

# 2010 Physical Biosciences Research Meeting



**Sheraton Inner Harbor Hotel  
Baltimore, MD  
October 17-20, 2010**



U.S. DEPARTMENT OF  
**ENERGY**

Office of  
Science

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



# 2010 Physical Biosciences Research Meeting

## Program and Abstracts

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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 2<sup>nd</sup> 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). Within DOE-BES there are two programs that fund basic research in energy-relevant biological sciences, Physical Biosciences and Photosynthetic Systems. These two Biosciences programs, along with a strong program in Solar Photochemistry, comprise the current Photo- and Bio-Chemistry Team.

This meeting specifically brings together under one roof all of the PIs funded by the Physical Biosciences program, along with Program Managers and staff not only from DOE-BES, but also other offices within DOE, the national labs, and even other federal funding agencies. Of course we also have some distinguished speakers who we hope will stimulate your thinking, and inform you about new tools and resources that will allow you to meet - or exceed - your current research objectives; more on that below.

Our objective in holding these research meetings is to provide an environment that (1) encourages free exchange of information on your DOE-funded work; (2) facilitates new collaborations between individual research groups with complementary strengths; (3) allows opportunities for discussions with DOE Program Managers and staff; (4) exposes you to new ideas and methodologies; and (5) supplies information on DOE User Facilities, and how one goes about gaining access to them.

In that regard, this year's agenda features several invited speakers from the DOE national labs. We are delighted to feature Allen Orville from Brookhaven National Laboratory as one of our featured "idea-generators" on the physical science side, and Bryan Linggi and John Cort from the Pacific Northwest National Laboratory (PNNL) who will tell you about the exciting "-omics" capabilities at PNNL's Environmental Molecular Sciences Lab (EMSL) – and how one goes about accessing them. Ian Carmichael from the Notre Dame Radiation Laboratory has also been invited to provide a brief talk on how he and his colleagues are trying to address the serious problem of radiation damage to protein structures that is caused by x-ray beam lines, and why it is a critically important problem to address.

While we extend a warm welcome and our sincere appreciation to our invited speakers, the real star at this year's meeting is...**you**. It is your hard work, creativity, productivity, and commitment to world-class science that comes across in your submitted abstracts. Whether you are delivering a talk or presenting a poster, we are sincerely appreciative of your contribution to this meeting. The depth and breadth of the DOE-BES Biosciences portfolio is what makes a meeting like this not only exciting, but also – we hope – a very fun and rewarding one to attend.

It has been an extraordinary period of time in DOE's Office of Science, and we want to thank you for your many contributions to the successful execution of the many calls we have put out over the last two years. Finally, we also wish to thank Diane Marceau from DOE-BES and Connie Lansdon from Oak Ridge Institute for Science and Education (ORISE) for their invaluable help in planning and successfully executing the many logistical tasks associated with putting on this meeting.

**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**  
2010 Physical Biosciences Research Meeting  
Sheraton Inner Harbor Hotel, Baltimore, MD  
October 17-20, 2010

**Sunday, October 17, 2010**

- 3:00 – 6:00 p.m. Registration  
5:30 – 6:30 Reception (No Host)  
6:30 – 7:30 Dinner at Sheraton Inner Harbor Hotel  
7:30 – 8:00 Welcome, Opening Remarks, and DOE Update/News  
**Robert Stack**, Program Manager, Physical Biosciences, DOE-BES  
**Eric Rohlfig**, Director, Chemical Sciences, Geosciences &  
Biosciences Division, DOE-BES

**Monday, October 18, 2010**

- 7:15 – 8:00 a.m. Continental Breakfast

*Session I: Physical Science Tools for Energy Transduction Studies*

- 8:00 – 8:30 a.m. Welcome and Physical Biosciences Program Update  
**Robert Stack**, Program Manager, Physical Biosciences  
8:30 – 9:30 More than Simply Atomic Structure: Correlated Single-Crystal  
Spectroscopy and X-ray Diffraction  
**Allen Orville**, Brookhaven National Laboratory

- 9:30 – 10:00 Break

*Session II: Hydrogen Metabolism and Electron Flux in Microbial Systems*      *Joe Krzycki, Moderator*

- 10:00 – 10:30 Enzymology of Methanogenesis: Mechanism of Methyl-Coenzyme M  
Reductase  
**Stephen Ragsdale**, University of Michigan  
10:30 – 11:00 Electron Bifurcation and Novel Pathways of Electron Flow from Formate in  
a Model Hydrogenotrophic Methanogen  
**John Leigh**, University of Washington  
11:00 – 11:30 Genetics and Molecular Biology of Hydrogen Metabolism in Sulfate-  
Reducing Bacteria  
**Judy Wall**, University of Missouri  
11:30 – 12:00 Genetic Analysis of Hydrogenotrophic Methanogenesis in *Methanosarcina*  
Species  
**William Metcalf**, University of Illinois  
12:00 – 1:00 Lunch

*Session III: Plant Growth and Regulation*

*Gloria Coruzzi, Moderator*

- 1:00 – 1:30 Regulation of Actin Filament Ends: The Role of Capping Protein in  
Stochastic Dynamics and Organelle Behavior  
**Christopher Staiger**, Purdue University

- 1:30 – 2:00 Cellulose Synthesis and the Control of Growth Anisotropy  
**Tobias Baskin**, University of Massachusetts
- 2:00 – 2:30 Exploring Molecular Mechanisms of Lignin Biosynthesis and Its Regulation  
**Chang-Jun Liu**, Brookhaven National Laboratory
- Session IV: Special Guest Lecture/Life Sciences Research Fellow* *Bob Stack, Moderator*
- 2:30 – 3:00 Spatial and Temporal Organization of Cyanobacterial Metabolism  
**David Savage**, Harvard Medical School
- 3:00 – 6:00 Free/Discussion Time (Put up your poster too!)
- 6:00 – 6:30 Reception (No-Host)
- 6:30 – 7:30 Dinner at Sheraton Inner Harbor Hotel
- Poster Session I*
- 7:30 – 9:30 Odd Numbered Posters (No-Host)

## Tuesday, October 19, 2010

- 7:15 – 8:00 a.m. Continental Breakfast
- Session V: 'Omics Tools for Energy Transduction Studies* *Bob Stack, Moderator*
- 8:00 – 9:00 EMSL: A National Scientific User Facility for State-of-the-Art Molecular and Environmental Research  
**Bryan Linggi & John Cort**, EMSL, Pacific Northwest National Laboratory
- 9:00 – 9:30 Break
- Session VI: Signal Transduction in Plants* *Elizabeth Vierling, Moderator*
- 9:30 – 10:00 A Proteomic Study of Brassinosteroid Responses in Plants  
**Zhiyong Wang**, Carnegie Institute
- 10:00 – 10:30 The Role of Auxin in Ambient Temperature Growth Regulation  
**Mark Estelle**, University of California, San Diego
- 10:30 – 11:00 Plant Response to LCO/CO Signals  
**Gary Stacey**, University of Missouri
- 11:00 – 11:30 The Crystal Structure of a Self-Activating G $\alpha$  Protein Reveals a New Mechanism of Signal Initiation  
**Alan Jones**, University of North Carolina
- Session VII: The Archaeal Proteasome* *Rick Vierstra, Moderator*
- 11:30 – 12:00 Proteasomes and Post-translational Modification of *Haloflexax volcanii* Proteins  
**Julie Maupin-Furlow**, University of Florida
- 12:00 – 1:00 Lunch

*Session VIII: Pumps, Transporters, and Trafficking in Plants* *Julian Schroeder, Moderator*

- 1:00 – 1:30 Molecular Mechanism and Biological Function of the Plasma Membrane Proton Pump (H<sup>+</sup>-ATPase) of *Arabidopsis thaliana*  
**Michael Sussman**, University of Wisconsin Biotechnology Center
- 1:30 – 2:00 FKBP-Mediated Maturation and Sterol Packing of the *Arabidopsis* ABCB19 Auxin Transporter are Distinct Processes  
**Angus Murphy**, Purdue University
- 2:00 – 2:30 Trafficking to the Plant Storage Vacuoles in Plants  
**Natasha Raikhel**, University of California, Riverside
- 2:30 – 3:00 Functional Analysis of Plant Sucrose Transporters  
**John Ward**, University of Minnesota
- 3:00 – 7:30 Free/Discussion Time and Dinner on Your Own

*Poster Session II*

- 7:30 – 9:30 Even Numbered Posters (No-Host)

## Wednesday, October 20, 2010

- 7:15 – 8:00 a.m. Continental Breakfast

*Session IX: Meeting the Challenges of X-ray Studies* *Bob Stack, Moderator*

- 8:00 – 8:30 Radiation Damage in Macromolecular Crystallography  
**Ian Carmichael**, Notre Dame Radiation Laboratory
- 8:30 – 9:00 Energetics and Structure of the ZIP Metal Transporter  
**Dax Fu**, Brookhaven National Laboratory
- 9:00 – 9:30 Break

*Session X: Bio-inspired Structural Design* *Jay Groves, Moderator*

- 9:30 – 10:00 Engineering Functional Scaffolds by Supramolecular Self-Assembly  
**David Lynn**, Emory University
- 10:00 – 10:30 Nanotube-Supported Phospholipid Bilayers: Self-Assembly and Nanoscale Confinement  
**Alex Smirnov**, North Carolina State University
- 10:30 – 11:00 Engineering Cells to Grow Electronic Connections to Materials  
**Caroline Ajo-Franklin**, The Molecular Foundry, Lawrence Berkeley National Laboratory

*Session XI: Photosynthetic Systems Program Guest Lecture* *Gail McLean, Moderator*

- 11:00 – 12:00 Photosynthetic Phenomics  
**David Kramer**, DOE-MSU Plant Research Laboratory
- 12:00 – 1:00 Lunch (**DON'T FORGET TO TAKE DOWN YOUR POSTER!**)

*Session XII: Discussion and Closing Comments* *Bob Stack, Moderator*

- 1:00 – 3:00 **Richard V. Greene**, Lead, Photo and Bio-Chemistry Team, DOE-BES



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*Session I*  
*Physical Science Tools for*  
*Energy Transduction Studies*



## **More than Simply Atomic Structure: Correlated Single-Crystal Spectroscopy and X-ray Diffraction**

Allen M. Orville, Principal Investigator

Deborah Stoner-Ma, Lonny Berman, Annie Héroux, Howard H. Robinson, Dieter K. Schneider,  
John M. Skinner, Alexei S. Soares, and Robert M. Sweet, Co-PIs

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Understanding the relationship between atomic and electronic structure is crucial for obtaining fundamental mechanistic insights. However, the more complex the reaction and/or catalyst are, the more difficult this task becomes. For example, consider that biological systems have evolved to the point that photons from the Sun drive the transformation of H<sub>2</sub>O and CO<sub>2</sub> (plus a few more elements) into ecosystems ranging from microbes to redwood forests to human civilization! Moreover, because of the remarkable progress within the MX field, the current frontier challenges for MX include structures of trapped reactive intermediates, large macromolecules and viruses, membrane proteins, protein-protein complexes, and protein-nucleic acid complexes.

Approximately one-third of all macromolecules expressed by all organisms contain an essential cofactor. This includes processes central to cellular energetics; nearly all biological macromolecules involved in these types of reactions contain essential cofactors or other modifications. Cofactors with color, such as metal ions and/or organic molecules, provide us with important spectroscopic access to reaction cycles. The spectroscopic signature(s) usually change as a function of catalysis. Roughly 11,000 entries in the current PDB archive contain colored cofactors. However, biological samples are altered by solvated, x-ray-derived photoelectrons. Consequently, unanticipated electron density or “observations” of color changes during x-ray diffraction studies are often reported, but rather poorly explained based upon the structure alone. Furthermore, only a few crystal structures with colored cofactors have been correlated with single-crystal spectroscopy to date. This raises questions about many important structures in the PDB. In contrast, single-crystal spectroscopy correlated with x-ray diffraction provides real data that is capable of removing the “mystery” from the interpretation of “mystery density”. Without question, the best correlations of atomic and electronic structure derive from results that are obtained from the same crystalline samples, which ultimately yield the most profound mechanistic insights.

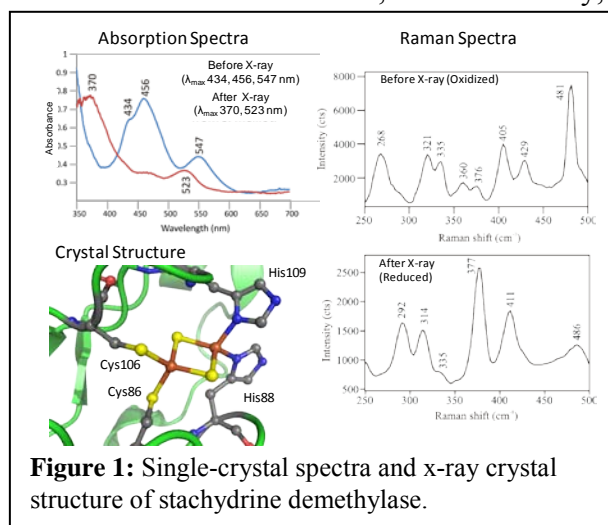
Therefore, our research philosophy is that correlated results yield more significant insights than results obtained by individual techniques. Consequently, a multi-disciplinary approach utilizing several complementary techniques to probe the structure and function of macromolecules is essential. To that end, beamline X26-C of the National Synchrotron Light Source (NSLS) has been developed recently into the only full-time facility in the United States that offers the user community the ability to collect correlated measurements of up to three types of complementary data from the same sample and under nearly identical experimental conditions: X-ray diffraction to high resolution, optical absorption spectroscopy, and Raman spectroscopy. The potential impact of this research strategy is enormous, ranging from processes that underlie human diseases, to those that will inspire and contribute to the development of new clean energy sources. Results of this nature will also provide unique insights into macromolecules relevant to metal transport and homeostasis, photosynthetic and light sensing systems, the fluorescent protein superfamily, nitric oxide synthesis and signaling,

cofactor redox-state and stress-response systems, and managing the toxicity of reactive oxygen and nitrogen species.

We routinely collect single-crystal electronic absorption spectra correlated with x-ray diffraction data at beamline X26-C. It is integrated into the beamline controls so that the spectroscopic data is obtained during the readout time of the X-ray detector from a  $\sim 25\mu\text{m}$  diameter region of the crystal that intersects the X-ray beam. We recently added Raman spectroscopy with two excitation  $\lambda$  (785 and 532nm), which provides a means to collect bond vibration-sensitive data from the same region of the crystal that intersects the X-ray beam and the electronic absorption optical path. An off-line laser spectroscopy laboratory immediately adjacent to the beamline is also under construction.

We have recently published several examples of correlated studies at beamline X26-C as applied to flavoproteins (1–3) and heme proteins (4). For example, choline oxidase from *A. globiformis* is a FAD-dependent enzyme that catalyzes the two-step, four-electron oxidation of choline (N,N,N-trimethylglycine, an osmoprotectant) to glycine betaine, with betaine aldehyde as a bound intermediate. In the two oxidative half-reactions, two molecules of  $\text{O}_2$  are converted into two  $\text{H}_2\text{O}_2$  molecules. We performed several x-ray-dependent spectroscopic measurements on a number of choline oxidase crystals. The results describe the first observations of a flavin C4a-OOH or C4a-OH enzyme reaction intermediate (1). Typically these reactive oxygen intermediates exhibit half-lives of only several milliseconds in solution, but remarkably, it is stable in the choline oxidase crystal at 100K.

Another example is illustrated in Figure 1 with the enzyme stachydrine demethylase from *S. meliloti*. Legumes produce a number of betaines that impact soil microorganisms, are important in the establishment of symbiosis, and necessary for normal nodulation. Stachydrine (N,N-dimethylproline or proline betaine) can be used by the microbe *S. meliloti* as an osmoprotectant or a source of carbon and nitrogen. The demethylase activity is catalyzed by an oxygenase complex similar to those used in aromatic hydrocarbon degradation in *Pseudomonas*. The active site of these  $\alpha_3\beta_3$  enzymes lays within each  $\alpha$  subunit and contains a Rieske [2Fe-2S] center and mononuclear iron atom. Our results show that the Rieske center in stachydrine demethylase is reduced at cryogenic temperatures during x-ray diffraction data collection. Moreover, the spectroscopy data shows that the His residues coordinate the  $\text{Fe}^{2+}$ .



Support: NIH National Center for Research Resources and the US Department of Energy, Office of Biological and Environmental Research

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*Session II*  
*Hydrogen Metabolism and*  
*Electron Flux in Microbial*  
*Systems*





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

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Overall research goals: Methanogens are masters of CO<sub>2</sub> reduction. They conserve energy by coupling the reduction of CO<sub>2</sub> to CH<sub>4</sub>, a clean fuel that is the primary constituent of natural gas, which accounts for 22 percent of the energy consumption of the U.S. The research objective is to determine the enzymatic mechanism of methyl-CoM reductase (MCR), the key enzyme in methane synthesis. MCR catalyzes the formation of methane and a heterodisulfide (CoB-S-S-CoM) from methyl-Coenzyme M (methyl-CoM) and Coenzyme B (HSCoB). Research over the past funding period supports a hybrid catalytic mechanism for MCR that involves both organometallic methyl-Ni(III) and methyl radical intermediates. These studies are expected to uncover unique biochemical and bioinorganic mechanisms and novel roles of metals (and their ligands) in biology that are important to scientists in the areas of bioinorganic chemistry, enzyme mechanisms and microbiology. Understanding the MCR mechanism offers insights that can be used to develop catalytic approaches and bioinspired catalysts that may approach the turnover number and efficiency of the enzymatic process.

Significant achievements 2008-2010: Over the funding period, we characterized organometallic and radical intermediates formed during the reaction of the active Ni(I)-MCR<sub>red1</sub> with substrate analogs (bromoethanesulfonate, BES; bromopropane sulfonate, BPS) and coenzyme B (HSCoB) (1-4) and with methyl-SCoM with an HSCoB analog (HSCoB<sub>6</sub>) (in preparation). Based on these studies, we proposed a hybrid mechanism (Fig. 1) that involves both organometallic and radical intermediates (4). We have determined the X-ray crystal structure of the methyl-Ni species formed by the reaction by reacting MCR with methyl iodide and high resolution structures of the Ni center in the alkyl-Ni and Ni(I) states by XAS (5) and spectroscopic (1) methods (Fig. 2). We also determined structures of the complexes of MCR with HSCoB and several analogs (6). We discovered that CO efficiently and rapidly activates MCR in vivo and propose a pathway for the cellular activation of MCR by H<sub>2</sub> or CO (in preparation). Finally, we have implemented a genetic system for methanogens, expressed the well-characterized MCR from *M. marburgensis* in *M. acetivorans*, and purified the active enzyme, opening the door for mutagenesis studies of MCR.

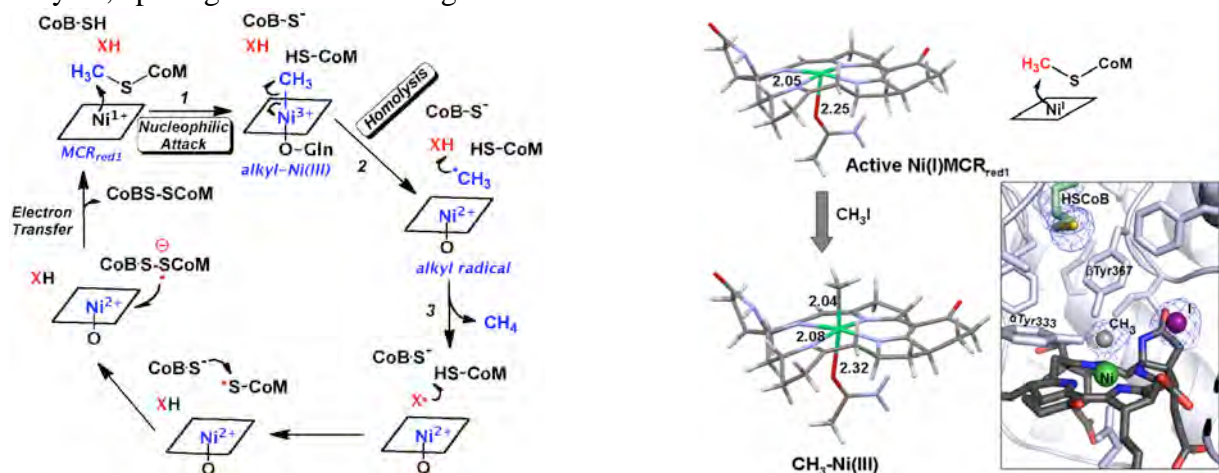


Figure 1. (left panel) Novel hybrid mechanism, modified from (4). Figure 2 (Right panel). Structures of the Ni(I) and CH<sub>3</sub>-Ni states of MCR as determined by XAS (5) and X-ray crystallography (inset on right) (in preparation).

### Science objectives for 2010-2011:

The research objectives are aimed at determining the enzymatic mechanism of methyl-CoM reductase (MCR), the key enzyme in methane synthesis. Recent studies suggest a hybrid catalytic mechanism for MCR that involves both organometallic methyl-Ni(III) and methyl radical intermediates. Objectives for 2010-2011 are to:

- test this mechanistic hypothesis by using substrate analogs and MCR variants to trap reaction intermediates in the catalytic cycle
- elucidate the crystal structure of the Ni(I) state of MCR and the structures of catalytic intermediates in the catalytic cycle
- determine how MCR is activated in vivo
- examine the role of a radical SAM (S-adenosyl-L-methionine) protein in activation and/or posttranslational modification of MCR.

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1. Dey, M., Kunz, R. C., Lyons, D. M., and Ragsdale, S. W. (2007) Characterization of Alkyl-Nickel Adducts Generated by Reaction of Methyl-Coenzyme M Reductase with Brominated Acids, *Biochem.* *46*, 11969-11978.
2. Dey, M., Telser, J., Kunz, R. C., Lees, N. S., Ragsdale, S. W., and Hoffman, B. M. (2007) Biochemical and Spectroscopic Studies of the Electronic Structure and Reactivity of a Methyl-Ni Species Formed on Methyl-Coenzyme M Reductase, *J. Am. Chem. Soc.* *129*, 11030-11032.
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4. Li, X., Telser, J., Hoffman, B. M., Gerfen, G., and Ragsdale, S. W. (2010) Observation of organometallic and radical intermediates formed during the reaction of methyl-coenzyme M reductase with bromoethanesulfonate, *Biochem.*, in press.
5. Sarangi, R., Dey, M., and Ragsdale, S. W. (2009) Geometric and Electronic Structures of the Ni(I) and Methyl-Ni(III) Intermediates of Methyl-Coenzyme M Reductase, *Biochem.* *48*, 3146-3156.
6. Cedervall, P. E., Dey, M., Pearson, A. R., Ragsdale, S. W., and Wilmot, C. M. (2010) Structural Insight into Methyl-Coenzyme M Reductase Chemistry using Coenzyme B Analogues, *Biochem. in press.*

## Electron Bifurcation and Novel Pathways of Electron Flow from Formate in a Model Hydrogenotrophic Methanogen

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Overall research goals: The goal of this project is to understand electron flow and energy conservation in hydrogenotrophic methanogens, an important group of organisms essential for anaerobic degradation and methane production. How these organisms achieve net energy conservation has been an unsolved problem for decades. In addition, the roles of multiple hydrogenases and other enzymes in electron flow from hydrogen and formate (two alternative electron donors) are poorly understood. We focus on *Methanococcus maripaludis* as a model species because of its superior laboratory growth properties and the availability of facile genetic tools.

Significant achievements 2008-2010: In hydrogenotrophic methanogens chemiosmotic energy is generated at a methyl transfer step (Mtr in Fig. 1) but is thought to be depleted at the initial step where CO<sub>2</sub> reduction to formylmethanofuran occurs. This leaves little net energy for ATP synthesis. The answer to this conundrum has long been thought to lie in the exergonic step where a heterodisulfide is reduced to two sulfhydryl coenzymes which provide electrons for the final methane production step. However, the mechanism has been elusive (the chemiosmotic electron transport chain of methylotrophic methanogens is absent from hydrogenotrophic methanogens). In 2008 Thauer et. al. (Nat Rev Microbiol 6:579-91) proposed a model involving electron bifurcation, in which electrons flowing through heterodisulfide reductase (Hdr, Fig. 1) go in two directions, exergonic heterodisulfide reduction driving endergonic reduction of the ferredoxin that in turn reduces CO<sub>2</sub> to formylmethanofuran. Since Hdr may be key to energy conservation, we sought to determine its interactions with other proteins. We his-tagged two different Hdr enzymes in *M. maripaludis*, grew cultures under hydrogen-excess and hydrogen-limiting conditions, prepared cell extracts, and carried out anaerobic Ni-affinity chromatography. Mass spectrometric analysis of the purified proteins relative to control samples consistently showed an association of Hdr with formylmethanofuran dehydrogenase (Fwd, Fig. 1), supporting the electron bifurcation model (1).

In addition, the complex included F<sub>420</sub>-nonreducing hydrogenase (Vhu) and formate dehydrogenase (Fdh), suggesting two independent inputs of electrons to Hdr, one from H<sub>2</sub> and one from formate. We additionally his-tagged Fdh and found that it also pulls down the entire four-enzyme complex. We further investigated electron flow from formate and found that it is entirely independent of H<sub>2</sub>. In a mutant deleting F<sub>420</sub>-nonreducing hydrogenase (Vhu), growth on H<sub>2</sub> was severely decreased but growth on formate remained normal. We generated a mutant lacking F<sub>420</sub>-nonreducing hydrogenase, F<sub>420</sub>-reducing hydrogenase (Fru), and the Hmd-Mtd cycle of F<sub>420</sub> reduction (Fig. 1) (2). The phenotype of the mutant suggests that methanogenesis from formate occurs without any generation of H<sub>2</sub> as an intermediate, supporting our earlier results showing that the rate of methanogenesis from formate exceeds the rate of H<sub>2</sub> production (3). Methanogenesis from formate without production of H<sub>2</sub> could prevent loss of reducing potential to competitors in the environment.

Science objectives for 2010-2011:

- We will demonstrate biochemically that electron bifurcation occurs. The purified complex should use either H<sub>2</sub> or formate and generate formylmethanofuran in a heterodisulfide dependent manner.
- How formate donates electrons to Hdr is unclear. We will test whether F<sub>420</sub> is needed using the purified complex. Also, it is unclear whether electrons from formate (via Fdh) and from H<sub>2</sub> (via Vhu) flow to the same site. Fdh is present only when the cells are grown under low H<sub>2</sub> or on

formate. We will measure stoichiometries of the components of the complex under different growth conditions and in different genetic backgrounds in an effort to determine whether Fdh displaces Vhu.

- Under high H<sub>2</sub> conditions, *M. maripaludis* and other hydrogenotrophic methanogens have a 3- to 4-fold decrease in growth yield (cell material synthesized per methane produced), i.e. growth becomes partially uncoupled from methanogenesis. It has been suggested that this occurs when the energy-conserving hydrogenase Eha produces excess reduced ferredoxin, depleting chemiosmotic membrane potential. One possible drain for the excess of reducing equivalents would be the production of CO via carbon monoxide dehydrogenase/acetyl CoA synthase (CODH/ACS). We will measure growth yields and CO production under high and low H<sub>2</sub> conditions in the wild type and in a mutant lacking CODH/ACS.

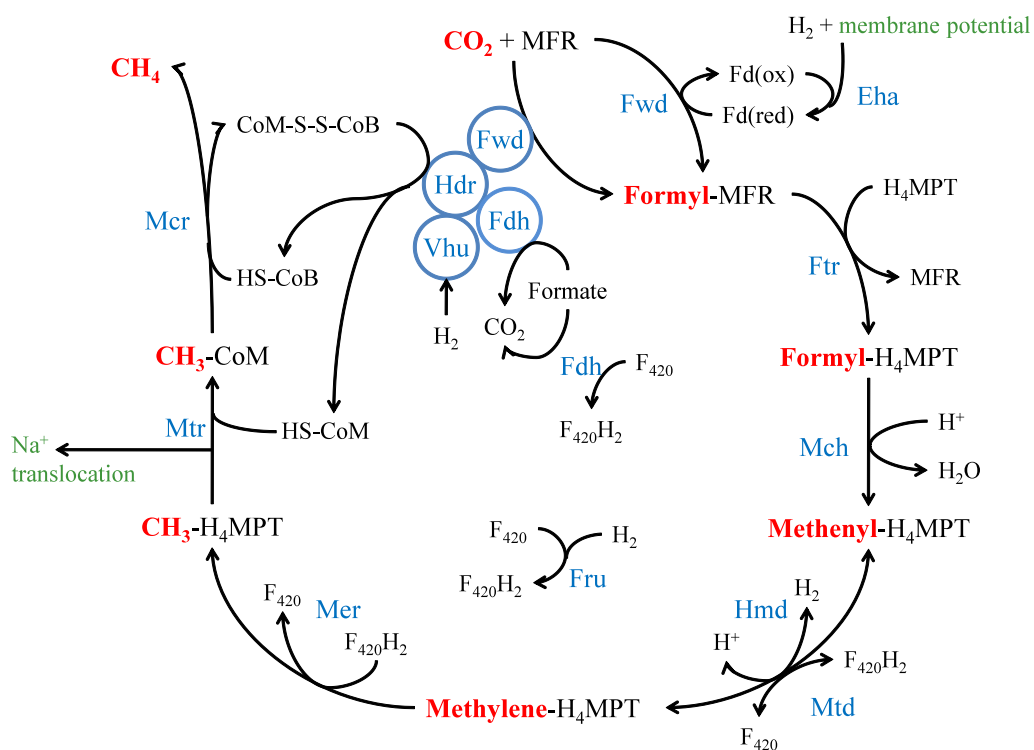


Figure 1. Methanogenic pathway showing the electron bifurcating Hdr complex and direct association of formate dehydrogenase. Drawn by Kyle Costa.

References to work supported by this project 2008-2010:

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2. **Hendrickson, E. L., and J. A. Leigh.** 2008. Roles of coenzyme F420-reducing hydrogenases and hydrogen- and F420-dependent methylenetetrahydromethanopterin dehydrogenases in reduction of F420 and production of hydrogen during methanogenesis. *J Bacteriol* **190**:4818-21.
3. **Lupa, B., E. L. Hendrickson, J. A. Leigh, and W. B. Whitman.** 2008. Formate-dependent H<sub>2</sub> production by the mesophilic methanogen *Methanococcus maripaludis*. *Appl Environ Microbiol* **74**:6584-90.



## Genetics and Molecular Biology of Hydrogen Metabolism in Sulfate-reducing Bacteria

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**Overall research goals:** The research objectives are to study the energy transduction systems of the anaerobic sulfate-reducing bacterium of the genus *Desulfovibrio*, strain G20, by: (1) improvement of the tools needed for facile creation of deletions and their analysis; (2) preparing knock out mutations of the various transmembrane complexes. These knock out mutants will reveal the physiological roles of the various complexes and provide insights into the energy transduction processes operative in *Desulfovibrio*.

### Significant achievements 2008-2010:

- 1) Studies with the *Desulfovibrio* G20 tetraheme cytochrome  $c_3$  (Tpl- $c_3$ ) mutant, I2, showed that growth of the mutant on pyruvate was limited to fermentation and that sulfate respiration was not supported with electrons from pyruvate (Giles et al. under revision).
- 2) The alternative respiration of fumarate by G20 is robust (Fig. 1) and analyses of excreted metabolites showed that some electrons are diverted to produce succinate even during sulfate reduction or pyruvate fermentation by the wild type G20 strain. I2 and a fumarate reductase transposon mutant are unable to respire fumarate.

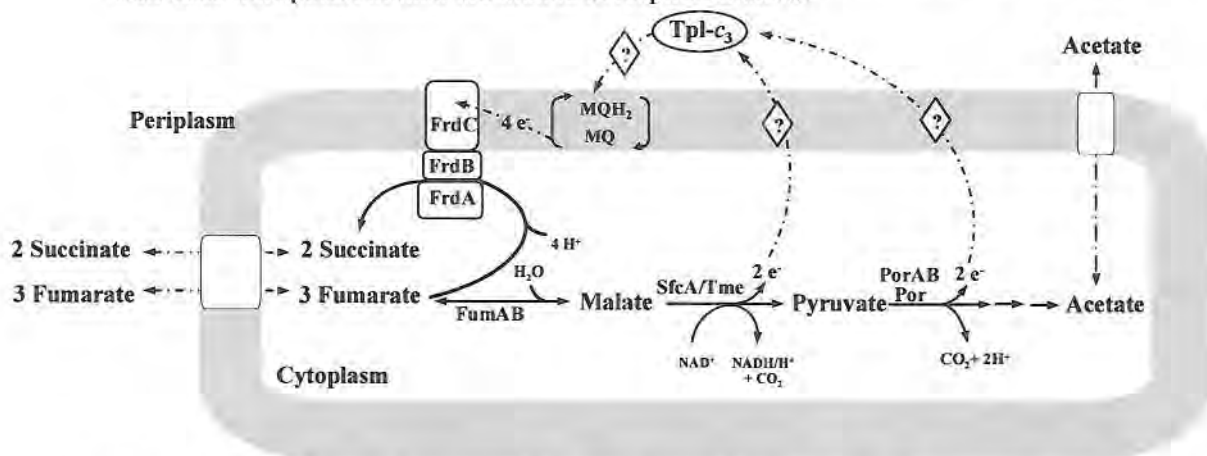


Figure 1. Model of electron flow during fumarate dismutation by *D. desulfuricans* G20. The electrons produced from fumarate oxidation are possibly delivered via Tpl- $c_3$  to the transmembrane fumarate reductase that carries out reduction of fumarate to succinate. Without Tpl- $c_3$ , enzymes for fumarate reduction were not detected in proteomics assays and growth on fumarate was not observed. End product analysis of G20 growth on fumarate reveals the predicted ratio of two succinates produced per acetate.

- 3) The Qmo complex was established to be the unique transmembrane pathway of electrons to adenyphosphosulfate reductase, the enzyme responsible for sulfate reduction to sulfite, in *Desulfovibrio vulgaris* Hildenborough (Zane et al., 2010). Because of the close synteny of the genes encoding the Qmo and Aps reductase in *Desulfovibrio* strains, we suggest that the Qmo complex functions in the same way in G20.
- 4) Microarray and proteomics (Fig. 2) analysis of G20 and I2 revealed the surprising result that genes for fumarate respiration and, therefore, the enzymes were significantly decreased in the I2 mutant regardless of the growth mode. This suggests a redox control on transcription of these genes affected by the absence of the periplasmic cytochrome.



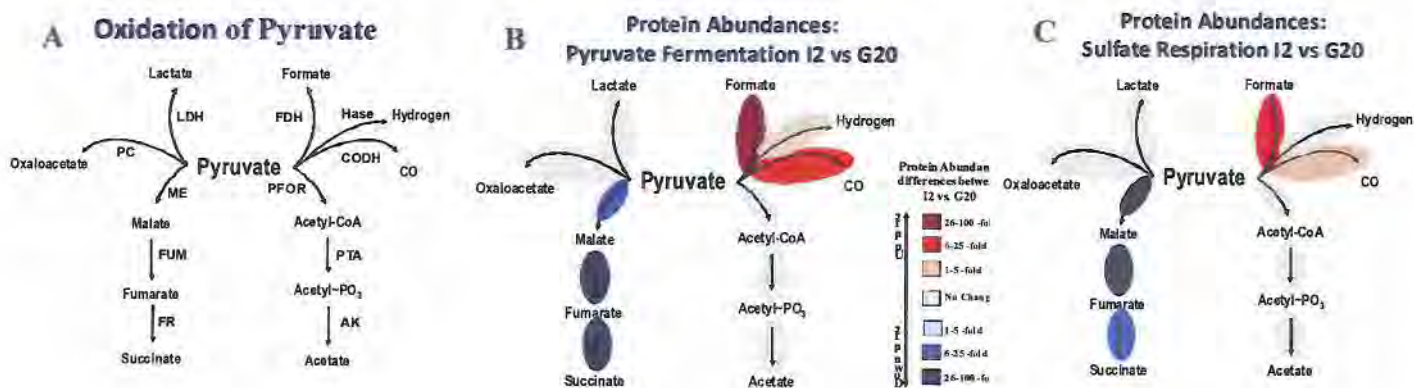


Fig. 2. A) Metabolic fates of pyruvate in *Desulfovibrio*. LDH, lactate dehydrogenase; PC, pyruvate carboxylase; ME, malic enzyme; FUM, fumarate hydratase; FR, fumarate reductase; FDH, formate dehydrogenase; Hase, hydrogenases; CODH, CO dehydrogenase; PFOR, pyruvate formate oxidoreductase; PTA, phosphotransacetylase; AK, acetate kinase. B) Oval color represents the magnitude and direction of difference between protein abundances in I2 versus G20 for pyruvate fermentative cells. Grey is no difference. C) Colors as in B) for I2 versus G20 for lactate/sulfate grown cells.

#### Science objectives for 2010-2011:

- 1) Explore microarrays and proteomics data for fumarate grown cells to seek candidates for regulatory genes.
- 2) Access the ~11,000 member transposon library of *Desulfovibrio* G20 (A. Deutschbauer) for knock outs of genes that will be tested for improvements in the genetic accessibility of G20 as was done for *D. vulgaris* (Keller et al. 2009). Insertions in 5 genes associated with restriction modification systems have already been obtained. Explore transformation with linear DNA to generate marker exchange mutants.
- 3) Determine the cause of formate inhibition of fumarate growth of G20.

#### References to work supported by this project 2008-20010:

- 1) Wall, J.D., A. P. Arkin, N. C. Balci, and B. J. Rapp-Giles, Genetics and genomics of sulfate respiration in *Desulfovibrio*. Springer-Verlag, Berlin, Heidelberg, Microbial Sulfur Metabolism (1):1-12 (2009)
- 2) Rapp-Giles, B. J., K. L. Keller, I. Porat, S. D. Brown and J. D. Wall. The role of cytochrome *c*<sub>3</sub> in electron flow for sulphate reduction and pyruvate metabolism in *Desulfovibrio* G20. In revision for, Appl. Environ. Microbiol.

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- 1) Keller, K. L., K. S. Bender, and J. D. Wall. Development of a markerless genetic exchange system in *Desulfovibrio vulgaris* Hildenborough and its use in generating a strain with increased transformation efficiency. Appl. Environ. Microbiol. 75:7682-7691 (2009).
- 2) Zane, G. M. H-C Yen, and J. D. Wall. Effect of the deletion of qmoABC and the promoter-distal gene encoding a hypothetical protein on sulfate reduction in *Desulfovibrio vulgaris* Hildenborough. Appl. Environ. Microbiol. 76:5500-5509 (2010)

## Genetic Analysis of Hydrogenotrophic Methanogenesis in *Methanosarcina* Species

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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 key aspect of this study involves examination of the genotypic and phenotypic differences between *M. barkeri*, an organism that grows well on H<sub>2</sub>/CO<sub>2</sub>, and *M. acetivorans*, a closely related organism that is incapable of growth on H<sub>2</sub>/CO<sub>2</sub>. These differences lie at the center of the energy-conserving electron transport chains of the two organisms. Insight into the molecular, genetic, biochemical and physiological traits that underpin these traits 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 2008-2010: We have made substantial progress in elucidating the genetic and metabolic traits that allow, or disallow, the use of hydrogen by the two *Methanosarcina* species. In particular, our recent data clearly show that hydrogen is a central intermediate in methanogenesis from all known growth substrates in *M. barkeri*, whereas *M. acetivorans* has evolved to specifically exclude hydrogen as an intermediate. The data indicate surprising differences in the electron transport chains of these closely related methanogens, which we believe reflect the adaptation to freshwater and marine environments, respectively.

Using a variety of mutant strains, we showed that under most growth conditions *M. barkeri* utilizes H<sub>2</sub> as obligate electron carrier for methanogenesis regardless of the substrate be used: *i.e.* all substrates are converted to H<sub>2</sub> during methanogenesis. H<sub>2</sub> is produced in the cytoplasm and then diffuses out of the cell where it is reoxidized with transfer of electrons into the energy conserving electron transport chain. This “hydrogen cycling” metabolism leads directly to production of a proton motive force that can be used by the cell for ATP synthesis. However, *M. barkeri* does have the flexibility to utilize other electron transport chains, as shown by our construction of mutants that lack all five hydrogenases. These mutants are viable, but show a severe growth defect. Our data support a model in which the very rapid enzymatic turnover of hydrogenases allows a competitive advantage via faster growth rates in this freshwater organism.

Our data indicate that the marine strain *M. acetivorans* lacks functional hydrogenases, despite the fact that it possesses genes for two of the three types of hydrogenase found in *M. barkeri*. Examination of these genes revealed the conservation of all known active site residues in the putative hydrogenase enzymes. A series of genetic experiments revealed that the *M. acetivorans* genes are not expressed under any growth conditions tested. Further, strains carrying genetic constructs that drive expression of the hydrogenase genes from strong, constitutive promoters fail to grow on hydrogen. Taken together, these data suggest that *M. acetivorans* has recently evolved to specifically inactivate its hydrogen producing/consuming metabolic processes.

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. In particular, there must be an electron transport chain connecting oxidative reactions of the various methanogenic pathways with reduction of the CoB-S-S-CoM disulfide produced during the final step of methanogenesis. 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) and both are absent in other *Methanosarcina* species. Mutants lacking *erh* have no measurable growth phenotypes; however, *rnf* mutants are unable to grow on acetate and have reduced yields on all other substrates. 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.

We have also examined the terminal step in the *Methanosarcina* electron transport chain, which is catalyzed by heterodisulfide reductase (Hdr). Previous biochemical investigations revealed a soluble Hdr in the *Methanobacteriales* (HdrABC) and a membrane-bound, cytochrome-containing HdrED that is only present in *Methanosarcinales*. However, genome sequencing revealed the presence of multiple genes for both classes of Hdr in *Methanosarcinales*. A combination of phylogenetic analyses and genetic experiments show that the HdrED enzyme is essential under all conditions tested, while distinct alleles of the HdrABC proteins play roles in either methylotrophic or acetoclastic methanogenesis. Transcriptomic experiments using *ΔhdrA/C1/B1* mutants revealed up-regulation of genes required for CoB-SH and CoM-SH biosynthesis and scavenging, and implicate (di)methylsulfide production as a strategy for overcoming CoM-SH limitation. In the past year we have constructed a series of double and triple mutants lacking *rnf*, *ehr*, and *hdrABC*. These results clarify the roles of each in gene locus in the energy-conserving electron transport chain on *M. acetivorans*.

#### Science objectives for 2010-2011:

- In the final year of this project we intend to focus our efforts on completing the biochemical and physiological analysis of the quintuple hydrogenase mutant of *M. barkeri*.
- We will also complete a series of transcriptomic experiments to characterize the changes in gene expression that result from mutation in the electron transport components of both *M. barkeri* and *M. acetivorans*.

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3. Kulkarni, G., D. M. Kridelbaugh, A.M. Guss and W.W. Metcalf. 2009. Hydrogen is a preferred intermediate in the energy conserving electron transport chain of *Methanosarcina barkeri*. *Proc. Natl. Acad. Sci. USA.* **106**:15915-20.
4. Buan, N.R. and W.W. Metcalf. 2010. Methanogenesis by *Methanosarcina acetivorans* involves two structurally and functionally distinct classes of heterodisulfide reductase. *Mol. Microbiol.* **75**:843-853.
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8. G. Kulkarni, A.M. Guss, N.R. Buan and W.W. Metcalf. 2010. Genetic analysis of energy-conserving electron transport in *Methanosarcina acetivorans*. *(in preparation)*
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***Session III***  
***Plant Growth and Regulation***



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

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Overall research goals: Our overall research 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 include: 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.

Significant achievements 2008-2010: Previously, we had characterized the biochemical and biophysical properties of the filament end-binding protein, AtCP. 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 as well as a simple reconstituted system for motility, was developed to describe this **stochastic dynamic** behavior.

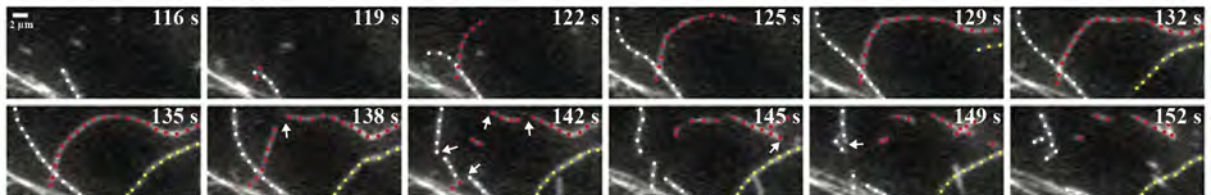


Figure 1. Actin filament stochastic dynamics in the cortical array of *Arabidopsis* epidermal cells. Individual filaments (red, white and yellow dots) elongate at rates of 1.7  $\mu\text{m/s}$  and are disassembled by prominent severing activity (arrows).

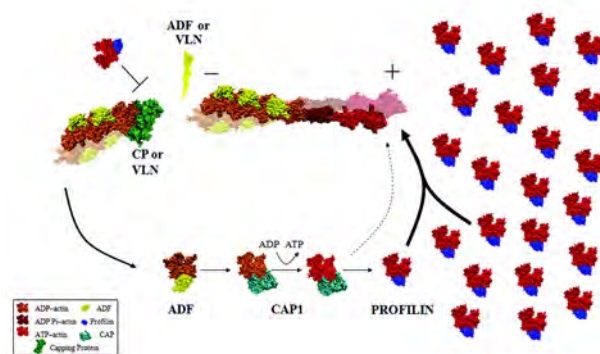


Figure 2. A simple model for regulation of actin stochastic dynamics by known plant ABPs, including the heterodimeric capping protein (green). See refs. [2,3] for full details.

### Science objectives for 2010-2011:

- We will spend considerable effort to get the genetic resources and tools in place to test a role for CP in stochastic dynamics. T-DNA insertion lines have been collected, homozygous mutants

isolated and level of transcript reduction analyzed by RT-PCR. Double mutants with disruption of both subunits have been generated, and all mutant lines will be marked with GFP-fABD2 to allow examination of actin dynamics by VAEM imaging. The preparation of RNAi as well as over-expression lines is also underway. All materials will be examined for growth and actin-based phenotypes, and CP protein levels analyzed quantitatively.

- A simple reconstituted system will be established using purified proteins and evanescent wave microscopy, to test the role of CP in stochastic dynamics *in vitro*.
- Preliminary data obtained by subcellular fractionation and immunolocalization indicate that a substantial amount of CP is associated with an endomembrane compartment, presumably the Golgi apparatus. We will confirm this by isolating intact Golgi with independent approaches and develop fluorescent fusion protein reporters of CP to examine co-localization with Golgi markers in living cells.
- To test for functional redundancy or synergies with other potential capping factors, homozygous mutant lines for villin isovariants and AIP1 will be isolated and characterized. Double mutants between CP and VLN, or CP and AIP1, will be recovered, marked with GFP-fABD2 and examined for growth phenotypes and perturbation of actin dynamics.

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- 1) **Staiger, C.J., Sheahan, M.B., Khurana, P., Wang, X., McCurdy, D.W., and Blanchoin, L.** (2009). Actin filament dynamics are dominated by rapid growth and severing activity in the *Arabidopsis* cortical array. *J. Cell Biol.* **184**, 269-280.
- 2) **Staiger, C.J., Poulter, N.S., Henty, J.L., Franklin-Tong, V.E., and Blanchoin, L.** (2010). Regulation of actin dynamics by actin-binding proteins in pollen. *J. Exp. Bot.* **61**, 1969-1986.
- 3) **Blanchoin, L., Boujemaa-Paterski, R., Henty, J.L., Khurana, P., and Staiger, C.J.** (2010). Actin dynamics in plant cells: A team effort from multiple proteins orchestrates this very fast-paced game. *Curr. Opin. Plant Biol.* in press
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## Cellulose Synthesis and the Control of Growth Anisotropy

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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 are to accomplish the following specific aims:

1. Isolate and 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. Image tagged cellulose synthase complexes in living *B. distachyon* roots.

