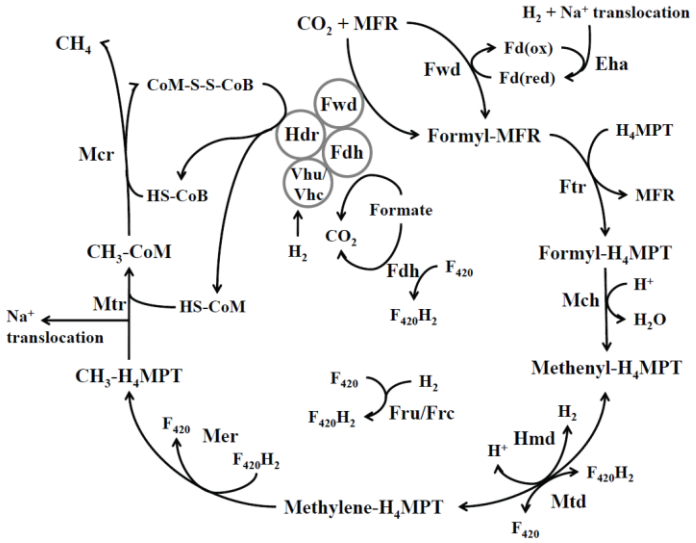


Figure 1. The methanogenic pathway in hydrogenotrophic methanogens.

Significant achievements 2010-2012: (1) We found that mutations abrogating three hydrogenase activities in a single strain resulted in a $H_2$ requirement for growth even when formate was also provided. Hence, $H_2$ is required. The three hydrogenase activities that were genetically eliminated were the $F_{420}$-reducing hydrogenases Fru and Frc, the $H_2$-dependent methylene-$H_4$MPT dehydrogenase Hmd, and the heterodisulfide reductase-associated hydrogenases Vhu and Vhc (Figure 1). $H_2$ was required only when all three hydrogenase activities were eliminated, suggesting that there are three different pathways of electron flow between formate and $H_2$ (with reduced $F_{420}$ acting as an intermediate). (2) The $H_2$ requirement was quantitatively small, suggesting that formate alone provided the stoichiometric electron equivalents for methanogenesis. In addition, in vitro experiments using a mutant lacking all hydrogenases except Eha showed that H2 was required for methanogenesis only when the intermediate $CH_3$-CoM was not provided. Hence, the requirement for $H_2$ and for Eha for methanogenesis is anaplerotic. Since $H_2$ is required

in sub- stoichiometric amounts, Eha cannot provide enough reducing equivalents to drive methanogenesis; therefore, electron bifurcation has to function. (3) CO could replace $H_2$ as an electron donor to anaplerotically stimulate methanogenesis. In addition, in a strain overexpressing the gene for glyceraldehyde-3-phosphate:ferredoxin oxidoreductase (GAPOR), Eha could be genetically eliminated (so no hydrogenases remained) and $H_2$ was not required. All of these processes reduce a ferredoxin and thus, three alternative electron flows appear to exist for the anaplerotic reduction of the ferredoxin associated with formyl-MFR dehydrogenase: from $H_2$ via Eha, from CO via carbon monoxide dehydrogenase, and from formate via the GAPOR cycle (Figure 2). (4) In the strain lacking all hydrogenases and expressing GAPOR, the reintroduction of $F_{420}$-reducing hydrogenase resulted in the production and accumulation of large amounts of $H_2$ from formate. This suggests that reduced $F_{420}$ is in excess and opens the door to a novel approach to the production of $H_2$ as a biofuel.

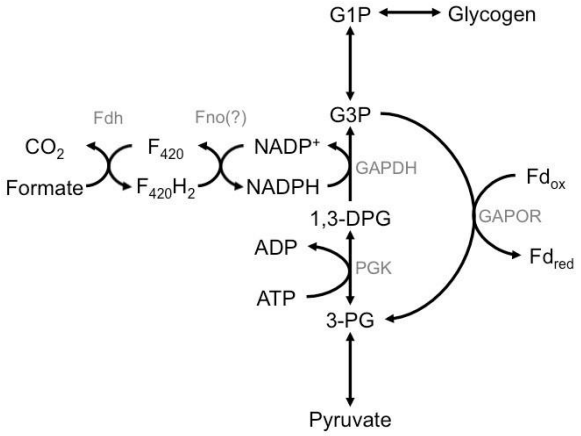


Figure 2. The GAPOR cycle with input from reduced $F_{420}$ ($F_{420}H_2$). Fdh, formate dehydrogenase; Fno, $F_{420}H_2$:$NADP^+$ oxidoreductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAPOR, glyceraldehyde-3- phosphate:ferredoxin oxidoreductase; PGK, phosphoglucokinase. G1P, glucose-1-phosphate; G3P, glyceraldehyde-3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; 3-PG, 3-phosphoglycerate.

Science objectives for 2012-2014:

- Test the potential role of Eha in energy spilling, whereby growth yield decreases markedly when electron-donating substrates are in excess. Reduction of $CO_2$ to formyl-MFR via electron bifurcation could be replaced by reduction via Eha, depleting chemiosmotic energy.
- Genetically determine the importance for Eha activity of multiple genes encoding subunits of unknown function.
- Use ion-specific ionophores to determine if Eha is a $H^+$ or a $Na^+$ translocator.
- Using purified Hdr complex, test the hypothesis that formate and reduced $F_{420}$ as well as $H_2$ are direct electron donors.
- Determine the subunit composition of the Hdr complex. Test the hypothesis that hydrogenase (Vhu) and formate dehydrogenase (Fdh) occupy the same site.

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**Modulating Lignin Biosynthesis Using Engineered Monolignol *para*-Methyltransferase**

Chang-Jun Liu, Principal Investigator
Ke-Wei Zhang, Yuanheng Cai

Biology Department, Brookhaven National Laboratory (BNL), Upton, NY 11973
Email: cliu@bnl.gov; Web: http://www.bnl.gov/biology/People/Liu.asp

Overall research goals: Lignin is an irregular rigid cell wall polymer formed via oxidative polymerization of three primary monolignols. The content and ratio of different lignin subunits dictate the degree of lignin condensation, and thus, the degradability of the cell wall. The overall goals of our research program are to understand the molecular mechanism of lignin biosynthesis, especially the lignin precursors' deposition and polymerization processes, as well as to elucidate the mechanism of their metabolic regulation. Our current research objectives include 1) understanding the structure-function and the regioselectivity of phenolic *O*-methyltransferases in modifying lignin biosynthetic precursors and the related analog phenolics; 2) using structure-based protein engineering strategy to evolve novel enzymes to produce lignin monomeric analogs; thereafter, 3) probing lignin polymerization mechanism, meanwhile developing novel biotechnological strategy for the rational manipulation of plant lignification.

Significant achievements in 2010-2012: Previously, we generated a set of monolignol 4-*O*-methyltransferase variants via structure-based protein engineering. The created variants showed broad substrate-specificity and significant *para*-methylation activity to both guaiacyl and syringyl lignin precursors. We then expressed the variants in *Arabidopsis*. During the last two years period, 1) we thoroughly examined the metabolic behavior of the *para*-methylated monolignols in oxidative polymerization process in vitro and the effect of *para*-methylation of monoligols on their translocation across cell membrane. 2) We then comprehensively analyzed the obtained *Arabidopsis* transgenic plants expressing monolignol 4-*O*-methnyltransferase; and revealed that expressing the evolved enzyme in *Arabidopsis* substantially disturbs lignification, reducing the total lignin content in the cell walls (Fig. 1). Concomitantly, the transgenic plants accumulate *de novo* synthesized 4-*O*-methylated soluble and "wall-bound" phenolic esters. Our study proves that etherifying the *para*-hydroxyl of monolignol *in planta* precludes the derived compound's participation in the subsequent polymerization process, thereby offering a potentially valuable biotechnological solution to manipulate lignin biosynthesis. 3) To further explore the effect of *para*-methylation of monolignols on plant lignification in different species, we transferred the engineered monolignol 4-OMT into poplar and conducted preliminary analyses on the generated poplar transgenic plants.

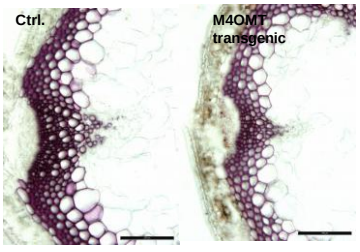


Fig. 1 Phloroglucinol-HCl staining of the stem section indicating the reduction of lignin content in vasculature of monolignol 4-OMT expression Arabidopsis.

Science objectives for 2012-2013:

- The detail cell wall polymer content and composition analyses, soluble phenolic profiling, and physiology evaluation will be conducted on the obtained poplar transgenic plants harbouring the expressed monolignol 4-OMT; this work will be compared with the previous data obtained from Arabidopsis. The study on poplar transgenic plants will provide more comprehensive evaluation on the interference of modification of lignin precursors on plant lignification in heavily lignified wood species.
- In concert with expressing the engineered monolignol 4-OMTs in poplar, and as a result, the producing large amount of transgenic biomass, we will examine the potential effects of the altered lignin content or composition on their bioconversion into ethanol or into different bioproducts under different pretreatment conditions.
- The current monolignol 4-OMT variants work effectively on both guaiacyl and syringyl lignin precursors, which affect lignin quantity but not the composition when they express *in planta*. Continuing protein evolution should create enzyme variants with further optimized catalytic efficiency and substrate specificity. Our goal is to obtain a subset of novel 4-OMTs with a highly restricted substrate-preference for particular monolignols, therefore to control specifically lignin structure.

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**Ubiquitin-like Proteins in Protein Conjugation and Sulfur Transfer in Archaea**

**Julie A. Maupin-Furlow, Principal Investigator**
Laurence Prunetti, Postdoctoral Research Associate
Hugo Miranda, Graduate Research Assistant
Nathaniel Hepowit, Graduate Research Assistant
Jonathan Martin, Graduate Research Assistant
University of Florida, Department of Microbiology and Cell Science, PO Box 110700, Bldg. 981, Museum Rd. Gainesville, FL 32611-0700. Tel: (352) 392-4095; Fax: (352) 392-5922;
Email: jmaupin@ufl.edu; Web: http://microcell.ifas.ufl.edu/personnel/faculty/maupin.shtml

**Overall research goals:**

Of the three domains of life, archaea are the least characterized yet represent tremendous potential to advance DOE missions toward a bio-based economy. Archaea are central to global organic and inorganic cycles including methanogenesis and contribute to a large fraction of the Earth's biomass. Archaea are ideal for biocatalyst development encoding enzymes that are stable and active in the harsh conditions encountered in industrial processes (e.g., extreme temperatures, high solvent) and are not pathogens. Archaea also provide new insight into metabolic biochemistry and the diversity of cell function with as much as 50% of their genes encoding novel proteins with no obvious counterparts in the other domains of life.

The **long-term goal** of our research is to understand how proteins are targeted to proteasomes for degradation and what pathways are regulated by this proteolytic system in archaea. Investigating central processes in archaea, such as the proteasome system, is important in furthering our understanding of this unusual group of organisms and in advancing our ability to modify pathways for applications (e.g., targeting proteins for post-translational modification, modulating protein stability/activity, altering protein partners). In this project, we will determine whether enzymes of archaeal Ubl-protein conjugation are required for sulfur transfer in the formation of: i) a dithiolene group in the biosynthesis of pyranopterin-based molybdenum cofactor (MoCo) for DMSO reductase and ii) 2-thiouridine in tRNA. We will also determine whether a newly discovered archaeal Ubl protein, SAMP3, forms isopeptide or other types of covalent bonds with its protein targets, identify the protein network modified by this Ubl-protein and elucidate whether SAMP3 is involved in sulfur transfer for biosynthesis. Finally, we will evaluate the distribution of Ubl systems among the archaea and determine a core set of proteins that are modified by SAMPs in this domain of life.

Significant achievements in 2010 – 2012:

Through our work, we have discovered that small ubiquitin-like (Ubl) archaeal modifier proteins or SAMPs are covalently attached to protein targets. This archaeal 'sampylation' system has deep evolutionary roots with ubiquitination, a pathway known to target proteins for proteasome-mediated destruction in eukaryotes. An archaeal Ub-activating E1 enzyme, UbaA, is shown to be required for the formation of SAMP protein-conjugates. We also provide evidence that components of the archaeal Ubl system are required for tRNA thiolation and dimethylsulfoxide (DMSO) reductase activity. While archaea do not encode ubiquitin-conjugating E2 or ubiquitin ligase E3 enzyme homologs, we have recently purified proteins that interact with the UbaA in a DTT-sensitive manner suggesting proteins that may interact with the archaeal E1-like enzyme via thioester or similar type of bonds. Much like eukaryotic ubiquitin, SAMP1-3 of *Haloferax volcanii* are shown to be conjugated to proteins through an isopeptide bond that is formed between the α-carboxyl group of a C-terminal glycine residue of the Ubl and the ε-amino group of lysine residues of target proteins. Target proteins and sites of sampylation were found to be diverse and include protein targets that mediate sulfur mobilization, transcription, stress response and other functions. Tandem-based affinity labeling and purification of select target proteins (*e.g.*, TpbE) confirmed their sampylation

and established a basis for elucidating the biological function of SAMP modification in archaeal cells. An archaeal JAMM (JAB1/MPN/Mov34 metalloenzyme) peptidase, termed HvJAMM1, was found to cleave the isopeptide bond formed between the SAMP and its target protein providing evidence that sampylation is reversible. Sampylation is also demonstrated to be functional in organisms of the phyla Euryarchaeota and Crenarchaeota revealing the system is relatively universal among the Archaea domain. Overall, our work reveals a universal system of protein conjugation in archaea that shares biochemical features with eukaryotic ubiquitylation, thus, providing a fundamental insight into the diversity of Ubl systems in living cells.

