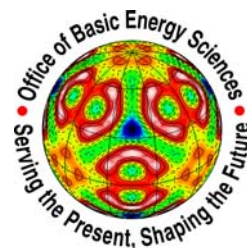


2008 Physical Biosciences Research Meeting *Program and Abstracts*

O'Callaghan Annapolis Hotel, Annapolis, MD October 28-31, 2008



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



2008 Physical Biosciences Research Meeting

DOE Contractors Meeting Program and Abstracts

O'Callaghan Annapolis Hotel
Annapolis, MD
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Foreword

This booklet provides a record for the inaugural meeting of the contractors (PIs) funded by the U.S. Department of Energy's Physical Biosciences Program, which is part of the Chemical Sciences, Geosciences, and Biosciences Division of Basic Energy Sciences. Other PIs in the Biosciences program will meet next year in a similar forum, where the emphasis will be photosynthetic systems.

Our objective in bringing you all together here in Annapolis is to provide an environment that: 1) Encourages free exchange of information regarding your DOE-funded research; 2) facilitates new collaborations between research groups having complementary strengths; 3) allows opportunities for discussions with DOE Program Managers and staff; 4) is conducive to sharing the latest techniques and clever adaptations of existing approaches to better address critical questions in energy bioscience research; and, 5) provides exposure to related fields and BES User Facilities through guest speakers. Accordingly, ample "down time" is scheduled for informal discussions and interaction with your peers.

The agenda features two invited speakers. We are honored to have Dr. Roger Kornberg present the Keynote Address on the molecular basis of eukaryotic gene transcription, and equally honored to have Dr. Dean Myles tell you all about neutron beams and their application to the study of biology in a follow-up Plenary Session. In addition, there will be 23 oral presentations and 43 posters presented over the course of this meeting by you - the funded PIs in the program. Taken in their entirety, your submitted abstracts are indicative of the strength and breadth of this research program.

We thank all of you – along with your coPIs and other lab personnel – for your participation in this meeting and for your dedication in advancing DOE basic research. It is your productivity that makes a meeting like this possible. It is our hope that this meeting will further enhance your creativity and overall research efforts. We shall look forward to meeting with you again in two years.

We express appreciation to our distinguished invited speakers, Drs. Kornberg and Myles, for their participation in this inaugural meeting. We also thank Diane Marceau of the Chemical Sciences, Geosciences and Biosciences Division, along with Margaret Lyday and Deneise Terry of the Oak Ridge Institute for Science and Education for their important logistical and other contributions to this meeting.

Richard V. Greene
Lead, Photo- and Bio-Chemistry Team

Robert J. Stack,
Detailer, Physical Biosciences Program

Agenda

Agenda
2008 Physical Biosciences Research Meeting
O'Callaghan Annapolis Hotel, Annapolis, MD
October 28-31, 2008

Tuesday, October 28, 2008

3:00 – 5:30 p.m. Meeting Registration
5:30 – 6:30 Reception (No Host)
6:30 – 8:00 Dinner at O'Callaghans Hotel

Session 1: Welcome, Opening Remarks

8:00 – 9:00 Welcome, DOE Update, Briefing on DOE Contractors Meetings
Rich Greene, Team Lead, Photo- and Biochemistry Team (DOE/BES)

Wednesday, October 29, 2008

7:00 – 7:30 a.m. Continental Breakfast

Keynote Session

Robert Stack, Chair

7:30 – 7:40 a.m. Introduction of Keynote Speaker
Eric Rohlfig, Director, Chemical Sciences, Geosciences & Biosciences
Division (DOE/BES)
7:40 – 8:40 **The Molecular Basis of Eukaryotic Transcription**
Roger Kornberg, Stanford University
8:40 – 9:00 Break

Session 2: Tools for Energy Transduction Studies and Their Application

Dax Fu, Chair

9:00 – 9:30 Use of ¹³C-Metabolic Flux Analysis to Elucidate Routes of Bio-Hydrogen
Production by the Bacterium *Rhodospseudomonas palustris*
Caroline Harwood, University of Washington
9:30 – 10:00 Measurement and Optimization of Enzyme Redox Properties using Single-
Molecule Imaging, Controlled Electrochemical Potential, and Nanoscale
Confinement
Eric Ackerman, Pacific Northwest National Laboratory
10:00 – 10:30 Break

Session 3: Plant Biochemistry - I

Judy Callis, Chair

10:30 – 11:00 a.m. Molecular Dissection of the Arabidopsis Holo-26S Proteasome
Richard Vierstra, University of Wisconsin
11:00 – 11:30 Trafficking to the Plant Storage Vacuoles in Plants
Natasha Raikhel, University of California, Riverside
11:30 – 12:00 Modification of Plant Lipids
John Shanklin, Brookhaven National Laboratory
12:00 – 12:30 Structural Analysis of Bacterial Virulence Factors to Understand Plant
Cellular Function
Jeff Dangl, University of North Carolina
12:30 – 1:30 Working Lunch
1:30 – 1:45 Put up posters for Poster Session - I

Session 4: Microbial Biochemistry - I

Judy Wall, Chair

- 1:45 – 2:15 Methyltransferase Reactions in Methanogenic Archaea
Joseph A. Krzycki, Ohio State University
- 2:15 – 2:45 Osmoregulation in Methanogens
Mary F. Roberts, Boston College
- 2:45 – 3:15 Selenocysteine Biosynthesis and Hydrogenase Engineering
Dieter Söll, Yale University
- 3:15 – 6:00 Interaction Time
- 6:00 – 7:30 Dinner at O'Callaghans
- 7:30 – 10:00 Poster Session - I (No-Host)

Thursday, October 30, 2008

- 7:00 – 7:30 a.m. Continental Breakfast

Plenary Session

- 7:30 – 7:40 Introduction of Plenary Speaker
Robert Stack, Physical Biosciences Program (DOE/BES; Detailee)
- 7:40 – 8:40 **Ask Not What You Can Do for Your Neutrons, Ask What Your Neutrons
Can Do For You**
Dean Myles, Oak Ridge National Laboratory
- 8:40 – 9:00 Break

Session 5: Microbial Biochemistry - II

Lee Krumholz, Chair

- 9:00 – 9:30 Engineering Interactive Biological/Material Interfaces with Nanotechnology
Jay Groves, Lawrence Berkeley National Laboratory
- 9:30 – 10:00 Syntrophy: Lifestyle of the Thermodynamically Challenged
Michael McInerney, University of Oklahoma
- 10:00 – 10:30 Break

Session 6: Plant Cell Walls

Ken Keegstra, Chair

- 10:30 – 11:00 a.m. The Plant Cell Wall – The First Line of Defence
Shauna Somerville, University of California, Berkeley
- 11:00 – 11:30 Structural Studies of Complex Carbohydrates of Plant Cell Walls
Alan Davill, Complex Carbohydrate Research Center, Univ. Georgia
- 11:30 – 12:00 p.m. Probing Lignin Primary Structure and Assembly: An Overdue Approach
Norman Lewis, University of Washington
- 12:00 – 12:30 Transcription Regulation of the Cellulase System of *Clostridium
thermocellum*
David Wu, University of Rochester
- 12:30 – 1:30 Working Lunch
- 1:30 – 1:45 Take down Session - I posters, Put up Session - II posters

Session 7: Ferredoxins, Hydrogenases, and Biological H₂ Production *Stephen Ragsdale, Chair*

- 1:45 – 2:15 Protein/Protein Interactions in the Ferredoxin/Thioredoxin Systems Studied by NMR Spectroscopy
David Knaff, Texas Tech University
- 2:15 – 2:45 Hyperthermophilic Multiprotein Complexes and Pathways for Energy Conservation and Catalysis
Michael W. W. Adams, University of Georgia
- 2:45 – 3:15 Photoproduction of Hydrogen: Biological Hydrogenase Gene Expression and Artificial Biomimetic Systems
Maria Ghirardi, National Renewable Energy Labs
- 3:15 – 6:00 Interaction Time
- 6:00 – 7:30 Dinner on Your Own (PIs will be provided with list of rec'd restaurants)
- 7:30 – 10:00 Poster Session - II (No-Host)

Friday, October 31, 2008

- 7:30 – 8:00 a.m. Continental Breakfast

Session 8: Plant Biochemistry - II *Jane Glazebrook, Chair*

- 8:00 – 8:30 Functions of HKT Transporters in Protecting Plant Leaves from Salinity Stress and in Sodium Uptake into Roots
Julian Schroeder, University of California, San Diego
- 8:30 – 9:00 Interdisciplinary Research and Training Program in the Plant Sciences
Michael Thomashow, DOE-MSU Plant Research Laboratory
- 9:00 – 9:30 The Structure and Regulation of the Acetyl-CoA Metabolic Network: the Fundamental Underpinning of High-energy Biorenewable Metabolites
Basil Nikolau, Ames Laboratory, Iowa State University
- 9:30 – 10:00 Break
- 10:00 – 10:30 Asparagine Synthetase Gene Regulatory Networks and Plant Nitrogen Metabolism
Gloria Coruzzi, New York University
- 10:30 – 11:00 From the Soil to the Seed: Metal Transport in *Arabidopsis*
Mary Lou Guerinot, Dartmouth College
- 11:00 – 11:30 Break

Session 9: Close Out Session –Rich Greene and Bob Stack, DOE/BES

- 11:30 – 12:30 Program Summary
- 12:30 – 1:30 p.m. Working Lunch
- 1:30 – 2:30 Open Discussion and take down posters

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Session 2
Tools for Energy
Transduction Studies and
Their Application

(no abstracts are available for Session 1)

Use of ^{13}C -metabolic flux analysis to elucidate routes of bio-hydrogen production by the bacterium *Rhodospseudomonas palustris*

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Overall research goals: To use ^{13}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 hydrogen production.

Significant achievements in 2007-2008: We used directed mutagenesis to generate stable mutants that produce pure hydrogen under all growth conditions. The mutants are derepressed for nitrogenase synthesis and activity due to mutations in the regulatory protein NifA. We call these NifA* mutants. Unlike the wild type, these constitutive hydrogen-producing mutants produce large amounts of hydrogen when grown anaerobically in light with acetate as a carbon source/electron donor and with ammonia as a source of nitrogen. Thus we are able to compare metabolic fluxes between a strain that is producing hydrogen (the hydrogen-producing mutant) and a strain that is not producing hydrogen (the wild type) when grown in the same growth medium.

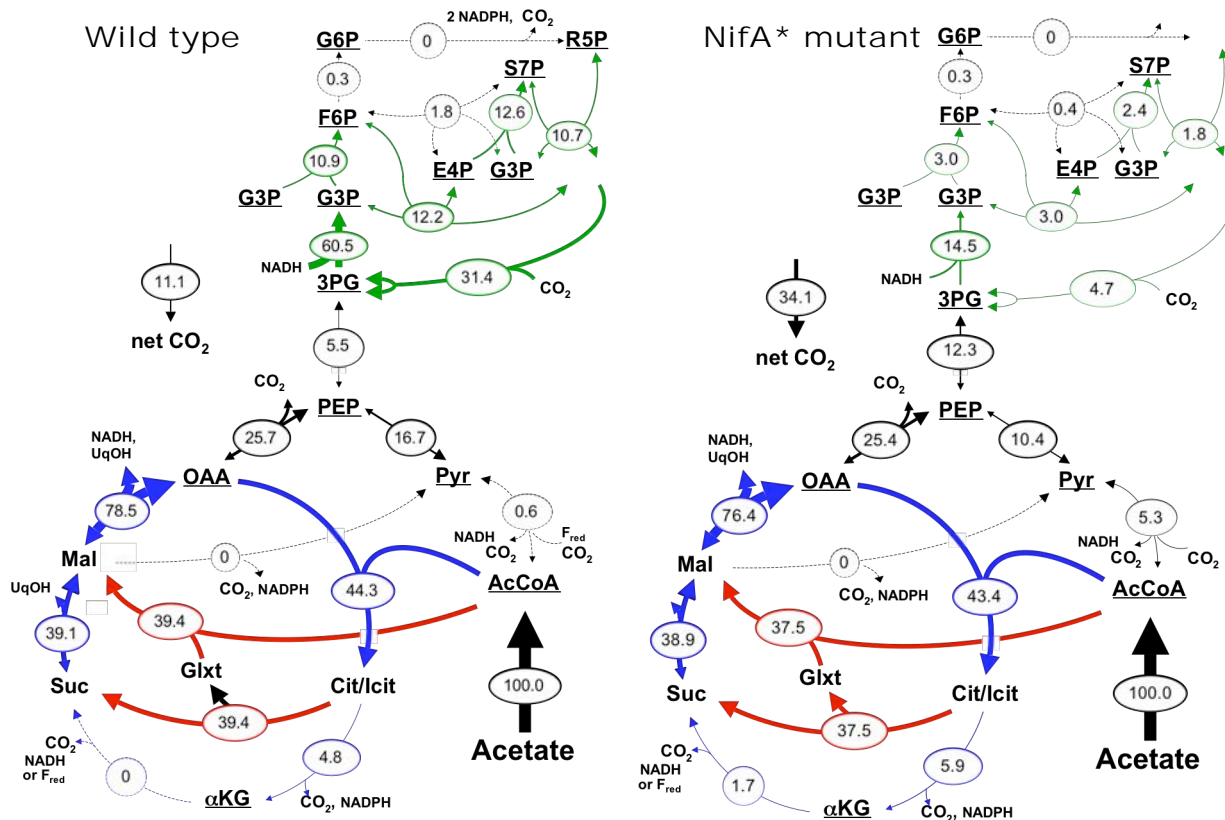


Figure 1. Metabolic flux maps for growing wild-type cells (non- H_2 -producing) and NifA* mutant cells (H_2 -producing). Arrowheads indicate the direction of the net flux. Arrow thickness is proportional to net flux magnitude. Underlined metabolites indicate that there are fluxes for biosynthesis that are not shown.

Our flux maps illustrate that wild-type *R. palustris* makes heavy use of metabolically generated carbon dioxide as an inorganic electron acceptor during photoheterotrophic growth on acetate when ammonia is the nitrogen source. By contrast the NifA* mutant does not make significant use of carbon dioxide as an electron acceptor and instead uses protons as an electron acceptor to form hydrogen gas through the agency of nitrogenase (Fig. 1). The two strains differed markedly in the amount of carbon that fluxed through the carbon dioxide-fixing Calvin cycle. The wild type, which was not producing hydrogen in these experiments, used ribulose biphosphate carboxylase (RubisCo) [Fig 1. $R5P + CO_2 \rightarrow 2 \times 3PG$] to capture about 73% of the carbon dioxide that it generated in central metabolic reactions involved in the oxidation of acetate. This amount of carbon dioxide fixation requires a large amount of reductant, as indicated by the large gluconeogenic flux from 3-phosphoglycerate (3PG) to glyceraldehyde-3-phosphate (G3P). Unlike the wild type, the NifA* mutant, which was producing hydrogen in these experiments, diverted electrons to nitrogenase to reduce protons to hydrogen. Instead of meeting this reductant demand by increasing fluxes through reactions that generate reductant (NADH, NADPH, F_{red} , UqOH), we found that the mutant decreased the flux through RubisCo and the reductant-utilizing Calvin cycle.

Our data indicate that the NifA* hydrogen-producing strain uses only 13% of the possible electrons that can be derived from acetate for hydrogen production during growth. Our flux maps suggests ways in which ways in which hydrogen production might be improved.

Science objectives for 2008-2009:

- We will further investigate our hypothesis that wild-type cells flux reductant through the Calvin cycle in proportion to the reduction state of the organic compound that serves as growth substrate. We expect cells grown on succinate (more reduced than acetate) to have less of a Calvin cycle flux and on fumarate (more reduced than acetate) to have a greater Calvin cycle flux.
- Will mutate key Calvin cycle enzymes in the NifA* mutant and test for increased hydrogen production.
- Will devise engineering strategies to decrease the glyoxylate shunt flux (Fig 1. $Icit \rightarrow Suc + Glxt$; $Glxt + AcCoA \rightarrow Mal$) and increase flux through reactions such as α -ketoglutarate dehydrogenase (Fig 1. $\alpha KG \rightarrow Suc$) to increase hydrogen production.

References to work supported by this project 2007-2008

1. Schaefer, A. L., E. P. Greenberg, C. M. Oliver, Y. Oda, J. J. Huang, G. Bittan-Banin, C. M. Peres, S. Schmidt, K. Juhaszova, J.R. Sufrin and **C. S. Harwood**. 2008. A new class of homoserine lactone quorum-sensing signals. *Nature* **454**:595-599.
2. Pan, C., Y. Oda, P.K. Lankford, B. Zhang, N.F. Samatova, D. A. Pelletier, **C. S. Harwood**, R. L. Hettich. 2008. Characterization of anaerobic catabolism of p-coumarate in *Rhodopseudomonas palustris* by integrating transcriptomics and quantitative proteomics. *Mol Cell Proteomics*.**7**:938-948.
3. **Harwood, C.S.** 2008. Nitrogenase-catalyzed hydrogen production by purple nonsulfur photosynthetic bacteria. p. 259-271. IN: A. Demain, C.S. Harwood and J. Wall (eds.) *Bioenergy*. American Society for Microbiology Press.

Measurement and Optimization of Enzyme Redox Properties using Single-Molecule Imaging, Controlled Electrochemical Potential, and Nanoscale Confinement

Eric J. Ackerman, Principal Investigator

Chenghong Lei, Dehong Hu, Charles F. Windisch, Jr. Co-Principal Investigators

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Overall research goals: The research objectives are to study electron transfer dynamics of enzymatic redox reactions by: (1) developing an instrument capable of single-molecule fluorescence spectroelectrochemistry; (2) mutagenesis, where necessary, of appropriate enzyme targets; (3) nanoscale confinement. Controlling the electrical and spatial environments surrounding redox enzymes should provide a path towards greater control and enhancement of enzymatic reactions. Extending study of these reactions to the level of single molecules should reveal novel insights into reaction trajectories and dynamics, perhaps eventually yielding higher reaction rates with enhanced stabilities.

Significant achievements in 2006-2008: Initial investigations utilized the quasi-reversible oxidation/reduction reaction of the dye cresyl violet under controlled cycles of electrical potential; *i.e.* cyclic voltammetry. The reduced non-fluorescent form of cresyl violet became fluorescent when oxidized and this reversible reaction yielded consistent results for both ensemble and single-molecule measurements. Experiments were done with both freely diffusing dye molecules and dye adsorbed to clays; *i.e.* freely diffusing and tethered single molecules.

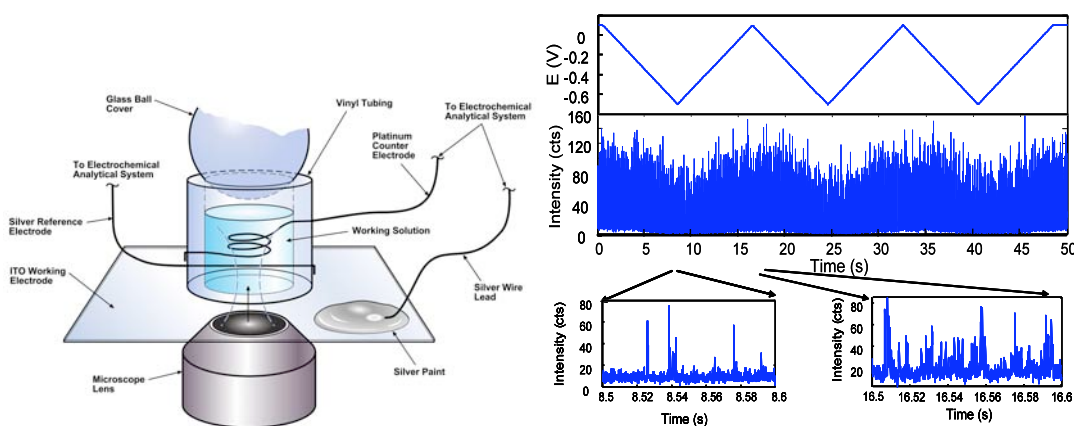


Figure 1. Left Panel: schematic of the electrochemical cell coupled with scanning confocal fluorescence microscopy; Right Panel: single-molecule fluorescence spectroelectrochemistry of cresyl violet where upper panel is potential vs. time plot of the cyclic voltammetry scan; middle panel is the single molecule fluorescence of cresyl violet with the number of fluorescence bursts per 0.1 sec vs. time calculated from the fluorescence time trajectory.; bottom panel is two 0.1-second segments of the fluorescence time trajectory with a bin time of 0.1 ms.

Science objectives for 2008-2009:

- The project must be transitioned from its initial proof-of-principal demonstrations working with isolated dyes to working with redox enzymes. It is not yet clear which enzyme system will be most suitable. A cyclic-voltammetry controlled enzyme:substrate combination must generate a sufficiently strong signal for single-molecule work. A mediator dye:enzyme:substrate system

and/or a recombinantly-modified enzyme with a covalent dye attached constitute alternative systems .

- Previous work demonstrates a remarkable effect of electrical potential on enzyme folding during translation, consistent with the importance of electrical potentials in cells. This effect may be explored with both ensemble and single-molecule experiments.
- Our previously published work demonstrate the remarkable ability of functionalized nanopores to enhance enzyme specific activity and stability. Studying the effects of confinement on enzyme reaction dynamics at the single-molecule level will help to address whether all molecules are enhanced or if a subset becomes super-activated. The challenge here is to find a nanomaterial providing the required molecular crowding effect without quenching the fluorescent signal.

•

References to work supported by this project 2006-2008(style=Stand alone text heading):

1. C. Lei, T. A. Soares, Y. Shin, J. Liu, E. J. Ackerman, "Enzyme Specific Activity in Functionalized Nanoporous Supports." **19**: 125102 (1-9) (2008).
2. C. Lei, D. Hu, and E. J. Ackerman, "Single-Molecule Fluorescence Spectroelectrochemistry of Cresyl Violet," Chem. Comm. In press, will be designated a 'Hot Paper' and may be on the cover (2008).
3. C. Lei, D. Hu, and E. J. Ackerman, "Nanoclay-based Single-Molecule Fluorescence Spectroelectrochemistry. Angewandte Chemie, submitted (2008)

Session 3
Plant Biochemistry - I

MOLECULAR DISSECTION OF THE *ARABIDOPSIS* HOLO-26S PROTEASOME

Principal Investigator: **DR. RICHARD D. VIERSTRA**

Research Personnel: **Adam J. Book, Lisa M. Farmer, James Holmes, Kwang-Hee Lee and Atsushi Minami**

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Overall Research Goals: The 26S proteasome is an ATP-dependent protease complex responsible for degrading many important cell regulators in plants, especially those conjugated with multiple ubiquitins (Ubs). It is composed of two subparticles, a 20S core protease (CP) that encloses the protease active sites and a 19S regulatory particle (RP) that binds to both ends of the CP and recruits appropriate substrates. Whereas the structure and proteolytic activities of the CP are understood at the atomic level, little is known about the structure of the RP and the functions of many of its principal subunits. It is also becoming clear that the currently defined RP actually represents the nucleus of an even more elaborate and dynamic complex that provides multiple routes for substrate recognition. These routes may be defined by specific isoforms of individual RP subunits, the association of the RP with a host of co-factors, the reversible binding of target shuttle proteins, and, in the most extreme cases, by the complete replacement of the RP with alternative subparticles. Genomic analyses suggest that plants exploit this heterogeneity to generate a wide array of proteasome types, each with distinct compositions and unique functions/specificities. The goal of this project is to more fully define the 26S proteasome function and diversity in plants by a complete analysis of the particle from *Arabidopsis thaliana*.

Significant Achievements in 2006-2008: From the analysis of 26S proteasomes purified from *Arabidopsis* by conventional methods, we identified by mass spectrometry most of the central RP and CP subunits. To determine the function(s) of the twelve RP subunits that together likely control target specificity, we developed a comprehensive library of T-DNA insertion mutants affecting the expression of each. In-depth analysis of mutants for several subunits revealed strikingly different phenotypes, suggesting distinct functions/targets for these subunits in the holo-proteasome. For example, homozygous null mutants in both *RPT2a* and *RPT2b* are female gametophyte lethal. Single *rpt2a* and *rpt2b* mutants assemble aberrant proteasome complexes in which the CP and RP dissociate more easily than wild-type complexes *in vitro*. While *rpt2b* mutants grow normally, *rpt2a* mutants display a host of phenotypic defects, including slower root growth, fasciation, increased trichome branching, enhanced sensitivity to DNA damaging agents and amino acid analogs, delayed flowering, and reduced seed production. Both *RPT2a* and *RPT2b* can rescue the *rpt2a* defects, indicating that the pair are functionally redundant. In contrast, homozygous null mutants in both *RPN5a* and *RPN5b* are both female and male gametophytic lethal. *rpn5b* mutants are normal while *rpn5a* mutants display a de-etiolated phenotype in the dark, are severely dwarfed and have lanceolate leaves as green seedlings, and are nearly sterile. Strong defects related to the expression of a host of meristematic genes were also evident. The *rpn5a* mutants could be rescued by expression of either *RPN5a* or *RPN5b*, indicating functional redundancy. Finally, while RPN10 appears to be the major Ub receptor in the 26S complex, recent data from yeast have also implicated RPN13. However, null mutants in the single *Arabidopsis* gene encoding RPN13 are normal, suggesting that this Ub receptor has a minor role in plants.

Studies with the yeast 26S proteasome indicated that the holo-complex contains a host of additional factors that help with complex assembly and substrate specificity. Most, if not all, of these factors are absent in our conventionally purified preparations even though the corresponding genes could be detected in the *Arabidopsis* genome, suggesting that they dissociate during purification. To circumvent this problem, we developed an affinity method to more rapidly isolate the 26S proteasome from *Arabidopsis* seedlings. The strategy involved complementation of a mutant in the single essential gene encoding the $\alpha 6$ subunit of the CP (PAG1) with a Flag-tagged version. By using anti-Flag antibody beads, the 26S proteasome can be affinity purified from homozygous *pag1-1:PAG1-Flag* lines in a single step directly from crude soluble extracts (Fig. 1). Both SDS-PAGE and

mass spectrometric analyses revealed that these preparations are more intact and include a host of other proteins. Included are PA200, a 200-kDa protein that may substitute for the RP to broaden the substrate specificity of the complex, Hsp70 that could assist in substrate unfolding, and several RAD23 isoforms that may shuttle ubiquitinated proteins to the 26S proteasome. In addition, we detected several post-translational modifications that may be important, including phosphorylation, acetylation, and N-terminal processing of specific subunits. We also complemented the *pag1-1* plants with a *PAG1-GFP* construction that now allows real-time imaging of the *Arabidopsis* 26S proteasome by confocal fluorescence microscopy. The particle was detected in the cytoplasm and was highly enriched in the periphery of the nucleus with small punctate structures in the middle, suggesting that the localization of the 26S proteasome can be dynamic.

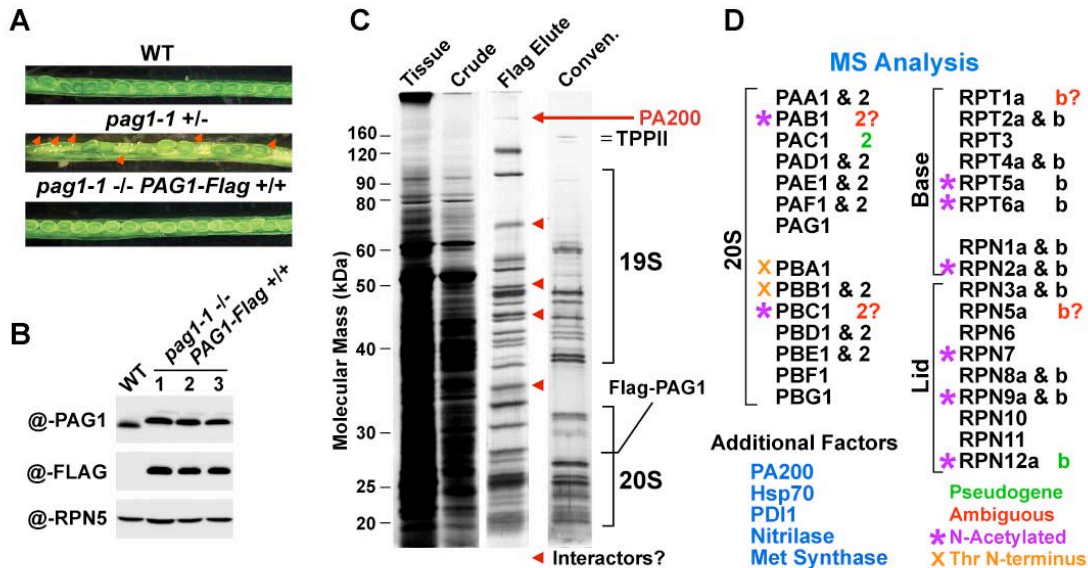


Figure 1. Flag affinity purification of the *Arabidopsis* 26S proteasome. **(A)** Complementation of an embryonic lethal *pag1-1* mutant with Flag-PAG1. Red arrowheads locate aborted seeds. **(B)** Expression of Flag-PAG1 in the 3 independent *pag1-1* lines transformed with *35S:Flag-PAG1*. Crude extracts were subjected to SDS-PAGE and immunoblot analysis with anti-PAG1, anti-Flag and anti-RPN5 antibodies. **(C)** Affinity purification of 26S proteasome with anti-Flag beads. Conven. = proteasome purified conventionally. Arrowheads identify potential accessory proteins. Arrow locates PA200. **(D)** 26S proteasome subunits and accessory proteins in the Flag preparations identified by mass spectrometry (MS). Ambiguous indicates situations where the identity of one isoform is uncertain.

Science Objectives for 2008-2009: Work will focus on:

1. The continuing genetic description of individual 26S proteasome subunits to deduce their functions within the particle.
2. The genetic analysis of several accessory factors to the 26S proteasome identified by our mass spectrometric analysis of affinity purified preparations.
3. Attempt to affinity purify 26S proteasomes from various *Arabidopsis* tissues exposed to a number of environmental challenges to see if the composition of the 26S proteasome changes dynamically in response to developmental cues and stress.

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ABSTRACT
DOE Physical Biosciences Contractors Meeting, October 28-30, 2008

Title: Trafficking to the Plant Storage Vacuoles in Plants

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Overall Research Goals:

Our goal is to examine endomembrane trafficking to the plant storage vacuoles in different tissues, to understand the interaction of the plant endomembrane system with various aspects of plant development and signal transduction, and to test our hypothesis that plant storage compartments in vegetative tissues may serve as reservoirs for developmentally related molecules, and a combination of endogenous and environmental signals may regulate the trafficking, sequestration and release of these factors.

Significant Achievements in 2006-2008:

Higher plants are unique in storing proteins in specialized protein storage vacuoles (PSVs), which are contained in seeds and vegetative tissues. Although plants utilize proteins that are stored in PSVs during germination, before photosynthesis is fully functional, the functions of PSVs in adult vegetative tissues are not understood. Transport pathways to the PSVs appear to be distinct from those to lytic vacuoles. Lytic vacuoles are analogous to yeast and mammalian lysosomes, such that some transport pathways to this compartment have been delineated based in part on homologies. It is not clear whether transport to the PSV has any analogy to pathways in yeast or mammals, nor is the ultrastructure of the PSV in Arabidopsis vegetative tissue known. Therefore, novel approaches are required to identify the components of this pathway.

Interfering with transport to PSVs has been shown to result in secretion of cargo. Therefore, secretion of a suitable marker could be used as an assay to identify mutants in this pathway. CLV3, a negative regulator of shoot stem cell proliferation, is an extracellular ligand that is rendered inactive when targeted to vacuoles (Rojo et al., 2002). We devised an assay in which trafficking mutants secrete engineered CLV3 that is targeted to the vacuole. Mutants with defects in vacuolar targeting displayed reduced meristems, a phenotype easily detected by visual inspection of plants.

First, we provided proof-of-principle for the efficacy of our Vac2 assay line. In this scheme, plants expressing Vac2, a fusion of CLV3 to the vacuolar sorting signal derived from barley lectin, a plant storage protein, were crossed to the known trafficking mutants *vti11* and *vti12*. We determined that trafficking of Vac2 required the SNARE VTI12 but not its close homologue, the conditionally redundant VTI11 protein. Furthermore, the *vti12* mutant is specifically altered in transport of storage proteins, whereas *vti11* is affected in transport of a lytic vacuole marker. These results demonstrate the specialization of pathways to plant vacuoles and validate the Vac2 secretion assay as a robust method to isolate genes that mediate trafficking to the PSV. Using the Vac2 assay line we developed a novel genetic screening approach where we can identify genes whose products comprise the trafficking machinery to the plant PSV. One of the mutants that disrupted trafficking to the PSV was identified as the *TERMINAL FLOWER 1* (*TFL1*) gene, a shoot meristem identity gene as a component of the PSV trafficking machinery. The *tfl1-19/mtv5* (for *m*odified *t*ransport to the *y*acuole) mutant is specifically defective in transport of proteins targeted to the PSV. TFL1 localizes in part to endomembrane compartments and co-localizes with the putative d-subunit of the AP-3 adapter complex. Our results

demonstrate an unexpected and novel developmental role for the protein storage vacuole in vegetative tissues.

The vacuole occupies most of the volume of plant cells; thus the fluorescent tonoplast reporter TIP-GFP clearly delineates cell shape in multiple tissue types. This permits the rapid identification of mutants with altered vacuoles and cellular development. Using this strategy, we identified the *cell shape phenotype 1 (csp-1)* mutant in Arabidopsis. Beyond the absence of lobes in pavement cells, *csp-1* featured dramatic phenotypes such as reduced trichome branching, altered leaf serrations, altered stem branching, and increased stomatal density. These characteristics result from a point mutation in the *AtTPS6* gene, which encodes a polypeptide with a conserved amino-terminal domain thought to catalyze trehalose-6-phosphate synthesis, and a carboxy-terminal phosphatase domain that catalyzes the conversion of trehalose-6-phosphate to trehalose in a two-step enzymatic process. Expression of *AtTPS6* in the corresponding yeast deletion mutants *tps1* (encoding a synthase domain) and *tps2* (synthase and phosphatase domains) indicates that *AtTPS6* is a bi-functional enzyme. *AtTPS6* fully complemented defects in *csp-1*. Mutations in Class I *TPS* genes (*AtTPS1-4*) have suggested a role in the regulation of starch storage, resistance to water deficits, and inflorescence architecture. Class II genes (*ATPS5-ATPS11*) encode multi-functional enzymes that have synthase and phosphatase activities. We show that the Class II family member *AtATPS6* regulates not only plant architecture but also the shape of epidermal pavement cells and the branching of trichomes. Thus, beyond a role in plant development, we demonstrate that the Class II gene *AtTPS6* is important for controlling cellular morphogenesis, a role not previously assigned to Class II *TPS* genes.

Science Objectives for 2008-2009:

- Continue analysis of the TFL1-mediated trafficking pathway to determine how PSV-specific endomembrane trafficking impacts plant developmental processes.
- Clone and characterize two mutants obtained as a result of EMS mutagenesis and one of the Vac2 T-DNA collection.