Significant achievements 2009-2010: The main thrust over the first year of this project has been establishing techniques for handling *B. distachyon*, which is a new system for the PI, and focusing on forward and reverse genetics. Screening mutant phenotypes as well as evaluating reverse genetic effects is, and will be, done primarily on roots. This is because roots grow rapidly, accessibly, and have highly organized cellulose microfibrils in their primary cell wall.

### **Forward genetics:**

We are developing 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 10,000, Bd21 seeds and approximately 5,000 germinated seedlings were transferred to soil and their seeds collected. Because recessive alleles are expected, M2 material needs to be screened. We have planted 6 to 8 seeds from each M1 family to bulk seed for screening in the M2 generation. Of the initial 5,000 M1 families, 1,600 M2 families have been bulked and harvested. Another 2,000 families are now flowering in the greenhouse. We have also been working to establish the growth and screening conditions to isolate temperature-sensitive root morphology mutants. Wild-type (Bd21) seedlings grow well on nutrient-agar in vertical Petri dishes and light from the photoperiod is not problematic. Rapid root elongation rate occurs on half-strength Murashige and Skoog medium, and sucrose is not required. Roots grow well at 19°C and 30°C, allowing those to be used for permissive and restrictive conditions, respectively. As a positive control, we used chemical inhibitors to examine root-swelling phenotypes. We have obtained dose-response curves for two cellulose synthesis inhibitors, isoxaben and DCB, and for the microtubule inhibitor, oryzalin. These experiments indicate that, as expected, interference with either cellulose synthesis or microtubules will alter root morphology in *B. distachyon* and assure us that these phenotypes can be readily detected.

### **Reverse genetics:**

For reverse genetics, we began with CESA genes. First, we determined which of the ten CESA genes present in the *B. distachyon* genome are expressed in roots. Based on sequence homology with *A. thaliana* genes, we identified BdCESA sequences most likely to be involved in primary cell wall synthesis. Using both RT-PCR and microarrays, we found that the most highly

expressed genes in roots are: *BdCESA1*, *BdCESA3*, *BdCESA6*, and *BdCESA9*. We have generated a series of artificial microRNA (amiR) constructs to silence expression of these four genes. Thus far, we have successfully cloned constructs against them individually, as well as against certain double and triple combinations (*BdCESA1*, 6; *BdCESA1*, 9; *BdCESA6*, 9; and *BdCESA1*, 6, 9). We also have generated constructs against the other putative primary cell wall *BdCESA* genes; including clade 6, which targets *BdCESA5*, 6, 9 and *BdCESA2*, 3. In addition, we identified a homologue to COBRA, a protein known to be involved in the control of growth anisotropy in *A. thaliana*, which is highly expressed in roots and have generated an amiR construct to silence it. In total, we have transformed *B. distachyon* embryonic calli with these ten amiR constructs. We have already regenerated plants from four independent transformation events with the *BdCESA1* amiR construct, which is the most abundantly expressed *BdCESA* gene in roots. Verification and evaluation of these lines is underway.

### **Natural accessions**

In collaboration with several labs in Europe, as well as the Caicedo and Hazen labs here at UMass, we have begun collecting and bulking diploid and polyploid accessions of *B. distachyon*. These accessions will be used to examine intra-specific and ploidy-specific differences in cellulose biosynthesis rates.

### **Imaging CESAs**

To image *B. distachyon* CESAs as they work, we have cloned the full-length coding sequences of *BdCESA1*, 3, 6, and 9, the four main CESAs we hypothesize to be most important for primary cell wall synthesis in the root. To facilitate this approach, as well as to speed the analysis of silencing effects, we aim to work with single elongating cells that regenerate from protoplasts. To this end, we have developed a protocol to isolate protoplasts at high yield from *B. distachyon* leaves. We are currently determining conditions for efficient transformation and for elongation. Once these conditions are in hand, and we have a better handle on the functionality of the *BdCESAs*, we will construct N-terminal fusions of various *BdCESAs* to GFP for in vivo imaging.

Science objectives for 2010-2011: For forward genetics, we aim to screen all 5000 currently available M2 families for temperature-dependent root swelling as well as to generate additional M2 families. For reverse genetics, we aim to have regenerated plants expressing all of the amiRNA constructs described and to have assessed their phenotypes. For natural accessions, collecting and bulking up will continue to support a simultaneous assay in the third year. Finally, we aim to develop a culture protocol so that CESAs can be imaged in single, elongating cells regenerated from protoplasts.

## Exploring Molecular Mechanisms of Lignin Biosynthesis and Its Regulation

Chang-Jun Liu, Principal Investigator  
Mohammed W. Bhuiya, Ke-Wei Zhang

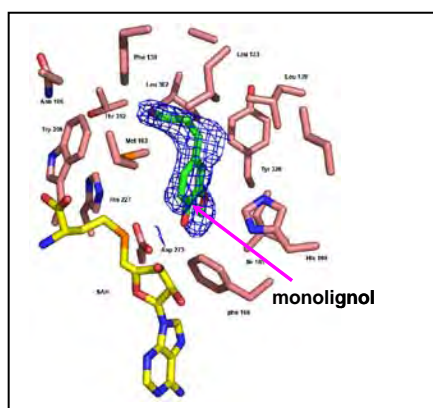
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### Overall research goals:

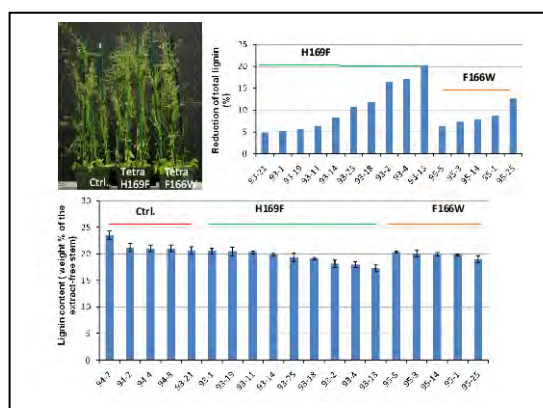
Lignin is a complex, irregular biopolymer derived from the oxidative coupling of three monolignols. According to current speculation, the phenoxy coupling starts with the deprotonation of the *para*-hydroxyls of monolignols to yield one electron radicals. The overall goals of our research project are to develop and apply novel biological tools/approaches to probe the molecular mechanisms underlying the synthesis of lignin polymer and its related phenylpropanoids, and consequently, to establish a scientific underpinning for the rational manipulation of plant lignification. Our major research objectives in present study are to explore the biochemical mechanism of the regiospecific methylation of the phenylpropanoid *O*-methyltransferases. Thereafter, by applying a structure-based protein-engineering approach, we will create a set of novel monolignol *para*-methyltransferases so to introduce non-natural precursors into lignin biosynthesis *in planta*, and subsequently, to explore the perturbation or disruption of the lignification of the cell wall.

### Significant achievements in 2008-2010:

Using phylogenetic analysis, protein-homology modeling, and site-directed mutagenesis, we identified a batch of evolutionarily “plastic” amino-acid residues from a set of plant phenolic *O*-methyltransferases. Following the strategy of structure-based iterative site-saturation mutagenesis, we created a series of mutant libraries from a parental enzyme responsible for methylating phenylpropenes. Screening mutant libraries, we obtained a set of enzyme variants that exhibited the ability for *para*-methylating lignin monomeric precursors, primarily monolignols, (termed monolignol 4-*O*-methyltransferases). Subsequent determination of the crystal structure of a triple-mutant variant revealed the molecular basis for its broadened substrate preferences and its regiospecific *para*-methylation (**Fig. 1**).



**Fig. 1.** Close-up view of the active site of the triple-mutant variant in complex with the monolignol coniferyl alcohol and methyl donor/product SAH.



**Fig. 2.** Over-expressing the monolignol 4-OMT in *Arabidopsis* resulted in the accumulation of novel 4-*O*-methylated soluble phenolics, and the reduction in the cell wall's lignin content (AcBr lignin).

Subsequently, we transferred a set of created monolignol 4-*O*-methyltransferases (with double-, triple- or tetra- mutations) into *Arabidopsis* and/or tobacco. Expressing the novel enzymes in the model plants revealed their broad effects on phenylpropanoid biosynthesis and lignin polymerization. Transgenic plants accumulated a large amount of non-nature soluble UV-screening phenolics in leaves and stems, and incorporated the novel “wall-bound” phenolics in the cell walls; moreover, we noted large decreases in both the content of soluble oligolignols, and the total content of insoluble lignins (**Fig. 2**).

#### Science Objectives for 2010-2011:

- Continue iterative saturation mutagenesis on the created monolignol 4-*O*-methyltransferases to further optimize their catalytic efficiency and substrate specificity. Our goal is to obtain a set of novel 4-OMTs with a highly restricted substrate-preference for particular monolignols, *e.g.*, either for coniferyl alcohol or sinapyl alcohol, or for other lignin biosynthesis-related monomeric precursors.
- Conduct detailed biochemical- and biophysical-analyses of the novel catalysts to determine their substrate specificity and to dissect the mechanisms governing their substrate preferences.
- Comprehensively analyze monolignol 4-OMT transgenic plants. We plan to focus particularly on analyzing lignin composition and structure, and on detailing the effects of the expression of the 4-OMT on synthesis of other phenolics, such as soluble compound lignan, and the overall effects on plant physiology.
- Elucidate the *in vivo* mechanisms of *para*-methylated compounds in disturbing lignin- and lignan-synthesis.
- Design a new expression strategy to better integrate the novel monolignol 4-OMT into the lignin-biosynthetic pathway in transgenic plants.
- Generate transgenic poplar plants harbouring the expressed novel enzymes to prepare materials for an NMR-based analysis of lignin and cell-wall structure, and to test the potential of biotechnological application.

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*Session IV*  
*Special Guest Lecture:*  
*Life Sciences Research*  
*Fellow*



## Spatial and temporal organization of cyanobacterial metabolism

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Overall research goals: The research objects are to study the spatial and temporal regulation of cyanobacterial carbon fixation by: (1) using fluorescence microscopy to investigate the role of the carboxysome, a self-assembling protein microcompartment, in facilitating the carbon fixation reaction and how this structure interacts with other cytoplasmic components; (2) using metabolomics to investigate the circadian regulation of photosynthetic metabolism; (3) developing synthetic biology strategies for the overproduction of industrially relevant commodity chemicals.

### Significant achievements 2007-2009:

We have developed a fluorescent microscopy assay for tracking the assembly and position of protein microcompartments *in vivo*. Using this technique we have shown that carboxysomes are spatially constrained by the cytoskeleton in cyanobacteria and that this organization is vital to organismal fitness. In other work, we have engineered the overproduction of sugars and lactic acid by developing efficient transport systems in cyanobacteria.

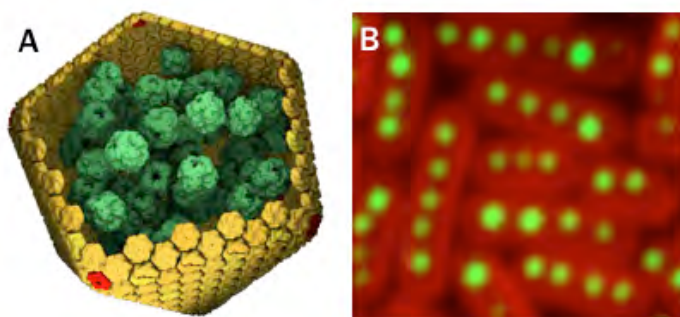


Figure 1. (A) Molecular model of the carboxysome from cyanobacteria. Diameter is 100 nm. (B) Fluorescent images of carboxysomes in live cells. Thylakoids are shown in red, carboxysomes in green.

### Scientific objectives for 2009-2010:

- Continue to develop carboxysome fluorescence analysis and extend to quantifying the assembly and degradation process.
- Continue metabolomics analysis of circadian rhythm and couple analysis with microscopy analysis of cell division
- Continue to develop hydrocarbon production in cyanobacteria by metabolic engineering of fatty acid biosynthesis.

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*Poster Session I*



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

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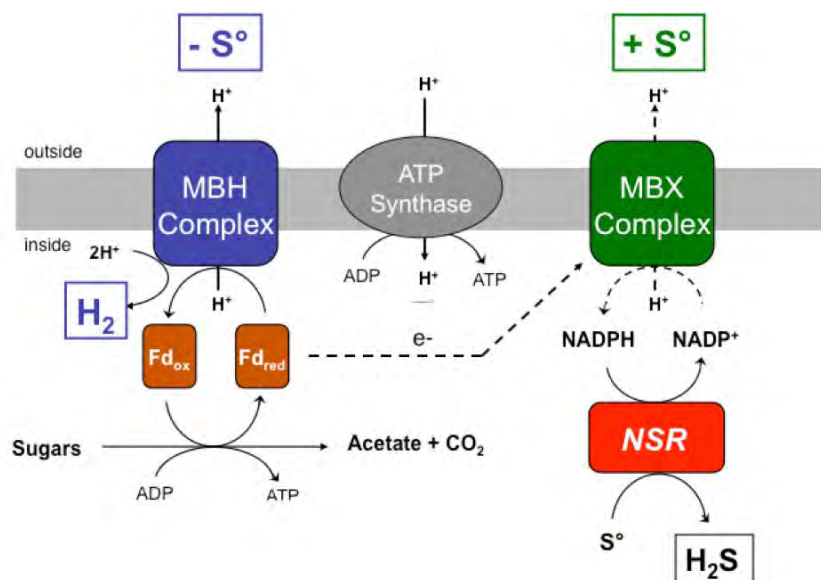
Overall research goals: The focus of this research is non-covalent multiprotein complexes that are involved in novel mechanisms of energy conservation and catalysis. The protein complexes under study have the remarkable property of being synthesized (self-assembling) at temperatures near 100°C in so-called 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 2008-2010: The model organism is *Pyrococcus furiosus* (Pf), which grows optimally at 100°C. Pf obtains carbon and energy for growth by fermenting carbohydrates and by producing H<sub>2</sub> or by reducing elemental sulfur (S<sup>0</sup>) to H<sub>2</sub>S. Pf has a respiratory metabolism in which a ferredoxin-dependent, membrane-bound hydrogenase (MBH) catalyzes the production of H<sub>2</sub> and couples this to the pumping of protons (and possibly Na<sup>+</sup> ions). The membrane potential that is generated is utilized by ATP synthase to synthesize ATP (Figure 1). Using DNA microarray, PCR and biochemical analyses we have shown that when S<sup>0</sup> is added to a growing Pf culture, the biosynthesis of MBH is halted within minutes and instead the synthesis is induced of a highly homologous membrane complex termed MBX. MBX is proposed to oxidize ferredoxin and reduce NADP (Figure 1) and to generate a proton (Na<sup>+</sup>) motive force. MBX and MBH are integral multiprotein complexes of 300 kDa, each encoded by at least 13 genes. The addition of S<sup>0</sup> to Pf also induces the synthesis of a cytoplasmic flavoprotein termed NADPH sulfur reductase (NSR). Pf was shown to directly interact with insoluble S<sup>0</sup> using a broad-spectrum antimicrobial agent that causes cell lysis. Membrane-associated S<sup>0</sup> is thought to be accessible from the cytoplasm and to be reduced by NSR. NSR is a multiprotein complex of 100 kDa and is proposed to be responsible for oxidizing the NADPH generated by MBX and for reducing S<sup>0</sup> to H<sub>2</sub>S in a coenzyme A-dependent reaction. The sulfide that is produced by NSR is sequestered in an iron-dependent manner by a novel protein termed SipA. This exists as large homomultimeric megacomplexes up to 80 MDa in size containing ~300,000 iron and sulfur atoms. Production of SipA in Pf is also induced by oxidative (peroxide) stress. The transcriptional response of MBH, MBX and NSR, but not of SipA, to S<sup>0</sup> is mediated by the regulator SurR, which undergoes a redox-dependent conformation change in the presence of S<sup>0</sup>.

Science objectives for 2010-2011:

- To characterize the native and recombinant forms of the homomeric protein megacomplex SipA, including mechanisms of assembly and incorporation of iron and sulfide.
- To characterize deletion mutants of MBH, MBX, NSR and SipA. This builds upon our recent development of a genetics system in Pf (in collaboration with J. Westpheling and R. Scott, U. Georgia).
- To characterize the novel energy conserving complex MBX using both native and recombinant tagged sub-complexes generated in Pf.

**Figure 1.** Bioenergetics and proposed pathways of electron flow from reduced ferredoxin ( $\text{Fd}_{\text{red}}$ ) either to protons to produce  $\text{H}_2$  or to elemental sulfur ( $\text{S}^\circ$ ) to produce  $\text{H}_2\text{S}$  in *P. furiosus*.



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## Role of HydF in Hydrogenase Maturation

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Overall research goals: The main goal of this project is to elucidate the biosynthetic pathway for the [FeFe]-hydrogenase active site metallocluster, the 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<sup>-</sup>) ligands as well as an as-yet unidentified 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 of [FeFe]-hydrogenases.

Significant achievements 2008-2010: HydA (the hydrogenase structural protein) has been shown to harbor only the [4Fe-4S] subcluster of the H-cluster when expressed in the absence of the accessory enzymes. HydF has been shown to be a scaffold protein for assembly of the 2Fe subcluster of the H-cluster; in the presence of the radical SAM enzymes HydE and HydG, a [2Fe-2S] cluster on HydF is converted to a CO and CN<sup>-</sup> ligated species that is transferred to HydA to effect activation. HydG has been shown to utilize tyrosine as a substrate for production of CO and CN<sup>-</sup>.

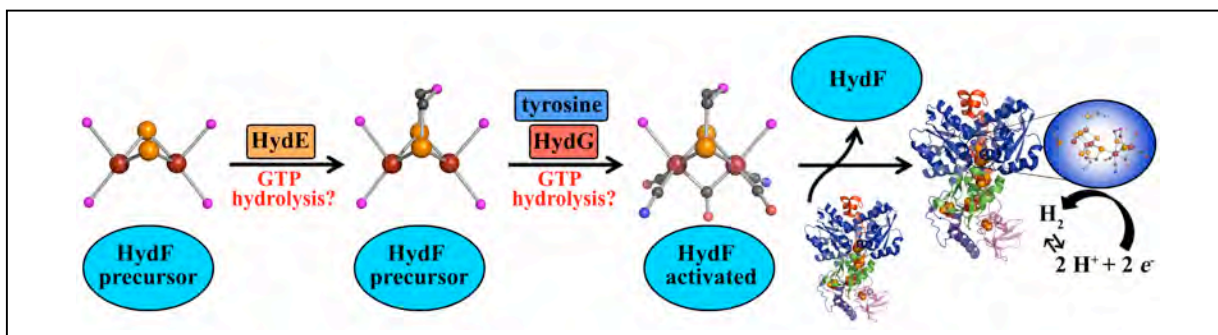


Figure 1. Hypothetical model for H-cluster assembly. In this model, the radical SAM enzyme HydE acts on a [2Fe-2S] cluster on HydF, resulting in the insertion of the bridging sulfides of the cluster into C-H bonds of an as-yet unknown substrate to generate the bridging dithiolate ligand. The radical SAM enzyme HydG then cleaves tyrosine to produce CO and CN<sup>-</sup>, which are delivered to the 2Fe subcluster on HydF. Preliminary evidence suggests that the GTPase activity of HydF may be involved in these interactions between HydF and the two radical SAM enzymes. In the final step of activation, the CO and CN<sup>-</sup> ligated 2Fe subcluster is transferred to HydA to effect activation.

### Science objectives for 2010-2013:

- The specific progression of steps in H-cluster assembly will be defined. The hypothetical progression of steps shown in Figure 1 will be examined by defining the requirements for the modifications catalyzed by HydE and HydG using a combination of biochemical and spectroscopic approaches.
- H-cluster precursors assembled on HydF by the other accessory proteins will be characterized. Genetic systems in which HydF is expressed alone or in various combinations with the other accessory proteins will be utilized to generate assembly intermediates on HydF. These intermediates will be characterized using spectroscopic approaches.
- The role of GTP hydrolysis in H-cluster assembly will be probed by examining the interactions between GTP and the iron-sulfur cluster on HydF, as well as by utilizing specific mutants of HydF that are unable to generate an active HydA. A fully *in vitro* activation system will be developed in order to provide well-defined systems for probing the role of GTP hydrolysis.

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

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Dr. Barney J. Venables, University of North Texas, Denton, TX  
Dr. Ruth Welti, Kansas State University, Manhattan, KS

### Overall research goals:

- *N*-Acylethanolamines (NAEs) are fatty acid derivatives that vary in acyl chain length and unsaturation. They are neutral in charge with the ethanolamine group amide-linked to the acyl moiety. The metabolism of NAEs is conserved substantially among plants and animals with *N*-acylated phosphatidylethanolamines (NAPEs) serving as the precursors for these metabolites. NAEs are converted to free fatty acids by fatty acid amide hydrolase (FAAH), and in mammals the formation and hydrolysis of NAEs is tightly regulated as part of the “endocannabinoid” signaling pathway. With DOE support, we are combining a molecular genetic approach to complement our ongoing research on the biochemistry and cell biology of NAE metabolism, in an effort to elucidate the functional role(s) of this lipid pathway in higher plants. Specific objectives include the following:
  - 1. Probe the Domain Structure/ Function Relationships of *Arabidopsis* Fatty Acid Amide Hydrolase (AtFAAH1).
  - 2. Identify and Characterize Alternative/ Additional Amidases Important in the Regulation of NAE Metabolism.
  - 3. Detail the Interaction(s) between Different NAE Types and ABA Signaling Pathways under Different Developmental/ Stress Conditions.

Significant achievements in 2008-2010: Pharmacological approaches by application of NAEs to plants have generally supported a role for these compounds as negative regulators of growth, especially in seedlings. A FAAH homologue of the rat enzyme was identified and characterized at the molecular level in several plant species. Manipulation of FAAH expression in *Arabidopsis* has led to new insights into the potential roles of this lipid metabolic pathway in plant growth, development and responses to environmental stressors. 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 participating through some protein-protein interactions.

In other work we have identified a second 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. Seed aging studies with *Arabidopsis* *faah2* and *faah1/2* double mutants suggest that FAAH and NAE18:2 accumulation may play a role in seed longevity. 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*. Our evidence suggests that FAAH and LOX cooperate to metabolize endogenous NAEs and we are investigating the potential function of NAE18:3 oxidation

metabolites 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 LOXes and may modulate plant growth and development through the regulation of oxylipin formation.

In addition to developing new analytical methods for the analysis of ethanolamide oxylipins, we have also worked with the Ruth Welti laboratory to develop new MS-based approaches to identify and quantify the metabolic precursors of NAEs—the NAEs. This new methodology has far reaching applications and we demonstrated its utility in mammalian systems (FAAH knockout mice) as well as plant systems. 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 2009-2010:

- Work is continuing to identify the mechanisms by which FAAH proteins participate in the regulation of plant growth, development and responses to environmental stresses. We have identified two candidate proteins that interact with FAAH1 in yeast 2-hybrid assays, and we are following up with determination of specific domain interactions and relevance in vivo.
- Assays to visualize cytosolic Ca<sup>2+</sup> signature in vivo were developed recently in the coPI's laboratory and we are assessing the effects of different NAE types for their influence on Ca<sup>2+</sup> signaling specifically in the context of seedling growth.
- We continue to probe the interaction of ABA and NAEs surveying different NAE types for similarities and differences in growth regulation and gene expression, with reference to the synergistic interactions between NAE 12:0 and ABA.
- Additional enzymes/pathways for NAE metabolism are being 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 are attempting to express and characterize the FAAH2 protein in vitro and have generated various knockouts and overexpressors of FAAH2 in Arabidopsis to assess the action(s) of this protein in vivo.

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## Molecular Mechanisms of Plant Cell Wall Loosening

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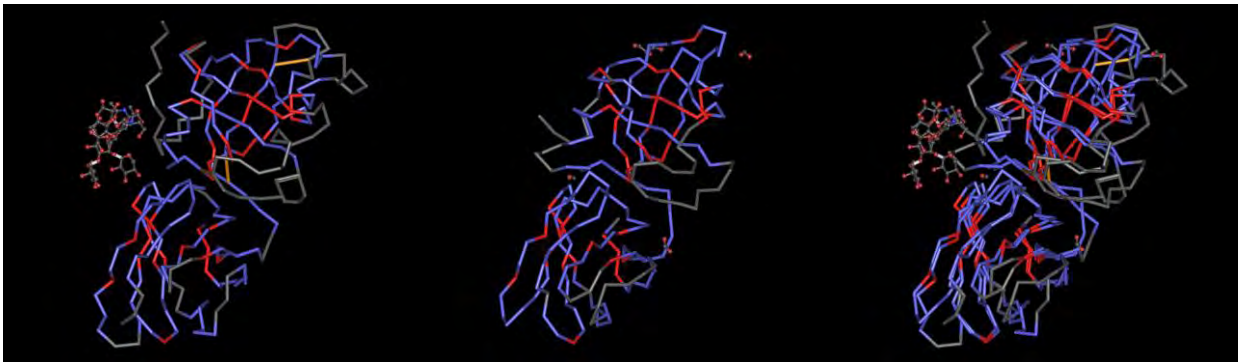
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Overall research goals: to elucidate the molecular controls and mechanisms of plant cell wall enlargement, with particular focus on the molecular structure and action of expansins. We discovered these wall-loosening proteins as the key mediators of pH-dependent cell wall extension ('acid growth') in plants, but the molecular basis for their loosening activity remains elusive. We are combining structural, biochemical, biophysical, imaging, and genetic approaches to understand how expansins interact with cell walls to induce loosening and polymer creep.

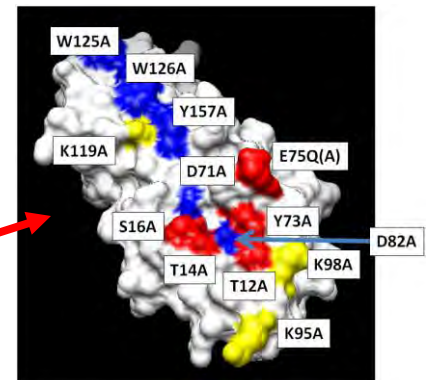
Significant achievements in 2008-2010: An extracellular protein (BsEXLX1) from the soil bacterium *Bacillus subtilis* has distant sequence similarity to plant expansins, its crystal structure exhibits the same domain and fold structure as plant expansins, it has (weak) wall loosening activity similar to that of plant expansins, and it promotes the colonization of plant roots by *B. subtilis* (ref. 2 below). Like plant expansins, BsEXLX1 consists of two domains: D1 has structural similarity to family-45 endoglucanases, while D2 is a 9-stranded  $\beta$ -sandwich. It does not exhibit lytic activity against the major polysaccharides of the plant cell wall.



Comparison of the X-ray crystal structures of a plant  $\beta$ -expansin (ZmEXPB1, left) with the bacterial expansin from *Bacillus subtilis* (middle), and their structural superposition (right).

BLAST searches of the NCBI database show that bacterial expansins are absent in most bacterial genomes, but found in a phylogenetically diverse group of bacteria that cause plant diseases (*Xanthomonas*, *Xyllela*, *Clavibacter*, *Erwinia*), suggesting that this protein functions in plant-microbe interactions, sometimes leading to disease. Additionally, we:

- cloned the genes for many of these bacterial expansins, expressed them in *E. coli*, and tested for expansin-like wall loosening activity. Some had activity, others not.
- swapped domains between plant expansins and bacterial expansins, and tested for wall-loosening activity (hybrid proteins lacked activity).
- tested the activity of single BsEXLX1 domains: negligible wall-loosening activity was detected in single domains, but D2 is largely responsible for cellulose binding.
- mutated specific BsEXLX1 residues to assess their importance for binding to cell walls, binding to cellulose, and for wall-loosening activity. Cellulose binding is large determined by three linear



aromatic residues in D2 (W125, W126, Y157), but binding to plant cell walls is largely a function of protein charge. Converting basic residues on the protein surface to neutral residues reduces binding to cell walls, but increases wall-loosening activity. This indicates that pectins bind the protein, whose target of action is a different wall component. BsEXLX1 weakens cellulose-based composites, suggesting it reduces cellulose-cellulose binding.

Turning to another group of expansins, we found that the **plant  $\beta$ -expansin, ZmEXPB1**, releases matrix polymers from the maize silk cell wall (its native target). These include a highly-substituted feruloylated arabinoxylan (~70% of the material), homogalacturonan (~20%), and smaller amounts of mixed-link glucan, cutin polymer (C:16; C:18 monomers), and arabinan. The mechanism of release does not appear to be polymer lysis. We designed in-vitro experiments to test for the release of cell wall polymers bound to cellulose; ZmEXPB1 could release a variety of polymers; however, the highest activity seen was only ~2X higher than control proteins. ZmEXPB1 was tagged with the Alex-488 fluorophore and used as a fluorescent stain. It bound to cell walls in general, but not lignified cell walls. It also bound intensely to  $\beta$ 1,3-glucans and to the cuticle. The protein exhibits little or no mobility once it binds to the cell wall (based on FRAP experiments, Fluorescence Recovery After Photobleaching). Preliminary calorimetry experiments indicate negligible enthalpy change upon binding to whole cell walls or cello-oligosaccharide, suggesting an entropy-driven binding mechanism. These molecular activities and binding affinities are consistent with the results of RNAi knock-down experiments, wherein maize lines deficient in EXPB1 expression showed reduced fertility and pollen tube penetration of the stigma and style (ref. 6 below).

Lastly, we combined mutants with gene-silencing techniques to reduce the expression of endogenous  **$\alpha$ -expansins** in *Arabidopsis*. The results show a linear relationship between in-vitro acid-induced wall extensibility and  $\alpha$ -expansin gene expression. This result further supports the conclusion that  $\alpha$ -expansins mediate the acid-growth response of plant cell walls.

#### Science objectives for 2010-2011:

- Use isothermal titration calorimetry to define BsEXLX1's target polysaccharide and quantify binding thermodynamics;
- Attempt to make crystals of an expansin:oligosaccharide complex suitable for X-ray analysis and structure solution.
- Complete the structure-functional analysis of BsEXLX1 by mutagenesis and activity assays.

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## The Role of the Primary Cell Wall Polysaccharide Xyloglucan in Plant Growth and Development

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Overall research goals: To determine the role of the cell wall in plant growth and development by: (i) Isolating and structurally characterizing cell wall components from wild-type and mutant plants; (ii) Developing high-sensitivity spectroscopic and immunocytochemical methods to structurally characterize cell wall components and their location *in planta*. In addition, we structurally characterize wall components from avascular (mosses, hornworts and liverworts) and vascular (lycophodiphytes, ferns, gymnosperms, and angiosperms) plants to gain insight into how wall composition and structure has changed during land plant evolution.

Significant achievements 2009-2010: The Arabidopsis *MUR3* gene encodes a galactosyltransferase involved in xyloglucan (XyG) synthesis. *MUR3* catalyzes the addition of a  $\beta$ -D-Galp residue to O2 of the  $\alpha$ -D-Xylp residue that is linked to O6 of a backbone  $\beta$ -D-Glcp residue adjacent to an unbranched  $\beta$ -D-Glcp residue. This Gal is often fucosylated to give  $\alpha$ -L-Fucp-(1 $\rightarrow$ 2)- $\beta$ -D-Galp-(1 $\rightarrow$ 2)- $\alpha$ -D-Xylp(1 $\rightarrow$ 6)- $\beta$ -D-Glcp (F side chain). *MUR3* mutations (e.g., *mur3-3*) that completely abolish this regiospecific galactosyltransferase activity result in a dwarf phenotype (Fig. 1A). This phenotype has been suggested in the literature to result from defective endomembrane and actin filament organization rather than abnormal XyG structure.

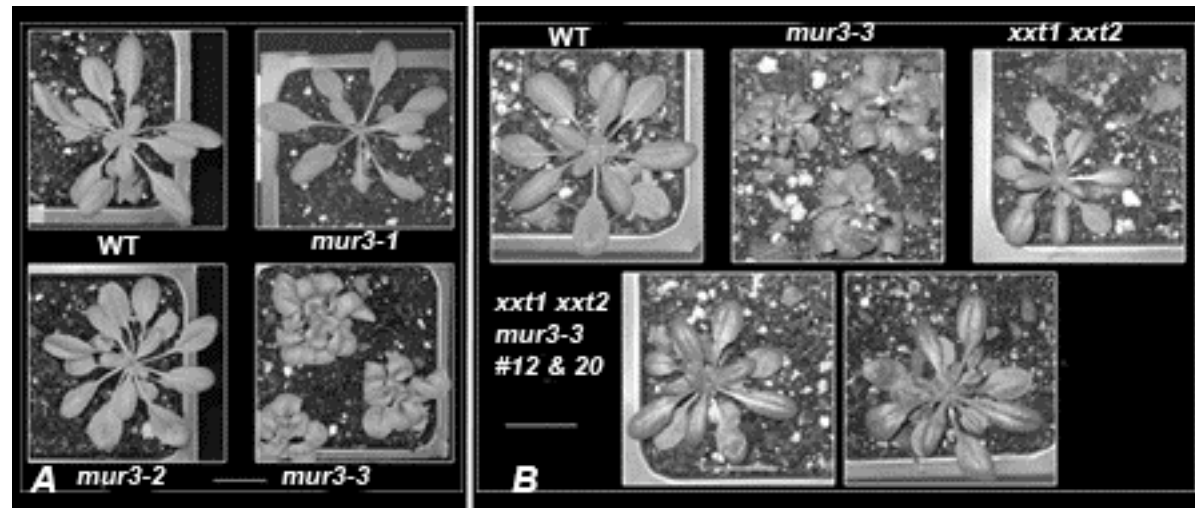


Figure 1. **A**, The phenotypes of wild type Arabidopsis and *mur3* mutant plants. **B**, The phenotypes of wild-type, *mur3-3*, the XyG-deficient *xtt1 xtt2* double mutant and the *xtt1 xtt2 mur3-3* triple mutant.

To test this hypothesis we used  $^1\text{H-NMR}$  spectroscopy, MALDI-TOF-MS, and glycome profiling with XyG-directed monoclonal antibodies to characterize the XyG synthesized by several *mur3* alleles. Plants with leaky point mutations (*mur3-1* and *mur3-2*) have wild-type phenotypes (Fig. 1A) and synthesize XyG containing discernible amounts of the F side-chain. By contrast, no fucosylated side-chains were detected in the XyG synthesized by *mur3-3*, a dwarf loss of function mutant (Fig. 1A). We also determined the phenotypic effect of eliminating XyG from the *mur3* background by crossing *mur3-3* with an Arabidopsis double mutant (*xtt1 xtt2*) whose walls contain no discernible amounts of XyG yet has a wild-type phenotype. The aerial portions of the *mur3-3 xtt1 xtt2* triple mutant are indistinguishable from the *xtt1 xtt2* double mutant (Fig. 1B)

and have walls with no discernible amounts of XyG. Our results establish that the *mur3-3* phenotype results from the synthesis of structurally abnormal XyG rather than from the absence of the MUR3 protein and support the notion that the galactosyl residue added by MUR3 is required for normal XyG function.

We have established that structurally abnormal XyG is itself responsible for the dwarf phenotype of the aerial portions of *mur3-3* plants. However, the root hairs of *mur3-3* and wild-type plants have comparable lengths (Fig. 2A) and are labeled with CCRC-M1 (Fig. 2B), a monoclonal antibody that recognizes fucosylated xyloglucan.

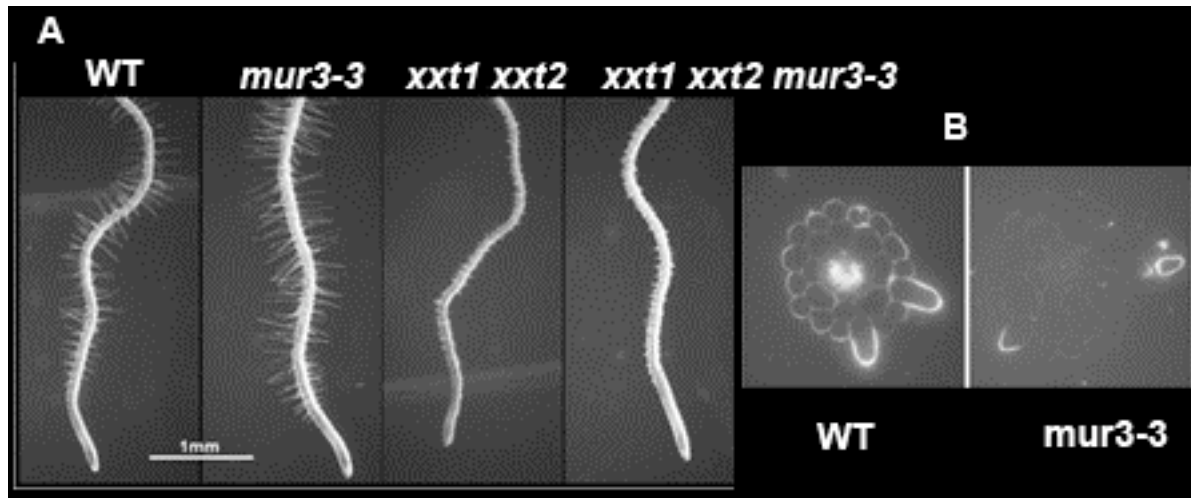


Figure 2. **A**, Wild-type and *mur3-3* plants have root hairs of comparable length. The *xxt1 xxt2* and *xxt1 xxt2 mur3-3* mutants synthesize no XyG and have very short root hairs. **B**, *mur3-3* root hairs are labeled with CCRC-M1, a monoclonal antibody that recognizes fucosylated xyloglucan.

In other xyloglucan studies we used MALDI-TOF-MS and  $^1\text{H-NMR}$  spectroscopy to identify two unique GalA-containing sidechains,  $\alpha\text{-L-Fucp-(1,2)-}\beta\text{-D-GalpA-(1,2)-}\alpha\text{-D-Xylp-(1}$  and  $\beta\text{-D-GalpA-(1,2)-}\alpha\text{-D-Xylp-(1}$ , in the XyG from root hair cell walls of *mur3-3* and wild-type *Arabidopsis* plants. GalA-containing xyloglucan was also detected in the cell walls of tomato, cucumber, and morning glory roots. The GalA-containing xyloglucan is likely present only in root-hair cell walls as it was not detected in the root cell walls of *Arabidopsis* mutants that lack root hairs or in *Arabidopsis* leaf and stem cell walls. No GalA-containing xyloglucan was detected in the roots of *Arabidopsis* plants carrying a null mutation in a *MUR3* homolog that is highly expressed in root-hairs, suggesting that the gene encodes a XyG-specific GalA transferase. The GalA residue is likely fucosylated by the xyloglucan-specific fucosyltransferase FUT1 as no fucose was detected in the anionic xyloglucan synthesized by the *Arabidopsis fut1* mutant. These results together with our demonstration that GalA-containing XyG is present in the cell walls of *Physcomitrella patens* protonema (Peña *et al.*, 2008 *Glycobiology* **18**: 891-904) suggest that anionic XyG is characteristic of the walls of cells that elongate by tip growth. Indeed, we have identified a second MUR3 homolog that is expressed in pollen cells, which also elongate by tip growth. Our data show that there are subtle differences in XyG structure in the cell walls of root and aerial tissues. Thus, future research will emphasize detailed studies of the composition and structure of root cell walls. Such studies will contribute to increasing our understanding of root biology, which is essential for improving plant productivity (Gerwin, 2010, *Nature*, **466**:552-553).



## Attachment of Living Cells to Material Surfaces Through DNA-Mediated Cell Adhesion

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**Overall research goals:** The overall goal of this collaborative program is the construction of well-defined interfaces between living cells and non-biological surfaces. In order to achieve this in the broadest possible context, we have developed techniques to introduce synthetic DNA strands as artificial adhesion groups on the cytoplasmic membrane of living cells.<sup>1</sup> Once the oligonucleotides have been attached, the cells bind to complementary sequences extending from a desired surface with very high efficiency and selectivity. Multiple DNA sequences can be used to generate complex patterns of several cell types,<sup>2</sup> and the method can be adapted to create defined clusters of cells in solution.<sup>3</sup> Relative to other adhesion techniques, the DNA-based approach offers compatibility with virtually any desired cell type, greater shear force resistance,<sup>2</sup> lower activation of endogenous signaling pathways,<sup>4</sup> and the potential for substrate reuse.<sup>2</sup> It currently stands as the most versatile method available for the integration of living cells into device structures.

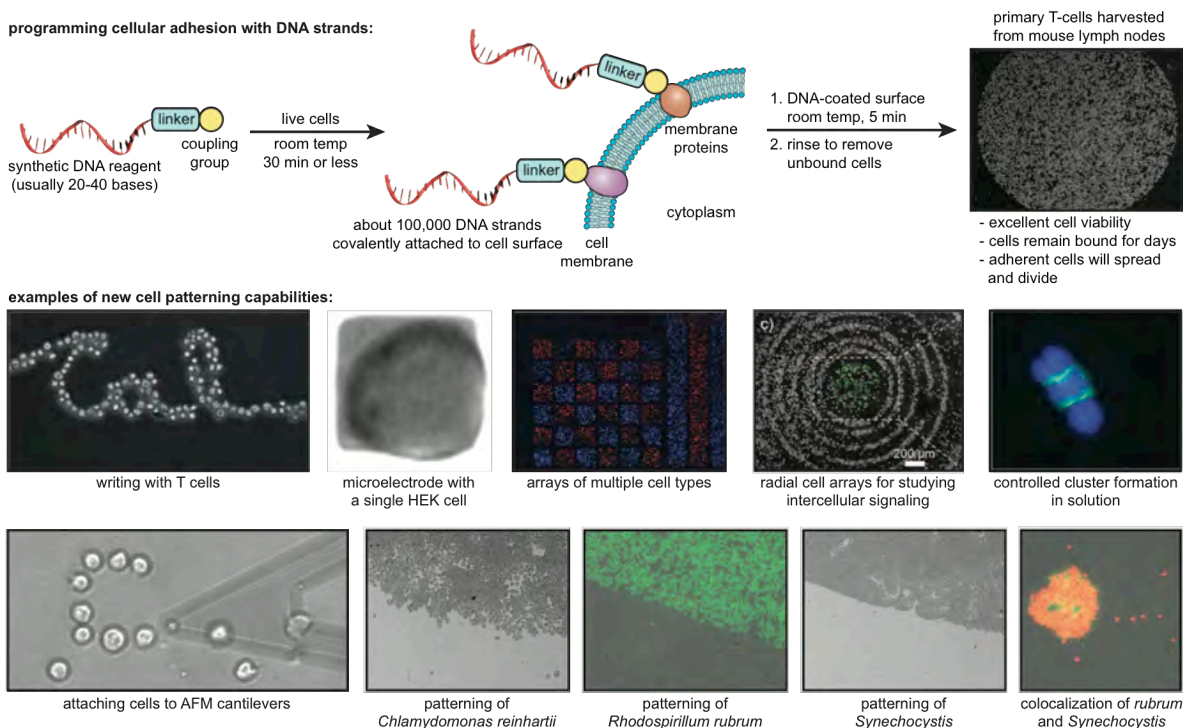


Figure 1. The attachment of synthetic DNA strands to the surfaces of living cells allows their facile incorporation into a number of different device formats. This technique is compatible with a wide range of different cell types and can be used in conjunction with well-developed DNA printing techniques.

**Significant achievements 2008-2010:** Our first demonstration of DNA-based cell adhesion<sup>1,2</sup> was limited to cultured mammalian cells and required the use of relatively complex reagents to attach synthetic oligonucleotide strands to the cell membranes. Since that time we have developed significantly more streamlined approaches that can achieve cell modification in a matter of minutes with much simpler reagents.<sup>4</sup> With these advances, the technique can now be used to attach all mammalian cells (both adherent and nonadherent, cultured and primary) to surfaces, and the

method is now compatible with algae, bacteria, yeast, and thermophilic archaeobacteria. We have used this method to (1) attach human T-cells to AFM probes for force measurement,<sup>5</sup> (2) connect primary and cultured cells to pH microelectrodes to measure metabolic rates in real time,<sup>6</sup> (3) capture single cells in microfluidic devices capable of carrying out ultrasensitive RT-PCR analysis,<sup>7</sup> (4) generate controlled patterns of cardiac myoblasts that can differentiate into oriented myotubes,<sup>4</sup> (5) prepare arrays of multiple cell types for the purpose of studying intercellular signaling, (6) capture photosynthetic bacteria and algae that are capable of hydrogen production, and (7) connect electroactive bacteria to metal surfaces for current measurement and collection.

#### Science objectives for 2010-2011:

- We are using the DNA-based adhesion method to anchor photosynthetic hydrogen producing organisms on patterned platinum electrodes. This will allow us to measure their hydrogen output in real time, providing a convenient and accurate way to study the organisms in controlled environments. Using this setup, we will gain a better understanding of how oxygen, nutrient, and light flux can be controlled and cycled to achieve optimum hydrogen production.
- One unique advantage of the DNA-based patterning method is its ability to create complex arrangements of multiple cell types through the use of several different anchoring sequences. We will use this capability to generate intimately interspersed combinations of photosynthetic microorganisms with complementary light absorption characteristics and nutrient requirements. We will then determine if new symbiotic relationships emerge, in which two cell types work together to produce more overall hydrogen than a single cell type alone.
- Several species of anaerobic bacteria have been observed to reduce extracellular metal ions by directly coupling them to their electron transport chains. The DNA-based adhesion method offers an effective way to study these organisms by attaching them directly to electrodes. We will continue to work with Dr. Caroline Ajo-Franklin to attach natural and engineered bacteria to metal surfaces, with the goal of directly harnessing the electrical current that they produce.
- In terms of mammalian cells, we will use micropatterned cell-capture surfaces to study the ways in which mammalian cells self-organize into complex functional structures. We will begin by positioning cardiac myoblasts and fibroblasts in specific locations on glass slides. We will then observe how the initial formations restructure into coated myotubes upon the addition of electrical currents and other developmental cues.

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## Regulation of H<sub>2</sub> and CO<sub>2</sub> Metabolism: Factors Involved in Partitioning of Photosynthetic Reductant in Green Algae

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Overall Research objectives: The overall objective of this project is to develop fundamental understanding about the regulation of the partitioning of photosynthetic reductants between the H<sub>2</sub>-production and the CO<sub>2</sub>-fixation pathways. Specific objectives are to: (a) identify additional protein factors whose expression may be required for optimal hydrogenase expression; (b) identify active promoter regions and transcriptional elements for the two algal hydrogenases; and (c) determine whether the recently identified FIXL homologs in *C. reinhardtii* play a role in O<sub>2</sub>-sensing mechanism and mediate components of the anoxic regulatory response that leads to hydrogenase expression.

### Significant Achievements in 2010

- Using high-quality sensors developed by Dr. Posewitz, we identified 12 candidate mutants displaying altered hydrogen production from insertional libraries provided by Dr. Hamel. We have successfully assigned the interrupted loci of 4 of the insertional mutants to proteins of unknown functions, and the molecular characterization of the remaining mutants is in progress.
- To investigate the potential role of the FIXL homologs in O<sub>2</sub>-sensing in *C. reinhardtii*, we successfully purified two recombinant polypeptides containing the heme-binding domains of FIXL1 and FIXL5, as well as mutants of these proteins in the heme-binding domain. Spectrophotometric titration of FIXL1 and FIXL5 with hemin showed saturation at a ratio of 1:1. Under non-denaturing electrophoresis, heme-specific staining for FIXL1 and FIXL5 showed a single, diffuse band similar in specificity to that of hemoglobin. Oxygen binding measurements on the reduced peptide–heme complexes showed that they bind O<sub>2</sub> and CO. The dissociation constants (*K<sub>d</sub>*) of O<sub>2</sub> for FixL1 and FixL5 were 116 and 187 μM, respectively. However, CO has much higher affinity for both proteins, with *K<sub>d</sub>* values of 35 and 40 μM, respectively.
- To understand the mechanism that underlies the anoxic regulation of hydrogenase in *C. reinhardtii*, we fused the truncated promoters of HYDA1 and HYDA2 to the SNAP reporter gene. Transformants expressing the SNAP protein with truncated promoters were shown to be transcribed only under anaerobic condition. The results indicate that region between position -144 and -1 for HYDA1 and -149 to -1 for HYDA2 with respect to the transcription start site is required for anaerobic specific gene expression.
- To probe the regulation and metabolic function of novel algal hydrogenases, we assessed H<sub>2</sub> production in the trebouxiophyte *Chlorella* NC64A, which is the first alga known to encode both the H and F-clusters of hydrogenases. We show for the first time that F-cluster-containing hydrogenases are coupled to both anoxic photosynthetic electron transport and dark fermentation in a green alga. H<sub>2</sub> photoproduction in *Chlorella* NC64A is as sensitive to O<sub>2</sub> inactivation as in *C. reinhardtii*. Phylogenetic analysis indicates that all known algal HYDA enzymes are monophyletic, suggesting that they emerged once within the algae. Furthermore, phylogenetic reconstruction indicates that the multiple *HYDA* copies in the algal taxa are the result of gene

duplication events that occurred independently in each algal lineage, and that the ancestor of the Trebouxiophyceae and Chlorophyceae likely encoded a single, H and F-cluster-containing HYDA.