Science objectives for 2012 – 2014:

- To further understand sampylation, we are characterizing components of this system using biochemical, genetic and proteomic methods. Components include SAMP1-3, UbaA, HvJAMM1, select target proteins (e.g., GlpR, TbpE) and partners of SAMP-UbaA that may interact via thioester bond.
- Components of the sulfur transfer pathways are also under investigation including the Ncs6 tRNA thiolation protein homology HVO_0580 that likely interacts with SAMP2, the MoCo biosynthesis protein MoaE that likely interacts with SAMP1 and interacting partners of SAMP3.

**Select** references related to work 2010 – 2012: (with corresponding author underlined)


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## Genetic Analysis of Hydrogenotrophic Methanogenesis in Methanosarcina Species

William W. Metcalf, Principal Investigator
Department of Microbiology, University of Illinois
B103 C&LSL, 601 S. Goodwin, Urbana, IL 61801
Email: metcalf@uiuc.edu; Web: http://mcb.illinois.edu/faculty/profile/1191

Overall research goals: The long-term goal of our research is to expand our knowledge regarding hydrogen-dependent (hydrogenotrophic) methanogenesis by members of the genus *Methanosarcina*. A central aspect of the study is examination of the genotypic and phenotypic differences between *M. barkeri*, an organism that grows well on $H_2/CO_2$, and *M. acetivorans*, a closely related organism that is incapable of growth on $H_2/CO_2$. The differences in hydrogen metabolism lie at the center of the energy-conserving electron transport chains of the two organisms. Examination of the molecular, genetic, biochemical and physiological traits that underpin these differences is expected to deepen our overall understanding of methanogenesis, hydrogen production/consumption and anaerobic metabolism; all of which are central themes in the DOE Energy Biosciences research program. **The specific goals are; (1) characterization of energy-conserving electron transport in *M. barkeri* via a proposed hydrogen-cycling mechanism, (2) characterization of hydrogen-independent energy-conserving electron transport in *M. acetivorans*, and (3) assessment of the roles of the multiple heterodisulfide reductase (Hdr) isozymes in *M. barkeri* and *M. acetivorans*.**

Significant achievements 2011-2012: Growth of *M. barkeri* on $H_2/CO_2$ is believed to require three functionally discrete hydrogenases: the ferredoxin-dependent Ech hydrogenase, the methanophenazine-dependent Vht hydrogenase and the cytoplasmic $F_{420}$-dependent Frh hydrogenase. *M. barkeri* encodes two copies each of Frh (the *fre* and *frh* operons) and Vht (the *vhx* and *vht* operons). To examine the role of these hydrogenases during growth on various substrates, we constructed *M. barkeri* mutants lacking each of the five hydrogenase operons, as well as ones lacking the hydrogenase maturation operon (*hyp*). Mutants lacking various combinations, including one deleted for all five operons, were also made. We characterized the growth of these mutants on all known methanogenic substrates showing that *M. barkeri* utilizes $H_2$ as electron carrier for methanogenesis ***regardless*** of the substrate be used: *i.e.* all substrates are converted to $H_2$ during methanogenesis. This "hydrogen cycling" metabolism leads directly to production of a proton motive force that can be used by the cell for ATP synthesis. To complete the study of hydrogenase function, we are measuring the relative contributions of each hydrogenase by quantitative assay of enzyme activity in cell extracts of the mutants. Preliminary data indicate that the membrane-bound, phenazine-linked hydrogenase accounts for approximately 80% of total activity, consistent with it's proposed role in hydrogen recapture during hydrogen cycling. We are also assessing whether substrate-channeling is required for hydrogen-cycling. To do this, we are using fluorescently tagged proteins and high-resolution microscopy to localize the cytoplasmic and membrane-bound hydrogenases. Preliminary results show that the tagged cytoplasmic hydrogenase Frh is fully functional and localized to the cell membrane, consistent with channelling of $H_2$ to the extracellular Vht hydrogenase.

The lack of hydrogen metabolism in *M. acetivorans*, coupled with the apparent obligate role of hydrogen in *M. barkeri,* suggests that *M. acetivorans* has evolved a hydrogen-independent energy-conserving electron transport chain. Based on our analysis of the *M. acetivorans* genome, we identified two gene clusters, designated *rnf* and *erh* as potential players in this presumptive electron transport chain. Both gene clusters encode proteins with homology to the proton-pumping sub-units of NADH hydrogenase (a key player in the electron transport chain of mitochondria and aerobic bacteria). Moreover, both operons are unique to *M. acetivorans* and not found in other *Methanosarcina* species.

Knock-out mutants of the *rnf* and *erh* operons were constructed and examined their ability to grow on, and produce methane from, various substrates. The *erh* mutant had no measurable growth phenotypes that we could identify. However, the *rnf* mutant was unable to grow on acetate and had reduced growth yields on all other substrates. Resting cell suspension experiments indicate that these phenotypes are due to an inability to make methane, consistent with a role for *rnf* in the electron transport chain (which ends in methane). In the past year we have constructed a series of double and triple mutants lacking *rnf*, *ehr*, and *hdrABC*. Genetic analysis and transcriptional profiling show that *rnf* is essential for growth on acetate and is an energy-conserving protein. Rnf is also involved in methylotrophic methanogenesis, where *rnf* and *hdrABC* are the main ferredoxin oxidoreductases in the cell. An *Δrnf ΔhdrABC* double deletion mutant was viable, indicating the existence of a third pathway for ferredoxin oxidation. These data show *M. acetivorans* has unexpected physiological adaptability for energy conservation.

Finally, each of three aims described above include the use of transcriptional profiling via a relatively new method called RNA-seq. Because this method has not been previously used in *Methanosarcina*, we have spent much of the last year in developing and testing a standardized protocol for use in *M. barkeri* and *M. acetivorans*. We have validated a simple and robust method for isolation of high-quality, DNA-free RNA and tested it in several RNA-seq experiments. The results show that the method is reliable and reproducible. However, as expected, the vast majority of RNA-seq reads (>90%) map to the ribosomal RNAs, rather than the desired mRNA reads. Thus, to generate sufficient numbers of mRNA reads without excessive cost we will need to develop a method for rRNA removal prior to the RNA-seq experiments. We are currently exploring two methods for rRNA removal and expect to begin our planned experiments by the end of the current budget year.

Science objectives for 2012-2013:

- Complete the characterization of hydrogenase function, including localization, pull-down experiments and characterization of enzyme activity
- Perform RNA-seq experiments to assess changes in gene expression found when $H_2$-cycling is disrupted.

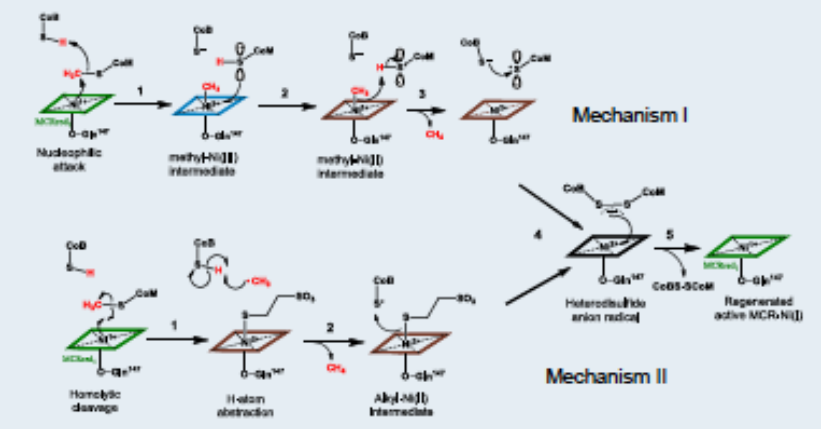
References to work supported by this project July, 2010-present: Three manuscripts are currently being prepared for submission.


1. G. Kulkarni, Guss, A.M., Buan, N.R. and W.W. Metcalf. 2012. Discovery of latent alternative electron transport pathways in the methane-producing archaeon *Methanosarcina acetivorans*. (*in preparation*)
2. G. Kulkarni and W.W. Metcalf. 2012. Experimental demonstration of hydrogen cycling as a mechanism of energy conservation in methanogenic Archaea. (*in preparation*)
3. G. Kulkarni and W.W. Metcalf. 2012. Characterization of mutants lacking each of the three distinct hydrogenases in *Methanosarcina barkeri*. (*in preparation*).

## Enzymology of Methanogenesis: Mechanism of Methyl-Coenzyme M Reductase

Stephen W. Ragsdale, Principal Investigator
Dariusz Sliwa, Yuzhen Zhou, Nathan Sheskey
Department of Biological Chemistry, Univ. of Michigan Medical School, Ann Arbor, MI 48109-0606
Email: sragsdal@umich.edu, Web: http://www.biochem.med.umich.edu/?q=ragsdale

Overall research goals: Methyl-coenzyme M reductase (MCR) from methanogenic archaea catalyzes the terminal step in the biological synthesis of methane. Using coenzyme B (HSCoB) as the two-electron donor, MCR reduces methyl-coenzyme M to methane. Two catalytic mechanisms have been proposed for methane synthesis: one involves an organometallic methyl-Ni(III) intermediate, while the other includes a methyl radical. Our research goals are (a) to elucidate the mechanism of methane formation by MCR, (b) to determine the mechanism of activation of MCR and (c) determine the crystal structure of the Ni(I) state of MCR and the structures of catalytic intermediates.




Significant achievements 2010-2012: We performed the first pH-dependent studies of MCR mechanism with the native substrates by steady-state and presteady-state kinetics and with CoBSH analog (CoB6SH) in which heptanoyl moiety was replaced with a hexanoyl group. These studies allow isolation of the first and second half reaction and to observe for the first time the decay of Ni(I) in reaction with native substrates. Pre-steady state kinetics indicate two ionization events with kobs reaching a maximum value at pH between 7.5-8.0, which suggest that only mono-protonated enzyme is active. Residues/substrates/intermediates with pKa1 = 6.2 and pKa2 = 8.9 essential for catalysis yet need to be identify. Possible candidates include: Tyr333, Tyr367, CoMSH, CoBSH, $H_20$, and F430.

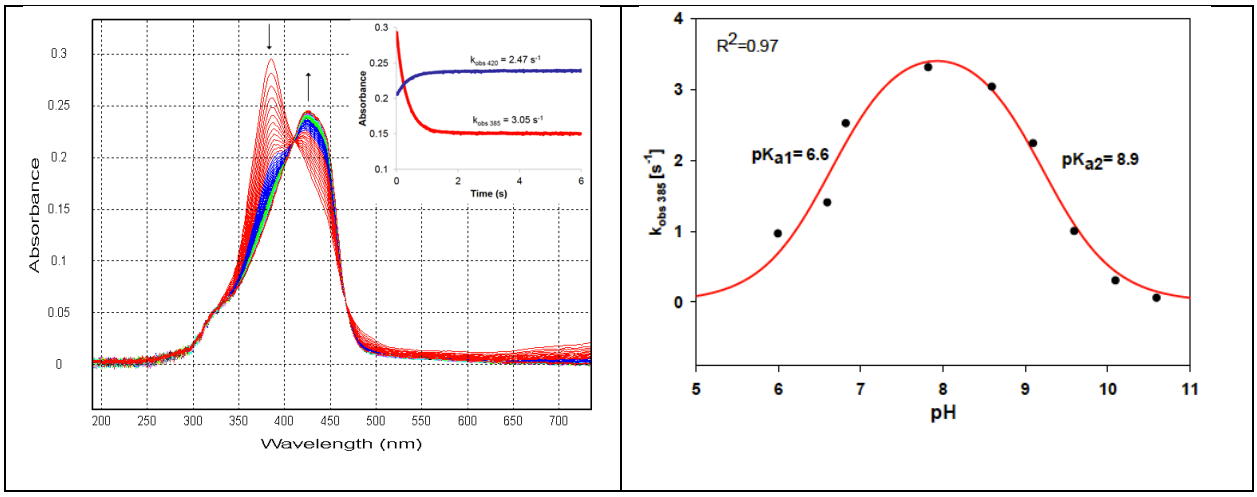
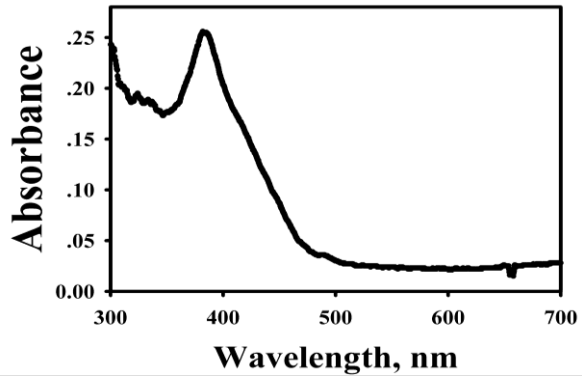
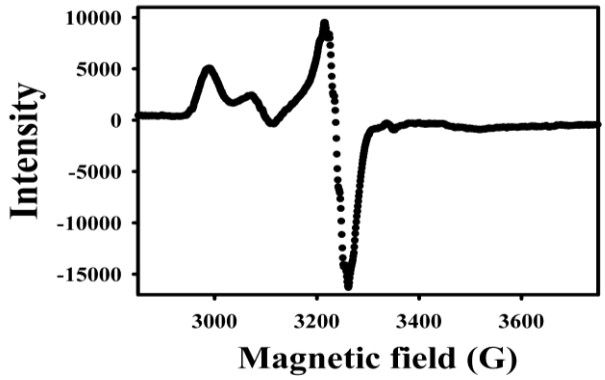



Fig. 1. pH dependence studies of MCR in the pre-steady state conditions was performed in 50mM GTP buffer in a pH range 5.6 -10.6.