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Modification of Plant Lipids

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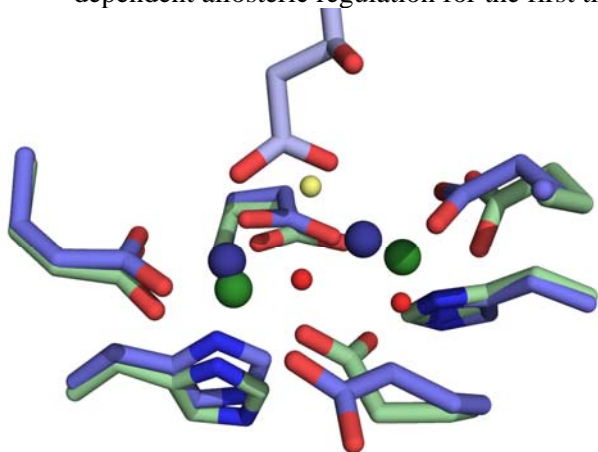
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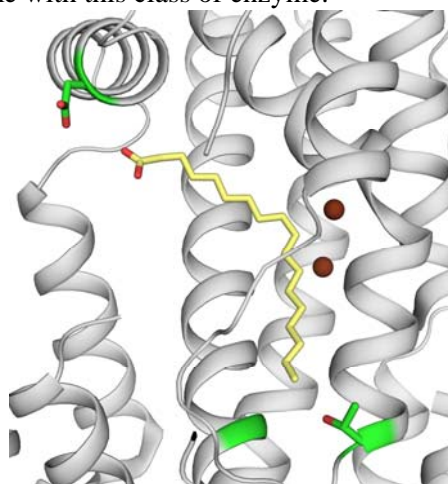
Overall research goals: Lipids and oils are a vital class of compounds. In eukaryotes, saturated fatty acids are modified post-synthetically by the introduction of *cis* double bonds or by the introduction of a variety of functional groups. The focus of this research is to understand the molecular basis 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 2006-2008:

- Identified an 18:0-acyl carrier protein (ACP) desaturase variant that synthesizes the allylic alcohol 9-OH 18:1 Δ^{10} and used labelled substrates to determine its reaction mechanism.
- Changed a desaturase enzyme's activity to mimic its hypothesized oxidase ancestor by a single mutation, T199D, i.e., by removing the proton donor from the active site.
- Solved the ivy 16:0- Δ^4 -desaturase crystal structure to 1.95 Å.
- Converted the Composition of a Arabidopsis i.e., a Temperate Seed Oil to that of a Palm-Like Tropical Oil by modulating levels of the β -Ketoacyl-ACP Synthase II.
- Published the first desaturase crystal structure with the diiron site in the oxidized diiron (FeIII-FeIII) state revealing the largest carboxylate shift seen for a diiron enzyme
- Demonstrated the introduction of a second double bond by the ivy desaturase to yield 18:2 $\Delta^{4,9}$ diene product.
- Determinants of specificity of plant thioesterases were determined with the use of our bioinformatics program the Conserved Property Difference Locator (CPDL).
- Established the specificity of 5 desaturase isoforms from Arabidopsis and showed product-dependent allosteric regulation for the first time with this class of enzyme.



Superposition of the diiron site ligands from the castor reduced crystal structure, green, and the ivy oxidized crystal structure in blue. The dramatic carboxylate shift is clearly seen at the bottom of the image.



Castor desaturase showing the position of a modeled substrate, yellow, and the three residues mutated to produce the allylic alcohol forming enzyme, green.

Science objectives for 2008-2009:

- Use 18:0-acyl carrier protein (ACP) desaturase variant that synthesizes the allylic alcohol 9-OH 18:1 Δ^{10} as a model system to further dissect the mechanism of “unusual fatty acid” biosynthesis.
- Investigate the regulation of desaturases, by investigation of posttranslational modifications with the use of macromolecular mass spectrometry.
- 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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Structural analysis of bacterial virulence factors to understand plant cellular function

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Overall Research Goals

Plant pathogens use a variety of virulence factors to suppress plant defense mechanisms. One important and large class of virulence factors from plant pathogenic bacteria are “type III effector proteins”. These are delivered into the plant cell, where they are addressed to localizations specific to their function. Type III effector proteins are often sequence convergent structural mimics of eukaryotic regulatory proteins. Hence, discerning the function of the large population of type III effectors (over 100 protein families, encoded by over 300 different genes in ~20 strains of *Pseudomonas syringae* alone) is not possible using merely computational means.

Our main hypothesis, supported by data from several labs, is that this collection of type III effector proteins will define a collection of plant targets that are themselves critical regulators of defense and response to infection.

Our main approach in our DOE funded project is to use structural biology to define likely functions for a collection of type III effector proteins, starting with those for which we know at least some of the plant targets, and proceeding outward to a wider solution of the structures to predict both type III effector function and to infer plant targets based on the structure. In essence, we view type III effector proteins as independently evolved probes of plant cellular biology.

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 2006-2008

We focused on three areas, two of relate to the targeting of a small Arabidopsis protein called RIN4 by three sequence unrelated type III effectors: AvrB, AvrRpm1, and AvrRpt2. The first two lead to RIN4 hyperphosphorylation, while the third is a protease that cleaves RIN4. We presume that these events contribute to bacterial virulence, though genetic evidence suggests that each of these effectors have additional targets in the plant cell.

We determined the structure of AvrB complexed with an AvrB-binding fragment of RIN4 at 2.3 Å resolution. We also determined the structure of AvrB in complex with ADP bound in a binding pocket adjacent to the RIN4 binding domain. AvrB residues important for RIN4 interaction are required for full RPM1 activation. AvrB residues that contact ADP are also required for initiation of RPM1 function. Nucleotide-binding residues of AvrB are also required for its phosphorylation by an unknown Arabidopsis protein(s). We conclude that AvrB is activated inside the host cell by nucleotide binding and subsequent phosphorylation and, independently, interacts with RIN4 (Desveaux et al, 2006).

Subsequent to this work, we have mutagenized the relevant RIN4 sites that are contacted by AvrB. We made a series of transgenic Arabidopsis in a *rps2 rin4* mutant background and we will soon analyze these for their ability to function in response to infection with bacteria expressing *avrB*. We anticipate that we will thus identify the key residues on RIN4 that are targeted by AvrB (A. Wu, et al, unpublished).

Third, we also used molecular modeling to structurally model the HopX type III effector and we were able to show that it encodes a likely transglutaminase (Nimchuk et al., 2007). Despite many attempts, we were unable to identify a plant cellular target for HopX and have returned our focus to the three type III effectors that target RIN4.

Finally, we had previously shown that over-expression of AvrB causes a novel chlorotic phenotype on plant leaves of genotypes unable to “recognize” AvrB. We had hoped that this phenotype represented a novel surrogate for virulence function of AvrB but in fact when we cloned the gene responsible for the chlorotic phenotype, it turned out to encode a second, weakly functioning NB-LRR class disease resistance gene. While disappointing in one sense, this result is

important as it demonstrates that the levels of virulence protein expression inside the plant can have profound effects on the outcome of an interaction with the plant host. Thus, evolution will likely fine tune virulence function levels to provide what is needed to serve the pathogen at levels below those that might activate significant host defense responses (Eitas et al., 2008).

Science Objectives 2008-2009

(1) We have work on the structure of AvrRpm1. This protein was, for several years, difficult to express and purify in the requisite amounts needed for crystallization. We solved this problem. We will also use *in vitro* binding assays to map the domain of RIN4 required for AvrRpm1 binding. We used molecular modeling to make a reasonable guess as to the function of AvrRpm1; we think it encodes a poly-ADP-ribosylation protein. The relevant candidate catalytic residues of AvrRpm1 have been mutated and will be tested for both their virulence function and their ability to trigger RPM1-mediated HR.

(2) The AvrB binding site on RIN4 overlaps the AvrRpt2 cleavage site. There are ~15 other short Arabidopsis proteins that carry a version of this site, known as the NOI domain, all of which also carry a RIN4-like carboxy terminal acylation/prenylation site that suggests they will all be embedded in the plasma membrane. Their functions are unknown, but given that we have genetic evidence that RIN4 is not the only target of AvrB, AvrRpm1 and AvrRpt2 in the plant cell, we are analyzing the NOI domain-containing protein family. We demonstrated that at least two of these are targeted by AvrB (but, interestingly, not by AvrRpm1) and that mutation of them in Arabidopsis compromises RPM1-mediated response to bacteria carrying these AvrB. We will assess how these two NOI proteins function with, or independently of, RIN4 itself (A. Wu et al.)

(3) Scott Peck's group noted in a shotgun mass spectroscopy examination of plasma membrane phospho-proteins, that RIN4 has two S/T residues that are phosphorylated in the presence of flagellin peptide, a potent PAMP in the plant immune system. We have mutated these residues and built the appropriate transgenic lines to test whether they function in a suite of RIN4 functions: (a) RPM1 localization and function, (b) basal defense function via callose deposition and (c) FLS2 dependent basal defense.

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Session 4
Microbial Biochemistry - I

Methyltransferase reactions 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 UAG codon as pyrrolysine. We are currently examining the function of pyrrolysine in the methylamine 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.

Significant achievements in 2006-2008: We have focused on the dimethylamine (DMA) and trimethylamine (TMA) methyltransferases in this DOE project period, with the primary aim to uncover the role of the 22nd amino acid in methylamine methyltransferases. We have proposed that pyrrolysine is the primary catalytic residue that binds the methylamine in order to present an oriented methylammonium adduct to the supernucleophilic Co(I) of the cognate corrinoid protein. We have taken two major routes toward demonstrating a catalytic role for pyrrolysine in the methyltransferases. The first is inhibitor studies with nucleophiles that would react with the electrophilic imine bond of pyrrolysine, and which we postulate is required for pyrrolysine binding, the second is site-directed mutagenesis of pyrrolysine. We have also continued our work on the function of RamA, an activation protein for the methyltransferase proteins.

(1) Reduction of MtbB (DMA methyltransferase) reveals an essential role for pyrrolysine imine bond. Previously, we had shown that borodeuteride treatment of the MtbB (the DMA methyltransferase) inhibits CoM methylation by MtbB, MtbC (the DMA corrinoid protein), and MtbA (the CoM methylase). In this period we confirm that, with 90% of MtbB sequenced, pyrrolysine was the only residue modified. We further found that MtbB treated with borodeuteride lost the ability to methylate cob(I)alamin. We used this assay to perform an active-site titration of MtbB, and found that the ratio of deuterated pyrrolysyl-peptide to non-deuterated pyrrolysyl-peptide correlated inversely with the methylation activity. This is strong evidence that pyrrolysine is a requirement for activity of the DMA methyltransferase.

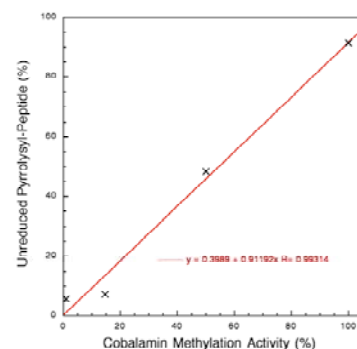


Fig. 1. Active site titration of MtbB with borodeuteride. Loss of the imine bond of pyrrolysine correlates with the loss of methylation activity.

(2) Site directed mutagenesis of MtbB and MttB. A major advance of this project period has been the development of a system for site-directed mutagenesis of the DMA and TMA methyltransferases in a recombinant methanogen host. We have cloned both methyltransferases from *M. barkeri* and expressed them in *Methanosarcina acetivorans*. We have

purified both methyltransferases and found they can comprise up to 1% of the total protein in *M. acetivorans*. We have also performed site-directed mutagenesis of each protein, and replaced the pyrrolysine residue with alanine. Our preliminary experiments have indicated that loss of pyrrolysine in both the DMA and TMA methyltransferase severely compromises CoM methylation.

(3) RamA mediates reduction of methylamine corrinoid proteins to the Co(I) state.

We have purified a not-previously described protein from *M. barkeri* that is named RamA. RamA is capable of activating the MMA, DMA and TMA methyltransferase reactions. RamA is a 60-kDa monomeric iron-sulfur protein. The *ramA* gene encodes a C-terminal ferredoxin domain, which is consistent with iron and acid-labile sulfide analysis, and is found in a cluster of genes encoding the genes necessary methylamine metabolism. We have now shown that purified RamA can reduce purified either MtmC and MtbC to the Co(I) state in an ATP-dependent reaction. RamA is part of a recognized group of proteins called COG3894, which is annotated as electron-transfer proteins of unknown function. We have surveyed the genomes and found the majority of COG3894 members are found near genes for corrinoid-dependent methyltransferases. RamA provides the first functional member of this COG, suggesting that we have identified a large family of corrinoid protein reductases. RamA will be useful in probing the function of pyrrolysine in the methylamine methyltransferases.

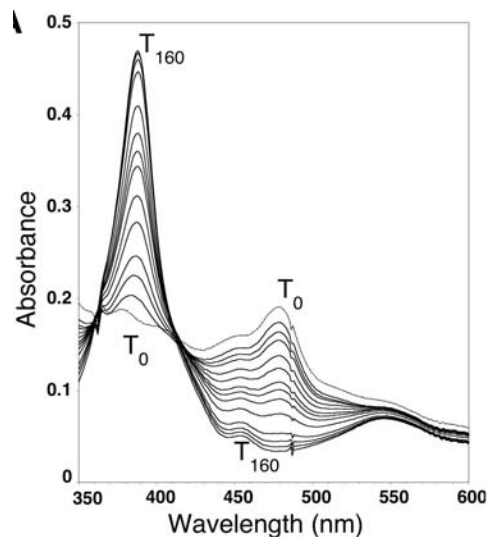


Fig. 2. RamA mediated reduction of Co(II)-MtmC to Co(I).

Science objectives for 2008-2009:

- We are currently cloning RamA for expression in *E. coli* and *M. acetivorans* in order to test if this protein alone is sufficient for corrinoid protein reduction.
- We are conducting further tests of the effects of alanine replacements of pyrrolysine with the DMA and TMA methyltransferases. These will include MMA, DMA and TMA binding by the mutant methyltransferases, cob(I)alamin methylation, and the methylation of the cognate corrinoid proteins.
- In collaboration with Michael Chan, we recently obtained the crystal structure of the TMAmethyltransferase, this has revealed residues surrounding pyrrolysine that we intend to also target for site directed mutagenesis.

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2. W. Gong, B. Hao, Z. Wei, D.J. Ferguson Jr., T. Tallant, J.A. Krzycki, and M.K. Chan. 2008. Structure of the $\alpha 2\epsilon 2$ Ni-CODH component of the *M. barkeri* ACDS complex. *PNAS*:105(28):9558-63.
3. T. Ferguson, J.A. Soares, T. Lienard, G. Gottschalk, and J. A. Krzycki. RamA, a protein required for activation of corrinoid-dependent methylamine methyltransferase reactions in methanogenic Archaea. 2008. (submitted, *J. Biol. Chem.*)

Osmoregulation in Methanogens

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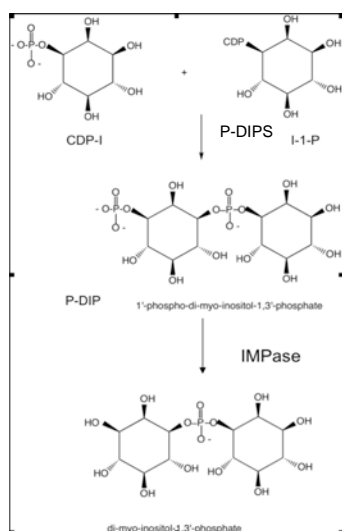
Dr. Boguslaw Stec & Dr. Andrei Osterman, The Burnham Institute, La Jolla CA 92037

Dr. Mikhail Bubunencko & Dr. Donald L. Court, NCI-Frederick, N.I.H., Frederick MS 2170

Overall research goals: Our research objectives are (1) to characterize, using NMR and MS methods, how small molecule solute pools (the ‘osmotosome’) in methanogens (and closely related bacteria) are controlled in the response to osmotic and thermal stress; (2) to obtain structures for key enzymes involved in unusual solute production; and (3) to explore the mechanism by which several of the unique solutes act as thermoprotectants for different enzymes.

Significant achievements in 2006-2008: Isotope-edited NMR studies have been finished of stress-induced solute synthesis in the mesophile *Methanococcus maripaludis* (α -glutamate and N ϵ -acetyl- β -lysine) and in the hyperthermophile *Methanocaldococcus jannaschii* (α - and β -glutamate). In both organisms, hyperosmotic stress induces a rapid synthesis of α -glutamate. However, synthesis and accumulation of the β -amino acid solute depended on the organism. *M. maripaludis* exhibited a 0.5 to 1 generation time lag in ^{13}C uptake into N ϵ -acetyl- β -lysine, and used α -glutamate as a temporary solute. *M. jannaschii* synthesized both glutamate isomers, but only after a lag time. There was also an increase in α -glutamate turnover as it was fixed into macromolecules. In the hyperthermophile, heat stress caused a readjustment of α - to β -glutamate ratios with export or metabolism of the β -glutamate. In *M. jannaschii* the trends in the small molecule pool were also connected to changes in the mRNA coding for selected enzymes likely responsible for the synthesis of different osmolytes. Solute synthesis in *Thermotoga maritima* in response to osmotic and heat stress was also monitored (using mass spectrometry as well as NMR) since this was the optimal organism for exploring DIP biosynthesis.

A major accomplishment was the identification, cloning, and overexpression of the enzymes in the pathway for biosynthesis of di-myoinositol-1,3'-phosphate (DIP) where D-glucose-6-phosphate is converted to a phosphorylated form of DIP and then DIP (Figure 1). This work was done with



collaborators at The Burnham Institute. A bioinformatics analysis sorted DIP-accumulating organisms into two classes: those like *T. maritima* that use four separate enzymes (IPS (inositol-1-phosphate synthase) to form L-inositol-1-P from D-glucose-6-P, IMP-CT (CTP:I-1-P cytidyltransferase) to activate I-1-P by forming CDP-inositol, P-DIPS (P-DIP synthase) which condenses L-I-1-P and the CDP-I to form 1'-phospho-di-myoinositol-1,3'-phosphate (P-DIP), and inositol monophosphatase (IMPase) that removes the phosphomonoester to generate DIP), and those like *Archaeoglobus fulgidus* that combine the IMP-CT and P-DIPS into a single membrane-bound fusion protein. In *T. maritima* the genes for the IPS, IMP-CT and P-DIPS are arranged in an operon; the gene for the IMPase is also nearby. We have also solved the structure of the IMPase from *T. maritima*. This tetramer occupies an interesting niche as an intermediate between allosterically regulated FBPase enzymes and the dimeric IMPases.

Figure 1. Novel steps in the biosynthetic pathway for DIP in *T. maritima*: D-glucose-6-P is first converted to L-inositol-1-P, which in turn is activated to CDP-inositol (CDP-I) by IMP-CT; P-DIPS condenses L-I-1-P with CDP-I to form a phosphorylated precursor of DIP (P-DIP); the P-DIP is then hydrolyzed to DIP by IMPase.

We have initiated RT-PCR studies of selected mRNA in *T. maritima* to investigate possible transcriptional regulation of DIP biosynthesis. We devised primers for the mRNA from the four genes in the DIP pathway along with primers for mRNA from *glnA*, glutamine synthetase, as a housekeeping activity (reasonable here since none of the osmolytes have nitrogen in them), and from a gene for a lysine aminomutase (*lam*). Although *T. maritima* does not make N ϵ -acetyl- β -lysine it does generate mRNA for the enzyme that converts α -lysine to β -lysine. Both increased salt and heat stress dramatically increase *ips* message with much smaller effects on mRNA for other DIP enzymes. The increase in DIP accumulation paralleled the timescale for *ips* mRNA increase. *T. maritima* does not make inositol-containing lipids (as far as we can detect), so the IPS is a logical step for transcriptional regulation of carbon flow into DIP biosynthesis. In contrast, *A. fulgidus* does use inositol lipids in its membranes, so the transcription of IPS can not be the target for regulation of DIP accumulation. For that hyperthermophilic archaeon, we could show that the temperature dependence of the IMPase K_m for I-1-P could contribute to controlling DIP biosynthesis. Below 75°C, the K_m for substrates was 10-20 fold higher than above 80°C, ensuring any P-DIP synthesized would not be rapidly converted to DIP.

We have also finished the synthesis of L,L- and L,D-DIP compounds (in collaboration with Dr. Scott Miller at Yale), and are presently exploring the ability of these pure solutes to protect enzymes from thermal denaturation. Compatible solutes act as chemical chaperones by allowing proteins to partially unfold but not irreversibly. When the temperature is decreased and in some cases, substrates or other ligands added, the protein can adopt a native conformation and regain activity.

Science objectives for 2008-2009:

- Thermoprotection studies of *A. fulgidus* IMPase, *E. coli* IMPase (for which we have determined a structure and explored its function in *E. coli*, which doesn't make inositol compounds), and RNase (assessed by residual enzyme activity, CD analysis of secondary structure, and fluorescence to monitor tertiary interactions) using synthetic DIP; crystallization of IMPase with DIP added to assess any specific interactions.
- Further RT-PCR studies of DIP biosynthetic enzymes in *T. maritima* under different stress conditions. RT-PCR studies looking at mRNA for the IPS, IMPase and IMP-CTP/P-DIPS fusion enzyme in *A. fulgidus*.
- Crystallization of the *T. maritima* IMP-CTP enzyme.

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3. Y. K. Wang, A. J. Morgan, K. A. Stieglitz, B. Stec, B. Thompson, S. J. Miller, and M. F. Roberts, "Temperature dependence of the inositol monophosphatase K_m correlates with di-myoinositol-1,1'-phosphate accumulation in *Archaeoglobus fulgidus*," *Biochemistry* **45**, 3307-3314 (2006).
4. D. A. Rodionov, O. V. Kurnasov, B. Stec, Y. Wang, M. F. Roberts, and A. Osterman, "Genomic identification and *in vitro* reconstitution of a complete biosynthetic pathway for the osmolyte di-myoinositol phosphate," *Proc. Natl. Acad. Sci. U.S.A.* **104**, 4279-4284 (2007).
5. K. A. Stieglitz, M. F. Roberts, W. Li, and B. Stec, "Crystal structure of the tetrameric IMPase (TM1415) from the hyperthermophile *Thermotoga maritima*," *FEBS J.* **274**, 2461-2469 (2007).
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Selenocysteine: Biosynthesis and Significance for Hydrogen Utilization

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Overall research goals: The current project has two specific aims: (i) Significance of selenocysteine in the F420-reducing [NiFeSe] hydrogenase, and (ii) Selenocysteinyl-tRNA formation in archaea.

Significant achievements since begin of the project in September 2007: **Aim (i):** The Sec residue (U391; U is the one letter code for selenocysteine, Sec is the three letter code) in *Methanococcus maripaludis* NiFeSe-hydrogenase is located at the catalytic center of the enzyme. Together with three cysteines (C63, C66 and C394) it coordinates a nickel molecule. The goal of this project is to elucidate the role of Sec residue in the hydrogenase. The proposed experiments include deletion of both Fru and Frc operons in *M. maripaludis*; introduction of plasmid-borne Fru mutants into the double-deletion cells; and characterization of the mutant hydrogenases. We have achieved the deletion of both Fru and Frc operons in *M. maripaludis* with a markerless deletion strategy developed by John Leigh (University of Washington, Seattle). **Aim (ii):** Continuing work (also partly supported by the National Institute for General Medical Sciences) we have characterized the two enzymes (PSTK and SepSecS) shown (Yuan, J., Palioura, S., Salazar, J. C., Su, D., O'Donoghue, P., Hohn, M. J., Cardoso, A. M., Whitman, W. B. and Söll, D. [2006] RNA-dependent conversion of phosphoserine forms selenocysteine in eukaryotes and archaea. *Proc. Natl. Acad. Sci. USA* **103**, 18923-18927) to be involved in archaeal/eukaryal Sec formation.

Science objectives for 2008-2009:

- **Aim (i):** Finish deletion strains and first *in vivo* test of mutant hydrogenase.
- **Aim (ii):** Isolation and characterization of complexes of PSTK, SepSecS and tRNA^{Sec}

References to work supported (in part) by this project since September 2007:

1. K. Sheppard, J. Yuan, M. Hohn, B. Jester, K. M. Devine, and D. Söll, "From one amino acid to another: tRNA-dependent amino acid biosynthesis," *Nucl. Acids Res.* **36**, 1813-1825 (2008).
2. Y. Araiso, S. Palioura, R. Ishitani, R. L. Sherrer, J. Yuan, H. Oshikane, N. Domae, J. DeFranco, D. Söll, and O. Nureki, "Structural insights into RNA-dependent eukaryal and archaeal selenocysteine formation," *Nucl. Acids Res.* **36**, 1187-1199 (2008).
3. R. L. Sherrer, P. O'Donoghue, and D. Söll, "Characterization and evolutionary history of an archaeal kinase involved in Sec-tRNA^{Sec} formation," *Nucl. Acids Res.* **36**, 1247-1259 (2008).
4. R. L. Sherrer, J. Ho, and D. Söll, "Divergence of selenocysteine tRNA recognition by archaeal and eukaryotic *O*-phosphoseryl-tRNA^{Sec} kinase," *Nucl. Acids Res.* **36**, 1871-1880 (2008).
5. J. Yuan, K. Sheppard, and D. Söll, "Amino acid modifications on tRNA," *Acta Biochim. Biophys. Sin. (Shanghai)* **40**, 539-553 (2008).

Session P1
Poster Session - I

Attachment of Living Cells to Material Surfaces Through DNA-Based Linkages

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Overall research goals: A powerful new technique has been developed for the attachment of virtually any cultured mammalian cell to material surfaces through the intermediary of DNA hybridization. Advances in surface printing techniques have allowed the generation of complex patterns and the recruiting of cells to electrode surfaces. The research objectives are to expand this method to encompass all cell types through the direct attachment of DNA strands to their native surface functional groups. Hydrogen producing photosynthetic organisms will then be immobilized into micropatterned fuel cells to study environmental influences on energy production and cell survival. Cardiac myocytes will be studied to determine the influence of spatial arrangement and colony size on collective synchronous contraction behaviour.

Significant achievements in 2006-2008: *Increasing the Efficiency of Substrate Printing.* In our preliminary studies, we prepared material substrates through the exposure of thiol-terminated DNA strands to gold surfaces. While this yielded sufficient coverage to confirm cell adhesion, we only obtained a low coverage of cells on the metal surfaces. At the beginning of this cycle, we adapted a previously reported DNA printing strategy that relied on a reductive amination reaction. The full collaboration of the Francis, Bertozzi, and Mathies groups has been absolutely crucial to the success of these efforts. In this method, aldehyde-coated surfaces are treated with solutions of amine-labeled DNA strands that have been applied using pipets, microinjectors, micropatterned stamps, or ink jet printers, Figure 1a. After heating the sample to evaporate the solvent and drive imine formation, the substrates are exposed to aqueous solutions of NaBH₄ to reduce the imine linkages to stable amine groups. This process permanently links the DNA strands to the surface with high efficiency, as can be confirmed through hybridization to fluorescent strand complements. In locations that lack the DNA strands, the borohydride treatment step serves to reduce the aldehydes to simple alcohol groups.

Attachment of Living Cells to AFM Cantilevers. In principle, the reductive amination procedure for DNA printing could be extended to any aldehyde-coated surface, allowing a number of physical probes to be attached to cell surfaces. As a particularly useful example, we have attached the aldehyde groups to the thin silica layer that coats the surface of silicon nitride AFM cantilevers, Figure 1b. Subsequent DNA strand attachment has been carried out using the reductive amination strategy detailed above, as has the attachment of concanavalin A and anti-CD3 antibodies. In the case of DNA-mediated attachment, we have found that the resulting AFM tips bind to live cells bearing the complementary sequences within 5 seconds of contact. The cells can then be lifted from the surface and moved to new locations with exquisite positional control. Interestingly, the cells can be transferred to new surfaces bearing longer DNA sequences, so that the complementary regions form greater overlap than that experienced with the AFM cantilever. Again, only 5 seconds are required for the transfer to take place. Figure 1c shows a pattern of cells that was produced using this one-cell-at-a-time transfer technique. We have found that a single AFM tip coated with DNA strands can be reused over 200 times with no apparent loss in cell binding ability, highlighting the reversibility advantage of DNA hybridization.

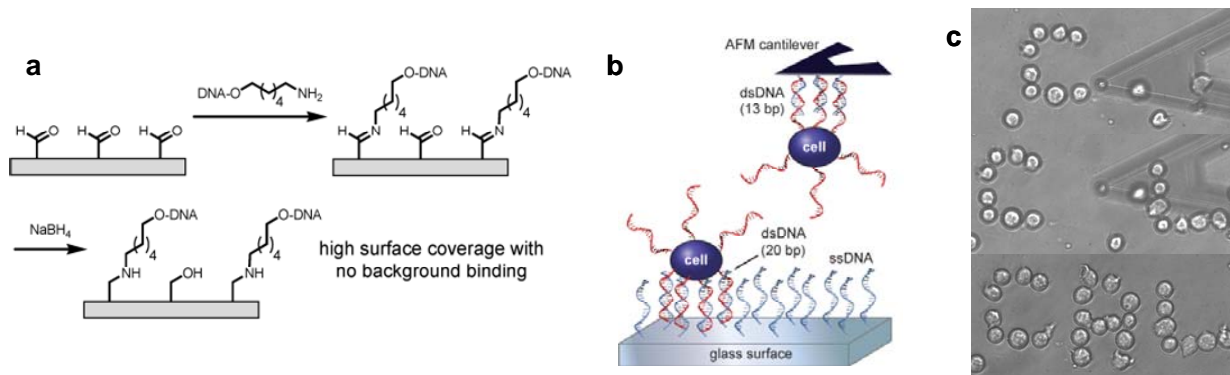


Figure 1. (a) Improved DNA printing techniques have increased cell binding efficiency. A reductive amination strategy is now used to attach amine-terminated DNA strands to surfaces coated with aldehydes. (b) By attaching a shorter DNA strand (13 bases) to a cantilever and a longer strand (20 bases) to the glass slide, a single living cell can be transported by the AFM and directly printed at a desired location on the glass slide. This process is shown stepwise for the formation of a single pattern of cells in (c).

Science objectives for 2008-2009:

- Expansion of the DNA Modification Technique to Include All Cell Types. In initial experiments we have found that this can be accomplished through the preparation of DNA-NHS ester conjugates, which modify lysine residues on the surface of mammalian cells.
- Incorporation of Photosynthetic Organisms into Device Platforms. In the next cycle, we will use direct cell surface modification techniques to expand significantly the types of cells that can be incorporated into device formats.
- Using Patterned Surfaces to Generate Artificial Symbiotic Systems. As the DNA-based attachment strategy continues to become more general, new opportunities will arise for the intimate juxtaposition of cell types that have not been in contact previously.

References to work supported by this project 2006-2008:

1. Chandra, R. A., E. S. Douglas, R. A. Mathies, C. R. Bertozzi, and M. B. Francis. 2006. Programmable cell adhesion encoded by DNA hybridization. *Angew Chem Int Ed.* 45:896-901.
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Defense Responses Affecting Resistance to *Alternaria brassicicola*

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Dr. Morten Petersen, Copenhagen University

Overall research goals: The goals of our project funded from 9/2005 to 9/2006 were to study plant defenses important for resistance to necrotrophic plant pathogens, using the *Alternaria brassicicola* – *Arabidopsis thaliana* model system. We focused on synthesis of camalexin, a phytoalexin produced by *Arabidopsis* that is important for resistance to *A. brassicicola*, and on the roles of genes and metabolites identified in expression profiling and metabolite profiling projects.

Significant achievements in 2006-2008: We identified a cytochrome P450 monooxygenase, CYP71A13, which is required for camalexin synthesis. Mutations in this gene compromised resistance to *A. brassicicola*. In collaboration with Barbara Halkier of Copenhagen University and Erich Glawischnig of the Technical University of Munich, we found that CYP71A13 catalyzes the conversion of indole acetaldoxime to indole acetonitrile in the camalexin biosynthetic pathway. This work has been published, and will not be described on the poster.

In a reverse genetic study of the roles of *A. brassicicola*-induced genes in resistance, we found that loss of the transcription factor WRKY33 resulted in greatly reduced camalexin levels after infection by *A. brassicicola* or the bacterial pathogen *Pseudomonas syringae*. Plants with *wrky33* mutations also had reduced expression of CYP71A13 and another gene encoding an enzyme involved in camalexin synthesis, PAD3. We collaborated with the group of Dr. Morten Petersen, who found that WRKY33 binds to the PAD3 promoter. It also interacts with MKS1, a protein that interacts with MAP kinase 4. This work has been published and will not be described on the poster.

In collaboration with Dr. Oliver Fiehn, we carried out metabolite profiling of plants infected with *A. brassicicola*. While levels of many metabolites were altered during infection, few of these changes were affected by defense signaling mutants blocking the salicylic acid, jasmonic acid, or ethylene-dependent pathways, suggesting that other signals have a major role during the response to *A. brassicicola*. The metabolites that underwent changes in concentration during infection included several sugar alcohols. We found that application of mannitol or myo-inositol together with fungal spores increased plant susceptibility. This was not due to provision of a carbon source to the fungus, as application of glucose had no effect. Wild-type *Arabidopsis* plants are resistant to *A. brassicicola*. In response to fungal attack, they produce reactive oxygen species (ROS) at the site of attack. Using diaminobenzidine staining, we found that the concentration of ROS was greatly reduced in the presence of mannitol or myo-inositol. Necrotrophic fungi are known to produce mannitol, which can function as an antioxidant. The *Arabidopsis* genome encodes several mannitol dehydrogenases. Mutations in one of these genes, *ELI3-2*, resulted in increased loss of chlorophyll during *A. brassicicola* attack, although fungal growth was not affected. Our results suggest that mannitol produced by the fungus promotes fungal growth by functioning as an anti-oxidant while removal of mannitol by plant mannitol dehydrogenases promotes resistance. This work will be described on the poster.

Science objectives for 2008-2009:

- Several mutants in which resistance to *A. brassicicola* is compromised have been identified using reverse genetics. These will be characterized using expression profiling and other approaches in an effort to understand factors important for resistance to *A. brassicicola*.
- Some proposals for improving cellulosic ethanol production involve producing plants with altered cell walls. Some cell wall alterations have deleterious effects on disease resistance. We will collect *Arabidopsis* mutants with altered cell walls and test them for defects in resistance to a panel of pathogens. The results are expected to allow predictions of how particular cell wall alterations are likely to affect disease susceptibility.

References to work supported by this project 2006-2008:

1. Qiu, J.-L., Fiil, B.K., Petersen, K., Nielsen, H.B., Botanga, C.J., Thorgrimsen, S., Palma, K., Suarez-Rodriguez, M. C., Sandbech-Clausen, S., Lichota, J., Brodersen, P., Grasser, K.D., Mattsson, O., Glazebrook, J., Mundy, J., and Petersen, M. *Arabidopsis* MAP Kinase 4 Regulates Gene Expression Via Transcription Factor Release in the Nucleus. *EMBO J.*, in press.
2. Nafisi, M., Goregaoker, S., Botanga, C.J., Glawischnig, E., Olsen, C.E., Halkier, B.A. and Glazebrook, J. *Arabidopsis* Cytochrome P450 Monooxygenase 71A13 Catalyzes the Conversion of Indole-3-Acetaldoxime in Camalexin Synthesis. *Plant Cell* 19:2039-2052 (2007).

Engineering metabolism of phenolics with transcription factors

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Overall research goals: The research objectives are to establish the function of a group of maize R2R3-MYB genes (the R2R3-MYB^{P-toA} clade) in the regulation of phenolics compounds in maize, using a combination of gain- and loss-of-function approaches. In addition, we will identify the genes that these R2R3-MYB regulators control with the broader objective to better define the biosynthetic pathways that lead to the formation of various phenylpropanoids in maize.