- To define the role of individual hydrogenases in green algae, we screened a *Chlamydomonas hyda2* mutant background for mutants lacking all hydrogenase activity. A double *hyda2hyda1* mutant has been identified and is being further characterized.

#### Science objectives for 2011

- Selected *C. reinhardtii* mutants showing aberrant H<sub>2</sub> production will be further analyzed with respect to HYDA1 and HYDA2 transcript and protein levels. Complementation of the the phenotype with the transformed gene will be performed and physiological studies will be carried out to determine the role of the disrupted gene in algal metabolism.

- To understand the involvement (if any) of FIXL proteins in signal transduction in response to O<sub>2</sub>, promising FIXL candidates will be tagged (streptavidin or histidine tag) and over expressed in *C.reinhardtii*. Tagged FIXL proteins will be used to search for interacting proteins involved in the signal transduction pathway.

- To understand the mechanism that underlies the anoxic regulation of hydrogenase, we will express SNAP proteins under the regulation of the HYDA1 and HYDA2 promoters into *hyda1/hyda2* single or double mutants. Additionally, we will perform DNA binding assays to identify the transcription factors binding to the promoter regions of each hydrogenase.

- The two *hyda1* mutants that were isolated are being crossed into single mutant backgrounds. Once completed, comprehensive analysis of the individual function of each enzyme in algal metabolism will be examined.

#### References to work supported by this project, 2008-2010

1. Seibert, M., P. King, M.C. Posewitz, A. Melis, and M.L. Ghirardi. 2008. "Photosynthetic Water-Splitting for Hydrogen Production," in Bioenergy (J. Wall, C. Harwood, and A. Demain, Eds.) ASM Press, Washington DC, pp. 273-291.
2. Posewitz, M.C., A. Dubini, J.E. Meuser, M. Seibert and M.L. Ghirardi. 2008. "Hydrogenases, hydrogen production and anoxia" in The *Chlamydomonas* Sourcebook, Vol 2: Organellar and Metabolic Processes (D. Stern, ed.) Elsevier Scientific 217-255.
3. Ghirardi, M.L., A. Dubini, J. Yu and P.C. Maness. 2009. "Photobiological hydrogen-producing systems". Chem. Soc. Rev. 38, 52-61.
4. Meuser, J.E., G. Ananyev, L.E. Wittig, S. Kosourov, M.L. Ghirardi, M. Seibert, G.C. Dismukes and M.C. Posewitz. 2009. "Phenotypic Diversity of Hydrogen Production in Chlorophycean Algae Reflects Distinct Anaerobic Metabolisms". J. Biotechnol. 142, 21-30.
5. Maness, P.C., J. Yu, C. Eckert and M.L. Ghirardi. 2009. "Photobiological hydrogen production – prospects and challenges" Microbe 4, 275-280.

#### Research papers in preparation/submitted

1. Narayana Murthy, U.M., Matt Wecker, Mathew C. Posewitz and Maria L.Ghirardi (2010). Cloning, expression and physical characterization of novel FixL-heme binding domains of oxygen sensing proteins in *Chlamydomonas reinhardtii*
2. Narayana Murthy U.M. and Maria L. Ghirardi (2010). "Algal Hydrogen production" in *Encyclopedia of Biological Chemistry*, submitted.
3. Jonathan E. Meuser, Eric S. Boyd, Gennady Ananyev, Devin Karns Narayana Murthy U.M. Randor Radakovits G. Charles Dismukes, Maria L. Ghirardi, John W. Peters, and Matthew C. Posewitz . Presence of accessory FeS clusters in the *Chlorella* NC64A [FeFe]-hydrogenases: new details into the evolution and diversity of algal hydrogen metabolism. *Planta*, submitted.

## Understanding the Mechanism by which Non-Catalytic Carbohydrate Binding Modules Contribute to Plant Cell wall Degradation

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**Overall research goals:** The research objectives of this program will test the following hypotheses:

(i) The biological rationale for the diversity of bacterial non-catalytic carbohydrate binding modules (CBMs) found in nature is to maximize the range of plant cell walls that can be degraded by the cognate enzymes. (ii) The essential synergy between glycoside hydrolases that display complementary activities is promoted by CBMs through the targeting of these biocatalysts to the same region of the plant cell wall. (iii). Specific CBMs, which target the ends of polysaccharide chains, direct glycoside hydrolases to regions of cell walls that are particularly susceptible to enzyme attack. (iv) The catalytic module linked to a specific CBM has evolved biochemical properties that are complementary to the targeting function of the appended CBM.

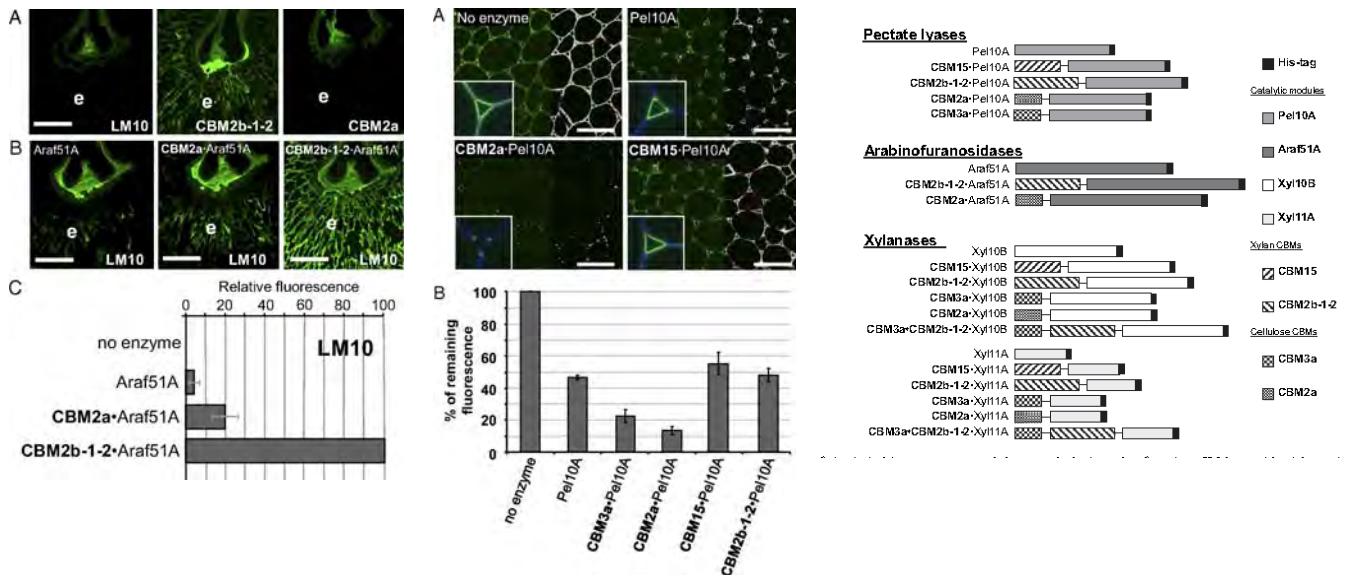


Figure 1. Left Panel immunofluorescence microscopy of CBMs and monoclonal antibodies binding to transverse sections of wheat grain endosperm cell walls. (A) The lack of binding of LM10 indicated that the endosperm cell walls are heavily arabinosylated. (B) Detection of the LM10 epitope in wheat grain sections after treatments with the arabinofuranosidase Abf51A alone, appended with CBM2a and linked to CBM2b-1-2. (C) Histogram showing relative LM10-associated fluorescence in wheat grain endosperm cell walls after no treatment and treatments with Abf51A constructs. Central Panel: Effect of pectate lyase (Pel10A) treatments on the detection of the JIM5 pectic HG epitope in primary cell walls of transverse sections of tobacco stem pith parenchyma. (A) Indirect immunofluorescence microscopy of JIM5 binding after Pel10A (no treatment, Pel10A alone, CBM2a-Pel10A, CBM15-Pel10A). JIM5-tagged FITC fluorescence is shown as observed on the left half of each micrograph. The right half of each micrograph shows the same micrograph with overlain false colours reflecting fluorescence intensities. Inset images of JIM5-fluorescence combined with Calcofluor White fluorescence (blue) show higher magnification of cell walls in the region of intercellular spaces. Scale bar, 200  $\mu$ m. (B) Histogram showing relative fluorescence intensities reflecting Pel10A treatments on JIM5 binding to the sections. Means  $\pm$  s.e.m. Right Panel: The constructs used in the experiments.

Significant achievements since January 2010: Initial studies have focused on the potential enzyme-targeting function of CBMs in the context of intact primary and secondary cell wall deconstruction. The capacity of a pectate lyase to degrade pectic homogalacturonan in primary cell walls was potentiated by cellulose-directed CBMs but not by xylan-directed CBMs. Conversely, the arabinofuranosidase-mediated removal of side chains from arabinoxylan in xylan-rich and cellulose-poor wheat grain endosperm cell walls was enhanced by a xylan-binding CBM but less so by a crystalline cellulose-specific module. The targeting actions of CBMs therefore have strong proximity effects within cell wall structures, explaining why cellulose-directed CBMs are appended to many non-cellulase cell wall hydrolases. In addition to the functional analysis of the CBM targeting function *in vivo* we have also explored the specificity of two novel CBMs that bind to a range of polysaccharides, and the structural basis for this broad specificity, a novel CBM family that binds to diverse ligands such as rhamnogalacturonan I and levan.

Science objectives for 2010-2011:

- Explore the mechanism by which the rhamnogalacturonan I specific CBM binds to its ligands. The 3D structure of the module will be sought in complex with rhamnogalacturonan-derived oligosaccharides.
- The CBM that binds to levan causes a 100 fold increase in catalytic activity of the appended levanase against the polysaccharide. This elevation in activity is extremely high compared to other CBMs (2-5 fold enhancement in activity). It is possible that the CBM, by binding to individual levan chains dissociates the proposed helical bundle formed by these polymers. This will be tested by trans experiments (incubating enzyme and CBM as discrete entities with levan), SAXs (small angle X-ray scattering) and scanning microscopy.
- Our previously published work demonstrates the capacity of cellulose-specific CBMs to potentiate the activity of both xylanases and pectinases. We will extend this work by investigating the capacity of mannan and cellulose-specific CBMs to enhance the activity of mannanases against intact cell walls containing high concentrations of galactomannan and glucomannan, respectively. By deploying catalytic modules that, respectively, hydrolyze mannan and glucomannan we will explore the interplay between specificity of the enzyme and CBMs.
- We have previously shown that a family 35 CBM is able to target glucuronic acid in xylans and anhydrogalacturonic acid in lyase treated pectins. We will explore how the specificity of the CBM contributes to the potential synergy between pectate lyase and xylanase action.

References to work supported by this project 2007-2009:

1. Hervé C, Rogowski A, Blake AW, Marcus SE, Gilbert HJ, Knox JP. (2010) Carbohydrate-binding modules promote the enzymatic deconstruction of intact plant cell walls by targeting and proximity effects. *Proc. Natl. Acad. Sc. USA* **107**:15293-15298
2. Montanier C, Flint JE, Bolam DN, Xie H, Liu Z, Rogowski A, Weiner D, Ratnaparkhe S, Nurizzo D, Roberts SM, Turkenburg JP, Davies GJ, Gilbert HJ (2010) Circular permutation provides an evolutionary link between two families of calcium-dependent carbohydrate binding modules. *J. Biol. Chem.* **285**: *In the press*



## **Functional Genomics Analysis of Plant Resistance to Pathogens-Impact of the Cell Wall**

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### Overall research goals:

- For metabolites that undergo changes during infection of Arabidopsis by *Alternaria brassicicola*, investigate functional roles in this interaction using plant reverse genetics.
- Survey Arabidopsis mutants with defects in cell wall structure to determine the types of cell wall changes that impact resistance to microbial pathogens.

### Significant achievements in 2008-2010:

- Previously, we identified numerous metabolites whose levels are altered during infection of Arabidopsis by the fungal necrotroph *Alternaria brassicicola*. Exogenous treatment of plants with several of these compounds, including ascorbate, gamma-amino butyric acid (GABA), and mannitol, enhanced fungal growth. Treatment with trehalose inhibited fungal growth. Treatment with glucose had no effect, so it is unlikely that effects on fungal growth resulted from metabolites serving as a carbon source for the fungus. We have attempted to alter levels of some of these metabolites using plant mutants, which we have tested for altered susceptibility to *Alternaria*. In the case of mannitol, we found that mutants with defects in a putative mannitol dehydrogenase gene (*At4g37970*) are more susceptible to *Alternaria*. However, we have not detected mannitol dehydrogenase activity in the encoded enzyme, suggesting that the mutant phenotype may not result from loss of mannitol dehydrogenase activity. In the case of ascorbate, we have studied *vtc* mutants, which have reduced levels of ascorbate. These mutants are more susceptible to *Alternaria*, which was unexpected since addition of exogenous ascorbate increases susceptibility. It is known that *vtc* mutants have elevated levels of salicylic acid, and this may in fact be the cause of the susceptibility phenotype. In the case of GABA, we have studied *pop2* mutants which have elevated levels of GABA. The susceptibility of these mutants does not differ from wild-type plants. Considering all this data, we conclude that these metabolites can affect the Arabidopsis-*Alternaria* interaction, but that genetic manipulation of the plant does not alter metabolite levels enough to cause a measurable change in plant susceptibility to the pathogen.
- We collected approximately 75 mutants thought to have alterations in cell wall structure. Most of these came from a functional genomics project led by Nick Carpita in which many mutants were studied using Fourier transform infrared spectroscopy (FTIR). We selected mutants that had altered FTIR spectra. Some other mutants were added based on published evidence of altered cell walls. In assays using the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* ES4326 (*Pma* ES4326), we have detected modest, but statistically significantly enhanced growth in mutants lacking function of 1 of 6 enzymes of phenylpropanoid metabolism, 1 of 2 cellulose synthases, 6 of 16 glycosyl transferases, 1 of 2 callose synthases (a 3<sup>rd</sup>, *pmr4* is known to be more resistant, and we confirmed this), 1 of 4 expansins, 3 of 6 xyloglucan endotransglucosylases, 2 of 8 glycanases, 1 of 2 rhamnogalacturonan I lyases, 1 of 2 structural proteins, and 6 of 11 pectinesterases. We have also screened for altered susceptibility to *Alternaria*, and found 4 mutants that appear more resistant than wild-type.

### Science objectives for 2010-2011:

- To be sure of our *Pma* ES4326 disease phenotypes, it is necessary to demonstrate similar phenotypes in at least one additional mutant allele of each gene. This work is in progress. We are also constructing several combinations of double mutants with defects in two pectinesterase genes. These will be tested to determine whether or not they show stronger susceptibility phenotypes.
- The *Alternaria* screen that we have already done was under conditions more likely to reveal enhanced-resistance than enhanced-susceptibility mutants. We are repeating the screen under different conditions to test for enhanced susceptibility.
- Some alterations in the cell wall are known to affect levels of major defense signaling hormones, salicylic acid and jasmonic acid. Levels of these hormones before and during infection will be determined in mutants with confirmed changes in pathogen susceptibility. For a small number of mutants with the strongest pathogen phenotypes, we will use expression profiling to search for and characterize changes in defense signaling processes.

References to work supported by this project 2006-2008:

1. Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J., and Katagiri, F. Network Properties of Robust Immunity in Plants. *PLoS Genetics* 5(12): e1000772. doi:10.1371/journal.pgen.1000772 (2009).



## **From the Soil to the Seed: Metal transport in Arabidopsis**

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Dr. David Salt, Purdue University

Overall research goals: Deficiencies of micronutrients such as Fe and Zn commonly limit plant growth and crop yields. The long-term goals of our program are to understand how plants acquire metal micronutrients from the soil and distribute them throughout while protecting themselves from the potential damage metals can cause to living tissues. With an increased emphasis on growing plants for biofuel production, our studies are helping to illuminate how to improve seedling growth in soils with limited micronutrient availability. Our lab has previously identified the major Fe transporter responsible for Fe uptake from the soil, IRT1, a founding member of the ZIP family of metal transporters. Arabidopsis is predicted to have 16 ZIP genes and we are systematically characterizing all the family members. In addition to the ZIP transporters, we are also exploring the function of a Fe transporter, VIT1, that localizes to the vacuolar membrane. We are particularly interested in determining how various ZIP transporters and VIT1 influence the metal content of the seed.

Significant achievements in 2008-2010: We are continuing to examine metal distribution using XRF in seeds from a variety of metal transporter mutants. We have been awarded beam time at three different DOE-supported facilities: the National Synchrotron Light Source (NSLS) at Brookhaven National Lab, the Advanced Photon Source (APS) at Argonne National Lab and the Stanford Synchrotron Radiation Lightsource (SSRL). We have recently added a Vortex® Silicon Drift Detector to NSLS beamline X26A that has greatly enhanced our imaging capability and shortened our capture time. This detector is a single element X-ray detector produced from high purity silicon using state-of-the-art CMOS production technology. It features excellent energy resolution (<136 eV FWHM at Mn K $\alpha$  is typical) and a high count rate capability (input rate >1 Mcps) that are particularly well suited for our applications. At a very short peaking time of 0.25  $\mu$ s, an output count rate of 600 kcps is achieved. A unique feature of these detectors is their ability to process high count rates with virtually zero loss in energy resolution and no peak shift with count rate. We have used the system to enhance detection of low energy X-ray emissions such as those from Ca K $\alpha$ , K K $\alpha$  and S K $\alpha$ . Enhanced detection of these elements by fluorescence microtomography is crucial for our studies on the elemental composition of plants, but is not possible using existing detectors at the beamline due to self-absorption effects. Installation has not only eliminated self-absorption, but has also allowed the switch to fly-scanning, making scan times appreciably shorter; tomographic sections of Arabidopsis seed now take less than 2 hours to complete, in comparison with approximately 7-10 hours for a high resolution image (see an example of a high resolution image in Kim et al., 2006 Science 314:1295). Fly-scanning allows for greater replication, greater accessibility to three dimensional imaging, high-throughput analysis and analysis of fresh, hydrated tissue such as stems, leaves and roots. This fall, we will begin imaging seed within the developing siliques, both at NSLS and SSRL.

We have just finished characterizing an Arabidopsis line that overexpresses the vacuolar transporter VIT1 and have compared its iron responsiveness to wild type and as well as to a *vit1* loss of function line (Kim et al., in preparation). Ectopic expression of VIT1 leads to increased iron accumulation in leaves. This increase is accompanied by increased ferric iron chelate activity and increased levels of IRT1 protein, the high affinity iron uptake components in Arabidopsis roots. The loss of *VIT1* attenuates the induction of IRT1 protein, resulting in delayed responses to high metal treatment. 35S-VIT1 plants deposit less iron in seeds and display altered iron distribution in the embryo. Not surprisingly, 35S-VIT1 seedlings grow less well than wild type on iron limiting soil. Our results suggests that VIT1, by moderating vacuolar iron content, plays a pivotal role in cellular iron homeostasis and the regulation of iron uptake. We are also characterizing loss of function and over expression lines for the two VIT orthologs in rice.

From our continuing analysis of the ZIP family members, it is becoming increasingly clear that single, loss of function mutants seldom have strong metal-related phenotypes. A quadruple *zip* mutant is unable to set seed unless watered with high levels of zinc, suggesting roles for these particular four ZIP transporters in seed development.

Science objectives for 2010-2011:

- Continue to capture images using X-ray fluorescence spectroscopy at NSLS, APS and SSRL.
- Continue characterization of *zip* loss of function lines, including triple and quadruple mutants.
- Continue characterization of Arabidopsis and rice lines with altered *VIT* expression, including lines where *VIT* expression is being driven by a seed-specific promoter.

References to work supported by this project 2008-2010:

- Guerinot, M.L., I. Baxter and D.E. Salt. 2009. From the ionome to the genome: Identifying gene networks that control the mineral content of plants. *In Plant Systems Biology*. G. Coruzzi and R. Gutierrez (eds.), Wiley-Blackwell. Annual Plant Reviews 35: 290-303.
- Punshon, T., A. Lanzirotti and M.L. Guerinot. 2009. Using synchrotron x-ray fluorescence microprobes to study metal(loid) homeostasis in plants. *Annal. Bot.* 103:665-672.
- Palmer, C. and M.L. Guerinot. 2009. Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Nature Chem. Biol.* 5:333-340.
- Morrissey, J. and M.L. Guerinot. 2009. The good, the bad and the ionome. *Chem. Rev.* 109:4553-4567.

## P-type ATPase Ion Pumps in Plants

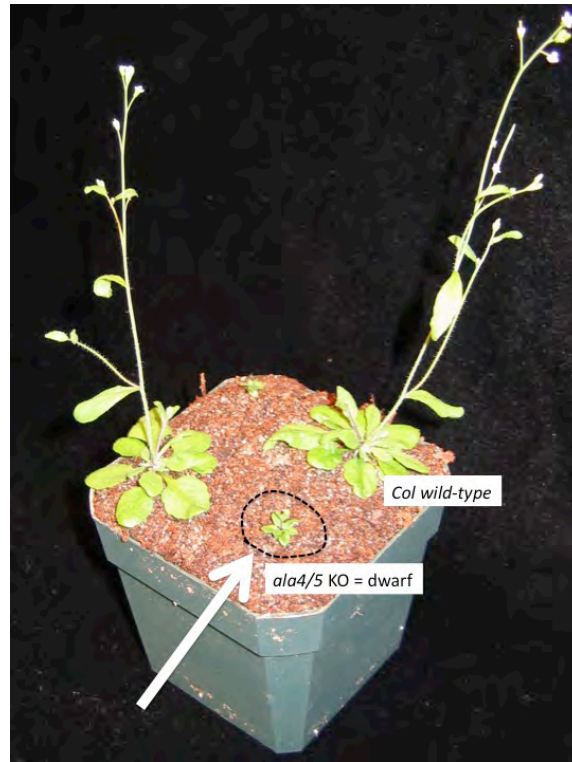
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Overall research goals: The long-range objectives of the research are to understand the biochemistry and biological functions associated with different P-type ATPases (ion pumps). Different subgroups of P-type ATPases have different ion translocating specificities, including  $H^+$ ,  $Ca^{2+}$ ,  $Na^+$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Cd^{2+}$ , and lipids. In eukaryotic cells, these pumps are thought to utilize between 25 to 50% of the cellular ATP (e.g. Principles of Biochemistry, 2006, 4<sup>th</sup> edition, Horton et al., pg 282).

Significant achievements in 2008-2010: Of the 46 P-type ATPases in Arabidopsis, 12 belong to a subfamily of aminophospholipid-dependent ATPases (ALAs), which are thought to function as flippases to reorient specific lipids across a phospholipid bilayer. Gene knockouts have been used to uncover important functions of these unusual ATPases in plant growth, reproduction, and responses to the environment. For example, a double knockout of the *ala4* and 5 subgroup results in a dwarfed, but otherwise healthy plant (*see figure*). A double knockout of the *ala6* and 7 subgroup results in plants with poor seed set, due to a pollen defect that causes tubes to be short and slow growing. A single knockout of *ala3* results in plants that are highly sensitive to various growth conditions and stresses, both in reproductive and vegetative development. For example, hot days and cold nights exacerbate a defect in mutant pollen transmission efficiency by an additional 10-fold, when assayed in competition with wild-type pollen. Similarly *ala3* (-/-) root growth is blocked by relatively mild temperature increases or decreases from 22°C (e.g. down to 15°C, or up to 30°C).



Our current focus is on using pollen to uncover cellular functions for different ALAs. Pollen is an experimentally accessible plant cell that undergoes a rapid program of desiccation and rehydration, and is among the fastest growing plant cells known. Pollen tube tip growth provides an ideal model system to study the secretory pathway, which is highly organized to selectively deposit new materials at the apical tip. Our working model is that ALA-type flippases function to induce membrane curvature, or to change the abundance of specific head groups on the surface of a membrane bilayer. To define the cellular defects associated with knockouts for *ala3* and the *ala6/7*

mutants, we have begun to study changes to the organization and dynamics of the secretory pathway in growing pollen tubes.

The Harper Lab has also begun to explore the potential of engineering ALA proteins, and other P-Type ATPases, to remodel endomembrane systems as a strategy to develop lipid-rich biofuel feedstocks. Target constructs have been made and introduced into *Arabidopsis*. TLC (thin layer chromatography) analyses are being used to evaluate changes in the abundance of various lipid classes.

#### Science objectives for 2010-2011:

- Test the hypothesis that ALA3 is required for proper association of membrane vesicles with the actin cables that guide secretory vesicles to the pollen tube apical tip.
- Test the hypothesis that ALA6 and 7 are required at the plasma membrane for rapid rates of endocytosis.
- Determine whether modifications to P-type ATPases can be used to remodel the architecture of endomembrane systems to increase the lipid content of a typical plant cell.

#### References to work supported by this project, 2008-2010:

1. Poulsen, LR, RL López-Marqués, S McDowell, J Okkeri, T Pomorski, JF Harper, and MG Palmgren (2008) The Arabidopsis P4-ATPase ALA3 localizes to the Golgi and requires a beta-subunit to function in lipid translocation and secretory vesicle formation. **Plant Cell** 20:658-76
2. Li X, Chanroj S, Wu Z, Romanowsky SM, **Harper JF**, Sze H (2008) A Distinct Endosomal Ca<sup>2+</sup>/Mn<sup>2+</sup> Pump Affects Root Growth through the Secretory Process. **Plant Physiol.** 147: 1675-89
- 3.
4. Chang, IF, A Curran, R Woolsey, D Quilici, J Cushman, R Mittler, A Harmon, and JF Harper (2009) Proteomic profiling of tandem affinity purified 14-3-3 protein complexes in *Arabidopsis thaliana*. **Proteomics.** 9:2967-85
- 5.
6. Bousiac, Y, SM Lee, S Romanowsky, R Blank, C Sladek, WS Chung, JF Harper. (2010) Disruption of a vacuolar calcium ATPase in Arabidopsis results in the activation of a salicylic acid-dependent programmed cell death pathway. **Plant Physiology** (in press).

## Macromolecule studies using time-resolved, multi-spectral, imaging

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Collaborators: Prof. Haw Yang, Dept. of Chemistry, Princeton University, NJ  
Dr. Darryl Sasaki, Sandia National Laboratories, Livermore CA  
Dr. Jeanne Stachowiak, Sandia National Laboratories, Livermore CA

Overall research goals: In this research program we study local chemical environments and their fluctuations in systems from single macromolecules to proteins bound on lipid membranes. Our work focuses on studies of macromolecular interactions with lipid membranes and the relationship between reactivity and macromolecule structure fluctuations. Critically important to this program are collaborations that enable studies of biophysical systems from the molecular to the macroscopic scale. With this approach we attempt to directly relate molecular scale processes to macroscopic phenomena. For this work we use a variety of imaging techniques including time-resolved multispectral confocal imaging that simultaneously measures fluorescence spectra and lifetimes with single-molecule sensitivity. This instrument is designed to extract the maximum information about the local environment from fluorescent probes. It records the wavelength ( $\lambda$ ), and emission time relative to excitation ( $\tau$ ) for each detected fluorescence photon along with its absolute detection time ( $\Delta$ ) so that correlations among all the fluorescence properties are maintained. Fully correlated photon records of  $\lambda$ ,  $p$ ,  $\tau$ , and  $\Delta$  improve the capability of fluorescence measurements to characterize local chemical environments. The record of fluorescence photon emission provides detailed information on chemical processes, including energy transfer and conformational changes, on time scales from picoseconds to minutes. The experimental capabilities are combined with the development of rigorous analysis based on maximum information methods to allow us to more clearly resolve the fluctuations of macromolecules and the chemical environments around them.

Significant achievements 2008-2010: Many important biological processes are enabled by cooperation between proteins and lipid membrane structure. We are developing structured membranes that strongly bind proteins to study this cooperation. The attachment method uses lipids that selectively chelate  $\text{Cu}^{+2}$  which then strongly binds his-tags commonly used on recombinant proteins to purify them after expression. Two forms of the copper chelating lipid, Figure 1, have been synthesized by Dr. Darryl Sasaki, at Sandia National Laboratories. Both forms use an iminodiacetic acid headgroup to bind copper, but one lipid, DSIDA, forms a gel phase membrane while the other, DOIDA, forms a fluid phase membrane. The availability of gel and fluid phase lipids provides opportunities to tune the membrane structural characteristics. We have

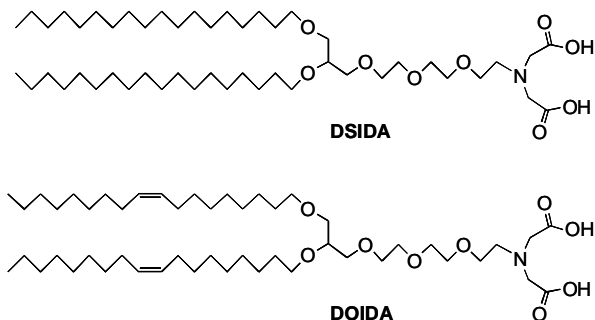


Figure 1. Structures of IDA-functionalized lipids.

studied supported membranes made from mixtures of a fluid phase lipid POPC and DSIDA and found that upon  $\text{Cu}^{+2}$  addition submicron gel phase membrane domains form that are extremely high affinity sites for his-tagged protein binding, shown on the left of Figure 2. Fluorescence correlation spectroscopy measurements on these domains show them to be gel phase imbedded in a fluid matrix. In

contrast, mixtures of POPC and DOIDA form completely fluid phase membranes. These membranes uniformly bind his-tagged proteins that freely diffuse on the surface. We have also incorporated DSIDA into giant unilamellar vesicles. Remarkably, upon binding of protein to the resulting DSIDA domains long, rigid lipid nanotubes develop spontaneously. The results are shown on the right of Figure 2.

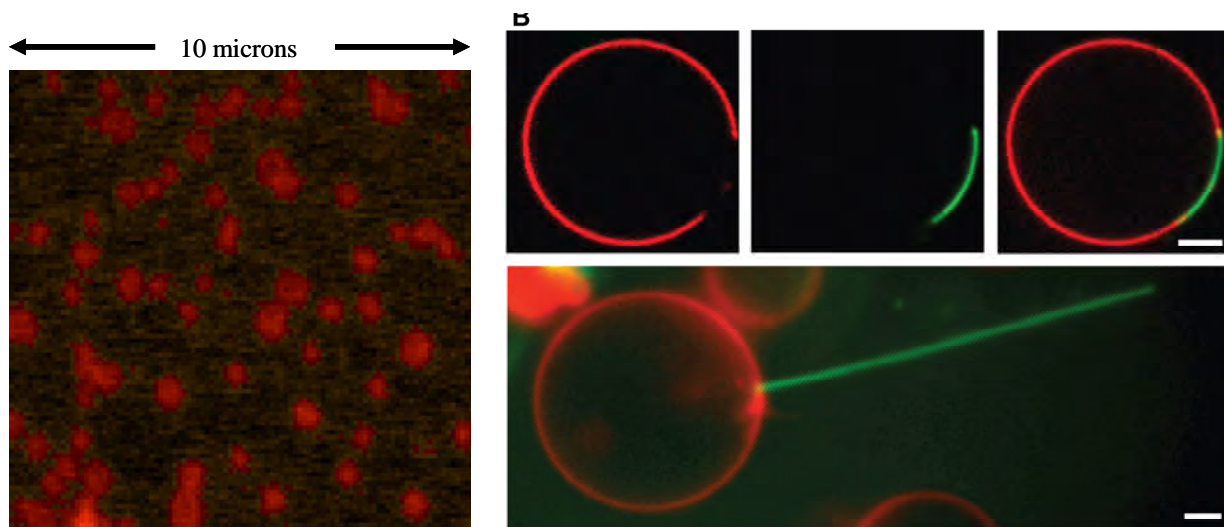


Figure 2. Left, supported bilayer of DSIDA/POPC shows submicron domains with high protein affinity. Right, DSIDA domains binding proteins in a giant unilamellar vesicle are curved into lipid nanotubes.

The results show that the high affinity binding of proteins in a confined area is a powerful mechanism to produce membrane curvature. Studies of several aspects of this lipid-protein system are ongoing.

We have been studying the relation between structure and ligand binding in small DNA aptamers. DNA aptamers that bind proteins such as thrombin have multiple structures in the unbound state. Using FRET labelling of the DNA strands we have measured structural changes in these aptamers as a function of solution ionic composition and strength. We are currently extending these studies to determine how the structures change upon protein binding and the importance of the solution structure to the reactivity of the aptamers.

#### Recent publications:

1. Stachowiak, JC, CC Hayden, DY Sasaki. (2010). Steric confinement of proteins on lipid membranes can drive curvature and tubulation. *Proceedings of the National Academy of Sciences of the United States of America* 107:7781.
2. Hayden, CC, JS Hwang, EA Abate, MS Kent, DY Sasaki. (2009). Directed formation of lipid membrane microdomains as high affinity sites for His-tagged proteins. *Journal of the American Chemical Society* 131:8728.
3. Liu, H, GD Bachand, H Kim, CC Hayden, EA Abate, DY Sasaki. (2008). Lipid nanotube formation from streptavidin-membrane binding. *Langmuir* 24:3686.
4. Xu, CS, H Kim, CC Hayden, H Yang. (2008). Joint statistical analysis of multi-channel time series from single quantum dot-(Cy5)<sub>n</sub> constructs. *Journal of Physical Chemistry B* 112:5917.
5. Xu, CS, H Kim, H Yang, CC Hayden. (2007). Multiparameter fluorescence spectroscopy of single quantum dot-dye FRET hybrids. *Journal of the American Chemical Society* 129:11008.



## Characterization of an H<sub>2</sub> Producing Biological System operating at 1 nM H<sub>2</sub> Concentration

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Overall research goals: Research objectives are to study genes within *Desulfovibrio* strain G20 that are involved in syntrophic interactions in coculture with a Syntrophic butyrate degrading bacterium and a during lactate oxidation in coculture with a hydrogenotrophic methanogen. Specifically, this has involved identifying genes in *Desulfovibrio* needed for syntrophic growth by screening mutants for loss of ability to grow in coculture. Mutants and mutated genes are being characterized to determine their specific function during syntrophic growth. Characterization involves a determination of the effect of the mutation on growth characteristics as well as the properties of the protein encoded by that gene.

### Significant achievements 2008-2010:

*Characterization of the role of Tetraheme Cytochrome C<sub>3</sub>, Fe only Hydrogenase and Quinone Reactive complex during syntrophic H<sub>2</sub> production.* These three mutants were shown by our group to be unable to grow on H<sub>2</sub> and formate and a mechanism by which the three enzymes are coupled to generation of a proton motive force has been proposed (Li et al, 2009). Mutants in each of these genes were shown to be unable to grow syntrophically with a methanogen on lactate and were subsequently shown to be unable to produce H<sub>2</sub> when washed cells were incubated with lactate, but could effectively produce H<sub>2</sub> when washed cells were incubated with pyruvate. This showed that each of these proteins has a role in (energy dependent) H<sub>2</sub> production (as H<sub>2</sub> production from lactate requires a PMF). A schematic for a proposed role for these proteins is shown below (Fig. 1).

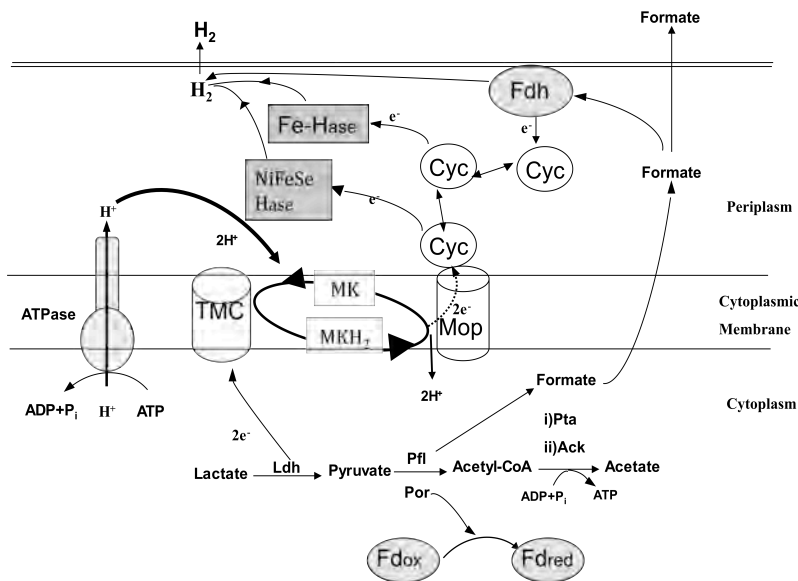


Figure 1. Model for the oxidation of lactate during syntrophic growth. Abbreviations for enzymes are as follows: TMC, uncharacterized transmembrane complex; LDH, lactate dehydrogenase; Qrc, Quinone reductase complex; Tplc<sub>3</sub>, cytochrome c<sub>3</sub>; Hase, hydrogenase.

*Formation of 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 they formed large complexes. Gels run with a mutant in the QMO complex had three absent bands in comparison to the parental strain (Fig. 2). Bands were excised and proteins were identified by mass spectroscopy. One of the bands (C) appears to be the qmo complex while another band (B) appears

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. As well, the QMO mutant will grow on sulfite but not on sulfate (Zane et al., 2010). This is the first evidence showing a physical interaction between these proteins.

Figure 2. Blue native gel and peptide identification data for QMO mutant and WT.

*Identification of genes involved in interaction among syntrophic bacteria.* Based on a screen of *Desulfovibrio* mutants during growth with *Syntrophomonas wolfei*, several genes were identified to be required for syntrophic growth. These include *flhA* involved in flagellum biosynthesis, *FliF* a component of the flagellum and *TadC*, a pilus assembly protein. These data suggest a role for chemotaxis or some additional flagellar function during syntrophy.

#### Science objectives for 2010-2011:

- Further experiments will be done involving purification of enzymes to determine the specific role of the electron transport proteins and their chemical interactions.
- Addition work will be done to demonstrate that QMO is physically linked to APS reductase and experiments will be done to address similar questions with other electron transport mutants.
- Work on flagellum and Pilus mutants will be continued to determine the roles for these proteins in syntrophic growth.

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## The Lignin Pathways: Towards Establishing Lignin Primary Structures and Redirecting Carbon Flux into Lignins Through Upstream Aroenate Dehydratase and Related Manipulations

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**Introduction:** This contribution summarizes some of the recent exciting work from our laboratory: the pioneering work: with aroenate dehydratases (ADTs), the final step in phenylalanine biosynthesis, and its importance in differentially controlling carbon allocation to lignins and proteins; in recent progress in defining lignin primary structures; in extending our discoveries of how flavor/fragrance plant chemicals, such as eugenol/isoeugenol are formed, and their potential role as new commodity chemicals. This includes our discoveries and progress to date in generating new woody plant lines accumulating selected sustainable petrochemical replacements.

**Aroenate dehydratases: An upstream biochemical step differentially modulating carbon allocation into the downstream lignin pathway.** Pioneering work by Roy Jensen and colleagues,<sup>1</sup> as well as by Eric Conn and coworkers,<sup>2</sup> provisionally established that the final step in phenylalanine biosynthesis in plants was catalyzed by aroenate dehydratase (ADT), although attempts to purify the enzyme(s) involved were unsuccessful. Following the genome sequencing of *Arabidopsis* in 2000, we then both discovered and validated that a 6-membered multigene family in this plant species encoded genes for ADTs.<sup>3</sup> One reason for our interest in this enzymatic step was as to whether individual isoforms might differentially control carbon allocation into phenylpropanoid (lignin) metabolism vs. that of, for example, protein synthesis. In our more recent work,<sup>4</sup> we have now generated a variety of ADT mutants, in order to probe and resolve this question. In this regard, ADT T-DNA mutants were obtained for 5 of the 6 ADT genes, with the sixth not being fully suppressed. Each of the 5 ADT mutant lines, following screening to obtain them in homozygous form, was initially examined to assess effects on lignin deposition. In addition, double mutants were generated in all possible permutations, as well as triple and quadruple mutants. The analysis of these different plant lines, over the life-span of growth and development of *Arabidopsis*, has now provided exciting new insights into how carbon allocation can be differentially controlled into the lignin-forming apparatus and the corresponding vasculature.

Specifically, the various mutants so obtained gave phenotypes that were differentially reduced in lignin content, from up to nearly ~73% reduction of lignin content in some lines to others having essentially little change in overall lignin amounts when compared to the wild type (WT) line. As a result, this is the first example of not only differential reduction in lignin contents through manipulation of different members of a multi-gene family, but perhaps even more interestingly that carbon can be differentially allocated by manipulation of an upstream enzymatic step (namely aroenate dehydratase, ADT). In addition, histochemical staining for the presence/absence of guaiacyl (G) and syringyl (S) lignins was also most informative. In this regard, histochemical staining using phloroglucinol-HCl for guaiacyl (G) lignin indicated that the interfascicular fiber (*if*) regions may have been most greatly affected in these manipulations in terms of G-lignin deposition. Furthermore, pyrolysis GC/MS analyses of micro-dissected (*if*) and vascular bundle (*vb*) tissues were also carried out, with this providing novel insight into the nature of the lignins in these different cell wall types.

**Towards definition of lignin primary structure:** The *Arabidopsis* mutant *Atomt1* lignin differs from native lignin in wild type plants, in terms of sinapyl (S) alcohol-derived substructures in fiber cell walls being substituted by 5-hydroxyconiferyl alcohol (5OHG)-derived moieties. During programmed lignin assembly, these engender formation of benzodioxane substructures due to intramolecular cyclization of their quinone methides that are transiently formed following 8-*O*-4' radical-radical coupling. Thioacidolytic cleavage of the 8-*O*-4' inter-unit linkages in the *Atomt1* mutant, relative to the wild type, indicated that cleavable sinapyl (S) and coniferyl (G) alcohol-derived monomeric moieties were stoichiometrically reduced by a *circa* 2 : 1 ratio.<sup>5</sup> Additionally, lignin degradative analysis resulted in release of a 5OHG-5OHG-G trimer from the *Atomt1* mutant, which then underwent further cleavage. Significantly, the trimeric moiety released provides new insight into lignin primary structure: during

polymer assembly, the first 5OHG moiety is linked *via* a C8–O–X inter-unit linkage, whereas subsequent addition of monomers apparently involves sequential addition of 5OHG and G moieties to the growing chain in a 2 : 1 overall stoichiometry. This quantification data thus provides further insight into how inter-unit linkage frequencies in native lignins are apparently conserved (or near conserved) during assembly in both instances, as well as providing additional impetus to further resolve how the overall question of lignin macromolecular assembly is controlled in terms of both type of monomer addition and primary sequence.<sup>5</sup>

**Plant phenolic flavor/fragrance and related aromatics as sources of commodity chemicals/petrochemical substitutes in cell-culture and transgenic woody plants:** Monomeric allylphenols/propenylphenols and related phenyl derivatives are important constituents of essential oils/flavors of several herbs, spices, flowers, and many woody species. As products of the phenylpropanoid (C6C3) pathway, we have been investigating whether the genes and enzymes involved can be used as a source of commodity chemicals/petrochemical substitutes. In this regard, isolation of a creosote bush (*Larrea tridentata*) cinnamyl alcohol acyltransferase (CAAT) catalyzing conversion of monolignols into their corresponding monolignol esters, as well as an allylphenol synthase (APS) and a propenylphenol synthase (PPS) converting monolignol esters into their corresponding allyl- and propenyl-phenols, respectively, has been achieved.<sup>6-8</sup> In this context, the co-expression of CAAT/APS and CAAT/PPS in *Escherichia coli* was carried out, where it was established that various monolignol substrates examined could be efficiently converted into their allyl/propenyl phenol counterparts without addition of cofactors (e.g., acetyl-CoA or NADPH). This work is discussed, in terms of their potential as sources of petrochemical substitutes.

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## Photodynamics of Single Antenna Proteins in Solution by Suppression of Brownian Motion

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

**Significant achievements 2008-2010:** We have achieved major strides forward, both in ABEL trap development and in single antenna protein measurements with the ABEL trap. In the area of trap development, we dramatically extended the abilities of the trap in several ways: first, we converted the older and slower camera-based system to a rotating beam hardware feedback design with  $\sim 100$  times faster update rate (1), and then we implemented this design using a fast Field-Programmable Gate Array (FPGA) (2). To provide a trap design with additional capabilities to go beyond extraction of fluorescence parameters alone (i.e., brightness, lifetime, etc.), we have invented and validated by simulation a novel method of beam scanning (a Knight's tour, see Figure 1) which allows measurement of the diffusion constant and achieves enhanced trapping using Kalman filtering (3), a recursive algorithm for optimal state estimation with Gaussian measurement noise. In the area of physical measurements of target systems, we began by trapping single B-phycoerythrin molecules with multiple fluorophores to prove the principle, and then had particular success in employing the ABEL trap to explore conformational and photo-dynamics of single APC antenna proteins via simultaneous intensity and lifetime measurements (Figure 2) and statistical extraction of state changes and kinetic pathways (4).

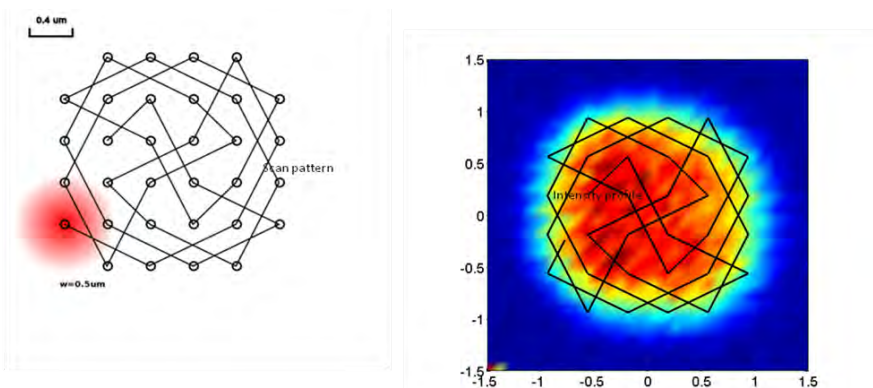


Figure 1. Left Panel: Schematic of the Knight's tour beam scanning pattern for our new advanced ABEL trap design (3). The laser beam spot (red) traverses the points shown in the plane of the microfluidic cell. Whenever a photon is detected, the position of the laser spot at that moment represents a probability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

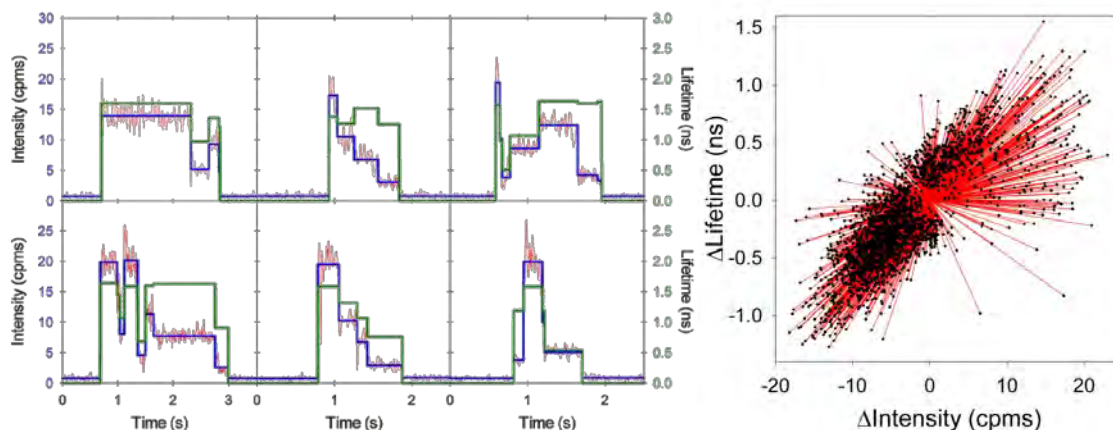


Figure 2. Left Panel: Examples of photodynamics of six APC antenna proteins in the ABEL trap (4). Red: Intensity trajectory (left ordinate). Blue: Brightness states extracted by a change-point finding algorithm. Green: Excited state lifetime during each state (right ordinate). Correlated changes in brightness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

#### Science objectives for 2010-2011:

- We will build and experimentally validate the new advanced design of the ABEL trap based on Knight's tour scanning and Kalman filtering. The goal will be to extract transport parameters (diffusion coefficient and mobility) directly from the motion of the protein held in the trap, which will supplement the fluorescence-based parameters.
- Because the fascinating photodynamics we observed with single APC antennas suggest a range of different quenching states of the complex, we will measure time-dependent spectral dynamics for each molecule in the trap.
- We will begin study of single copies of the important Fenna-Mathews-Olson (FMO) antenna complex in the ABEL trap in order to study the excitonic interactions between the chromophoric units and the effect of added reductant, in collaboration with R. Blankenship.
- We will extend our ABEL trap experiments to include measurements of the conformational and chemical dynamics of the redox enzyme Nitrite Reductase containing a fluorescent reporter near one copper site, in collaboration with G. Canters. This will serve as a new class of biomolecules which can be probed during enzymatic action without surface attachment or encapsulation.