We also showed that incubating growing cells with CO can also activate MCR *in vivo*. The rate of CO-dependent activation is about 15 times faster than with $H_2$ (130 $min^{-1}$ and 8 $min^{-1}$ for CO and $H_2$ activation, respectively) and CO can activate MCR to a higher percentage (~ 2-fold more $MCR_{red1}$ produced). Unlike $H_2$-dependent activation, which exhibits a 10-h lag time, there is no lag for CO-dependent activation. CODH activity is required for the activation of MCR by CO based on the observation that $CN^-$, a CODH inhibitor, blocks this *in vivo* activation. The purified MCR from CO purged cells has the same enzymatic and spectroscopic features as the enzyme purified from $H_2$-purged cells. The hydrated form of CO, formate, which also is a strong reductant, cannot activate MCR in *M. marburgensis in vivo*. Electrons from CO are not transferred from ferredoxin directly to MCR.




UV-visible spectrum of MCR purified from CO-purged cells




EPR spectrum of MCR purified from CO-purged cells

We have developed methods to generate and crystallize the active Ni(I) form of $MCR_{red1}$. We also can mount and freeze the crystals in the Ni(I) state, as confirmed by EPR- and UV-visible micro-spectroscopic studies of the crystals. In addition, we have been able to determine the structure of the methyl-Ni(III) form of MCR by reacting the Ni(I) crystals with the methyl donor (1); thus, the Ni(I) state must have been present, as the Ni(II) state is unreactive with methyl donor.

Science objectives for 2012-2014:

- The pH dependent studies allow isolation of the first half reaction in the MCR mechanism. This characteristic should allow trapping of the radical or the methyl-Ni intermediate in the mechanism and permit determination of the mechanism of MCR.
- Solvent isotope effect studies should permit determination if proton transfer occurs during the first step in the mechanism.

References to work supported by this project 2010-2012:

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**Engineering self-assembled bioreactors from protein microcompartments**

David Savage, Principal Investigator
Departments of Molecular & Cell Biology and Chemistry
University of California, Berkeley
Berkeley, CA 94720-5230
Email: dsavage@berkeley.edu Web: www.savagelab.org

Overall research goals: The goal of our work is to understand the assembly, function, and engineering potential of the carboxysome bacterial microcompartment by: (1) using a genetic and cell biological approach to explore the functional role of each component microcompartment protein in the native host *Synechococcus elongatus* and (2) reconstitution of carboxysome formation in a heterologous host as a means of defining the essential molecular features relevant to carboxysome size, shape, and assembly. Together, these approach aim to define the fundamental principles of carboxysome structure and enable the future engineering of microcompartments with novel function, such as synthetic organelles with enhanced metabolic function.

Significant achievements 2011-2012. Initial investigation revealed that portion of the carboxysome operon from *Halothiobacillus neapolitanus* was sufficient to yield expression of functional carboxysomes in *E. coli*. By optimizing operon construct and biochemical protocols, we have demonstrated that *E. coli* is capable of synthesizing carboxysomes highly similar to the native host. We have also demonstrated these microcompartments are functional both *in vivo* and *in vitro*.

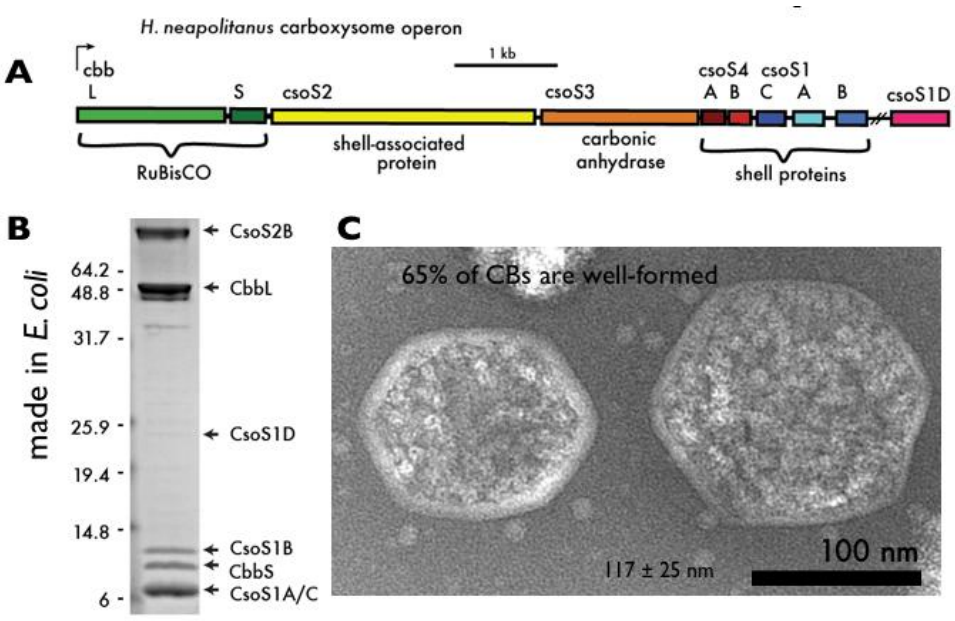


Figure 1. Heterologous expression of carboxysomes in *E. coli*. (A) Schematic of the operon transferred from *E. neapolitanus* into *E. coli*. (B) Entire carboxysomes run on an SDS-PAGE gel (with coommassie stain) showing component stoichiometry. (C) Electron microscopy of heterologous carboxysomes.

Scientific objectives for 2012-2014.

- There is, as of yet, no molecular explanation for protein targeting to the carboxysome complex. We are currently developing a fluorescent-tagging approach for screening chimeric 'signal sequences' to identify protein domains capable of targeting.

- We have identified the set of genes sufficient for carboxysome formation. Future work will use this construct to identify the minimal number of genes required both *in vivo* and *in vitro* for formation, as well as how genetic perturbation affects quaternary structure of the complex.

- Work in our group suggests that the polymer polyphosphate may play a role in carboxysome function in cyanobacteria. We are currently taking a genetic approach to identify genes linking polyphosphate storage with cellular metabolism and to understand the impact of this connection using growth assays, metabolomics, and fluorescence microscopy of the cellular ultrastructure.

References to work supported by this project 2011-2012*:*

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## Modification of Plant Lipids

John Shanklin, Principal Investigator
Edward Whittle, Ying Lou, Carl Andre, Xiaohong Yu, Hui Liu, Qin Liu and Allen Orville.
Biology Dept. Building 463, Brookhaven National Laboratory, Upton, NY 11973
Email: shanklin@bnl.gov; Web: http://www.bnl.gov/biology/people/shanklin.asp

Collaborators: Dr. Ylva Lindqvist and Dr. Jodie Guy, Karolinska Institute, Stockholm, Sweden.

Overall research goals: Lipids and oils are a vital class of compounds. In eukaryotes, saturated fatty acids are modified post-synthetically by the introduction of *cis* double bonds or by the introduction of a variety of functional groups. The focus of this research is to understand the molecular basis for these chiral lipid-modification reactions by using the process of fatty acid desaturation as a model. We will test our hypotheses regarding structure-function relationships by creating new activities. These enzymes with novel activities will be introduced into model plants for validation and subsequently into for commercially useful plants that will accumulate renewable sources of industrial feedstocks currently derived from petrochemicals.

Significant achievements in 2010-2012:

- Determined the structure of the castor Δ9-18:0-ACP desaturase in complex with its substrate.
- Identified the binding helix of the ACP residues and their corresponding interaction residues on the surface of the desaturase enzyme
- Identified a key determinant of the mechanism of regioselectivity for the ivy and castor acyl-ACP desaturases; i.e., that the nature of the charge on the sidechain at residue 280 is a critical determinant which stabilizes one of two distinct binding orientations of ACP differing by ~ 60°. One binding orientation predisposes Δ9 desaturation, the other Δ4 desaturation (see Fig. 1).
- Developed a variant of RNAi in which a hairpin and an antisense portion of the gene together downregulate a target gene; producing stronger suppression than either method alone.
- Determined the stereochemistry of 10-sulfoxidation catalysed by a soluble $\Delta^9$ desaturase.
- Determined the oligomeric state of the membrane desaturases are dimers, like the soluble desaturases.
- Performed metabolic engineering of Arabidopsis to increase the accumulation of ω-7 fatty acids from 2-71%, equivalent to that of the naturally ω-7 accumulating species *Doxantha*.

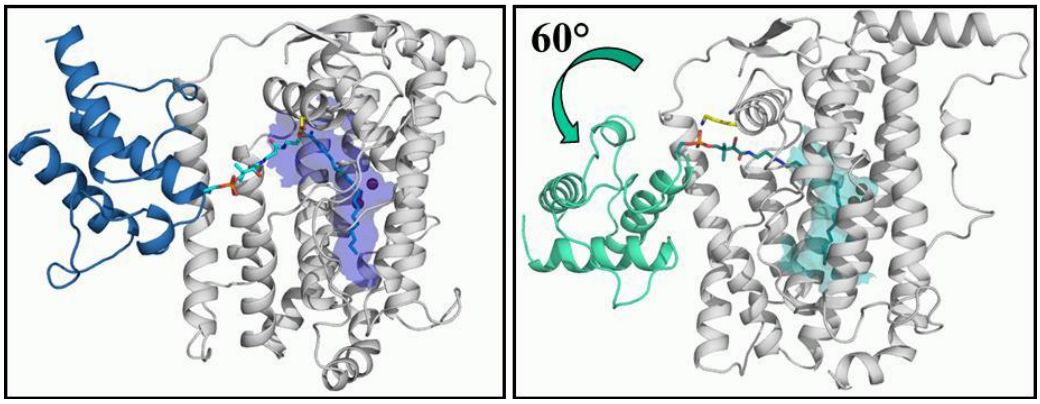


**Fig. 1.** Binding orientations of ACP relative to the castor Δ9 desaturase (left) and ivy Δ4 desaturase (right). Desaturase enzyme is shown in grey, ACP in green or blue, residue 280 in yellow.

Science objectives for 2010-2011:

- Test the effects of various mutations on the castor desaturase surface that potentially interact with ACP or the pantetheine group of the substrate to create novel regioselectivities.
- Perform correlated UV-Vis and Raman spectral and structural determinations to detect reaction intermediates for the soluble desaturase enzymes using NSLS beamline X27C.

- Determine a crystal structure of the *Thunbergia* Δ6-16:0-desaturase both alone and in combination with 16C substrate.
- Engineer ω-7 fatty acid accumulation into the emerging crop plant camelina.
- Use thermofluor analysis to determine the melting temperatures of various novel and archetypal desaturases to test the hypothesis that newly evolved enzymes are less stable that archetypal ones.
- Test the hypothesis that metabolic channelling occurs in membrane bound desaturase heterodimers of FAD2 and FAD3.

References to work supported by this project 2010-2012:


1. Tremblay, A.E., N. Tan, E. Whittle, D. J. Hodgson, B. Dawson, P. H. Buist, and J. Shanklin. 2010. Stereochemistry of 10-sulfoxidation catalyzed by a soluble $\Delta^9$ desaturase. Organic & Biomolecular Chemistry 8: 1322-1328.
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11. Fan, J; Yan, C; Andre, C; Schwender, J; Shanklin, J; Xu, C. 2012. Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in *Chlamydomonas reinhardtii*" In review, Plant and Cell Physiology 53:1380-90.

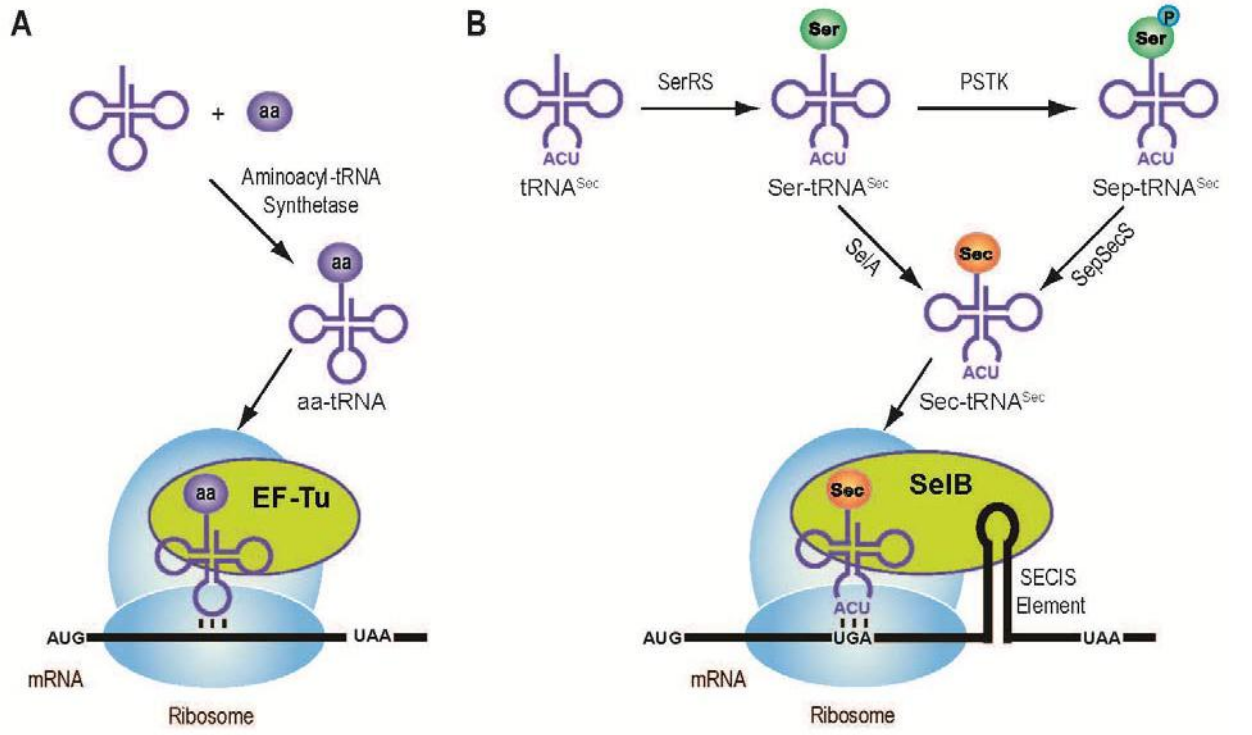
## Engineering Selenoproteins for Enhanced Hydrogen Production

Dieter Söll, Principal Investigator
Michael Hohn, Postdoctoral Associate; Caroline Aldag, Postdoctoral Associate
Department of Molecular Biophyscis and Biochemistry, Yale University, 266 Whitney Avenue, New Haven, CT 06511
Email: dieter.soll@yale.edu; Web: http://trna.research.yale.edu/

Overall research goals: Selenoenzymes (enzymes containing selenocysteine - Sec) are known to have superior catalytic efficiency compared to their sulfur homologs (containing cysteine - Cys). Between 2005-2009 we worked out the pathway and mechanism of Sec biosynthesis in archaea and eukaryotes. As in bacteria, no aminoacyl-tRNA synthetase exists that attaches Sec to tRNA; instead Sec is formed in an RNA-dependent reaction that generates Sec-tRNA (Palioura *et al*, *Science* **325**, 321 (2009)). Co-translational Sec insertion proceeds only with a modified translation apparatus (Scheme 1) that rules out conventional protein engineering with Sec.