Significant achievements in 2007-2008: We have identified three additional members of the R2R3-MYB^{P-toA} (bringing the currently available to 6 from the 10 total previously predicted to be present in maize). For ZmMYB-IF35 and ZmMYB-IF25, we have generated p35::IF35 and p35S::IF25 constructs, which are ready for transformation into maize Black Mexican Sweet cells and plants. We are in the process of generating similar constructs with the pUbi promoter. We have completed the construction of dsRNAi constructs for both of these TFs, and constructs have been submitted maize transformation.

The systematic analysis of the promoters of all the genes in the maize phenylpropanoid pathway have been initiated. Based on the maize genome sequences being released, we are identifying and cloning the promoters (1 - 2 kb upstream of the transcription start site when known, otherwise from the ATG) of these genes. This analysis is central for the identification of targets for these TFs using chromatin immunoprecipitation (ChIP). In addition, we are cloning these promoters in yeast vectors to carry out yeast one-hybrid analyses, in case that we need to identify additional regulators. So far, promoters for 14 genes have been cloned and several others are in progress. We have standardized ChIP methods for maize using epitope-tagged transcription factors, and using antibodies against the endogenous regulators. Antibodies against ZmMYB-IF35 and ZmMYB-IF25 are currently being generated using as antigens protein fragments unique for each of the two proteins.

Science objectives for 2008-2009

- Generation of dsRNAi and over-expression constructs for other R2R3-MYB^{P-toA} genes. Transformation into maize plants.
- Identification of direct targets for ZmMYB-IF35 and ZmMYB-IF25 by ChIP
- Analysis of gain- and loss-of-function ZmMYB-IF35 and ZmMYB-IF25 transgenic maize plants

References to work supported by this project 2007-2008:

1. Grotewold, E. "Transcription factors for predictive plant metabolic engineering: Are we there yet?" *Curr. Opin. Biotech.* **19**, 138-144 (2008).

Exploration of phenylpropanoid regiospecific *O*-methylation and engineering of novel monolignol 4-*O*-methyltransferases to probe lignin biosynthesis

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Overall research goals: Our research objectives are to explore the molecular mechanisms of the regiospecific methylation of phenylpropanoid *O*-methyltransferases by applying a structure-based protein engineering approach, thereby, to create a set of novel monolignol 4-*O*-methyltransferases that will introduce the non-natural precursors to lignin biosynthesis *in planta*, and subsequently, to explore the perturbation or disruption on plant cell wall lignification.

Significant achievements in 2007-2008:

In order to explore the nature of phenolic *O*-methyltransferase in substrate preference and regio-selective methylation, we isolated two caffeic acid 3-*O*-methyltransferases (COMTs), and three phenylpropene 4-OMT homologues from poplar and basil, respectively. Together with the collection of phenylpropene 4-*O*-methyltransferases from *Clarkia* and sorghum, we determined or re-evaluated their transmethylation activities on a range of phenylpropenes, monomeric phenolics, and monolignols using highly sensitive LC-MS to characterize the products. A functional plasticity of those phenylpropene 4-*O*-methyltransferases and lignin 3/5-*O*-methyltransferases were observed. Subsequently, we initiated crystallization on phenylpropene 4-*O*-methyltransferases from both *Clarkia* and sorghum.

As the high similarities between *Clarkia* phenylpropene 4-*O*-methyltransferase and COMTs of poplar and alfalfa, we conducted protein homology modeling and substrate docking analyses on both *Clarkia* and poplar enzymes based on the alfalfa COMT's crystal structure (PDB code: 1KYW); and we identified seven amino acid residues different in the active sites of two types of enzymes, we then conducted the rational mutagenesis to evaluate the roles of those amino acid residues in governing their regio-selectivity and substrate discrimination. Subsequently, we initiated saturation mutagenesis with targeting on each of the identified amino acid residues of both *Clarkia* and poplar enzymes, and created a large number of mutant variants. In order to functionally screen the created mutants, we developed a cost effective colorimetric assay method for quick determining the activity of phenolic *O*-methyltransferases. Together with highly sensitive LC-MS and radio-activity detection, we established a throughput mutagenesis-function screening procedure and examined a number of mutant libraries harboring the signal site saturation mutation. Successfully, we obtained a few mutant variants showing activity of 4-*O*-methylation of monolignols.

Science objectives for 2008-2009:

- We will perform homology modeling and docking analysis and the detailed biochemical determination for the obtained novel mutant enzymes, i.e., monolignol 4-*O*-methyltransferases; by comparing to the wild type parental enzyme, we will probe the mechanistic disparity of regio-specificity and extract information to guide the further mutagenesis to improve the enzyme catalytic efficiency.

- We will continue to create and screen the saturation mutants of phenylpropene 4-*O*-methyltransferases and lignin 3/5-*O*-methyltransferases. The obtained single site mutant enzymes will also be used as template for the next round mutagenesis on the remaining other residues.
- Meanwhile, we will initiate DNA family shuffling to create chimeric phenolic *O*-methyltransferase genes. The random chimeragenesis will be performed using different pairs of phenolic *O*-methyltransferases that exhibit distinct regio-specificity and substrate preferences.
- We will continue to perfume the crystallization and structural determination on phenylpropene OMTs. The diffraction patterns of the generated crystals will be detected and crystallization condition will be optimized. The crystals in complex with different substrates will be generated and determined. Meanwhile, we will crystallize the created novel mutant variants that exhibit activities for *O*-methylation of monolignols to fully elucidate the mechanisms governing their distinct regio-specific methylation and substrate specificity.
- We will generate transgenic tobacco and Arabidopsis plants toward evaluating the effects of the novel mutant enzymes on lignin biosynthesis. Whenever they are available, the created monolignol *O*-methyltransferase genes will be inserted into plant expression vectors driven either by the constitutive promoter or xylem-specific promoter.

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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. Understanding how secondary walls are produced would not only contribute to our knowledge of basic plant biology but also have economic and environmental implications because wood is the most environmentally cost-effective and renewable source of energy and widely used for lumber and pulping. We proposed to carry out the functional characterization of glycosyltransferases (GTs) involved in the biosynthesis of xylan, the second most abundant polysaccharide produced by plants, and of the regulatory genes controlling secondary wall synthesis.

Significant achievements in 2006-2008: We have uncovered key roles of several glycosyltransferases (GTs) in xylan biosynthesis and of transcription factors in regulating secondary wall biosynthesis. Xylan is composed of a linear backbone of (1,4)-linked β -D-xylosyl residues with substitutions of glucuronic acid, 4-O-methyl-glucuronic acid, and/or arabinose. The reducing end of xylan contains a distinct tetrasaccharide sequence. We found that the Arabidopsis GTs, including FRA8, IRX8, PARVUS, are required for the biosynthesis of the reducing end sequence of xylan, and another GT, IRX9, is essential for xylan backbone elongation. These findings provide important framework for assigning biochemical functions of various GTs in the different steps of xylan biosynthesis. We also discovered that the coordination of expression of genes involved in the biosynthesis of xylan together with cellulose and lignin during secondary wall formation is regulated by a cascade of transcription factors, including SND1 and MYB46. This finding marks an important step toward molecular dissection of transcriptional networks regulating wood formation.

Science objectives for 2008-2009:

- Our findings that FRA8, IRX8, IRX9, and PARVUS are required for xylan biosynthesis provide an unprecedented opportunity to uncover the biosynthetic pathway of xylan. We will continue to elucidate the biochemical functions of these glycosyltransferases.
- Our findings that the NAC domain protein SND1 is a key transcriptional switch for the developmental program of secondary wall deposition and that MYB46 is a direct target of SND1 led us to hypothesize that a transcriptional network is involved in the regulation of secondary wall biosynthesis. We will continue to investigate how SND1 and MYB46 regulate the biosynthetic pathways of secondary wall components.

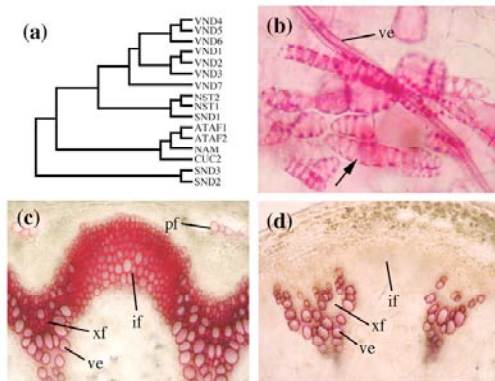


Figure 1. NAC genes are key switches for activation of secondary wall biosynthesis. (a) Phylogenetic relationship of secondary wall-associated NACs compared with a few other known NACs. (b) Ectopic deposition of helical secondary walls in leaf mesophyll cells (arrow) induced by overexpression of SND1. Note the normal vascular strand with helical secondary wall thickening (ve). (c) Cross section of a wild-type *Arabidopsis* inflorescence stem showing the lignified secondary walls (stained as red) of interfascicular fibers, vessels and xylary fibers. (d) Cross section of a stem of the *snd1/nst1* double knockout line showing a complete loss of secondary wall thickening in interfascicular fibers and xylary fibers but no change in vessel walls. if, interfascicular fiber; pf, phloem fiber; ve, vessel; xf, xylary fiber.

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Roles of Microtubule-based Motors in Plant Growth

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Overall research goals: The long-term goal of this project is to understand how microtubule-based motor kinesins contribute to ordered synthesis and deposition of cellulose microfibrils during rapid cell enlargement in flowering plants. The immediate research objectives are to elucidate the role of Kinesin-13A in activities of the Golgi apparatus; to understand how Kinesin-4 members regulate cell elongation; and to characterize molecular mechanisms that regulate the interaction between the cellulose synthase complex and microtubules.

Significant achievements in 2006-2008: The rapidly elongating cotton fibers have continuously allowed us to discover proteins like kinesins which may play critical roles in anisotropic cell enlargement. Among identified kinesin motors are members of the Kinesin-4 and Kinesin-13 subfamilies. Functions of these motors began to be revealed by reverse genetics in *Arabidopsis thaliana* and fluorescent microscopy.

There are three Kinesin-4 members encoded by the *A. thaliana* genome, AtKinesin-4A, -4B, and -4C. Using promoter-GUS fusion, it was determined that Kinesin-4A and Kinesin-4C were highly expressed in the vascular tissue of expanding leaves, and in the root elongation zone. However, Kinesin-4B showed strong expression in the root apical meristem and lateral root primordia, and very little if any overlapping expression with the other two motors. Multiple alleles of T-DNA insertional mutations at the three kinesin loci have been isolated. While the *kinesin-4a* mutation significantly impaired shoot elongation, mutations of either *kinesin-4b* or *kinesin-4c* alone did not exhibit a noticeable growth defect. Possible redundant functions have been analyzed in homozygous double mutants. The most severe phenotype was detected in the *kinesin-4a;kinesin-4c* double mutant which exhibited an enhanced inhibition of cell elongation compared to the *kinesin-4a* single mutant. Moreover, the double mutant exhibited abnormal growth patterns of the leaf trichomes with stunted appearance and increased branches.

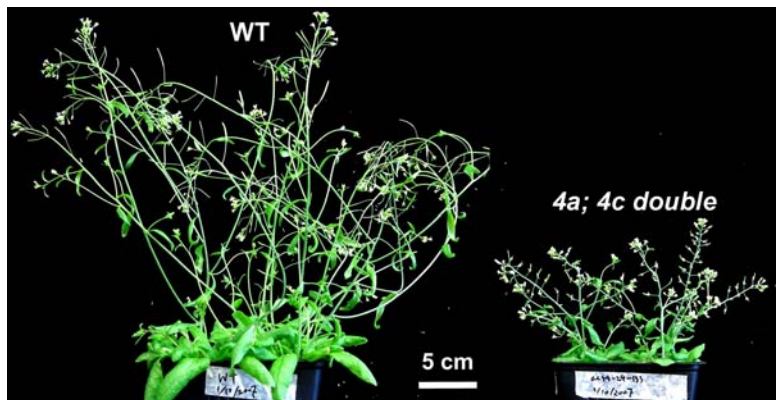


Figure 1. Growth phenotype exhibited by the *kinesin4a; kinesin-4c* double mutant.

Kinesin-13A is probably the most abundantly expressed kinesin in developing cotton fibers and cells in *A. thaliana*. Our earlier results have shown that this internal motor kinesin decorates Golgi stacks, and plays a critical role in regulating the initiation of new growth point during anisotropic cell expansion. We started to dissect the functions of non-motor domains of Kinesin-13A in order to understand molecular mechanisms that regulate motile activities surrounding the Golgi apparatus, and consequently cell morphogenesis. When the N-terminus (K13AN) was expressed as a fusion protein with GFP and S-tag (K13AN-GFP-S), transgenic lines phenocopied the null *kinesin-13a* mutant in which the gene was inactivated by T-DNA insertions. This result supported the notion that over expression of K13AN alone created a dominant negative environment in which functions of the endogenous Kinesin-13A was significantly inhibited. Moreover, using an anti-GFP

monoclonal antibody the K13AN-GFP-S fusion protein was detected exclusively at Golgi stacks. This line of evidence suggested that K13AN was likely the determinant for Kinesin-13A to be localized to the Golgi apparatus. Conversely, the K13AC-3XFLAG-S fusion did not exhibit an obvious localization pattern when probed with an anti-FLAG monoclonal antibody. However, the transgenic lines over-expressing K13AC-3XFLAG-S exhibited an enhanced phenotype compared to *kinesin-13a* null mutants, and increased trichome branches. The K13AC-3XFLAG-S lines formed mini-branches on top of increased branches in leaf trichomes. The data suggested to us that Kinesin-13A may function in controlling new growth points leading to trichome branches through interaction with one or more effector protein(s). Over expression of K13AC might have inhibited the function of such an effector so that the inhibitory effect on trichome branching was alleviated to a higher degree than just knocking out *Kinesin-13A*.

We have attempted to purify proteins which might be associated with K13AC-3XFLAG-S in transgenic lines. An affinity chromatographic approach was applied using an anti-FLAG antibody column and an S-protein affinity column. Proteins eluted from the anti-FLAG column using excess of 3XFLAG peptide had K13AC-3XFLAG-S greatly enriched. K13AC-3XFLAG-S was further purified from eluted proteins *via* S-protein column. With little contamination, K13AC-3XFLAG-S and a polypeptide of ~30-kDa were detected. The identity of this co-purified polypeptide will be determined using masses obtained by mass spectrometry.

Science objectives for 2008-2009:

- Functions of members of the Kinesin-4 subfamily will be further analyzed by determining their intracellular localization patterns using mono-specific antibodies to be purified from anti-sera. A complementary experiment is to apply the epitope-tagging approach to express functional Kinesin-4 fusion proteins for localization using tag-specific antibodies.
- We will examine how activities of the cellulose synthase CESA may be affected in various *kinesin-4* mutants.
- We aim to identify proteins which specifically interact with the N-terminus and the C-terminus of Kinesin-13A. Functions of the resulting proteins will be attempted by reverse genetics.

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Modification of Lignin by Protein-Crosslinking to Facilitate Production of Biofues from Poplar

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Overall research goals: The overall research goal is to develop poplar plants whose secondary cell walls exhibit little or no change in lignin quantity but whose wood will be more accessible to lignin extraction by pretreatment with proteases. Research objectives include design of tyrosine-rich peptide genes, generation of transgenic hybrid poplar plants carrying the tyrosine-rich peptide genes, and characterization of transgenic plants in terms of fitness, cell wall structures, lignin digestibility, and small scale of ethanol production efficiency.

Significant achievements in 2007-2008: A total of 28 transgenic lines were generated for our first tyrosine-rich peptide gene (Fig. 1). Characterization of the transgenic plants is in the process. Preliminary data indicate that the transgenics had no change in total lignin content or overall plant morphology relative to wildtypes (Fig. 2 and 3). Wood storage modulus was analyzed with a Dynamic Mechanical Analyzer DMA 2980 on wildtype and five transgenic poplar plants. Two different stem samples for each transgenic line were included, each from the same sapling. The preliminary data indicated reduced storage modulus in transgenic lines (Fig.4), except in transgenic line T22 ($\alpha=0.05$, as tested by ANOVA with the Tukey pairwise comparisons test). Digestibility of the stem tissue was investigated by using a sequential treatment of protease K followed by cellulase/hemicellulase. The concentration of reducing sugar in each sample was detected by tetrazolium blue method and represented as milligrams of reducing sugar per milliliter per gram of stem tissue. Seven transgenic lines were included, and there were at least two experimental repeats for each sampling plant. Of the lines being surveyed, two (lines T1 and T7) showed significant differences in the amount of sugar released from stem digestions pre-treated with protease K, relative to those without protease treatment (Fig. 5) ($p=0.0017$, 0.0380 , respectively for T1 and T7). In one of these cases (line T1), the digestibility of the non-protease treated stem tissue was similar to the wildtypes.

Base on the first construct being tested in poplar (Fig. 1), a new set of constructs have been made. A His-tag was fused to the tyrosine-rich gene (TRG) in the vector. The four different insertions are whole TRG gene with 15% tyrosine content, the first 276bp of TRG gene with 18% tyrosine content, the second 228bp of TRG gene with 17% tyrosine content, and a synthetic gene with 20% tyrosine content. Three of them have been transformed into *Arabidopsis thaliana* wild type, and T1 seeds have been collected.

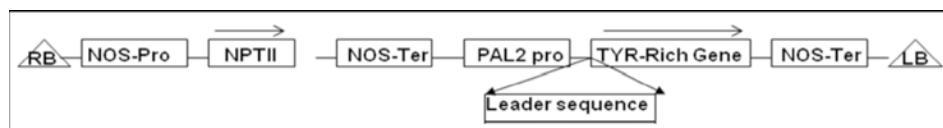


Fig.1 A vector map of the pPAL:TYR. The TYR transgene was fused to a leader sequence derived from a lodgepole pine xylem b-glucosidase to direct secretion into the cell wall and a poplar phenylalanine ammonia-lyase gene (PAL2) promoter to facilitate transgene expression in lignifying tissues. The map is not drawn to scale.

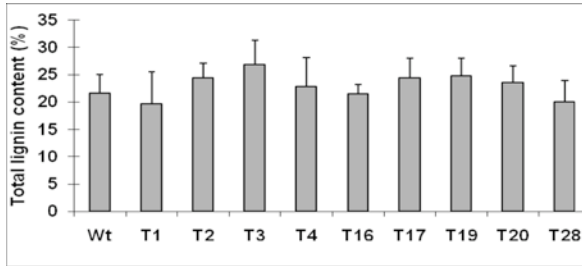


Fig.2. Lignin content analysis from hybrid poplar wildtype and transgenic lines. Lignin was extracted from stem tissues by the Klason method (H₂SO₄ extraction) and lignin content was represented as percentage of stem dry weight. Bars are means ± standard deviation (SD) of three biological replicates.

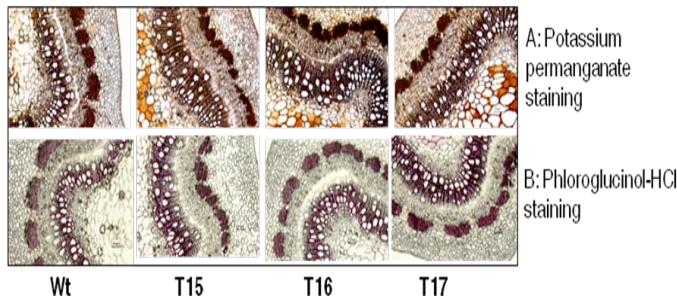


Figure 3. Histochemical staining of lignin of wildtype hybrid poplar and transgenic lines with (a) potassium permanganate and (b) phloroglucinol-HCl. Transverse stem tissue from internodes between leaf plastochron index 8 & 9 (growth stages) were sectioned and stained.

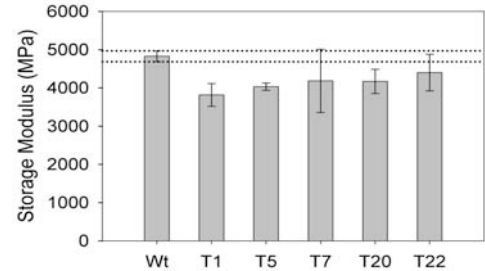


Fig.4. Dynamic mechanical analysis data showing storage modulus results from wildtype and transgenic hybrid poplar. Bars represent means ± standard deviation (SD).

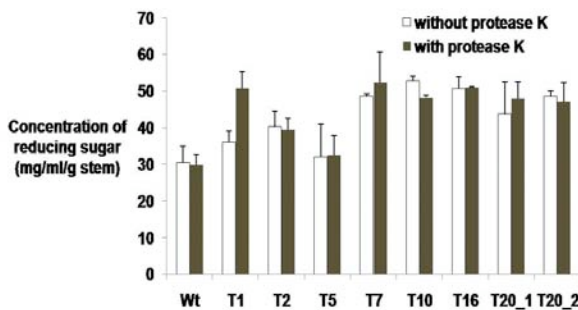


Fig. 5. Reducing sugar concentrations in stem tissue extracts of hybrid poplar “Ogy” wildtypes and transgenic lines. For each line, a portion of ground tissue was incubated with sequential incubations of protease K followed by cellulase and hemicellulase (shaded bars), while another portion of tissue was incubated only with cellulase and hemicellulase (open bars). Bars are means + SD of 2-3 replicates of individual saplings.

Science objectives for 2008-2009:

- Developing TMAH (Tetramethylammonium hydroxide) method to analysis our transgenic plant. First, we will conduct some polymerization assays in test tube with coniferyl alcohol which is a kind of monomer of lignin and tyrosine under the catalysis of peroxidase, and then the polymerization product was used to conduct TMAH thermochemolysis/GC-MS. With this experiment, we might figure out with what kinds of bonds tyrosine and lignin are binding;
- Immuno-gold labeling TEM to localize TRG at sub-cellular level. Antibody has been generated. After fixation, embedding, polymerization, staining and washing, specimens will be observed with TEM;
- Pathogen and herbivore tests on the available transgenic poplar lines;
- Characterization of the *Arabidopsis* plants transformed with the newly designed tyrosine-rich gene constructs;
- Generation of transgenic poplar lines for the newly designed tyrosine-rich gene constructs.

Hydrogen Metabolism and Energy Conservation In *Methanococcus maripaludis*

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Overall research goals: This research examines the enzymes and pathways in H₂ metabolism in methanogens, especially as they relate to energy conservation. In addition, the metabolism of formate, from which H₂ is produced, is investigated.

Significant achievements in 2006-2008: Efforts were centered in three areas: global regulation by H₂, production of H₂ during growth and methanogenesis from formate, and identification of protein complexes that may function in energy conservation.

Transcriptomics on cells of *Methanococcus maripaludis* raised by continuous culture led to the identification of genes that had increased expression with H₂-limitation. Continuous culture was essential in order to maintain defined growth conditions. H₂ limitation was compared with two other conditions, phosphate limitation and leucine limitation, while growth rate and cell density were held constant. The results showed that mRNA levels for genes encoding steps in methanogenesis that involve the electron carrier F₄₂₀ were uniformly increased with H₂ limitation.

Growth and methanogenesis by *M. maripaludis* were studied using formate as electron donor. The oxidation of formate results in the reduction of F₄₂₀. Growing cultures produced H₂, and genetic studies indicated that H₂ could be produced from reduced F₄₂₀ by either of two pathways, direct production by F₄₂₀-linked hydrogenase or indirect production via reduced F₄₂₀-utilizing methylene-tetrahydromethanopterin dehydrogenase coupled to H₂-forming methylene-tetrahydromethanopterin dehydrogenase. Resting cells produced H₂ from formate at high rates. Mutants that had decreased rates of H₂ production were unaffected in their rates of methane production, suggesting that H₂ is not a direct intermediate in methanogenesis from formate.

A critical energy-conserving step in methanogenesis is thought to be carried out by the enzyme heterodisulfide reductase (HDR). We initiated experiments to identify proteins that associate with HDR and thus might also be involved in energy conservation. Initial results indicated that two additional enzymes of methanogenesis associate with HDR: non-F₄₂₀ reducing hydrogenase and formate dehydrogenase. These observations suggest models in which protein complexing facilitates electron flow from either H₂ or formate to HDR.

Science objectives for 2008-2009:

- Generate mutants to test the roles of hydrogenases and other enzymes in electron flow and energy conservation. One focus will be to determine whether H₂ is a required intermediate during growth and methanogenesis on formate.
- Conduct additional experiments to identify protein complexes that may form with heterodisulfide reductase and function in energy conservation.
- Implement the use of inverted membrane vesicles to monitor the generation of chemiosmotic membrane gradients linked to pathways of electron flow.

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Photophysical Behavior of Single Antenna Proteins in Solution by Suppression of Brownian Motion

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Overall research goals: Our primary research goal is to study the optical properties of photosynthetically relevant proteins at the single-molecule level without the use of a surface or host matrix that can significantly influence the molecule's photophysics. Our measurements address fluorescence lifetime, photobleaching pathway, access to dark states and the molecule's conformational dynamics.

Significant achievements in from Sept. 2007 to 2008: Recent efforts in our lab¹ have resulted in the construction of a microfluidic device capable of compensating for the Brownian motion of a single fluorescent object in solution through the careful application of electrokinetic feedback forces, the Anti-Brownian ELectrokinetic or ABEL trap (Fig. 1). The object is excited by a rotating laser beam which allows the particle's position to be determined from analysis of the time modulation of the fluorescence signal. The feedback loop is closed by voltages applied to the solution which counteract the object's Brownian motion away from the trap's center. In this manner, objects down to a single chromophore can be confined *in solution* for essentially as long as they are capable of maintaining a fluorescent signal. Unlike laser tweezers which rely on optical forces on a large bead, here there is no need and much smaller objects can be trapped for study.

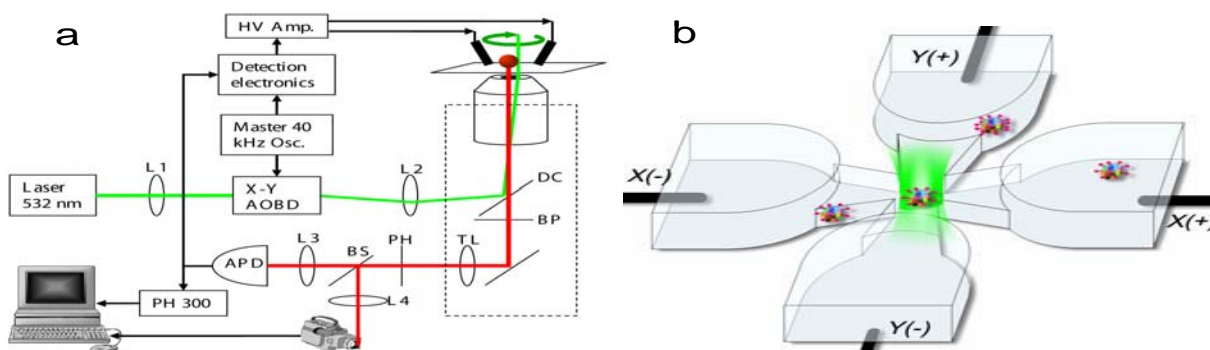


Figure 1: a) Schematic of the ABEL trap including rotating excitation laser and feedback electronics¹. b) Cartoon of the microfluidic component of the ABEL trap including electrodes, excitation laser, and trapped fluorescent object at center

As a first test (Fig. 2), the ABEL trap has been successfully used to confine single copies of the biliprotein, B-Phycocerythrin (B-PE). Importantly, replacement of a CW excitation source with a mode-locked pulsed laser (515nm) also allows trapping to be maintained. In this manner, measurement of fluorescence lifetimes and Brownian motion cancellation were conducted simultaneously. Time traces showing distinct fluorescent bursts from trapped molecules were accumulated, with long dwell times exhibiting one advantage of the ABEL trap over traditional open volume methods, such as Fluorescence Correlation Spectroscopy. The use of Time Correlated Single Photon Counting allowed the arrival time of each photon to be recorded with respect to the excitation pulse. By examining all photon arrival times over a trapped object's dwell time, fluorescence lifetimes were extracted after fitting via a maximum likelihood algorithm and deconvolution of the instrument response. Each burst was assigned a fluorescence lifetime and these lifetimes were used to populate a histogram, where the shape of the histogram can be used to identify heterogeneity normally obscured at the ensemble level. The center value of the histogram, 2.2 ± 0.4 ns compares favorably with the literature ensemble averaged value of 2.5 ns.

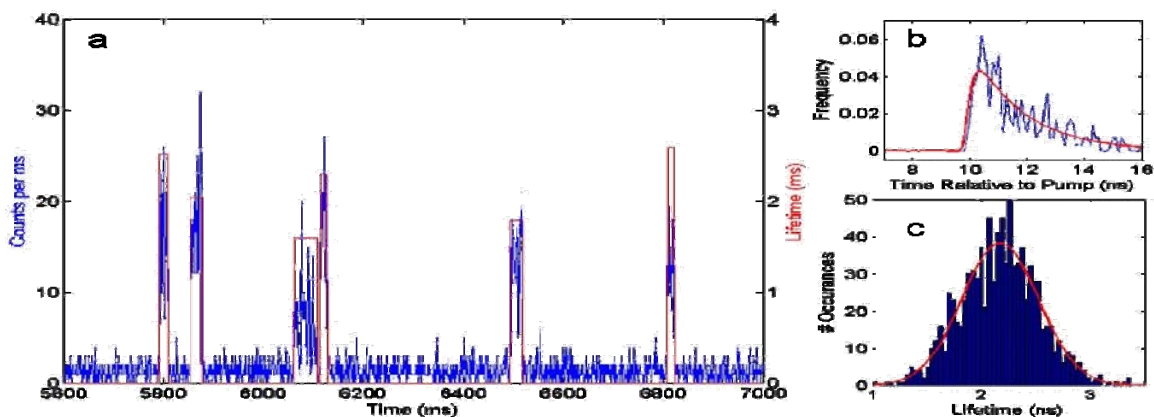


Figure 2. a) Time-dependent emission trace of trapped B-PE (left) with fluorescence lifetimes associated with each burst (right). b) Plot of the arrival times of photons relative to the pump pulse arrival time with fit for the burst at 6500 ms. c) Histogram of measured lifetimes with a Gaussian fit

We have initiated preliminary investigations of another biliprotein, the Allophycocyanin trimer (APC). APC is a particularly interesting candidate for study with the ABEL trap, as previous single-molecule studies in different host media (agarose, polyvinylalcohol, glass slide) demonstrated different sequences of photobleaching steps, suggesting that the medium has a significant impact on the protein's conformation and electronic structure. We also observed photobleaching steps upon examination of APC's on glass slides. The ABEL trap was utilized with CW and pulsed excitation, and data similar to Fig. 2 was recorded. However, rapid photobleaching was observed. To address this, we replaced the 515nm pulsed laser by pumping a non-linear photonic crystal fiber to generate a pulsed supercontinuum (SC). The SC can be spectrally filtered to flexibly generate pulses over a wide spectral range. 515 nm pulses are at the blue edge of APC's absorption spectra and are more likely to promote photodestructive pathways. Generation of more spectrally appropriate 605 nm pulses was accomplished and their incorporation into the ABEL trap setup is underway.

Our immediate goal is to use the SC source to trap single APC and allow accurate determination of fluorescence lifetimes and identification of inhomogeneity across the ensemble. We will add a second detector for measurement of the emission polarization anisotropy to allow us to explore rotational and conformational dynamics. We will also label APC with a robust second chromophore and perform two-color excitation to enable trapping on the timescale of many seconds, the same timescale as the previous work on glass slides and in polymer hosts

Science objectives for 2008-2009:

- Complete the augmentation of the ABEL trap with the SC source and investigate trapped APC's
- Measure polarization information and use it to study conformational dynamics of trapped objects
- Build the capacity for two-color experiments and synthesize labeled photosynthetic proteins for long-time trapping experiments

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Title: Integrating Cation Transport and pH across Endomembranes with the Secretory System

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Rajini Rao, J. Hopkins Univ., Baltimore, MD

Overall research goals

The overall goals are to understand how pH and ion, like Ca^{2+} , are integrated with plant growth, reproduction and adaptation. Endosomal membrane trafficking, including exocytosis and endocytosis, is now recognized to be integral to cytokinesis, cell expansion, cell polarity, guard cell movement, cell wall formation, and gravitropism; though little is known about the cellular and molecular bases for membrane trafficking. A fundamental question is: How do plant cells mediate exocytosis and endocytosis in a spatially- and temporally-regulated manner. One working hypothesis: is that the process depends in part on regulation of both Ca^{2+} and pH dynamics. Our studies have revealed the diversity of Ca^{2+} -pumps in plants, and highlight the critical roles that H^+ and Ca^{2+} play in plant growth and tolerance to stress. One goal is to determine the role of cation/ H^+ exchangers that potentially regulate pH and K^+ homeostasis, and understand how these transporters are integrated with guard cell movement and pollen tube guidance.

Significant achievements in 2007-2008

a) The function of a novel $\text{Ca}^{2+}/\text{Mn}^{2+}$ pump (AtECA3) was determined after functional expression of the gene in yeast and analyses of Arabidopsis mutants. The results demonstrate that AtECA3 supports Ca^{2+} -stimulated root growth and the detoxification of high Mn^{2+} , possibly through activities mediated by post-Golgi compartments that coordinate membrane traffic and sorting of materials to the vacuole and the cell wall (Li X et al 2008).

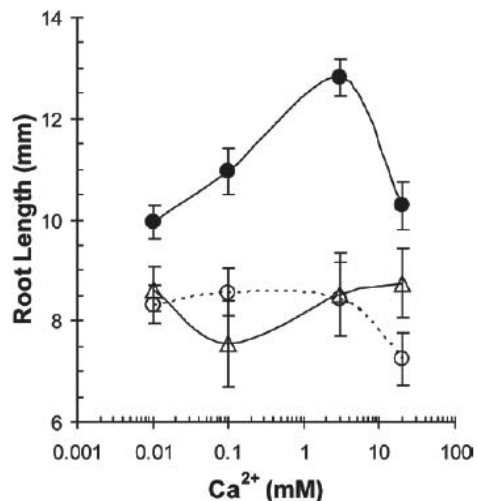


Figure 1. Ca^{2+} -stimulated root growth is abolished in *Ateca3* mutants.

Seeds from wild-type (black circles), *eca3-1b* (white circles), and *eca3-4* (white triangles) plants were germinated on plates containing half-strength MS with either no added Ca^{2+} or supplemented with Ca^{2+} to final concentrations of 0.1, 3, and 20 mM. Each experiment consisted of 20 seedlings per treatment. Primary root length was measured at 3 to 4 d. Data represent at least three independent experiments.

b) We demonstrate the first functional study of a CHX gene. CHX20 was the only member of its family to be expressed in guard cells. We provide genetic and biochemical evidence that this CHX protein plays a critical role in osmoregulation through K⁺ fluxes and possibly pH modulation of an active endomembrane system in guard cells. (Padmanaban et al. 2007).

c) We show for the first time that CHX genes affect male sterility. Pollen carrying T-DNA insertions in two CHX genes fail to produce progeny. Mutant pollen grain germinates and extends a tube, but fail to find the ovule, suggesting these transporters are involved in tube guidance and reorientation of polarized tip growth. (Lu Y & H Sze)

Science objectives for 2008-2009

We will define the cellular and biochemical bases for the osmoregulation of guard cells and for pollen tube guidance. Both processes are likely mediated by coordinated membrane trafficking, including exocytosis and endocytosis. As several CHX are localized to endosomes, we plan to determine the transport functions of selected CHX, test if they regulate intracellular pH using pH-sensitive reporters/dyes, and determine how activity is regulated. 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 guard cells and in pollen tubes.