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## Mass Spectrometric Imaging of Plant Metabolites

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Overall research goals: We are developing mass spectrometric imaging techniques to spatially map metabolite distributions within plant tissues, and eventually among individual plant cells. We are using these techniques to address specific biological questions concerning the distribution of metabolites from cell to cell, cooperative and antagonistic effects among the metabolites, and environmental and genetic influences on metabolism. Such details are required for a predictive understanding of the mechanisms that multicellular organisms use to regulate metabolic processes.

### Significant achievements in 2008-2010:

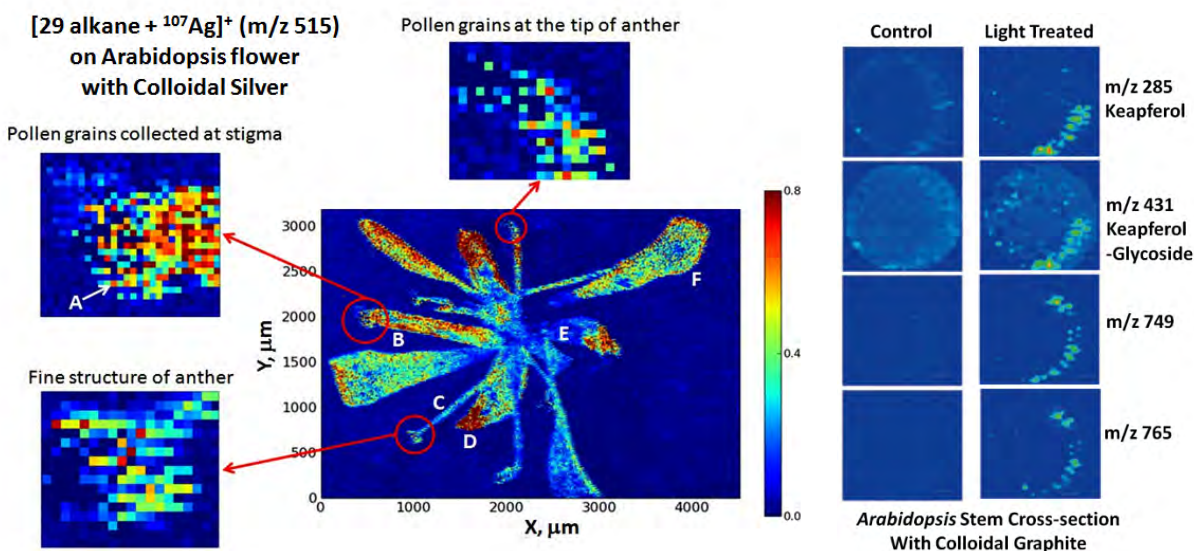
The research has focused on profiling the spatial distribution of metabolites during the development of *Arabidopsis* flowers. Flowers were selected for this research due to the challenges and opportunities that this organ provides for deciphering new knowledge concerning the tissue-specific differences in metabolism that is not otherwise accessible to visualization without the development of such high-resolution imaging technology. Moreover, flowers are crucial to the development and evolution of plants, as they are the organs where haploid gametogenesis occurs resulting in the propagation of the organism to the zygote of next generation, which ultimately develops in to the product of a large portion of commodity agriculture, namely seeds.

We have employed colloidal-silver laser desorption ionization (LDI) mass spectrometry (MS) to directly profile and image metabolites on different flower tissues of *Arabidopsis thaliana*. Initially, surface lipid compounds, such as very long chain fatty acids, alcohols, alkanes, and ketones were successfully imaged as silver adduct ions. The effect of specific mutations known to influence surface lipid chemistry of *Arabidopsis* (e.g., *cer1*, *cer2*, *cer5*) were investigated. The spatially resolved surface metabolite profiling data of these mutants has provided new insights into the complexity of the establishment of the extracellular matrix of the plant.

In parallel, we have begun to evaluate different methods for imaging the distribution of metabolites among the internal tissues of *Arabidopsis*. These subsurface imaging experiments are being conducted with graphite-assisted laser desorption ionization (GALDI). These studies have focused on imaging flavonoid metabolites. The rationale for this choice is two-fold: 1) flavonoid metabolites can be independently visualized via electromagnetic spectral methods, which provides a means of validating mass-spectrometric based imaging data; and 2) genetic variants that affect flavonoid metabolism are known, and the data that we are generating is providing new high spatial resolution knowledge concerning the regulation of these metabolic networks. The location and the degree of light-induced flavonoid accumulation in stem sections have been probed, and the effect of *transparent testa* mutants on flavonoid distribution is being evaluated.

To date, high resolution mass spectrometric images have been obtained with an instrument that was upgraded from a previous MALDI LTQ instrument to MALDI LTQ-Orbitrap. Technical capabilities for the project should receive an additional boost with the acquisition of a Bruker solariX FTMS, which is scheduled to be installed by November, 2010.





**Figure 1. (Left) Single cell level high spatial resolution MS image of C29 alkane on the surface of *Arabidopsis* flower.** LDI MS imaging was acquired with laser beam size of 12 $\mu$ m and raster size of 12 $\mu$ m and colloidal silver as additive. **(Right) Chemically selective images of *Arabidopsis* stems grown under a normal light regime as control (left column) and under a high-light regime (right column).**

#### Science objectives for 2010-2012:

- We will strive to achieve high spatial resolution down to 5- $\mu$ m or smaller by replacing current N<sub>2</sub> laser with Nd-YAG laser and using new laser optics.
- Methodology for the quantification of metabolites in cellular and subcellular level will be developed.
- A number of *Arabidopsis* mutants, including *tt7*, *cer1*, and *cer5*, will be studied using the developed MS imaging technique.
- APMALDI imaging will be attempted with LTQ-Orbitrap and Bruker solarix FTMS mass spectrometers.

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## Osmoregulation in Methanogens: Do Compatible Solutes Interact Directly with Protein Surfaces?

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Overall research goals: Our research is aimed at understanding how different classes of compatible solutes affect macromolecular stability in response to osmotic and thermal stress. Compatible solutes are thought to protect proteins from thermal and osmotic stresses by being excluded from the surface, allowing critical water molecules to interact with the protein. This implies there are no specific binding interactions between osmolytes and proteins. However, we and others have often observed very specific solute effects for proteins that suggest a more direct interaction between solute and protein is likely can occur. Measuring such a weak interaction is extremely difficult. We are currently developing a solution NMR method, high-resolution field cycling, that can measure spin-lattice relaxation rates as a function of magnetic field from 11.7 (the field of a 500 MHz spectrometer) to 0.003 T. The methodology is ideal for nuclei in small molecules with moderately long relaxation times at high fields – phosphate groups ( $^{31}\text{P}$ ), enriched carbonyls ( $^{13}\text{C}$ ), or methyl groups ( $^1\text{H}$ ). The protein of interest is spin-labeled to introduce a large dipole on it that will dominate the relaxation of nuclei on any small molecules that bind transiently. The key is to measure relaxation below 1-2 T (and extract nuclei-spin label distances in the bound complex) where the small molecule relaxation will be dominated by dipolar mechanisms with a correlation time indicative of the large protein complex.

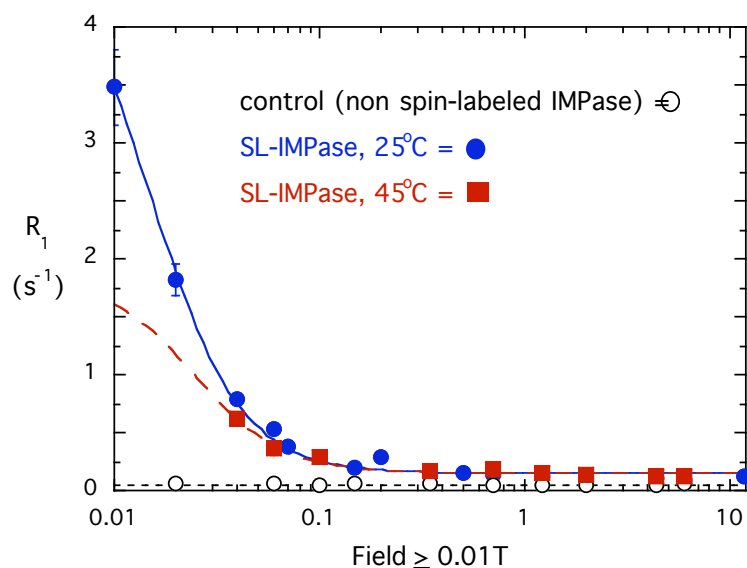


Figure 1. Variation of  $^{31}\text{P}$  relaxation rate ( $1/T_1$ ) as a function of field for 20 mM Pi and 33  $\mu\text{M}$  IMPase ( $\circ$ ) or IMPase spin-labeled on its two surface Cys at 25°C ( $\bullet$ ) and 45°C ( $\blacksquare$ ). Fits of the dipolar relaxation can be used to extract parameters related to the averaged distance between the Pi nucleus and the spin-labels on the protein. Note: Under these conditions there is no effect of non-spin-labeled protein on the Pi relaxation rate.

As a test of the methodology, our initial  $^{31}\text{P}$  results show we can easily detect binding of inorganic phosphate (Pi) to the *Archaeoglobus fulgidus* inositol monophosphatase (IMPase) active site at a ratio of Pi/SL-IMPase = 660:1. The effect is large enough so it could be detected at low field for even 6000:1 free to bound ligand.

Currently, we are carrying out similar field cycling studies looking for evidence for the binding of phosphodiester solutes with compatible solute properties to this protein. In concert with the field cycling we are also generating selected single mutation variants of this IMPase (replacing Val, Ile or Leu with Ala to destabilize hydrophobic clusters) with reduced thermostability to see if relaxation effects detected with spin-labeled wild type protein are altered. The idea is to see if discrete sites for solutes do occur on proteins. Pi binding serves as a proof-of-concept control since it binds (although not very tightly) to the active site.

#### Significant achievements 2009-2010:

- Proof-of-concept field cycling to detect weak binding of Pi to the active site of spin-labeled *A. fulgidus* IMPase has been carried out.
- The crystal structure of the inositol-1-phosphate synthase from *A. fulgidus* with an intermediate along the reaction pathway, 5-keto-glucose-6-phosphate; this is the regulatory point in synthesis of the osmolyte 1,1'-di-myo-inositol-phosphate (DIP) by this organism.
- Characterization of mutant IMPases with altered acidic residues in a mobile loop that can occlude the active site has provided specific insights into identifying where Li<sup>+</sup> binds to and inhibits members of the IMPase superfamily.

#### Science objectives for 2010-2011:

- High resolution <sup>31</sup>P field cycling will be explored with DIP/ $\alpha$ -diphosphoglycerol mixtures (extracts from *Thermotoga maritima*) in the absence and presence of spin-labeled IMPase to see if evidence for protein/osmolyte interaction can be found.
- We will explore <sup>13</sup>C field cycling with [1-<sup>13</sup>C]glutamate and IMPase since this amino acid has also been shown to have thermoprotective effects on the protein.
- We will characterize solute protection of single mutation IMPases with reduced thermostability; spin-labeling of these will be used in <sup>31</sup>P field cycling NMR experiments to see if a discrete binding site(s) can be detected.

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## Calcium-mediated regulation of proton-coupled sodium transport

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Overall research goals: Our long term goal is to understand mechanisms that regulate energy coupling by ion currents in plants. By passing from one side of a membrane to the other, a current of ions can link metabolism to work, playing a major role in energy capture and supporting a range of physiological processes. Molecular genetic studies in *Arabidopsis thaliana* (*Arabidopsis*) have shown that the activity of a plasma membrane  $\text{Na}^+/\text{H}^+$  exchanger, *SALT OVERLY SENSITIVE1 (SOS1)* is essential for regulation of sodium ion homeostasis during plant growth in saline conditions. SOS1 is a secondary active transport system coupling movement of sodium ions out of the cell using energy stored in the trans-plasma membrane proton gradient, thereby preventing the build-up of toxic levels of sodium in the cytosol. Our studies are focused on understanding how SOS1 activity and sodium ion homeostasis are regulated.

Significant achievements in 2008-2010: SOS1 is regulated by a complex containing the SOS2 serine/threonine protein kinase and the CALCINEURIN B-LIKE10 (CBL10) EF-calcium sensor proteins. In collaboration with Dr. Yan Guo, we have shown that SOS2 specifically phosphorylates CBL10 at its C terminus. In vitro, SOS2 phosphorylation of CBL10 is enhanced by the bimolecular interaction of SOS2 and CBL10. In vivo, this phosphorylation is induced by salt stress, takes place at the membrane, stabilizes the CBL10-SOS2 interaction, and enhances plasma membrane  $\text{Na}^+/\text{H}^+$  exchange activity. When a Serine at position 237 in the CBL10 protein (the SOS2 phosphorylation target) is mutated to Alanine, CBL10 is no longer phosphorylated by SOS2 and the mutant protein does not rescue the *cbll0* salt-sensitive phenotype.

Science Objectives for 2010-2012: Our studies indicate that, in addition to its regulation post-translationally, CBL10 is also regulated at the post-transcriptional level. When CBL10 expression was monitored in wild-type *Arabidopsis* grown in control conditions (no NaCl), an additional band appeared above the expected size of the *CBL10* transcript and was found to represent an alternatively spliced form of *CBL10* with a retained intron. This transcript (*CBL10LA*) likely encodes a truncated protein due to a premature stop codon within the retained intron. When seedlings were grown in the presence of salt, accumulation of the *CBL10LA* transcript was reduced. A yeast two-hybrid assay revealed that both CBL10 and CBL10LA interact with the SOS2 kinase. Based on these results, in combination with the response of wild-

type Arabidopsis to growth in salt and the salt-sensitive phenotype of the *cb110* mutant, we have developed the following working model. In control conditions, *CBL10* and *CBL10LA* are transcribed and translated. In response to increased cytosolic sodium, levels of *CBL10LA*, a negative regulator, are reduced so that SOS2 predominately interacts with CBL10, activating the SOS pathway. Several predictions arose from this model: (1) *CBL10LA* is translated into a protein, (2) CBL10LA competes with CBL10 for interaction with SOS2, (3) the CBL10LA protein is not functional during salt stress and only CBL10 is needed to initiate a response to salt, and (4) down-regulation of *CBL10LA* is critical for a salt stress response. The following experiments are in progress to test these predictions. (1) An N-terminal tagged *CBL10* genomic construct (driven by the *CBL10* promoter) has been generated to detect the presence of CBL10 and CBL10LA proteins. The construct has been expressed in and found to rescue the salt-sensitive phenotype of the *cb110* mutant. Assays are underway to monitor protein accumulation via immunoblot analysis. (2) *In vitro* pull-down assays are being used to determine if CBL10LA is able to interact with SOS2 and if its interaction decreases CBL10-SOS2 interaction. (3) The *cb110* mutant has been transformed with *CBL10* and *CBL10LA* cDNAs to monitor complementation of the *cb110* mutant phenotype. (4) *CBL10LA* has been over-expressed in wild type to determine if plants that are unable to down-regulate *CBL10LA* transcript levels become sensitive to sodium.

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Nah G, Pagliarulo CL, Mohr PG, Luo M, Sisneros N, Yu Y, Collura K, Currie J, Goicoechea JL, Wing RA, Schumaker KS. 2009. Comparative Sequence Analysis of the *SALT OVERLY SENSITIVE1* Orthologous Region in *Thellungiella halophila* and *Arabidopsis thaliana*. *Genomics* 94, 196-203.

Yang Y, Qin Y, Xie C, Zhao F, Zhao J, Liu D, Chen S, Fuglsang AT, Palmgren MG, Schumaker KS, Deng XW, Guo Y. 2010. The *Arabidopsis* chaperone J3 regulates the plasma membrane H<sup>+</sup>-ATPase through interaction with the PKS5 kinase. *Plant Cell* 22, 1313-1332.

## Modification of Plant Lipids

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

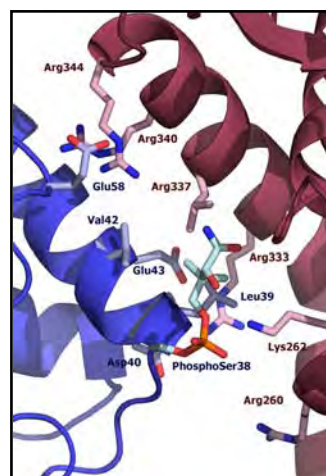
Overall research goals: Lipids and oils are a vital class of compounds. In eukaryotes, saturated fatty acids are modified post-synthetically by the introduction of *cis* double bonds or by the introduction of a variety of functional groups. The focus of this research is to understand the molecular basis for these chiral lipid-modification reactions by using the process of fatty acid desaturation as a model. We will re-engineer desaturases with tailored specificities for introduction into commercially useful plants that will accumulate renewable sources of industrial feedstocks currently derived from petrochemicals.

### Significant achievements in 2008-2010:

- Used 18:0-acyl carrier protein (ACP) desaturase variant that synthesizes the allylic alcohol 9-OH 18:1 $\Delta^{10}$  as a model system to further dissect the mechanism of “unusual fatty acid” biosynthesis.
- Identified position 280 as a critical determinant of regioselectivity in the castor desaturase.
- Solved the structure of the castor desaturase in complex with its acyl-ACP substrate.
- Identified the binding helix of the ACP residues and their corresponding interaction residues on the surface of the desaturase enzyme
- Identified the helix within the desaturase that appear to interact with the pantetheine of the acyl-ACP substrate.
- Identified the determinants of the soybean desaturase that relates to the high stearate soy line.
- 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 catalyzed by a soluble  $\Delta^9$  desaturase.
- Found the oligomeric state of the membrane desaturases, like the soluble desaturase is a dimer.
- Performed metabolic engineering of Arabidopsis to increase the accumulation of  $\omega$ -7 fatty acids from 2-71%, equivalent to that of the naturally  $\omega$ -7 accumulating species *Doxantha*.
- Performed radical clock experiments on the alkane  $\omega$ -hydroxylase from *Pseudomonas oleovorans* and demonstrated that the reaction proceeds via a radical intermediate.



View down the four helix bundle of the castor desaturase showing the diiron site. Image selected as journal cover for ref. 5 below.



The desaturase-ACP interface. Shown are desaturase chain B in pink and ACP blue.

### 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 simultaneous UV-Vis and Raman spectral and structural determinations to detect reaction intermediates for the soluble desaturase enzymes using NSLS beamline X27C.
- Perform biochemical characterization and determine a crystal structure of the newly identified soluble diiron enzyme aldehyde decarboxylase in complex with its substrate.
- Engineer the aldehyde decarboxylase to produce short and medium chain alkanes.
- Perform protein expression and purification trials on the integral membrane class of desaturases and related enzymes that are capable of abstracting hydrogens from unactivated methylene groups with a goal of obtaining crystals for this class of enzymes.

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## Regulation of Cellulose Synthesis

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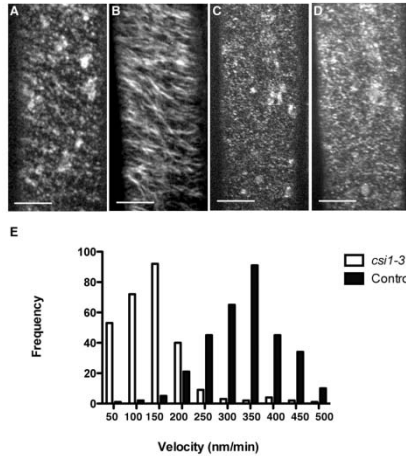
Email: [crs@berkeley.edu](mailto:crs@berkeley.edu)

Overall research goals: The research objectives are to characterize the factors that regulate the pattern, amount and quality of cellulose deposition in higher plants. We are pursuing this goal in four ways: (1) by searching for proteins that interact with the cellulose synthase complex using novel bioinformatics tools to identify genes which exhibit gene expression patterns that are highly correlated with cellulose synthase components, (2) by using cellulose synthase domains as bait in 2-hybrid screens, and then characterizing any proteins that are identified by both gene expression network analysis and 2-hybrid screens (3) by testing the function of all known post-translational modifications, (4) by identifying and characterizing kinases that carry out most of the known post-translational modification of cellulose synthase components.

Significant achievements 2009-10: Cellulose synthase interactive protein 1 (CSII) was identified in a two-hybrid screen for proteins that interact with CESA isoforms involved in primary plant cell wall synthesis (Gu et al., 2010). CSII encodes a novel 2150 amino acid protein that contains 10 predicted Armadillo (ARM) repeats and a C2 domain. Mutations in *CSII* cause defective cell elongation in hypocotyls and roots and reduce cellulose content. Two color confocal imaging of cellulose synthase and the CSI protein in live cells showed that CSI is associated with CESA complexes, and *csi1* mutants affect the distribution and movement of CESA complexes in the plasma membrane. The very large size and the unusual structure of the CSI protein suggest a structural role that supports assembly or function of cellulose synthase.

The CESA1 component of cellulose synthase is phosphorylated at sites clustered in two hypervariable regions of the protein. Mutations of the phosphorylated residues to Ala (A) or Glu (E) alter anisotropic cell expansion and cellulose synthesis in rapidly-expanding roots and hypocotyls (Chen et al., 2010). Expression of T166E, S686E, or S688E mutants of CESA1 fully rescued the temperature sensitive *cesA1-1* allele (*rsw1*) at a restrictive temperature while mutations to A at these positions caused defects in anisotropic cell expansion. However, mutations to E at residues surrounding T166 (i.e., S162, T165, and S167) caused opposite effects. Live-cell imaging of fluorescently labeled CESA showed close correlations between tissue or cell morphology and patterns of bidirectional motility of CESA complexes in the plasma membrane. In the wild type, CESA complexes moved at similar velocities in both directions along microtubule tracks. By contrast, the rate of movement of CESA particles was directionally-asymmetric in mutant lines that exhibited abnormal tissue or cell expansion, and the asymmetry was removed upon depolymerizing microtubules with oryzalin. This suggests that phosphorylation of CESA differentially affects a polar interaction with microtubules that may regulate the length or quantity of a subset of cellulose microfibrils and that this, in turn, alters microfibril structure in the primary cell wall resulting in or contributing to the observed defect in anisotropic cell expansion.

In order to identify kinases that phosphorylate cellulose synthase, we synthesized peptides representing all known phosphorylation sites, with and without phosphorylation, and assayed extracts for kinase activity. Several of the peptides were phosphorylated, raising the possibility of using the activity to purify the corresponding kinases by conventional methods. However, as a first step, we expressed in microbial hosts a number of kinases that had been identified as CESA interacting proteins in the yeast 2-hybrid screen or which were expected to have substrate specificities similar to known CESA phosphorylation sites. This resulted in identification of clones for several kinases that phosphorylate CESA peptides.



**YFP-CESA6 dynamics are altered in *csi1-3* mutant.** YFP-CESA6 localization in dark-grown hypocotyls cells in wild type (A and B) and *csi1-3* (C and D). (A) Single optical section acquired at the plane of plasma membrane in wild type. (B) Average of 61 frames (Duration: 2 min; 2 s interval) showing movement of labeled particles along linear trajectories. (C) Single optical section of YFP-CESA6 in *csi1-3*. (D) Average of 61 frames (Duration: 2 min; 2 s interval) in *csi1-3*. (E) Histogram of measured particle velocities. The mean is 365 nm/min in control (n=318) and 132 nm/min in *csi1-3* (n=225). Bar= 5 $\mu$ m.

#### Science Objectives for 2010-2011:

- We have produced mutations of all the phosphorylation sites in CESA3 that convert the phosphorylated residues to either alanine or glutamate. During 2010 postdoc Shaolin Chen will complete the analysis of the effects of all phosphorylation sites in CESA3 using the same approaches used to measure the effects of the mutations in CESA1 (i.e., using live-cell imaging of the CESA complexes). The S227A mutation exhibits a strong growth phenotype. We will focus on investigating what aspect of CESA function is altered (eg., rate of catalysis, lifetime, secretion, other).
- Postdoctoral fellow Ian Wallace will complete the characterization of the kinetic properties of one or more of the identified CESA kinase proteins, determine when and where the kinase's are expressed and located, characterize mutants of Arabidopsis lacking the kinases, and carry out an analysis of how the kinases are regulated.
- The length of cellulose microfibrils is thought to be an important factor in specifying the physical properties of cell walls. Our current understanding of cellulose synthesis suggests that the length of cellulose microfibrils is controlled by the lifetime of CESA complexes. However, because the field of view is very complex when imaging cellulose synthase complexes in live cells, and we are imaging very faint signals, we have not been able to obtain satisfactory measurements of lifetime. Postdoctoral fellow Nadav Sorek will explore the use of laser-induced photobleaching to simplify the field of view (by bleaching part of the cell to create an empty field into which CESA particles can move and be observed). Nadav will also develop photoactivatable GFP-CESA constructs and FLASH-tagged constructs and use them for lifetime analyses.

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## Endomembrane Cation/Proton Exchangers: role in membrane sorting and signal transduction

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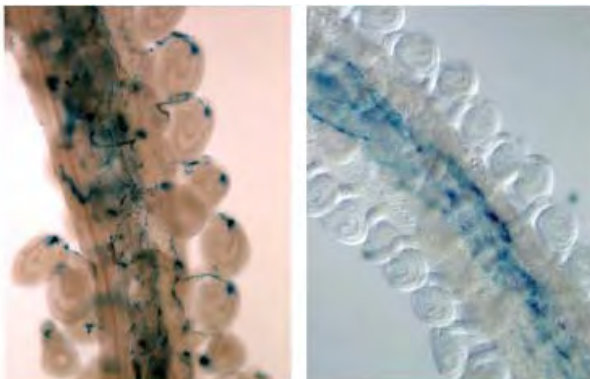
### Overall research goals

Membrane trafficking, including biosynthesis, endocytosis, and membrane recycling, are integral to cytokinesis, cell expansion, cell polarity, and cell wall remodeling; though the cellular and molecular bases for membrane trafficking in plants are poorly understood. Diverse membrane compartments are thought to provide distinct environments in a temporal manner to modulate secretion and/or endocytosis. One working hypothesis: is that the process depends in part on regulation of  $\text{Ca}^{2+}$  and pH dynamics. Our studies have revealed the importance of endomembrane  $\text{Ca}^{2+}$ - and  $\text{H}^+$ -pumps in plants in growth and stress tolerance. The current goal is to determine the biological roles of a novel family of uncharacterized cation/ $\text{H}^+$  exchangers. Results show that CHX17 and CHX20 associated with compartments, like ER and PVC, modulate  $\text{K}^+$  and pH homeostasis and affect protein sorting differentially. Analysis of mutants show that two CHX affect signaling in pollen tube guidance, and one affect guard cell osmoregulation. We hypothesize that membrane trafficking in root cells influence cell wall remodeling, and are testing this idea.

### Significant achievements in 2008-2010

#### a) **Pollen tubes lacking a pair of CHXs fail to target ovules in Arabidopsis.**

Flowering plant reproduction requires the precise delivery of the sperms to the ovule by a pollen tube. We have shown that 2 CHX genes in Arabidopsis are essential for pollen tube guidance. Double mutant pollen grains germinated and grew tubes down the transmitting tract, but the tubes fail to turn towards the ovules. Moreover, double mutant pollen tubes failed to enter the micropyle of excised ovule. As GFP-tagged CHX23 is localized to ER of pollen tubes, and E coli expressing the gene take up more  $\text{K}^+$ , we propose that CHX adjusts cation homeostasis to affect steps in signal reception or transduction that are critical for directing pollen tube growth towards the ovule (Lu Y).



**Figure 1.** *Double mutant pollen fail to reach the ovule in vivo.* Wild type pistils were hand-pollinated with single chx or double mutant grains. After 24 h, Gus-stained pollen of single mutants have targeted ovule and discharged Gus (left). Pollen tubes of double mutants remain in the transmitting tract (right).



**b) CHX associated with ER and endosomes: differential activities and role in protein sorting.** Several CHXs (16-20) showed two types of activities when functionally expressed in yeast. CHX17 restored growth of yeast mutants sensitive to alkaline pH, and conferred HygB resistance similar to NHX1. Using pH-sensitive fluorescent reporters, we found that CHX17 had no effect on yeast  $\text{pH}_{\text{cyt}}$  or  $\text{pH}_{\text{vac}}$ . In contrast, CHX20 partially restored yeast growth on alkaline medium, but failed to confer HygB resistance. It caused acidification of  $\text{pH}_{\text{cyt}}$  and alkalinized  $\text{pH}_{\text{vac}}$ . Both CHX17 and CHX20 mediated  $\text{K}^+$  uptake in E coli, but were differentially localized to PVC and ER, respectively, in plant cells. In yeast KTA strain, vacuolar enzyme CPY is missorted and secreted, however CHX17, but not CHX20, reduced this secretion. Together, these results indicate that multiple CHXs differentially affect  $\text{K}^+$  and pH homeostasis, and CHX17 in PVC is important for proper protein sorting (Chanroj S).

#### Science objectives for 2010-2011

Two major questions are (i) what is the mode of transport mediated by CHX, and (ii) what are the molecular bases for the dwarf phenotype of chx mutants. We are testing (i) by expressing CHX in E coli mutants and conducting flux assays to determine the affinity, velocity and cation specificity. We also propose to test if it might have channel activity. We suspect that triple or quad mutants of chx16-19 might have defective cell wall assembly due to altered secretion. We want to test this in collaboration with experts studying cell wall synthesis and remodeling. If time permits, we want to identify potential partners that interact with the transporters as a step to understand how CHXs are integrated with signaling networks in pollen tubes.

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- Chanroj S, S Padmanaban, Y. Lu, N Uozumi, R. Rao, H. Sze. Multiple cation/ $\text{H}^+$  exchangers on endomembranes: differential activities and role in protein sorting. (manuscript in preparation)



## Hsp100/ClpB Chaperone Function and Mechanism

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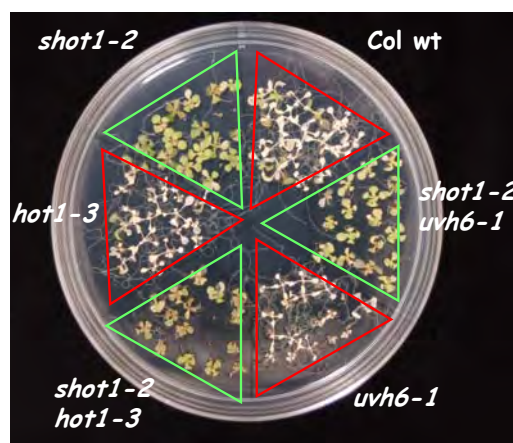
Overall research goals: The goal of the funded research is to investigate the mechanism of action of one class of molecular chaperone in higher plants, the Hsp100/ClpB proteins, which are reported to be effective protein disaggregases, but may have other roles. Hsp100/ClpB proteins occur in the plant cytosol and also in the energy-generating organelles of plants, the chloroplasts and mitochondria. The chaperone activity of the Hsp100/ClpB proteins impacts how plants generate and assemble components, as well as allowing for their self repair. Furthermore, Hsp100/ClpB protein function is required for optimal utilization of biological energy and is involved in mechanisms that control the architecture of energy transduction systems. We are using genetic and molecular approaches to investigate Hsp100/ClpB proteins, including studies of both cytosolic Hsp101 and chloroplastic ClpB-p, in the model plant *Arabidopsis thaliana*. The ultimate goal is to define mechanistically how these chaperones influence plant growth, development, stress tolerance and productivity.

Significant achievements 2009-2010: We have identified a putative regulator of mitochondrial gene expression which results in plants with reduced oxidative stress. If we can understand the mechanism by which oxidative stress is reduced, it may be possible to prevent or reduce this type of stress in plants.

We isolated a mutant, named *shot1* (suppressor of *hot1*), as a suppressor of an Hsp101 mutant allele. Importantly, we found that *shot1* suppresses other heat sensitive mutations and is also more resistant to heat than wild type (Fig. 1). Therefore, the absence of Shot1 function must prevent basic damage that occurs during heat stress, reducing the need for repair functions and resulting in more stress resistant plants.

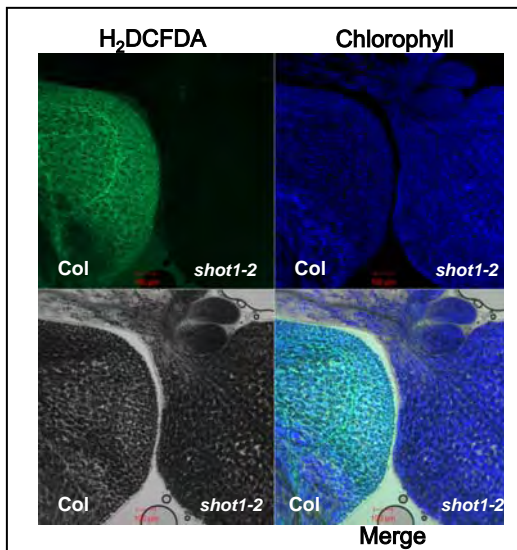
The *shot1* mutant shows dramatically reduced production of reactive oxygen species (ROS) during heat stress (Fig. 2). This result suggests that either Hsp101 facilitates repair of oxidative stress damage, or that in the absence of oxidative damage, Hsp101 function is no longer essential. This relationship of Hsp101 to oxidative stress was previously unknown.

Shot1 belongs to a family of 34 genes in *Arabidopsis*, almost all of which are predicted to be chloroplast or mitochondrial-localized; they are called mitochondrial transcription termination factor-related proteins (mTERFs). *In silico* structure predictions indicate that *Arabidopsis* mTERFs are all alpha-helical proteins with structures related to known nucleic acid binding proteins (Fig. 3). This predicted structure was recently validated by a paper in press describing the structure of a human mTERF. We hypothesize that

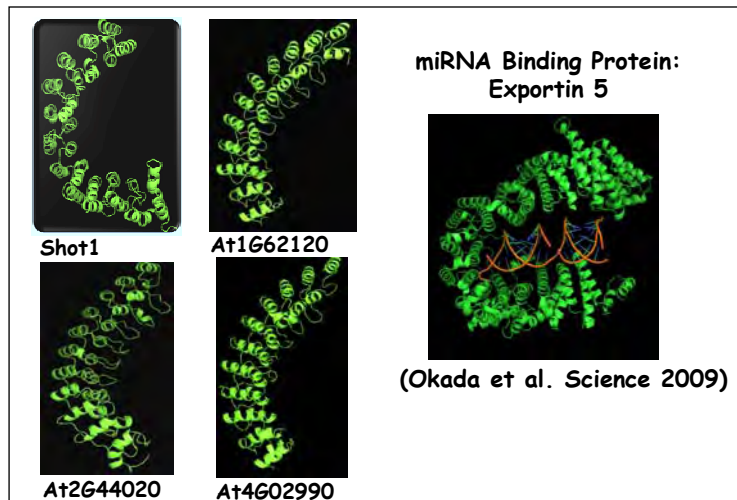


**Figure 1.** The *shot1-2* mutant survives high temperature treatment better than wild type (Col) and suppresses the heat sensitivity of *hot1-3* (Hsp101 mutant) and other heat sensitive mutants (*uvh6-1*) and *hot5* (not shown).

plant mTERF proteins are a large family of understudied regulators of organelle gene expression.



**Figure 2.** Seedlings of Arabidopsis wild type (Col) and the *shot1-2* mutant stained with 2',7'-dichlorodihydrofluorescein diacetate (*H2DCFDA*), a fluorogenic probe commonly used to detect ROS. Seedlings were heated at 45 C for 1 hr and then recovered for 1 hr at 22 C.



**Figure 3.** Predicted structure of Shot1 and three other Arabidopsis mTERF proteins. All resemble all helical proteins that are known to bind nucleic acid, such as Exportin 5.

#### Science objectives for 2010-2011:

- Work towards determining the target of mTERF activity in plant mitochondria.
- Determine if other mTERF protein mutants have similar phenotypes.
- Complete further studies of Hsp101-interacting proteins to understand the protective function of Hsp101
- Test if the chloroplast Hsp101 homolog can substitute for Hsp101 in the cytosol and vice versa.

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## Secondary Wall Formation in Fibers

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**Overall research goals:** The goal of this DOE-funded project is to study the molecular mechanisms underlying secondary wall formation. Secondary walls are the major constituent of tracheary elements and fibers in wood, which is the most abundant biomass produced by plants. Secondary walls from dicot wood are mainly composed of cellulose, xylan, and lignin. Although the biosynthetic genes for cellulose and lignin have been well characterized, our understanding of genes participating in xylan biosynthesis is still limited. We proposed to investigate functional roles of glycosyltransferases involved in the biosynthesis of xylan. Identification of genes involved in xylan biosynthesis is essential for our understanding of the complex process of wood formation.

### Significant achievements in 2009-2010:

We have uncovered that four Arabidopsis GT43 members (namely IRX9, I9H, IRX14 and I14H) are involved in xylan biosynthesis and that they form two functionally non-redundant groups essential for the normal elongation of xylan backbone, which provides important new insight into the biochemical mechanism of xylan biosynthesis. We have found that the Arabidopsis glycosyltransferase F8H function redundantly with FRA8 in the biosynthesis of the xylan reducing end sequence during secondary wall biosynthesis. We have extended our findings on xylan biosynthesis in Arabidopsis to unravel the biosynthesis of wood components in a tree species and have characterized several glycosyltransferases involved in xylan biosynthesis in poplar.

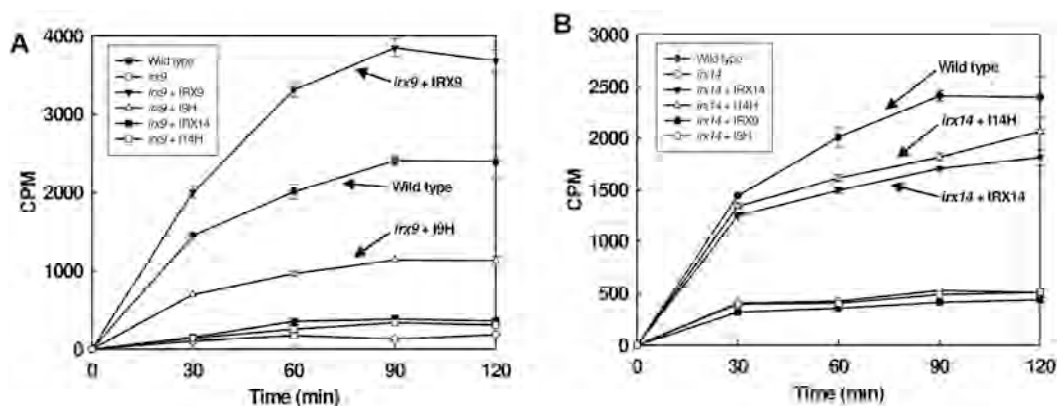


Figure 1. Time course of the xylan xylosyltransferase activity in the wild type, *irx9*, *irx14*, and the mutants overexpressing GT43 members. (A) Overexpression of I9H or IRX9 but not IRX14 or I14H in *irx9* rescued the deficiency in the xylosyltransferase activity caused by the *irx9* mutation. (B) Overexpression of I14H or IRX14 but not IRX9 or I9H in *irx14* rescued the deficiency in the xylosyltransferase activity caused by the *irx14* mutation.

### Science objectives for 2010-2012:

- Our recent finding that two functionally non-redundant GT43 members are necessary for the normal elongation of xylan backbone provides an important insight into the biochemical mechanism of xylan backbone elongation. The next challenging steps are to

- assign the biochemical functions of GT43 glycosyltransferases. We hypothesize that GT43 glycosyltransferases are strong candidates for xylan synthase and will continue our efforts to investigate the biochemical activity of GT43.
- 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.

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*Session V*  
*‘Omics Tools for Energy*  
*Transduction Studies*



## **EMSL: A National Scientific User Facility for State-of-the-Art Molecular and Environmental Research**

Presenters: Bryan E. Linggi and John R. Cort  
Web: <http://www.emsl.pnl.gov/>

Overall goals: EMSL, the Environmental Molecular Sciences Laboratory, is a national scientific user facility located at Pacific Northwest National Laboratory and funded by the Department of Energy's Office of Biological and Environmental Research. For over a decade, EMSL has provided thousands of researchers worldwide with unique computational and experimental capabilities that are focused on solving important problems in biology, geochemistry, subsurface science and interfacial chemistry. EMSL is accessed, typically at no charge, through a peer-reviewed proposal process.



Current capabilities: Capabilities at EMSL include Chinook, a 163-teraflop, supercomputer, and NWChem, DOE's premier computational chemistry software. EMSL also has a suite of state-of-the-art mass spectrometers, high-field nuclear magnetic resonance instruments, ultra-resolution electron and optical microscopes as well as a highly skilled staff who are experienced in using these technologies to solve extremely challenging scientific problems.

EMSL's cutting-edge capabilities and in-house expertise are supporting the critical and growing area of bioenergy research. In particular, our unparalleled capabilities in molecular-level analysis and high-throughput biology allow the molecular structure of native and modified biomolecules, such as cellulose to be directly observed. Dynamic measurements of enzyme conformations by NMR and single-molecule microscopy provide essential experimental data on theories of enzyme action and a basis for reengineering them for increased catalytic efficiency. EMSL capabilities in high-throughput proteomics, transcription profiling and metabolomics allow for the reconstruction of metabolic networks and degradation pathways of cells and cellular communities and assist in the identification of novel mechanisms of biomass conversion. Importantly, EMSL's vast suite of analytical capabilities is located under one roof, allowing a wide variety of different technologies to be applied to solve difficult problems.



Our evolving capabilities: EMSL evolves with the needs of its scientific users and the American Recovery and Reinvestment Act is accelerating this process. New instruments and capabilities are being acquired to extend and enhance EMSL's ability to support research in biochemistry, biophysics and molecular biology. Facilities for experimental biology and cell culture are also being enhanced with the acquisition of automatic sampling bioreactors as well as parallel microbial culturing systems with individual monitoring and control of O<sub>2</sub>, pH and temperature.

Investments in microscopy are being made to push the limits of resolution and allow *in situ* observations of cells and single molecules. New capabilities include a helium ion microscope, a low temperature scanning probe microscope and several multimodal, high-resolution confocal and environmental microscopes. An imaging/mass microscope system will allow for the spatial analysis and identification of proteins and small molecules in biological samples. This suite of instruments will allow dynamic real-time observation of biological and complex systems and analysis of complex materials at extremely high spatial resolution and unprecedented depth of field.

Other instruments include new mass spectrometers for metabolomics characterization, isotopic mass spectrometers for elemental analysis and ion mobility mass spectrometers for proteomics. These instruments will provide unprecedented dynamic range, resolution, and throughput of complex mixtures as well as allow for global and targeted metabolic analysis and a comprehensive identification of post-translational modifications of proteins. NMR capabilities are being upgraded with the acquisition of an 850MHz wide bore instrument as well as a variety of MAS probes and various console upgrades. A high spatial resolution imaging secondary Ion Mass Spectrometer (NanoSIMS) for biological, geochemical and materials science is being acquired that will combine high sensitivity with high spatial resolution, allowing the simultaneous detection of up to seven different ions.

How to become an EMSL User: Access to EMSL resources is granted through a peer-reviewed proposal process. In general, users whose open research proposals are accepted may use EMSL resources free of charge. Open research is loosely defined as science and engineering research for which the resulting information is published and shared broadly within the scientific community.

EMSL provides a variety of application opportunities throughout the year. To submit a proposal, follow this link: <http://www.emsl.pnl.gov/access/>. Depending on the scope of the proposal project and the proposal call being targeted, this process may take days to a few months.

*Session VI*  
*Signal Transduction in Plants*



## A proteomic study of brassinosteroid responses in plants

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**Overall research goals:** The research objective is to study brassinosteroid signal transduction mechanisms by quantitative proteomic analysis of BR-regulated proteins and affinity purification of BZR1-interacting proteins, followed by genetic and molecular characterization of these proteins. The specific aims of this project include: (1) Functional study of DREPP, a BR-induced plasma membrane protein that promotes cell elongation; (2) identification and functional studies of the BZR1-dephosphorylating phosphatase (BRP); (3) Identification of additional BR early response proteins using prefractionation followed by 2-D DIGE and quantitative mass spectrometry. Brassinosteroid (BR) is a growth-promoting hormone that controls a wide range of developmental and physiological processes in plants. This study advances our knowledge of signal transduction mechanisms in plants and provides molecular tools for genetic engineering plant growth to improve plant productivity.

### Significant achievements 2008-2010:

**I. Functional study of DREPP.** We identified the DREPP1 protein as a BR-induced protein in the plasma membrane fraction, and we showed that overexpression of DREPP1 partly suppressed the dwarf phenotype of the BR deficient *det2* mutant (Tang et al., 2008, Mol Cell Proteomics 7, 728-738). In the current project, we have characterized the function of DREPP1 in more detail. First, we have found that *DREPP1* is a target gene of the BZR1 transcription factor, and is activated by BR signaling at the transcriptional level. We have generated *drepp1/drepp2* double loss-of-function mutant lines by transforming a *drepp1* T-DNA knockout line with an RNAi construct of *DREPP2*. These plants show apparently reduced size, confirming the role of DREPP family members in promoting plant growth. In the roots, DREPP is expressed specifically in the endodermis in a BR-dependent manner. To understand the biochemical function of DREPP, we are identifying DREPP1-interacting proteins using affinity purification followed by mass spectrometry analysis.

### **II. Functional study of other early BR-response proteins – BRP in BR signal transduction.**

We have replaced the BSU1 phosphatase upstream of BIN2 in the BR signaling pathway (Kim et al., 2009), raising a question of what phosphatase dephosphorylates BZR1. We initially identified a phosphatase (BRP) as BR-regulated proteins in the plasma membrane fraction. We subsequently identified BRP as part of the BZR1 complex using tandem affinity purifying BZR1 interacting proteins. We therefore studied the functions of BRP in BR signaling.

An affinity purification of BZR1-myc-6His fusion protein expressed in transgenic Arabidopsis identified known BZR1-interacting proteins such as a 14-3-3 protein and homologs of BIN2, as well as BRP. Subsequent analysis demonstrated that BZR1 interacts directly with BRP. The immunopurified BRP effectively dephosphorylated BZR1 *in vitro*, whereas the BSU1 phosphatase was unable to.