Our research objectives are (1) to develop a system allowing for site-specific insertion of Sec into any position of a protein, and (2) to re-engineer the $F_{420}$-reducing [NiFeSe] hydrogenase.

Significant achievements 2011-2012: Establishment of a method to site-specifically incorporate Sec into any position of a protein.




**Scheme 1**. Aminoacyl-tRNA formation and first steps of protein synthesis. (**A**) Canonical amino acids: Aminoacyl-tRNA gets formed and delivered by EF-Tu to the ribosome. (**B**) Selenocysteine gets formed while bound to tRNA; Sec-tRNA transfer to the ribosome and accurate codon recognition are achieved by SelB, the Sec-specific elongation factor, and the SECIS element, an RNA structure within the open reading frame of bacterial selenoprotein mRNAs.

We developed a synthetic tRNA (tRNA*) that is an acceptable substrate for three major *E. coli* proteins: seryl-tRNA synthetase forming Ser-tRNA*, selenocysteine synthase SelA gener- ating Sec-tRNA*, and EF-Tu for Sec-tRNA* transport to the ribosome. This tRNA is orthogo- nal to the translation machinery allowing unrestricted site-specific Sec insertion into recombi- nant proteins including *E. coli* formate dehydrogenase H and selenoglutaredoxin, and human glutathione peroxidase.

References to work supported by this project 2011-2012:

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**Enhancing photosynthetic energy conversion efficiency through increased plastoquinone pool size**

*Sowmya Subramanian[1], Kelly M Gillespie[2], Ellen Marsh[2], Edgar B. Cahoon[3] and Richard T. Sayre[1]*

*[1]New Mexico Consortium/Los Alamos National Laboratory*

*[2]Donald Danforth Plant Science Centre*

*[3]Department of Biochemistry at University of Nebraska-Lincoln*

Improving photosynthetic efficiency for biomass production is one of the many approaches that are critical to the development of an economical and environmentally sustainable biofuel. One of the bottle-necks in the efficient conversion of sunlight into available energy is the oxidation of reduced plastoquinone (PQ) by the cytochrome $b_6f$ complex. We propose to buffer this constraint by increasing the PQ pool size. To accomplish this goal, we have engineered transgenic algae to over-express yeast Prephenate dehydrogenase (PDH) and *A. thaliana* Homogentisate solanesyltransferase (HST). PDH converts prephenate directly into homogentisate circumventing competing reactions for tyrosine synthesis, while HST catalyzes the prenylation of homogentisate with solanesyl diphosphate, and subsequent decarboxylation. Preliminary results indicate that transgenics expressing the PDH and HST gene have slower variable chlorophyll fluorescence raise kinetics than wild-type cells indicative of an increase in the PQ pool size. Experiments are underway to determine the effect on growth rates and quantify PQ pool size. The merits of the strategy to increase the PQ pool size will be discussed in the light of these results.

**Putting a Handle On The Pump: Molecular Properties and *In Planta* Functions of the Plasma Membrane Proton Pump ($H^+$-ATPase)**

Michael R. Sussman, Principal Investigator
University of Wisconsin Biotechnology Center, 425 Henry Mall, Madison, WI 53706
Email: msussman@wisc.edu
Web: http://www.biochem.wisc.edu/faculty/sussman

Overall research goals: The plasma membrane is the point of contact between a cell and its external environment and plays a critical role in the growth and development of all organisms, including higher plants. The proteins within this membrane that act as pumps, carriers, and channels together convert the chemical energy of ATP into gradients of organic and inorganic solutes that support life. In higher plants and fungi, foremost among these transport proteins is the proton pump ($H^+$-ATPase) since it alone generates the protonmotive force (composed of both a transmembrane electrical potential and a proton chemical gradient) that drives the transport of solutes essential for cell growth. This enzyme appears to be one of the major ATP consumers in a living plant and despite many years of study, a molecular genetic approach to understand its function has, until recently, been less forthcoming. The overall research goal of this project is to use genetic, biochemical and physiological technologies for understanding the molecular functions of this protein, both in terms of what precise physiological and developmental roles it is playing in everyday plant life, as well as in learning how it converts chemical energy into electrical energy. Our specific approach is to isolate Arabidopsis mutants and study the phenotype resulting from the genetic defects. We are also using the mutants to perform structure-function studies via rescuing the mutants with site directed mutations via full length genomic clones containing the regulatory sequences. A major recent focus has been on identifying posttranslational modifications and interacting partners of the enzyme, and substantial progress has been made in developing and using the tools required for understanding the role of kinase-mediated phosphorylation, in particular.

We have three specific objectives: (1) *In planta* gene replacement of AHA1/2 with mutants via complementation of the double AHA1/2 knockout plants, (2) Targeted AQUA mass spectrometric assay for measuring changes in stoichometry AHA1, 2, 3 phosphorylation under many environmental and genetic perturbations and (3) Nontargeted mass spectrometric analysis of purified AHA 1/2 to identify interacting proteins, lipids and additional posttranslational modifications.

Significant Achievements of 2009-2011: We have demonstrated that in Arabidopsis, this enzyme is in fact an essential gene (Haruta et al., 2010). In Arabidopsis and all other plants, this protein is encoded by a family of 11 genes called AHA (**A**rabidopsis **H**-$^+$**A**TPase)'s 1-11. AHA1 and 2 appear to be functionally redundant and together produce mRNA and protein corresponding to ca. 70% of the enzyme found in plant extracts. Last year we published the results of a comprehensive study of the effects of genetically reduced plasma membrane proton pump activity for AHA1 and AHA2 and demonstrated for the first time, that this is an essential enzyme for vegetative growth. We developed growth assays that demonstrate effects of single gene defects on growth and established, for the first time, a molecular

mechanism for compensation at the posttranslational level, rather than at the transcriptional or translational level. We also developed and used highly sensitive and accurate assays with heavy isotope labeled synthetic peptides (phosphorylated and nonphosphoryalted) for measuring the stoichometry of phosphorylation in AHA1, 2 and 3 using a triple quadrupole mass spectrometer. Initiated complementation studies with the lethal double mutant, for a longterm structure-function study and to establish a system for obtaining a tagged pump that rescues growth of the lethal double mutant. We have recently used this translationally fused tag attached to the pump to purify the active enzyme from plants treated for short periods of time with various effectors (e.g., auxin, fusicoccin etc). Thus, with this 'TAP-tagged' enzyme and isotope-assisted methods for quantitation with an LTQ-Orbitrap tandem mass spectrometer, we are identifying and measuring changes in the proteins that remain associated with the enzyme after detergent solubilization and purification.

Science Objectives for 2012-2013:

- Using different epitope tags, continue to refine and improve gAHA1 and gAHA2 complementing clones for pulldown's to purify the pump and identify posttranslational modification, its interacting protein and lipid partners and for microscopic studies with GFP fusions, aimed at understanding endo- and exo-cytosis at the plasma membrane
- Using site directed mutagenesis, continue extensive structure-function studies with rescued AHA1 and AHA2 genomic clones with double lethal mutant in Arabidopsis.
- Using our newly developed targeted method for measuring stoichometry of phosphorylation at penultimate threonine with synthetic peptides (see Haruta et al, 2010) using triple quadrupole, for AHA 1, 2 and 3 under many environmental and genetic perturbations, with accurate time courses, for correlating pump activation with specific physiological processes.

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## A proteomic study of steroid regulation of plant growth

Zhiyong Wang, Principal Investigator
Alma L. Burlingame, Co-PIs, UCSF
Shouling Xu, Postdoctoral Research Associate
Sunita Patil, Research Associate
Department of Plant Biology, Carnegie Institution for Science, Stanford, CA 94305
Email: zywang24@stanford.edu; Web: http://dpb.carnegiescience.edu/labs/wang-lab

Overall research goals: The research objectives are to study brassinosteroid hormone regulation of plant growth and development by: (1) developing a quantitative proteomic methods to identify brassinosteroid-induced proteomic changes, (2) identify nuclear proteins that show changes of posttranslational modifications upon brassinosteroid treatment; (3) study the function of the Nuclear Brassinosteroid-Regulated 1 (NBR1) protein in brassinosteroid regulation of flowering in plants; (4) study how NBR1 mediate brassinosteroid regulation of flowering through epigenetic and posttranscriptional mechanisms. Brassinosteroid is a major growth-promoting hormone in plants and is considered a major target for increasing biomass productivity. Brassinosteroid controls many major processes including chloroplast development, cell wall synthesis, and transition from vegetative to reproductive development, which are all critical for biomass accumulation. We have identified NBR1 as a key component that mediates brassinosteroid regulation of flowering, the transition from vegetative to reproductive development. NBR1 is a component of a nuclear complex involved in RNA processing and epigenetic regulation. Further studying the mechanism of NBR1 function will connect brassinosteroid signal transduction to epigenetic and posttranscriptional regulation and flowering. Our study not only generates fundamental knowledge of the molecular mechanisms controlling photosynthetic energy storage, but also provides means for specifically manipulating brassinosteroid-regulated developmental processes that are important for improving plant productivity.

Significant achievements 2011-2012:

Brassinosteroid has been shown to promote flowering by repressing the expression of the flowering repressor FLC gene through changes in histone acetylation. Our proteomic analysis has identified NBR1 as a nuclear protein that becomes dephosphorylated upon brassinosteroid treatment. NBR1 shares sequence homology with the human Apoptotic Chromatin Inducer in the Nucleus (Acinus), which is a component of the apoptosis and splicing–associated protein (ASAP) complex. ASAP is considered a hub in the protein interaction network that regulates gene expression, and its components have been implicated in histone deacetylation, transcriptional regulation, and preRNA splicing. Based on the late flowering and FLC-overexpression phenotypes of *nbr1-2* and BR-deficient mutants, we propose that NBR1 mediates BR-regulation of histone modification or RNA splicing of the FLC gene to control flowering.

We have employed mass spectrometry to analyze the NBR1 phosphorylation sites and NBR1-associated proteins. More than 10 phosphorylation sites have been identified, several of which are BL regulated according to label-free mass spectrometry quantifications. Site-directed-mutagenesis is being performed to determine the functions of these phosphorylation sites. Mass spectrometry analyses have also identified 80 proteins as components of the NBR1 complex purified by immunoprecipitation. Fifty (62.5%) of these NBR1-intaracting proteins are predicted to be involved in RNA processing and five are involved in Chromatin remodeling. These include homologs of the other two components of the ASAP complex (RNP1 and SAP18). At least three of the identified NBR1-interacting proteins have been shown to regulate flowering time and we have confirmed the interaction for one of them by co-immunoprecipitations.

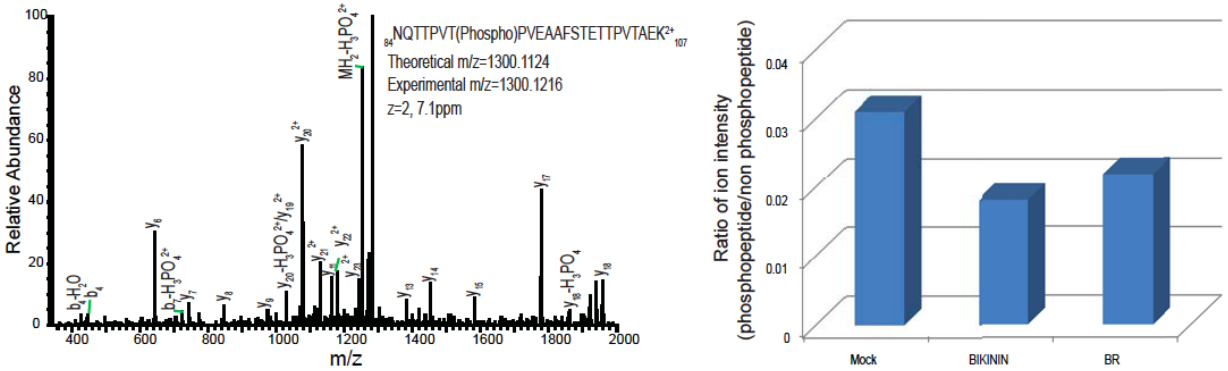


Figure 1. (A) CID spectrum of phosphopeptides of immunoprecipitated NBR1 protein digested by trypsin. Threonine 90 is phosphorylated.
(B) Label free quantification shows phosphorylation on Thr 90 of NBR1 is reduced upon bikinin and BR treatments. NBR1-GFP proteins were immunoprecipitated from mock-, bikinin- or BR-treated samples and analyzed by mass spectrometry analysis. The ion intensities of phosphorylated peptide NQTTPVT(Phospho)PVEAAFSTETTPVTAEK and its cognate dephosphorylated peptide were extracted and ratios were calculated.

To understand how brassinosteroid regulates NBR1 and BNR1-complex, we are currently performing a quantitative proteomics analysis of the BR-induced dynamic changes in protein composition and protein modification in the NBR1 complex.