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Energy Transduction at the Plasma Membrane of *Arabidopsis thaliana*

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Overall research goals: The plasma membrane of all plants and fungi is dominated by the function of a proton pump (H^+ -ATPase), comprised of a single $M_r=100,000$ dalton protein, which generates the electrochemical gradient that in turn drives the concentrative uptake and transport of all nutrients and other solutes. This protein is very similar to the sodium pump of animal plasma membranes. Unlike animals, however, plants and fungi do not utilize a sodium gradient at the cell surface. Instead, plants and fungi use this pump to actively secrete protons out of the cell, and because all of the other transporters (carriers and channels) are coupled to the proton electrochemical gradient rather than the sodium gradient, the plasma membrane proton pump in plants and fungi is the primary active transport system. With this unique enzyme, plants and fungi are capable of creating one of the largest electric potentials found in nature, reaching minus 300 millivolts, negative inside. The proton pump of plants and fungi consumes as much as one third of all cellular ATP, a reflection of its extreme importance, particularly in metabolically active or rapidly dividing specialized cells, such as in the meristem, phloem, root hairs and guard cells. In plants, the proton gradient is also thought to play an important role in the function of the cell wall, although there is little direct genetic data confirming this potentially critical function. The research objectives in this project are to elucidate the in situ biological roles and mechanistic function of the plasma membrane proton pump in higher plants. We are using *Arabidopsis thaliana* as a model organism and our overall approach is to use genomic technologies, including reverse genetics and site directed mutagenesis, to create and study plants in which the proton pump has altered catalytic and regulatory properties.

Significant achievements in 2006-2008: In all known plants, there is a large gene family encoding the plasma membrane proton pump and in *Arabidopsis*, this family contains eleven gene members (abbreviated AHA 1-11, for *Arabidopsis* H^+ -ATPase genes). We have focused most of our recent work on AHA's 1,2 and 3 since these collectively produce over 90% of the total pump protein in the plant. We have previously found that AHA3 is specialized for providing the proton gradient in sieve companion cells, one of the most metabolically active cells in the plant specialized for the function of long distance sucrose transport (Ref.1). This gene also provides an essential function for pollen development since homozygous knockouts are lethal for male gametogenesis (Ref.1). Although AHA1 and 2 are the most active AHA genes in terms of total RNA and protein abundance in vegetative tissue of roots, stems, flowers and leaves, single knockouts of either gene are healthy under a normal laboratory growing regime, although aberrant phenotypes can be detected under certain conditions (extremes of pH, temperature). In contrast, a plant containing null mutations in both genes has never been found, and our recent work has proven that this is because in the double mutant, embryo development is aborted at an early stage (Figure 1). We have complemented the phenotype back to wildtype by transforming the double mutant with a wildtype gene, demonstrating that it is the lack of the AHA1 and AHA2 genes that is causing lethality, rather than some secondary mutation elsewhere in the genome. This is an important result because not only is it the first genetic evidence for an essential function of this protein, but it also allows us to generate transgenic plants in which 90% or more of the AHA plants containing a protein 'tagged' with a fusion protein such as GFP or poly HIS, greatly facilitating all future biochemical work with this enzyme.

We have also been exploring the regulatory properties of this enzyme since it has been predicted to be an important target of various hormones, pathogens and other effectors that induce rapid changes in cell wall structure and function. In rapidly dividing yeast, large amounts of this enzyme can be isolated and in collaboration with Prof. Carolyn Slayman at Yale Medical School, we have made important progress this

year with an exhaustive biochemical and genetic study of all of the *in vivo* phosphorylation sites found in the protein *in vivo* with the plasma membrane proton pump of yeast (Ref.5).

Science objectives for 2008-2009: In the past two years we have published several papers that were mostly technological in nature, concerned with careful quantitation of changes in posttranslational modifications in *Arabidopsis* and fungi. The quantitative proteomic methods we have recently developed, using heavy isotopes such as ^{18}O , ^{13}C and ^{15}N are the basis for future work we will be using to quantify changes in the phosphorylation status of the pump and other enzymes. Thus, these initial studies have been very important in laying the groundwork for the technology we will be employing in the next two years, with this DOE project. With the AHA 1/2 double knockout mutants of *Arabidopsis* rescued with a translationally fused tagged version of the protein and our recent purchase and installation of an ESI-LTQ-orbitrap mass spectrometer within my lab, we are now poised to purify and characterize similarly large amounts of the plant enzyme to ensure that all possible *in planta* AHA phosphorylation sites have been identified and changes quantified in response to various hormonal and environmental effectors (e.g., pathogens). Coupling this with site directed mutagenesis followed by complementation of the AHA 1/2 double knockout mutants, we will be able to genetically confirm or refute the predicted *in vivo* roles of these phosphorylation sites as well as determine whether the resultant changes in pump activity are causally related to hormone and/or pathogen action. In addition, we are exploring the use of a conditional promoter to allow us to examine more exhaustively the function of amino acids important for biogenesis and catalytic activity. Having a tagged version of the protein will be important to help verifying that the mutant proteins are being made and targeted to the proper cellular locations.

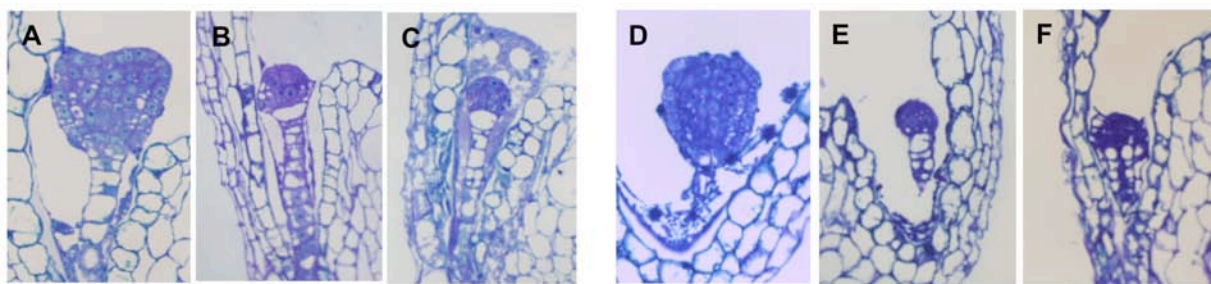


Figure 1. Phenotypic analyses of putative *aha1-6/aha1-6/aha2-4/aha2-4* embryo. (A) Normal growing embryo from self-pollinated *aha1-6/aha1-6 AHA2/aha2-4* plants. (B and C) Arrested embryos from the same silique as in (A). (D) Normal growing embryo from self-pollinated *AHA1-6/aha1-6 aha2-4/aha2-4* plants. (E and F) Arrested embryos from the same silique as in (D). Scale bar = 20 μm .

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

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Overall research goals: The research objectives are to understand the energy transduction systems of the anaerobic sulfate-reducing bacterium of the genus *Desulfovibrio*, strain G20. We will apply genetic and physiological approaches. Critical for achieving our objectives is the improvement of the genetic accessibility of the G20 strain. Once achieved, the transmembrane complexes proposed to be critical for the reduction of sulfur oxides, sulfate, sulfite, and thiosulfate, will be deleted and the mutant strains analyzed.

Significant achievements accomplished: Genetic development in the PI's lab has resulted in the application of a number of techniques to the G20 strain including conjugation, shuttle vectors, mutagenesis by plasmid insertion, and transposon mutagenesis. The PI has been involved in the genome sequencing projects for both *Desulfovibrio vulgaris* and especially G20 where she provided DNA and is the contact person for the project. The G20 strain has been deposited at both the ATCC and the DSMZ and

release awaits reclassification of the species designation to *D. alaskensis* (Wall et al., in preparation). Figure 1 is a map displaying the arrangement of ORFs maintained by my lab where gene annotations can be visualized by expanding selected sections of the genome. Clicking on a given gene or ORF opens the gene information present in the Microbesonline website (www.microbesonline.org). Our collaborator, Steve Brown, has generated oligo microarrays for this strain and we have an approved DOE EMSL proposal for proteomics analysis by high-performance mass spectrometry. Thus we are poised for a systems analysis of mutants in

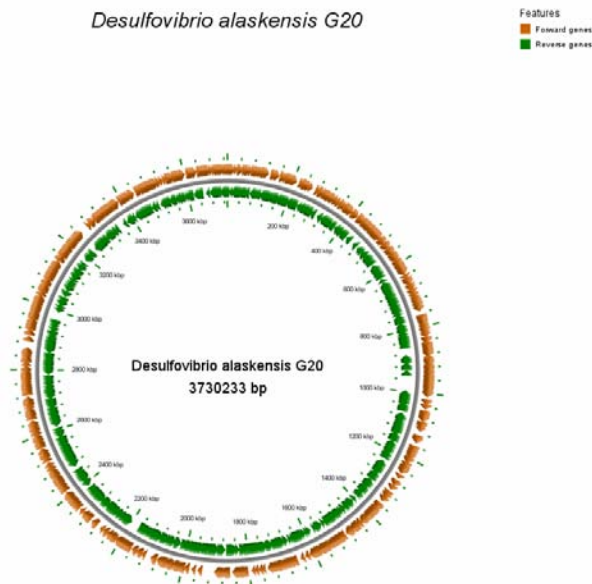


Figure 1. Diagram of the *D. alaskensis* G20 genome ORF arrangement <http://desulfovibriomaps.biochem.missouri.edu/>. This map is expandable, revealing the gene annotations currently available.

electron transport pathways.

A G20 mutant lacking the type 1 tetraheme cytochrome *c*₃, the protein accounting for over 80% of the periplasmic *c*-type cytochrome, has been constructed (designated I2) and studied for its growth capabilities. Remarkably, we have found that I2 is unable to respire sulfate if the source of electrons is pyruvate. While I2 ferments pyruvate as well or better than the wild type, the end products of the fermentation suggest that electron flow to fumarate for succinate generation is impaired in I2 and that more electrons are lost as formate and hydrogen. Preliminary proteomics data show an increase in formate dehydrogenase and CO dehydrogenase enzymes, supporting alternative routes for electron dispersal in I2. We have interpreted these results to mean that the pathway of electrons from pyruvate to cytoplasmic electron acceptors involves periplasmic cytochrome *c*₃. In contrast, respiration of sulfate by I2 with electrons from lactate is nearly wild type in rate and extent. These observations suggest that the routes of electrons from lactate and pyruvate and their delivery to various electron acceptors are distinct.

We plan to explore these pathways and their uniqueness through deletion of the genes for transmembrane protein complexes (TMCs). First to be deleted are those that have been proposed as conduits of electrons from the periplasm to sulfate and sulfite, *qmoABC* and *dsrMkJOP*, respectively. To accomplish these deletions, we are striving to improve the genetic accessibility of G20. We seek to make multiple mutations in the same strain so that the possibility of compensation by alternative isozymes can be explored. We have identified a counterselectable marker, the gene for pyrimidine salvage in *D. vulgaris*, that we have used to construct in-frame, markerless deletions that will be applied to G20.

Science objectives for 2008-2009:

- The system using the uracil phosphoribosyltransferase gene (*upp*) as a counterselectable marker for unmarked, in-frame deletions will be adapted for use in G20. A Δupp host strain will be constructed.
- Restriction systems of G20 will be sequentially eliminated.
- Deletions of TMCs will be constructed and characterization initiated.

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Phenylpropanoid metabolism in Arabidopsis: the role of REF4

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Overall research goals: We have identified a number of *reduced epidermal fluorescence* (*ref*) mutants of Arabidopsis with alterations in the sinapate esters found in leaves. The focus of this study is the *ref4* mutants which exhibits substantial reductions in the levels of all phenylpropanoids, including flavonoids, sinapate esters, and lignin, suggesting that REF4 is essential for normal secondary metabolism and cell wall biosynthesis. We have cloned the *REF4* gene and have found that it encodes an apparent integral membrane that belongs to a novel, plant-specific family. The Arabidopsis genome encodes one additional member of this family that we have named *RFR1* for *REF4-Related1*. The objectives of our work is to analyze knock out and site-directed mutants of *REF4* and *RFR1*, identification of genes involved in the *ref4* phenotype, and identification of REF4-interacting proteins.

Significant achievements in 2007-2008: We have generated a number of essential basal constructs with which to complete this objective. First we have generated *REF4* and *RFR1* entry clones for site directed mutagenesis and subsequent Gateway based binary construct generation. Second, we have generated *REF4* promoter and *RFR1* promoter binary Gateway constructs that will be used to drive expression of site-directed mutant *REF4* and *RFR1* genes. Wild-type versions of these constructs have been generated, and site directed mutagenesis is underway.

To most effectively identify the genes that are mis-regulated in the *ref4* mutant, we have established a collaboration with Alan Qi, a new faculty member in the Departments of Computer Science and Statistics at Purdue University. An exciting result of this new collaboration is that Dr. Qi was successful in obtaining funding from Microsoft Corporation to develop new algorithms for the identification of gene networks using preliminary data on the *ref4* mutant.

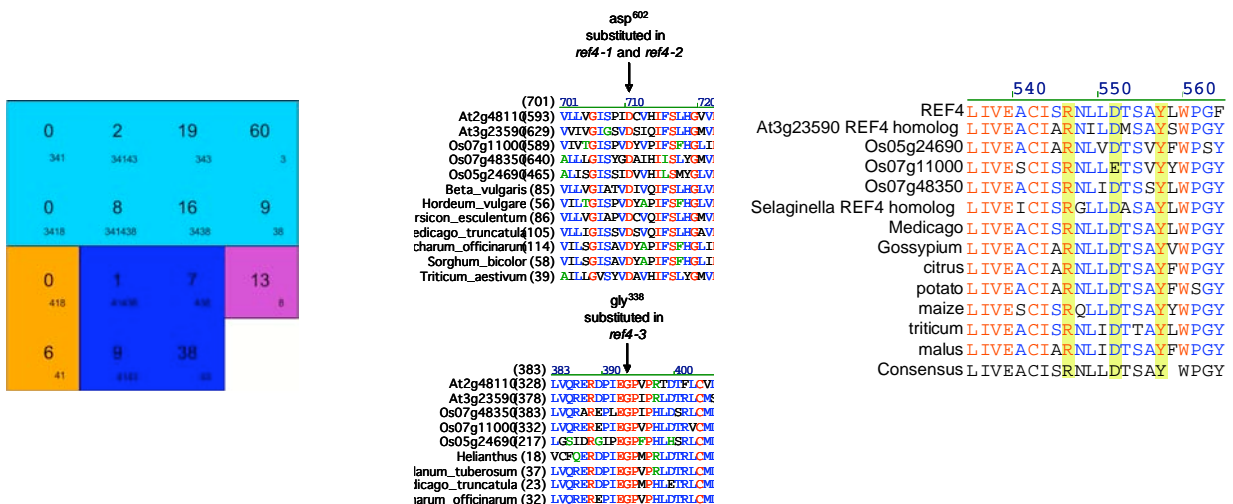


Figure 1 (left). Venn-type diagram representing the number of genes called present by Affymetrix software in wild-type, *ref3*, *ref4-1*, *ref4-3* and *ref8* plants that are up-regulated in the mutants relative to the wild type. Squares are coded to represent the mutants, or combinations thereof, in which the given number of genes is up-regulated. Thus, the squares designated 41, 43, and 4143 represent the number of genes up-regulated in *ref4-1*, *ref4-3* and both *ref4-1* and *ref4-3*, respectively. Figure 2 (center) Alignments of portions of the REF4 and homologous protein sequences near the *ref4* mutations. Figure 3 (right). Alignments of the conserved portions of the REF4 and homologous protein sequences that contain the putative tyrosine phosphorylation sequence RX3D/EX3Y.

We have completed a two-hybrid screen for proteins that interact with REF4. Predicted non-membrane-spanning domains of REF4 were fused to the Gal4 DNA-binding domain and used as bait using the Invitrogen Gateway-based two hybrid system. Although potential interacting partners were identified, none of these passed the rigorous tests that are designed to eliminate false-positives. As an alternative approach, we have generated the constructs necessary for REF4 to be screened using the split-ubiquitin yeast 2-hybrid assay. This analysis is being conducted collaboratively in the lab of Dr. Wolf Frommer at the Carnegie Institute of Washington at Stanford University who is funded by an NSF 2010 project to determine the membrane protein interactome (<http://www.associomics.org/>).

Science objectives for 2008-2009:

- Our microarray analysis (Fig. 1) suggests that the decreases in lignin, flavonoids, and sinapate esters in the *ref4* mutants may be the result of the increased expression of pathway repressors (AtMYB4 and possibly AtMYB7) and decreased expression of pathway activators (AtERF108). Our data also indicates that at least seven additional genes are uniquely up-regulated in the *ref4* mutants, but it is not clear whether their enhanced expression is a result of, or a cause of, the *ref4* phenotype. To test the hypothesis that over-expression of AtMYB4 is critical for phenylpropanoid pathway down-regulation in *ref4*, we will generate double mutants between *ref4-3* and AtMYB4 loss-of-function alleles. Similar experiments will be conducted using T-DNA insertional alleles of the other genes up-regulated in both *ref4* mutants to determine whether they are required for *ref4* phenotypes, and whether mutations in these genes independently lead to phenotypes observed in *ref4* mutants, or any other phenotypes of interest.
- Mutations corresponding to the D602N and G338S substitutions in our dominant *ref4* mutants (Fig. 2) will be engineered into the RFR1 ORF by site-directed mutagenesis, and introduced into the *rfr1-1* knock-out line under the control of the *RFR1* promoter. If the resulting plants display *ref4* phenotypes, we would conclude that *RFR1* likely acts in the same genetic pathway as *REF4*; whereas, if the phenotype of the transgenics is distinct from those of *ref4* plants, they may shed light on the pathways in which the *REF4* homologue is involved.
- REF4, RFR1 and putative orthologs from a range of species contain a conserved 22 amino acid sequence which contains an absolutely conserved tyrosine phosphorylation consensus motif RX3D/EX3Y (Fig. 3). To test the hypothesis that tyrosine phosphorylation regulates or is required for REF4 function, we will generate wild-type and *ref4* mutant alleles in which this tyrosine is mutated to a phenylalanine residue. If phosphorylation at this site is required for the activity of the dominant *ref4* alleles, we would predict that transformation of wild type or *ref4-4* knock out plants with constructs containing the Y491F and D602N (*ref4-1* and *ref4-2*) or G338S (*ref4-3*) mutations would not lead to phenylpropanoid phenotypes. Conversely, if phosphorylation of REF4 is required to negatively regulate REF4 activity, we might expect that the double mutant alleles would be even stronger suppressors of phenylpropanoid metabolism.

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Quantitative Analysis of Carbon and Nitrogen Allocation in Developing Seeds

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Overall research goals: Although quite abundant data on the genetic and molecular makeup of plants are available, the understanding of the functioning of plants at a molecular level is still limited. For example, in developing seeds which produce storage compounds, the mechanisms that govern partitioning of common carbon and nitrogen precursors into the synthesis of storage oil, protein or starch are still an enigma. With such knowledge, plants could be manipulated to produce more of a desired storage product. The overall goal of this project is to understand the simultaneous formation of seed storage products in the oil crop *Brassica napus* (also called rapeseed, canola) at the level of the biochemical reaction network of central metabolism from a systems biology point of view. This includes construction of computational models of plant cellular metabolism and validation of these models with experimental data. In order to model and understand how carbon precursors are processed by growing seeds into different storage products, embryos are cultured under different nutritional conditions that allow for storage synthesis. Parallel measurement of metabolic fluxes, extractable activity of multiple enzymes of central metabolism and of metabolite levels provides the metrics necessary to characterize the adaptation of central metabolism to different nutritional conditions.

Significant achievements in 2006-2008: By feeding ^{13}C -labeled isotope tracers (glucose) to canola embryos growing in culture, metabolic fluxes were measured dependent on different nitrogen sources present in the culture medium. By comparison of the different resulting flux distributions, re-routing of carbon flow was observed (Figure1), documenting the metabolic adjustments taking place to use the different nitrogen sources for protein production in the seed. In parallel, 21 enzyme activities related to central metabolism were determined. In general, the changes in activities of enzymes of central metabolism were small. At the same time all the measured enzyme activities were higher by up to three orders of magnitude than the measured associated *in-vivo* net fluxes (Junker *et al.*, 2007). Therefore it is unlikely that genetic top-down regulation has the main control over the observed metabolic adjustments. It rather appears that metabolic regulation, i.e. self-regulation of the biochemical reaction network, is sufficient to accomplish the flux adjustments seen in Figure 1. In conclusion, the experimental approach described here proved to be very well suited for development of predictive models of plant / seed central metabolism.

Science objectives for 2008-2009:

- Additional flux, metabolite and enzyme profiles will be measured.
- In an integrative approach, using the flux maps, metabolite levels, and enzyme kinetic data, we will construct and validate kinetic models, able to predict the behavior of the system upon change in environmental conditions or due to genetic perturbation.
- Flux analysis of seeds of different *A. thaliana* mutants severely impaired in seed storage formation.
- A stoichiometric model of metabolism will be used to explain the behavior of central metabolism in terms of optimality.
- Based on the models, strategies for genetic engineering of carbon partitioning in seeds will be generated and tested.

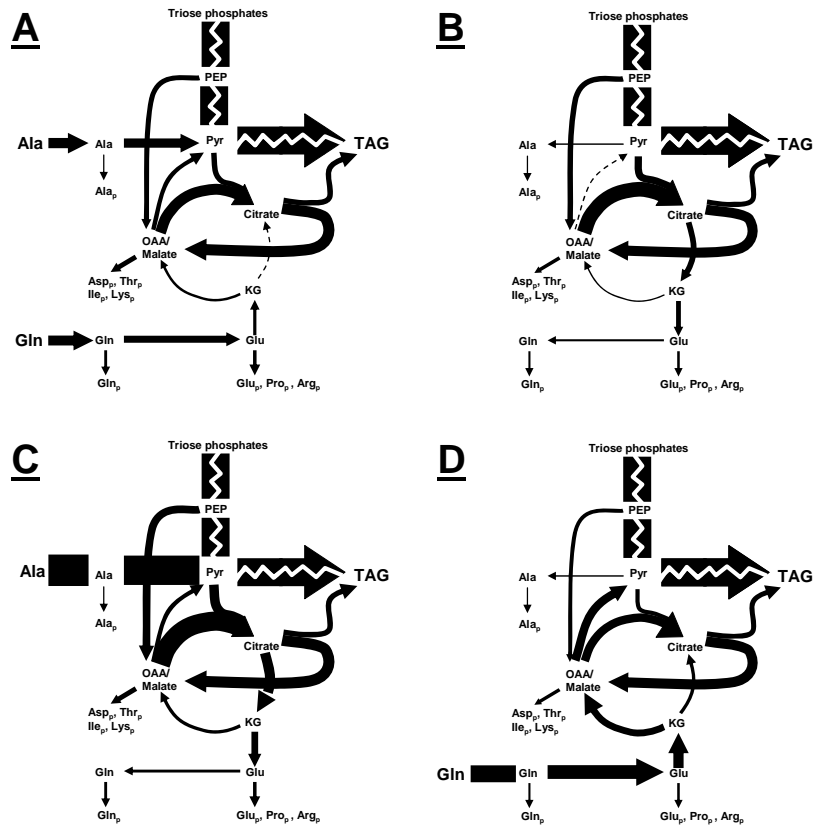


Figure 1. Visualization of net Fluxes related to lower glycolysis and the citrate cycle in developing *B. napus* embryos during formation of storage lipids (TAG) and storage proteins (represented by Ala_p, Asp_p, ... Thr_p). Fluxes were determined by flux parameter fitting after steady state labelling with ¹³C-labeled glucose: (A) Embryos grown with Gln/Ala as nitrogen source and labeled with a combination of several ¹³C- and ¹⁵N- labeling experiments (flux values taken from Schwender *et al.*, 2006). Embryos were also grown with NH₄NO₃ (B), alanine (C) or glutamine (D) as sole nitrogen source. Arrow thickness is scaled to net carbon flux, except for fluxes between triose phosphates, PEP, Pyr and FAS for which arrow thickness is downsized about 5 times. TAG, triacylglycerol; PEP, phosphoenol pyruvate; pyr, pyruvate; OAA, oxaloacetate; KG, ketoglutarate. The index **p** denotes protein-bound amino acids.

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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 the domain *Archaea*, one of the least characterized in terms of the biological networking systems used to respond to the environment. Recent advances in systems biology reveal post-transcriptional mechanisms are important in modulating protein levels as archaeal cells respond to environmental cues. Regulatory proteases, such as proteasomes, are likely to be central to the quality control and regulated turnover of proteins during these cellular responses. The objectives of this research project are to identify native protein substrates that are specifically targeted for turnover by regulatory proteases and, ultimately, elucidate the roles these proteases play in archaeal cell physiology.

Significant achievements in 2006 – 2008:

Using the extremophilic haloarchaeon *Haloferax volcanii* as a model, recent evidence in our lab suggests protein phosphorylation plays an important role in proteasome-mediated substrate recognition and/or turnover in archaea, where ubiquitin is absent. This evidence has led us to perform a comparative analysis of the proteomes and, more specifically, the phosphoproteomes of wild-type and isogenic proteasome mutant strains of this archaeon. Comparative proteomic analyses have also been conducted using wild-type cells treated with an irreversible inhibitor of proteasomes, *clasto*-lactacystin- β -lactone (*cL* β L). Thus far, a sizeable list of over 100 putative substrates of proteasomes have been identified using various methods of phosphopeptide and phosphoprotein enrichment (*i.e.* fluorescent affinity labeling of *O*-modification sites, immobilized metal affinity chromatography, and metal oxide affinity chromatography) coupled with multidimensional protein identification technology (MudPIT), 2D-PAGE, and tandem mass spectrometry (ESI-QTOF and LTQ linear ion trap MS/MS). Many of the differential proteins identified were related in primary structure to those functioning in: phosphate assimilation, polyphosphate biosynthesis, translation, cell division, DNA replication and repair, protein phosphorylation, signal transduction, and central metabolism [*e.g.* dihydroxyacetone kinase- (DHAK-) linked phosphoenolpyruvate: protein phosphotransferase (PTS) system]. A number of phosphosites were also identified with supporting MS/MS spectra, including those reproducibly exclusive or more abundant in a proteasome-activating nucleotidase A (*panA*) mutant (*e.g.* pyruvate kinase, Cdc6, Cdc48). Pulse-chase was performed with custom-made antibodies to determine the half-lives of a subset of differential proteins in wild-type and proteasome mutant strains. Using this approach, proliferating cell nuclear antigen (PCNA) was shown to be stabilized by deletion of the *panA* gene with an increase in protein half-life from less than 10 min to over 1 h. This combined use of global methods (proteomics, transcriptomics) with traditional biochemical approaches has and will continue to provide greater insight into the roles proteasomes play in the archaea.

Science objectives for 2008 – 2010: The proposed DOE renewal remains focused on our **long-term goal** to identify protein substrates and pathways regulated by *Hfx. volcanii* proteasomes. To achieve this goal, the following **specific tasks/aims** will be performed:

- global transcript levels of proteasome (*panA*) mutant, cLβL-treated, and wild type cells will be compared using high-density microarrays recently developed and optimized for *Hfx. volcanii*,
- half-lives and phosphorylation status of candidate substrate proteins will be determined using substrates defined via comparison of the proteomic and microarray studies, and
- transcriptome- and proteome-guided phenotypic and biochemical analyses of proteasome mutant and wild type cells will be performed. This latter task will include: a) determining the global rate of protein synthesis and degradation as well as overall oxidation and aggregation state of proteins, b) determining the level and types of polyphosphate polymers, c) assessing the sensitivity of cells to osmotic, oxidative and DNA damaging stresses and the types of proteins interacting with proteasomal components under these conditions, and d) evaluating carbon flux through and interacting components of a proposed DHAK-linked PTS system. Isogenic proteasome mutant strains available include: *panA*, *panB*, *psmA* ($\alpha 1$), *psmC* ($\alpha 2$), and *psmC panB*.

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Regulation of Actin Filament Ends: The Role of Capping Protein in Plant Growth and Lipid Signaling

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Overall research goals: Our major research objective is to understand whether the heterodimeric capping protein (CP) is a major regulator of actin filament dynamics in plant cells. Specifically, we will test the hypothesis that AtCP is responsible for maintaining a large pool of unpolymerized actin subunits, by preventing the assembly of profilin-actin complex, and functions to limit the dynamic behavior of actin filaments. We will also test whether this filament end-binding protein is regulated by specific phospholipids, like phosphatidic acid.

Significant achievements in 2006-2008: A reverse-genetic approach, taking advantage of T-DNA insertion lines available in the community, has been initiated. *CPA* and *CPB* are single genes in Arabidopsis. Six insertion lines, representing putative disruptants for both subunits of the CP heterodimeric complex were obtained from ABRC. These were genotyped to verify the insertion and backcrossed 1 or 2 times. Initial characterization of the homozygous mutant plants indicated that several disruptants (e.g. *b1*, *b3*) had modest effects on seed germination and root growth. Immunoblotting with affinity-purified antisera against both subunits suggested a marked reduction in protein levels for several of these lines (e.g. *a1*, *b3* and *b1*). Because of the low levels of endogenous CP heterodimer, we can not be certain that we have eliminated or reduced levels of CP below 10% of wild-type. Methods are being developed to accurately determine CP protein levels in wild-type and homozygous mutant *cp* lines, using quantitative immunoblotting. However, RT-PCR of these lines suggests that none of them are true knock-outs. Consequently, we decided it would be prudent to construct double mutants, disrupted for both subunits (A and B, or α and β) of the heterodimer to further reduce levels of functional protein. Doubles containing the *a1*, *b1* and *b3* alleles have been constructed, genotypes verified by PCR, and transcript levels quantified. The *a1;b3* and *a1;b1* doubles have transcript levels for each subunit that are reduced by 50–80% compared to wild-type and the β subunit protein appears to be completely absent on semiquantitative immunoblots. Both doubles (as well as the singles) are being analyzed for defects in actin-based function, like cell and tissue expansion, cell morphogenesis and response to biotic and abiotic stimuli. In parallel, we are generating overexpression lines that produce both subunits as well as putative dominant-negative constructs missing the actin-binding C-terminal 'tentacle'.

Work is also underway to test whether the *cpa/cpb* double mutants have altered cytoskeletal organization or actin filament levels. Our prediction is that actin filament levels will increase in these lines due to the availability of actin filament ends for polymerization from the huge pool of profilin-actin. This is being examined by localization of actin filaments with rhodamine phalloidin or anti-actin antibodies and confocal fluorescence microscopy. If semi-quantitative differences in average pixel intensity are observed, we will adapt our previous method for bulk analysis of actin filament levels on intact tissues using fluorescent phalloidin and the fluorimeter. Finally, GFP-fABD2, the best available reporter of actin filament in live plant cells, has been crossed into the *cpa/cpb* mutant lines. We have recently adapted a novel imaging technique, variable-angle epifluorescence microscopy (VAEM), and demonstrated that it is ideally suited for examining the dynamic behavior of cortical cytoskeletal events in live plant cells, particularly the epidermal cells of hypocotyls. Timelapse images of single actin filaments reveal that they have a chaotic or random organization, as opposed to the transversely-oriented and parallel arrays of microtubules in the same cells visualized with GFP-TUB5. Moreover, the dynamic behavior of filaments is incredibly fast,

with polymerization rates at the barbed ends of 1.7 $\mu\text{m}/\text{sec}$ and prolific severing activity rapidly destroying newly-created filaments. Consequently, most individual filaments have lifetimes $< 30\text{s}$, whereas bundles appear to be rather stable. We believe that such single filament dynamics have only rarely been observed in any eukaryotic cell. Moreover, the stochastic dynamics of single filaments is not consistent with the prevailing view of actin filament turnover by treadmilling. Pertinent to CP protein function, we observe that very few ends (one or two events for every thousand seen) grow after they have been severed. This suggests that newly-created ends are rapidly capped following severing. We predict this is due either to abundant capping protein or the activity of a villin, which remains bound to filament barbed ends subsequent to severing. Single actin filament dynamics in *cpa/cpb* double mutants will be quantitatively examined using VAEM and the GFP-fABD2 reporter.

Science objectives for 2008-2009:

- Expand the analysis of actin filament dynamics *in vivo* to relevant mutant backgrounds to test molecular mechanisms underpinning rapid growth rates, high severing frequency and capping of available filament ends.
- Adapt the existing TIRFM platform for multicolor, fast (ms) image acquisition and analysis of actin filament interactions with other key components of the cortical cytoplasm (e.g. microtubules, ER, secretory vesicles).
- Continue the reverse-genetic and phenotypic analysis of CP insertion lines, as well as overexpression and dominant-negative plants.
- Identify the subcellular localization of CP with antibodies, fluorescent fusion protein reporters and subcellular fractionation.

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- 7.

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

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Overall research goals: The research goals of this project are to use high-resolution structural information to provide an understanding of the structure, mechanism and specificity of the starch biosynthetic enzymes.

Significant achievements in 2006-2008: The lab is working on all three of the enzymes required for the biosynthesis of starch in plants and glycogen in bacteria, as the pathway is the same. The three enzymes are ADP-glucose pyrophosphorylase, which is responsible for the formation of the ADP-glucose building blocks from ATP and Glucose-1-phosphate; Glycogen or starch synthase, which elongates the starch or glycogen chain by adding glucose units from ADP-glucose to the 4-position of the growing starch chain; Branching enzyme, which breaks the α -1-4-linkages and re-adds them to the 6-position of the starch or glycogen molecule to produce the very important branching chains. Our group published the 1st structure of an ADP-glucose pyrophosphorylase and the 1st structure of a branching enzyme. We have recently determined the structure of *E. coli* glycogen synthase (EcGS) in its catalytically active, closed conformation that for the 1st time shows the bonafide active site of the enzyme. We have also determined the structure of EcGS bound to ADP, glucose and the sugar acceptor mimic Heppso. We have also determined the structure of EcGS in its open conformation when not bound to any substrates. We have in addition determined the structure of a catalytically inactive EcGS mutant (E377A EcGS) bound to a malto-oligoside. This structure shows how a glycan acceptor binds the active site of a glycogen or starch synthase. Together these structures provide a detailed picture of many of the steps of the starch or glycogen synthase catalytic cycle. We are able to make significant insights into the mechanism and specificity of the enzyme as well.

We have very recently also determined the structures of branching enzyme bound to several oligomaltoside substrates. These structures identify completely novel glycan binding sites on the surface of the enzyme that are likely involved in determining the specificity of the branching enzymes. Three distinct glycan binding sites have been identified, some as far as 20 Å from the active site of the enzyme. Together, these structures give us critical information regarding the role of Branching Enzyme's surface in its specificity and activity.

We are also making progress in our efforts to determine the structure of potato tuber ADP-glucose pyrophosphorylase in its catalytically active conformation. We are developing an improved protocol for the overexpression and purification of the enzyme using a novel expression vector we are developing that combines the utility of the Sumo protein tag for specific cleavage with the superior expression and solubility of the maltose binding protein. This will allow us to express higher levels of the pyrophosphorylase and to remove the purification tag using the very specific and active Sumo Protease. Though we have produced crystals of pyrophosphorylase in the absence of its inhibitor sulfate or phosphate, We have been hampered by the low expression levels of the protein using older methods. The new system has significantly improved the expression of a number of proteins in our lab and we are hopeful that it will significantly help in the production of Potato tuber Pyrophosphorylase.