Overexpressing BRP reduced the sensitivity to the BR-biosynthetic inhibitor BRZ and suppressed the BR-insensitive mutants *bri1* and *bin2*. In contrast, loss-of-function mutants of *brp* show dwarf phenotypes and accumulate phosphorylated BZR1, similar to the BR insensitive mutants.

BZR1 was initially identified by the dominant *bzr1-1D* mutation (P234L), which causes BZR1 activation and BR-independent growth responses. Immunoblotting experiments showed an increased dephosphorylation of the *bzr1-1D* mutant protein in plant. *In vitro* assays showed that the *bzr1-1D* mutation significantly increases the affinity of BRP-BZR1 interaction. Two intragenic *bzr1-1D* suppressor mutants (*bzs247* and *bzs248*) contain mutations within seven amino acid (aa) residues away from the *bzr1-1D* mutation, and these *bzs* mutations reduced the binding to BRP *in vitro* and BZR1 dephosphorylation *in vivo*. Binding assays of

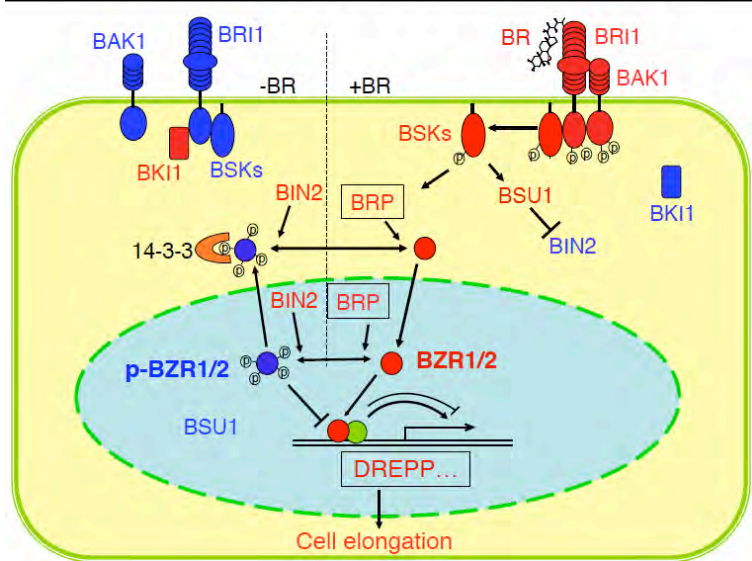
truncated BZR1 fragments demonstrated that BRP interacts with a 35-aa region containing the *bzr1-1D* and *bzs* mutations. Deletion of 20 aa at the center of this region abolished the interaction with BRP, and greatly reduced *in vivo* BZR1 dephosphorylation upon BR treatment, demonstrating that interaction with BRP is essential for BR-induced dephosphorylation of BZR1. These results clearly demonstrate that BRP catalyzes the dephosphorylation of BZR1 in the BR signaling pathway.

Our work on BRP identifies the last major component of the BR signaling pathway and establishes a complete BR signaling pathway from BR perception by receptor kinase BRI1 to dephosphorylation/activation of transcription factor BZR1 (Figure 1). BR binding to the extracellular domain of BRI1 activates BRI1 kinase (with help of the co-receptor kinase BAK1), BRI1 phosphorylates BR-signaling kinase1 (BSK1) at Ser230 to increase its interaction with BSU1, BSU1 dephosphorylates BIN2 at Tyr200 to inhibit BIN2 phosphorylation of BZR1, while BRP dephosphorylates BZR1. Unphosphorylated BZR1 accumulates in the nucleus and alters expression of target genes such as *DREPP1*, leading to growth responses.

**Figure 1. The BR signal transduction pathway.**

Genetic studies by many labs identified BRI1, BAK1, BSU1, BIN2, and BZR1 as key components for BR signal transduction. Proteomic studies of my group identified BSKs, BRP, and DREPP as new components of the pathway, and illustrate a fully connected BR-signaling pathway. When BR levels are low, BRI1 is inactive but BIN2 is active and phosphorylates BZR1 and its homolog BZR2 (BZR1/2). Phosphorylation inactivates BZR1/2 by blocking DNA binding and causing cytoplasmic retention due to binding to the 14-3-3 proteins. When BR level is high, BR binding to BRI1 activates BRI1 by recruiting the BAK1 coreceptor. Activated BRI1 phosphorylates BSK Ser230, and this phosphorylation promotes BSK binding to the BSU1 phosphatase and presumably activates BSU1. BSU1 dephosphorylates BIN2 at Tyr200 to inactivate BIN2 and stop phosphorylation of BZR1/2. Subsequently BRP dephosphorylates BZR1/2, which move to the nucleus and regulate gene expression. One of the BZR1 target genes encodes the plasma membrane associated protein DREPP, which promotes cell elongation.

**The BR signal transduction pathway**



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## The role of auxin in ambient temperature growth regulation

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Overall research goals: The sessile lifestyle of plants requires that they adapt rapidly to a constantly changing environment. Fluctuations in light, temperature, as well as water and nutrient availability all require dynamic changes in growth and development. These issues are particularly pressing at a time when human activity is causing unprecedented changes in climate. In this study we focus on the role of the plant hormone auxin in plant responses to changes in ambient temperature. In our earlier studies we showed that auxin is perceived by an F-box protein called TIR1 and its' close relatives, the AFB proteins. Auxin interacts with TIR1 and promotes the binding of transcriptional repressors called Aux/IAs, ultimately leading to their degradation. We have shown that the TIR1/AFB auxin receptors participate in the plant response to elevated temperature and that AFB4 has a particularly crucial role. The goal of our project is to understand the mechanisms of auxin perception and response in the context of temperature-dependent growth regulation.

Significant achievements 2007-2009:

- Auxin is perceived by a unique mechanism that does not involve changes in receptor conformation. Rather, auxin promotes binding of TIR1 to the Aux/IAA proteins by interacting with both proteins.
- Biochemical studies show that auxin binding requires both TIR1 and the Aux/IAA protein indicating that the two proteins function as co-receptors. Since both the F-box protein (TIR1/AFB) and Aux/IAA family are large, many co-receptor pairs are possible, suggesting that the plant may have a repertoire of distinct auxin sensors.
- The AFB4 and AFB5 proteins are primary targets of the picolinate class of auxinic herbicides. We have shown that loss of AFB4 and AFB5 results in insensitivity to picloram and related molecules suggesting that these two co-receptors are the primary targets of the picolinates. Consistent with this we show that picolinates compete for IAA for binding to AFB4/5 but not to the TIR1 protein.
- Members of the TIR1/AFB proteins have distinct functions throughout development. The AFB4 protein in particular plays a key role the response of the plant to elevated temperature.

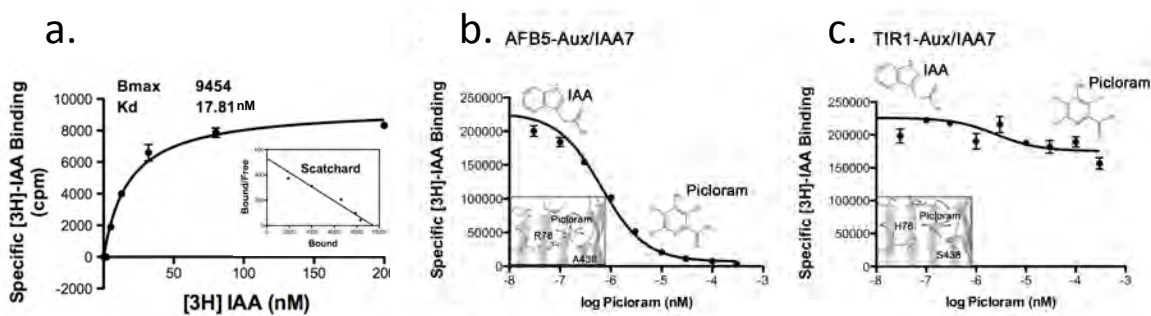


Figure 1. IAA binding to auxin co-receptors. (a) Saturation of curve of IAA binding to TIR1-IAA7 (b,c) Picloram does not compete for IAA binding to AFB5-IAA7 but does to TIR1-IAA7.

Science objectives for 2009-2010: In the coming year we will focus more directly on the role of auxin and auxin signaling during the plant response to elevated temperature.

- The response of seedlings to elevated temperature with high temporal resolution, both with respect to growth and transcriptional changes using an automated imaging system and microarray analysis.
- In collaboration with Steve Kay we will identify transcription factors that mediate the auxin-dependent growth response.
- Biochemical experiments will be performed to further characterize AFB4 and AFB5. The basis for picloram selectivity will be determined and the special role of AFB4 in the temperature response will be analyzed.

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## Plant Response to LCO/CO Signals

### Gary Stacey, PI

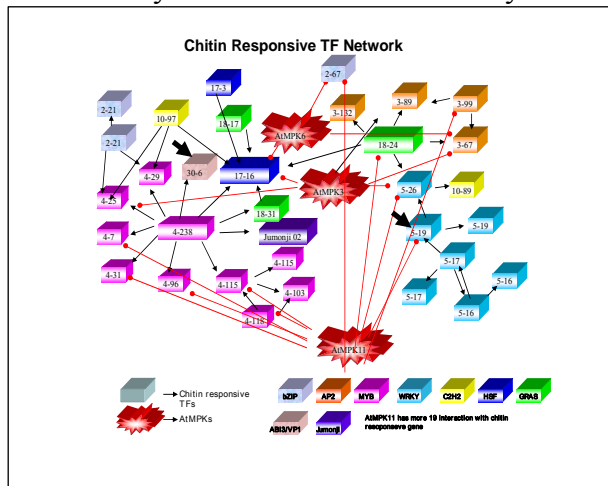
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**Overall research goals:** Chitoooligosaccharide (CO) signals, produced by pathogenic fungi, trigger a strong plant defense response. In contrast, structurally similar lipo-chitoooligosaccharide (LCO) signals, produced by beneficial symbiotic bacteria, initiate de novo organogenesis on legume plants. Our long term goal is to understand how such similar signals can elicit such different plant responses. In recent work, we identified the plant CO receptor. The proposed work focuses on understanding the mechanism of CO recognition and the downstream signaling processes. Past work from our own lab, as well as others, has shown that such CO/LCO signals impact plants in a multitude of ways. For example, similar molecules have recently been shown to be key signals in the plant-mycorrhizal symbiosis. Treatment of plants with LCO signals has been reported to affect photosynthetic efficiency, seed germination, root growth, etc. Therefore, the proposed research relates to the mission of DOE to understand the capture of light energy and how this energy is harnessed to produce plant biomass. The research is taking a systems approach to understand how CO/LCO signalling pathways are integrated into the overall regulatory pathways that ultimately affect photosynthetic conversion.

**Significant achievements in 2009-2010:** In the past year, we made major advancements in understanding how the CO receptor, encoding by the Arabidopsis *ATLYK1/CERK1* gene (1-3), functions to induce the downstream signaling pathways. LysM receptor-like kinases play a critical role in the perception of lipo-chitin (LCO) nodulation signals essential for the establishment of the legume-rhizobium symbiotic interaction. The LysM domain is a peptidoglycan-binding module



**Figure 1.** The network constructed from tested yeast two-hybrid data using chitin responsive transcription factors as bait, as well as three MAP kinases.

originally identified in bacterial enzymes that hydrolyze the peptidoglycan component in bacterial cell walls. Peptidoglycan is a chitin-like polymer. Interestingly, LysM RLKs are also found in non-legume plants. Due to the structural similarity between chitin, LCO Nod signals and peptidoglycan, it was reasonable to hypothesize that some LysM RLKs may also play a role in chitin signaling. Consistent with this hypothesis, we found that the CO receptor also is encoded by a LysM RLK.

In other work, using a yeast 2-hybrid (Y2H) approach, we identified a large, interacting network of transcription factors that control the chitin response (Figure 1). These data have now been confirmed by co-immunoprecipitation, as well as *in planta* fluorescence resonance energy

transfer (FRET) experiments. We view this interacting network of transcription factors as something similar to a spiderweb, which can integrate a wide variety of environmental cues to elicit general sets of physiological outcomes. For example, Wan et al (3) showed that, while plant recognition of various elicitors is mediated through different receptors, they all induce a similar gene expression response. How does biotic and abiotic stress affect things such as flowering time, photosynthetic efficiency, growth, etc? Our hypothesis is that, at least in part, these responses are integrated through this interconnected network

of transcription factors. We are now studying mutations in each of these transcription factors, as well as combination mutants, to decipher the role in CO signalling, as well as other plant processes.

We are also continuing our work to identify other components of the CO signalling system. This work includes an analysis of other LysM proteins in *Arabidopsis* and other proteins that interact with the CO receptor. We have also conducted general mutant screens to identify other *Arabidopsis* genes important to CO signalling. These results also reinforce our view that the plant has the ability to integrate its response to biotic and abiotic stresses (e.g., through manipulation of hormonal pathways). Our work on CO signaling provides an avenue to investigate these system level responses, which ultimately affect major plant processes, such as photosynthesis.

**Science objectives for 2010-2011:** We will continue our work to define the chitin signaling pathway in the model plant *Arabidopsis thaliana*. However, we also plan to extend this work into crop plants, including those (e.g., soybean) used for biofuel production. Specific objectives include:

- Examine the phenotypes of plant mutants defective in CO signaling, as well as investigate the role of the CO-responsive transcription factor network in integrating the plant response to a variety of biotic and abiotic stresses
- Explore the role of additional LysM proteins in CO recognition. This includes the initiation of a collaboration to deduce the X-ray crystal structure of the AtLYK1 CO receptor and other LysM proteins.

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## The crystal structure of a self-activating G $\alpha$ protein reveals a new mechanism of signal initiation

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Project Title: G-Protein-Coupled Sugar Sensing in Arabidopsis

Background and Rationale: G protein-coupled receptors (GPCRs) convert extracellular signals to intracellular responses by activating the G $\alpha$  subunit of the G $\alpha\beta\gamma$  heterotrimer. GPCRs activate G proteins by promoting GDP release from the G $\alpha$  protein in favor of GTP. When bound to GTP, the G $\alpha$  protein undergoes conformational changes that result in heterotrimer dissociation. Both the active G $\alpha$  protein and the free G $\beta\gamma$  dimer can then stimulate or inhibit downstream effector enzymes. Signal propagation is halted after the G $\alpha$  subunit hydrolyzes GTP and returns to the inactive GDP-bound heterotrimeric state.

High-resolution three-dimension structures of animal G $\alpha$  proteins show that the guanine nucleotide is buried at the interface of two domains: (i) an all  $\alpha$ -helical domain and (ii) a domain that resembles the Ras monomeric G protein. The Ras domain and domain linkers contain the residues that contact the magnesium ion, GPCR, Regulator of G protein signaling (RGS) protein, G $\beta$  subunit, as well as residues that form the guanine nucleotide binding pocket and catalyze GTP hydrolysis. Thus far the only function ascribed to the helical domain is interaction with the GoLoco motif from RGS14 (2).

Some organisms, including *Arabidopsis thaliana*, lack canonical GPCRs and therefore rely on a distinct, unknown mechanism for G protein activation. Unlike animal G $\alpha$  proteins, AtGPA1 has an extremely fast spontaneous rate of nucleotide exchange (50-fold faster than the next fastest G $\alpha$  protein) and thus does not require a GPCR or guanine nucleotide exchange factor to become activated.

Arabidopsis contains a 7 transmembrane RGS protein (designated AtRGS1) and a variety of evidence is consistent with AtRGS1 being a glucose receptor that controls the active state of AtGPA1.

The evidence also points to Arabidopsis G proteins complex serving as a sugar sensor to control efficiencies of sugar catabolism and metabolism. Photosynthesis efficiency in mutants lacking the G protein mutants is significantly different.

Overall goals. Aim 1. Determine how the active state of AtGPA1 regulates photosynthesis efficiency. Aim 2: Determine the structural requisites for AtGPA1 activation.

Significant Achievement for 2009-2010. Animal GPCRs initiate signaling by activating the G $\alpha$  subunit of heterotrimeric G proteins. However, the plant *Arabidopsis thaliana* lacks canonical GPCRs and instead employs a distinct mechanism of G protein

activation. To investigate how AtGPA1 becomes activated, we determined its crystal structure. AtGPA1 is structurally similar to animal  $G\alpha$  proteins (Fig 1), but our crystallographic and biophysical studies reveal unique properties. Notably, the AtGPA1 helical domain displays pronounced intrinsic disorder and a tendency to dissociate from the Ras domain. Domain substitution experiments show that the AtGPA1 helical domain is necessary for self-activation and sufficient to confer self-activation to an animal  $G\alpha$  protein. These findings reveal a mechanism for G protein activation in *Arabidopsis* distinct from the well-established mechanism found in animals.

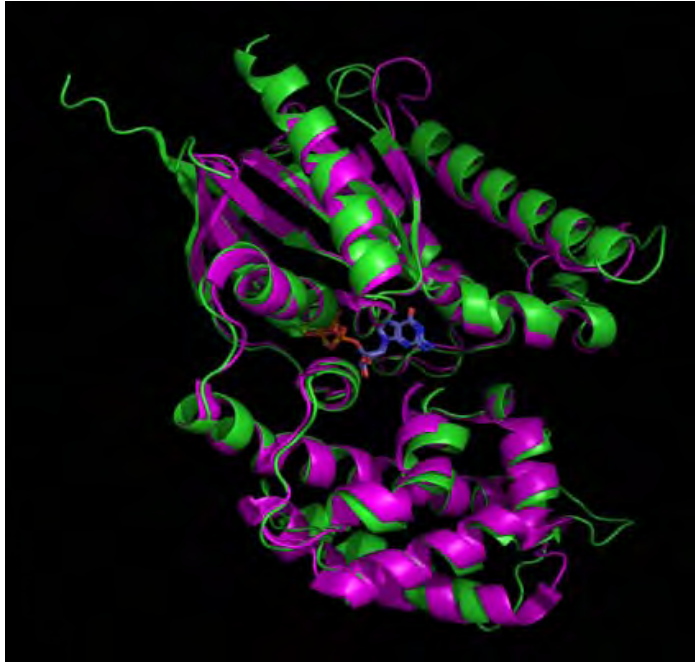


Figure 1. The crystal structure of ATGPA1 (green) at 2.3 Å resolution overlaid onto the structure of mammalian  $G\alpha_1$  (magenta)

Science Objectives for 2010-2011. We have solved the structure of AtGPA1 and thus completed Aim 2. We determined an important requisite necessary and sufficient to confer the rapid nucleotide exchange property of AtGPA1. We have discovered how to make a fast exchanger into a slow exchanger. We now need to determine how altering this exchange property affects sugar sensing in vivo. Aim 1 is to test the effect directly on photosynthesis efficiency, one manifestation of sugar sensing in plants.

*Session VII*  
*The Archaeal Proteasome*



## Proteasomes and Post-translational Modification of *Haloferax volcanii* Proteins

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### Overall research goals:

Microbes that thrive in extreme environments (*e.g.* high temperature, high salinity, low water availability, extreme pH) are useful for biotechnology applications of DOE mission including the production of renewable fuels and chemicals. Many of these extremophilic organisms are classified to *Archaea*, one of three major evolutionary lineages of life. Advances in systems biology reveal post-translational mechanisms are important in mediating the levels and activities of proteins in archaea, yet our understanding of these processes is limited. Energy-dependent proteases are central to the quality control and regulated turnover of proteins. Like eukaryotes, one of the major energy-dependent proteases of archaea is the proteasome composed of 20S core particles and regulatory particle triple-A ATPases. In contrast, ubiquitin-like proteins with a  $\beta$ -grasp fold are less conserved in archaea and are thought to be reserved for sulphur activation in processes including cofactor biosynthesis and tRNA thiolation. This has complicated our understanding of the origin and evolution of ubiquitination and its connection to proteasomes. The major objectives of this research project are to identify native protein substrates that are specifically targeted for turnover by proteasomes and, ultimately, elucidate the roles these proteases play in archaeal cell physiology. Post-translational mechanisms that alter proteasomes and their protein substrates are also of interest.

### Significant achievements in 2008 – 2010:

Using the extremophilic archaeon *Haloferax volcanii* as a model for understanding global regulatory mechanisms, we provide evidence that ubiquitin-like-protein conjugation and phosphorylation are important in mediating the recognition and turnover of proteins by proteasomes in archaeal cells. Here we report the identification of two small archaeal modifying proteins, SAMP1 and SAMP2, with a  $\beta$ -grasp fold and C-terminal diglycine motif similar to ubiquitin, that are differentially conjugated to proteins in *H. volcanii*. SAMP-conjugate levels were altered by nitrogen-limitation, were perturbed by proteasomal gene knockout and spanned a variety of functions from metabolism, translation and transcription to sulphur-chemistry including components of the Urm1 pathway. LC-MS/MS-based collision-induced dissociation (CID) enabled the identification of isopeptide bonds between the C-terminal glycine of SAMP2 and the  $\epsilon$ -amino group of lysine residues of a large number of proteins including Lys58 of SAMP2 itself, revealing the formation of poly-SAMP chains. The widespread distribution of these ubiquitin-like proteins among the archaea and diversity of pathways modified by these SAMPs suggest this type of protein-conjugation system is central to archaeal cell function.

### Science objectives for 2010 – 2011:

- To further understand the pathway of SAMPylation, a series of deletion strains will be generated in genes encoding proteins associated with SAMP-protein conjugation: SAMP1 (HVO\_2169), SAMP2 (HVO\_0202), SAMP3 (HVO\_2177, see below), MoeB/E1-like UbaA (HVO\_0558), RHD proteins (HVO\_0559, HVO\_0024, HVO\_0025), MobB-MoaE protein (HVO\_1864) and a Ncs2/Ncs6 homolog (HVO\_0580).



- Mutant strains will be examined for growth phenotypes (*e.g.*, growth on DMSO, low salt, high temperature, N-limitation) and their ability to form SAMP-protein-conjugates by Western blot of Flag-SAMP1,-SAMP2 and -SAMP3 genes expressed *in trans*. Strains unable to form SAMP-protein conjugates (*ubaA* mutant, in progress) will be compared to wild-type by LC-MS/MS-based CID (LTQ-Orbitrap) of total proteome and Flag-SAMP purified fractions. This will be correlated to transcriptome by microarray (Nimblegen). Phenotypic differences will provide a foundation for future studies that perform a more detailed analysis of how these genes may be involved in global regulation and/or sulphur-activation for MoCo biosynthesis and tRNA thiolation.

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*Session VIII*  
*Pumps, Transporters, and*  
*Trafficking in Plants*



## Molecular Mechanism and Biological Function of the Plasma Membrane Proton Pump ( $H^+$ -ATPase) of *Arabidopsis thaliana*

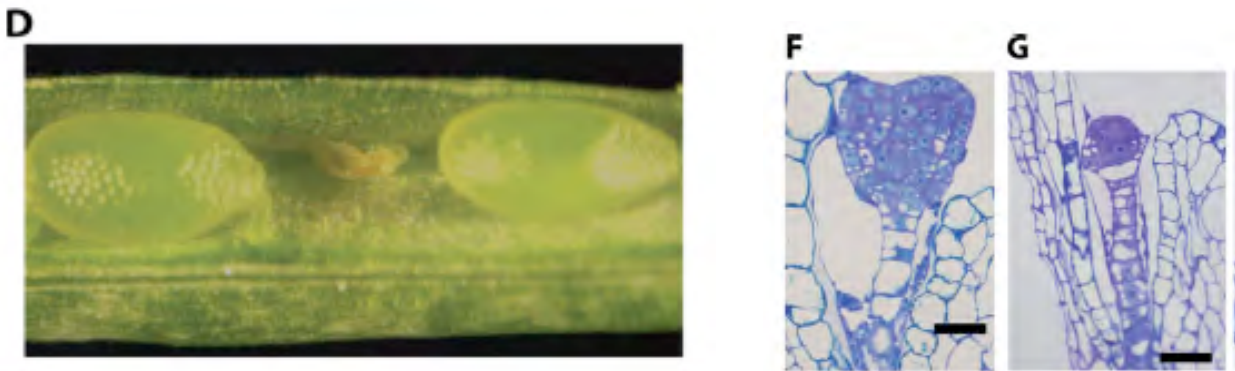
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Overall research goals: The plasma membrane is the point of contact between a cell and its external environment and plays a critical role in the growth and development of all organisms, including higher plants. The proteins within this membrane that 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 since it alone generates the protonmotive force (composed of both a membrane potential and a pH gradient) that drives the transport of solutes which is fundamental to cell growth. In plants, this enzyme appears to be one of the major ATP consumers and despite many years of study, a molecular genetic approach to understand its function has, until recently, been less forthcoming. Recently, we have demonstrated that in *Arabidopsis*, this enzyme is in fact an essential gene (Fig. 1 below, from Haruta et al., 2010)



**Figure 1.** Left (D) shows an aborted seed surrounded by two normal seeds, in a silique of a selfed plant containing a homozygous knockout mutation in *AHA1* and a heterozygous knockout mutation in *AHA2*. As predicted for an essential gene, one fourth of normal seeds are absent in the silique, and the homozygous double mutant embryos are aborted at an early stage of embryogenesis. On the right is shown a stained, microscopic thin section of a normal (F) and an aborted (G) embryo from the same plant.

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 approach is to isolate *Arabidopsis* mutants and study the phenotype resulting from the genetic defect. We are also using the mutants to perform structure-function studies via rescuing the mutants with site directed mutations within the genomic clones. Most of our work is focused on two particular members of the 11-membered *AHA* (*Arabidopsis*  $H^+$ -ATPase) gene family encoding this enzyme since these two are the most highly expressed

and together appear to constitute ca. 70% of the enzyme produced in vegetative tissue. A major recent focus has been on identifying posttranslational modifications and interacting partners of the enzyme, and substantial progress has been made in developing 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 stoichiometry AHA1, 2, 3 phosphorylation under many environmental and genetic perturbations and (3) Nontargeted mass spectrometric analysis of purified AHA 1/2 to identify additional interacting proteins and any additional posttranslational modifications.

Significant Achievements of 2007-2009: Published 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. Developed growth assays that demonstrate effects of single gene defects on growth. Established, for the first time, a molecular mechanism for compensation at the posttranslational level, rather than at the transcriptional or translational level. Developed highly sensitive and accurate assays using heavy isotope labeled synthetic peptides (phosphorylated and nonphosphorylated) for measuring the stoichiometry 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.

Science Objectives for 2009-2010:

- Obtain a double mutant rescued plant (AHA1/2) containing either AHA1 or AHA2 tagged at a terminus or internally, with a peptide sequence that can be used for pulldown's (e.g., polyhis, GFP or c-myc e.g.) to purify the pump and its interacting partners.
- Using our newly developed method for measuring stoichiometry 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.
- Using site directed mutagenesis, continue extensive structure-function studies with rescued AHA1 and AHA2 genomic clones with double lethal mutant in Arabidopsis.

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# FKBP-mediated maturation and sterol packing of the Arabidopsis ABCB19 auxin transporter are distinct processes

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Overall research goals: Plant ABC transporters function in the cellular export of a wide range of substrates including hormones, organic acids, extracellular waxes, and small molecules that contribute to secondary cell wall structure. As such, genetic manipulation of these transporters is an important strategy for biomass improvement. However, the function of some ABC transporters is dependent on cellular folding/maturation mechanisms and association with specific membrane domains. The specific goals of this project are to 1. resolve how folding and sterol interactions regulate ABCB maturation and function. 2. Identify the domains and residues that confer substrate specificity in plant ABCB transporters. 3. Determine the sequences and structures underlying transport directionality in plant ABCBs. 4. Determine the role of the putative linker domain in plant ABCB full transporters.

## Significant achievements 2007-2009:

1. Completed testing of auxin transport activity and specificity of additional auxin transport proteins from the AUX1/LAX family and representative transporters from the ABCG transporter family in *S. pombe* and in Arabidopsis. Showed that ABCG/PDRs exhibit broader substrate specificity than ABCB class. As such, ABCG substrate interaction sites are good models for comparison with ABCB substrate interaction sites for analyses of substrate specificity. This work is described in a PNAS article and a second manuscript in review.
2. In collaboration with Klara Hoyerova at the Czech Academy of Sciences, established the functional domains that mediate concentration-dependent directionality of transport in Arabidopsis ABCB4. This work also identified unique activity of the auxinic herbicide 2,4-D in preventing reversal of transport directionality in ABCB4. This work is described in a manuscript in preparation.
3. An auxin sensor based on the use of an ion-specific microelectrode and new deconvolution software has provided a means to measure auxin movement at the root surface. This electrode was tested using the maize ABCB1 mutant *br2* and was shown to be capable of measuring apparent influx and efflux along the root surface. This work is published in *Plant Journal*.
4. Created the first round of domain swapping, deletion, and point mutation constructs described in the renewal proposal for testing in *Schizosaccharomyces pombe*. However, before proceeding with functionality assays, we sought to resolve whether functionality in *S. pombe* would be enhanced by co-expression of Arabidopsis FKBP42. Expression of FKBP42 at low levels appears to increase functionality. A stable FKBP42-expressing line has been created.
5. The Arabidopsis ABCB19/PGP19/MDR1 ATP binding cassette class B transporter. functions coordinately with ABCB1 and PIN1 to motivate long distance transport of the phytohormone auxin from the shoot to root apex. ABCB19 exhibits a highly stable, predominantly apolar plasma membrane localization in sterol-enriched domains and is not trafficked by the dynamic GNOM-dependent mechanism that mobilizes PIN1. ABCB19 folding is monitored in the ER lumen as expected, but also involves interactions at the ER surface similar to those required for maturation of mammalian ABCB1 and CFTR. ABCB19 maturation is mediated by FKBP42, an Arabidopsis paralog of the mammalian FKBP38 immunophilin co-chaperone protein. However, plasma membrane localization of ABCB19 is also dependent on integration into sterol-enriched membrane vesicles in post-Golgi compartments during anterograde trafficking. ABCB19 association with these membranes is not related to maturation processes in the ER and Golgi, and does not appear to involve endocytosis. Previous co-purification of Sec14 and ABCB19

from microsomal membranes suggests that this process takes place in the *trans*-Golgi network (TGN). These results indicate that ABC protein maturation processes in the early secretory pathway are largely conserved between the plant and animal kingdoms, but sterol packaging of these proteins in plants is spatially distinct. This work is described in a manuscript in review at *Frontiers in Physiology*.

Science objectives for 2010-2011:

1. Identify the domains and residues that confer substrate specificity in plant ABCB transporters. We hypothesize that the substrate specificities of ABCB 4, 14, and 19 are primarily determined by the putative “gate” domains formed by transmembrane helices (TMH) 2/3 and TMH 8/9 in the inner leaflet region of the proteins. Mutation of critical residues for substrate interactions coupled with activity assays in *S. pombe* will be used to test this hypothesis. ABCB14 and ABCB19 subdomains will also be swapped to determine if this exchange is sufficient to alter substrate specificity for IAA and malate. Mutational results from *S. pombe* will be confirmed with complementation of Arabidopsis *abcb* mutants.

2. Determine the role of the putative linker domain in plant ABCB full transporters. We hypothesize that the linker domain connecting the two halves of ABCB transporters functions in stabilizing the nucleotide binding domains and is required for normal function of plant ABCB transporters. The linker domain of ABCB4, 14 and 19 will be deleted or replaced with “generic” Gly-Ser linker. The mutated half or full transporters will be expressed and characterized in *S. pombe*, then used to complement Arabidopsis *abcb4*, *abcb14*, and *abcb19*.

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## Trafficking to the Protein Storage Vacuoles in Plants

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Overall research goals: Occupying up to 90% of the volume of an individual cell, the vacuole is the most prominent compartment of the plant cell. It is responsible for storing sugars, pigments, ions, proteins, and volatile compounds, as well as for cellular homeostasis maintenance and regulation of the cytosolic pH. In plants, two types of vacuoles with different properties have been identified, the lytic vacuoles that carry out many of the same processes as yeast and mammalian lysosomes such as the breakdown and recycling of cellular components, and the protein storage vacuoles (PSVs) that carry out an additional number of plant-specific functions such as the accumulation and sequestration of toxic compounds and the storage of defense molecules such as alkaloids, phenolics, and protease inhibitors. Although components of the trafficking machinery to lytic vacuoles are well defined and appear to be conserved in yeast and multicellular eukaryotes, very little is known about the cellular machinery required for transport to PSVs and the mechanisms that regulate the transit of vacuolar soluble proteins containing C-terminal and N-terminal vacuolar sorting determinants (VSDs) to the PSV are only starting to be unveiled. To determine the nature of the PSV in vegetative tissues and seeds we used genetics and proteomics approaches.

Significant achievements 2007-2010: *Screen of the Vac2 T-DNA lines.* We designed a transgenic line Vac2 to isolate new components of the plant-specific CTPP sorting machinery in *Arabidopsis thaliana*. Vac2 contains a genetically engineered CLAVATA3 (CLV3) fused to the barley (*Hordeum vulgare*) lectin C-terminal vacuolar sorting signal (CLV3:CTPPBL) in the *clv3-2* mutant background. Once generated, Vac2 was successfully used in different approaches such as genetic crosses and ethyl methanesulfonate (EMS) mutagenesis for the identification of components involved in the specific sorting of CTPP proteins. Those studies, demonstrated the specialization of pathways to plant vacuoles, established a function for the PSV in development, and validated the Vac2 secretion assay as a robust method to isolate genes that mediate trafficking to the PSV.

Having demonstrated the robustness of our Vac2 meristem assay to identify and characterize mutant lines defective in trafficking to the PSV we performed large-scale screens to identify other components of this vital pathway using a T-DNA tagged population. An important advantage of the T-DNA tagged collection versus the EMS mutagenized lines is the relatively fast identification of the insertion sites using Thermal Asymmetric Interlaced PCR (TAIL-PCR), plasmid rescue, and Inverse PCR, and the generation of knockouts. For this screen, 11000 T-DNA tagged lines in the Vac2 background were generated in collaboration with Dr. Bressan (Purdue University) and the selection of mutants was performed similarly to the EMS population.

As a result, nine mutant lines exhibited complementation of the *clv3* phenotype after the tertiary screen and the identities of the genes in which we detected T-DNA insertions were classified into three categories: **(I)** putative endomembrane system related genes, **(II)** putative direct suppressors of the CLV3 pathway and **(III)** genes that have no obvious characteristic.

Among the putative endomembrane-related genes are: a FYVE motif containing Zinc finger protein (At1g22510) involved in endosomal docking, a putative GAP (glycosylphosphatidylinositol-anchored protein) belonging to the COBRA family (At4g16140); Sec1B, which belongs to the KEULE family of proteins that regulate the formation of SNARE complexes (At4g12120), the autophagy-related gene *ATG8h* (At3g06420), and the secondary cell wall thickening promoting factor (At3g61910). A putative suppressor of the CLV3 pathway was also identified, the TOPLESS related protein involved in floral meristem determination (At2g25420) and, as in most genetic screens, we identified unexpected candidates involved in transport pathways to the protein storage vacuole including the ribosomal protein of the large subunit RPL4 (At3g09630), the cytosolic proton pump interactor PPI2 (At3g15340), and the ABC transporter P-GLYCOPROTEIN 16 (At3g28360). The direct involvement of these mutants in

vacuolar trafficking highlights the role of basic cellular mechanisms such as translational regulation, H<sup>+</sup> homeostasis, and auxin transport for the proper delivery and accumulation of vacuolar cargoes. The improved understanding of endomembrane trafficking to the PSV that emerges from these findings will further contribute to improve plant and crop fitness and production of biomass as we unravel the machinery required for loading reserves into PSVs.

*Isolation of specific vesicles and cargo identification.* The vacuoles belong to the large endomembrane system, which includes the endoplasmic reticulum (ER), Golgi apparatus, trans-Golgi network (TGN), prevacuolar compartments (PVCs or multivesicular bodies, MVBs), the endosomes, and the plasma membrane (PM). Traffic between the compartments that form the endomembrane system is accomplished by vesicle trafficking. To date, several approaches have been established for the identification of limited proteomes of cellular organelles in plants. However, in order to fully understand the mechanisms of PSV-targeted protein trafficking within cells, it is necessary to determine the protein composition of vesicles that relay proteins between organelles. A valuable approach to analyze their identity is to affinity purify these vesicles and identify its cargo using proteomic analysis. Towards this aim vesicle isolation tailed with a proteomic analysis allowed us to gain additional insights in the SYP61 vesicles content and behavior. The identified proteome demonstrated the presence of a complete SYP61 SNARE complex with its regulatory proteins without contamination from other compartments validating this approach. The analysis of the vesicles content identified several proteins involved in vesicle, fusion, tethering and trafficking and uncovered similarities and differences between mammalian and plants concerning their subcellular location and role in the endomembrane system. The isolated vesicles also revealed the presence of cargo destined to the plasma membrane and suggested a secretory role of SYP61 vesicles. More specifically, our co-localization studies demonstrated that the SYP61 vesicles are involved in trafficking of cellulose synthases demonstrating a role for these vesicles in cell wall biosynthesis and biotic stress responses. As in the case of the Vac2 T-DNA screen, several proteins of unknown function were identified in the SYP61 compartment and demonstrated that the specific vesicle isolation is an effective approach towards the understanding of the vacuolar trafficking pathways and provides a starting point for the systematic dissection of the plant endomembrane system.

#### Science objectives for 2010-2011:

To determine the nature of the protein storage vacuole in seed and vegetative tissues it is necessary to identify the components involved in their cognate trafficking pathways, we will continue our genetics and proteomics approaches.

- We will finish analysis of the SYP61 vesicles cargo and publish these data.
- We will clone and analyze two more interesting mutants were obtain as a result of Vac2 T-DNA mutants screen.

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## Functional Analysis of Plant Sucrose Transporters

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Overall research goals: The main research goal is to understand the transport activity and physiological function 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 Arabidopsis as a model. The ability to modify SUT activity will be important for engineering changes in carbon partitioning.

Significant achievements 2008-2010: Plants encode three types of SUTs, they are distinguished phylogenetically and by transport function. We produced the first detailed analysis of a type III SUT (1). Type III SUTs are localized to the vacuole membrane, are proton coupled symporters and our results suggest that they function in sucrose transport from the vacuole lumen into the cytoplasm. In addition to sucrose, type III SUTs transport many other glucosides and this may be physiologically significant. We identified functions for AtSUC1, a sink-expressed sucrose transporter (2). We showed by analysis of Arabidopsis mutants that AtSUC1 is required for pollen function and sucrose-induced anthocyanin production in seedlings. In contrast to eudicots, monocot plants lack type I SUTs and utilize type II SUTs for phloem loading. We analyzed two type II SUTs from rice and show that, compared to type I SUTs, they have a very high specificity for sucrose (3). This work revealed the range of transport activities of the three types of SUTs. Most significantly, fluorescent coumarin glucoside substrates were identified that are transported by type I but not type II SUTs (Fig. 1) and this allows us to use fluorescence-based techniques to study transporter activity.

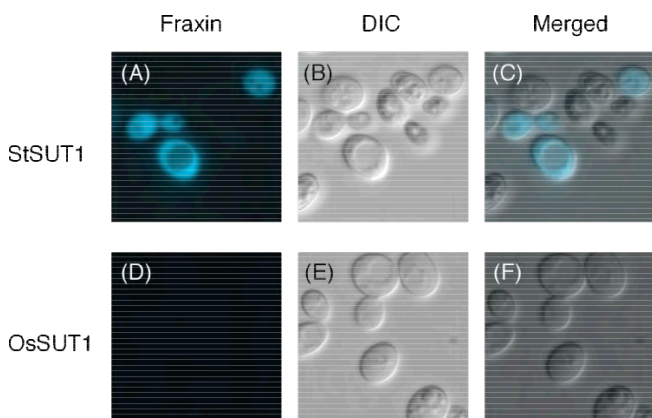


Fig. 1 Uptake of the coumarin glucoside fraxin in yeast differentiates between Type I and Type II SUTs. StSUT1 confers fraxin uptake activity while OsSUT1 does not.

### Science objectives for 2010-2011:

- Determine amino acid positions that function in substrate binding. We are using gene shuffling between type I and II SUTs and exploiting strong differences in substrate specificity between type I and II SUTs to identify amino acids that control substrate specificity. Yeast expressing

type I SUTs accumulate coumarin glucosides (Fig. 1). Using gene shuffling with OsSUT1 and a synthetic OsSUT1 cDNA encoding 64 amino acids conserved in type I SUTs, we used fluorescence activated cell sorting (FACS) to select yeast expressing recombinant cDNAs based on uptake of coumarin glucosides. Two amino acid positions that may be important for substrate binding were identified and will be studied in detail.

- Test the validity of 3D structural predictions. We are using site directed mutagenesis to test predictions based on the structure of lac permease. The only amino acids that are irreplaceable in lac permease are charged and located within membrane spans. We mutated several charged amino acids predicted in our model to be in membrane spanning domains and show several positions required for activity.
- Further develop *atsuc2* mutant complementation. In Arabidopsis AtSUC2 is required for loading sucrose into the phloem. The *atsuc2* mutant has stunted growth, and accumulates starch and sugar in leaves. Several type I and type II SUTs complement the mutant. To develop this system to study mutated SUTs in the plant, we need additional biochemical assays to study phloem sap in complemented mutants. So far metabolite analysis of Arabidopsis phloem sap has not been successful.

References to work supported by this project 2008-2010:

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2. Sivitz, A. B., Reinders, A. and Ward, J. M. (2008) Arabidopsis sucrose transporter AtSUC1 is important for pollen germination, lateral root development and sucrose-induced anthocyanin accumulation. Plant Physiol. 147: 92-100.
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*Poster Session II*



## Structural and Kinetic Studies of Novel Cytochrome P450 Small-Alkane Hydroxylases

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**Overall research goals:** The goal of this project is to investigate the changes in structure and biophysical properties of members from two families of cytochrome P450s that have been evolved in the laboratory to catalyze the hydroxylation of gaseous alkanes. By studying these changes, we aim for a basic understanding of the improved activity on small alkanes, which should aid further evolution or design of a methane-hydroxylating cytochrome P450 and P450 catalysts for selective alkane oxidation.

**Significant achievements 2009-2010:** We report the first finding of direct methane to methanol conversion by a heme porphyrin catalyst, the soluble cytochrome P450 from *Mycobacterium sp*, CYP153A6 (A6). By utilizing terminal oxidants to circumvent the requirement for substrate binding in dioxygen activation in the canonical P450 catalytic cycle, we were able to interrogate five P450s as to whether their active heme-ferryl oxidant (compound I) is capable of inserting oxygen into the 104.9 kcal/mol C-H bond of methane. This strategy essentially dissected the influence of substrate binding in generating compound I from the oxidizing ability of compound I and allowed for the identification of A6 as a promising candidate for directed evolution towards a P450-based methane monooxygenase. Iodosylbenzene-mediated hydroxylation of methane by A6 was validated by isotope labeling experiments with  $^{13}\text{CH}_4$  and  $^{18}\text{OH}_2$ . Attempts to reconstitute this activity under turnover conditions by means of whole-cell bioconversions or reconstituted reductase proteins were unsuccessful, however, due to low affinity of the native enzyme for methane. We have devised and validated an *in vitro* high-throughput colorimetric screen for the dehalogenation of iodomethane as a surrogate to methane oxidation, which will enable screening for enhanced iodomethane, and methane, affinity.

Table 1: Alkane hydroxylation by P450s utilizing terminal oxidants.

	product formed, $\mu\text{mol} (\mu\text{mol P450})^{-1} \text{ a}$										
	CYP102A1			PMO	CYP101			CYP153A6			A6 BMO-1
	PhIO	MCPBA	H <sub>2</sub> O <sub>2</sub>	PhIO	PhIO	MCPBA	H <sub>2</sub> O <sub>2</sub>	PhIO	MCPBA	H <sub>2</sub> O <sub>2</sub>	PhIO
Methane	-	-	-	-	-	-	-	<b>0.05</b>	-	-	<b>0.02</b>
Ethane	-	-	-	-	-	-	-	2.37	0.35	0.20	2.4
Propane	0.97	0.35	0.27	0.74	0.83	0.86	1.06	3.86	1.65	0.45	2.97
Hexane	1.95	0.19	0.29	0.78	0.55	0.39	0.05	0.50	0.57	0.23	N.D
Octane	1.37	0.12	0.22	0.32	0.32	0.05	0.07	0.51	0.14	0.32	N.D

<sup>a</sup>Alkanes (2.5 mM, or saturated at 20 psi) were incubated with P450 (100  $\mu\text{M}$ ) and terminal oxidant (5 mM) at 25 °C for 10 min. The data represent the averages of at least three experiments and are not corrected for P450 destruction, with all data for each parameter being within 15 % of the mean.



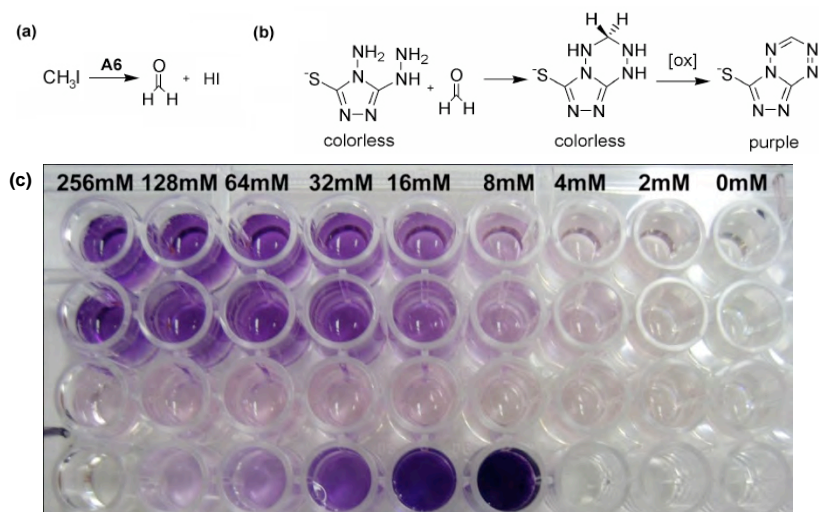


Figure 1: Iodomethane dehalogenation assay (a) Hydroxylation of iodomethane produces formaldehyde. (b) Purpald reaction: Purpald reacts with formaldehyde to form a purple adduct upon air oxidation. (c) Reactions of various concentrations of iodomethane (row 1 and 2) and  $\text{d}_3$ -iodomethane (row 3) with  $0.5 \mu\text{M}$  CYP153A6 with Purpald addition after 5 minutes at  $25^\circ\text{C}$ .

We have also crystallized and determined the structure of P450 variant 7-7, a key intermediate on the evolutionary pathway to our highly efficient propane hydroxylase, P450<sub>PMO</sub>. This structure, upon further refinement, will allow us to identify specific structural changes that accompanied the acquisition of high activity on propane.

#### Science objectives for 2010-2011:

- Conversion of CYP153A6 into a methane monooxygenase will be our top priority. We will implement a ‘substrate walk’ strategy, evolving for improved iodomethane dehalogenation as a surrogate for methane hydroxylation. We have previously demonstrated the success of such a strategy in evolving CYP102A1 from a medium-chain (C12-C18) fatty acid hydroxylase into P450<sub>PMO</sub> with parent-like catalytic efficiency for propane hydroxylation.
- Our effort to solve the crystal structures of P450 variants will expand to CYP153A6 and derived variants. With a crystal structure, we will be able to study the second-sphere coordination differences that distinguish the compound I of CYP153A6 from homologs that cannot hydroxylate methane and determine how these differences contribute to the unique preference of A6 for otherwise stronger terminal C-H bonds in octane, hexane and propane.

#### References to work supported by this project 2009-2010:

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3. C. A. Tracewell and F. H. Arnold, “Directed Enzyme Evolution: Climbing Fitness Peaks One Amino Acid at a Time,” *Current Opinion in Chemical Biology* **13**, 3-9 (2009).
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## Dissection and Manipulation of LRR Domains in Plant Disease Resistance Gene Products

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Overall research goals: Leucine-rich repeat domains offer a readily diversifiable platform (literally, an extended protein surface) for specific binding of diverse ligands. Individual plants contain hundreds of different LRR-containing proteins, often receptors, that mediate a wide array of biological processes. This research addresses the following overlapping research questions:

- How do leucine-rich repeat (LRR) proteins recognize their cognate ligands?
- What are the intra- and inter-molecular transitions that occur that cause transmembrane LRR proteins to switch from “off” to “on”?
- How do plants use LRR receptor proteins to activate disease resistance?
- Can we synthetically evolve new LRR proteins that have acquired new ligand specificities for extant, relevant ligands?