Science objectives for 2012-2013:

- Quantitative proteomic analysis of the NBR1 complex is critical for understanding how phosphorylation affects the function of the NBR1/ASAP complex, and for identifying the kinase and phosphates of BNR1 and the phosphorylation site(s), which provide targets for manipulating the complex activity and flowering time.
- Genetic interactions between BR mutants, *nbr1-2*, and other flowering mutants affecting FLC expression will be completed to determine the relationships between BR, NBR1 and other flowering regulators.
- Genomic analysis using RNA-Seq to identify target genes regulated by NBR1 at the levels of transcription accumulation and alternative splicing.
- Quantitative proteomic analysis of the BR-regulated protein in the nucleus and nucleosome will identify additional proteins that mediate BR regulation of gene expression.

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**Intracellular Lipid Transfer Involved in the Biogenesis of Photosynthetic Membranes and Storage Triacylglycerol**

Changcheng Xu, Principal Investigator

Biology Department, Brookhaven National Laboratory (BNL), Upton, NY 11933

Email: cxu@bnl.gov; Web: http://www.bnl.gov/biology/People/Xu.asp

Overall research goal: The goal of this project is to understand the molecular basis of intracellular lipid transfer and triacylglycerol (TAG) biosynthesis in vegetative tissues of plants. The specific objectives of the proposed work are to: 1) isolate and characterize Arabidopsis mutants defective in interorganellar lipid trafficking; 2) complete the map-based cloning to identify the genes responsible for deficiencies in lipid transfer in the two confirmed mutants; 3) determine the functional role of the two gene products by biochemical, cell biological and molecular means; 4) determine the pathways and enzymes involved in TAG biosynthesis in vegetative tissues of plants.

Significant achievements 2011-2012: We have finished the proposed lipid mutant screen. Based on the initial genetic and biochemical analysis, eight mutants have been confirmed to be defective in lipid transfer necessary for the ER pathway of thylakoid lipid biosynthesis. Among them are mutants allelic to previously described *tgd1, tgd2, tgd3 and tgd5* and mutants that do not accumulate trigalactosyldiacylglycerol (TGDG). Two of these mutants originally named as *rtl1-1 and rtl2-1* was characterized in great detail. The *RTL1* gene has been identified by a map-based cloning approach and it encodes a putative membrane protein of unknown function. Detailed lipid profiling revealed that *rtl1-1* is deficient in ER-derived thylakoid lipids and accumulates triacylglycerol (TAG) and TGDG in leaves similar to previously described four *tgd* mutants. This mutant was thus renamed as *tgd5-1*. Additional analyses with two independent alleles containing T-DNA insertion mutations in the *TGD5* gene confirmed our findings with *tgd5-1*. Green fluorescent fusion and proteinase protection assay revealed that TGD5 is localized in the inner envelope membrane of chloroplasts. The *rtl2* locus was mapped on Chromosome II around the *TGD2* gene and genomic sequencing revealed *rtl2* is allelic to the *tgd2* mutant. Additional work was focused on the analysis of the pathway of TAG biosynthesis and its physiological significance in vegetative tissues of plants. By double mutant analysis, we found that phospholipid: diacylglycerol acyltransferase (PDAT), but not diacylglycerol (DGAT), is critical in mediating TAG biosynthesis in rapidly growing tissues such as young leaves and floral organs. Disruption of PDAT1 in the *tgd1-1* mutant background causes serious growth retardation, gametophytic defects and premature cell death in developing leaves.

Science objectives for 2012-2013:

- The localization of TGD5 in the chloroplast and the accumulation of TGDG in *tgd5* mutants raise the question of the possible functional relationship between TGD5 and other TGD proteins in lipid trafficking. Accordingly, we will test their potential

interaction by *in vitro* pull-down and yeast-two hybrid assays and the split yellow fluorescent protein BiFC analysis.

- Our previous work demonstrates the possible saturation of the *tgd* mutant collection and indeed a major portion of the mutants isolated in our genetic screen are new alleles of *tgd* mutants. Therefore, characterizing the lipid trafficking mutants that do not accumulate TGDG may lead to the isolation of novel components of lipid trafficking pathway involved in the biogenesis of photosynthetic membranes.

- Our initial results suggest that PDAT1 plays a pivotal role in TAG synthesis in vegetative tissues of plants. Characterizing the double mutants between *tgd* mutants and the mutant defective in PDAT will help to address the functional significance of TAG synthesis in nonseed tissues and the mechanism underlying premature cell death induced by deficiencies of TAG synthesis in plant model systems. In addition, the *tgd* mutants will be instrumental in dissecting the molecular factors controlling TAG accumulation in vegetative tissues of plants.

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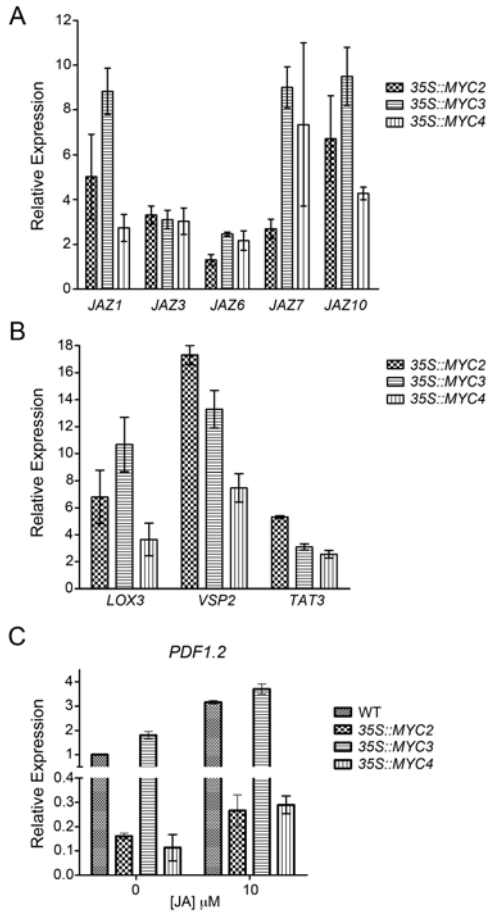
# *Session VII*

# Jasmonate Hormone: Regulating Synthesis of Reduced Carbon Compounds in Plants.

John Browse, Principal Investigator
Pablo Figueroa, Postdoctoral Research Associate
Jeremy Jewel and Amanda Wager, Graduate Students
Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340
Email: jab@wsu.edu; Web: http://cahnrs-cms.wsu.edu/ibc/research/browse/Pages/default.aspx

Overall research goals: Our original interest in understanding the role of JA in regulating the final stages of stamen and pollen development led to our discovery of the JAZ repressors, and the molecular mechanism of jasmonate (JA) hormone action is now a second important focus of our research. The specific goals for this grant period are to: **1.** Investigate the generation and clearance of the hormone with emphasis on the regulation of the OPR3 enzyme and the hydrolysis of JA-Ile. **2.** Use dominant-negative and overexpression constructs to explore the role of the MYC5 transcription factor in initiating and regulating JA responses. **3.** Investigate specific JAZ protein interactions that will help us to recognize and understand the extended network of processes, such as sulfur nutrition, that interface with JA signaling.

Significant achievements 2011-2012: Our ongoing research on this project aims to identify the transcription factor(s) that is inhibited by JAZ and mediates *MYB21* expression in stamens in response to jasmonate. We conclude that MYC3 (At5g46760) and MYC4 (At4g17880) are JAZ-interacting transcription factors that act together with MYC2 to activate jasmonate responsive genes, including the *JAZ* genes (Fig. 1). In our yeast-two-hybrid and pulldown assays, MYC5 interacted poorly with JAZ proteins (see 2011 Report). However, plants overexpressing MYC5 exhibited enhanced and constitutive jasmonate responses similar to plants overexpressing MYC3 or MYC4.




The lack of jasmonate phenotypes in *myc3* and *myc4* mutants suggested that redundancy may exist among the JAZ-regulated transcription factors. One strategy to possibly overcome such redundancy is to express a cDNA that accomplishes the addition of an EAR motif (e.g., LDLDLELRLGFA) to the target transcription factor. This can convert transcriptional activators to repressors by recruiting the TOPLESS corepressor to the transcription complex. We produced transgenic plants expressing MYC2-EAR, MYC3-EAR, MYC4-EAR, and MYC5-EAR constructs. The MYC2-EAR plants exhibit a *myc2* mutant phenotype, while plants expressing MYC3-EAR or MYC4-EAR were not distinguishable from wild-type.

**Fig. 1.** MYC3 and MYC4 regulate expression of JA-responsive genes. (A) and (B) qRT-PCR expression analysis of *JAZ* genes and wound-responsive genes in 10-day-old wild-type and *MYC* overexpression seedlings without JA treatment. Wild-type samples served as a calibrator for the calculation of relative expression levels (arbitrarily set to one). (C) Relative transcript levels of *PDF1.2* gene in 10-day-old wild-type and overexpression transgenic seedlings with or without 10 μM JA treatment (incubated for 6 hours). Wild-type samples without JA treatment served as a calibrator, and relative expression was determined from replicate measurements in two independent biological replicates. Data are mean ± s.e.

However, several lines expressing MYC5-EAR were partially or completely male-sterile. We are performing experimental tests to examine whether or not expression of the MYC5-EAR protein is responsible for the male-sterile phenotype, and whether or not the effect is through blocking jasmonate responses. These include cosegregation analysis, pollen germination assays and experiments to test whether MYC5-EAR plants are blocked in other jasmonate responses.

Science objectives for 2012-2013: The synthesis of JA-Ile via the oxylipin pathway and the JAR1 amino acid conjugase is the initial event in JA signaling. Several lines of circumstantial evidence indicate that the OPR3 enzyme, 12-oxo-phytodienoic acid reductase, is the site of regulation by upstream wound and hormone inputs. First, the ratio of JA-Ile to its immediate precursor, (3R, 7S) JA remains relatively constant as these two compounds increase >30-fold following wounding. Characterization of the *jar1* mutant also indicates that the JAR1 conjugase is not the regulated step. By contrast OPDA is present at high concentrations in many plant tissues relative to the downstream products, JA and JA-Ile, indicating the possibility that this provides a precursor pool for rapid synthesis of the active hormone. Finally, during systemic wound signaling the rise in JA and JA-Ile concentrations are associated with a corresponding disappearance of OPDA. In this next year, we will attempt to identify post-translational modifications of OPR3 using mass spectrometry. Time course experiments involving purification of OPR3 from plants before and after wounding will be used to identify dynamic post-translational modifications which change following a wounding stimulus.

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## Suppression of Photosynthesis by the Plant Stress Hormone Jasmonate

Gregg A. Howe, Principal Investigator
Jin Chen, Sheng Yang He, David Kramer, Co-PIs
Elham Attaran, Jeff Cruz, Ian Major, Bruce Rosa, Postdocs
MSU-DOE Plant research Lab, Michigan State University, East Lansing, MI 48823
Email: howeg@msu.edu; Web: http://www.prl.msu.edu/

Overall research goals: Efficiency in energy capture and conversion is profoundly influenced by the way in which photosynthetic organisms interact with the environment. Myriad environmental stresses reduce photosynthetic efficiency and suppress plant growth. Strong repression of photosynthesis-associated genes (PAGs) has been linked to stress-induced production of the phytohormone jasmonate (JA), but the molecular basis of this response is largely unexplored. The overall goals of the research are to understand how photosynthetic efficiency is dynamically modulated by changing environmental conditions, and to determine how changes in photosynthesis divert carbon flux from growth- to defense-related processes. The specific objectives are: 1) to develop a fine-scale temporal map of PAG repression in response to coronatine, a potent agonist of the JA receptor; 2) to identify specific points in photosynthetic electron transfer and carbon metabolism that are affected by the JA pathway; and 3) to identify signaling nodes that control photosynthetic output in response to biotic stress.

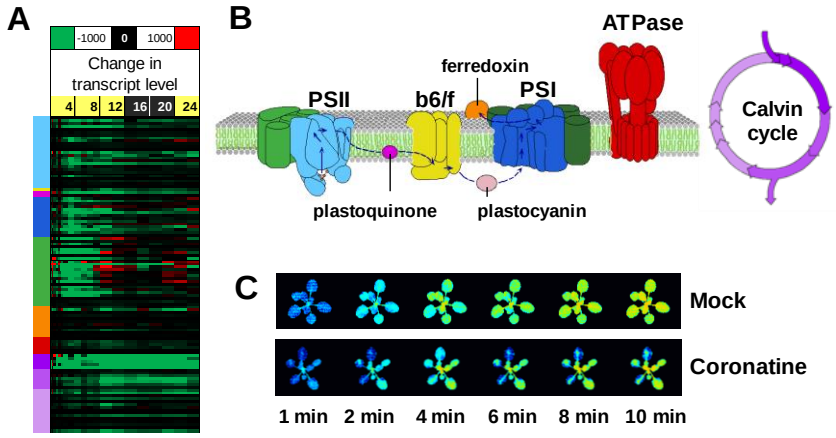


**Figure 1. Repression of photosynthesis in response to activation of the JA receptor by coronatine.** **(A)** Heat map showing coronatine-induced changes in the level of transcripts encoding components of the photosynthetic electron transport chain and Calvin cycle. Color-coding on left of the map corresponds to photosynthetic components diagrammed schematically in **(B)**. **(C)** Chlorophyll fluorescence imaging of coronatine-treated Arabidopsis seedlings. Images were collected at the indicated times after the start of the light cycle.