Science objectives for 2008-2009:

- Crystallize and determine structures of a number of EcGS mutants that will add further to our mechanistic insights into the enzyme. These include the E377Q mutant which is completely inactive even though Gln is isosteric with Glu and would seem to be able to provide much of the function of the Glu in the structure. We will also determine structures of the wild-type enzyme bound to malto-oligosaccharides, as we are able to soak these molecules into our crystals. We will attempt to produce intermediates using quick soak/freeze protocols similar to work done for other similar enzymes. This may allow us to produce a well-resolved intermediate in the active site of the enzyme.
- With significantly higher amounts and better purified, Potato ADP-glucose pyrophosphorylase in hand, we will improve our crystals and determine the structure of the enzyme in the absence of the allosteric inhibitors Sulfate and phosphate. This will provide us with critical information into the nature of the allosteric regulation of the enzyme.
- We will make mutants of Branching enzyme based on the structures we have determined to understand the effects our glycan binding sites have on the activity and specificity of the enzyme. We will also continue to work on crystallizing a full-length branching enzyme and a branching enzyme from a plant source.

References to work supported by this project 2006-2008(style=Stand alone text heading):

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CBL10 alternative splicing regulates salt tolerance in Arabidopsis

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Overall research goals:

Calcium ions (Ca^{2+}) have emerged as an essential component of many pathways in plants, underlying growth and development by linking perception of physiological and environmental cues to cellular responses. Models for specificity in Ca^{2+} -mediated signaling suggest it may reside in the array of molecules that sense alterations in Ca^{2+} levels (Ca^{2+} sensors). We are continuing our studies to understand how one member of the Calcineurin B-Like (CBL) family of Ca^{2+} sensors in Arabidopsis, CBL10, interprets changes in cellular Ca^{2+} levels and participates in the generation of specificity during plant growth in saline conditions.

Significant achievements in 2006-2008: Several CBL family members are involved in signal transduction in response to environmental stresses. To determine if CBL10 is important for the response of the plant to salt stress, growth of a CBL10 mutant (*cbll0-1*) was characterized in the presence and absence of NaCl. Roots of *cbll0-1* did not show any reduction in growth in response to NaCl. However, growth of aerial portions of mutant plants was reduced and leaves developed chlorosis at concentrations of NaCl as low as 100 mM. The severity in the reduction in leaf growth and of the chlorosis increased with higher concentrations of NaCl. When *cbll0-1* was transformed with a construct containing the CBL10 promoter and whole genomic fragment, a wild-type response to salt was restored indicating that disruption of full-length *CBL10* expression is responsible for the salt-sensitive phenotype in the *cbll0-1* mutant. These results suggest that CBL10 serves as a positive regulator of salt tolerance in aerial portions of the plant.

When CBL10 expression was determined in wild-type Arabidopsis grown in control conditions (no NaCl) using RT-PCR, an additional band appeared above the expected size of the *CBL10* cDNA. The bands were isolated and cloned and cDNAs were sequenced. A variant of *CBL10* mRNA with a retained intron was found and was analyzed based on its deduced amino acid sequence. This cDNA (*CBL10LA*) contains a premature termination codon likely resulting in a truncated protein with one less Ca^{2+} -binding motif (EF-hand) than the full-length CBL10 protein. The expression of *CBL10LA* appears to be significantly reduced during salt stress. Based on the response of wild-type Arabidopsis to growth in salt and the salt-sensitive phenotype of the *cbll0-1* mutant, we have proposed the following working model: in control conditions, *CBL10LA* is present and interacts with but does not activate a downstream target of CBL10, the protein kinase Salt-Overly-Sensitive2 (SOS2); the result is a reduction in the effective concentration of *CBL10*. During salt stress, *CBL10LA* is degraded so that *CBL10*, the major transcript that remains, encodes a full-length, functional protein. The full-length CBL10 protein then interacts with SOS2 to initiate a response to salt stress.

Science Objectives for 2008-2009: We will focus our studies on the following experiments to test our model.

1. As a direct test of our model, we have generated wild-type plants over-expressing *CBL10LA* (*CBL10LA* under the control of the Cauliflower Mosaic Virus 35S promoter). Our hypothesis is that over-expression of *CBL10LA* will result in the continued production of the truncated CBL10 protein

in wild type leading to a reduction in the ability of the plant to respond to salt and to increased salt sensitivity. We have tested twenty independent transformed lines and 17 show increased sensitivity to salt when grown on 200 mM NaCl. Preliminary experiments examining *CBL10* expression in several of the lines suggests that over-expression of *CBL10LA* does not induce silencing of the endogenous *CBL10* gene. Experiments will be performed to confirm *CBL10* expression in the transformed lines and to verify *CBL10LA* overexpression.

2. Another important prediction of our model is that *CBL10LA* does not code for a functional protein. To test this, we have taken *CBL10* cDNA encoding the full-length CBL10 protein and *CBL10LA* cDNA under the control of the *CBL10* promoter and transformed them into the *cbll0-1* mutant. Phenotypic assays will be performed to determine if: (1) full-length *CBL10* cDNA is sufficient to confer salt tolerance and (2) if *CBL10LA* can complement the salt-sensitivity of *cbll0-1*. If, as we expect, full-length *CBL10* alone is able to fully complement the mutant phenotype and *CBL10LA* is unable to do so, the conclusion that *CBL10LA* likely serves to regulate CBL10 activity will be further supported.

3. One method that has been used to demonstrate Arabidopsis *CBL10* function involves a yeast Na⁺ transport mutant that is unable to grow on media with salt due to an inability to remove Na⁺ from its cells. When *CBL10* was expressed in this mutant in combination with the Arabidopsis proteins SOS1 (a plasma membrane Na⁺/H⁺ exchanger) and SOS2, the SOS pathway was activated; SOS1 transported Na⁺ out of the yeast cells and growth of the yeast on media with salt was restored. We are collaborating with Dr. José Pardo (IRNASE/CSIC) to determine if CBL10LA interferes with CBL10 activity by introducing *CBL10LA* into these yeast Na⁺ transport mutants in combination with *CBL10*, *SOS2*, and *SOS1*. Our model is that CBL10LA will compete with CBL10 for interaction with SOS2, reducing the effective concentration of CBL10, preventing activation of the SOS pathway and preventing yeast growth on salt.

4. As part of our studies to understand how CBL10LA regulates CBL10 activity, we have performed yeast two-hybrid assays with CBL10, CBL10LA and SOS2. These studies indicate that both CBL10 and CBL10LA are able to interact with SOS2. In vitro pull down assays will be performed to verify this interaction.

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Control of energy metabolism and carbon catabolite repression in the bacterial nitrogen-fixing symbiont *Sinorhizobium meliloti*

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Background: The industrial production of nitrogenous fertilizers is energetically expensive, and the wide-spread application of fertilizers to agricultural sites often has far-reaching, negative, impacts on the environment. Problems include eutrophication, the formation of anoxic dead-zones in estuaries, and the formation of reactive nitrogen species and greenhouse gases. Biological nitrogen fixation provides a way to lessen dependence on industrially produced nitrogenous fertilizers. Many agriculturally important legumes are able to fix nitrogen in conjunction with symbiotic nitrogen-fixing bacteria (Rhizobia) from the genera *Sinorhizobium*, *Rhizobium*, *Bradyrhizobium* and *Mesorhizobium*. Intercropping using legumes, and using legumes to amend soil or provide “green fertilizer”, can lessen the environmental impacts associated with application of industrial ammonia-based fertilizers.

Overall research goals: Those Rhizobial activities which are not uniquely required to establish a productive symbiosis, but are still important for symbiosis, are research areas of on-going interest. Our work is designed to further understanding of one of these key processes: global control of gene expression and physiology by succinate-mediated catabolite repression (SMCR). Rhizobia are able to utilize a large variety of compounds for growth, but succinate, and other C₄-dicarboxylic acids, are important carbon sources during both free-living and symbiotic states. C₄-dicarboxylic acids are used to fuel nitrogen fixation, they support high growth rates, and they are used in preference to many other (secondary) carbon sources. In bacteria, catabolite repression controls carbon utilization patterns and many other physiological traits including cell division, virulence and biofilm formation. Our long term goal is to identify how SMCR is established in *S. meliloti* and to identify the molecular mechanisms that connect SMCR to global regulation of gene expression and physiology.

Progress toward understanding SMCR:

- 1. We have developed tools and protocols which allow the study of SMCR.** Raffinose and lactose are transported into *S. meliloti* by proteins encoded by the *mela-agp* and *lac* operons respectively. These operons, tools derived from them, and the protocols developed to use them, have been instrumental in dissecting SMCR.
- 2. We have shown that SMCR operates, in part, by preventing secondary carbon sources from entering the cell (inducer exclusion).**
- 3. We have shown *S. meliloti* uses an incomplete PTS system to regulate SMCR.** Phosphotransferase systems (PTS) transport sugars and regulate gene repression in *E. coli* and many other well-studied bacteria. *S. meliloti* is missing genes required to assemble a complete PTS. Even though the partial PTS of *S. meliloti* cannot transport carbon sources, it has been retained as a signal transduction system. We have shown through

genetics and physiological experiments that the PTS regulates: SMCR, other aspects carbon metabolism, exopolysaccharide synthesis and cell viability.

4. We have shown that Hpr kinase (HprK) regulates SMCR and symbiotic function.

The protein kinase HPrK, phosphorylates the PTS protein Hpr, and regulates global gene expression and physiology in many Gram positive bacteria, but is lacking in most Gram negative bacteria. It is, however, encoded in the genome of *S. meliloti* and other α -proteobacteria. We have found that in *S. meliloti* HprK is an important regulator of SMCR. *hprK* mutants exhibit abnormally strong catabolite repression, altered expression of carbon utilization genes and very slow growth. In addition, *hprK* mutants can induce and occupy root nodules, but cannot fix nitrogen.

5. We have identified a 2-component system that regulates catabolite repression and energy metabolism. A genetic screen designed to isolate mutants defective in SMCR identified the gene *sma0113* as a regulator of SMCR in *S. meliloti*. *sma0113* encodes a histidine kinase with five PAS domains. The gene immediately downstream, *sma0114*, encodes a CheY-like response regulator. Deletion mutants of *sma0113* show a relief of catabolite repression, compared to wild-type. Dye reduction experiments with *sma0113* mutants show that *sma0113* mutants have excess reducing capacity during exponential growth. We believe that this 2-component system may use sensory information about redox levels, or energy state, gathered from the PAS domains of Sma0113 to control phosphorylation of Sma0114, which in turn regulates catabolite repression, directly or indirectly, via protein-protein interactions.

6. Protein purification. We have cloned and His-tagged: the PTS proteins of *S. meliloti*, Sma0113 and Sma0114. We have also constructed site-directed mutants of many of these.

Future work

1. Biochemistry of phosphotransfer using purified components of the PTS and the Sma0113/114 pair.
2. Structural characterization of PTS proteins and the Sma0113/0114 pair
3. Biochemical and genetic analysis of the 5 tandem PAS domains of Sma0113.
4. Understand the connection between redox/energy metabolism and Sma0113/0114.
5. Identification of downstream partners of Sma0114 and the key PTS protein Hpr.

Papers

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- Garcia, P.P., Bringhurst, R.M., and D. J. Gage (2008). Characterization of a two-component regulatory system which regulates succinate-mediated catabolite repression in *Sinorhizobium meliloti* (submitted)
- Arango-Pinedo, C., Bringhurst, R.M. and D. J. Gage (2008). *Sinorhizobium meliloti* mutants lacking phosphotransferase system enzyme HPr or EIIA are altered in diverse processes, including carbon metabolism, cobalt requirements, and succinoglycan production. *J. Bacteriol.* 190:2947
- Cardon, Z. G. and D. J. Gage (2006) Resource exchange in the rhizosphere – molecular tools and the microbial perspective. *Ann. Rev. Ecology, Evolution and Systematics* 37: 459-488
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Plant Response to LCO/CO Signals

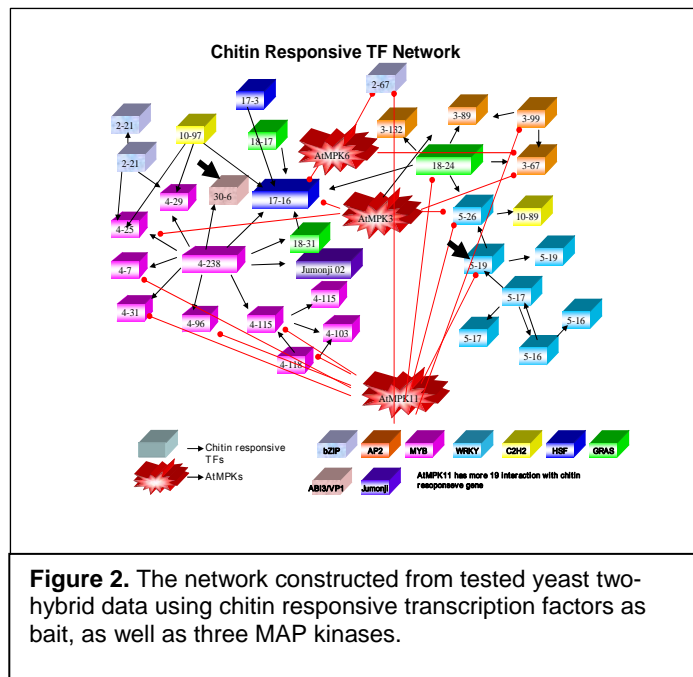
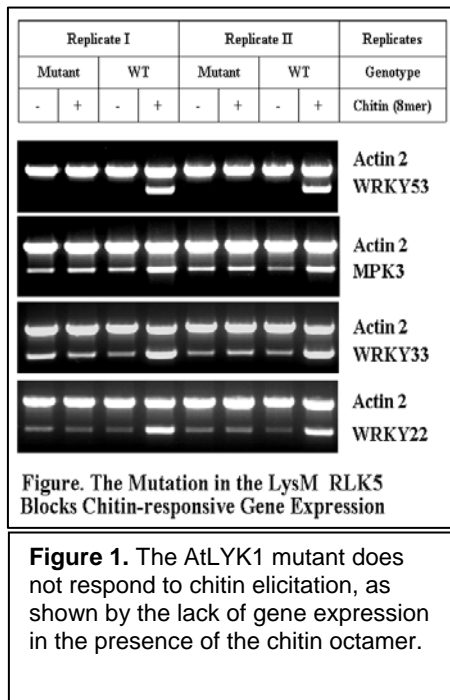
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Overall research goals: Chitoooligosaccharide (CO) signals, produced by pathogenic fungi, trigger a strong plant defense response. In contrast, structurally similar lipo-chitoooligosaccharide (LCO) signals, produced by beneficial symbiotic bacteria, 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. The proposed research relates to the mission of DOE since it impacts plant yield and biomass. Elicitors also play a major role in modulating the expression of many plant secondary product pathways, some of



which have important uses. The work is also pertinent to an understanding of the infection mechanism of rhizobia on legume plants leading to biological nitrogen fixation. The work is also relevant to understanding and controlling symbiotic (i.e., mycorrhizal) and pathogenic fungal infection of plants.

Significant achievements in 2006-2008: In the past year, we made a major breakthrough by identifying the AtLYK1 (LysM RLK) as the major chitin receptor in Arabidopsis (Wan et al., 2008). This now opens the way for a full analysis of the chitin signaling pathway.

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 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.

We screened mutants defective in each of the five LysM RLK genes in the Arabidopsis genome. Three of these genes were shown to be significantly induced by chitin, but mutations in only one of these genes (*AtLyk1*) led to loss of the response to chitin elicitation (Figure 1). As would be expected, loss of the chitin response also led to increased susceptibility to fungal infection. AtLYK1 is only the third pattern recognition receptor (PRR) identified in plants. The other two PRRs are both leucine-rich repeat receptor-like kinases (LRR RLKs). Therefore, our findings also add a new class of proteins to the family of PRRs.

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 2). In addition, we showed direct interaction between some of these transcription factors and MAP kinases known to mediate the chitin response. Y2H experiments are also being used to define interacting partners with the AtLYK1 protein. Mutants in these interacting partners are showing the expected phenotypes of altered response to chitin elicitation and fungal infection. The net effect of this work will be define in full detail the signalling pathways operable during chitin elicitor action.

Science objectives for 2008-2009: 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:

- Confirm using other methods (such as co-immunoprecipitation) the protein-protein interactions identified by yeast two hybrid methods
- Examine the phenotypes of plant mutants defective in those genes identified using Y2H methods. This will include an examination of chitin and fungal response.
- Examine the role of *AtLyk1* orthologues in selected crop plants.

References to work supported by this project 2006-2008:

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Hsp100/ClpB Chaperone Function and Mechanism

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Overall research goals: The proposed research will use genetic and molecular approaches to investigate the Hsp100/ClpB proteins, including studies of both cytosolic Hsp101 and chloroplastic ClpB-p, using the model plant *Arabidopsis thaliana*. The ultimate goal is to define mechanistically how these chaperones influence plant growth, development, stress tolerance and productivity. **The chaperone activity of the Hsp100/ClpB proteins clearly impacts how “plants generate and assemble components” as well as “allowing for their self repair”.** Furthermore, **Hsp100/ClpB protein function in plants is directly required for optimal “utilization of biological energy”, and is involved in “mechanisms that control the architecture of energy transduction systems”.**

Significant achievements in 2006-2008: We have previously shown that the cytosolic molecular chaperone Hsp101 is essential for the ability of plants to acclimate to high temperatures. We have made progress on multiple fronts to determine how Hsp101 facilitates stress protection. The homologous proteins, ClpB-p, which is found in the chloroplast, is essential for normal chloroplast development and plant growth. Data from gene and protein expression studies is beginning to reveal how this chaperone promotes proper assembly of chloroplast structures.

Cytosolic Hsp101: To define the role of different domains of the Hsp101 protein, N- and C-terminal deletions of cytosolic Hsp101 have been introduced into *Arabidopsis* plants that are null for Hsp101. Results of heat stress tests indicate that neither of these domains are essential for heat tolerance at the seedling stage of growth. Further quantitative analysis of these plants as well as tests of defects in other phenotypes will be carried out on homozygous lines.

To identify potential substrates or partners in Hsp101 action, transgenic plants have been generated with an N- and C-terminal affinity tag attached to Hsp101. These constructs are expressed well in the transgenic plants and preliminary data indicate that they rescue the heat sensitivity of the Hsp101 null. Therefore these plants can be used for affinity studies.

Progress has been made towards producing plants with N- and C-terminal GFP fusions for protein localization studies. We are also generating plants with an inducible dominant-negative allele of Hsp101.

Four extragenic suppressors of a dominant-negative mutant allele of Hsp101 are being characterized. One, *shot1*, we previously cloned and found it to be a member of the “mitochondrial transcription factor related proteins” (mTERFs) family. Genetic analysis has now shown that *shot1* can suppress the heat sensitive phenotype of other mutants, distinct from Hsp101. Therefore, *shot1* appears to be a general suppressor of some function that results in plant heat sensitivity. The other three extragenic suppressors are being subjected to further genetic analysis to separate them from the Hsp101 mutant allele and determine if they are dominant or recessive.

To investigate the connection of Hsp101 to drought and heat tolerance we have collected samples across different accessions and treatments, and will quantify *hsp101* expression. Dr. S. Tonsor at University of Pittsburgh will perform ANOVA and other analyses to determine if there is any relationship with Hsp101 expression level and plant growth and seed production.

Chloroplast ClpB-p: ClpB-p null mutants must be grown initially on sucrose-containing media at low light, but can eventually be transferred to soil, where they will continue to grow as potted plants, producing little or no normal seeds. Microarray analysis of chloroplast gene transcripts was performed comparing *clpB-p* and wt plants after they reached the 10 leaf stage. Results show that *clpB-p* mutants have no defect in

transcription, and even produce elevated levels of some transcripts, including ribosomal protein and Ndh transcripts. Precise measurements of rRNAs indicate the mutant accumulates fewer chloroplast ribosomes than the wt. Western analysis indicates that there are severe defects in accumulation of photosystem proteins, although clpP and Ndh proteins appear to accumulate like wt. Rubisco protein is also very low in the mutant, despite normal mRNA levels. Possible explanations for these results are that there is either selective translation of transcripts, or assembly and degradation of proteins complexes are differentially affected in the mutant.

Science objectives for 2008-2009:

- To complete analysis of the phenotypes of plants expressing N- and C-terminal deletion mutants of Hsp101. Phenotypes of growth and development in addition to heat tolerance at different stages of development will be tested.
- To finalize generation of other transgenic plants carrying 101:GFP translational fusions, an inducible dominant-negative allele of Hsp101, and constructs investigating if the chloroplast ClpB-p protein can complement the Hsp101 mutant.
- To utilize affinity tagged Hsp101 transgenic plants to search for biochemical partners or substrates of Hsp101.
- To complete basic genetic analysis of other extragenic suppressors of Hsp101.
- To investigate the potential function of the Hsp101 suppressor SHOT1 in organelle gene expression or function.
- Complete analysis of relationship between Hsp101 expression and whole plant phenotypes in response to drought and heat.
- To complete polysome analysis of chloroplast gene expression in *clpB-p*

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* Reviews that include work supported by other funding in addition to DOE funds.

Energetics and Structure of the ZIP Metal Transporter

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Overall research goals:

Chloroplasts represent one of the most metal-enriched organelles in all life systems. Plants employ a host of *metal transport proteins* that acquire metal ions from the soil, and then move them to the chloroplast sink where metal ions are incorporated into photosynthetic apparatus as catalytic/structural co-factors. The long-term goal of our research is to understand the fundamental chemical principles governing selective binding and energized movement of metal ions mediated by metal transporters. We will use direct *biophysical measurements* to characterize the kinetic process of metal transport in millisecond timescale, and use *x-ray crystallography* to reveal the molecular architecture of metal transporters in atomic detail. As an important step toward these goals, the current proposed research will focus on ZIP (ZRT, IRT-like Protein) metal transporters, a family of ubiquitous membrane proteins found in bacteria, plants and mammals. At present, only a bacterial ZIP homolog, ZupT from *Escherichia coli*, can be purified in sufficient quantities for structural analysis. Thus, we will use ZupT as a model protein to study the energetics and structure of the ZIP transporter. The proposed studies may reveal how structures of metal transporters are built around metal coordination chemistry, thereby facilitating protein engineering of metal transport systems that play a critical role in the biogenesis and function of photosynthetic apparatus.

Significant achievements in 2008:

This is a newly funded BES program starting from 7/1/2008. A post-doc has been recruited and experiments have been initiated.

Science objectives for 2008-2009:

- Over-express and purify ZupT
- Reconstitute the purified ZupT into proteoliposomes and characterize its transport kinetics.
- Begin crystallization of ZupT

References to work supported by this project 2007-2008:

Lu M. and Fu D. Structure of the zinc transport YiiP. *Science*, 317: 1746-8 (2007).

Session 5
Microbial Biochemistry - II

Engineering Interactive Biological/Material Interfaces with Nanotechnology

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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. Progress within this program over the last funding cycle is advancing towards this goal. This program seeks to develop basic scientific understanding and synthetic capabilities to ultimately direct the behavior of cells down to the molecular level.

Significant achievements in 2006-2008: At the inception of this program, the supported membrane platform was chosen as the primary strategy for interfacing synthetic substrates with living cells based on its successful application in studies of the T cell immunological synapse (Grakoui, Bromley et al. 1999). Functional signaling interfaces have been successfully established between three different live cell systems and appropriately functionalized inorganic substrates in 2005 and 2006. These include the repatterning of the T cell junction using electron beam patterned substrate constraints (Mossman, et al. 2005), induction of synaptogenesis in neurons (Pautot et al. 2005; Baksh et al. 2005), a general strategy for the surface-bound display of soluble signaling molecules (Nam et al. 2006). Our collective progress in this general area was recognized through an invitation to write a perspective for Science's STKE (*Science's STKE* **2005**, 301, pe45: "Learning the chemical language of cell surface interactions", Jay T. Groves). A more recent development, introduces the use of photocaged peptides to fabricate optically patternable surfaces. The key to this development is that substrates can be patterned in real time, while they are interacting with living cells. We refer to this technique as '*in vivo photolithography*', and see it emerging as a critical new capability in controlling the interface between living cells and inorganic substrates. Our most recent work extends this line of investigation to the use of nanometer scale "molecular mazes" to probe the processes and mechanisms behind protein transport and sorting in cell membranes (DeMond et al. 2008).

The generation of synthetic surfaces that can direct cellular behavior requires a diverse repertoire of signaling. Thus general strategies of generating these ligands, most of which are proteins, in forms that enable linkage to membranes is an essential component of this program. Nickel-chelating lipids are general tools for anchoring polyhistidine-tagged proteins to supported membranes. The ease of preparing histidine-tagged proteins makes chelator lipids an appealing tool for supported membrane functionalization, but a stable surface anchor is necessary for most realistic applications. Significant controversy exists over the stability and ultimate utility of this protein lipid attachment strategy. Therefore we have undertaken a systematic study of the desorption kinetics of decahistidine-tagged proteins from supported membranes containing nickel-chelating lipids (Nye and Groves 2008). Our results demonstrate that even low concentrations of chelator lipid (1%) are sufficient for building protein-functionalized surfaces, and that a range of protein surface densities are obtainable with a single concentration of chelator lipid. Supported membrane bound species exist in multiple association states, from monovalently to polyvalently bound. Significantly, our results revealed that equilibrium is unlikely to be reached under any

usual experimental circumstances, providing a possible explanation for discrepancies in the literature. The polyvalently bound species are quite stably associated with the substrate and the total surface density of these can be kinetically controlled during incubation. We have developed quantitative models of these binding kinetics that agree with experimental observations and provide a predictive framework for supported membrane functionalization. This system serves as the backbone of many of our exploratory experiments and has replaced the use of genetically-encoded GPI linkages used in earlier experiments.

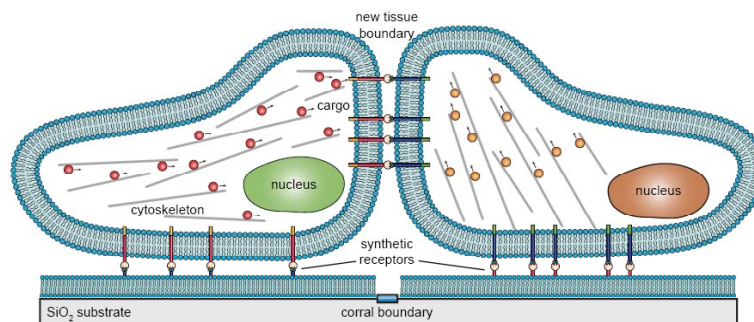


Figure 1. Generating artificial self-organizing boundaries between different cell types through surface patterning.

Science objectives for 2008-2009(style=Stand alone text heading):

- One fundamental goal of this is to develop a library of membrane anchoring motifs that lead to differing degrees of dynamic clustering of their tethered cargo in supported membranes. This library should contain anchors leading to mutual association as well as mutual avoidance, thus spanning the full spectrum of organizational behaviors observed *in vivo*. Such a library of anchors would aid the implementation of multiple signaling systems into a single supported membrane by providing control over how the signaling ligands interact.
- Another goal of this program is to create surfaces displaying patterns of signals that direct multiple cell types to assemble into complex configurations with tissue-like levels of order, but following a designed plan of our choosing (see Figure 1). Applications for such capabilities include using cells to deliver cargo to specified locations with high spatial and orientational precision. It may also be possible to hijack the internal organization of the cell to, for example, arrange a series of nanoparticle devices on the nanometer scale. One very important challenge, which may be solvable by this strategy, is the oriented assembly of solar-electric and solar-fuel converting nanoparticles into macroscopic functioning devices.

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Syntrophy: Lifestyle of The thermodynamically Challenged

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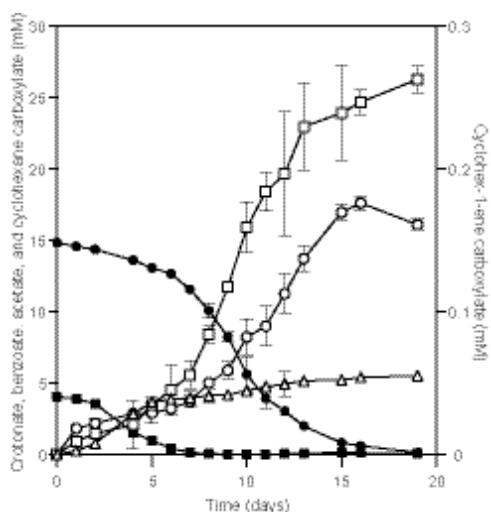
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Overall research goals: We wish to understand an essential but poorly characterized process critical for carbon cycling called syntrophy and how bacteria operate at free energy changes close to equilibrium. The specific objectives of project are to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

Significant achievements in 2006-2008: The genomes of *Syntrophus aciditrophicus*, *Syntrophomonas wolfei*, and *Syntrophobacter fumaroxidans* have been sequenced, manually annotated, and deposited in GenBank. These genomes are the first, completely sequenced and gapped-closed genomes for syntrophic metabolizers that require reverse electron transport. An inventory of expressed proteins of *S. aciditrophicus* grown in pure culture with crotonate and with crotonate plus benzoate suggests that novel strategies are used for benzoate reduction and ATP synthesis. Proteins for a novel benzoyl-CoA reductase and acetyl-CoA synthase (ADP-forming), the typical route from for ATP synthesis in acetate-forming archaea, were detected. The proteome lacked proteins for acetate kinase and phosphotransacetylase, the typical route for ATP synthesis from acetyl-CoA in bacteria. We found that *S. aciditrophicus* forms a diene intermediate from 3-fluorobenzoate. In collaboration with Prof. Boll, the cyclohex-1,5-diene-1-carboxyl-CoA hydratase from *S. aciditrophicus* was heterologously expressed in *Escherichia coli* and its activity confirmed. Our data show that a two-electron reduction occurs during aromatic ring reduction in strict anaerobes.

We discovered that *S. aciditrophicus* forms benzoate and cyclohexane-1-carboxylate from crotonate and elucidated the main metabolic steps in the pathway. Cyclohexane-1-carboxylate formation represents one of the few cases in biology where a saturated ring compound is made from a straight-chain organic acid. The pathway for cyclohexane-1-carboxylate from crotonate appears to be a



reversal of the benzoate degradation pathway. This suggests that the direction of metabolism is controlled by thermodynamic regimes and operates at high efficiency. We discovered that benzoate is respired by *S. aciditrophicus* (Fig. 1). The increase in molar growth yield with crotonate and benzoate and the formation of [ring-¹³C]-cyclohexane-1-carboxylate from [ring-¹³C]-benzoate in the presence of crotonate are consistent with benzoate serving as an electron acceptor. Diverse fates for benzoate metabolism exist in methanogenic environments including syntrophic metabolism, fermentation, and respiration.

Figure 1. Metabolism of crotonate in the presence of benzoate by *S. aciditrophicus* pure cultures. Symbols: ●, crotonate; ■, benzoate; □, acetate; △, cyclohexane carboxylate; ○, cyclohex-1-ene carboxylate. The data are averages ± standard deviations of triplicate cultures.

Science objectives for 2008-2009:

- The mechanism of interspecies electron transfer, e.g., by the transfer of hydrogen, formate, or other molecules, or directly by electrically conductive filaments, will be determined for fatty- and aromatic acid-degrading syntrophic consortia. We will determine if inhibitors of hydrogenase and formate dehydrogenases affect the rate of syntrophic fatty and aromatic acid metabolism. We will also determine the effect of other potential extracellular redox carriers on the rate of syntrophic metabolism.
- Membrane complexes potentially involved in reverse electron transport will be identified. We will use blue native gel electrophoresis to identify membrane protein complexes differentially expressed during syntrophic fatty aromatic acid metabolism.
- We will determine which of the multiple hydrogenase and formate dehydrogenase genes are expressed and whether candidates for reverse electron transport such as *fix*, *rnf*, and genes for a novel iron-sulfur protein complex are expressed differentially during syntrophic metabolism. We will use RT-PCR and QRT-PCR to measure expression when syntrophic cocultures are grown with butyrate, benzoate and cyclohexane carboxylate (conditions that require reverse electron transport) compared to crotonate-grown cocultures (conditions that do not require reverse electron transport).

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Session 6
Plant Cell Walls

The Plant Cell Wall – The First Line of Defence

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Overall research goals: We have used mutational analysis to identify host plant factors that are required for optimal powdery mildew infections. A majority of the mutations cause changes in the host cell wall highlighting the importance of the cell wall as an important barrier to pathogen ingress. Our goal is to determine the nature of the specific changes in the plant cell wall that occur in these mutants and understand why these changes influence pathogen success on its host plant.

Significant achievements in 2006-2008:

Pectins represent some of the most compositionally complex and diverse polysaccharides in the plant cell wall (Somerville et al. 2004). Albersheim and colleagues proposed that some of the complexity in cell wall polysaccharides and glycoproteins serves a reservoir of latent signalling molecules (Darvill et al. 1994, Hahn et al. 1981). These signalling molecules, oligosaccharins, may be released by plant polysaccharide degrading enzymes to affect development or by pathogen-derived enzymes to elicit plant defence responses (Darvill et al. 1994).

Plants are able to perceive oligogalacturonide elicitors and respond with the number of defence-associated processes (e.g., increased Ca^{++} flux, alkalinization of the medium, production of phytoalexins, induction of defence-related gene expression) (Vorwerk et al. 2004). Other features of the plant cell wall that could influence plant-pathogen interactions include its rigidity, porosity and ionic charge, which could affect penetration and the movement of pathogen- or plant-derived elicitors or effectors through the wall. The pectin fraction is a major determinant of these properties via the degree of esterification and cross-linking of pectin components (Vinken et al. 2003, O'Neill et al. 2001).

The Arabidopsis *pmr5* (*powdery mildew resistant 5*) mutant affects cell wall pectin composition and result in resistance to the fungal pathogen, powdery mildew, suggesting that these mutants will provide the genetic materials to study the role of pectins as signalling molecules and/or determinants of wall properties in plant-pathogen interactions (Vogel et al. 2004).

PMR5 is annotated as a plant-specific expressed protein of unknown function and contains a domain of unknown function (DUF231) in the terminal third of the predicted amino acid sequence. The first 22 amino acids are predicted to serve as a signal sequence targeting *PMR5* to the endoplasmic reticulum. *PMR5* is a member of a large gene family in Arabidopsis (n~45). Mutations in at least one other gene family member displayed increased resistance to powdery mildew. Our rationale for surveying additional gene family members is that a common change in the cell wall may be suggested by the changes in all of the mutants of *PMR5*-like genes.

One hypothesis we have is that the changes in cell wall in the *pmr5* and *pmr6* (a pectate-lyase-like gene) mutants may lead to the activation of defence responses via a novel signal transduction pathway, and these defence responses are responsible for the powdery mildew resistant phenotype of the mutants. Among the 54 genes that are constitutively elevated in *pmr5* and *pmr6* relative wild type are a number of potential defence-related genes. Thus, although the well-known defence pathways, salicylic acid and ethylene/jasmonic acid, do not seem to

contribute to *pmr5*- or *pmr6*-resistance, it is possible that defence responses are activated via a novel pathway in these mutants.