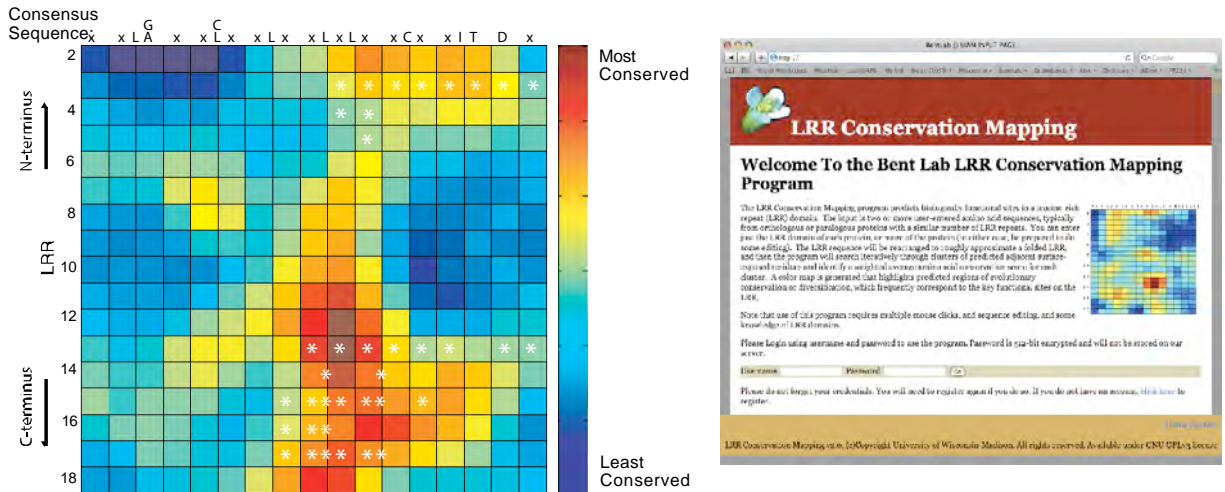
Significant achievements in 2008-2010:

Arabidopsis FLS2 and EFR are two deeply studied plant transmembrane LRR receptor kinases. They directly bind bacterial flagellin (FLS2) or bacterial EF-Tu (EFR), or flagellin- or EF-Tu-derived peptides, and activate plant defenses. We recently dissected four aspects of FLS2 protein function. We find that some FLS2 is present in **FLS2-FLS2 associations *in vivo***, and the overall abundance of those associations is not ligand-dependent. We find that overexpression of the intracellular kinase domain of FLS2 exerts **dominant-negative impacts**. These findings inform physical models for FLS2 signaling activity. Thirdly, we find that FLS2 operates relatively effectively without many of the **glycosylations** that normally decorate the FLS2 receptor, while function of EFR (a similar receptor that is of more recent evolutionary origin) is highly sensitive to altered glycosylation. Lastly, we find that the **paired cysteines** at the amino-terminal end of the LRR (common in most plant and animal extracellular LRR proteins) are crucial for successful ER processing and delivery of the receptor to the cell surface. Intriguingly, the second Cys-pair that is at the carboxy-terminus of the LRR is not required for receptor processing and localization, but mutation of it can cause overactive signaling activity. The above constitutes one manuscript that is under review.

We have also identified autophosphorylation sites of recombinant FLS2 protein, as well as novel signaling partners of FLS2, and further studies are underway. An extensive multi-year collaboration to express and crystallize FLS2 or portions thereof was unsuccessful, although the FLS2 autophosphorylation sites were identified through that project.

In a separate major effort, we have designed algorithms that use generic knowledge of LRR structures to predict, in the absence of known crystal structures or ligands for a given LRR domain, the most highly conserved patches of amino acids on the LRR surface. For most proteins, these most highly conserved patches are the key functional sites. A public, web-based interface for use of this **“LRR Conservation Mapping” (LCM)** has been under development and v.1 is close to being released (Figure 1, next page). Known co-crystals of LRR+ligand have served as positive controls to validate the method (Figure 1).

As a separate but central part of this work, we have carried out wet-lab testing of the predictions made by LCM computational analysis, using “unknowns” (the plant innate immunity receptors FLS2 and EFR) to demonstrate use of the method as a tool for new biological discovery. For example, use of our LCM program with Arabidopsis EFR and a confirmed EFR ortholog from *Brassica napus* predicted highly conserved sites and “negative control” sites that were not particularly conserved. We then constructed and tested double-alanine mutations of EFR in these conserved and less conserved locations. Mutations in less conserved regions never broke function, whereas mutations in conserved regions severely abrogated function in 2/3 of the tested cases. A comparison to the ‘desolvation energy’



**Figure 1. LRR Conservation Mapping to predict functional sites.** Predicted surface residues on the cylindrical (solenoid) LRR domain are shown in a 2D representation, with heat-map coloring of conservation across related proteins. **a)** Validation example: TIR and AFB (auxin receptor) proteins were compared to generate heat map. White asterisks show known contact points with ligands (auxin and InsP<sub>6</sub>), from co-crystal structure, which overlap substantially with the sites predicted by LCM. **b)** A web-based public interface for LRR Conservation Mapping is functional and almost ready for release.

approach to ligand binding site prediction suggests that LCM is a more accurate predictor. Novel FLS2 alleles have been generated that push FLS2 modestly toward hyper-responsiveness or specificity for new ligands. This work is ongoing.

Science objectives for 2009-2010:

- Test the biological relevance of the autophosphorylation sites that were identified *in vitro*.
- Investigate novel candidate signaling partners of FLS2 that have been identified.
- Eliminate known minor bugs in the LRR Conservation Mapping program and release it for public use.
- Add new LRR dissection tools to the LRR Conservation Mapping program.
- Continue work on new FLS2 alleles with novel functions.
- Continue development of novel systems for LRR evolution and screening.

References to work supported by this project 2007-2009:

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## **Jasmonate Signaling and Stamen Development in *Arabidopsis***

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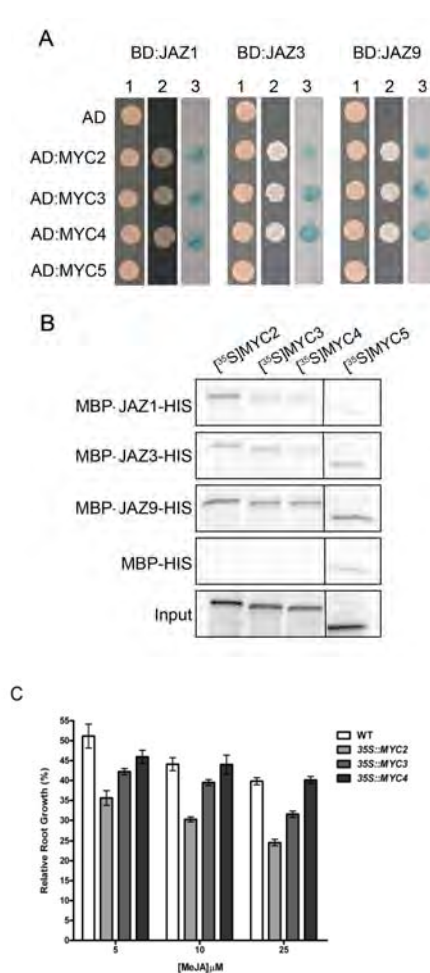
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Overall research goals: Our original interest in understanding the role of JA in regulating the final stages of stamen and pollen development led to our discovery of the JAZ repressors, and the molecular mechanism of jasmonate (JA) hormone action is now a second important focus of our research. The specific goals for this grant period are: **1.** Identify the functions and regulatory relationships among the transcription factors involved in JA-mediated stamen maturation using mutants, dominant-negative constructs and regulated expression of the genes encoding them. **2.** Collaborate on solving the crystal structure of COI1 to provide information on the atomic-level interactions governing COI1/JA-Ile/JAZ binding. **3.** Use structural information from the COI1 crystal structure and results from measurements of protein expression and assays of protein-protein interactions to refine our understanding of the molecular mechanism of JA hormone signaling. **4.** Employ specific assays and yeast-two-hybrid screens to identify additional transcription factors that interact with the JAZ proteins. Mutant analysis, overexpression and dominant-negative constructs will be used to probe the roles of these transcription factors in mediating JA responses.

Significant achievements 2007-2009: The plant hormone jasmonate (JA) plays important roles in regulating the synthesis and accumulation of plant secondary products, and is also required for fertility. JASMONATE ZIM-DOMAIN (JAZ) proteins inhibit transcription factors that regulate early JA-responsive genes, and JA-induced degradation of JAZ proteins thus allows expression of these response genes. To date, MYC2 is the only transcription factor known to directly interact with JAZ proteins and regulate early JA responses, but the phenotype of *myc2* mutants suggests that other transcription factors also activate JA responses. Yeast-two-hybrid and pulldown assays identified MYC3, MYC4 and MYC5 as additional transcription factors that interact with the JAZ repressor proteins (Fig. 1A,B). Although *myc3* and *myc4* loss-of-function mutants showed no phenotype, transgenic plants overexpressing *MYC3* and *MYC4* had higher levels of anthocyanin compared to the wild-type plants. In addition, roots of *MYC3* overexpression plants were hypersensitive to JA (Fig. 1C). Quantitative real time RT-PCR analysis of nine JA-responsive genes revealed that eight of them were induced in *MYC3* and *MYC4* overexpression plants, except for a pathogen-responsive gene, *PDF1.2*. Similar to MYC2, MYC4 negatively regulates expression of *PDF1.2*. Together, these results suggest that MYC3 and MYC4 are JAZ-interacting transcription factors that regulate JA responses.

In the presence of the active JA hormone, jasmonoyl-isoleucine (JA-Ile), JAZ proteins bind to the F-box protein, COI1, and this results in their ubiquitination and degradation in the 26S proteasome. The crystal structure of the JAZ1 degron (amino acids 200-220) bound to COI1 in the presence of JA-Ile has been solved by our collaborator, Ning Zheng (U. of Washington). The resulting structure and other results indicate that COI1 and JAZ are co-receptors for JA-Ile and that the structural details of the receptor are remarkably distinct from the closely related auxin system.

Science objectives for 2009-2010: We have identified additional bHLH proteins that appear to have important roles in regulating JA responses. During the next year, we will characterize these by reverse-genetic and overexpression approaches to test their involvement in stamen/pollen development and other processes controlled by JA signaling.



**Figure 1.** MYC3 and MYC4 interact with JAZ repressors and regulate JA responses. **(A)** MYC3 and MYC4 interact with JAZ proteins in Y2H. (1) YRG2 yeast cells expressing both bait and prey fusions were grown on yeast synthetic minimal SD medium with omission of leucine and tryptophan, and (2) on yeast SD medium with omission of leucine, histidine and tryptophan for examination of HIS3 reporter gene (3) assayed for lacZ activity. **(B)** Pull-down of [<sup>35</sup>S]bHLHs (MYC2, MYC3, MYC4 and MYC5) produced by in vitro transcription/translation, using magnetic beads containing MBP-His or MBP-JAZ-His fusion proteins. The input lane shows 10% of [<sup>35</sup>S]-Met labeled products used in each pull-down assay. **(C)** Relative root growth of wild-type and overexpression transgenic seedlings. Seedlings were grown on normal MS plates for 4 days and transferred to new MS plates containing 0, 5, 10 and 25 μM JA. Root length was measured 6 days after transfer. Root growth in the absence of JA was set to 100%. Data are mean ± s.e. for n=20 seedlings.

We have already identified a series of MYB transcription factors that contribute to the development of fertile stamen and pollen during reproductive development. One of these, MYB21, is required for plant fertility – *myb21* null mutants are substantially sterile. To find out if MYB21 is *sufficient* for male fertility in the absence of JA signaling, we will express a *MYB21* transgene in mutants deficient in JA synthesis (*opr3*) or JA perception (*coi1*). If *MYB21* expression rescues the sterile phenotype of these mutants, it will indicate that this transcription factor is the master

regulator of the very large gene-expression cascade that is required for correct stamen and pollen development in *Arabidopsis*.

References to work supported by this project 2008-2010:

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## Asparagine synthetase gene regulatory networks and plant nitrogen metabolism

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Overall Research Goals: Our long-term goals are to identify the regulatory components that coordinate metabolic, photosynthetic, energy and developmental processes affecting N-use efficiency in plants. Using a combination of genetics, genomics and systems biology, we aim to model and alter gene regulatory networks affecting N-assimilation into asparagine (Asn) a key amino acid used to transport and store nitrogen (e.g. in seeds). Studies performed and patents issued under this DOE grant, have shown that N-assimilation into Asn, are mediated via changes in transcription of the major asparagine synthetase gene (ASN1). Altering ASN1 transcription can be used to affect increases in N-content of seed in Arabidopsis and are translated to field trials in corn. Our goal is to now alter the gene regulatory networks affecting ASN1 in concert with other related metabolic processes (e.g. photosynthesis) to coordinately effect changes in N-use efficiency, an energy issue whose importance has grown in the era of biofuels.

Significant achievements from 2008-2010:

**Aim 1. Role of histone methylation in the control of ASN1 regulation by light and carbon.** 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 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 (*cli*) mutants impaired in ASN1 repression by C and L signals (Thum et al 2008). We recently showed that the *cli186* mutation is in a gene encoding a histone lysine methyltransferase (H3K4/K36) involved in the remodeling of chromatin associated with actively regulated genes (Mukherjee et al., 2010). *cli186* is a new deletion allele of EFS/SDG8, previously shown to control FLC, a repressor of flowering (Kim et al., 2005; Zhao et al., 2005). Transcriptome analysis of C and/or L treated *cli186* flowering plants (light-grown or etiolated) showed that the misregulated set of genes are significantly overrepresented in GO processes including photosynthesis, nitrogen compound biosynthesis, response to stimulus and several other related metabolic functions. To identify the genome-wide targets of H3K4/K36 methylation by EFS/CLI186, we performed Chromatin-IP-Solexa analysis using antibodies to the trimethyl group of H3K4 or the dimethyl group of H3K36. Preliminary analysis of the deep-seq Solexa data demonstrates the direct targets of H3K4/K36 methylation by EFS/CLI186 include a set of regulatory and signaling genes which we will now target for mutant studies. We are also studying several predicted downstream transcription factor targets of EFS/CLI186 for their role in the regulation of ASN1 and related metabolic genes. Preliminary results using a 35S::TF line suggest that one such downstream TF (HAT22 an HD-Zip transcription factor) appears to mediate the light regulation of ASN1 and related genes in N-metabolism. **Aim 2. Identification of an ASN1 regulatory network involved in seed development.** We have previously shown that manipulation of ASN1 expression in transgenic plants leads to increased N-content of seed (Lam et al 2004). More recent network analysis of transcriptome data has uncovered a gene regulatory network that coordinates ASN1 induction during seed development, which reflects the role of Asn as an N-storage compound. In this predicted regulatory network, ASN1 showed high correlation between several transcription factors, especially two bZIP transcription factors, bZIP1 and bZIP11. Our studies on bZIP1 knockout lines have shown the bZIP1 mutation affects not only N and L regulation but also that this regulation can have different patterns or modes of regulation (Obertello et al., 2010). This analysis demonstrates that bZIP1 tunes L and N relationships, and can suppress regulatory mechanisms required at different developmental stages and/or environmental conditions. The T-DNA and over-expressor lines of bZIP11 will be analyzed for the regulation of ASN1 expression in seedlings and in seeds of mature plants. **Aim 3. N-use and seed N/yield via manipulations of ASN1 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 thus begun to measure changes in temporal levels of the primary N-assimilation products (Glu, Gln, Asp, Asn) using  $^{15}\text{N}$  labeling as a function of time, first in wild-type and later in mutants in Asn metabolic genes (both synthesis and degradation) and in putative regulatory genes. This  $^{15}\text{N}$  tracer study will enable us to study the flux of newly assimilated nitrogen through the N-assimilation pathway and stored as Asn, compared to changes in internal  $^{14}\text{N}$  pools.

Science objectives for 2010-2011: Our scientific aims for 2010-11 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.

**Publications and patents from this project 2004-2010:**

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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.

## Pathogen Virulence Factors: Unique Probes of Plant Cell Structure and Function

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### Overall Research Goals

Plant pathogens use a variety of virulence factors to suppress plant defense. One important and large class of virulence factors from plant pathogenic bacteria is the type III effector (TTE) proteins. These proteins are injected directly into the plant cytoplasm, where they interact with a set of host targets to promote virulence. We have sequenced a set of ~20 *Pseudomonas syringae* strains that infect a variety of plant hosts and found a large population of TTEs: ~60 protein families, encoded by over 400 different genes.

While most TTEs remain uncharacterized, an emerging trend is that they are often structural mimics of eukaryotic proteins. This mimicry is only apparent by examining them structurally, as the primary sequence is often uninformative. Thus, discerning the function of the large population of type III effectors is not possible using simple primary sequence analysis such as BLAST.

Our main hypothesis, supported by data from several labs, is that the targets of this collection of TTEs will define a collection of plant processes that are critical regulators of defense and response to infection. Importantly, effectors can serve as a discovery tool to identify processes unamenable to traditional forward genetics due to lethality and/or redundancy. In essence, we view TTEs as evolved probes of plant cellular biology.

Our main approach in our DOE funded project has been to use structural biology to define likely functions for a collection of TTE proteins, starting with those for which we know at least some of the plant targets (AvrRpm1, AvrB). We are now proceeding outward to a wider set of proteins using structures to both predict TTE function and to predict effects on putative host targets (HopAF1, HopH1, HopC1, HopBA1).

Pathogens cause the loss of up to 30% of the world's crops, including bioenergy crops present and future. Thus, our work serves DOE's mission by trying to minimize the loss of primary production due to yield loss from infection.

### Significant Achievements 2008-2010

**(1) AvrRpm1 is a putative ADP-ribosyltransferase.** AvrRPM1 is one of the best characterized TTE proteins. It, along with AvrB and AvrRpt2, is known to target the host protein RIN4, which in turn is guarded by the R-protein RPM1. The molecular function of AvrRpm1 is still unclear. By using tertiary structure prediction, we found that AvrRpm1 is similar to the catalytic domain of poly-ADP-ribosyl polymerase. The predicted catalytic residues are conserved and required for function. We have recently found two candidate targets for AvrRpm1 ADP-ribosylation via biotin affinity tagging.

**(2) HopAF1 is a putative deamidase that targets ethylene accumulation.** HopAF1 is a widely distributed TTE without primary sequence similarity to known proteins. By using tertiary structure prediction, we found that HopAF1 is similar to deamidases. In a separate approach, we have found putative targets for HopAF1 via Y2H screening. At least one of these targets is required for normal ethylene



accumulation. We have found that bacterial delivery of HopAF1 can alter ethylene accumulation *in planta*. This ethylene effect requires the predicted catalytic residues.

**(3) AvrB and AvrRpm1 lead to the phosphorylation of a specific residue of their host target protein RIN4.** The co-crystal structure of AvrB and RIN4 we generated suggested several potential phosphorylation sites relevant to binding/activity. Accordingly, we have created a set of transgenic Arabidopsis *rin4* plants complemented with RIN4 variants mutated at predicted phosphorylation sites. We have found that at least one of the phosphomimic variants, T166D, is capable of activating RPM1-dependent cell death in the absence of TTEs. The non-phosphorylatable mutant T166A is unable to respond to AvrB. We have generated a phospho-specific antibody to verify that T166 is indeed phosphorylated in response to AvrB and AvrRpm1. We hypothesize that AvrB-dependent phosphorylation of RIN4 T166 is activates RPM1.

#### Science Objectives 2010-2012

**(1) Identify the biotin-labeled, putative ribosylation targets of AvrRpm1.** We are currently attempting to identify ADP-ribose biotin-tagged proteins that are candidate substrates of AvrRpm1. We expect an eventual mechanistic convergence between AvrRpm1 function and RIN4 T166 phosphorylation.

**(2) Identify the targets of HopAF1, determine deamidase activity.** We have purified HopAF1 as a GST fusion and are currently purifying *in vitro* expressed protein for the candidate substrates (Y2H interacting proteins). After an *in vitro* coincubation, we'll perform tandem mass spectrometry analysis to find HopAF1-dependant modifications of the substrates. We are also testing if HopAF1-dependent ethylene phenotypes are dependent on the putative substrates.

**(3) Identify substrates of the hopC1/hopH1 protease cluster.** HopH1 is a protease with structural similarity to botulinum toxin, a bacterial virulence effector known to cleave mammalian SNARE proteins. Since SNARE proteins are known to be required for secretion of anti-microbial compounds we are testing if HopH1 is able to cleave SNARE proteins *in planta* and *in vitro*. We are also attempting to identify substrates for these proteases via mRNA display.

**(4) Characterization of the novel effector HopBA1.** HopBA1 is a TTE of unknown function we recently discovered via promoter trapping. We have constructed transgenic lines that inducibly express HopBA1 and are conducting a forward genetic screen to identify Arabidopsis mutants that lose HopBA1-induced hypersensitive cell death. We are also in the process of attempting to solve the crystal structure of hopBA1 and are characterizing loss of function phenotypes for hopBA1 *Pseudomonas* strains that infect a variety of crop species.

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## Resolving protein-semiquinone interactions by advanced EPR spectroscopy

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Overall research goals: Our focus is on use of modern, high-resolution EPR spectroscopy to explore the catalytic domains trapped in states with semiquinone (SQ) as an intermediate. The catalytic sites we propose to study, - the  $Q_A$  and  $Q_B$ -sites of the reaction center, the  $Q_I$ -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 2008-2010:

*Semiquinone in the  $Q_H$  site of cytochrome  $bo_3$  ubiquinol oxidase.* *E. coli* cytochrome  $bo_3$  ubiquinol oxidase (cyt  $bo_3$ ) catalyzes the oxidation of ubiquinol in the cytoplasmic membrane with reduction of  $O_2$  to water. An SQ intermediate is generated at a high affinity quinone binding site ( $Q_H$ ). The X-ray structure of cyt  $bo_3$  does not contain any bound quinone, but site-directed mutagenesis studies have suggested a role for residues R71, D75, H98, and Q101, which modulate the properties of the  $Q_H$  site. Our results described below provide new contributions to the development of a structural model of the site.

*Identification of the nitrogen donors hydrogen bonded with the semiquinone.* Selective  $^{15}N$  isotope labeling of R71, H98 and Q101 was used to probe the hydrogen bonds between nitrogen donors and the  $Q_H$  SQ in the cyt  $bo_3$ . We demonstrated that the  $Q_H$  SQ has a strong hydrogen bond to the  $\epsilon$ -nitrogen of R71 and has very weak interactions with H98 and Q101 (ref. 1).

*The  $^{13}C$  labeling of the SQ.* We have applied a biochemical approach for selective  $^{13}C$  labeling of methyl groups in methyl and methoxy substituents in the SQ using L-methionine (methyl- $^{13}C$ ) as the only source of  $^{13}C$  in the wild-type  $bo_3$  and D75E mutant. The  $^{13}C$  labeled quinone isolated from cyt  $bo_3$  was used for the generation of the anion-radical in model alcohol solutions.  $^{13}C$  2D ESEEM spectra of the model system have shown significantly different hyperfine couplings with the  $^{13}C$  in methyl and methoxy groups in comparison with the wild-type cyt  $bo_3$ , and D75E and D75H mutants. The hyperfine couplings in proteins will characterize the redistribution of the unpaired spin density induced by new environment as well as the conformation of the methoxy groups.

*Semiquinone in cytochrome  $aa_3$ -600 from *Bacillus subtilis*.* Four polar residues indicated above have been implicated in binding to the quinol at the  $Q_H$ -site in cyt  $bo_3$ . Within the > 400 sequences of quinol oxidases, R71, D75 and H98 are totally conserved. Q101 is totally conserved in sequences from proteobacteria, but is often replaced by a glutamic acid (E97) in the homologues in the firmicutes, including the *B. subtilis*  $aa_3$ -600 menaquinol oxidase. Our studies of cyt  $bo_3$  were extended on cyt  $aa_3$ -600 in order to decipher the differences between the protein-quinol interactions of the  $bo_3$ -type ubiquinol oxidase and the  $aa_3$ -600 menaquinol oxidase. Cyt  $aa_3$ -600 has been cloned and expressed in a his-tagged form in *B. subtilis*. Following isolation of the enzyme in dodecylmaltoside, it is shown that the pure enzyme contains one equivalent of menaquinone-7 and stabilizes a menasemiquinone with significant asymmetry in the distribution of the unpaired spin density. Pulsed EPR showed that the SQ possesses weaker hydrogen bonding in cyt  $aa_3$ -600 in comparison with cyt  $bo_3$ . In addition, the electronic structure of the SQ in cyt  $aa_3$ -600 has more anionic character compared to the neutral SQ state in cyt  $bo_3$  (ref.2).

**Semiquinone in  $Q_A$  and  $Q_B$  site of bacterial reaction center.** Photosynthetic reaction centers from *Rhodobacter sphaeroides* have identical ubiquinone-10 molecules functioning as primary ( $Q_A$ ) and secondary ( $Q_B$ ) electron acceptors. Since the first reaction center crystal structure in 1985, a plethora of subsequent structures have suggested potential hydrogen bonding features. Despite this fact, significant uncertainties in the conformations of the two quinones, and in the significance of the variable location of  $Q_B$  in the protein, still exist. Our 2D ESEEM experiments clearly show that the  $Q_B$  SQ is involved in the interaction with one histidine and one peptide nitrogen. Both of them carry unpaired spin density transferred through hydrogen bond bridges from the SQ. However, the hyperfine couplings, especially for peptide nitrogen, are smaller than the corresponding couplings for the  $Q_A$  SQ, suggesting weaker binding in the  $Q_B$ -site. We have also characterized the exchangeable protons, presumably involved in hydrogen bonding with carbonyls. They are characterized by weaker anisotropic hyperfine coupling in comparison with those previously determined for  $Q_A$ -site from ENDOR experiments, and confirmed in our experiments. Our spectra allow us to suggest the presence of at least one more proton carrying unpaired spin density, which could belong to the hydrogen bond between the oxygen of a methoxy group and a peptide nitrogen, as proposed in a recent X-ray structure (ref.3).

#### Science objectives for 2010-2011:

- Construction of a comparative structural model showing the H-bonding in wild-type and D75H  $bo_3$ , based on the hyperfine couplings with side-chain nitrogens, will be completed after quantitative analysis of  $^{15}\text{N}$  2D ESEEM spectra. .
- Further analysis of  $^{13}\text{C}$  methyl couplings will provide insight into the role of the methoxy groups on the SQ binding to the protein. We will apply a biochemical approach for selective  $^{13}\text{C}$  labeling of ring carbons in the  $Q_H$  SQ. The quinones biochemically labeled in  $bo_3$  enzyme will also be used in studies of the SQs in bacterial reaction center and  $bc_1$  complex.
- H-bonds with nitrogens of His M219 and Ala M260, respectively, were reported for  $Q_A^-$  in reaction centers of *Rb. sphaeroides*. Previous studies have left uncertainties about hyperfine couplings with these nitrogens, and this has frustrated the simulations exploiting different structural models of the  $Q_A$ -site in the SQ state. We will complete a characterization of the nitrogen couplings for the  $Q_A$ -site SQ using  $^{14}\text{N}$  and  $^{15}\text{N}$  2D ESEEM data.

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## Structure, function and regulation of the enzymes in the starch biosynthetic pathway.

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Overall research goals: Our overarching goal is to begin to delineate in molecular detail the structure, mechanisms, specificity and regulation of the enzymes that make up the pathway for starch and glycogen biosynthesis in bacteria and plants, respectively. This knowledge will enable the rational redesign of these enzymes to both increase starch production and to alter the properties of starch.

Significant achievements 2009-2010: We have obtained clones for several of the rice starch biosynthetic enzymes and have begun over-expressing and purifying these enzymes to homogeneity. So far we have over-expressed the  $\alpha$  subunit of an ADP-glucose pyrophosphorylase and have begun cloning the  $\beta$  subunit for over-expression. We have also obtained the rice Branching enzyme and have over-expressed this enzyme and purified it to about 70% homogeneity. Clones for starch synthases have also been obtained and cloning of these into over-expression vectors has begun.

Branching enzyme is responsible for all branching of glycogen and starch. It is an unusual member of the  $\alpha$ -amylase family because it has both  $\alpha$ -1,4 amylase activity and  $\alpha$ -1,6-transferase activity. It also, quixotically, does not bind the common short chain inhibitors such as acarbose, though it will bind much longer substrates and substrate mimics.

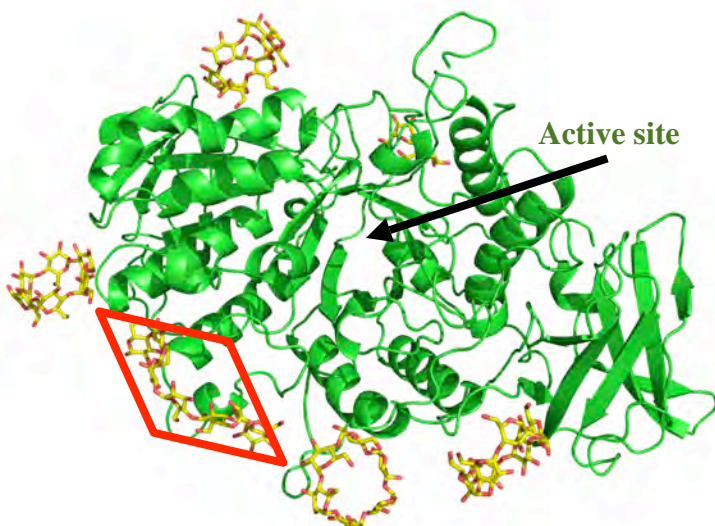


Figure 1. The *E. coli* BE structure (green ribbon) bound to  $\alpha$ -cyclodextrin and maltoheptaose, made by composite of the two structures.

the enzyme's active site.

Science objectives for 2009-2010:

In an effort to better understand how Branching enzyme interacts with its polymeric substrate we have determined the structure of *E. coli* Branching enzyme bound to  $\alpha$  cyclodextrin and maltoheptaose (Figure 1). Together these structures define seven distinct potential oligosaccharide binding sites on the surface of *E. coli* Branching Enzyme. Most of these binding sites surround the edge of the  $\beta$ -Barrel domain and are quite far from the active site. Surprisingly, there is no evidence for oligosaccharide binding in the active site of the enzyme. The closest bound oligosaccharide resides almost 20 Å from the active site. These structures have led us to propose a mechanism for Branching Enzyme chain transfer specificity that makes use of glycan binding sites at disparate locations relative to

- Further mutational studies will be used to confirm and elaborate our new theory of branch chain selectivity in *E. coli* BE.
- The Branching Enzyme work will be transitioned to the plant enzyme. Crystals of rice BE will be grown and BE's from a variety of species will also be pursued. Structures of the complexes with malto-oligosaccharides will also be pursued.
- Several Starch Synthase enzymes, first from rice, will be over-expressed, purified and screened for crystallization.
- ADP-glucose pyrophosphorylase from potato in its active conformation will be completed.

References to work supported by this project 2009-2010:

L. Feng, J. H. Geiger "The structure of Branching enzyme bound to linear polysaccharides." *J. Biol. Chem. Submitted.*

## Structural, Functional, and Integration Studies of Solar-Driven, Bio-Hybrid, H<sub>2</sub>-Producing Systems

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Overall research objectives: The overall goal of the project is to develop an understanding of [FeFe]-hydrogenase (H<sub>2</sub>ase) structure-function, integration with photoelectrochemical cell, and parameters controlling hydrogen phototransformation efficiencies. The research objectives include: (i) developing theoretical models of H<sub>2</sub>ase to understand electron/proton-transfer (ET and PT) and catalysis; (ii) experimental studies of H<sub>2</sub>ase using mutagenesis and infra-red (IR) spectroscopy; (iii) optical and electronic studies of H<sub>2</sub>ase-carbon nanotube and nanocrystal complexes; and (iv) understanding H<sub>2</sub>ase interactions with photoactive materials and electrode surfaces. These efforts will provide fundamental knowledge of H<sub>2</sub> production catalysts and how to functionally integrate them as components of artificial hydrogen production schemes.

Significant achievements 2008-2010: (i) Computational chemistry studies of H-cluster models with perturbed diatomic ligands revealed a potential role of the bridging ligand in buffering charge upon reduction. (ii) A quantum chemical H<sub>2</sub>ase model encompassing the H-cluster, accessory [4Fe-4S]-clusters and surrounding protein was constructed. Custom computational methods allow for breaking the spin symmetry within accessory [4Fe-4S]-clusters, and integration of the initial gas-phase calculation into a QM/MM model of the complete H<sub>2</sub>ase. The free energies along PT pathways were investigated using QM/MM and umbrella sampling techniques. Several important residues were identified and *pK<sub>a</sub>* values estimated by thermodynamics integration method (iii) Brownian dynamics and molecular dynamics simulation techniques predicted the binding structures between H<sub>2</sub>ase and SWNTs or bulk carbon surfaces, and ET rates. ET appears to be at least a 100-fold faster for SWNTs than for a bulk carbon surface, and independent of SWNT diameter. (iv) The native H<sub>2</sub>ase has been successfully adsorbed to Au electrodes bearing self-assembled thiol-based monolayers (SAMs) and retains activity. Binding is via interactions between positively charged patches on the enzyme and carboxylate groups on the SAM. Single-molecule images have been obtained in an electrochemical STM and suggest tunneling currents increase under an applied bias. Removal of adsorbed H<sub>2</sub>ase from the STM surface helped confirm that the features observed with the STM are H<sub>2</sub>ase. (v) Electrostatically guided assemblies of H<sub>2</sub>ase and mercaptopropionic acid CdTe nanocrystals (NC) were successfully formed in solution. Catalytically competent complexes could be isolated by electrophoresis, and showed compositional heterogeneity. Photoluminescence and hydrogen evolution studies suggested that efficiencies were proportional to molecular ratios, NC quantum yields and donor composition.

Science objectives for 2010-2011:

- Tunneling currents will be examined between accessory [4Fe-4S]-clusters and the H-cluster in the H<sub>2</sub>ase model. Dynamics trajectories will also be analyzed to determine protein conformational features correlating with large couplings, as approximated by empirical

pathways analysis. The free energies along the PT pathways will be further refined based on the *pKa* calculation results.

- We will continue to investigate the binding free energies for H<sub>2</sub>ase with SWNTs and bulk carbon surfaces using MD simulation techniques, and develop these models to predict binding modes, orientations and ET processes.
- We will continue to characterize native and mutant H<sub>2</sub>ases in different redox states using IR spectroscopy to learn how protein structure contributes to the control of the H-cluster ligand shell during the catalytic cycle.
- Single-molecule electrochemistry of immobilized H<sub>2</sub>ase on Au-electrodes will continue to be developed to characterize the binding interaction, tunneling currents and the operating potentials of the enzyme. Mutated H<sub>2</sub>ases lacking individual accessory iron-sulfur clusters functioning in electron-transfer will be studied in order to learn more about the conductive path through the protein.
- We will continue to characterize the assembly and charge-transfer processes for H<sub>2</sub>ase-SWNT and H<sub>2</sub>ase-NC complexes under photoexcitation using steady-state and time-resolved optical techniques.

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## Chemo-mechanical interfaces with living cells

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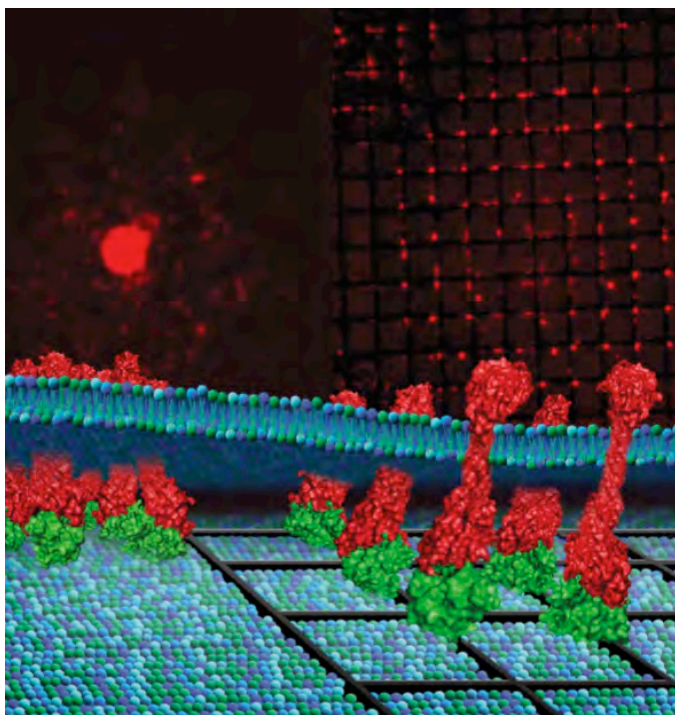
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Overall research goals: The fundamental goal of this program is to tap the world of biological nanotechnology by constructing molecular level, functional interfaces between living systems and synthetic materials. The key to domesticating life at the cellular and molecular level is communication. Living cells have tremendous ability to follow directions and perform functions on demand. In order to effectively communicate with cells, we must be able to produce synthetic materials that present the signals necessary to elicit technologically useful behaviors from cells. Critical to this goal is to decode and learn to implement the chemical language through which living cells naturally communicate.

### Significant achievements in

#### 2008 – 2010:

In recent work along these lines, we have tapped into the EphA2 signaling system. EphA2 is a member of the Eph family of receptor tyrosine kinases, which play a major role in guiding the assembly of cells into organized tissues. Tapping into the Eph system is a key step towards enabling use of living cells to generate self-organizing devices, such as the controlled spatial positioning of nanoparticles. An important discover in our recent work is that the EphA2 system is responsive to mechanical forces. The hybrid live cell – synthetic substrate junctions, central to this program, proved critical in this discovery and also offer a plausible way of utilizing these findings in energy related applications.



**Figure:** Schematic image of a hybrid live cell – synthetic device junction (lower portion). Receptors in the live cell membrane engage reconstituted ligands in the substrate supported membrane and structures on the substrate control protein movement. Fluorescence images of genuine system shown in background (red). Adapted from Groves et al., *Science* **327**, 1380 (2010).

Science objective for 2010 – 2011: There are several immediate goals we seek to reach in the coming year. First, we will use this system to explore – and hopefully discover – what is the molecular mechanism by which the cells are sensing force. This will be done by imaging the spatial localization of a library of candidate proteins, labeled as fluorescent fusion proteins in live cells. Force dependent localization with EphA2 signaling clusters will be the search parameter to identify leads. Second, we will begin a broader exploration of these sorts of phenomena in other cellular systems. In particular, the ability to form stable and controllable interfaces with species such as photosynthetic bacterial is a long term goal.

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## An assessment of the biofuel properties of crop plants fixed in the juvenile phase of development through over-expression of the *Corngrass1* gene

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Overall research goals: The research objectives are to enhance biofuel properties of crop plants by controlling expression of the maize microRNA gene *Corngrass1* (*Cg1*) by: (1) over-expressing *Cg1* in several transgenic plant species; (2) assaying biofuel properties of the transformants using FTIR and NMR to measure lignin content and sugar and starch levels. In addition, saccharification assays and digestibility assays after ionic pre-treatment of the resulting biomass should affirm the ultimate suitability of the transgenic plants as biofuel crops. Together, these experiments will test the hypothesis that constitutive over-expression of *Cg1* in transgenic plants will increase biomass production, as well as create biomass with a greater energy yield.

Significant achievements 2007-2009: The *Cg1* cDNA was expressed behind constitutive promoters in four different plants species, including the monocots *Brachypodium* and switchgrass, as well as the dicots *Arabidopsis* and poplar. All transformants displayed a similar range of phenotypes, including increased biomass from extended leaf production, and increased vegetative branching (Fig. 1). The switchgrass transformants were chosen for in depth analysis of biofuel characteristics using FTIR microscopy, as well as NMR. It was found that overall lignin content was reduced, the ratio of glucans to xylans was increased, and surprisingly, that starch levels were greatly increased. These plants are currently undergoing field trials to determine whether these properties can be maintained on a larger scale, outdoors. In addition, we identified a target of the *Cg1* gene called *tasselsheath4* (*tsh4*), and determined that it represses leaf initiation. Thus, when *Cg1* is over-expressed, *tsh4* is down-regulated, leading to excess leaf initiation and increased biomass.

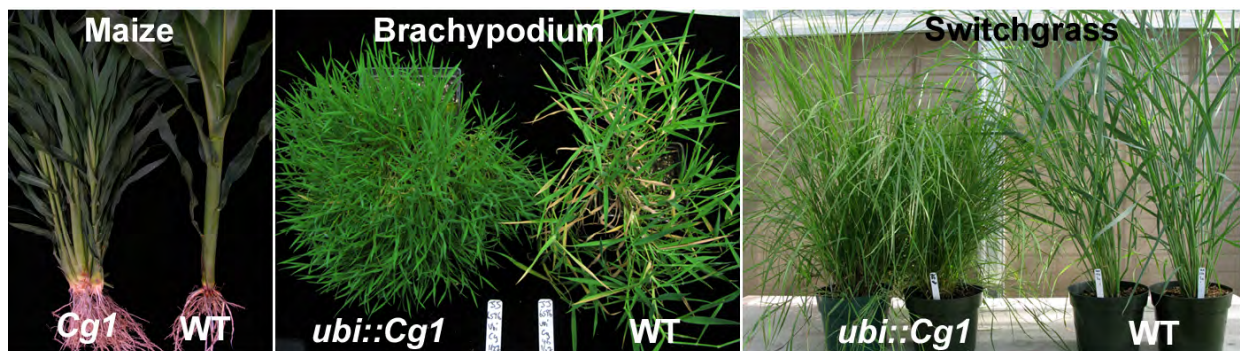


Figure 1. Left Panel: Maize *Cg1* mutant compared to wild type; middle panel shows transgenic *Brachypodium Cg1* over-expression line compared to wildtype; right panel shows transgenic switchgrass *Cg1* over-expression line compared to wildtype.

### Science objectives for 2009-2010:

- Six other targets of the *Cg1* gene need to be functionally characterized to determine their roles in biomass accumulation and acquisition of plant cell identities.
- The biofuel characteristics of the *Brachypodium* and poplar *Cg1* transgenic lines need to be assayed to determine if their biomass is similarly enhanced.
- Since overexpression of *Cg1* appears to have a negative effect on root growth, transgenic lines expressing *Cg1* behind aerial tissue specific promoters will be created.

- Deep sequencing of the transcriptome of *Cg1* transgenic lines will be done to identify additional downstream *Cg1* targets, as well as the genes responsible for the changes in biomass characteristics.

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**Use of  $^{13}\text{C}$ -metabolic flux analysis to determine how electrons flow from organic donors to nitrogenase where they are combined with protons to form hydrogen gas.**

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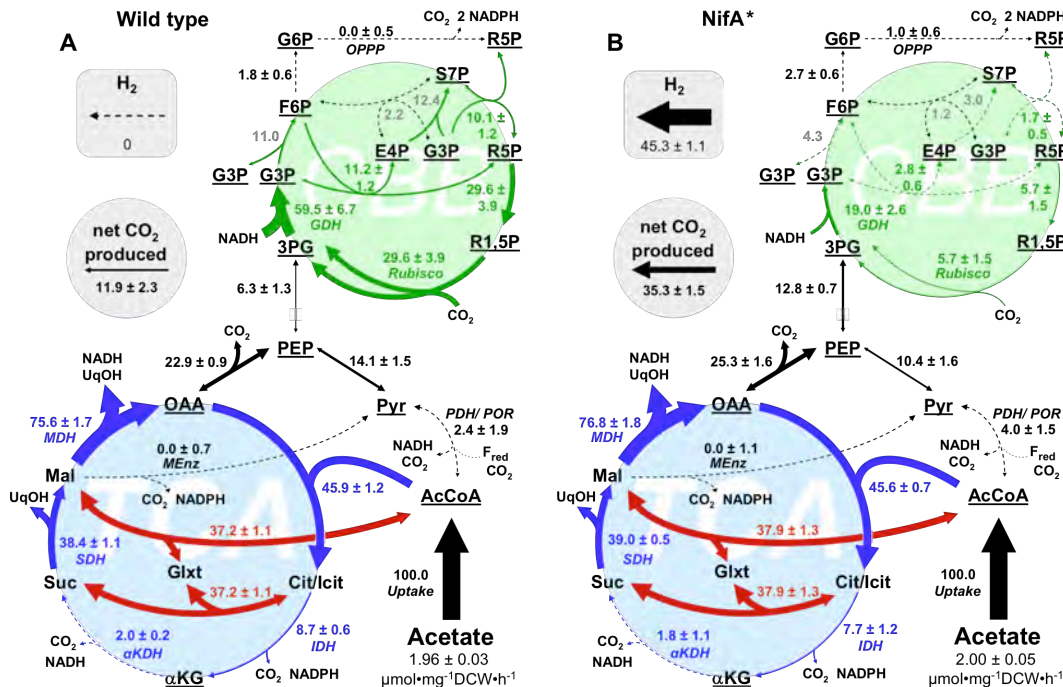
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Overall research goals: To use  $^{13}\text{C}$  metabolic flux analysis to identify metabolic pathways and enzymes important for removal of electrons from electron-donating organic compounds and for their delivery to nitrogenase, the biocatalyst for  $\text{H}_2$  production.

Significant achievements in 2008-2010:  $\text{H}_2$  is a promising fuel, having about three-times the energy content of gasoline, and can be produced biologically. The phototrophic bacterium *Rhodospseudomonas palustris* uses energy from light and electrons from organic compounds to produce  $\text{H}_2$  via nitrogenase. Its growth mode is photoheterotrophic. It grows anaerobically and obtains all its ATP by cyclic photophosphorylation and all its carbon for growth from organic compounds. It diverts some electrons from organic compounds that it is degrading to nitrogenase when they are combined with protons (present in water) to produce  $\text{H}_2$ .

To better understand this process we used  $^{13}\text{C}$ -acetate to track and compare central metabolic fluxes in non- $\text{H}_2$  producing wild-type *R. palustris* and a  $\text{H}_2$ -producing mutant. Wild-type cells released approximately 22% of the acetate carbon as  $\text{CO}_2$ . They then fixed 67% of this  $\text{CO}_2$  (combined RubisCo and GDH reactions) to reoxidize reduced cofactor generated mainly in the conversion of malate (Mal) to oxaloacetate (OAA) (Fig 1A). When nitrogenase is active (the NifA\* strain) a similar amount of acetate was oxidized to  $\text{CO}_2$ , but the reduced cofactor was reoxidized by  $\text{H}_2$  production (by nitrogenase) rather than by  $\text{CO}_2$  fixation (Fig 1B). Microarray and Q-PCR analyses showed that the shift of electrons from the Calvin cycle to  $\text{H}_2$  production was controlled at the level of transcription.





**Fig. 1. Metabolic flux maps for wild-type cells (non-H<sub>2</sub>-producing when grown with ammonium) and NifA\* mutant cells (H<sub>2</sub>-producing when grown with ammonium) grown with acetate and ammonium.** Arrowheads indicate the direction of the net flux. Arrow thickness is proportional to net flux magnitude.

In a second study we compared Calvin cycle fluxes in *R. palustris* cells provided with <sup>13</sup>C-labeled substrates (fumarate, succinate, acetate, and butyrate) ranging from more oxidized to more reduced. The flux distributions showed a general trend of increasing Calvin cycle flux with increasing substrate electron content. In all cases a large proportion of the reduced cofactor pool could not be reoxidized in biosynthetic reactions and thus was reoxidized by the Calvin cycle reactions. Surprisingly, growth on succinate required more Calvin cycle flux than growth on acetate, which is more reduced than succinate. The flux distributions explained this by showing that the route of succinate metabolism generates more reduced redox cofactor than the route of acetate metabolism. When H<sub>2</sub> production was used as an alternative means of recycling redox cofactors by the H<sub>2</sub>-producing (NifA\*) mutant strain, Calvin cycle flux decreased to varying extents depending on the substrate.