Significant achievements 2010-2012: Although it is well established that JA rapidly activates the expression of defense genes, the precise timing of stress-induced PAG repression has not been described. We performed RNA-seq experiments with Arabidopsis seedlings treated with the bacterial toxin coronatine, which was identified as a potent and stable agonist of the JA receptor (Sheard et al., 2010; Koo et al., 2011). The results showed that PAGs account for the vast majority of transcripts in photosynthetically active Arabidopsis leaves, and that exposure to coronatine results in massive reduction in PAG transcript levels within 4 hr of treatment (Fig. 1A). Hierarchical clustering revealed that functional subgroups of PAGs exhibit distinct patterns

of expression. Genes involved in the Calvin cycle, for example, were among the most rapidly and strongly repressed PAGs. The timing of PAG repression suggests that this is a genetically programmed and highly coordinated response that is controlled directly by the JA receptor. RNA-seq data were used to design an algorithm for optimizing time points in high-throughput gene expression experiments, which may allow us to further dissect the mechanism of stress-induced PAG repression (Rosa et al., 2012).

Using a unique whole plant imaging system—the Photosynthetic Phenometrics Array— to monitor photosynthesis and growth in situ, we found that coronatine induced a transient effect on the quantum yield of Photosystem II (ΦII) at the beginning of the subjective day (Fig. 1C). The coronatine-induced reduction in ΦII was preceded by PAG repression, suggesting that photosynthesis is not the direct target of coronatine, but rather responds to defense-related signaling that is associated with a sustained repression of plant growth.

Science objectives for 2012-2013: To elucidate how JA represses the expression of genes involved in photosynthesis, we will test a repertoire of JA signaling mutants for their ability to down-regulate PAG marker genes in response to coronatine and other forms of biotic stress. The objective of these experiments is to identify specific transcription factors and other regulatory components that modulate photosynthetic efficiency in response to environmental stress, and to understand how changes photosynthetic efficiency contribute to stress-induced growth repression (Yang et al., 2012). A second objective is to determine the physiological basis of coronatine-induced reduction in PSII efficiency. Among the specific hypotheses to be tested is that reduced ΦII results from a defect in regulation of stomata, as suggested by preliminary in situ thermal imaging and $CO_2$-dependence experiments. We are also testing an alternative hypothesis that coronatine affects the $CO_2$ assimilation reactions as suggested by the sustained repression of genes involved in the Calvin-Benson cycle.

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## The Rhizobial Nitrogen Stress Response and Effective Symbiotic Nitrogen Fixation

Michael L. Kahn, Principal Investigator
Svetlana N. Yurgel, Co-PI
Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340
Email: kahn@wasu.edu; Web: http://cahnrs-cms.wsu.edu/ibc/research/kahn/Pages/default.aspx

Overall research goals: The overarching research objective is to understand how the metabolism of symbiotic nitrogen fixation is organized in order to couple high levels of bacterial nitrogen fixation to the plant's need for nitrogen. A current focus is on a *Sinorhizobium meliloti* Rm1021 mutant that has a deletion in the N-terminus of the major nitrogen sensor, GlnD, and forms nodules with an unusual $Fix^{+}Eff^{-}$ phenotype; the bacteria fix nitrogen at a normal rate but the symbiosis is ineffective. The hypothesis is that altered regulation in the GlnD mutant leads the bacteria to incorporate fixed nitrogen into a compound the plant cannot metabolize, thus leading to a nitrogen-starved plant phenotype. We have recently incorporated proteomics and structural protein biology into our efforts, collaborating with scientists at Pacific Northwest National Laboratory, Brookhaven National Laboratory and the Albert Einstein College of Medicine.

Significant achievements 2011-2012:

GlnD mutant: We established that the organization of nitrogen stress response (NSR) regulation initiated by GlnD in *S. meliloti* is different from the NSR in enteric bacteria like *E. coli.* In *E. coli*, GlnD uridinylylates the two PII proteins, GlnB and GlnK, in response to nitrogen stress. Moreover, in enteric bacteria a Δ*glnB*Δ*glnK* mutant has similar phenotypes in either a *glnD*+ or *glnD* background, suggesting that GlnD can only communicate with the cell by modifying these proteins. In *S. meliloti,* the *glnD* allele does make a difference. As shown below, it is dominant to Δ*glnB* Δ*glnK* for some phenotypes and recessive for others.

| mutations | Nitrogen stress response | GSII Expression ($NH_4$/glutamate) | Growth on minimal media | Symbiosis |
|---|---|---|---|---|
| Wild type | Yes | No/Yes | yes | $Fix^{+}Eff+$ |
| *glnD* | Severe impairment | No/No | slow | $Fix^{+}Eff^{-}$ |
| Δ*glnB* Δ*glnK* | Impairment | Yes/Yes | slow | $Fix^{+}Eff+$ |
| *glnD* Δ*glnB* Δ*glnK* | Severe impairment | Yes/Yes | none | $Fix^{+}Eff^{-}$ |

The unusual induction of glutamine synthetase II when the Δ*glnB*Δ*glnK* mutant is grown on ammonium has been linked to the phosphate stress response. We have made progress in purifying a compound that appears at high levels in nodules formed by the *S. meliloti glnD* mutant but do not yet have convincing NMR structural information. The compound has a MW of 204 and a provisional formula of C5H12O5N2. One class of compounds that is a strong candidate is a diacid opine but so far the chromatographic behavior of the isolated compound is not exactly like any of the diacid opines or dipeptides we have synthesized.

Proteomics and structural protein biology: In collaboration with Mary Lipton in the Proteomics group at PNNL, we have identified over 1600 proteins in free-living and symbiotic bacteria. The degree of specialization of the symbiotic bacteroids is impressive— the NifH, NifD and NifK nitrogenase subunits account for approximately 10% of total bacteroid protein! There is also strong expression of the FixABCX proteins thought to be involved in electron transfer and major shifts in the abundance of several chaperonins and cold shock proteins between free-living cells and bacteroids. 424 proteins are classed as nodule-specific, 399 as free-living specific. 817 are found in both growth environments The Albert Einstein group has over 2500 *S. meliloti* proteins in the pipeline and so far has deposited 50 crystal structures in the PDB.

Science objectives for 2012-2013:

- GlnD: A major problem has been the difficulty of getting sufficient amounts of the MW=204 compound for NMR characterization. We are collaborating with the NMR group at PNNL and hope to figure this out soon. If the compound is a diacid opine, there is a clear genetic path to characterizing the biology of its synthesis. Understanding the synthesis and degradation of the compound in mutant and wild-type nodules also depends of knowing the structure and improving the purification/quantitation scheme. We are also trying to trace the link between phosphate and nitrogen stress responses, a task made more interesting by the observation that the number of differences seen between the transcriptome of Rm1021 and the Δ*glnB*Δ*glnK* mutants is much greater than between Rm1021 grown on ammonium or glutamate, which suggests that the GlnB/GlnK proteins are active in regulating processes other than the NSR.
- Proteome: We are eager to see what differences exist in the proteomes of the wild-type and mutant bacteria and in nodules formed by the GlnD mutant. This is in progress.
- Structural biology: The Einstein and Brookhaven efforts offer a major asset for working with the symbiosis in the form of purified proteins in quantities sufficient for enzyme characterization and for use as antigens. While we provide the organism expertise, we operate in the collaboration as opportunists since the progress in crystallography is only partially guided by the importance of the proteins and is very dependent on the group's success in overexpressing and purifying active protein. A number of the metabolic proteins they have isolated so far are interesting, especially the redox proteins potentially involved in energy production.

References to work supported by this project 2011-2012:


1. S. N. Yurgel, J. Rice, M. L. Kahn. (2012) Nitrogen metabolism in *Sinorhizobium meliloti*-alfalfa symbiosis: dissecting the role of GlnD and PII proteins. Mol Plant Microbe Interact. 25:355-62.
2. S. N. Yurgel and M. L. Kahn. (2012) Protein differences between rhizobial free-living and symbiotic lifestyles. Workshop on the Genomics of N-Fixing Organisms, Munich, Germany
3. S. N. Yurgel, J. Rice, M. L. Kahn. (2011) GlnD and symbiosis: Control beyond the nitrogen stress response? Invited presentation at 17th International Nitorgen Fixation Congress, Freemantle, Australia
4. S. N. Yurgel, J. Rice, M. Mulder, M. L. Kahn, V. S. Belova, and M. L. Roumiantseva· (2012) Truncated *betB2*-144 plays a critical role in *Sinorhizobium meliloti* Rm2011 osmoprotection and glycine-betaine catabolism. European Journal of Soil Biology (in revision)

**CHX transporters at dynamic endomembranes: roles in pH homeostasis critical for vegetative and reproductive success of land plants**

Principal Investigator: Heven Sze
Dept. Cell Biol & Mol Genetics, Bioscience Research Bldg, University of Maryland, College Park, MD 20742
Email: hsze@umd.edu; URL: http://www.clfs.umd.edu/cbmg/faculty/sze/lab/

Collaborators: Rajini Rao (*J. Hopkins U.*); N. Uozumi (Tohoku U.); Alice Cheung (U. Mass.)

Overall research goals

All organisms have evolved mechanisms to regulate ion and pH homeostasis in order to respond to developmental cues and adapt to a constantly changing environment. The central goal of my laboratory has been to identify transporters that regulate cation (e.g. $Ca^{2+}$, $K^+$) and pH homeostasis, and understand their roles in plant growth, development and adaptation. The dynamic endomembrane system of eukaryotes is emerging as a critical and central coordinator of processes, including signaling, cell wall modeling and stress tolerance in plants. A key question is: how do plant cells mediate membrane trafficking and cargo sorting in a spatially- and temporally-regulated manner. One hypothesis is that mechanisms regulating the internal and external environment of endomembrane compartments provide the conditions for specific biochemical reactions and protein-protein association and/or disassociation. Our recent effort has focused on a novel gene family predicted as Cation/$H^+$ eXchanger (CHX) that we show are important for vegetative and reproductive success of flowering plants.

Significant achievements in 2010-12

a) ***A novel gene family*, *CHX in plants***. A phylogenetic analysis revealed that one subfamily (CHX) of the monovalent-cation antiporter (CPA) superfamily diversified in land plants possibly from an ancestral prokaryote gene, EcNhaA or Synechocystis NhaS4. In contrast, genes sharing homology to the plant Na/H exchanger (NHX) family are found in metazoa and have been conserved from single-celled algae to higher plants. KEA genes are conserved from green algae to angiosperms, and their presence in red algae and secondary endosymbionts suggest a role in plastids. The great diversity of CHX genes in plants compared to metazoa would imply a significant role of these transporters in flowering plants (Chanroj et al. 2012).

b) ***pH homeostasis, membrane trafficking and cargo sorting***: *Arabidopsis thaliana* CHX16-CHX20 proteins are implicated in pH homeostasis because their expression rescued the alkaline pH-sensitive growth phenotype of the host yeast strain. However, CHX17 did not alter cytoplasmic or vacuolar pH in yeast when a pH-sensitive GFP and BCECF were used to monitor cytoplasmic and vacuolar pH respectively. CHX17 mediated $^{86}Rb$ transport when they are expressed in an E coli strain deficient in $K^+$-uptake pathways, and both showed selectivity for $K^+$=$Rb^+$ over $Na^+$ and Li. In yeast, CHX17 or CHX20 tagged with GFP were active and localized to endomembranes. As there is no detectable increase in $K^+$ uptake in yeast expressing CHX17, we suggest that $K^+$ transport take place at endomembranes to mediate pH homeostasis in these compartments. Consistent with this idea, CHX17 has been localized to endosomes such as the PVC in plant cells (Chanroj et al. 2011).

Significantly, alkaline pH-sensitive yeast mutants secrete vacuolar CPY into the medium, however expression of CHX17 reduces the secretion. Results suggest CHX17 affects the pH homeostasis of endosomes thus affecting membrane trafficking and protein/cargo sorting. This

idea is further supported by the ability of CHX17 to confer tolerance to hygromycin B in mutant yeast. Aminoglucosides enter cells by endocytosis, and resistance is thought to be mediated by its sorting to vacuoles for degradation.

c) ***Role in male fertility.*** Many CHX genes are expressed in pollen though their functions are not known. We showed that pollen carrying insertional mutations in both *CHX21* and *CHX23* failed to target the ovules leading to impaired male fertility. As CHX23 is expressed in endomembranes, resembling the ER in pollen tubes, we suggest that cation and pH homeostasis is critical to either perception, response or both to guidance cues from the ovule (Lu et al. 2011).

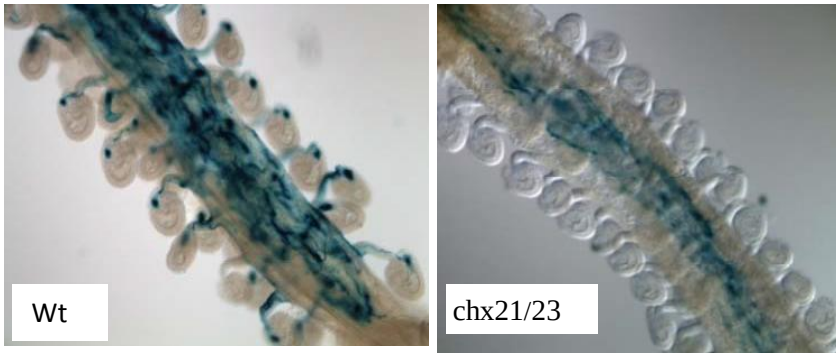


Fig. 1. ***chx21chx23* double mutant pollen failed to reach the ovule *in vivo***. Wild-type pistils were hand pollinated with pollen expressing GUS genes.
(Left) Tubes showing GUS discharge by $Wt_{GUS}$ in ovules
(Right) chx21/23 mutant tube grows but do not turn or discharge GUS

Objectives

-We are using homology modeling to identify critical residues in CHX17.
-We want to determine the mode of transport and test the hypothesis that CHX can act as a cation/$H^+$ antiporter and as a cation channel.
-We want to test whether CHXs are involved in the synthesis and sorting of cell wall materials.