In the *pmr5*-suppressor screen, a mutagenized population was screened directly for restoration of powdery mildew susceptibility. Twenty suppressor mutants were recovered (*p5s1* to *p5s20*), some of which affect both stature and disease resistance and others that affect disease resistance only. The richness of *pmr5* suppressor mutant collection suggests that this is a promising source of new tools for understanding the role of PMR5 in cell wall biogenesis and the role of the plant cell wall in powdery mildew disease resistance.

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Science objectives for 2008-2009:

- To determine specific changes in the cell walls and their properties from the *pmr5* mutant.
- To determine a biochemical role for the PMR5 protein.
- To clone and initiate characterization of at least one of the *pmr5* suppressor genes.

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Structural Studies of Complex Carbohydrates of Plant Cell Walls

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Much of the solar energy captured by land plants is converted into the polysaccharides (cellulose, hemicellulose, and pectin) that are the predominant components of plant cell walls. These walls, which account for the bulk of plant biomass, have numerous roles in the growth and development of plants, as well as in the plants' interactions with their environment. Moreover, these walls have a major impact on human life, as they are a renewable source of biomass, a source of diverse commercially useful polymers, a major component of wood, and a source of nutrition for humans and livestock. Thus, understanding the molecular mechanisms that lead to wall assembly and modification, and how cell walls and their component polysaccharides contribute to plant growth and development, is essential to improve and extend the productivity and value of plant materials. We develop and apply advanced analytical and immunological techniques to study specific changes in the structures and interactions of the hemicellulosic and pectic polysaccharides that occur during differentiation and in response to genetic modification and chemical treatments that affect wall biosynthesis. These techniques make it possible to accurately characterize minute amounts of cell wall polysaccharides, so that subtle changes in structure that occur in individual cell types can be identified and correlated to the physiological or developmental state of the plant.

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Probing Lignin Primary Structure and Assembly: An Overdue Approach

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Overall research goals: The research objectives are: to develop and apply methodologies for determination of lignin primary sequences (structures) in selected wild type, mutant and transgenic plant lines; to apply further a proteomics/metabolomics approach to study lignifying plant cell walls; to identify and characterize the enzymes/proteins involved in cell-wall assembly(ies) proper.

Significant achievements in 2006-2008: Our earlier metabolic flux analyses established that carbon allocation to the monomers resulting in formation of lignins were essentially (normally) regulated in three distinct ways: phenylalanine supply, and the two hydroxylation steps at C4- and C-3, respectively, leading to the monolignols, *p*-coumaryl, coniferyl and sinapyl alcohols. Additionally, the biochemical pathway to phenylalanine in plants has only recently been determined with discovery/characterization of arogenate dehydratases.

While representing nature's second most abundant organic natural products, however, the study of lignin primary structure and assembly proper has essentially only now begun. Much of the long standing confusion regarding lignin structure/assembly has resulted from the serious deficiencies in current lignin analytical protocols and quantification, as well as in the general lack of experimental approaches/design to probe lignin primary structure(s). Our development and application of new biophysical/analytical chemical technologies to distinct cell wall and/or tissue types of the vascular apparatus have begun to yield incisive information on both lignin structure and assembly processes. For example, using laser micro-dissection (Figure 1), together with various chemical analyses, of specific cell wall/tissue types in *Arabidopsis*, alfalfa and poplar stem vascular tissues from wild-type, mutant and transgenic plant lines, we have established a very limited substrate degeneracy during the proposed template polymerization leading to lignification. We also summarize herein the new chemistries that have been developed to begin to fragment/structurally define lignin biopolymer primary structure(s). These data also provide important evolutionary insights into the lignification process, and why monolignols and not other phenylpropanoids are used to biosynthesize lignins. Moreover, the data obtained is in stark contrast to the proposed combinatorial chemistry and random assembly that others have proposed for lignification.

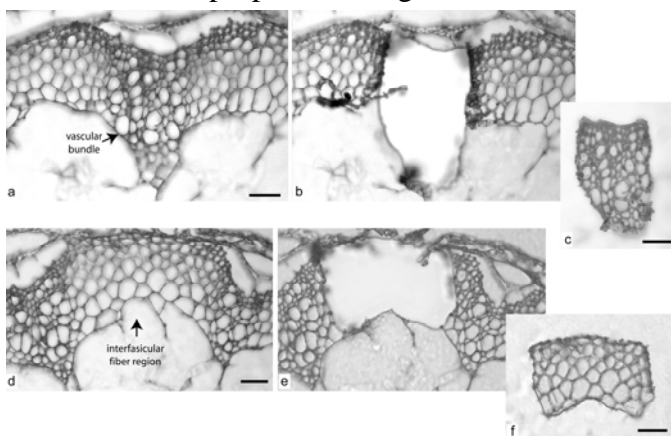


Figure 1 An example of laser capture microdissection of *Arabidopsis*. The vascular bundles (a) were laser excised (b) and isolated (c), as were the interfascicular fiber regions from the base of the stem of a wild type inflorescence stem at 26 dpf for subsequent chemical and proteomic analyses.

In addition, the effects on overall growth/development/cell wall properties of various plant lines modified in lignin contents/compositions are discussed from several different angles; these include effects on tensile strength properties in the stem vascular apparatus, in differing effects

on growth/ development under distinct environmental conditions, as well as on cell wall assembly processes themselves leading to, for example, different anatomical defects.

In related studies, the proteomes for the initial stages of vascular development (protoxylem and metaxylem), as well as for later stages (including vascular bundles and interfascicular fibers) have been determined. These data, in turn, are now beginning to shed light on both early and late stages of vascular development/lignification and the precise metabolic processes involved.

Science objectives for 2008-2009: To continue establishing lignin primary sequences; to further apply proteomic/metabolomic approaches to study specific forms of lignifying plant cell walls, and to identify and characterize the enzymes/proteins involved in cell-wall assembly(ies) proper.

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Transcription Regulation of the Cellulase System of *Clostridium thermocellum*

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Overall research goals: The overall goal of the project is to study the structure and regulation of the cellulase system of *C. thermocellum* at the molecular level. Specifically, we propose to identify transcription factors controlling key cellulase components by EMSA, MALDI-TOF, nanoLC/MS/MS, and *in vitro* transcription analysis.

Significant achievements in 2007-2008: We profiled system level changes in *C. thermocellum* cellular protein expression using two-dimension separation and analysis on the ProteomeLab™ PF2D platform, a 2D chromatographic technique (ion exchange + hydrophobic interaction) established at the University of Rochester. Separation with PF2D is analogous to traditional 2-D gels, but is superior in effective resolution, sensitivity, reproducibility, and ease of follow up analysis by mass spectrometry platforms. In pilot experiments, *C. thermocellum* was grown on cellobiose or cellulose. Fig. 1 shows virtual 2-D gels generated from the chromatograms of PF2D resolving cellular proteins from each culture at the exponential growth phase. The central “gel” represents differentially detected protein peaks. Blue bands indicate proteins expressed on cotton and green bands indicate those on cellobiose. Comparison of the protein expression profiles indicates more genes are expressed on cotton (note the prevalence of blue “bands” in “Differential Expression” analysis). One of the few examples of a protein whose expression is down-regulated by cotton is highlighted by a red box. The fractions of interest can be subject to mass-spectrometer based analysis to identify proteins such as Proteins 1 and 2. Further analysis of fractions of interest with FT-MS gives the added ability to identify post-translational modifications (PTMs). These proteins will be identified with an emphasis on finding the transcription factors and their expression profile related to carbon source and cellulase production.

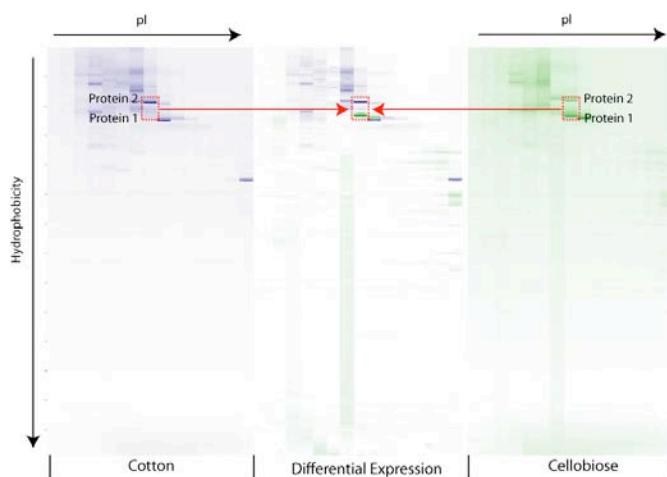


Figure 1. PF2D analysis of 2.5 mg total cellular proteins from cells grown on cellobiose or cotton.

Science objectives for 2008-2009:

- We will continue to identify the cellular proteins species that are expressed under different culture conditions with an emphasis on identifying transcription factors.

- We will continue to determine the transcription factors that bind to the promoter regions of the genes encoding key cellulase components.

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Session 7
***Ferredoxins, Hydrogenases,
and Biological H₂ Production***

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 2006-2008: The interaction domain on the [2Fe-2S] ferredoxin from the cyanobacterium *Synechocystis* sp. PCC 6803 for the ferredoxin:thioredoxin reductase (FTR) from the same species was mapped using NMR spectroscopy. The use of a ferredoxin derivative in which the native [2Fe-2S] cluster was replaced by a single non-paramagnetic Ga²⁺ eliminated the paramagnetic broadening of NMR signals arising from 21 amino acids close to the iron-sulfur cluster and allowed the first complete mapping of an enzyme-binding domain on a plant-type ferredoxin.

All eight tryptophan residues present in spinach nitrite reductase were replaced, one at a time, by both non-aromatic and aromatic amino acids using site-directed mutagenesis. The effects of these replacements on steady-state kinetic parameters with ferredoxin serving as the electron donor and on activity with the non-physiological electron donor, reduced methyl viologen, were measured. The effects of these substitutions on K_d's for substrate binding and on the redox properties of the enzyme's prosthetic groups were also measured. Acrylamide fluorescence quenching measurements were carried out to assess the solvent accessibility of tryptophan residues. The results of this study indicate that none of the tryptophan residues is directly involved in electron transfer.

Spinach glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was crystallized for the first time without NADP⁺ bound at the active site. A comparison of the structure for this form of the enzyme to a form containing bound NADP⁺ revealed that significant conformational changes in the enzyme result from co-factor binding.

The oxidation-reduction properties of FAD and two disulfide/dithiol couples in Erv1, a sulfhydryl oxidase that functions in the import of cysteine-rich proteins in the

mitochondrial intermembrane space of *Saccharomyces cerevisiae*, were measured. Erv1 forms a 1:1 complex with cytochrome c, within which reduced Erv1 reduces ferri-cytochrome c. Reduced Erv1 also reduces oxygen, generating hydrogen peroxide, which is then reduced to water by cytochrome c peroxidase. Oxidized cytochrome c peroxidase is then reduced by the cytochrome c that had been reduced by Erv1.

Science Objectives for 2008-2009:

- To use NMR spectroscopy to characterize the ternary complex between ferredoxin, thioredoxin and ferredoxin:thioredoxin reductase (FTR) and to determine the solution structure of a Ga-substituted ferredoxin.
- To use site-directed mutagenesis to identify amino acid residues in nitrite reductase involved in substrate binding.
- To explore the specific role(s) of different chloroplastic ferredoxins in the green alga *Chlamydomonas reinhardtii*.
- To use flash photolysis and nitrite analogs to identify reaction intermediates in the reaction catalyzed by nitrite reductase.
- To characterize the complex formed between ferredoxin and phycobilin reductase.

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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 the concomitant conservation of energy in the form of ion gradients. The 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.

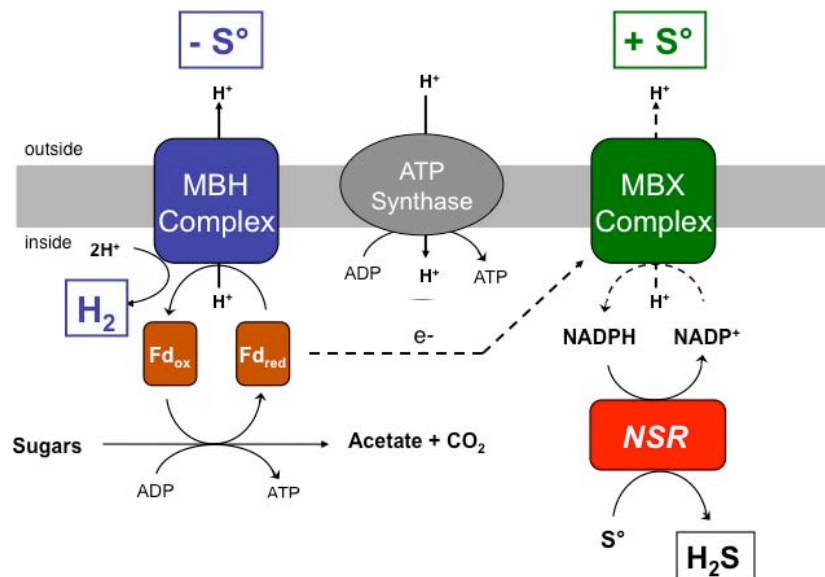
Significant achievements in 2006-2008: The model organism for the proposed studies is *Pyrococcus furiosus* (Pf), which grows optimally at 100°C. Pf obtains carbon and energy for growth by fermenting carbohydrates and by producing H₂ or by reducing elemental sulfur (S⁰) to H₂S. Pf has a respiratory metabolism in which a ferredoxin-dependent, membrane-bound hydrogenase (MBH) catalyzes the production of H₂ and couples this to the pumping of protons (and possibly Na⁺ 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 S⁰ 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, like MBH, to conserve energy in the form of a proton (Na⁺) motive force. MBX and MBH are both integral multiprotein, metal-containing complexes of ~300 kDa, each encoded by at least 13 genes. The addition of S⁰ to Pf also induces the synthesis of a cytoplasmic flavoprotein termed NADPH sulfur reductase (NSR). This multiprotein complex of 100 kDa is proposed to be responsible for oxidizing the NADPH generated by MBX and for reducing S⁰ to H₂S in a coenzyme A-dependent reaction. The sulfide that is produced by NSR is thought to be sequestered by a novel protein termed SipA, which exists as a large homomultimeric complex of ~600 kDa. S⁰ appears to enter the cell by another membrane complex termed HST (~120 kDa) and this complex may facilitate the interactions of S⁰ both with coenzyme A and with a key regulatory protein, PF0095, which appears to mediate the primary transcriptional response of Pf to S⁰.

Science objectives for 2008-2009:

- To characterize the novel energy conserving complex MBX using both native and recombinant sub-complexes, including mechanisms of catalysis, bioenergetics and self-assembly
- To characterize the novel S⁰-reducing enzyme NSR and to determine the role of CoASH in catalysis
- To determine the mechanisms by which insoluble S⁰ becomes an enzyme substrate (for NSR) and an intracellular signal (for PF0095).

The results of this research will provide a fundamental understanding of how the metabolism of S⁰ leads to energy conservation and biological syntheses in Pf using novel catalytic mechanisms.

Figure 1. Bioenergetics and proposed pathways of electron flow from reduced ferredoxin (Fd_{red}) either to protons to produce H_2 or to elemental sulfur (S^0) to produce H_2S in *P. furiosus*.



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Photoproduction of Hydrogen: Biological Hydrogenase Gene Expression and Artificial Biomimetic Systems

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Overall research goals: The potential light conversion efficiency to H₂ by biological organisms is theoretically about 10%. One of the limitations to meeting this efficiency is the low availability of reductants to the hydrogenase due to the existence of competing metabolic pathways such as CO₂ fixation. The objective of this proposed research is to continue to develop fundamental understanding about the regulation of partitioning of photosynthetic reductants (at the level of ferredoxin) between the H₂-production and the CO₂-fixation pathways. Our analysis of *C. reinhardtii* mutant libraries in the previous funding periods for strains having attenuated H₂ production has led to the discovery of maturation proteins that are required for the proper assembly of hydrogenases, of the STA7 isoamylase protein, and of the putative 3-hydroxybutyrate dehydrogenase gene, reflecting the importance of starch and lipid metabolic pathways for H₂ production. We have recently shown the presence of heme-binding, FixL-like proteins in *C. reinhardtii*. Such proteins likely play a role in the O₂-sensing mechanism and may mediate components of the anoxic regulatory response. The proposed work will continue to (a) determine what other protein factors may be required for optimal hydrogenase expression and activity, (b) identify O₂-sensor proteins in *C. reinhardtii* that regulate the expression of the reversible hydrogenase, (c) conduct analyses of the promoter regions for *HYDA1* and *HYDA2* genes, encoding the two algal hydrogenases; and (d) understand the simultaneous or complementary regulation of the competitive CO₂ fixation pathway under anaerobic conditions.

Significant achievements in 2006-2008: The flanking DNA for three attenuated hydrogen production mutants have been obtained and the disrupted genes appear to include: 1) a putative 3-hydroxybutyrate dehydrogenase (*3-HBD*); 2) a potential quinone oxidoreductase; and, 3) a disruption neighboring a potential transcriptional regulator, ferredoxin, or kinase. A mutation in 3-HBD indicates that lipid metabolism is involved in H₂ production, a finding that could correlate with chloroplast membrane lipid degradation upon sulfur deprivation and ensuing H₂ production in *C. reinhardtii*. The 3-HBD mutant has attenuated hydrogen production in early to mid exponential growth phase, but normal H₂ production in older cultures. PCR and reverse transcription PCR assays indicate a duplication of the *3-HBD* gene during insertion mutagenesis. One of the duplicated genes is disrupted by the insert, while the other gene appears to be intact, with mRNA for *3-HBD* at approximately wild-type levels.

In a second research area, *C. reinhardtii* proteins with homology to PAS O₂-sensing proteins were identified by our lab. Analysis of the *Chlamydomonas reinhardtii* genome indicates a number of closely-related homologs to eubacterial FixL proteins. Nine of these FixL like homologs (FXL) have a core PAS domain that is homologous to the FixL O₂-sensing PAS domains of the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, *Sinorhizobium meliloti* and *Rhizobium leguminosarum*. Two of the *Chlamydomonas* FixL heme-binding domains were cloned as truncated genes and the corresponding expressed proteins in *E. coli* were shown to coordinate heme. Each of the nine putative homologs has multiple transmembrane-spanning domains, which are typical of the bacterial FixL homologs. Additional homology includes conserved α -helical loops, β -sheet structures, and critical residues required for binding a heme moiety transmitting the presence of heme-bound O₂ to an autophosphorylation site of a conserved histidine residue within a neighboring histidine kinase domain. However, the *Chlamydomonas* FixL homologs do not have the typical histidine autophosphorylation site.

An intriguing aspect of *Chlamydomonas* metabolism is how the cell senses O₂ levels and initiates the appropriate transcriptional and translational responses. As *Chlamydomonas* is capable of generating significant quantities of photosynthetic O₂, the expression of O₂-sensitive proteins must be tightly controlled to ensure that cellular energy is not expended on the inappropriate

synthesis of highly O₂-intolerant proteins. For example, the [FeFe]-hydrogenases are irreversibly inhibited by O₂. Similar challenges are faced by N₂-fixing Rhizobia, some of which use the heme-based O₂-sensing FixL proteins to detect O₂ levels and initiate signal transduction events that ensure nitrogen fixation proteins are synthesized only when O₂ levels are sufficiently low to prevent enzyme inactivation.

Science objectives for 2008-2009:

- Back-cross the putative 3-hydroxybutyrate dehydrogenase mutant 5-29 against a wild type cell line prior to further analysis of hydrogen production. An additional library of insertion *ApaH* paromycin mutants is being created in order to streamline some of the difficulties encountered in the original library of mutants having attenuated levels of hydrogen production. These experiments will help to further define the biochemical pathways required for H₂ production in *C. reinhardtii*.
- Identify the promoter regions and transcriptional elements activating the expression of hydrogenase in *C. reinhardtii*.
- Test the ligand binding characteristics of the FXL PAS domains to determine the effects of O₂ coordination. These data will be correlated with the transcriptional level of the FixL homologs resulting from exposure to O₂ and other gases during culture. Analysis of these FixL homologous genes may prove invaluable for understanding the regulation of fermentative metabolism and the production of H₂ under conditions of low O₂ tension.

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Session P2
Poster Session - II

Fundamental Aspects of Microbial Cellulose Utilization DOE Grant DE-FG02-02ER15350

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Overall research goal: Advance fundamental understanding of cellulose utilization by *Clostridium thermocellum*, and in the future perhaps other cellulolytic microorganisms. A particular focus is examination of features operative during microbially-mediated cellulose solubilization that are not operative during cellulose solubilization by enzymes acting independently of microorganisms. Such features include, among several, bioenergetics, catalytic efficacy of the cellulosome, and consequences of cell adherence to cellulose.

Significant achievements in 2006-2008.

Cell-enzyme synergy in *Clostridium thermocellum* was investigated. Specific cellulose hydrolysis rates (g cellulose/g cellulase/h) were shown to be substantially higher (2.7 to 4.7-fold) for growing cultures of *Clostridium thermocellum* as compared to purified cellulase preparations from this organism in controlled experiments involving both batch and continuous cultures. This “enzyme-microbe synergy” requires the presence of metabolically active cellulolytic microbes, is not explained by removal of hydrolysis products from the bulk fermentation broth, and appears due to surface phenomena involving adherent cellulolytic microorganisms. Results support the desirability of biotechnological processes featuring microbial conversion of cellulosic biomass to ethanol (or other products) in the absence of added saccharolytic enzymes. Our results are the first quantitative demonstration of enzyme microbe synergy known to us.

A new functionally based kinetic model for enzymatic hydrolysis of pure cellulose by the *Trichoderma cellulase* system was presented. The model represents the actions of cellobiohydrolases I, cellobiohydrolase II, and endoglucanase I; and incorporates two measurable and physically interpretable substrate parameters: the degree of polymerization (DP) and the fraction of β -glucosidic bonds accessible to cellulase, F_a (Zhang and Lynd, 2004). Initial enzyme-limited reaction rates simulated by the model are consistent with several important behaviors reported in the literature, including the effects of substrate characteristics on exoglucanase and endoglucanase activities; the degree of endo/exoglucanase synergy; the endoglucanase partition coefficient on hydrolysis rates; and enzyme loading on relative reaction rates for different substrates. This is the first

cellulase kinetic model involving a single set of kinetic parameters that is successfully applied to a variety of cellulosic substrates, and the first that describes more than one behavior associated with enzymatic hydrolysis.

Additional achievements during the reporting period include reporting a method for synthesis of radiolabeled cellodextrins using cellobiose and cellodextrin phosphorylase from *C. thermocellum*. This method has potential utility in investigation of the substrate capture hypothesis (below). A paper also appeared on proteomic analysis of *C. thermocellum* from Herb Strobel's group, formerly a co-P.I. Finally, a substantial book chapter was written and published that summarizes recent progress in understanding microbial cellulose utilization, frames new challenges for investigation and examines similarities and differences between adhered cellulolytic microbes and biofilms on inert surfaces.

Science objectives for 2008-2009.

- 1) Test the substrate capture hypothesis for cellulose utilization by *C. thermocellum* – that is, the hypothesis that most cellulose solubilization products are consumed by cellulose-adhered cells without leaving the boundary layer surrounding biomass particles.
- 2) Demonstrate a functional gene knockout system in *C. thermocellum*.
- 3) Use the gene knockout system to investigate the importance of individual cellulosome components in microbial cellulose utilization by *C. thermocellum*.

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Dissection and Manipulation of LRR Domains in Plant Disease Resistance Gene Products

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Collaborators: Drs. Simon Gilroy, Eric Shusta and George Phillips, Univ. Wisconsin – Madison
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Overall research goals: This research addresses the following overlapping research questions:

- How do leucine-rich repeat (LRR) proteins recognize their cognate ligands?
- How do plants resist disease?
- What are the intra- and inter-molecular transitions that occur that cause transmembrane LRR proteins of the plant immune system to switch from off to on?
- Can we synthetically evolve new LRR proteins for biofuel and food crops in order to defend against problematic pathogens?

Rationale: Plant diseases are a consistent energetic and economic drain on plant productivity. Proteins carrying leucine-rich repeat domains are a primary target in traditional plant breeding because they make substantial contributions to plant health. LRRs often form specific ligand binding sites and they occur in a broad array of proteins, including a few hundred receptor types in any individual plant that control development and disease resistance (Kobe and Kajava, 2001; Bell et al., 2003; Morillo and Tax, 2006) (Jones and Dangl, 2006; Bent and Mackey, 2007). Hence a general understanding of LRR structure/function relationships, and development of new paradigms for study and manipulation of LRR domains, will have relevance well beyond plant disease resistance. FLS2 is one of the best-studied plant transmembrane LRR receptor kinases; it directly binds bacterial flagellins or flagellin-derived peptides and in response, activates plant defenses. Our work has touched on a number of LRR proteins but focuses in particular on Arabidopsis FLS2.

Significant achievements in 2006-2008:

We recently published a paper describing a combined phylogenetic and mutational dissection of the LRR domain of FLS2 (Dunning et al. 2007). The paper provided information about FLS2 diversity across the Brassicaceae, and identified the probable binding site for flagellin. A high-throughput survey of over 1200 mutant alleles altered at specific surface-exposed residues identified residues at which variation does or does not alter flagellin responsiveness. The paper also described a relatively rapid approach to scanning the concave face of the LRR domain to find functional sites that merit further study. This can be done in the absence of a known/purified ligand or a receptor crystal structure, making it a widely accessible approach for researchers studying other LRR proteins.

We have subsequently developed a LRR conservation mapping program that can be used to identify functional sites *in silico*. Our approach has been validated using data for LRR proteins for which ligand-receptor co-crystals and/or other functional data are available. An example is shown on the next page. Using this approach, we are currently carrying out further dissection of FLS2 and other receptors.

We are also studying other structure-function aspects of FLS2 function, with particular emphasis on the LRR domain. We have found that FLS2, unlike the analogous LRR-kinase EFR, is highly tolerant of mutation of putative glycosylation sites. We have found, in general, that mutation of most solvent-exposed residues leaves FLS2 highly functional. We have found that only one of the cysteine-pairs that flank the LRR is crucial to protein function,

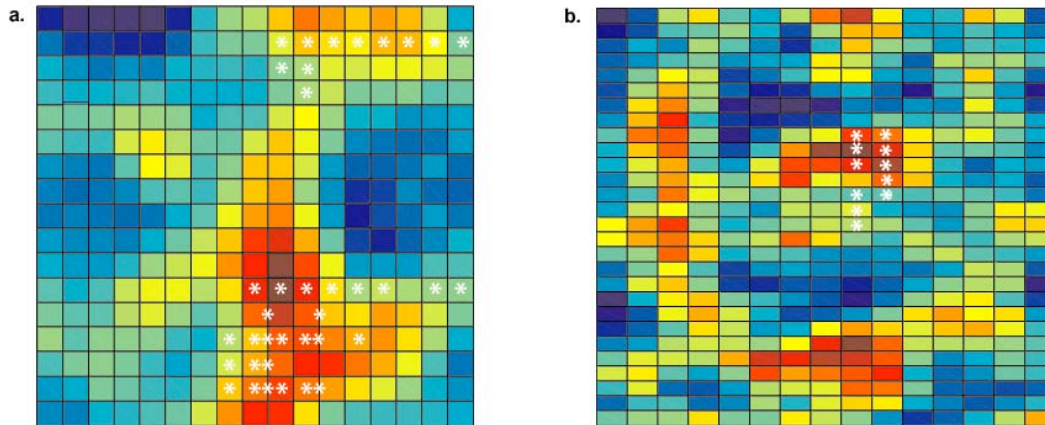


Figure 2. Conservation mapping highlights areas of functional significance for the auxin receptor TIR1 or for FLS2. Conservation is indicated by a heat map from least conserved (purple) to most conserved (dark red). Residues shown to be important for function by crystallography or mutagenesis are marked with asterisks. (a) Comparison of TIR1 with five paralogs all shown by crystallography to directly bind auxin or cofactor. b) Comparison of FLS2 and its ortholog from tomato (additional function experiments with FLS2 are in progress).

and that this cys-pair mutant is unstable but can still function if overexpressed. We have also identified LRR-kinase partners that interact with FLS2 *in vivo*, and have studied the ligand dependence of these interactions.

Some of our earlier work (Sun et al. 2006) focused in the ligand that stimulates FLS2. In a result with broad implications, we showed that PAMPs such as flagellin are not always a broadly conserved epitope detectable by basal immune systems, but rather, can vary between strains within a single pathogen species. For a set of *Xanthomonas* flagellins with significant sequence divergence across the entire protein, we identified the one naturally polymorphic amino acid that determines detectability by Arabidopsis FLS2. We also found that *Xanthomonas campestris* pv. *campestris* can evade FLS2-mediated defenses as long as the FLS2 system has not been activated by previous infections.

Science objectives for 2008-2009:

- Carry out *in vitro* evolution of FLS2 toward new ligand specificities. The goal is to develop general methods that target mutagenesis toward functional sites of the LRR domain, vastly shrinking the mutational universe of proteins that need to be screened to identify useful variants.
- Identify functional mechanisms of transmembrane LRR-kinase receptors through further structural and functional characterization. LRR-kinase receptors play a key role in a huge diversity of plant functions. Understanding the structural and functional mechanisms of, for example, ligand specificity and interaction with signaling partners, should facilitate our future ability to manipulate many aspects of plant growth and development.

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Engineering Functional Scaffolds by Supramolecular Self-Assembly

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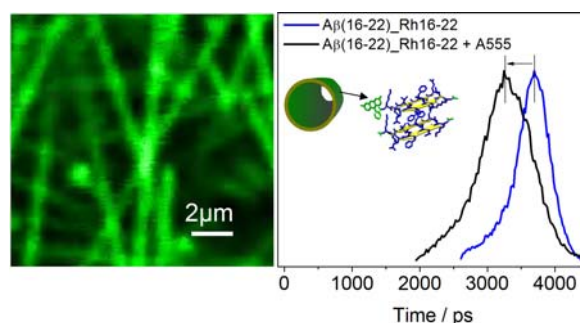
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Collaborators: Dr. Pappanan Thiyagarajan, APS, ANL
Dr. S.V. Pingali, ORNL

Overall research goals: The overall goal of this fruitful collaboration between the Center for Supramolecular Self-assembly at Emory University and the Argonne/Oak Ridge National Laboratories has been to development methods to direct the assembly of synthetic oligopeptide motifs into nano-scale objects such that the degree of order can emulate and extend that observed in biological systems.

Significant achievements in 2006-2008: Mechanistic understandings of the pathway for self-assembly of complex multi-component arrays are just beginning to emerge. While this understanding with β -sheet arrays may well be critical to the intervention strategies in many of the roughly 40 different amyloid diseases, it is the synthetic potential of these pathways that may well be most profound and useful. We now have metal switches that regulate the assembly morphology, kinetics and thermodynamics of both α -helical and β -sheet arrays, allowing us to construct robust fibrils, complex multilayered fibers, ribbons, sheets and vesicles, and nanotubes of well-defined cross-sectional area and wall thickness that display unique paracrystalline and catalytic activity. We have now shown that metal ions can be arrayed in high densities along the nanotube surfaces at one nanometer repeat intervals, that DNA bases can be used to control assembly morphology and functionality, and that specific chromophoric entities can be arrayed within the assemblies or layered specifically along their surface following assembly.

We are now positioned to take the critical step of organizing antenna complexes on a peptide bilayer surface for light harvesting and energy transfer. This achievement will constitute the critical first step in creating a simple bio-inspired self-healing assembly capable of emulating critical components of natural photosynthesis. Shown at the right is our initial attempt where we demonstrate Förster energy transfer between synthetically incorporated rhodamine and Alexa 555 bound specifically along the nanotube surface. This ability to both create 2D arrays along the nanotube surface as directed by the amyloid assemble and still use the amyloid groves to order other pigments appears very powerful and will be further explored and extended.



Science objectives for 2008-2009:

- *Define methods for creating and processing organized arrays of peptide-based supramolecular assemblies that may be interfaced with nano-scale devices.*
- *Development of methods to control the incorporation and spatial distribution of functionalized substrates within organized arrays.*

- *Combine these unique molecular recognition elements to create highly ordered functional assemblies.*

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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*. These studies are predominantly based on recently developed methods for genetic analysis of *Methanosarcina* species; however, physiological, biochemical and molecular approaches are also used. A central aspect of the study is examination of the genotypic and phenotypic differences between *M. barkeri*, an organism that grows well on H₂/CO₂, and *M. acetivorans*, a closely related organism that is incapable of growth on H₂/CO₂. The differences in hydrogen metabolism lie at the center of the energy-conserving electron transport chains of the two organisms. Examination of the molecular, genetic, biochemical and physiological traits that underpin these differences is expected to deepen our overall understanding of methanogenesis, hydrogen production/consumption and anaerobic metabolism; all of which are central themes in the DOE Energy Biosciences research program.

Significant achievements in 2006-2008: We have made substantial progress in understanding the differences in the abilities of the two *Methanosarcina* species to metabolize hydrogen. 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. Taken together, the data produced in the current grant period demonstrate very significant differences in the electron transport chains of the two organisms, which we believe reflects the adaptation of these species to freshwater and marine environments, respectively.

Using a variety of mutant strains, we showed that under most growth conditions *M. barkeri* utilizes H₂ as obligate electron carrier for methanogenesis *regardless* of the substrate be used: i.e. all substrates are converted to H₂ during methanogenesis. H₂ 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.

In contrast, *M. acetivorans* does not express hydrogenase activity despite having a nearly full complete set of hydrogenase genes. We showed that the promoters of these genes have been specifically inactivated in *M. acetivorans*, explaining the observed lack of activity. The lack of hydrogenase gene expression indicates that *M. acetivorans* has another mechanism for energy conserving electron transport. We showed that at least three novel membrane complexes are involved in this hydrogen-independent electron transport chain. Our data suggest that this marine organism has evolved to prevent “hydrogen cycling”, possibly to prevent competition with H₂-utilizing sulfate reducers, which are especially abundant in marine systems.

Finally, we have begun the study of the terminal step in the electron transport chain of *Methanosarcina*, which is catalyzed by heterodisulfide reductase (Hdr). In contrast to other methanogens, *Methanosarcina* species have two types of Hdr. Our data clearly show distinct roles for the two classes of Hdr. Although one of the Hdr copies (*hdrDE*) appears to be essential for energy conservation and methanogenesis, the other (*hdrABC*) appears to play a biosynthetic role.

Science objectives for 2008-2009:

- Test whether the *M. acetivorans* hydrogenases have the capacity to be functional by complementation of *M. barkeri* mutants
- Further characterization of hydrogen-independent energy-conserving electron transport in *M. barkei* via whole genome microarray experiments
- Characterization of hydrogen-independent energy-conserving electron transport in *M. acetivorans* via genetic analysis
- Assess the roles of the multiple heterodisulfide reductase (Hdr) isozymes in *M. barkeri* and *M. acetivorans* using a combination of genetics, biochemistry and whole genome microarray experiments .