These results point to the Calvin cycle as a competing pathway for electrons, and therefore a target for disruption to improve the H<sub>2</sub> yield. When Calvin cycle flux was disrupted completely by deleting the genes encoding ribulose 1,5-bisphosphate carboxylase, *R. palustris* was forced to use H<sub>2</sub> production alone to maintain redox balance. This mutant exhibited about 1.5-fold increases in H<sub>2</sub> yields from fumarate, succinate or acetate.

Science objectives for 2009-2010: We have started to shift gears, our objectives are to:

- Begin to dissect a complex regulatory system that controls production of the *R. palustris* of light harvesting 4 system genes in response to light intensity and cellular fixed nitrogen status. The LH4 genes are among the most highly expressed in the genome.
- Initiate studies to determine how the alternative vanadium nitrogenase that *R. palustris* encodes is regulated at the transcriptional level.

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## The Rhizobial Nitrogen Stress Response and Effective Nitrogen Fixation

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**Overall research goals:** Fixed nitrogen is an important input for plant growth. Nitrogen fertilizer production requires methane and produces carbon dioxide and consumes about 5% of world natural gas production or about 2% of world energy use. Some plants can grow without an input of nitrogen fertilizer because of symbiotic interactions with nitrogen-fixing bacteria. Nitrogen fixation carried out in the symbiotic relationship between bacteria and legumes is an important contributor to the productivity of these plants and also is a substantial input of fixed nitrogen to natural and agricultural ecosystems. Symbiotic nitrogen fixation is supported by a cooperative metabolism between the plant and bacteria. We want to understand how this metabolism works.

**Significant achievements in 2008-10:** Direct funding for this project began in summer, 2008, but the project is a resumption of research funded by the Energy Biosciences Program several years ago.

While studying *Sinorhizobium meliloti* mutants with defects in energy metabolism, our screening procedure identified a transposon mutant, TcW1, with a very unusual symbiotic phenotype. The mutant appeared to fix nitrogen at normal rates, as indicated by acetylene reduction activity and  $^{15}\text{N}_2$  incorporation into non-volatile compounds (it was Fix+), but the interaction did not stimulate plant growth to the level that would be expected (it was not effective, Eff<sup>-</sup>). The lesion leading to the Fix+Eff<sup>-</sup> phenotype is in the N terminus of *glnD*, a gene thought to encode the primary sensor of bacterial nitrogen status (Fig 1). GlnD initiates a signal cascade that controls the transcription and post-translational modification of diverse enzymes involved in responding to nitrogen stress. We believe that the mutated GlnD retains some functions but is unable to uridylylate its target effector proteins, GlnB and GlnK. The free-living mutant is unable to induce a nitrogen stress response.

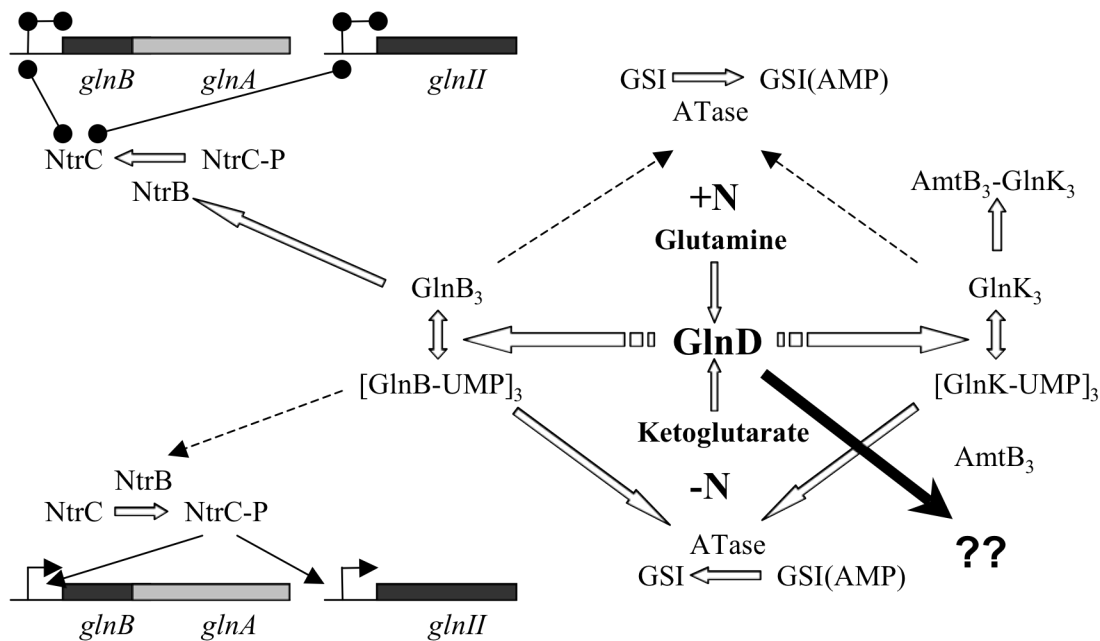
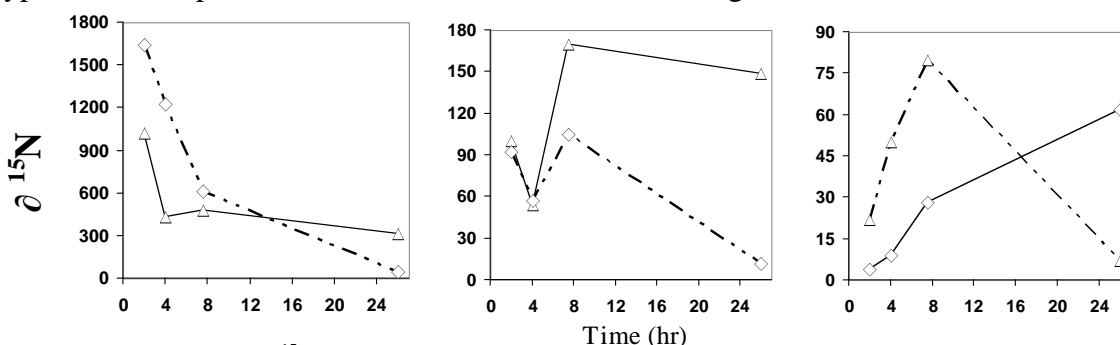


Figure 1. A generalized bacterial nitrogen stress response circuit with features specific to *S. meliloti*. GlnD uridylylates two PII proteins, GlnK and GlnB, which in turn control the levels of many nitrogen stress response related proteins by post translational modification (glutamine synthetase GSI, transcriptional regulator NtrC) or direct protein-protein interaction (ammonium importer AmtB). We have evidence for other levels of control, indicated by the question marks.



The existence of this *glnD* mutant and a deletion mutant we constructed that removes the same part of GlnD, was surprising since earlier work, which we confirmed, showed that *glnD* was essential for viability. We have confirmed in vitro that the mutant GlnD does not add UMP to the PII proteins. To trace the signal transduction, we generated and have characterized  $\Delta glnB$ ,  $\Delta glnK$  and  $\Delta glnB \Delta glnK$  mutants and have recently succeeded in making a  $\Delta glnB \Delta glnK glnD::Tn5-TcW1$  mutant. In *E. coli*, mutating GlnD in strains lacking PII proteins makes no difference. In *S. meliloti*, the  $\Delta glnB \Delta glnK$  mutant grows very slowly but is Eff<sup>+</sup>. The triple mutant is even more impaired and is ineffective. This is interesting since it implies that GlnD can affect cell growth and symbiosis without acting through the PII proteins. In addition, fast-growing pseudorevertants of the  $\Delta glnB \Delta glnK$  mutant include a *phoB* mutant, implying that there is a connection between phosphorous stress response and the nitrogen stress response.

The fate of the fixed nitrogen in nodules formed by the *glnD* mutant was examined using a <sup>15</sup>N<sub>2</sub> pulse-chase experiment (Fig 2). Fixed nitrogen leaves the nodules and leaves the plants. A hypothesis to explain this has been formulated and is being tested.



**Fig 2. Location of fixed <sup>15</sup>N in alfalfa plants.** Plants nodulated with wild-type (—□—) or the *glnD* mutant (---◇---) were labelled in 10% <sup>15</sup>N<sub>2</sub> for 2 hr then shifted to air and harvested at 0, 2, 5.5 and 24 hr. The enrichment of <sup>15</sup>N in each plant fraction (left—nodules; center—roots; right—shoots) was determined by ion ratio mass spectrometry.

#### Science objectives for 2010-2011:

- Determine identify nitrogen compounds overrepresented in the *glnD* symbiosis.
- Explore whether concepts about nitrogen allocation and feedback regulation in the symbiosis can be used to design symbioses that produce more fixed nitrogen.
- Understand how the mutant GlnD protein influences cell growth and symbiosis independently of the PII proteins.

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## Ferredoxin-Dependent Plant Metabolic Pathways

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Overall Research Goals: To study the mechanisms of ferredoxin-dependent enzymes in oxygenic phototrophs and map the interactions between ferredoxin and its target enzymes.

### Significant Achievements in 2008-2010:

In oxygenic photosynthesis, carbon metabolism is regulated by a pathway in which the availability of energy from light is signaled via a redox chain consisting of ferredoxin (Fd), ferredoxin:thioredoxin reductase (FTR), and thioredoxin (Trx). NMR spectroscopy has been used to investigate the interaction of Fd, FTR, and an *m*-type Trx. Titrations indicated that FTR can use distinct sites to bind Fd and Trx simultaneously, forming a non-covalent ternary complex. The orientation of Trx-*m* relative to FTR was determined from intermolecular paramagnetic broadening caused by FTR's [4Fe-4S] cluster. A model of the non-covalent FTR/Trx-*m* binary complex, based on the paramagnetic distance restraints, revealed both similarities to and differences from an x-ray structure of a covalent, disulfide-linked FTR/TRX-*m* complex. NMR spectroscopy was used to determine a high-resolution solution structure for a Ga-substituted Fd. The Ga-Fd, in which a single Ga<sup>2+</sup> replaces the [2Fe-2S] cluster found in the native protein, contains no sulfide. Instead, four cysteinyl sulfurs serve as the ligands for Ga. Ga-Fd has a structure very similar to that of native Fd and was shown to be a good non-paramagnetic analog for the native protein in binding studies. Ga-Fd was used to map the Fd interaction domain Fd for ferredoxin:NADP<sup>+</sup> reductase (FNR) in a more complete fashion than possible with native Fd..

A series of site-directed mutants of the ferredoxin-dependent spinach nitrite reductase (NiR) has been used to identify several amino acids involved in the interaction of the enzyme with Fd. In a complementary study, Fd/NiR binding constants and steady-state NiR kinetics, obtained with site-specific variants of Fd, were used to identify Fd amino acids involved in the interaction with nitrite reductase. The results have been interpreted in terms of an *in silico* docking model for the 1:1 complex of Fd with NiR.

Flash photolysis was used to study the kinetics of electron transfer from reduced Fd to spinach NiR. It was demonstrated that a His-tagged, recombinant form of the enzyme and NiR isolated directly from spinach leaf displayed essentially identical values for  $k_{cat}$  (450 electrons per second per NiR molecule) and for the rate constant for reduction by Fd (1200 s<sup>-1</sup>) despite the fact that the siroheme group of the recombinant enzyme has a slightly more negative  $E_m$  value than does the leaf enzyme. The rate constant for NiR reduction by Fd and the thermodynamics of Fd binding to NiR were shown to be the same for oxidized NiR and for its reduced, NO-bound intermediate. Spectral and kinetic analyses suggest that reduction of the NO intermediate results in reduction of the enzyme's [4Fe-4S] cluster with little, if any, reduction of NO, indicating that the enzyme must "wait" for the arrival of another electron from reduced Fd before further reduction of NO, possibly to hydroxylamine. In separate studies, with both spinach NiR and NiR from the green alga *Chlamydomonas reinhardtii*, the enzymes were shown to be able to reduce hydroxylamine to ammonia at rates consistent with hydroxylamine functioning as a true reaction intermediate in the NiR-catalyzed conversion of nitrite to ammonia.

Expression levels of the six Fd isoforms found in *C. reinhardtii*, were measured under a variety of nutritional and environmental conditions and it was demonstrated that each of the FDX

genes is differentially regulated in response to nutrient supply. Four of these Fds were localized to the chloroplast using isoform-specific antibodies (This portion of the work was carried out by Dr. Aimee Terauchi in the laboratory of Prof. Sabeeha Merchant at UCLA). Two chloroplastic Fds, Fd (the most abundant form) and Fdx2, were expressed and Fdx2 was shown to have an unusually positive redox midpoint potential. Steady-state kinetics for three target enzymes (FTR, NiR and FNR) suggest that the enzymes exhibit substrate specificity for these Fd isoforms.

Scientific Objectives for 2010-2011:

- To use site-directed mutagenesis to identify amino acid residues in nitrite reductase involved in prosthetic group binding to the apo-protein and nitrite to the holo-enzyme.
- To use site-directed mutagenesis to identify the ferredoxin-binding domain on a cyanobacterial glutamate synthase, with emphasis on a 27 amino acid-long loop that is present in all Fd-dependent glutamate synthases dependent enzymes but absent in the NADPH-dependent enzymes.
- To use flash photolysis to study electron transfer from ferredoxin to a cyanobacterial nitrate reductase and to a cyanobacterial phycobilin reductase.
- To use site-directed mutagenesis to study the roles of two absolutely-conserved lysines and two absolutely-conserved arginines in the mechanism of a cyanobacterial nitrate reductase.
- To use isothermal calorimetry to study the thermodynamics of Fd binding to target enzymes.

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## Transmethylation reactions during methylotrophic methanogenesis in methanogenic Archaea

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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, using acetate, methylamines, methylated thiols, and methanol to form methane. Our overall goal is 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 are currently examining the function of pyrrolysine in the TMA and DMA methyltransferases. This will help elucidate the mechanism of these key methyltransferases of methane formation, but also provide a rationale for why they required the addition of a novel amino acid to the genetic code of methanogens. Further, we are examining another key participant in the methyltransferase reactions, RamA. We have shown that RamA carries out an ATP dependent reductive activation of methylamine corrinoid proteins to the supernucleophilic Co(I) state necessary for catalysis.

Significant achievements in 2009-2010. We have proposed that pyrrolysine is key to the catalysis of methylamine methyltransferases and that it may bind the methylamine in order to present an oriented methylammonium adduct to the supernucleophilic Co(I) of the cognate corrinoid protein. Other mechanisms are also possible, e.g. pyrrolysine may accept the amine leaving group following transfer of the methyl group to the corrinoid protein.

Site directed mutagenesis of pyrrolysine in MtbB. Previously, we had shown that nucleophiles reducing the imine bond of pyrrolysine inactivated MtbB. We have now made the first site directed mutant of pyrrolysine in MtbB. We constructed an expression vector based on pWM311 in which *M. barkeri mtmB1* with a His-tag encoding sequence was driven by the *mcr* constitutive promoter. The *M. barkeri mtmB1* was then cloned into this vector replacing *mtmB1*, and the *mtmB1* expression plasmid transformed into *M. acetivorans*. Nickel affinity and mono-Q chromatography resulted in nearly pure His-tagged MtbB1. Recombinant MtbB1 in DMA:cob(I)almin methyl transfer is as active as the *M. barkeri* MtbB purified from *M. barkeri*. An MtbB1 mutant in which pyrrolysine had

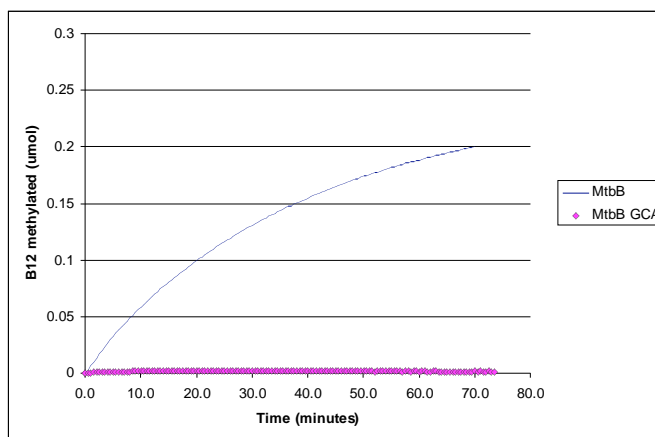


Fig. 1. Relative activities in DMA dependent cob(I)almin methylation of MtbB wild type (MtbB, blue line) and MtbB(O356A) (MtbB GCA, red line). Wild type activity is 45 nmol/min•mg MtbB, while no activity was detectable in the alanine mutant.

been substituted with alanine was shown to completely lack DMA methyltransferase activity (fig.1). Further studies showed the O256A mutant was also compromised for binding of the cognate corrinoid protein.

Structure of the TMA methyltransferase. The TMA methyltransferase gene *mttB* from *M. barkeri* was cloned into pDL05 to replace the *mtmB1* gene. The resulting *mttB* product could be isolated by Nickel-affinity column by virtue of the C-terminal His-tag followed by a mono-Q column after expression in *M. acetivorans*. We collaborated with Michael Chan's lab at OSU to obtain the structure of MttB, the first pyrrolysyl-protein solved following MtmB. The two methyltransferases have no sequence similarity, aside from the presence of the pyrrolysine encoded by the amber codon in their respective genes. In spite of their sequence dissimilarity, both proteins have hexameric quaternary structure. Each monomer is comprised of a TIM barrel, and in the center of each, a putative active site cleft is formed. However, pyrrolysine lies on opposite sides of the barrel cleft in MtmB and MttB. As in MtmB, the putative active site cleft surrounding pyrrolysine in MttB is one of the most highly conserved parts of the protein. However, very different amino acids surround pyrrolysine in MtmB and MttB. The pyrrolysine imine nitrogen is within H-bonding distance of two conserved glutamates in the former, but in the MttB structure only a water is H-bonded to the imine N, this water is further within H-bonding distance to two highly conserved residues. This arrangement has suggested different mechanisms for the two methyltransferases, which will be tested by site directed mutagenesis in the coming period.

Recombinant RamA carries out ATP dependent reduction of MtmC. RamA was previously isolated from *M. barkeri* following a lengthy column procedure. This left open the possibility that the observed Ti(III) and ATP dependent reduction of MtmC to Co(I) could require other proteins in addition to RamA. We recently succeeded in expressing *M. barkeri ramA* in *E. coli* under anaerobic conditions, and isolated it by virtue of an appended his-tag. The isolated RamA protein has a UV-Vis spectrum similar to that of the native RamA protein, and carried out the reduction of MtmC to the Co(I) state. As RamA activity is not found in *E. coli*, this demonstrates that RamA carries out the activity observed with natively isolated RamA, and that other factors from *M. barkeri* are not required.

#### Science objectives for 2010-2011.

- Mutants are in hand of the DMA methyltransferase with pyrrolysine changed to five different residues that will be characterized as to activity.
- TMA methyltransferase with substitutions of pyrrolysine, as well as the amino acids forming a hydrogen bonding network with the imine nitrogen via water, will be characterized as to TMA methyltransferase activity and binding to the TMA corrinoid protein.
- The recombinant RamA protein ATPase activity will be further characterized as to MtmC dependence and to test the possibility of a covalent phosphorylation event during catalysis.

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## Bioenergetic aspects of syntrophic fatty and aromatic acid metabolism

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Overall research goals: We wish to understand an essential but poorly characterized process critical for carbon cycling called syntrophy and how bacteria operate at free energy changes close to equilibrium. The specific objectives of project are to determine: (1) the membrane complexes involved reverse electron transfer, (2) the components involved in hydrogen and formate production, and (3) the membrane components involved in acyl-CoA oxidation.

Significant achievements in 2008-2010: We detected a new membrane-bound complex in *S. wolfei* involved in reverse electron transfer during syntrophic growth with butyrate (Figure 1). The complex was not detected when *S. wolfei* grew syntrophically with crotonate, a compound

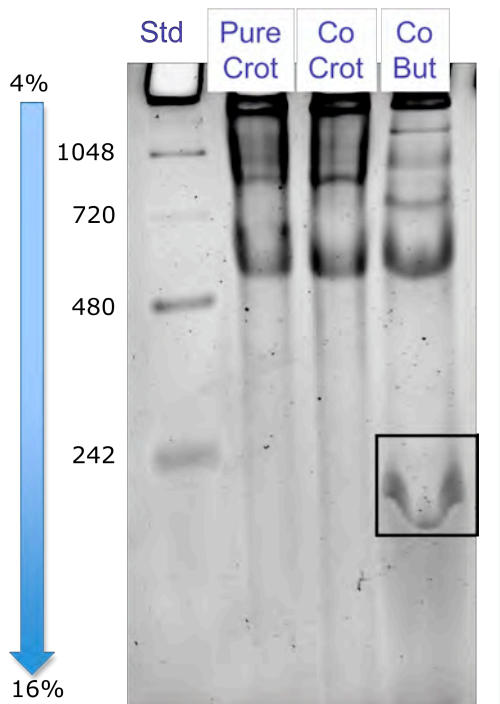


Figure 1. Membrane complexes of *S. wolfei* detected by blue-native gel electrophoresis.

Std: molecular weight markers;

Pure Crot: *S. wolfei* pure culture with crotonate;

Co crot: *S. wolfei*-*M. hungatei* coculture with crotonate;

Co but: *S. wolfei*-*M. hungatei* coculture with butyrate.

Box indicates novel membrane complex described in text.

that does not require reverse electron transfer for its metabolism, or axenically with crotonate. Mass spectrometry detected unique peptides of the membrane-bound, iron-sulfur oxidoreductase, the beta subunit of electron transfer flavoprotein and two subunits of a membrane-bound hydrogenase were detected. These data support the conclusion that hydrogen is formed from electrons generated during beta-oxidation by a novel membrane complex.

A combination of genomic analysis, gene expression, enzymatic analyses and inhibitor-based approaches were used to determine the importance of H<sub>2</sub> and/or formate transfer for syntrophic benzoate and cyclohexane-1-carboxylate metabolism by *Syntrophus aciditrophicus* and syntrophic butyrate oxidation by *Syntrophomonas wolfei*. *S. wolfei* expressed all three hydrogenase gene systems and two of five

formate dehydrogenase gene systems when grown syntrophically on butyrate. Butyrate metabolism and CH<sub>4</sub> production by washed cell suspensions of *S. wolfei* and the methanogen were inhibited by hydrogenase inhibitors, cyanide and carbon monoxide, but not by the

formate dehydrogenase inhibitor, hypophosphite. Genes for one hydrogenase and two formate



dehydrogenases were induced when *S. aciditrophicus* grew syntrophically on benzoate or cyclohexane-1-carboxylate. Syntrophic benzoate oxidation and CH<sub>4</sub> production was inhibited by hypophosphite, but not by cyanide and carbon monoxide. All three inhibitors were equally effective in halting syntrophic cyclohexane-1-carboxylate oxidation. These results demonstrate that syntrophic fatty, alicyclic and aromatic acid metabolism involves H<sub>2</sub> or formate transfer rather than direct electron transfer by nanowires. Secondly, the importance of H<sub>2</sub> versus formate transfer depends on the substrate and organism involved.

#### Science objectives for 2010-2011:

- Membrane complexes potentially involved in reverse electron transfer will be identified. We will use blue native gel electrophoresis to identify membrane protein complexes differentially expressed during syntrophic fatty acid and cyclohexane-1-carboxylate metabolism.
- We will determine biochemical activity of the novel membrane complex induced during syntrophic butyrate growth. We will determine if the complex couples electron flow from butyryl-CoA to H<sub>2</sub>.
- We will determine if candidates for reverse electron transfer such as *fix*, *rnf*, and genes for a novel iron-sulfur protein complex are induced during syntrophic metabolism. We will use RT-PCR and QRT-PCR to measure expression when syntrophic cocultures are grown with butyrate, benzoate and cyclohexane-1-carboxylate (conditions that require reverse electron transfer) compared to crotonate-grown cocultures (conditions that do not require reverse electron transfer).

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## The Structure of Pectins

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

Significant achievements in 2008-20010: The main approach to achieving the objectives is to selectively digest the pectin with cloned enzymes to allow isolation of fragments containing the linkages between two different regions of pectin for their characterization by mass spectrometry and NMR spectroscopy. 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. 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. An important enzyme activity, endoarabinanase, does not seem to be stably expressed in the *Pichia* system. However, Rolfe Prade's group has expressed a thermotolerant endoarabinanase in *E.coli*

Using a combination of the cloned enzymes mentioned above we have generated several oligosaccharides from apple pectin which give indications of how different regions of pectins are linked together. The oligosaccharide  $\alpha$ -GalAp (1-2)  $\alpha$ -Rha p (1-4)  $\alpha$ -GalAp (1-2)  $\alpha$ -Rha p (1-4)  $\alpha$ -GalAp could only have been generated by the action of endopolygalacturonase on polygalacturonan attached to the non-reducing end of the oligosaccharide and rhamnogalacturonanase acting on rhamnogalacturonan (RG) at the reducing end. Thus it appears that there is a linear linkage between homogalacturonan and rhamnogalacturonan.

We isolated a combination of two oligosaccharides containing two galactose residues attached to a segment from the RG backbone. From 2D NMR spectra we could quite readily deduce that one oligomer had a single galactose on two rhamnose residues whereas the other had a  $\beta$ -Galp (1-4)  $\beta$ -Galp disaccharide linked to the O4 of the non-reducing terminal rhamnose. Since we used endogalactanase in the production of this oligosaccharide which cannot hydrolyze the  $\beta$ -Galp (1-4)  $\beta$ -Galp disaccharide we presume that this means that the galactan chain which was attached to the RG was hydrolyzed down to a disaccharide by the galactanase. From knowledge of the mode of action of the enzyme and the nature of the disaccharide sidechain left behind it appears that galactan chains are  $\beta$ -(1-4) linked all the way to the rhamnose in RG.

Several more oligosaccharides were isolated which contain small segments of RG galactose and rhamnose. The exact structure of these oligomers has not yet been determined. However, the NMR spectra show that the galactose is linked to the rhamnose and that the arabinose residues are either linked to the galactose, not the rhamnose, or to arabinose residues. The oligomers containing multiple arabinose residues are probably representative of arabinan chains. Completing the characterization of these oligomers should tell us exactly how arabinans are linked to GR.

With the cloning of an active endoarabinanase (into E-coli) it has become possible to test the hypothesis that some of the xyloglucan (XG) in primary cell walls is covalently linked to pectin via an arabinan. We isolated an XG/RG complex from cotton suspension cultures by collecting the fraction of XG that binds strongly to an anion exchange column. After digesting this fraction with an arabinosidase to debranch 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.

Science objectives for 2010-2011:

- Characterize oligomers from apple pectin showing linkage between arabinans and RG.
- Characterize oligomers from sugar beet showing linkage between arabinans and RG.
- Continue efforts to obtain small characterizable fragments containing both RG and XGA regions.
- Characterize the arabinans fragment left on the XG after the arabinanase treatment.

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## Function and Control of Xyloglucan Galactosylation in Arabidopsis

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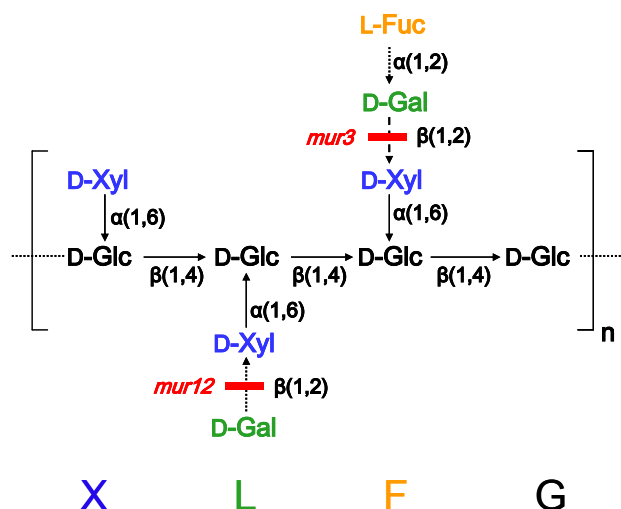
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Overall research goals: Most matrix polysaccharides in plant cell walls consist of simple backbone structures that are modified by the attachment of monosaccharides or oligosaccharides. The degree of modification varies substantially between plant organs and species suggesting that either the availability of nucleotide sugars or the activity of glycosyltransferases is rate-limiting during polysaccharide synthesis. We are investigating this issue by analyzing the effects of changes in precursor availability and glycosyltransferase activity on the degree by which the backbone of the hemicellulose xyloglucan is modified by the attachment of galactose residues. In Arabidopsis, the synthesis of the monosaccharide donor UDP-galactose is catalyzed by five isoforms of UDP-glucose 4-epimerase all of which are located in the cytosol. The attachment of a galactose residue to one of the two possible positions in the xyloglucan backbone is catalyzed by the Golgi-localized type II membrane protein MUR3 (Fig. 1). Based on genetic evidence, the evolutionarily related MUR12 protein attaches galactose specifically to the central xylose residue in the XXXG repeat unit of the xyloglucan backbone. To determine the rate-limiting step in xyloglucan galactosylation and obtain insight into the functional significance of this modification, we have taken the following approaches: (1) Phenotypic characterization of mutants with defects in the *MUR3* and *MUR12* genes, (2) overexpression of the two galactosyltransferases in wild type and mutant plants, and (3) overexpression of the UGE2 isoform of UDP-glucose 4-epimerase both in its native cytosolic form and as a fusion with a predicted Golgi-targeting signal. The latter strategy is designed to generate a pool of UDP-galactose in the Golgi without the need for nucleotide sugar transporters. Once the genetically modified lines are available, alterations in xyloglucan structure can be determined by mass spectrometry or NMR analysis of enzymatically released oligosaccharides.

Significant achievements 2008-2010: We completed the biochemical characterization of all five UGE isoforms in Arabidopsis, and found that all of them efficiently interconvert UDP-glucose and UDP-galactose as expected. UGE1, UGE3 and UGE5 are highly active as UDP-xylose 4-epimerases leading to the formation of UDP-arabinose. This helps explain the known cytosolic interconversion activity between the latter two nucleotide sugars. Since UGE2 and UGE4 had a comparatively low tendency to act on UDP-xylose, we selected UGE2 as the most promising isoform for overexpression experiments with UGE4 as an alternative.

Phenotypic characterizations of two null mutants in the *MUR12* gene did not reveal any visible phenotypes or alterations in the properties of the cell wall. Null mutations in the *MUR3* gene caused dwarfism, reduced fertility, disturbances in the organization of the endomembrane system, constitutive expression of defense-related genes, and a weakening of the cell wall. Studies on an allelic series of *mur3* mutants indicated that some but not all of the above phenotypes were rescued if XXLG and XXFG building blocks were not completely eliminated due to slight leakiness. We tentatively conclude that xyloglucan galactosylation at the MUR3-specific position plays a special role in determining whether the polysaccharide is correctly processed through the secretory pathway.

**Figure 1.** Structure of the hemicellulose xyloglucan. This matrix polysaccharide has a backbone of XXXG building blocks in which two of the three xylose residues can be modified by galactosylation mediated by the known or predicted galactosyltransferases MUR3 and MUR12 that have different acceptor site specificities. One of the two galactosyl residues can be further modified by fucose attachment. The modifications of the backbone structure are only partial, leading to the possible permutations XXXG, XXLG, XLXG, XLLG, XXFG and XLFG. These oligosaccharides can be enzymatically released from the intact polymer by digestion with  $\beta$ -glucanases that cleave the glycosidic bond after the unsubstituted glucose residue.



#### Science objectives for 2010-2011:

- Although the MUR12 protein has all of the hallmarks of a Golgi-localized glycosyltransferase, and disruptions of the *MUR12* gene lead to the complete absence of galactosylation at one of the two possible positions in the XXXG backbone (see Fig. 1), its subcellular location and biochemical function remain to be established. We are in the process of analyzing the location of MUR12:GFP fusions in *Arabidopsis* and *N. benthamiana* via laser scanning confocal microscopy to address the first point. His<sub>6</sub>- and *c-myc*-tagged versions of the coding region have been introduced into *P. pastoris* and *S. cerevisiae* to obtain recombinant protein for enzyme assays.
- Transgenic lines expressing UGE2, MUR3 and MUR12 as tagged and untagged versions have been generated in wild type and mutant backgrounds, and need to be assayed for enzymatic activities and genetic complementation of mutant lines to verify that the respective constructs have the intended functions. Furthermore, the predicted Golgi-localization of a modified version of UGE2 needs to be demonstrated.
- The *Arabidopsis* lines with functional transgenes need to be analyzed for changes in the galactosylation pattern of their xyloglucan and for potential visible or cell wall-related phenotypes.
- Crosses will be performed between promising transgenic lines to determine possible additive or synergistic effects on xyloglucan galactosylation. Alternatively, transgenic lines can be re-transformed with new constructs to accomplish this goal.

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## **Functions of HKT Transporters in Plant Sodium Transport and in Protecting Plant Leaves from Salinity Stress.**

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### **Overall Research Goals:**

Water limitation and salinity stress are progressively causing reduced plant growth in many regions. Basic knowledge of plant responses to environmental challenges is needed to cope with these constraints in plant growth and biomass production. In this regard, salinization of soils has become a major and increasing concern. Our DOE-supported research has shown that the AtHKT1;1 transporter mediates a major mechanism for protection of plants from salinity stress by excluding toxic Na<sup>+</sup> ions from leaves (Horie et al., 2009; Sunarpi et al., 2005; Horie et al., 2006). Recent mapping of salinity resistance quantitative trait loci (QTL) in grasses in several labs, including in wheat and rice, and analyzing candidate HKT genes in rough mapping domains suggest that the same HKT mechanisms that the P.I.'s lab has characterized in *Arabidopsis* are key salinity resistance mechanisms in grasses. Our DOE research focuses on the basic biophysical, physiological and genetic mechanisms through which HKT sodium cation transporters and K<sup>+</sup> transporters play central roles in mediating salt tolerance in plants.

### **Significant Achievements:**

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

Na<sup>+</sup> and K<sup>+</sup> homeostasis are crucial for plant growth and development. Two HKT transporter/channel classes have been characterized that mediate either Na<sup>+</sup> transport or Na<sup>+</sup> and K<sup>+</sup> transport when expressed in *Xenopus* oocytes and yeast. However, the Na<sup>+</sup>/K<sup>+</sup> selectivities of the K<sup>+</sup> permeable HKT transporters had not yet been studied in plant cells. We therefore analyzed two highly homologous HKT transporters in plant cells, OsHKT2;1 (Horie et al., 2007) and OsHKT2;2, that show differential K<sup>+</sup> permeabilities in heterologous systems. Using *oshkt2;1* knock out mutant lines in rice, we have found that OsHKT2;1 mediates large rates of sodium influx into rice roots in response to K<sup>+</sup> starvation. Upon stable expression in cultured *Nicotiana tabacum* Bright-Yellow 2 (BY2) cells, OsHKT2;1 mediated Na<sup>+</sup> uptake, but little Rb<sup>+</sup> uptake, consistent with earlier studies and new findings in oocytes (Yao et al., 2010). In contrast, OsHKT2;2 mediated Na<sup>+</sup>-K<sup>+</sup> co-transport in plant cells. Furthermore, at millimolar Na<sup>+</sup> concentrations OsHKT2;2 mediated Na<sup>+</sup> influx into plant cells. In addition, the presence of external K<sup>+</sup> and Ca<sup>2+</sup> down-regulated OsHKT2;1-mediated Na<sup>+</sup> influx in two plant systems, BY2 cells and intact rice roots (Yao et al., 2010). The present study shows that the Na<sup>+</sup>/K<sup>+</sup> selectivities of these HKT transporters in plant cells coincide closely with the selectivities in oocytes and yeast and furthermore that OsHKT transporter selectivities in plant cells are regulated by cationic conditions (Yao et al., 2010).

#### ***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 previous research has shown that AtHKT1;1 is a sodium (Na<sup>+</sup>) transporter that functions in mediating tolerance to salt stress (Horie et al., 2009). Our DOE-supported research has led to the model that AtHKT1;1 unloads sodium directly from xylem vessels to xylem parenchyma cells (Sunarpi et al., 2005; Horie et al., 2006; Horie et al., 2009). However, direct biophysical electrophysiological analyses of AtHKT1;1 transport function have not yet been pursued *in planta* for any plant HKT transporter. Using enhancer trap lines marking root xylem parenchyma cells, we have now analyzed the voltage dependence and ion selectivity properties of AtHKT1;1 in its native plant cells. These analyses show a sodium selectivity of AtHKT1;1 *in vivo* and also reveal new properties of plant HKT transporters as will be presented (unpublished data).

### **High-affinity K<sup>+</sup> transport in *Arabidopsis*: AtHAK5 and AKT1 are vital for seedling establishment and post-germination growth under low K<sup>+</sup> conditions**

Potassium (K<sup>+</sup>) is a major plant nutrient required and potassium uptake is an important contributor to salinity (Na<sup>+</sup>) resistance. Classical studies E. Epstein and colleagues showed that plant roots absorb K<sup>+</sup> through high-affinity and low-affinity transport isotherms. We previously characterized AtHAK5 as the major high-affinity K<sup>+</sup> uptake transporter in K<sup>+</sup>-starved roots (Gierth et al., 2005). Now we have analyzed double loss of function mutants in AtHAK5 and AKT1, two K<sup>+</sup> transporters active in roots, in a collaboration with Dr. Myeon Cho (Seoul, Korea). *athak5 akt1* double mutants failed to grow on media containing up to 100 μM K<sup>+</sup> (Pyo et al., 2010). Moreover, transfer of plants to low K<sup>+</sup> concentrations leads to growth defects and leaf chlorosis at 10 μM K<sup>+</sup> in *athak5 akt1* double mutant plants. High-affinity Rb<sup>+</sup>/K<sup>+</sup> uptake into roots is almost completely abolished in double mutants (Pyo et al., 2010). These results strongly indicate that AtHAK5 and AKT1 are the two major, physiologically relevant molecular entities mediating high-affinity and intermediate-affinity potassium uptake into roots (Pyo et al., 2010). This research was supported by a grant from the Department of Energy to J.I.S.

#### Science objectives for 2010-2011:

- Our recent *in vivo* biophysical transport analyses of HKT family members is suggesting new important properties of these transporters. We will further pursue the *in vivo* analysis of AtHKT1;1 in xylem parenchyma cells towards understanding the mechanism mediating salinity tolerance. We will further comparatively analyze the biophysical selectivity properties of several HKT transporters based on our recent findings.
- Two families of HKT transporters from plants have been characterized which differ in their ion selectivities. But plant disruption mutants have not yet been analyzed in the second potassium-transporting class of HKT transporters. We have isolated rice double mutant alleles in the later HKT transporter class, for functional analyses.
- Our research on HKT transporters provides evidence for rapid regulation of Na<sup>+</sup> transport activities *in planta* (Horie et al., 2007; Horie et al., 2006; Horie et al., 2009; Yao et al., 2010). However, no HKT protein interactors or regulators of plant HKT transporters are known. Strategies are being developed, to identify HKT protein interactors.

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## Quantitative Analysis of Central Metabolism and Seed Storage Synthesis

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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  $^{13}\text{C}$ -Metabolic Flux Analysis ( $^{13}\text{C}$ -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*, *Arabidopsis thaliana* or *Thlaspi arvense*, a potential non-food bioenergy crop ([www.pennycressbiodiesel.com/](http://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 2008-2010: (1) Two *A. thaliana* mutants severely impaired storage oil accumulation in seeds have been characterized by metabolic flux analysis of cultured developing embryos. As one major result, flux analysis demonstrated how a transcription factor regulating the expression of glycolysis and lipid biosynthetic enzymes controls lipid synthesis more effectively than mutation/down-regulation in a single step of the pathway. We also found that, in response to loss in enzyme capacity, compensatory metabolic bypasses are used only to a limited extent. (2) A large scale stoichiometric model of developing *B. napus* seeds was constructed based on biochemical literature. We could extend former findings on the special role of Ribulose 1,5 bisphosphate carboxylase/oxygenase (RubisCO) in photoheterotrophic seed metabolism in *B. napus*. (3) A kinetic model of the phosphoenol pyruvate (PEP) branchpoint in *B. napus* was constructed based on biochemical literature. With the model we will explore the significance of biochemical allosteric regulation of enzymes for carbon and nitrogen partitioning.

Science objectives for 2010-2011:

- Produce transgenic plants of *Thlaspi arvense* altered in particular enzyme targets of central metabolism as well as regulatory proteins. We will start to study the effect on carbon partitioning in developing seeds *in planta* as well as by flux-, enzyme- and metabolite profiles. In addition transgenics of *B. napus* generated by a industrial collaborator will be analyzed in a similar way.
- We will model the photosynthetic performance of *B. napus* embryos by use of our stoichiometric large scale model by integrating photosynthetic measurements (gas exchange measurements) and simulating different experimental light levels.
- Refinement of the enzyme kinetic model with focus on the phosphoenol pyruvate metabolic branchpoint. Enzyme profile, metabolite profile and flux data will be integrated by the model.
- Using the enzyme kinetic model studies, modifications of allosteric regulation of enzymes will be predicted that lead to changes in carbon partitioning. Respective protein modification on the amino acid sequence level will be designed and the detailed biochemical analysis by expression in *E. coli* and *Thlaspi arvense* will be started.



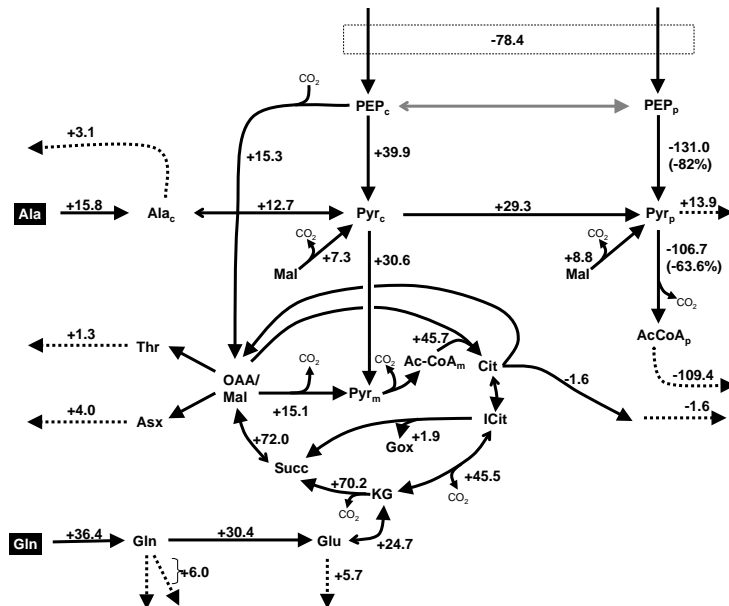


Figure 1. Effect of a mutation causing severe reduction in seed oil content in *A. thaliana*, shown by  $^{13}\text{C}$ -MFA of cultured developing embryos. Lower glycolysis leading into fatty acid synthesis and the tricarboxylic acid cycle are shown. Changes in flux are shown for the transcription factor mutant *wri1-1* vs. the wild type (Flux units: relative units normalized to the carbon of 100 moles hexose accumulated in biomass). For a few reactions the change is given as % reduction of the wild type value. For more details see Lonien and Schwender (2009).

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## Engineering Selenoproteins for Enhanced Hydrogen Production

Dieter Söll, Principal Investigator

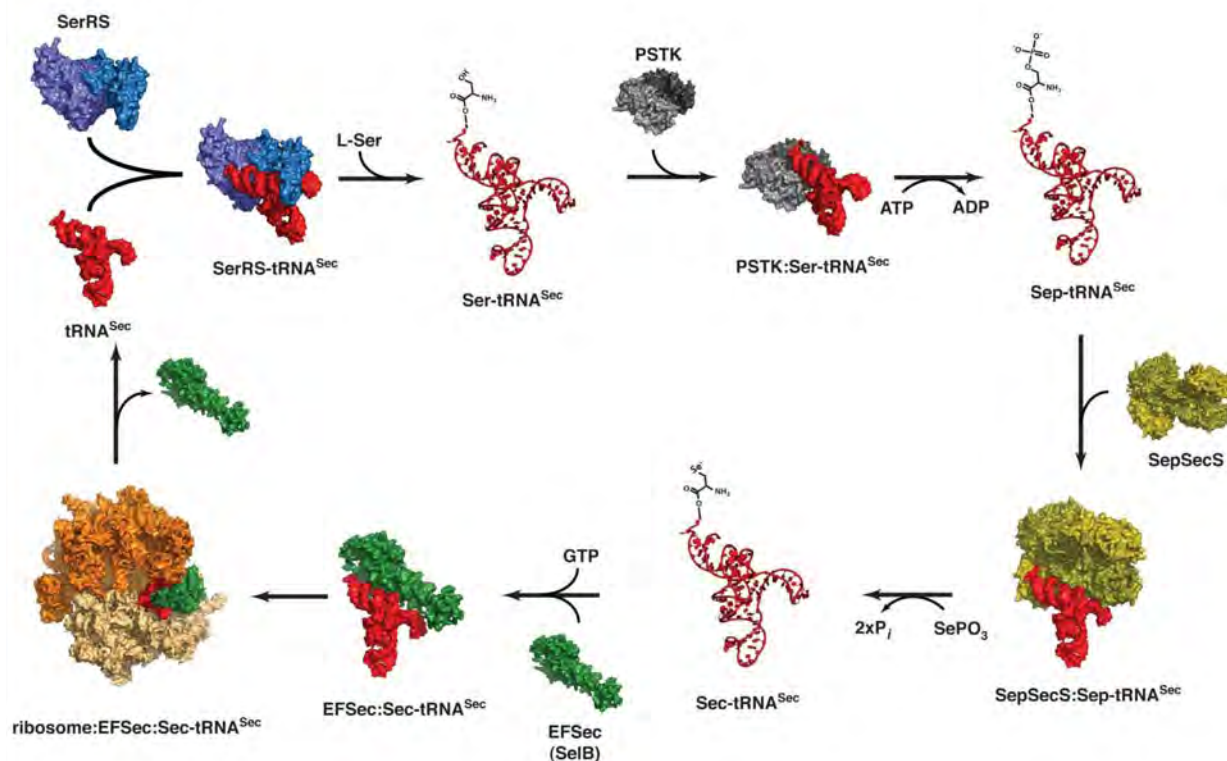
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Overall research goals: Selenoenzymes (containing selenocysteine - Sec) are known to have superior catalytic efficiency compared to their sulfur homologs (containing cysteine - Cys). During the past three years the pathway of Sec biosynthesis in archaea and eukaryotes has been worked out. (Fig. 1). Co-translational Sec insertion proceeds only with a modified translation apparatus that rules out mutational Cys → Sec mutagenesis. Our research objectives are (1) to develop a system allowing co-translational insertion of selenocysteine at any site in a protein, and (2) to re-engineer the F<sub>420</sub>-reducing [NiFeSe] hydrogenase.

Significant achievements 2009-2010: Crystal structures of archaeal SepSecS, complex of human SepSecS:tRNA<sup>Sec</sup>, human and archaeal tRNA<sup>Sec</sup>, and complex of archaeal PSTK:tRNA<sup>Sec</sup>.



**Fig. 1.** Schematic diagram of the synthetic cycle of selenocysteine in eukaryotes. The process begins with serylation of tRNA<sup>Sec</sup> (red) by SerRS (light and dark blue). PSTK (light and dark grey) then phosphorylates Ser-tRNA<sup>Sec</sup> and releases Sep-tRNA<sup>Sec</sup>. A SepSecS tetramer (gold and olive) subsequently binds Sep-tRNA<sup>Sec</sup> and catalyzes a two-step transformation of Sep into Sec using selenophosphate as the selenium donor. The final product, Sec-tRNA<sup>Sec</sup>, is delivered to the 80S ribosome (orange and beige) by the specialized elongation factor EFSec (green). Once Sec is inserted into the polypeptide chain, free tRNA<sup>Sec</sup> is released for another round of Sec synthesis. All molecules are in surface representation, whereas Ser-tRNA<sup>Sec</sup>, Sep-tRNA<sup>Sec</sup> and Sec-tRNA<sup>Sec</sup> are shown as ribbon diagrams. Except for the SepSecS-tRNA<sup>Sec</sup> complex, all other complexes are proposed models and not true structures. The archaeal pathway is similar using a 70S ribosome and the specialized elongation factor SelB.