References to work supported by DOE

Aranda-Sicilia MN, Cagnac O, Chanroj S, Sze H, Rodríguez-Rosales MP, Venema K. (2012). Arabidopsis KEA2 a homolog of bacterial KefC encodes a K+/H+ antiporter with a chloroplast transit peptide. **Biochim Biophys Acta** – Biomembranes 1818(9):2362-71

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Padmanaban S, Chanroj S, Kwak J, Li X, Ward JM, Sze H. (2007) Participation of an endomembrane cation/$H^+$ exchanger AtCHX20 in osmoregulation of guard cells. **Plant Physiol**. 144(1): 82-93

# *Session VIII*

## Quantitative Analysis of Central Metabolism and Seed Storage Synthesis

Jorg Schwender, Principal Investigator
Alistair Rogers, Co-PI
Jordan Hay, Postdoctoral Research Associate
Biology Department, Bldg. 463, Brookhaven National Laboratory, Upton, NY 11973
Email: schwend@bnl.gov; Web: http://www.bnl.gov/biology/People/Schwender.asp

Collaborators: Hardy Rolletschek, Ljudmilla Borisjuk, Department of Molecular Genetics, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany

Overall research goals: Plant biomass is of increasing importance as renewable resources for the production of fuels and of chemical feedstocks that replace petroleum based materials. Our goal is to increase the basic understanding of the functioning of storage metabolism in plants as a basis for rational engineering of seeds and other storage organs. To do this we combine experimental and computational approaches. By using methods of $^{13}$C-Metabolic Flux Analysis ($^{13}$C-MFA), Flux Balance Analysis, enzyme kinetic modeling as well as data from enzyme profiling and metabolite profiling we analyze cultured developing embryos of oilseeds from crucifer species like *Brassica napus* (oilseed rape), *Arabidopsis thaliana* or *Thlaspi arvense*, a potential non-food bioenergy crop (www.pennycressbiodiesel.com/). Different genotypes or different light and nutritional conditions can be compared using the embryo cultures and modeling approaches, revealing emerging properties of the central metabolism network. Together, this will increase understanding of the biochemical processes involved in partitioning carbon and nitrogen into seed storage compounds.

Significant achievements 2010-2012: (1) *In-silico* modeling of *in-planta* seed development: Based on *in-planta* physiological measurements in developing *Brassica napus* seeds we used our large scale stoichiometric model of developing seeds to infer metabolic flux in three parts of the embryo that differ in the amount of light they receive during seed development. Accordingly light substantially contributes via photosynthetic electron transport to storage biosynthesis in the outer layers (outer cotyledon), while in the inner more shaded layers of the seed photosynthetic processes have no significant contribution. (2) *In-silico* analysis of change in seed biomass composition: Using our large-scale stoichiometric model of developing seeds we simulated a trade-off between the protein and oil fractions of seed biomass. Resulting flux sensitivities identify the most relevant targets for engineering of seed composition. For example, mitochondrial respiration and ATP production were predicted to be most relevant for an increase in protein content. (3) $^{13}$C-Metabolic Flux Analysis was performed for cultured embryos of 11 *B. napus* genotypes that differ in seed composition and oil content. Flux values can be correlated with oil content. For example, flux through plastidic pyruvate kinase is highly correlated to oil content, confirming the supposed important function of the enzyme in lipid synthesis. (4) Overexpression of proteins that might increase seed oil content: We genetically transformed *Thlaspi arvense* with full-length cDNAs of the seed specific transcription factor WRINKLED1 (At3G54320) and of the plastid localized malic enzyme isoform 4 (AtME4, At1G79750), both cloned from *Arabidopsis thaliana*.

Science objectives for 2012-2013:

- *Thlaspi arvense* metabolic model: Based on transcript profiling (RNASeq) the expression of genes in central metabolism of *Thlaspi arvense* developing embryos will be analysed in order derive a metabolic model. The *B. napus* seed model will serve as a template.
- Effect of transgenic alteration in central carbon metabolism on oil accumulation in seeds: Analysis of transgenics of *Thlaspi arvense* (WRINKLED1, AtME4) with $^{13}$C-metabolic flux analysis and enzyme profiling of cultured embryos. Similar analysis for transgenics of *B. napus* (generated by an industrial collaborator) which are altered in central carbon metabolism.

References to work supported by this project 2010-2012:


1. J. Hay, J. Schwender, "Computational analysis of storage synthesis in developing *Brassica napus* L. (oilseed rape) embryos: flux variability analysis in relation to $^{13}C$ metabolic flux analysis." The Plant Journal **67**: 513–525 (2011). (See cover image Volume 67, Issue 5)
2. J. Hay, J. Schwender, "Metabolic network reconstruction and flux variability analysis of storage synthesis in developing oilseed rape (*Brassica* napus L.) embryos." The Plant Journal **67**:526–541 (2011).
3. J. O'Gready, J. Schwender, Y. Shachar-Hill, J.A. Morgan, "Metabolic cartography: experimental quantification of metabolic fluxes from isotopic labelling studies." J. Exp. Botany **63**: 2293-2308 (2012).
4. J. Schwender (2011) Experimental flux measurements on a network scale. Front. Plant Sci. 2:63. doi: 10.3389/fpls.2011.00063 (2011).
5. D. Koschutzki, B. H. Junker, J. Schwender, F. Schreiber, "Structural analysis of metabolic networks based on flux centrality." Journal of Theoretical Biology **265**: 261-269 (2010).

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

B. Markus Lange, Principal Investigator
Glenn W. Turner & Narayanan Srividya, Postdoctoral Associates, Siau Sie Voo, Graduate Student
Washington State University, Institute of Biological Chemistry, Pullman, WA 99164-6340
Email: lange-m@wsu.edu; Web: http://public.wsu.edu/~lange-m/index.html

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.

Significant achievements 2010-2012:

- 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.

  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).

  **Lange B.M.,** Rios-Estepa R. (2012) Kinetic modeling of plant metabolism and its predictive power – peppermint essential oil biosynthesis as an example. *Methods Mol. Biol.*, in press.
- 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.

  **Lange B.M.**, Mahmoud S.S., Wildung M.R., Turner G.W., Davis E.M., Lange I., Baker R.C., Boydston R.A., Croteau R.B. (2011) Improving peppermint essential oil yield and composition by metabolic engineering. *Proc. Natl. Acad. Sci. USA* 108, 16944-16949.
- We determined the regulatory characteristics of the monoterpene biosynthetic pathway at the transcriptional, posttranslational and metabolite accumulation level and were able to correlate these data sets with microscopic measurements of oil cavity volumes. As part of this effort we obtained cell type-specific transcriptome data from isolated *Citrus* peel epithelial cells lining secretory cavities and evaluated the biosynthetic capabilities of these specialized cells.

  Voo S.S., Turner G.W., Grimes. H.D., **Lange B.M.** (2012) Assessing the biosynthetic capabilities of secretory glands in *Citrus* peel. *Plant Physiol.* 159, 81-94.

Science objectives for 2012-2013:

- Perform structure-function analyses with (4S)-limonene synthase mutants to determine structural constraints for generating terpenoid hydrocarbon diversity in oils and resins (Fig. 1).

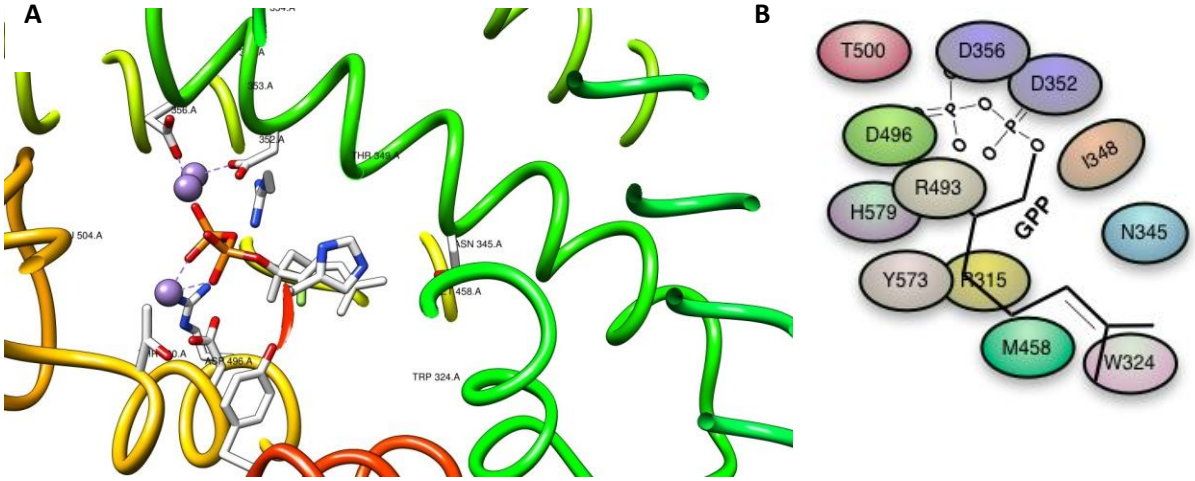


**Fig. 1.** Active site rendition of (4*S*)-limonene synthase. **A**, folding of the substrate analogue, 2-fluorogeranyl diphosphate, with divalent metal ions shown as purple spheres, based on the published crystal structure data; **B**, simplified scheme indicating residues positioned at the protein-substrate interface (the natural substrate, geranyl diphosphate (GPP), is depicted).

- Using microscopic images and 3-dimensional reconstruction models, we determined the distribution and storage capacity of the resin duct system of entire loblolly pines. The most important conclusion from these studies is that needles and small branches, in the forest management industry often termed slash and burned with only moderate benefit for the soil, are an underestimates source of resins that can be used to turn logging 'waste' into energy. We are now obtaining transcriptome data from epithelial cells surrounding emerging pine resin ducts to evaluate the regulation of duct formation.

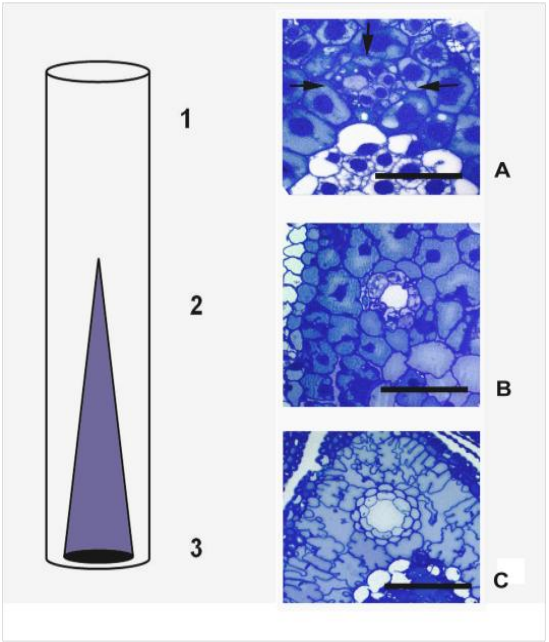


**Fig. 5.** Resin duct developmental stages in loblolly pine needles. The diagram on the left visualizes a developing resin duct, with developmental stages divided into three phases: (**1**) new resin duct initial cells are formed by mitosis in the intercalary meristem; (**2**) initiation of the secretory phase with the opening of the schizogenous resin storage space; and (**3**) maturation of the resin duct in the older portions of the needle. The microscopic images on the right show transverse sections of a developing needle resin duct from loblolly pine: **A**, resin duct initials (phase 1) that were recently derived through asymmetrical divisions of cells in the intercalary meristem (bar = 50 µm); **B**, secretory phase resin duct (phase 2) with actively secreting cells surrounding a recently opened portion of the resin storage space (bar = 60 µm); **C**, mature resin duct (phase 3) at the lateral side of a mature pine needle (bar = 130 µm).

- We have made significant progress toward localizing biosynthetic enzymes (in particular (+)-limonene synthase) for essential oil biosynthesis in *Citrus* peel to leucoplasts of epithelial cells lining secretory cavities. As part of these studies we have now made several unexpected observations. We demonstrated that, in contrast to other essential oil-synthesizing cell types such as peppermint secretory cells of glandular trichomes, the gland cells of *Citrus* secretory cavities form plastoglobules that, based on preliminary evidence, appear to contain terpenoids. The secretion of essential oil components in *Citrus* appears to proceed via an as yet unknown, novel mechanism, which we are now investigating experimentally.

**Regulation of Carbon Allocation to Phenylpropanoid Metabolism: The Role of Components of the Mediator Complex**

Clint Chapple, Principal Investigator
Nicholas D. Bonawitz, Postdoctoral Research Associate
Department of Biochemistry, Purdue University, 175 South University Street, West Lafayette IN 47907
Email: chapple@purdue.edu; Web: https://ag.purdue.edu/biochem/Pages/ProfilePages/chapple.aspx

Overall research goals: We have identified two previously uncharacterized components of the Mediator complex, REF4 and RFR1, that negatively influences the accumulation of phenylpropanoid metabolites by participating in transcriptional co-activation and/or co-suppression. To gain more insight into the biological roles of REF4 and RFR1 and the influence of phenylpropanoid metabolism on plant growth and morphology, we will (1) determine the components found in REF4- and RFR1-containing Mediator complexes, (2) identify the specific regions in the genome to which REF4- and RFR1-containing Mediator complexes are targeted, and (3) determine whether there are functional differences between REF4 and RFR1. By completing this set of experiments, we will learn how REF4 and RFR1 function to coordinate transcription of genes required for lignin deposition and gain insights into how this pathway can be manipulated for human energy needs.

Significant achievements 2011-2012: Lignin is a phenylpropanoid-derived heteropolymer found in plant secondary cell walls that is required for the strength and rigidity of water-conducting and supportive tissues. In most angiosperms, lignin is synthesized mainly from coniferyl alcohol and sinapyl alcohol, which upon polymerization give rise to G and S units in lignin, as well as trace amounts of *p*-coumaryl alcohol-derived H lignin. The presence of lignin in bulk plant biomass impedes fermentable sugar extraction, and thus genetic manipulation of lignin biosynthesis has been proposed as a strategy to improve bioenergy crops. However, many such manipulations result in stunted growth and developmental abnormalities via a currently unknown mechanism. For instance, disruption of the gene encoding the phenylpropanoid biosynthetic enzyme *p*-coumaroyl shikimate 3′-hydroxylase (C3′H), which is required for the production of G and S (but not H) lignin, results in dwarfing, sterility, and hyperaccumulation of flavonoids, leading to the suggestion that H lignin may not be sufficient for normal growth and development.