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Mechanisms of Plant Cell Wall Loosening

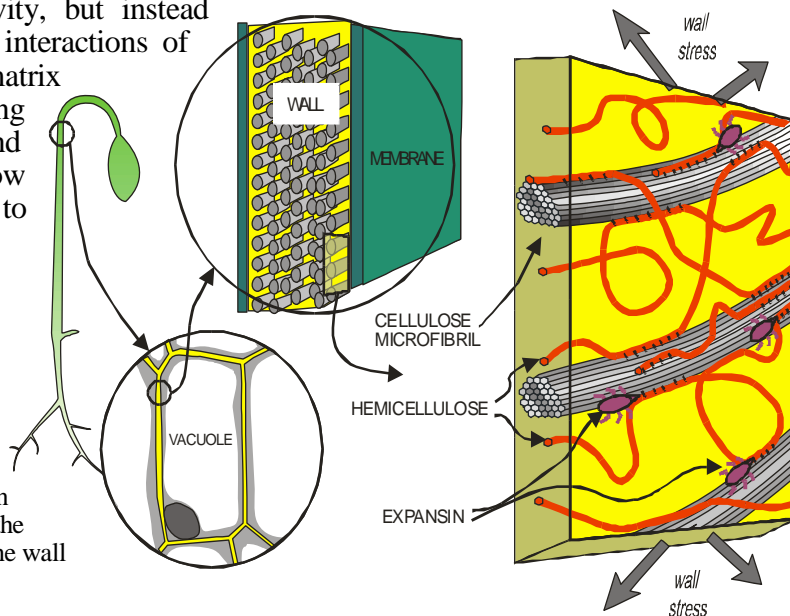
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Overall research goals: Our long-term goals are to elucidate the molecular controls and mechanisms of plant cell wall enlargement, with a special focus on the molecular structure and action of the cell-wall loosening proteins known as expansins. Expansins were discovered as the major mediators of pH-dependent cell wall extension ('acid growth') in plants. They are important for plant growth (cell enlargement) and other developmental events such as fruit softening, abscission and pollen tube invasion of the stigma and style. Their mechanism of loosening the cell wall is still obscure, as they lack classical enzymatic activity, but instead seem to modulate the noncovalent interactions of cellulose and glycan-binding matrix polysaccharides. We are combining structural, biochemical, imaging, and genetic approaches to understand how expansins interact with cell walls to induce loosening and polymer creep.

Figure 1. Plant growth entails cell expansion which is physically limited by the surface expansion of the cellulosic cell wall. In the cell wall cellulose microfibrils are tethered together by hemicelluloses (red strands) such as xyloglucan or arabinoxylan. Expansin (purple) induces cell wall creep by allowing the cellulose-hemicellulose network to yield to the wall stresses generated by cell turgor pressure.



Significant achievements in 2006-2008: We solved the crystal structure of beta-expansin protein from maize pollen (EXPB1), which consists of two small domains tightly packed onto each other to form a long planar surface, suitable for binding up to 10 sugar residues of a polysaccharide chain.

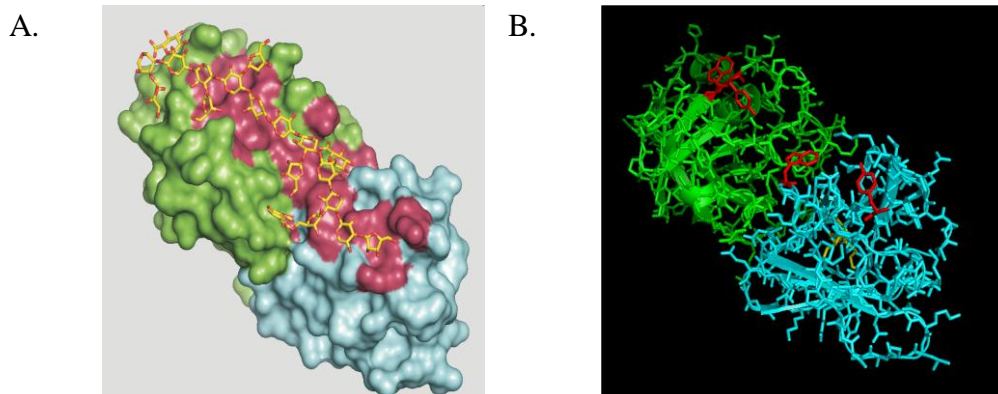


Figure 2. Crystal-based structure of beta-expansin (EXPB1). **A:** A space-filling model of the protein with an arabinoxylan filtered to its surface. The amino-terminal domain (green) has a double-psi β -barrel fold with partial conservation of the catalytic site found in family-45 glycosyl hydrolases. The carboxy-terminus (cyan) forms an 8-stranded beta-sandwich (an immunoglobulin-like fold). A strip of highly conserved residues (red) spans the two domains, forming an open surface with aromatic and polar residues suitable for polysaccharide binding. **B:** Wire-frame model showing the four aromatic residues that form part of the presumptive polysaccharide binding surface.

- Maize mutants deficient in pollen beta-expansin (EXPB1) production were identified. The mutant phenotype in the pollen included reduced reproductive success of the pollen when competed with wild-type pollen at high pollen loads. Microscopic observations indicate that the EXPB1-deficient pollen penetrate the stigma and style more slowly than wild type pollen. These observations provide an evolutionary explanation for the unusual abundance of beta-expansins in grass pollen, which is associated with a proliferation of beta-expansin genes in the maize genome.
- A protein named EXLX1 (gene=yoaJ) from *Bacillus subtilis* has a structure that is homologous to beta-expansin (structure analysis was a collaboration with F. Kerff and coworkers at the University of Liege). It binds to plant cell walls and has weak cell wall extension activity characteristic of beta-expansin, and also lacks lytic activity against bacterial and plant cell wall polysaccharides. Deletion of the gene encoding EXLX1 greatly reduced the bacterium's ability to colonize maize roots, suggesting that EXLX1 promotes plant surface colonization. The presence of EXLX1 homologs in a small but diverse set of plant pathogens further supports a role in plant-bacterial interactions.

Science objectives for 2008-2009:

- We will use isothermal titration calorimetry (ITC) and other methods to define EXPB1's target polysaccharide and attempt to make crystals of the EXPB1::oligosaccharide complex suitable for X-ray analysis and structure solution.
- We will use fluorescence recovery after photobleaching to assess EXPB1 mobility while bound to the cell wall, and use fluorescent derivatives of EXPB1 to localize its target binding sites in tissue cross sections.
- Another major focus will be on bacterial homologs of plant expansins, which we will express in *E. coli* and assess their activity and binding properties.

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Analysis of ABCB phosphoglycoproteins (PGPs) and their contribution to monocot biomass, structural stability, and productivity

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Overall research goals: To use plants and heterologous systems to elucidate the influx and efflux mechanisms of ABCB phosphoglycoproteins at the molecular level. The ultimate objective of this research is to manipulate ABCB function to increase useable plant biomass. Specific goals are to: 1) *Identify the specific domains and residues that confer substrate specificity to ABCB-mediated auxin transport.* 2) *Determine which functional domains regulate the directionality of ABCB-mediated auxin transport* 3) *Determine which ABCBs regulate monocot plant height and form and how they interact.*

Significant achievements 2006-2008

1. *PIN and ABCB auxin transport systems function co-ordinately.* Directional transport of the phytohormone auxin is established primarily at the point of cellular efflux and is required for the establishment and maintenance of plant polarity. Studies in whole plants and heterologous systems indicate that PINFORMED (PIN) and P-glycoprotein (ABCB) transport proteins mediate cellular efflux of natural and synthetic auxins. We showed that ABCB1 and 19 co-localized with PIN1 in the shoot apex, and with PIN1 and 2 in root tissues. Specific ABCB-PIN interactions were demonstrated in yeast two-hybrid and co-immunoprecipitation assays. PIN-ABCB interactions enhanced transport activity and substrate/inhibitor specificities. In contrast, no interactions between ABCBs and the AUX1 influx carrier were observed. *pin abcb* mutants exhibited phenotypes that are both additive and synergistic, suggesting that PINs and ABCBs characterize coordinated, independent auxin transport mechanisms that also function interactively.
2. *ABCB19 characterizes unique membrane microdomains that are distinct from domains characterized by ABCB4.* Inhibitor and mutant analyses showed that ABCB19 function is not regulated by the dynamic cellular trafficking mechanisms that regulate PIN1 in apical tissues. However, endocytosis of the styryl dye FM4-64 labels diffuse rather than punctuate intracellular bodies in *abcb19 (pgp19)*, aggregations of PIN1 induced by short-term brefeldin A treatment did not disperse after BFA washout in *abcb19*, and PIN1 plasma membrane localisation in *abcb19* roots was more easily perturbed by the detergent Triton X-100. ABCB19 was found to be stably associated with sterol/sphingolipid enriched membrane fractions containing BIG/TIR3 and partitions into Triton X-100 detergent-resistant membrane (DRM) fractions. In wild type, PIN1 was also present in DRMs, but was less abundant in *abcb19* DRMs. These data suggest that ABCB19 stabilises PIN1 localisation at the plasma membrane in discrete cellular subdomains.
3. *Development of an S. pombe system for analyses of recombinant plant transport proteins.* Several heterologous systems have been developed to study the transport activity of ABCB, PIN, and AUX/LAX transporters. *Saccharomyces cerevisiae* can be used for heterologous expression of some, but not all of these proteins. Most notably, the major Arabidopsis auxin transport proteins PIN1, AUX1, and ABCB19 cannot be functionally expressed in *S. cerevisiae*. The lack of cell wall, the necessity of viral transfection, and difficulty in performing kinetic assays limits the utility of mammalian systems used for this purpose. Other limitations have been found with *Xenopus* oocyte and insect systems. *Schizosaccharomyces pombe* has plant-like polar sterol-rich membrane domains and more plant-like N-glycosylation activity. *S. pombe* has only 11 ABC transporter genes and single copies of many other transporter genes. We have made an extensive effort to develop the *S. pombe* system for studies of plant transport proteins. This has included development of easy to implement gene knockout protocols and knockout lines for all ABC and a number of other membrane transporters. We expressed both AtABCB1 and AtABCB19 in *S. pombe* ABC transporter double mutant *mam1Δpdr1Δ* under the inducible nmt41 promoter. As expected, *S. pombe* cells expressing AtABCB1 and AtABCB19 accumulated less ³H-IAA compared to vector controls. Second, we deleted the auxin effluxer like 1 (*AEL1*) gene using disruption plasmids and expressed PIN proteins in the mutant *ael1Δ*. *ael1Δ* lines expressing PIN1, PIN2 and PIN7 accumulated less ³H-IAA than that controls, consistent with

auxin efflux function. We also expressed AUX1 in *vat3Δ S. pombe* mutants and observed auxin uptake activity of AUX1 that was competitively inhibited by 2, 4-D but not NAA, as expected. As a test to determine whether the system can be used for non-auxin transport studies, a putative nicotine uptake transporter from tobacco was expressed and successfully assayed for activity in the system.

4. **Modeling of ABCB structures.** To gain a clearer understanding of how eukaryotic ABCB proteins interact with their transport substrates, we developed computer models of plant and human ABCB transporters based on the Sav1866 half transporter crystal structure. Sequence and structural comparisons showed that characterized plant and mammalian ABCB transporters share a common architecture. Our analysis also identified candidate substrate binding sites in the transmembrane domains of the proteins near the inner leaflet of the plasma membrane. We proposed a general transport mechanism for ABCB proteins based on the predicted substrate binding pockets in the transmembrane domains (TMDs), the reported movement of nucleotide-binding domains (NBDs), and the deduced movement of TMDs driven by ATP hydrolysis. Re-examination of recent mutational analyses indicates that these new structural models are consistent with existing experimental data. Furthermore, the conserved "gate" sequences in the animal ABCB1 transporters explain their substrate promiscuity, while a divergent "gate" region in plant ABCBs accounts for their substrate specificity. Finally, these comparative models suggest potential domains that regulate the directionality of the plant ABCB transporters. However, they also suggest that eukaryotic uptake and conditional uptake/export transporters are structurally and evolutionarily distinct from prokaryotic uptake transporters. Our data provide a significant framework for the experiments in investigation of binding sites and directionality for ABCBs. This work will be reported in a commissioned review in *TIBS*.
5. **Dissecting the function of ABCBs in *Arabidopsis* using multiple RNAi.** In *Arabidopsis* genome there are 22 PGP/ABCB genes and in rice there are 17 ABCB genes. In *Arabidopsis*, ABCB1, ABCB19 and ABCB4 are auxin transporters and ABCB14 is a malate uptake transporter in stomatal guard cells. Among the uncharacterized ABCBs, most occur in highly similar clusters suggesting functional redundancy. RNAi constructs were designed using the inducible pOpOff system to knock down 1) *ABCB2, 10*; 2) *ABCB13, 14*; 3) *ABCB11, 12, 13, 14*; and 4) *ABCB15, 16, 17, 18* and were used to transform Col-0 plants. The homozygous lines of *ABCB2/10* RNAi are smaller than controls. *ABCB2/10* RNAi plants show wavy and skewed root phenotypes. *ABCB 11/12/13/14* RNAi plants show wavy but not skewing root phenotypes. As expected, *ABCB 13/14* RNAi plants were like wild type when grown under normal conditions, but are more sensitive to water stress. *ABCB 15/16/17/18* and *ABCB 15/16/17/18* RNAi transformants are currently being analyzed.
6. **Mutational analysis of *OsABCB10* rice mutants.** We made an extensive screen of putative *abcb* Tos17 insertion mutants from the RIKEN collection. Most of the mutations were either misreported or only a single allele could be identified. However, multiple alleles of *osabcb10* were identified and were extensively characterized. *OsABCB10* has also been cloned and has been localized *in planta* by *in situ* hybridization.

Science objectives for 2008-2009:

1. Mutational analysis of predicted binding sites in AtABCB4 and AtABCB19 in *S.pombe* and *in planta*
2. Functional characterization of AtABCB2, AtABCB10, and OsABCB10 in *S. pombe*.
3. Completion of phenotypic analyses of RNAi lines described above
4. RNAi transformation of rice targeting OsABCB14 and 22 putative auxin transporters

References to work supported by this project 2006-2008:

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Identification of Proteins Involved in Hydrogen Metabolism during Syntrophic Growth of *Desulfovibrio*.

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Overall research goals: Hydrogen is an important electron carrier used during syntrophic interactions among bacteria and therefore the production and oxidation of H₂ are critical processes during the degradation of organic compounds. The goals of this project are to: (a) Identify genes in *Desulfovibrio desulfuricans* G20 involved in mediating H₂ transfer between species; (b) identify and characterize proteins in strain G20 that are used for H₂ production and/or oxidation; and (c) optimize H₂ production by strain G20.

Significant achievements in 2006-2008:

The identities and roles of a number of proteins involved in syntrophic growth and H₂ metabolism have been addressed during the first part of this study. We first generated a transposon mutant library using a mini-Tn10 transposon-bearing plasmid that was shown to mutagenize *D. desulfuricans* G20 efficiently and randomly. The mutant library contained 5760 mutants and therefore provided about 1.5 fold coverage of the 3775 candidate protein-encoding genes found in the G20 genome. The mutant library was then screened for mutants unable to grow in syntrophic association with *Methanospirillum hungatei* on lactate as an electron donor. We identified 27 mutants that were either unable to grow syntrophically or exhibit slow syntrophic growth and present a small group of them in Table 1 along with results of growth experiments with them.

Genes within several functional groups were identified and shown to influence syntrophic growth. These included genes involved in H₂ oxidation (electron transfer for PMF generation), lactate oxidation for H₂ production, posttranslational modification (maturation of the hydrogenase) and signal transduction. The abundance of genes involved in H₂ oxidation suggest that H₂ oxidation may be an important process for energy production during lactate oxidation when syntrophic growth occurs likely allowing continuous electron flux to the methanogen for CO₂ reduction. However, H₂ production was observed for both parent and mutant strains of *Desulfovibrio* G20 suggesting that other electron carriers aside from H₂ and formate may shuttle the electrons during interspecies electron transfer.

Three mutants deficient in hydrogen/formate uptake were obtained through the screening process. Mutations were in the cytochrome *c*₃ (*cycA*), Fe-only hydrogenase (*hyd*) and molybdopterine oxidoreductase (*mop*) genes. The three mutants exhibit similar phenotypes in that (a) syntrophic growth is either completely abolished or slowed; (b) no detectable growth for *mop* and *cycA* occurs in pure culture with sulfate as the electron acceptor and either formate, H₂, or ethanol as the electron donor; *hyd* showed a slow growth rate in above conditions; and (c) low levels of growth on pyruvate as the electron donor with sulfate. Complementation of these mutants by transformation with a plasmid carrying the intact genes from parent strain allowed recovery of growth on H₂ and formate, showing that these specific genes are involved in H₂ oxidation. We hypothesize that these three proteins form an electron transfer chain in which electrons derived from formate, H₂, pyruvate or ethanol oxidation are transferred to the respiratory proteins involved in sulfate reduction. These proteins may be key components responsible for H₂ cycling.

Science objectives for 2008-2009:

- The selected genes will be isolated and recombinant protein products will be purified. If recombinant proteins cannot be produced in *E. coli* grown anaerobically, they will be purified from strain G20.
- Biochemical characterization of these proteins will be then conducted and protein complexes will then be prepared to determine interaction among proteins.
- mRNAs of functional genes from *D. desulfuricans* strain G20 grown with lactate either syntrophically with *M. hungatei* or in pure culture will be quantified using microarrays. This will allow identification of additional genes involved in H₂ production or electron transfer.
- The results from the above experiments will determine the mechanisms for H₂ production from organic compounds and identify cellular components needed for the reactions. Ultimately, parts or all of these enzymes may be used for industrial production of H₂.

Table 1. Mutated genes identified and shown to be involved in syntrophic growth along with results of growth experiments in pure culture with electron donors in the presence of sulfate. The ability of the mutants to grow syntrophically on lactate with *M. hungatei* and to produce H₂ during growth on lactate-sulfate (H₂ evolution) is given.

Locus	Predicted protein	Growth						
		Syntrophy	Lactate	H ₂	Format	Pyruva	EtOH	H ₂ evol
Parent strain		+	+	+	+	+	+	+
Dde_2933	Molybdopterin oxidoreductase, molybdopterin-binding subunit	-	+	-	-	±	-	+
Dde_3245	iron-sulfur cluster-binding protein	±	±	+	+	ND	ND	+
Dde_0082	Fe-hydrogenase small subunit	±	+	±	±	±	-	+
Dde_3775	ferredoxin I	±	±	±	±	ND	ND	+
Dde_0364	Hydrogenase expression/formation protein HypD	±	+	+	+	ND	ND	+
Dde_0555	(NiFe) hydrogenase maturation protein HypF	±	+	+	+	ND	ND	+
Dde_3182	cytochrome c3	-	+	-	-	±	-	+

+, grows similarly to the parent strain. ±, grows slower than parent strain. - no detectable growth or negative within a 10-d incubation at 37 °C. ND Not determined.

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Mechanistic Studies of Biological Methane Synthesis

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Overall research goals: The research objectives are to elucidate and understand the mechanism of biological methane synthesis by the nickel metalloenzyme, methyl-SCoM reductase. We are using a combination of biochemical and biophysical approaches to isolate and characterize key intermediates in the pathway of methane synthesis.

Significant achievements in 2006-2008: Methyl-coenzyme M reductase (MCR) from methanogenic archaea catalyzes the terminal step in biological synthesis of methane. Using coenzyme B (CoBSH) as the two-electron donor, MCR reduces methyl-coenzyme M (MeSCoM) to methane and the mixed disulfide, CoB-S-S-CoM. MCR contains coenzyme F430, an essential redox-active nickel tetrapyrrole at its active site. The active form of MCR (MCRred1) contains nickel in the Ni(I) redox state, Ni(I)-F430. The role of nickel in the catalytic cycle is controversial and two catalytic mechanisms have been proposed for methane synthesis: one involves an organometallic methyl-Ni(III) intermediate while the other includes a methyl radical.

We have trapped the key Ni(I)-F430 and alkyl-Ni(III)-F430 species and characterized them by various spectroscopic approaches, and showed that the alkyl-Ni species undergo reactions resembling the proposed intermediate steps in methanogenesis and anaerobic methane oxidation (1-3). Recently, we trapped the active Ni(I) and the methyl-Ni(III) states both in solution and in crystals. We also have demonstrated that the methyl-Ni species can be converted to methane (in the forward reaction), as well as to methyl-SCoM and active Ni(I)-MCRred1 (in the reverse direction). The observation of a methyl-Ni MCR species is thus of considerable significance in terms of both biochemistry and bio-organometallic chemistry. We also have trapped a radical intermediate that fits the criteria of being a catalytic intermediate in the pathway of methanogenesis. Isotope labeling studies indicate that this is a tyrosyl radical, which we surmise to be generated from one of the two Tyrosine residues located in the active site directly above the F430 cofactor. We propose a new mechanism of methane formation, which involves in successive steps: methyl-Ni(III), methyl radical, tyrosyl radical, CoB radical, and CoB-SSCoM radical anion.

Science objectives for 2008-2009:

- We will continue to characterize the various intermediates in the catalytic cycle by solution and single crystal X-ray absorption methods. This will provide a high-resolution structure of the nickel active site in its active states.
- We will determine the structure of MCR in the active states by X-ray crystallographic studies.

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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 - 50% of the cellular ATP.

Significant achievements in 2006-2008: Of the 46 P-type ATPases in Arabidopsis, 10 belong to a subfamily of calcium translocating pumps (ACAs) that are stimulated by calmodulin and located in the ER, tonoplast and PM. Knockouts of these pumps have uncovered important functions in plant development, reproduction, and responses to the environment. For example, a loss of function of plasma membrane calcium pump ACA9 results in pollen tubes that grow slow and are impaired in their ability to discharge sperm into ovules. This gene is also crucial to the plant's reproductive success under temperature stress. A disruption of plasma membrane calcium pump ACA10 (in the NO background) results in a reduced floral internode expansion defect, providing evidence that this pump functions in regulating cell growth. A double disruption of vacuolar calcium pumps ACA4 and 11 result in a high frequency of necrotic lesions and a severe reduction in growth. Preliminary result for a single set of knockout alleles for the 3 ER localized calcium pumps, ACA1, 2, 7, suggest that these pumps are also important for several aspects of plant growth and development, including pollen fitness.

The Harper lab has begun using pollen as a model cell in which growth and signalling functions can be easily quantified by a simple genetic segregation distortion analysis. 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. Using pollen fitness as an assay for stress sensitivity mutations, we have uncovered stress tolerance functions for multiple ion transport pathways, including cyclic nucleotide gated ion channels (CNGCs), a cation-chloride cotransporter (CCC), P-type Ca Pumps (ACAs and ECAs), and a P-type ATPase lipid pump (ALAs). Our biological focus is on the 4 most significant abiotic stresses: drought, cold, heat, and salt.

Science objectives for 2008-2009:

- Test the hypothesis that a plant can survive without any of the 4 plasma membrane targeted calmodulin-regulated calcium pumps.
- Test whether a H^+ -ATPase can piggy back target a Na-antiporter to the plant cell plasma membrane through an engineered leucine zipper protein-protein interaction.
- Identify the Na-ATPases from *Dunaliella salina*, a salt tolerant ("extremophile") algae being considered as a feedstock for an emerging biofuels industry. These pumps are hypothesized to enable *D. salina* to grow in saturated solutions of NaCl.

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Nanopore-supported Phospholipid Bilayers

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Overall research goals: Further development of substrate-supported lipid nanotube arrays. To attain fundamental understanding of effects of nanoscale confinement on the mechanism of self-assembly of lipid molecules and membrane proteins into functional and robust hybrid nanostructures that could assist in rational design of hybrid nanosystems for biomimetic solar energy conversion and/or bioinspired separation technology. Another significant aspect of our program is in developing new biophysical tools to advance structure-function studies of energy-transporting membrane proteins and the self-assembly mechanisms of membrane protein complexes.

Significant achievements in 2006-2008: Studies of structure and dynamics of lipid bilayers confined in nanoporous channels of anodic aluminum oxide (AAO) such as shown in Figure 1c have been continued. For lipids confined in nanopores with diameter from 40 to 200 nm the main phase transition temperatures determined by either DSC or spin-labeling EPR were found to be essentially the same as for unsupported bilayers. Lipids' rotational dynamics was also unaffected according to EPR. The main effect of the nanoscale confinement was found to be in a decreased van't Hoff enthalpy that characterizes lipid bilayer cooperativity properties. Such a confinement limits the growth of ordered domains that are characteristic of liquid crystalline phase. A calorimetric evidence that substrate-supported nanotubular bilayers exhibit a pre-transition suggesting formation of a ripple phase has been provided. To the best of our knowledge this is the first observation of the ripple phase in substrate-supported bilayers that points to little perturbations in our design.

Highly efficient protocols for aligning peptide-containing lipid bilayers inside AAO substrates for 1- and 2-D solid-state NMR studies have been developed. It was shown that such bilayers remain fluid and highly hydrated with surfaces of the both leaflets fully accessible to water-soluble molecules. These unique features that enable solvent flow through lipid nanotube arrays have been exploited for the first ever structure-function study of membrane proteins by solid-state NMR using physically the same sample. Figure 1a provides a demonstration of such NMR experiments to ascertain effects of ion binding to a model ion channel.

A method for incorporating a large electron-transfer membrane protein complex - bacterial reaction center (RC) protein from *Rhodospira rubra* - into substrate-supported lipid nanotubes has been developed. We described several

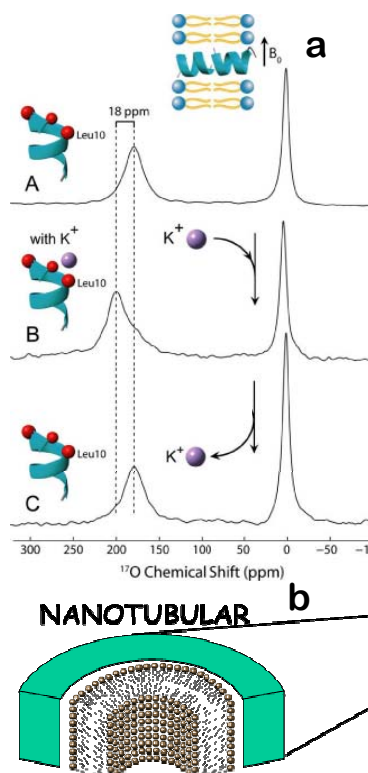


Figure 1. (a) ^{17}O NMR (19.6 T) spectra of ^{17}O -[D-Leu 10]-gramicidin A uniformly aligned in DMPC bilayers in the absence and presence of KCl (2.4 M) with a peptide:lipid ratio of 1:16 and excess hydration in AAO-aligned nanotubular bilayers.

(b) Cartoon of a nanotubular lipid bilayer.

(c) SEM image of a nanoporous AAO substrate fabricated by the PI group at NCSU.

incorporation techniques that result in formation of proteonanotubes that retain functionality over a period of two months. We propose that a large number of membrane proteins and their complexes could be incorporated into AAO-supported lipid nanotubes. The macroscopic lipid and protein alignment in such structures combined with solvent accessibility would assist structure-function studies with variety of spectroscopic techniques such as NMR, EPR, and optical spectroscopy without protein crystallization.

Science objectives for 2008-2009:

- Investigation of self-assembly of nanopore-confined lipids as a function of the lipid chain length with the goals of further tuning properties of the nanostructures formed; expansion of these studies to mixed lipid bilayer systems including mixtures of negatively charged lipids, cardiolipin (bisphosphatidyl glycerol), sphingolipids and other compositions as needed for studying membrane proteins involved in energy transduction.
- Mapping interfacial electrostatics of substrate-supported lipid bilayers using a series of pH-sensitive EPR-active lipids and utilization of the obtained knowledge for the rational design of the substrate-lipid interface through chemical modification of the nanochannel surface.
- Development of new types of hybrid nanostructures by investigating lipid polymorphic structures constrained by rigid nanopores with specific focus on template-constrained polymerizable lipid nanotubes.

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Understanding Acyl Chain and Glycerolipid Metabolism in Plants

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Overall research goals: Plant oils represent the largest source of renewable reduced carbon chains available from nature and are a major commodity with an annual value of over \$100 billion worldwide. In addition to providing 15% or more of dietary calories consumed by developed nations, plant oils provide a variety of non-food industrial products such as lubricants, polymers, and surfactants. These oils are produced in the seed by the fatty acid and glycerolipid pathways that are also essential for the assembly of membrane bilayers in all cells. Our lack of clear understanding of these pathways is reflected by many unexpectedly minor or unpredicted responses of plants to lipid metabolic engineering efforts. To provide the knowledge needed to better engineer oilseeds, we focus on several basic questions which remain as major gaps in our understanding. 1) Membrane transport processes control the flux of metabolites between organelles. We have characterized the transport of fatty acids across the plastid envelope and now are identifying the initial steps by which acyl chains enter glycerolipid metabolism. 2) Long chain acyl-CoA pools turn over rapidly and are dynamic regulators of metabolism and gene expression in animals, fungi and bacteria. We will determine the composition and turnover of acyl-CoA pools, and relate these studies to polar lipid turnover and the flux of acyl chains into membrane and storage lipids.

Significant achievements in 2006-2008:

Incorporation of newly synthesized fatty acids into cytosolic glycerolipids in pea leaves occurs via acyl editing. In expanding pea leaves, over 95% of fatty acids (FA) synthesized in the plastid are exported for assembly of eukaryotic glycerolipids. It is often assumed that the major products of plastid FA synthesis (18:1 and 16:0) are first incorporated into 16:0/18:1 and 18:1/18:1 molecular species of phosphatidic acid (PA), which are then converted to phosphatidylcholine (PC), the major eukaryotic phospholipid and site of acyl desaturation. However, by labeling lipids of pea leaves with [(14)C]acetate, [(14)C]glycerol, and [(14)C]carbon dioxide, we demonstrate that acyl editing is an integral component of eukaryotic glycerolipid synthesis. First, no precursor-product relationship between PA and PC [(14)C]acyl chains was observed at very early time points. Second, analysis of PC molecular species at these early time points showed that >90% of newly synthesized [(14)C]18:1 and [(14)C]16:0 acyl groups were incorporated into PC alongside a previously synthesized unlabeled acyl group (18:2, 18:3, or 16:0). And third, [(14)C]glycerol labeling produced PC molecular species highly enriched with 18:2, 18:3, and 16:0 FA, and not 18:1, the major product of plastid fatty acid synthesis. In conclusion, we propose that most newly synthesized acyl groups are not immediately utilized for PA synthesis, but instead are incorporated directly into PC through an acyl editing mechanism that operates at both sn-1 and sn-2 positions. Additionally, the acyl groups removed by acyl editing are largely used for the net synthesis of PC through glycerol 3-phosphate acylation.

Acyl-editing in initial eukaryotic lipid assembly in soybean seeds. To accurately understand the synthesis of plant oils we must identify not only the enzymes involved in triacylglycerol (TAG) synthesis but also the flow of substrates to each enzymatic step. To determine the flow of acyl chains and glycerol into TAG of developing soybeans we utilized an embryo culture system in which soybean development closely mimics *in planta* growth. Rapid *in vivo* labeling with

[¹⁴C]acetate and [¹⁴C]glycerol was used to analyze the early kinetics of acyl chain and glycerol backbone incorporation into PC, DAG and TAG. Additionally stereochemical and molecular species analysis of labeled lipids was used to determine the initial species of each lipid produced from *de novo* synthesis and acyl editing. The major flux of nascent [¹⁴C]18:1 FA out of the plastid was into the *sn*-2 position of PC alongside a previously synthesized FA, similar to the acyl editing we observed in pea leaves. Thus, the kinetics of glycerol backbone labeling and the stereochemistry of newly synthesized acyl chain labeling indicate that the major flux of acyl chains from the plastid in soybean seeds occurs first into PC, rather than into phosphatidic acid.

Identification and characterization of two *Arabidopsis thaliana* lysophosphatidyl acyltransferases with preference for lysophosphatidylethanolamine. Two *Arabidopsis* genes, At1g80950 and At2g45670, both annotated to encode proteins with acyltransferase regions and with sequence similarity to a recently identified lung lysophosphatidylcholine acyltransferase (LPCAT) were characterized. To identify their substrate specificity and biochemical properties, the two *Arabidopsis* acyltransferases, designated AtLPEAT1 and AtLPEAT2 were expressed in yeast knockout lines *ale1* and *slc1*, that are deficient in microsomal acyltransferases. Lysophosphatidyl acyltransferase activity is almost null in the yeast knockout *ale1* and expression of AtLPEAT1 in this background exhibited strong acylation activity of lysophosphatidylethanolamine (LPE) and lysophosphatidate (LPA) with lower activity on LPC and LPS. AtLPEAT2 was more selective for LPE > LPC > LPS > LPA. Both acyltransferases preferred 18:1-LPE over 16:0-LPE as acceptor and preferred palmitoyl-CoA as acyl donor in combination with 18:1-LPE. Both acyltransferases showed no or minor response to Ca²⁺, despite the presence of a calcium binding EF-hand region in AtLPEAT2. AtLPEAT1 was more active at basic pH while AtLPEAT2 was equally active between pH 6.0 - 9.0. These results represent the first description of plant acyltransferases with a preference for LPE.

Science objectives for 2008-2009:

- Characterize and determine subcellular localization of lysophosphatidylcholine acyltransferase in oilseeds
- Evaluate and test O18 labeling strategies for diagnosis of acyl editing mechanisms in oilseed metabolism.

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Physiology of Acetate Conversion to Methane

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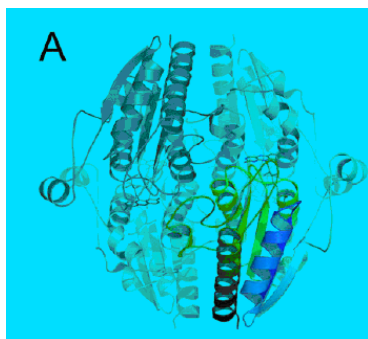
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Overall research goals: The conversion of renewable plant biomass to methane as a biofuel involves a microbial food chain comprised of at least three metabolic groups of anaerobes. Acetate is the principal intermediate in the food chain from which at least two-thirds of the methane is produced by acetotrophic methanogens. The conversion of acetate to methane is the rate limiting and least reliable step in the process. Although much is known concerning carbon transformations in the pathway, little is known of other factors that govern growth. The overall research goals of this research project are to advance an understanding of membrane-bound electron transport, energy conservation, and mechanisms of coping with oxidative stress.

Significant achievements in 2006-2008: Environmental stress is a major factor that influences the physiology of prokaryotes; however, relatively little is known how stress effects acetate-dependent growth of methanogens. A specific goal has been to elucidate the physiology and biochemistry of Isf, an iron-sulfur flavoprotein discovered in *Methanosarcina thermophila* that reduces O₂ to water (2). This research lead us to WrbA, a flavoprotein with sequence identity to Isf. WrbA was first identified in *Escherichia coli* and hypothesized to be involved in regulation of the tryptophan operon; however, our research showed that WrbA from *E. coli* (Bacteria) and *Archaeoglobus fulgidus* (Archaea) are NAD(P)H:quinone oxidoreductases (3). The enzyme reduces a variety of quinone-like compounds to the 2-electron state avoiding reaction of the semiquinone with O₂ and production of superoxide. To



address the mechanism of WrbA the crystal structure for the *E. coli* enzyme was determined in collaboration with Susana Andrade (University of Göttingen) (1). As shown in the accompanying figure, the structure shows a tetramer similar to Isf, the major difference being that WrbA lacks an iron-sulfur cluster. The results also showed that WrbA binds substrates at an active site that provides an ideal stacking environment for aromatic moieties, having a highly specific binding site for at least the ADP part of an NADH molecule in its immediate vicinity. Structures of WrbA in complex with benzoquinone and NADH suggest a sequential binding mechanism for both molecules in the catalytic cycle.