Science objectives for 2010-2011:

- Based on the structural information of PSTK and SepSecS complexes with tRNA<sup>Sec</sup> (see above), the two enzymes and the tRNA<sup>Sec</sup> molecule will be re-engineered in order to generate an efficient Sec-tRNA<sup>Sec</sup> molecule that will be a substrate for the normal elongation factor EF-Tu. This is tall order, and new biochemical and genetic assays need to be developed in order to test out/screen a large number of possible variants.

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## Principles of Energy Transduction: Basis for the Design and Synthesis of Hydrogen Catalysts

Thomas C. Squier, Principal Investigator

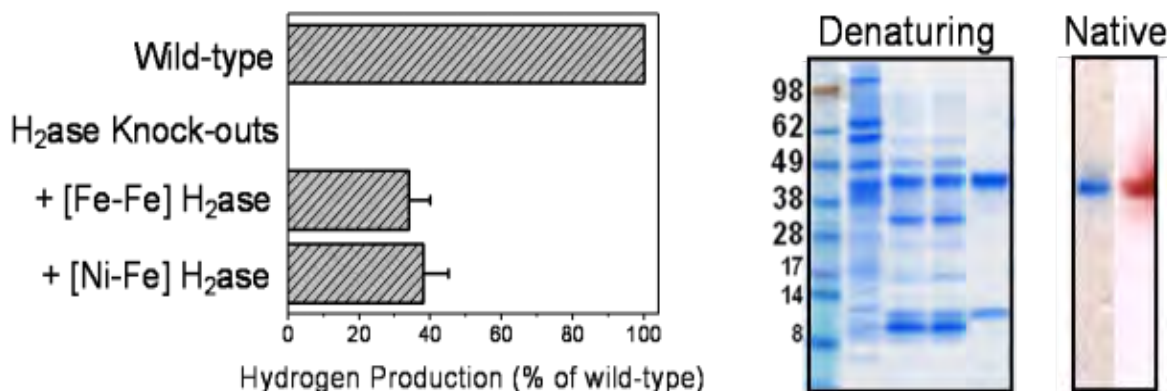
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Overall research goals: Our long-term goal is search to understand how hydrogenase ( $H_2ase$ ) enzymes catalyze proton-coupled electron transfer mechanisms to efficiently form hydrogen with high catalytic rates and reaction specificity. To this end we aim to: a) understand and test how active site geometry modulates hydricity and catalytic rates for hydrogen production; b) clarify how protein dynamics control proton-coupled electron transfer and rates of hydrogen production; and c) determine how orientation and dynamics of Fe/S clusters affects electron transfer rates and minimizes the formation of reactive oxygen species. Our approach includes: 1) expressing and purifying  $H_2ase$  mutants using a newly developed heterologous expression system in *Shewanella* to control active site properties and introduce tagging sites for labeling by spectroscopic probes; 2) carrying out hybrid QM/MM and molecular dynamics calculations to identify how electron densities of Fe/S clusters and dynamics affect electron transfer rates; 3) through the synthesis of molecular catalysts that incorporate protein structural elements that test how local environment and proton buffering affect rates of hydrogen formation. This program bridges enzymology and strengths in molecular catalysis at PNNL through linked hypothesis testing. The ability to modify hydrogenase structure and probe molecular dynamics through the newly developed expression system permits testing of ideas developed using Ni-phosphines in enzyme systems (e.g., V71C will compress diatomic ligands on active site iron to alter bite angle). Newly developed molecular probes and single molecule spectroscopic measurements permit direct comparison with computational predictions of linked protein motions and catalysis.

Significant achievements 2009-2010: Initial measurements have cloned and expressed wild-type and mutant hydrogenase that permit mechanistic measurements (Figure 1). Complementary QM/MM and molecular dynamics calculations have developed necessary force-fields and homology modeling approaches that permit the construction of homology models based on known structures and the determination of necessary force fields for realistic modeling of metal centers.



**Figure 1:** Heterologous Expression and Purification of Functional Hydrogenase Enzyme. Left Panel: Complementation assay for hydrogen production using *S. Oneidensis* MR-1 wild-type bacteria, following the construction of a double deletion mutant without either [Fe-Fe]-hydrogenase or [Ni-Fe]-hydrogenase (i.e., hydrogenase knock-outs), and following complementation with either the [Fe-Fe]-hydrogenase or [Ni-Fe]-hydrogenase. Right Panels: SDS-PAGE (denaturing) gel showing step-wise purification of [Ni-Fe]-hydrogenase, and native gels showing purified hydrogenase and associated hydrogen activity (red stain).

Science objectives for 2010-2011:

- Test how active site geometry (bite angles) modulates hydricity and catalytic rates of hydrogen production. Measurements will involve quantitative comparisons between catalytic rates and substrate (protons or hydrogen) binding affinities between i) different hydrogenase enzymes and following the introduction of site-specific mutations near the active site. Molecular dynamics calculations will permit how sequence differences alter the electronic structure of the active site to alter catalytic rates and reaction specificity.
- Understand how the local dielectric constants or polarity/hydrophobicity surrounding the active site affects catalytic activity. Catalysts will be synthesized involving the introduction of polar (e.g., serine) or hydrophobic (e.g., phenylalanine) amino acids or small peptides within current diphosphine molecular catalysts under development at PNNL, which catalyze hydrogen formation at rates near those observed for hydrogenase enzyme systems (albeit in organic solvents at high overpotentials). It is expected that modulation of the local environment around these molecular catalysts will permit facile testing of how changes in active site physical properties modulate catalysis.
- Clarify how protein dynamics controls proton-coupled electron transfer rates of hydrogen production. Measurements of changes in subunit interactions and molecular dynamics will be measured using fluorescent molecular probes that will be introduced at engineered sequences located within loop regions, permitting single molecule measurements of subunit reorientations and changes in molecular dynamics that are hypothesized to control electron transfer rates.
- Determine how orientation and dynamics of Fe/S clusters affects electron transfer rates. Following implementation of QM/MM geometry optimizations, we will assess how ligand coordination to the active site affects active site structure and the orientation and dynamics of Fe/S clusters to control electron transfer rates. It is our hypothesis, based on our earlier work on electron transfer rates in heme proteins, that subtle changes in the geometry of Fe/S clusters triggered by ligand binding (coordination) at the active site will have dramatic effects on electron transfer rates, acting to enhance reaction specificities by gating electron flux in response to bound protons in the active site.

## Interdisciplinary Research and Training Program in the Plant Sciences

Michael Thomashow, Principal Investigator

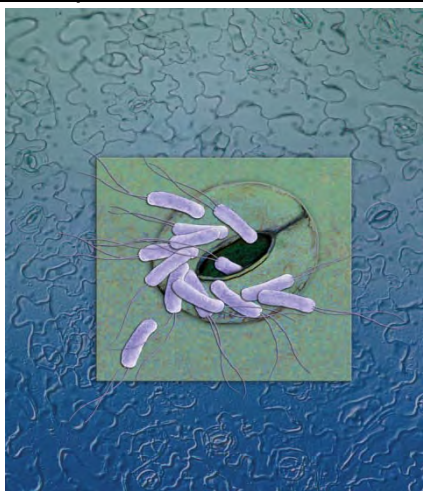
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Overall Goals: The MSU-DOE Plant Research Laboratory (PRL) was established in 1965 as a joint venture between the U.S. Department of Energy (then the Atomic Energy Commission) and Michigan State University to conduct basic interdisciplinary research on fundamental questions in plant biology and to train graduate students and postdoctoral researchers in this area of science. Over the years, the specific topics of research in the PRL have changed, but all have contributed to developing a greater understanding of the flow of energy from photon capture to its deposition in energy-rich molecules and how the associated processes are affected by plant interactions with the environment. Current research focuses in four areas: the mechanisms that enable plants and photosynthetic microorganisms to sense and respond to environmental factors that impact the overall efficiency of growth and development (He, Howe, Kramer, Montgomery, Thomashow, and Walton); the development and function of plant organelles (Brandizzi, Hu, Keegstra, and Larkin); the steps involved in converting fixed carbon into energy-rich cell walls (Keegstra and Walton); and the bio-production of hydrogen gas (Wolk). In addition, there is a group effort designed to map the energy regulatory network of plants. This effort, designated the PlaNet (Plant Energy Network) project, is initially focused on determining the global “regulatory logic” used to coordinate the expression of genes involved in photosynthesis.

Examples of recent achievements: From 2008 to the present, the PRL faculty has published more than 120 articles. A complete listing of these publications can be found at the PRL website (<http://www.prl.msu.edu/publications.html>). All of these findings cannot be summarized here, but a few highlights can serve as examples of current research efforts. In the area of plant and microbial interactions with the environment, research has provided new insights into the nature of the jasmonate stress-hormone receptor, the chemical specificity of jasmonate perception, and post-transcriptional mechanisms to increase versatility in gene regulation by jasmonate (1-3); has provided evidence that the flagellin receptor FLS2 has a critical role in regulating stomatal defense against bacterial invasion in *Arabidopsis* (4; see figure);



Artistic illustration of bacteria invading an *Arabidopsis* plant leaf through stomata (Credit: Marlene Cameron)

has led to the discovery of a novel light-dependent iron acclimation response in *F. diplosiphon* (5); has established a role for the histone deacetylase inhibitor, depudecin, in the virulence of *Alternaria brassicicola* (6); and has provided new insights into how plants sense and respond to low temperature (7, 8). In the area of organelle biology, research has shown that plastid dysfunction "rewires" light signaling to promote chloroplast biogenesis and function (9) and that the endoplasmic reticulum (ER) has a significant role in triggering components of the heat shock response leading to increased thermotolerance (10). In addition, a new component of the peroxisome division machinery was identified (11) and a forward genetics screen based on live cell imaging provided new insights into components having critical roles in the structure of the ER and its function in the plant secretory pathway (12, 13). Finally, in the area of network biology, a novel knowledge-based gene clustering approach has been developed that narrows the search for novel functional genes while increasing the likelihood that they are biologically active (14). Throughout the coming year, efforts will continue in these and the other focal areas of PRL research.

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## AFFINITY PURIFICATION OF THE ARABIDOPSIS 26S PROTEASOME REVEALS A DIVERSE ARRAY OF PLANT PROTEOLYTIC COMPLEXES

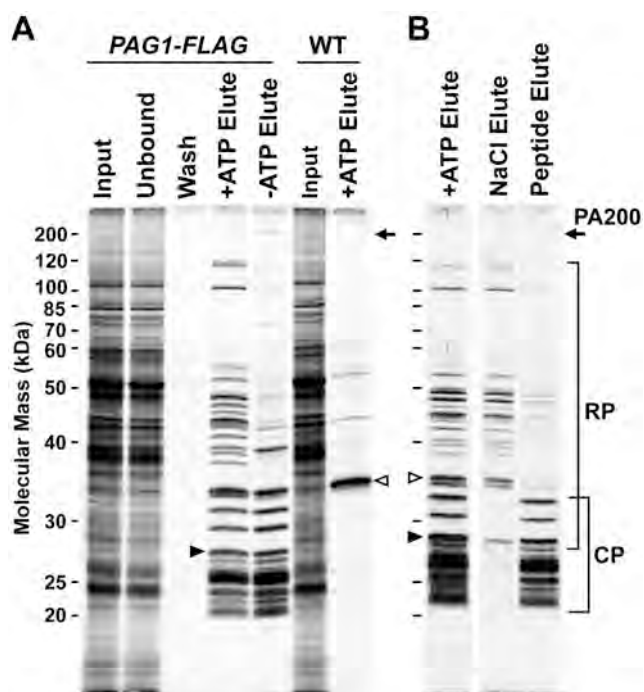
Vierstra, R.D.<sup>a</sup>, A.J. Book<sup>a</sup>, N.P. Gladman<sup>a</sup>, D.C. Gemperline<sup>a</sup>, S.-S. Lee<sup>a</sup>, M. Scalf<sup>b</sup>, and L.M. Smith<sup>b</sup>

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Central to the ubiquitin (Ub) proteolytic system is the 26S proteasome, a 2.5-MDa proteolytic complex with specificity for Ub conjugates. The 26S proteasome is regulated by various mechanisms, including the use of alternative subunit isoforms, capping with different regulatory complexes, association with accessory proteins, and/or various post-translational modifications. The complex 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.

To better define the 26S proteasome in plants, we developed an affinity method to rapidly purify the particle intact from *Arabidopsis thaliana* that involved genetic replacement of the alpha 7 subunit of the CP with an epitope-tagged version (Figure 1A). Subsequent salt washes then allows separation of the CP from the RP to enrich for these particles specifically (Figure 1B). In-depth mass spectrometric 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 are modified post-translationally by proteolytic processing, acetylation, and/or ubiquitylation. Ubiquitylation of RPN1 and RPN2 in particular appears to be an abundant modification. Several proteasome-associated proteins were also identified that likely assist in complex assembly and regulation, including UMP1/2, SEM2, the chaperonin PBAC2, and the DUB UBP16.

In addition, we detected a particle consisting of the CP capped by the single subunit PA200/Blm10 activator. 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 MG132. Reverse genetic analyses of several *Arabidopsis* CP and RP subunits revealed diverse roles for individual components in plant growth and development. From expression studies using promoter-GUS fusions, we also discovered that the synthesis



**FIGURE 1.** Affinity purification of proteasomes from *PAG1-Flag pag1-1* plants. **A**, SDS-PAGE analysis of the affinity purification steps. Total protein extracted from 10-day-old wild-type (WT) and *PAG1-Flag pag1-1* plants was incubated with anti-Flag affinity resin, washed, and competitively eluted with the Flag peptide. The procedure was performed in the presence or absence of ATP. The input, unbound, washed and eluted fractions were subjected to SDS-PAGE and the gel was stained for protein with silver. The arrow and closed arrowhead locate the PA200 and the *PAG1-Flag* proteins, respectively. The open arrowhead identifies nitrilase, which is non-specifically enriched during the purification. **B**, Salt dissociation of the 26S proteasome into the RP and CP subcomplexes. 26S proteasomes were bound to the anti-Flag resin in the presence of ATP and either eluted with the Flag peptide, or first eluted with 800 mM NaCl, followed by elution with the Flag peptide. The brackets locate subunits of the CP and RP subcomplexes. The arrow indicates PA200.

of the most CP and RP subunits is coordinately regulated by various conditions that increase proteolytic demand. Taken together, it appears that a diverse and highly dynamic population of proteasomes is assembled in plants, which may expand the target specificity and functions of intracellular proteolysis.

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*Session IX*  
*Meeting the Challenges of*  
*X-ray Studies*



## Radiation damage in macromolecular crystallography

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**Collaborator: Elspeth F. Garman**

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**Overall research goals:** For protein crystals at room temperature, radiation damage during the diffraction experiment is rapid even on a laboratory X-ray source. In the past, the required data typically had to be collected from several different crystals and then merged together. The intense X-ray beams produced by third-generation synchrotrons can destroy crystalline order in a matter of seconds. Over the last 20 years, the use of cryo-cooling techniques which allow X-ray data to be collected with the sample held in a stream of cooled nitrogen gas at around 100 K, has become the norm [1, 2]; at 100 K crystals can withstand many times the dose ( $\text{J kg}^{-1} = \text{Gy}$ ) [3] compared with room temperature (depending on the dose rate [4]), and the necessary data can usually be obtained from a single crystal. However, observations of degradation of crystal diffraction with increasing radiation dose at 100 K have now become commonplace at third-generation synchrotrons. This damage manifests itself in a number of different ways, including: changes in crystal color, decreasing diffraction power with dose, a small but measurable increase in unit cell volume, and specific structural damage to covalent bonds in the amino acids of the protein molecules [5]. Enzyme active sites seem particularly sensitive to damage, so this phenomenon can easily lead to incorrect conclusions on biological mechanisms being drawn. Thus the issue of radiation damage during diffraction experiments has recently come to the fore as a concern for all structural biologists. We aim to understand the physical and chemical processes involved in this damage (reviewed in [6,7]) and to develop mitigation strategies.

**Recent achievements:** Key findings include the discovery of a surprising inverse dose rate effect during data collection at room temperatures [4]. Specific radical scavengers were shown to prolong the useful lifetime of crystals under irradiation. The use of such radioprotectants was also demonstrated to alter the dose dependence of the total diffracted intensity [8].

**Dose rate effects** – The dose dependence of radiation damage to lysozyme crystals at room temperature (RT) was investigated in order to provide an accurate comparison with cryotemperature results as well as to enable the calculation of expected maximum room-temperature crystal lifetimes by future researchers **before** beginning their experiments. The results of intensity loss analysis ( $I/I_0$ ) for complete datasets unexpectedly showed that the dose that can be tolerated by a crystal is **inversely proportional** to the dose rate with a 99% correlation coefficient over the range studied. Alternative metrics of radiation damage were assessed from these room temperature data and shown not to be quantitatively reliable in biologically useful dose ranges. In the dose rate range tested cryocooling was demonstrated to offer a 26-113 times increase in the dose tolerance of the crystal.

**Scavenger effects** – An alternative mechanism to cryocooling for prolonging crystal lifetime is the use of radioprotectants which presumably scavenge the radical species formed in the medium upon X-ray irradiation. Three putative radioprotectants: ascorbate, 1,4-benzoquinone and 2,2,6,6-tetramethyl-4-piperidone (TEMP) were tested at RT. Again plots of  $I/I_0$  against dose were used as a metric to assess radioprotectant ability: ascorbate and 1,4-benzoquinone appear to be effective, whereas studies on TEMP were inconclusive. Ascorbate, which in irradiated

aqueous solution scavenges OH<sup>•</sup> radicals, doubled the crystal lifetime, whereas 1,4-benzoquinone, which rapidly scavenges both OH<sup>•</sup> radicals and hydrated electrons, offered an **8 fold increase** at the dose-rates used. Pivotaly, these scavengers also induced a striking change in the dose dependence of the intensity decay from a first order to a **zeroth order** process.

**Future plans:** Several key questions concerning the mechanism of radiation damage at both cryo and RT will be addressed.

Firstly, the RT inverse dose rate effect has been characterized only between 6-10 Gy s<sup>-1</sup> on a home X-ray source, and for only 2 dose rates (2.8 and 5 kGy s<sup>-1</sup>) using a synchrotron X-ray beam. Both to understand the underlying processes of radiation damage and to inform data collection strategies for macromolecular crystals for which cryodata collection is not feasible, these measurements will be extended both to determine the functional form of the diffraction pattern intensity decay with dose rate in the missing range, 20 Gy s<sup>-1</sup> and 5 kGy s<sup>-1</sup> and to experiments on crystals of other proteins. From this characterization, we anticipate that we will be able to extend our current radiation chemistry hypothesis covering the lower dose region, and determine if there is an optimum dose rate at which to collect RT diffraction data at third-generation synchrotrons.

Secondly, we have previously identified several radical scavengers which are effective in reducing the rate of both global and specific radiation damage in protein crystals: ascorbate and 1,4-benzoquinone at RT, and ascorbate and nitrate at 100 K. Our planned work in this area aims at elucidating in detail the mechanism of action of these scavengers so that an intelligent search for further effective compounds can be made. We also seek to understand the radiation chemistry at RT underlying the change in the functional form of the intensity decay with dose from first to zero order upon the addition of scavengers, the apparent form at 100 K. Experiments in this area will include assessment of the efficacy nitrate as a scavenger at RT, and 1,4-benzoquinone as a scavenger at 100 K.

Concomitant to this experimental work we have an ongoing effort to improve RADDOSE, a computer program written by the Garman group and widely used at synchrotrons, to calculate the dose absorbed by protein crystals during data collection for structure determination. Current developments include a quantitative treatment of the previously neglected energy loss due to Compton electrons [9]. This factor will become increasingly important if ideas to perform data collection at shorter X-ray wavelengths are in fact pursued. In addition, radiation chemical experience suggests that, at room temperature, the removal of oxygen will qualitatively alter radical mechanisms and rates. Such studies will also be systematically pursued using in-house radiation sources.

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## **Energetics and Structure of the ZIP Metal Transporter**

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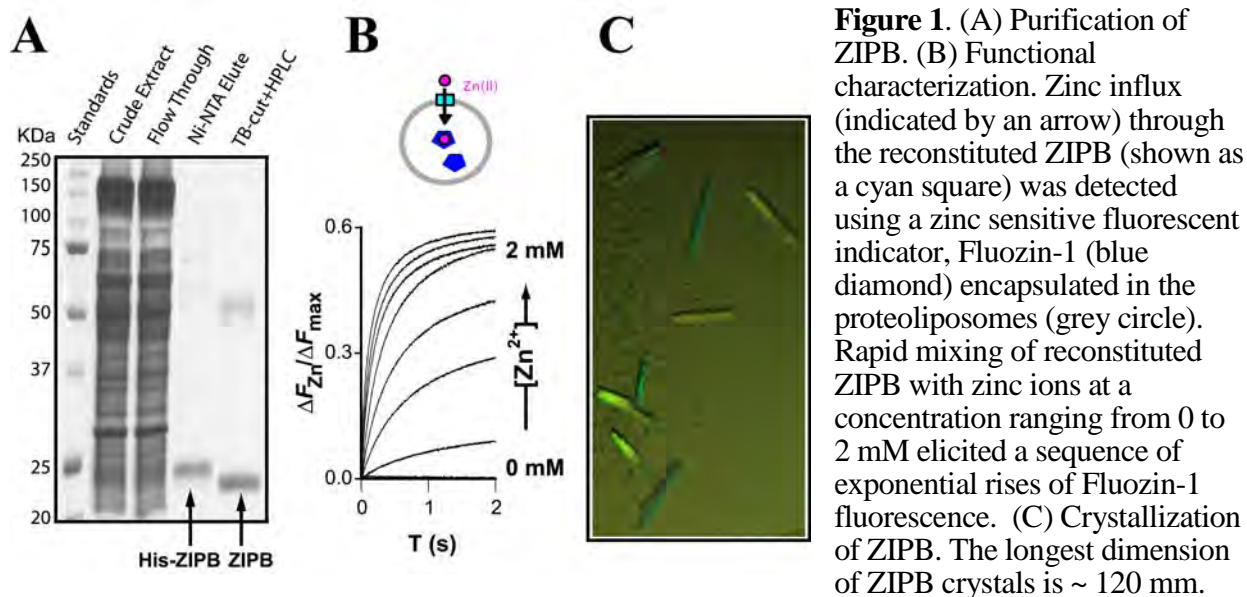
### **Overall research goals:**

Electron transfer reactions in photosynthesis require multiple metal centers for redox-catalyses. Metal uptake from the soil to the chloroplast sink involves multiple transport steps across a series of membrane barriers. The long-term goal of our research is to understand the chemical principles governing selective binding and energized movements of metal ions in metal transporters. We will use direct biophysical measurements and x-ray crystallography to study the ZIP (ZRT, IRT-like Protein) metal transporters. Specifically, we will (1) identify a ZIP model protein that can be purified in a sufficient quantity for biophysical and structural analyses, (2) determine the energetic mechanism for ZIPs, and (3) crystallize a prototypic ZIP for x-ray analysis. The proposed studies may reveal how metal coordination chemistry is exploited to move metal ions across the membrane barriers to reach their final destinations in the photosynthetic complexes. This project supports DOE's mission to understand and to adapt strategies used by plants and microorganisms to capture, store, and mobilize energy.

### **Significant achievements 2008-2010:**

(1) High-throughput expression screening of 52 ZIP homologs selected from 96 fully sequenced prokaryotic genomes led to the identification of only one ZIP homolog that can be purified in crystallographic purity and quantity. This result is consistent with the general observation that ZIPs tend to be highly refractory to over-expression and purification. The purified protein, known as ZIPB, is the first ZIP homolog to be purified, thus providing a unique opportunity for direct biochemical and structural analysis of ZIPs at the molecular level. (2) The energetic mechanism of ZIPB was studied by functional reconstitution, stopped-flow fluorometry and inductively coupled plasma mass spectroscopy. Zinc flux through ZIPB was found to be nonsaturable and electrogenic as predicted by the Nernst equation. Conversely, membrane potentials drove zinc fluxes with a linear voltage-flux relationship. Direct measurements of metal uptake demonstrated that ZIPB is selective for two group-12 transition metal ions, Zn(II) and Cd(II), while rejecting transition metal ions in groups 7 through 11. Our data suggest that ZIPB exploits the *in vivo* zinc homeostatic gradient to promote zinc uptake through a simple electrodiffusion mechanism. This result challenges a common assumption that cellular zinc uptake is an active process. (3) Optimization of the ZIPB stability in detergent micelles and initial crystallization screening yielded protein crystals with excellent reproducibility. Although the crystal sizes were small in the early stage of the optimization process, some crystals could diffract to 7 angstroms. Initial x-ray analysis suggested that the existing crystals belong to space group R32 with  $a=146$ ,  $b=146$ ,  $c=727$  angstroms,  $\alpha=\beta=90$ , and  $\gamma=120$  degree. This result established the feasibility for structure determination of ZIPB by x-ray crystallography.





**Figure 1.** (A) Purification of ZIPB. (B) Functional characterization. Zinc influx (indicated by an arrow) through the reconstituted ZIPB (shown as a cyan square) was detected using a zinc sensitive fluorescent indicator, FluoZin-1 (blue diamond) encapsulated in the proteoliposomes (grey circle). Rapid mixing of reconstituted ZIPB with zinc ions at a concentration ranging from 0 to 2 mM elicited a sequence of exponential rises of FluoZin-1 fluorescence. (C) Crystallization of ZIPB. The longest dimension of ZIPB crystals is ~ 120 nm.

**Science objectives for 2010-2012:**

- Optimize crystallization conditions to achieve an x-ray diffraction resolution better than 4 Å
- 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 the first protein electron density map.
- Build an atomic model into the electron density map
- 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 transport
- Carry out mutation-activity analysis to validate the structural model and test the proposed zinc transport mechanism.

**References to work supported by this project 2008-2010:**

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*Session X*  
*Bio-Inspired Structural*  
*Design*



## Engineering Functional Scaffolds by Supramolecular Self-Assembly

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Vincent P. Conticello, Co-PI

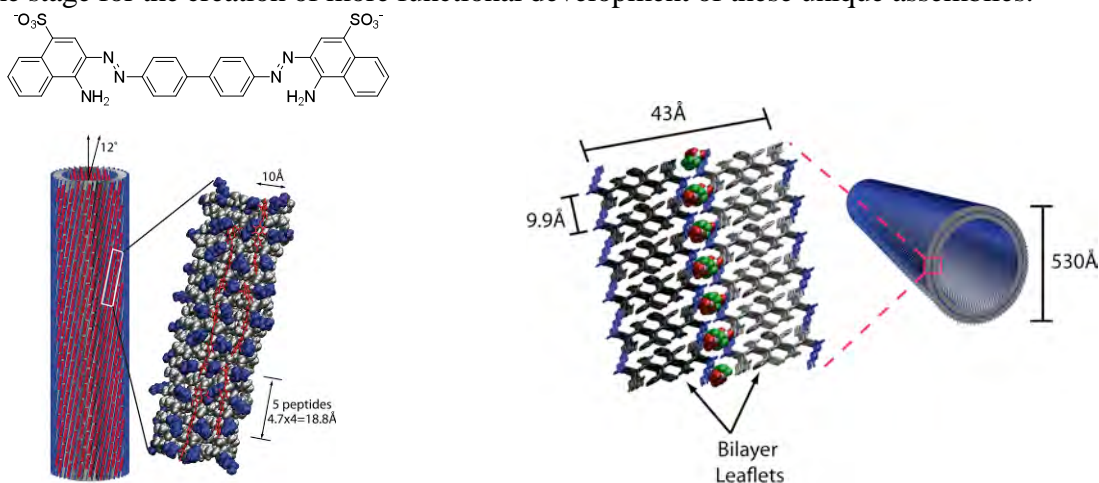
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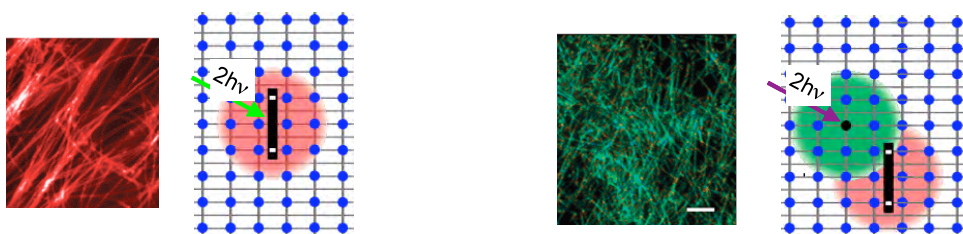
Overall research goals: Our overriding hypothesis is that organized arrays of functionalized nano-scale structures can be created from peptide self-assembly and these materials may be employed as platforms for the creation of nano-scale devices through selective functionalization with non-native substrates. Our design of self-assembled, structurally defined, supramolecular morphologies is based on features programmed into peptide sequences. We are exploring the potential of such synthetic peptide-based assemblies for energy capture/transduction and their ability to perform chemistry. Such a synthetic nano-scale device could retain the favorable properties of self-association that characterize biological systems, while allowing expansion of the functional capacity beyond the range of the native photochemical system through inclusion of non-native scaffolds and substrates.

Long-range molecular order is the hallmark of Nature's photosynthetic antenna, and we have created a thermodynamic self-assembled system that creates long-range order but is based on a completely different scaffold than the biological membranes. Further, we have now demonstrated the ability of this assembly to absorb photons and undergo Forster energy transfer along the surface of ordered arrays. This system is now poised for organizing new materials for a variety of functions including energy transfer and conversion to chemical energy.

Significant achievements 2008-10: We have accomplished the first step of directing our peptide assemblies to organize molecules into both covalent and non-covalent arrays (**Fig. 1**) and used them to capture light (**Fig. 2**). We have proven that the peptides organize as bilayers (**Fig. 1**), begun to define the unique functionality that exists at the leaflet interface, and are developing strategies for differentially functionalizing the inner and outer surfaces of the nanotubes. These achievements set the stage for the creation of more functional development of these unique assemblies.



**Figure 1. Left Panel:** Model of Congo red (red in cartoon) binding to KLVFFAL amyloid nanotubes.  $\beta$ -sheets run along the tube long-axis with a helical pitch of  $12^\circ$ . The positively charged side chains of the N-terminal lysines are shown as blue and form an organized array on the tube surface. The remaining hydrophobic residues are shown in gray. Anti-parallel  $\beta$ -strand organization results in lysine spacings of  $9.4 \text{ \AA}$  along the  $\beta$ -sheet. Congo Red binding site is located in the hydrophobic groove between two  $\beta$ -sheets separated by  $10 \text{ \AA}$ . **Right Panel:** Model of the KLVFFAE peptide bilayer and trapped TFA. Lysine residues coloured in blue and are located at the tube surface and the bilayer interface. TFA ions (space filling representation) are trapped within the bilayer interface.



**Figure 2: Left Panel** Two photon fluorescence imaging ( $\lambda_{\text{Ex}}=980\text{nm}$ ,  $\lambda_{\text{Em}}=565\text{nm}$ ) of Alexa555 bound to KLVFFAE tubes and cartoon of tube surface with lysines (blue dots) forming an  $\sim 10 \times 10$  Å array and binding of Alexa555 (black bar). **Right Panel:** Two-photon fluorescence lifetime image ( $\lambda_{\text{Ex}}=780\text{nm}$ ) of covalently linked Rh110-KLVFFAE donor nanotubes and bound Alexa555 acceptor. Two photon excitation ( $\lambda_{\text{Ex}}=780\text{nm}$ ) of Rh110-KLVFFAE results in energy transfer to Alexa 555 bound to the tube surface and emission at 565nm.

Science objectives for 2010-2011: Having organized antenna complexes on a peptide bilayer surface for light harvesting and energy transfer, we are now positioned to explore both the range of structures that peptides can be directed to form and the chemistry the assemblies can perform. Unlike passive lipid bilayer membranes, our peptide bilayer nanotube assemblies are composed of polypeptides that have the potential for protein-like function. Therefore, both the organizing ability and potential reactivity of the tube surface is inherently dependent on the functional groups that are displayed. We have created nanotube assemblies with many of the structural features of well-folded enzymes including metal binding sites, trapped ions, and displaying specific amino-acid side-chains. Similar to lipids, our preliminary results also indicate that peptide assemblies have temperature and solvent dependent phase transitions. Our goals are to understand:

- how solvent and temperature direct peptide assembly phase transitions,
- how cooperative behaviour can be controlled in mixed peptide assemblies, and
- how  $\alpha$ -helix and  $\beta$ -sheet functional properties can be integrated into self-assembling systems

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## Nanotube-Supported Phospholipid Bilayers: Self-assembly and Nanoscale Confinement

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Overall research goals: 1) Develop a series of nanoporous substrates and experimental procedures for forming nanopore-confined lipid bilayers of various compositions mimicking native cellular membranes and optimal for studying membrane proteins involved in energy transduction; 2) Employ a battery of spectroscopic techniques such as NMR, calorimetry, and spin-labeling EPR to investigate effects of nanoscale confinement on the lipid self-assembly and interfacial electrostatics of substrate-supported lipid bilayers; 3) Demonstrate the feasibility of using lipid nanotube technology for building hybrid nanostructures based on membrane proteins. The overall long term objective of this 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, including those involved in energy transduction.

Significant achievements 2008-2010: Developed and optimized in-house technology for fabricating nanoporous anodic aluminum oxide (AAO) substrates with variable pore diameter and length, and, most importantly, a high degree of pore ordering and uniform pore diameter as compared with those available commercially. Examination of new substrates revealed superior optical and mechanical properties and practically no surface defects – an essential requirement for forming macroscopically ordered nanotubular lipid arrays such as shown schematically in Fig. 1. A series of ion accessibility measurements has been completed using solid-state  $^{31}\text{P}$  NMR. Phospholipids with both saturated and unsaturated acyl chains, different polar head groups, as well as various acyl chain lengths have been investigated. Trivalent shift reagent  $\text{Pr}^{3+}$  and divalent relaxation agent  $\text{Mn}^{2+}$  have been used to access bilayer accessibility. It was concluded that the bilayer leaflet accessibility in AAO is primarily affected by the length of the lipid chain: the short lipids form fully accessible bilayers while longer lipids form sealed structures. Finally, efficient protocols for chemical modification of the AAO nanochannel surface have been developed in order to tune biophysical properties of the nanotubular bilayers. Specific modifications include introduction of lysine residues and nickel chelate, Ni-NTA (Ni-nitrilotriacetic acid), suitable to attaching His-tagged protein.

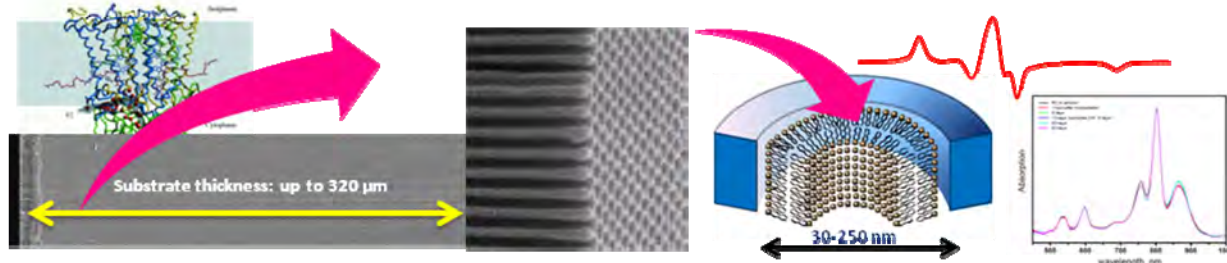


Figure 1. Left Panel: (Top) – a ribbon diagram of a photosynthetic reaction center (RC) protein from purple bacterium *Rhodospirillum rubrum*; (Bottom) – a scanning electron microscopy (SEM) image of the entire 320  $\mu\text{m}$  cross-section of the AAO substrate fabricated at NCSU. Center Panel: a 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 an EPR spectrum of spin-labeled peptide gramicidin A (top, red) showing a high degree of macroscopic alignment; stability of membrane proteins in such matrices could be accessed by conventional UV-vis spectroscopy as demonstrated for RC protein that remained at least 90% stable over the period of 63 days (bottom right).



### Science objectives for 2010-2011:

- Developing experimental protocols to improve efficiency of loading lipid bilayer membranes and membrane proteins into nanoporous substrates. High lipid and protein loading is essential prerequisite for structure-function NMR studies and efficient hybrid nanoscale devices. Preliminary TGI measurements demonstrated that the loading of up to 5-10% of lipid phase (by volume) could be achieved for commercial AAO when using a pressurized extruder. We are constructing a specialized extruder to investigate the conditions for optimal lipid deposition into the homogeneous substrates made in our lab. Deposition of both multilamellar and small unilamellar lipid vesicles will be investigated.
- Investigating lipid self-assembly mechanisms in pores of various surface composition (*e.g.*, comparing self-assembly in silica *vs.* alumina nanopores. Further manipulating self-assembly through surface modification of nanoporous surfaces and investigating interfacial electrostatic phenomena under conditions of controlled lipid bilayer curvature.
- Developing layer-by-layer deposition of lipids into complex nanotubular structure that would further mimic cellular membrane environment;
- Carrying out benchmark experiments to characterize self-assembly and self-association of membrane protein fragments within nanopore confined lipid bilayers using advanced pulsed EPR methods with the new X- and Q-band EPR spectrometers installed during summer of 2010.

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## Engineering Cells to Grow Electronic Connections to Materials

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**Overall Research Goals:** The fundamental goal of this program is to tap the world of biological nanotechnology by constructing molecular level, functional interfaces between living systems and synthetic materials. The key to domesticating life at the cellular and molecular level is communication. Living cells have tremendous ability to follow directions and perform functions on demand. In order to effectively communicate with non-living systems, cells must be able to send and receive signals in a language shared with materials. Critical to this goal is to the ability to re-route electron flow - the fundamental carrier of information and energy in both organisms and human-made devices – across the cell/material interface with molecular specificity.

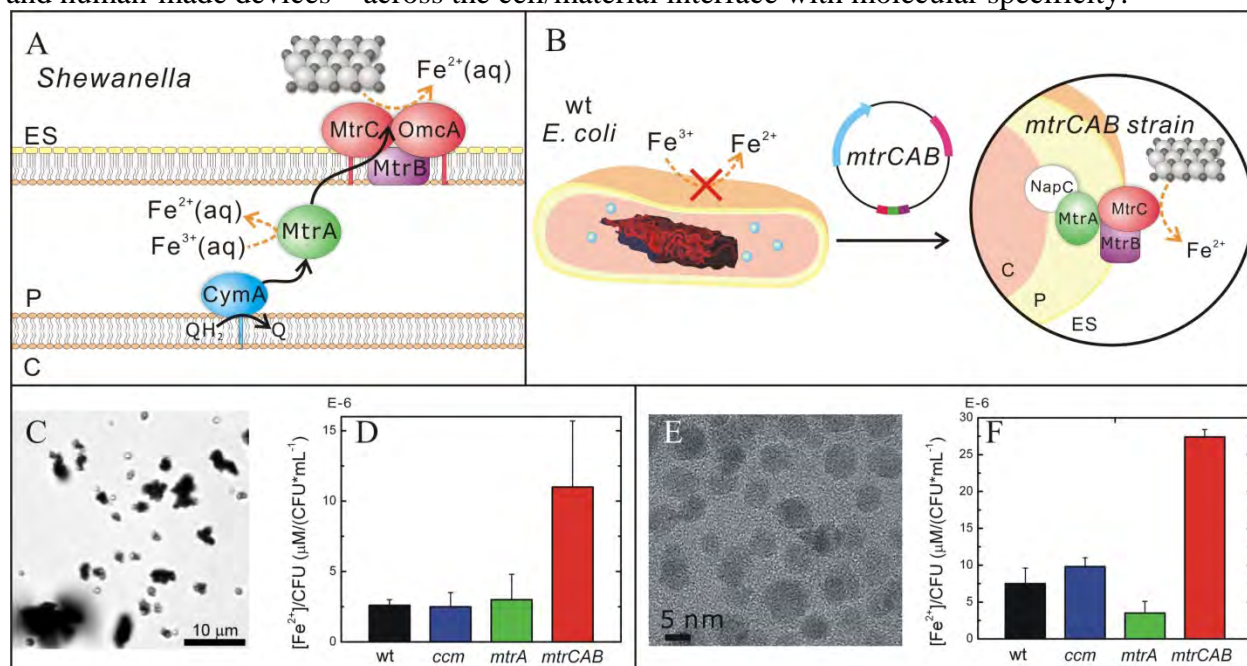


Figure 1. Genetically engineering cells to transfer electrons to inorganic materials. A) Schematic of the extracellular electron transfer pathway in *S. oneidensis* MR-1. Addition of a genetic cassette *mtrCAB* to *E. coli* (B) enables these cells to reduce micron-sized Fe<sub>2</sub>O<sub>3</sub> (C, D) and Fe<sub>2</sub>O<sub>3</sub> nanoparticles (E,F).

### Significant achievements: 2008-2010

Our central objective has been to explore a radically new, biologically-focused approach to create electronic connections between living/non-living systems: to use synthetic biology to introduce a new electron transfer pathway which would route electrons along a well-defined path from the cell interior to an extracellular inorganic material. Naturally occurring bacteria have evolved mechanisms for direct charge transfer to inorganic minerals, enabling them to use solid metal oxides as terminal electron acceptors during anaerobic respiration. The electron transfer pathway of *Shewanella oneidensis* MR-1, one of the best understood pathways, is comprised of

c-type cytochromes that shuttle electrons from cytoplasmic and inner membrane oxidizing enzymes to extracellular metal oxides via a series of intermolecular electron (Fig 1A). We selected the *mtrCAB* genes as a potentially minimal set required to create a synthetic electron conduit and chose to test this approach in the genetically tractable model organism *Escherichia coli*. Indeed, we find that *E. coli* cells expressing the MtrCAB proteins can now reduce both aqueous metal ions and solid metal oxides (Fig 1 C,D) at rates that are ~8x and 4x faster than the unmodified cells. We also find that metal oxide reduction is more efficient when the extracellular electron acceptor has nanoscale dimensions (Fig 1E,F) as would be expected for electron transfer between outer membrane and the solid surfaces. This work provides the first example of a predetermined, molecularly-defined route for electronic communication between living cells to inorganic materials<sup>1</sup>. More broadly, because this is an effective method to transduce electrical signals from cells to inorganic materials, we anticipate that our approach will find wide utility in bioenergy, biosensing and programmed cell behavior applications.

#### Science objectives for 2010-2011

In the coming year, our efforts will focus on expanding the extent and efficiency of electron flow across the living cell/material interface. With Profs. Francis, Bertozzi, and Mathies, we will use DNA-based adhesion to attach our engineered *E. coli* to an electrode surface and use that electrode to harness current from the living cell. Second, we will work with Prof. Groves to improve electron transfer by improving the design of our synthetic gene. If successful, this work will establish the first ever self-replicating, self-healing electronic connection between cells and technological devices and begin to make this process efficient enough to enable real-world technologies.

#### Reference to work supported by this project 2008-2010

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*Session XI*  
*Photosynthetic Systems*  
*Program Guest Lecture*



## Photosynthetic Phenomics

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### Overall Research Goals

This project aims to understand what limits photosynthesis, or why some plants and algae are more efficient than others. To accomplish this goal, we need to understand how the biophysical machinery of photosynthesis is integrated into the living organism, to provide the correct amount of energy, in the correct forms without self-destruction by photodamage under varying environmental conditions. Many of the key intermediates of photosynthesis are “ephemeral” or short-lived (e.g. excitons in antenna, the proton motive force) and cannot be isolated, so we need to measure them *in vivo*. This requires that we observe specific reactions and intermediates of photosynthesis operate *in vivo*, under natural conditions.

### Significant achievements 2007-2010

Our recent work (selected publications: 1-6, 7) established the importance of regulation of the proton and electron circuits of photosynthesis in setting the overall photosynthetic strategy, i.e. the sensitivity of regulatory networks that determines the balance between efficient light capture and avoidance of photodamage.

We have focused our recent work on the regulation mechanisms and roles of the ATP synthase and cyclic electron transfer around photosystem I (CEF1) in balancing photoprotection with efficiency as well as the output ratio of ATP/NADPH, to meet downstream needs. For example, we used a combination of novel genetics screening, *in vivo* spectroscopy and biochemistry to isolate a new class of mutants, called *hcef* for high CEF1, which shows dramatically higher CEF1. Characterization of these mutants shows that 1) elevated CEF1 flows through NADH:plastoquinone oxidoreductase (NDH) complex rather than other proposed pathways; 2) CEF1 is highly inducible and can achieve substantial rates; 3) CEF1 appears to be critical for maintaining ATP levels under stress; 4) the NDH complex likely pumps protons, like its homolog in the mitochondrion, Complex I. Most of our *hcef* mutants showed highly elevated H<sub>2</sub>O<sub>2</sub> production, and that infiltration or induction of H<sub>2</sub>O<sub>2</sub> in the chloroplast also induced CEF1, suggesting that the trigger for activating CEF1 is this reactive oxygen species. Intriguingly, we characterized a new mutant, *hcef3*, which appears to be in a new chloroplast H<sub>2</sub>O<sub>2</sub>-signal cascade.

We have also developed a novel PhotoBioreactor/Sensor Array (PBSA) for photosynthetic phenomics of algae under strains and growth conditions. This system will allow us to screen photosynthetic phenotypes using *in vivo* spectroscopy or destructive assays in large numbers of algal strains or mutants under a large number of conditions that mimic those algal cultures experience in raceways or bioreactors. We are currently using the PBSA to screen for

### Science objectives for 2010-2011:

My new position at MSU will allow us to establish a “photosynthetic phenomics” center, with state of the art facilities for high throughput measurements of key photosynthetic reactions *in vivo* and *in vitro*, in both plants and algae. We will then apply these facilities, in combination with genetics, biochemistry, computational biology and ‘omics to understand the connections between the light reactions and the biochemical and physiological systems it powers.

Our specific goals for 2010-2011 focus on establishing demonstrating and utilizing photosynthetic phenomics facilities:

- 1) Develop a system for advanced, high-throughput screening of key photosynthetic properties of plants under controlled environmental conditions, using a) video fluorescence (lifetime and yield) imaging, to probe quantum efficiency, antenna regulation and photodamage; b) absorbance changes using an automated, sensitive kinetic spectrophotometer, to probe

electron and proton transfer reactions, the xanthophyll cycle, the cytochrome *b<sub>6</sub>f* complex, plastocyanin, photosystem I turnover, efficiency and photodamage, etc.; and c) thermal imaging, to assess stomatal regulation.

2) Further develop our PBSA system for screening algal photosynthesis, allowing for continuous measurements of growth rates, CO<sub>2</sub> uptake, O<sub>2</sub> evolution, chlorophyll fluorescence (antenna efficiency, photosystem II activity), proton pumping and ATP synthase activity, intermediate electron transfer, and sophisticated control of light, temperature and CO<sub>2</sub> levels.

3) Demonstrate these systems by selecting for novel mutants and strains with complex photosynthetic phenotypes, in particular those with altered co-regulation of the light and dark reactions. We are particularly interested in mutants regulation of CEF1, ATP synthases, partitioning of the proton motive force into  $\Delta\psi$  and  $\Delta\text{pH}$ , and dependency of cytochrome *b<sub>6</sub>f* complex on lumen pH. We have devised a strategy that should select for these types of mutants. We will also use look for suppressors of our existing high CEF1 mutants, potentially allowing us to test hypotheses generated in our previous work.

4) Make the instrumentation and facility available to the plant biology community to address fundamental questions regarding the biophysics, biochemistry, genetics and regulation of photosynthesis.

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