Figure 1. Upper Panel: Elimination of REF4 and RFR1 restore the growth defects of the C3'H-deficient *ref8* mutant. Lower Panel: Despite their rescued growth, ref4 rfr1 ref8 triple mutants are still defective in sinapoylmalate biosynthesis as evidenced by their *ref* phenotype when observed under ultraviolet light.

We have previously shown that REF4 and RFR1 are required for phenylpropanoid homeostasis in Arabidopsis and may play a role in the repression of lignin biosynthesis. Remarkably, disruption of REF4 and RFR1 in the C3′H-deficient Arabidopsis mutant *ref8* rescues its growth and fertility to near wild-type levels without restoring C3′H function (Fig. 1). In contrast to the above suggestion, *ref4 rfr1 ref8* mutant plants do not show rescued levels of G and S lignin, and instead contain lignin that is composed almost entirely of H subunits. These results show that H lignin is sufficient for relatively normal growth and development in Arabidopsis, implicate Mediator as being directly involved in the dwarfism of plants with disrupted lignin biosynthesis, and suggest that manipulation of H lignin levels may be a promising strategy for improving biofuels feedstocks.

Science objectives for 2012-2013:

- To determine with which known subunits of plant Mediator REF4 and RFR1 interact, we will carry out a directed yeast two hybrid screen using the Invitrogen ProQuest two-hybrid system.
- To characterize the components of the Mediator complex more broadly, we will first generate multiple expression constructs for both REF4 and RFR1, in which the expressed proteins are fused to the epitope tags HA or FLAG, which have previously been shown to be suitable for use in Arabidopsis. These expression constructs will then be transformed into *ref4 rfr1* null plants, where their functionality can be verified based on their ability to rescue the high phenylpropanoid phenotype of these plants. We will then verify that these tagged proteins are incorporated into Mediator by incubating leaf whole cell extract with the appropriate epitope-directed antibody and protein A sepharose beads, precipitating these beads by centrifugation, and using mass spectrometry to identify co-immunoprecipitating proteins. Parallel approaches will employ biochemical purification of Mediator complexes from plants and immunoprecipitation.

References to work supported by this project 2011-2012:

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# *Author Index*

# Author Index

# *List of Participants*

# Participants


Michael Adams
University of Georgia
Department of Biochemistry
Athens, GA 30602
706-542-2060
adams@bmb.uga.edu

Dawn M. Adin
AAAS Science and Technology Policy Fellow
U. S. Department of Energy
Germantown, MD 20874
301-903-0570
dawn.adin@science.doe.gov

Charles Anderson
Penn State University
201 Life Sciences Building
University Park, PA 16802
814-863-4736
cta3@psu.edu

David Baker
University of Washington/HHMI
15th and Pacific
Seattle, WA 98112
206-543-1295
dabaker@uw.edu

Tobias Baskin
University of Massachusetts-Amherst
Biology Dept., 611 N. Pleasant St
Amherst, MA 01003
413-545-1533
baskin@bio.umass.edu

Elison Blancaflor
The Samuel Roberts Noble Foundation
2510 Sam Noble Parkway
Ardmore, OK 73401
580-224-6687
eblancaflor@noble.org

Jim Brainard
National Renewable Energy Laboratory
15013 Denver West Parkway
Golden, CO 80401-3305
303-384-6462
James.Brainard@nrel.gov

David Britt
University of California-Davis
Dept of Chemistry
Davis, CA 95616
530-752-6377
rdbritt@ucdavis.edu

Joan Broderick
Montana State University
Dept of Chemistry & Biochemistry, 103 CBB
Bozeman, MT 59717
406-994-6160
jbroderick@chemistry.montana.edu

John Browse
Institute of Biological Chemistry
Washington State University
Pullman, WA 99164-6340
509-335-2293
jab@wsu.edu

Clint Chapple
Purdue University
15 South University Street
West Lafayette, IN 47907
765-494-1607
chapple@purdue.edu

George Chuck
University of California-Berkeley
800 Buchanan St.
Albany, CA 94710
510-559-5922
georgechuck@berkeley.edu

Gloria Coruzzi
New York University
12 Waverly Place
New York, NY 10003
212-998-3963
gc2@nyu.edu

Daniel Cosgrove
Pennsylvania State University
208 Mueller Lab
University Park, PA 16802
814-863-3892
fsl@psu.edu

Alan Darvill
Complex Carbohydrate Research Center
315 Riverbend Road
Athens, GA 30602
706-542-4411
adarvill@ccrc.uga.edu

Sergei Dikanov
University of Illinois
190 MSB, 506 S. Mathews Av.
Urbana, IL 61801
217-300-2209
dikanov@illinois.edu

James Evans
Pacific Northwest National Laboratory
3335 Q. Avenue
Richland, WA 99354
509-371-6709
james.evans@pnnl.gov

James Ferry
Pennsylvania State University
205 South Frear Laboratory
University Park, PA 16801
814-863-5721
jgf3@psu.edu

Dax Fu
Brookhaven National Laboratory
50 Bell Avwe
Upton, NY 11973
631-344-4208
dax@bnl.gov

Bruce Garrett
Pacific Northwest National Laboratory
902 Battelle Boulevard (K9-90)
Richland, WA 99354
509-372-6344
bruce.garrett@pnnl.gov

Jane Glazebrook
University of Minnesota
1445 Gortner Ave
Saint Paul, MN 55108
612-624-5194
jglazebr@umn.edu

Ying Gu
Pennsylvania State University
262 North Frear Lab
University Park, PA 16802
814-867-3827
yug13@psu.edu

Caroline Harwood
University of Washington
1705 NE Pacific Street, Box 357242
Seattle, WA 98195-7242
319-594-8122
csh5@uw.edu

Michael Himmel
National Renewable Energy Laboratory
15013 Denver West Parkway
Golden, CO 80401
303-384-7756
mike.himmel@nrel.gov

Gregg Howe
Michigan State University
Michigan State University
East Lansing, MI 48824
517-355-5159
howeg@msu.edu

Cynthia Jenks
Ames Laboratory
311 TASF
Ames, IA 50011
515-292-8486
cjenks@ameslab.gov

Alan Jones
University of North Carolina
CB#3280 Coker Hall
Chapel Hill, NC 27599
919-962-6932
alan_jones@unc.edu

Michael Kahn
Washington State University
203 Clark Hall, Washington State University
Pullman, WA 99164-6340
509-335-4563
kahn@wsu.edu

Paul King
National Renewable Energy Laboratory
16253 Denver West Parkway
Golden, CO 80401
303-384-6277
paul.king@nrel.gov

David Knaff
Texas Tech University
Dept. of Chemistry and Biochemistry
Lubbock, TX 79409-1061
806-742-0288
david.knaff@ttu.edu

David Kramer
Michigan State University
Michigan State University
EAST LANSING, MI 48824-1312
517-432-0072
kramerd8@msu.edu

Lee Krumholz
University of Oklahoma
Dept. of Microbiology and Plant Biology
Norman, OK 73019
405-325-0437
krumholz@ou.edu

Joseph Krzycki
Ohio State University
484 West 12th
Columbus, OH 43210
614-292-1578
Krzycki.1@osu.edu

Paul Langan
Oak Ridge National Laboratory
Center for Structural Molecular Biology
Oak Ridge, TN 37831
505-412-0476
langanpa@ornl.gov

Bernd Markus Lange
Washington State University
PO Box 646340
Pullman, WA 99164-6340
509-335-3794
lange-m@wsu.edu

Young-Jin Lee
Ames Laboratory
3756 Gilman
Ames, IA 50011
515-294-1235
yjlee@iastate.edu

John Leigh
University of Washington
University of Washington, Box 357735
Seattle, WA 98195-7735
206-685-1390
leighj@uw.edu

Norman Lewis
Washington State University
PO Box 646340
Pullman, WA 99164-6340
509-335-2682
lewisn@wsu.edu

Chang-Jun Liu
Brookhaven National Laboratory
50 Bell Avenue
Upton, NY 11973
631-344-2966
cliu@bnl.gov

David Lynn
Emory University
1521 Emerson Hall
Atlanta, GA 30322
404-727-9348
dlynn2@emory.edu

Diane Marceau
U. S. Department of Energy
19901 Germantown Road
Germantown, MD 20874
301-903-0235
diane.marceau@science.doe.gov

Julie Maupin-Furlow
University of Florida
PO Box 110700, Dept. Microbiology & Cell Science
Gainesville, FL 32611-0700
352-392-4095
jmaupin@ufl.edu

Maureen McCann
Purdue University
Purdue University
West Lafayette, IN 47905
765-494-1610
mmccann@purdue.edu

Michael McInerney
University of Oklahoma
Dept Micro & Plant Biology, 770 Van Vleet Oval
Norman, OK 73019
405-325-6050
mcinerney@ou.edu

Gail McLean
U. S. Department of Energy
1000 Independence Ave SW, Germantown
Washington, DC 20585-1290
301-903-7807
Gail.McLean@science.doe.gov

William Metcalf
University of Illinois
B103 C&LSL, 601 S. Goodwin
Urbana, IL 61801
217-244-1943
metcalf@illinois.edu

William Moerner
Stanford University
MC 5080, Dept. Chemistry
Stanford, CA 94305
650-723-1727
wmoerner@stanford.edu

Andrew Mort
Oklahoma State University
246 NRC, Department of Biochemistry and Molecular Biology
Stillwater, Oklahoma 74078
405-744-6197
andrew.mort@okstate.edu

Erik Nielsen
University of Michigan
830 N. University Ave.
Ann Arbor, MI 48109
734-764-1324
nielsene@umich.edu

Basil Nikolau
Iowa State University
3254 Molecular Biology Building
Ames, IA 50011
515-290-3382
dimmas@iastate.edu

Jennifer Ogilvie
University of Michigan
450 Church St
Ann Arbor, MI 48109
734-615-0485
jogilvie@umich.edu

John Peters
Montana State University
215 Chemistry & Biochemistry Building
Bozeman, MT 59717
406-994-7212
john.peters@chemistry.montana.edu

Stephen Ragsdale
University of Michigan
1150 West Medical Center Drive
Ann Arbor, MI 48103
734-660-4067
sragsdal@umich.edu

Natasha Raikhel
University of California-Riverside
900 University Ave
Riverside, CA 92521
951-201-9180
nraikhel@ucr.edu

Wolf-Dieter Reiter
University of Connecticut
75 North Eagleville Road
Storrs, CT 06269-3125
860-486-5733
wdreiter@uconn.edu

Mary Roberts
Boston College
Merkert Chemistry Center
Chestnut Hill, MA 02467
617-552-3616
mary.roberts@bc.edu

David Savage
University of California-Berkeley
2151 Berkeley Way
Berkeley, CA 94720-5230
510-643-7847
dsavage@berkeley.edu

Richard Sayre
Los Alamos National Laboratory
4200 W Jemez Rd
Los Alamos, NM 87544
505-412-6532
rsayre@newmexicoconsortium.org

Julian Schroeder
University of California-San Diego
Division of Biological Sciences - 0116
La Jolla, CA 92093-0116
858-534-7759
jischroeder@ucsd.edu

Jorg Schwender
Brookhaven National Laboratory
Biology Department
Upton, NY 11973
631-344-3797
schwend@bnl.gov

John Shanklin
Brookhaven National Laboratory
Biology Dept., 50 Bell Ave
Upton, NY 11973
631-344-3414
shanklin@bnl.gov

Michael Sussman
University of Wisconsin-Madison
8761 Hidden Valley Road
Cross Plains, WI 53528
608-798-3174
msussman@wisc.edu

Alexej Smirnov
North Carolina State University
2620 Yarbrough Drive, Campus Box 8204
RALEIGH, NC 27695-8204
919-513-4377
Alex_Smirnov@ncsu.edu

Heven Sze
University of Maryland
Dept Cell Biol Mol Genetics, Biosci Res Bldg
College Park, MD 20705
301-405-1645
hsze@umd.edu

Dieter Soll
Yale University
Dept of Molec Biophys & Biochem
New Haven, Connecticut 06520-8114
203-432-6200
dieter.soll@yale.edu

Michael Thomashow
Michigan State University
MSU-DOE Plant Research Lab
East Lansing, MI 48824
517-355-2299
thomash6@msu.edu

Robert Stack
U. S. Department of Energy
19901 Germantown Rd
Germantown, MD 20874
301-903-5652
robert.stack@science.doe.gov

Richard Vierstra
University of Wisconsin-Madison
425-G Henry Mall
Madison, WI 53706
608-262-8215
vierstra@wisc.edu

Chris Staiger
Purdue University
Dept. of Bio Sciences, 201 S. University St.
W. Lafayette, IN 47907-2064
765-496-1769
staiger@purdue.edu

Zhiyong Wang
Carnegie Institution for Science
260 Panama Street
Stanford, CA 94305
650-387-3800
zywang24@stanford.edu

Sowmya Subramanian
Los Alamos National Laboratory
4200 W Jemez Road
Los Alamos, NM 87544
505-412-6525
subraman@newmexicoconsortium.org

John Ward
University of Minnesota
250 Biological Sciences Center
St. Paul, MN 55108
612-625-4763
jward@umn.edu

Creighton Wirick
Brookhaven National Laboratory
Building 463
Upton, NY 11973
631-344-3063
wirick@bnl.gov

Changcheng Xu
Brookhaven national Laboratory
50 Bell Ave
Upton, NY 11973
631-344-2183
cxu@bnl.gov

Zheng-Hua Ye
University of Georgia
2502 Miller Plant Sciences Bldg.
Athens, GA 30602
706-542-1832
zhye@plantbio.uga.edu