Another rationale for the crystal structure was to guide research aimed at determining how residues in the FMN binding site influence the redox properties of the flavin. Initial results show roles for several residues adjacent to the flavin binding site in modulation of redox potentials. As shown in the accompanying table, replacement of several residues adjacent to the FMN binding site with alanine perturb the one- and two-electron midpoint potentials (mV) relative to the wild-type (EcWT).

	First Transition		Second Transition	
EcWT	-81	± 11	-149	± 3.6
W98A	35	± 7.9	-168	± 1.8
Y143A	-63	± 4.3	-196	± 2.0
H133A	-104	± 5.7	-215	± 2.6
T78A	-139	± 2.7	-189	± 0.5
T116A	-167	± 1.2	-203	± 1.7

Another goal of our research is an improved understanding of acetate conversion to methane with a focus on electron transport and energy conservation in the conversion of acetate by *Methanosarcina acetivorans*. Thus, a flavoredoxin was investigated for its potential electron transport role in energy conservation or oxidative stress. Flavoredoxin is an FMN-containing electron transfer protein that functions in the energy-yielding metabolism of *Desulfovibrio gigas* of the Bacteria domain. Although characterization of this flavoredoxin is the only one reported, a database search revealed

homologs widely distributed in both the *Bacteria* and *Archaea* domains that define a novel family. A flavodoxin from *M. acetivorans* was produced in *E. coli* and biochemically characterized (4). The subunit molecular mass was 21 kDa and the native molecular mass was 45 kDa suggesting a homodimer. The protein contained one non-covalently bound FMN per monomer. Redox titration showed a E_m of -271 mV with $n = 2$ electrons consistent with no semiquinone observed in the potential range studied. A $2\times[4\text{Fe-4S}]$ ferredoxin donated electrons to the flavodoxin whereas NAD(P)H or coenzyme $F_{420}H_2$ were incompetent. The X-ray crystal structure determined at 2.05\AA resolution



revealed a homodimer containing one FMN per monomer. As shown in the accompanying figure, the structure showed the isoalloxazine ring of FMN buried within a narrow groove $\sim 10\text{\AA}$ from the positively charged protein surface that is proposed to facilitate interaction with the negatively charged ferredoxin. The FMN is bound with hydrogen bonds to the isoalloxazine ring and electrostatic interactions with the phosphate moiety. The structure, together with sequence analyses of homologs, indicate a novel FMN binding motif for the flavodoxin family.

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A proteomic study of brassinosteroid responses in plants

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Overall research goals: The research objectives are to identify brassinosteroid-regulated proteins using proteomics methods; and elucidate the roles of the brassinosteroid-regulated proteins in plant growth regulation.

Significant achievements in 2006-2008: Using subcellular fractionation followed by two-dimensional difference gel electrophoresis and mass spectrometry, we have identified a number of brassinosteroid (BR)-regulated proteins, including BSKs (BSK1, BSK2, BSK3), BRPP, BRN, and DREPP. We have demonstrated important functions of these proteins in BR regulation of plant growth and development.

We have demonstrated that BSKs are essential components of the BR signaling pathway. Loss-of-function mutation of BSK3 causes a BR insensitive phenotype and overexpression of BSK1, BSK2, BSK3 or BSK5 suppresses the *bri1* mutants but not the *bin2-1* mutant, indicating that BSKs function downstream of the BR receptor kinase BRI1 and upstream of the GSK3-like kinase BIN2 in the BR signaling pathway. BSKs are phosphorylated by BRI1 in vitro and interact with BRI1 in vivo. In contrast to BRI1-BAK1 interaction, which is induced by BR, the interaction between BSK1 and BRI1 is reduced upon BR treatment, suggesting a mechanism of signal amplification. We identified serine 230 of BSK1 as the major site for BRI1 phosphorylation. The study of BSKs fills a major gap in the BR signaling pathway and identifies the first signaling substrate of a receptor kinase in plants. This work has been recently published in Science. (Functional study of BSKs was supported by a grant from NSF).

We identified BRPP as a BR-regulated phosphatase in the plasma membrane fraction. We have shown that BRPP interacts with and dephosphorylates BZR1. Genetic studies provide evidence for an essential role of BRPP in BR signaling. The results together indicate that BRPP is another key component of the BR signaling pathway.

BRN1 is a BR-regulated protein identified in the nuclear phosphoprotein fraction. BRN1 is dephosphorylated upon BR treatment, which is similar to BZR1. A T-DNA insertion mutant *brn1-1* shows altered flowering time and branching pattern. Since several BR mutants, such as *bzr1-1D*, also show flowering and branching phenotype, BRN1 is likely to be another nuclear protein that mediates BR responses. Mechanism of BRN1 function and its interaction with other BR signaling components are being studied.

DREPP is a BR-regulated plasma membrane-associated protein. We have shown that DREPP promotes cell elongation and can partly suppress the BR-deficient mutant phenotypes. The sequence of DREPP shares no similarity with any other proteins with known functions. To understand the function of this novel protein, we are trying to identify other proteins that interact with DREPP. In the meantime, we have found that the expression of DREPP is directly regulated by BZR1. BZR1 directly binds to the promoter of DREPP to activate its expression.

Science objectives for 2008-2009:

- Complete genetic and transgenic studies of BRPP. Illustrate the role and functional mechanism of BRPP in BR signaling. Determine whether BR regulates BRPP or BRPP constitutively

dephosphorylates BZR1. Determine the upstream regulator of BRPP in the BR signaling pathway.

- Continue genetic and transgenic studies of the functions of BRN1; identify the kinase and phosphatase of the BR signaling pathway that regulate BRN1.
- Identify proteins that interact with DREPP and understand the biochemical mechanism by which DREPP promotes cell elongation.
- Continue proteomic studies and identify more BR-signaling proteins using improved fractionation methods.

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Regulation of Proteolysis

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Overall Research Goals: Our research objectives are to understand how the levels of proteins are regulated in cells. In addition to transcriptional control, regulated proteolysis is an equal partner in controlling protein concentration. By regulating the levels of key transcription factors, signaling molecules and rate limiting enzymes in biosynthetic pathways, the cell regulates growth and development. Plant growth and biomass production is an important area of research. Understanding how plants grow can contribute to plants better able to utilize solar energy for biomass production. The current interest in using plants as a renewable resource makes it even more imperative that we understand the fundamental mechanisms for plant growth and development. Once we have a better understanding, we may use this information to increase productivity, either by optimizing the growth environment or by genetic engineering mechanisms. Our lab is focusing on how proteolysis is regulating seedling growth, but what we learn is applicable to other phases of the life cycle, such as vegetative growth, flowering, fruit development, and seed set, which are other important aspects contributing to plant biomass.

The major mechanism to regulate protein abundance is by the ubiquitin pathway. Ubiquitin is a 76-aa protein that covalently attaches typically to the lysyl amino group of other proteins. The enzymes that catalyze ubiquitin attachment are E1 (ubiquitin activating), E2 (ubiquitin conjugating or UBC) and E3 (ubiquitin ligase). E1 and E2 covalently link to ubiquitin in a labile thioester linkage. The third enzyme, E3 or ubiquitin ligase, recognizes the substrate protein and also binds to the E2 carrying activated ubiquitin for transfer. Hence, E3s are key to understanding the specificity and can control the ubiquitination pathway.

One type of E3, called the CRL for cullin-RING-ligase, is a major type of E3 in all organisms, including plants, with likely over 700 different ligases of this type in one species. In plants, three different cullins have been described, CULLIN1, CULLIN3a/b and CULLIN4. While all share the same E2 binding subunit, the RING protein called RBX, each assembles with a different substrate interacting subunit that brings the substrate to be ubiquitinated close to the activated ubiquitin-E2. One major mechanism that serves to activate this ligase superfamily is modification of the cullin subunit by the ubiquitin-like protein, RUB for Related to Ubiquitin (called Nedd8 in animals). Our lab has focused on understanding the RUB pathway and how CULLINs are regulated. There is an E1-like, E2-like and E3-like activity that catalyzes RUB attachment. Recently, additional proteins in mammals have been described that regulate the RUB pathway. Their role in plants is unknown.

Significant Achievements in 2007-2008:

Studies on AXL1- In the Arabidopsis predicted proteome, we identified a protein related to the known RUB activating enzyme subunit AXR1, called AXL1 (AXR1-like). Is this an active gene and what role does this protein play in the RUB pathway? We developed an *in vitro* activity assay for RUB activation. We demonstrated that AXL and AXR have the same activity *in vitro*. To determine *in vivo* role, we characterized a line with T-DNA insertion in *AXL*, which does not express authentic *AXL* mRNAs. In contrast to loss of *AXR1* alone, loss of just *AXL1* function does not significantly affect gross plant growth-germination, organ size, flowering time,

or fertility. Loss of *AXR1* function, in contrast, does have multiple effects on growth, which has been reported previously. We crossed *axl1* plants to *axr1* plants to make a plant lacking both proteins. Double homozygous *axr1axl* seedlings are never produced. Double mutant embryos fail to complete embryogenesis. Some shrunken inviable seed are produced, indicating that arrest occurs at various times after fertilization.

Mutations in CUL1 C-terminus affect RUB modification- In a study of a *CULLIN1* mutation that we isolated in our laboratory, we discovered that it affected the ratio of RUB-modified to unmodified CUL1. This mutation is close to the RUB modification site in CUL1. We have determined that this mutant cullin protein, called *cul1-7* is capable of being Rub modified. However, the unmodified *cul-7* protein is degraded more rapidly than wild-type CULLIN and does not bind to RBX. We have discovered that RBX interaction may be important in regulating the levels of CULLIN in cells.

Mass spectrometry of rubylated proteins- During the past year, we have started to generate transgenic plant lines that express epitope tagged forms of RUB proteins. Given recent papers on the identification of additional Nedd8 modified proteins in mammals, this will be an important objective. Are there additional RUB modified proteins in plants besides the CULLIN?

Degradation of ARF1- We are interested in identifying additional target proteins of the ubiquitin pathway and with DOE support have analyzed the degradation of Auxin Response Factor 1, and important transcription factor in auxin signaling. We generated transgenic lines expressing tagged forms of ARF1 and measured their degradation rates. Two differently tagged ARF1 proteins had the same half-life of 3 hours. Using deletion derivatives, we were able to identify a region of ARF1 required for its observed degradation. Finally, we demonstrated that ARF1 degradation requires the proteasome, but did not appear to require CUL1. This suggests that ARF degradation is not mediated by ubiquitylation by a CUL1 type E3 ligase. This work was completed last year and is published (see below).

Science Objectives for 2008-2009:

- Determine the in vivo role for AXL1 by characterization of additional null alleles alone and in combination with the *axr1* mutation, and by determining whether AXL can substitute for AXR when expressed under control of the *AXR1* promoter in an *axr1* mutant background.
- Determine the role DCN (Defective in Cullin Neddylation) in the plant RUB cycle
- Identify additional RUB targets using mass spectrometry
- Characterize the effects of *CULLIN1* mutations

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Control of plant cell wall synthesis by precursor availability and glycosyltransferase activity

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Overall research goals: We are interested in determining whether the synthesis of plant cell wall material is limited by the availability of nucleotide sugars, glycosyltransferase activities or both. During the last couple of years we focused our research efforts on the identification of genes involved in nucleotide sugar interconversion pathways and genes encoding fucosyl- and galactosyltransferases in the biosynthesis of the hemicellulose xyloglucan. This polysaccharide is partially but not completely galactosylated, which raises the question whether the availability of the donor substrate UDP-galactose or the activity of the Golgi-localized galactosyltransferases XyGAT1 and XyGAT2 limit the degree of galactosylation. This question can be addressed by overexpressing the two galactosyltransferases and increasing the availability of UDP-galactose in the Golgi lumen followed by structural analysis of xyloglucan from the transgenic plants.

Significant achievements in 2006 – 2008: To lay the groundwork for the research goals summarized above, we functionally expressed several predicted nucleotide sugar interconversion enzymes from Arabidopsis in *E. coli*, and determined their enzymatic activity. The *RHM1* gene was shown to encode an enzyme that consists of two domains and catalyzes the conversion of UDP-glucose to UDP-rhamnose (1). Mutations in this gene suppress the root-hair phenotype of *lrx1* plants and lead to a reduction in the amount of rhamnogalacturonan-II (RG-II) in the root (2). Overexpression of a mutated form of RHM1 in wild type Arabidopsis caused a substantial decrease in the rhamnose content of cell wall material possibly because of a dominant negative effect of the mutant protein on two closely related isoforms. To determine the functional significance of the apiose residues in RG-II, virus-induced gene silencing was used to downregulate UDP-apiose/UDP-xylose synthase activity in *N. benthamiana*. This genetic manipulation resulted in swollen cell walls and disintegration of organelles presumably because of a defect in the assembly of side chains A and B of RG-II (3).

Using a combination of bioinformatics and reverse genetics, we isolated mutants in the *XyGAT2* gene of Arabidopsis that is predicted to encode a xyloglucan-specific galactosyltransferase attaching a galactose residue to the central xylose residue in the XXXG core structure of xyloglucan. This gene is a paralog of the *XyGAT1* (*MUR3*) gene that encodes a xyloglucan-specific galactosyltransferase that converts the XXXG building block to the galactosylated form XXLG. Null mutations in the *XyGAT1* gene cause a stunted phenotype that is more severe in *xygat1 xygat2* double mutants even though *xygat2* single mutants do not show any obvious growth abnormalities (Fig. 1).

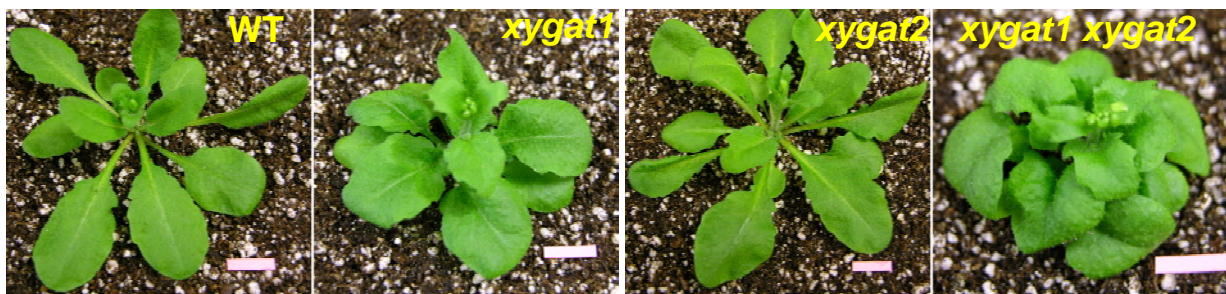


Figure 1: Growth habit of xyloglucan galactosyltransferase mutants of Arabidopsis

We constructed transgenic plants that overexpress XyGAT1 and XyGAT2, respectively, and found that some of the XyGAT1 overexpressing lines showed an approximately 50% increase in the fucose content of their cell wall material. This could be explained by a substantial increase in the conversion of XXXG to XXLG by XyGAT1 followed by fucosylation to XXFG by xyloglucan fucosyltransferase. The structure of xyloglucan from the overexpressing lines is currently being analyzed to test this hypothesis.

To address the issue of precursor availability for the two xyloglucan galactosyltransferases, we characterized the enzymatic properties of the five UDP-glucose 4-epimerase isoforms of Arabidopsis (UGE1 through UGE5). UGE2, UGE4 and UGE5 turned out to act almost exclusively on the UDP-Glc/UDP-Gal pair whereas UGE1 and UGE3 also interconverted UDP-Xyl and UDP-Ara. It is therefore reasonable to manipulate the expression of one of the first three enzymes to change the availability of UDP-Gal without affecting the UDP-Xyl/UDP-Ara pool.

Objectives for 2008 – 2009:

- Biochemical characterization of XyGAT2 via heterologous expression in *N. benthamiana* followed by enzyme assays.
- Construction and analysis of plants that overexpress both XyGAT1 and XyGAT2.
- Overexpression of UGE2 and/or UGE4 in the cytosol.
- Transformation of Arabidopsis plants with chimeric proteins where the catalytic domain of UGE2 and/or UGE4 is fused to Golgi-localization signals.

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Structural, Functional, and Integration Studies of Solar-Driven, Bio-Hybrid, H₂-Producing Systems

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Overall research objectives: The overall goal of the project is to advance basic knowledge essential for understanding hydrogenase structure-function; integrating hydrogenases with (nano)materials and on electrode surfaces; characterizing the performance of biocatalysts in artificial photobiochemical devices. The project involves four main areas of research: (i) theoretical studies of the electronic and physical structure of the [FeFe] hydrogenase; (ii) biochemical and vibrational studies of [FeFe] hydrogenase; (iii) relationship of the physical, electrical and catalytic properties of [FeFe] hydrogenases on electrodes and in photobiochemical devices; and, (iv) optical and electronic properties of hydrogenase-carbon nanotube complexes.

Significant achievements in 2005-2008: Theoretical investigations of the electronic and physical structure of [FeFe] hydrogenase have led to fully parameterized models of the FeS-clusters. The models allow for more accurate calculations of cluster motion in molecular dynamics, effects of redox states on protein dynamics and determination of electron transfer mechanisms. NBO based covalency analysis of fully bonded H-cluster has been completed. Comparisons between artificial and natural ligand arrangements (assigned based on crystallographic and vibrational studies) show the natural configuration has a distinctive HOMO-LUMO energy and a balanced bonding of the bridging CO.

An initial investigation on the interactions between carbon single-wall nanotubes (SWNTs) suspended by surfactants in aqueous solutions and [FeFe] hydrogenase led to a recent published manuscript. It was discovered that, under appropriate conditions, hydrogenase displaced surfactant and adsorbed onto individual SWNTs. The current understanding of the findings is that hydrogenase mediated electron injection into nanotubes having appropriately positioned LUMO levels when the H₂ partial pressure is high.

Results of [FeFe] hydrogenase immobilization on carbon electrodes, and integration in the PEC cell have been recently published. In summary, hydrogenase reversibly adsorbed to carbon electrodes, resulting in 'immobilized' hydrogenase films. Corrected for the electrochemically active surface area, a variety of carbon electrodes gave similar current densities. The voltammograms show a pronounced 'catalytic bias' towards proton reduction, manifest as larger cathodic than anodic currents at all overpotentials. Compared directly to that of Pt-electrodes under similar experimental conditions, the cathodic current densities for hydrogenase (based on the active surface area), were ~40 % of those for Pt. Hydrogenase/carbon felt electrode current densities were considerably higher than bulk Pt in terms of the two-dimensional surface area. In the PEC cell photoanode efficiency depends upon the potential of the cathodic H⁺/H₂ redox couple, which influences the occupancy of conduction band/trap states in the TiO₂, and hence the rate of electron recombination. At negatively imposed potentials the electron density in the TiO₂ conduction band increases, favoring electron recombination with oxidized sensitizer dyes, decreasing photocurrents. Wired to a hydrogenase-

cathode in a two-electrode configuration, the H^+/H_2 couple determines the potential and electron density in the TiO_2 in an analogous manner. Hydrogenase operates near the formal potential of the H^+/H_2 redox couple, with the cathode operating at these negative potentials, the TiO_2 conduction band was not sufficiently reducing to maintain the diode effect of the photoanode. This results in ohmic behavior with the efficiency of the photoanode highly sensitive to the accumulation of the H_2 product near the cathode.

Science objectives for 2008-2009:

- Theoretical investigations will: minimize hydrogenase structures of all relevant redox states using molecular mechanics; calculate DFT wavefunctions and electronic coupling of cluster pairs to probe for rate determining step(s) in electron transfer and catalysis; simulate the dynamic proton-transfer (PT) process; generate reaction coordinate maps and structural features of the PT pathway.
- Investigations on hydrogenases will: seek to determine the electrochemical, biochemical and vibrational properties of native and mutant hydrogenases; characterize PT-pathway mutants; develop vibrational spectroscopy to interrogate local environmental control of catalytic site redox states in native and mutant enzymes.
- Immobilization studies will: continue to assess the electrochemical and catalytic properties of hydrogenases absorbed onto a variety of surface-functionalized carbon and gold electrodes; test porous polymer overlayers as a means to alleviate enzyme desorption.
- Single molecule imaging microscopy will: identify the sites, densities and orientations of hydrogenases on electrode surfaces made from a variety materials and attachment chemistries; develop techniques to measure electrochemical signals of single-molecules.
- Conduct time-resolved optical and electronic measurements of hydrogenase-carbon nanotube complexes under photoexcitation.

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The Role of RabA4b in Polarized Secretion of Cell Wall Components In Arabidopsis Root Hair Cells

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Overall Research Goals:

The overall goal of this research proposal is to understand the role that RabA4b-labeled compartments play in delivery of cell wall components from the Golgi complex to the plasma membrane and to characterize the molecular machinery that allows for the polarized localization of RabA4b membranes to the tips of growing root hair cells.

Significant Achievements in 2006-2008:

PI-4P displays altered distributions in *rh4-1* mutant root hairs. Membranes labeled by RabA4b display altered dynamics in the tips of *rh4-1* root hairs. The *rh4-1* mutation was cloned and identified as the At3g51460 gene (AtSAC7) using map-based cloning methods. AtSAC7 is a predicted phosphoinositide phosphatase family protein similar to yeast SAC proteins, and we showed this protein contained PI-4P phosphatase activity. Further investigation of the mechanisms by which loss of RHD4 function resulted in defects in tip-restricted expansion in root hair cells indicated that *rh4-1* had significantly increased PI-4P levels in root tissues. Visualization of PI-4P dynamics in plants stably transformed with the fluorescent PI-4P biosensor, YFP-hFAPP1 by scanning laser confocal microscopy, revealed dramatic alteration of the subcellular localization of PI-4P between wildtype and *rh4-1* mutant root hairs. We interpreted this as indication that loss of the RHD4 PI-4P phosphatase resulted not only in higher cellular PI-4P levels but that PI-4P accumulation occurred in different subcellular compartments, and that this altered PI-4P distribution likely contributed to the defects in root hair tip-restricted expansion seen in the *rh4-1* mutant plants.

RabA4d is essential for normal pollen tube growth and guidance. To date, in root hairs we have been unable to directly test the requirement for RabA4b activity in tip-organized expansion due to an inability to determine which of the 26 RabA GTPase family members are exclusively expressed in this cell type. We showed that RabA4d, which is highly similar to RabA4b, was exclusively expressed in pollen and that loss of RabA4d activity in a T-DNA insertional mutant resulted in defects in pollination and pollen tube tip-growth. We stably transformed homozygous *raba4d* plants with EYFP-RabA4d under control of the native RABA4D promoter, and showed that expression of the EYFP-RabA4d fusion could fully restore normal pollen tube growth, confirming that RabA4d is required for normal pollen tube growth in vitro. Further, we identified a defect in the transmission of pollen containing the *raba4d* allele versus *WT* pollen have shown that in vivo, this transmission defect is associated pollen tube morphology and reduced micropyle targeting.

Isolation of temperature-sensitive *tl* mutants and initial mapping of *tl1-1*. To identify other proteins involved in proper localization of RabA4b compartments in root hairs we initiated a mutagenesis screen. An EMS mutagenized population of Arabidopsis seedlings homozygous for EYFP-RabA4b was generated and we have so far screened >2500 M2 seeds representing ~1/2 of an original population of ~6000 M1 plants. We have identified 33 confirmed constitutive loss-of-function mutations. While most of these do not complement known root hair mutations, we identified three new alleles of CSLD3 (cellulose-synthase-like D3), corresponding to the previously characterized *kjk* root hair mutant. In addition to constitutive loss-of-function mutations we have identified four independent ts-mutants that display normal root hair morphology at 20°C but are unable to form root hairs at 30°C. All four of these mutants are distinct and are not new alleles to the previously characterized root hair mutants *kjk1-kjk3*, *scn1*, *trh1*,

trh3, *srh* or *rhd1-rhd4*. We are presently mapping *ltl1-1* and have narrowed this mutation down to ~90 kilobases of chromosome 3 representing ~80 putative open reading frames. Further map-based cloning is underway as well as sequencing of candidate genes within this region.

To more precisely characterize the nature of the temperature-sensitive root hair growth defect we have developed a novel imaging technique that allows us to visualize root hair growth using time-lapse video microscopy and simultaneously control growth temperatures. While wildtype root hairs display normal growth rates at both permissive and non-permissive temperatures, upon transition to 30°C *ltl1-1* root hair elongation became completely inhibited within five minutes.

Science Objectives for 2008-2009:

- To identify cargo contained within the RabA4b membranes and establish the secretory nature of this membrane trafficking pathway.
- To examine the mechanisms by which both PI-4Kb1 and RHD4 control PI-4P levels on RabA4b compartments in growing root hairs.
- To investigate the molecular defect that results in pollen tube bulge formation in *raba4b* mutant pollen.
- To identify the locus for the temperature-sensitive *ltl1-1* mutation.
- To further characterize temperature-sensitive mutants, *ltl2-1*, *ltl3-1* and *ltl4-1*.

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Amidase Mediated Modulation of N-Acylethanolamine (NAE) Signaling

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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 proposed to use 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. Manipulate NAE metabolism by altering the endogenous expression of FAAH in *Arabidopsis*.
 2. Characterize NAE12:0 sensitivity of *Arabidopsis* seedlings with altered FAAH expression as an index of successful, targeted manipulation.
 3. Determine differences in FAAH expression levels, subcellular enzyme distribution, enzyme activity levels, and NAE profiles in transgenics and mutants compared with wildtype.
 4. Examine differences in physiological characteristics and NAE-regulated gene expression in plants with altered FAAH expression.

Significant achievements in 2006-2008: NAE metabolism was originally attributed to regulatory functions in the vertebrate central nervous system with NAE metabolites binding to cannabinoid receptors as endogenous ligands. Additional work now has revealed a wider array of physiological functions for this metabolic pathway including modulating embryo development, immune function, cell division, and feeding behavior, some of which are cannabinoid receptor-independent. In plant systems, NAE types generally mirrored the acyl composition of the corresponding tissues. Moreover, they appear to be formed from similar *N*-acylated phospholipid precursors and hydrolyzed by an analogous FAAH enzyme activity. 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, and manipulation of its expression in *Arabidopsis* has led to interesting insights into the potential roles of this lipid metabolic pathway in plant growth, development and responses to environmental stressors. To date, evidence indicates that ectopic overexpression of FAAH in *Arabidopsis* leads to enhancement of overall plant size, resulting in part from increased cell size/expansion. In addition, there is an accelerated growth phenotype that can be measured by timing of leaf and inflorescence development. Interestingly, the enhanced growth phenotype that is associated with ectopic overexpression of FAAH also is associated with marked hypersensitivity to a number of abiotic and biotic stresses, and molecular studies indicate a perturbation in phytohormone signaling in these FAAH overexpression lines. Efforts are continuing with strategies to further disrupt endogenous NAE metabolism in *Arabidopsis* (and other plants) to gain a better understanding how this lipid pathway influences the balance between plant growth and responses to stress.

Science objectives for 2008-2009:

Work is continuing to identify the mechanisms by which Fatty Acid Amidases participate in the regulation of plant growth, development and responses to environmental stresses. Specifically we will focus on the following objectives.

1. Probe the Domain Structure/ Function Relationships of *Arabidopsis* Fatty Acid Amide Hydrolase (FAAH1).
2. Identify and Characterize Alternative/ Additional Amidases Important in the Regulation of NAE Metabolism.
3. Detail the Interaction(s) between the NAE and ABA Signaling Pathways under Different Developmental/ Stress Conditions.

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The Rhizobial Nitrogen Stress Response and Effective Nitrogen Fixation

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Overall research goals: The production of reduced nitrogen within a nitrogen-fixing 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: (Direct funding for this objective was initiated in summer, 2008, but the project continues 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 with a very unusual symbiotic phenotype. In symbiosis, the mutant appeared to reduce 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⁻). Subsequent work showed that the lesion leading to the Fix+Eff⁻ phenotype was in *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.

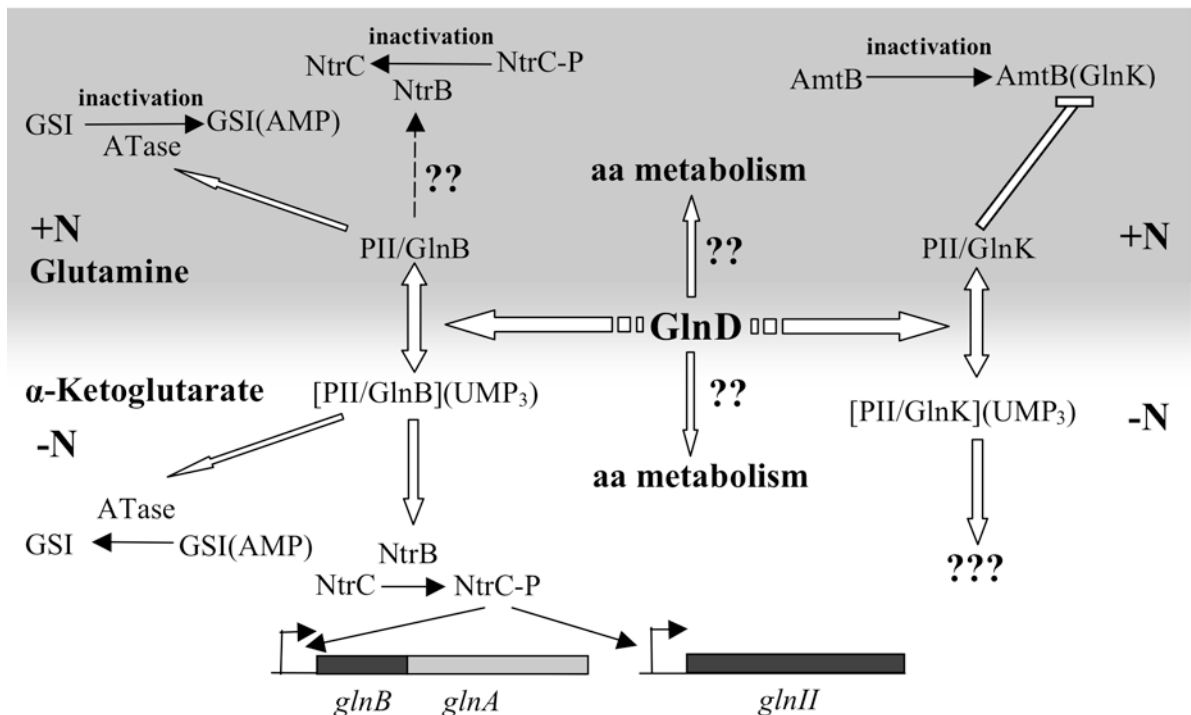


Figure 1. A generalized bacterial nitrogen stress response circuit with features specific to *S. meliloti*. GlnD uridylylates two PII proteins, GlnK and GlnB, which in turn control the levels of many nitrogen stress response related proteins by post translational modification (glutamine synthetase GSI,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

The existence of the mutant was somewhat surprising since earlier work, which we confirmed, reported that *glnD* was essential for viability. We therefore believe that the mutation has some

residual GlnD activity. The transposon insertion was predicted to remove the N terminus of GlnD and detach the remaining part of the protein from the regulatory signals that would normally control it. We constructed a “clean” mutant missing the N terminus and found this mutant was also Fix⁺Eff⁻. We have studied the free-living phenotype of the *glnD* mutants and of other mutants with defects in the hypothesized nitrogen stress response regulation cascade. The *glnD* mutants have a severe defect in using many different nitrogen sources (amino acids, nucleic acid bases, amino sugars) for growth. This is consistent with the idea that, because of the defect, the bacterial circuitry was reporting that there was sufficient nitrogen for growth, a condition that blocks the induction of nitrogen salvaging catabolic pathways that use these compounds. Previous characterization of the *S. meliloti* mutants had not revealed such a strong level of control.

The results show that the nitrogen stress response can influence the interaction and we propose that this may indicate a role for nitrogen signalling in coordinating the interaction between *S. meliloti* and the host plant. Additional data will be presented to examine some of the fundamental questions raised by the symbiotic phenotype.

Science objectives for 2008-2011:

- Determine the fate of nitrogen fixed in the *glnD* symbiosis.
- Understand how the mutant GlnD variants are controlling the rhizobial nitrogen stress response, in particular how the nitrogen stress response circuitry in *S. meliloti* differs from the stereotype represented in Fig 1.
- We speculate that nitrogen is being sequestered either physically or physiologically. We want to understand how this sequestration works and whether the relevant enzymes play a role in a wild-type symbiosis.

References to work supported by the previous related project 2004-2007:

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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 2006-2008: 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, Rolf Prade's group has expressed a thermotolerant endoarabinanase in *E.coli*. Thus, we should now have available all of the enzyme activities we need.

We have fully characterized oligosaccharides oligosaccharides from the xylogalacturonan (XGA) region of watermelon pectin by NMR and mass spectrometry. An oligosaccharide was obtained and characterized from cotton cell wall pectin which indicates that the link between arabinan and rhamnagalacturonan is via a single galactose residue on rhamnose. (See figure 1) We have also isolated oligosaccharides indicating that the (1-4) linked galactan in rhamnagalacturonans is linked directly to rhamnose O-4 as has been previously believed.

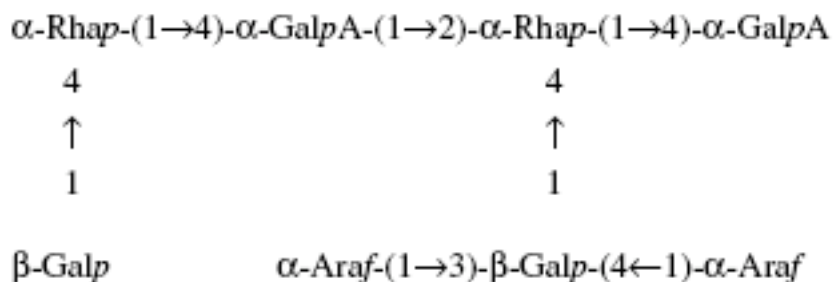


Figure 1.

Science objectives for 2008-2009:

- Follow up on the observation that xylogalacturonase allows separation of homogalacturonan (HG) fragments from the rhamnagalacturonan of apple pectin. This indicates that HG could be linked to RG via xylogalacturonan.

- Develop the use of nanospray/ MS/MS for rapid screening of fractions off HPLC columns.
- Continue efforts to obtain small characterizable fragments containing both RG and HG or XGA regions..
- Continue to try to obtain small characterizable fragments containing both xyloglucan and a region of pectin.

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Functional analysis of plant sucrose transporters

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Overall research goals: To understand the mechanism of carbohydrate transport in vascular tissue and to control the rate and selectivity of phloem loading by modifying transporter activities or regulation. We are interested in extending the range of transport activities by mutagenesis and to modify phloem loading in plants by replacing companion cell-expressed sucrose transporters.

Significant achievements in 2006-2008: Sucrose transporters (SUTs, also called SUCs) in plants belong to the glycoside-pentoside-hexuronide (GPH): cation symporter family. These are membrane proteins with twelve transmembrane spanning domains and cytosolic N- and C- termini. SUTs catalyze the coupled transport of glucosides with H^+ and are essential for the long-distance transport of sucrose in the phloem (vascular tissue). Plants have several SUT genes (nine in *Arabidopsis*, five in rice) that encode transporters with distinct physiological functions. The immediate objectives of this project are: 1) to analyze the biochemical transport activity of SUTs in plants; 2) to study the physiological function of SUTs; and 3) to modify phloem loading in plants using transgenic approaches.

Arabidopsis SUTs are classified as type I, II and III based on phylogeny. AtSUC2 is a type I SUT expressed in companion cells of the phloem, it is localized to the plasma membrane, and is required for phloem loading. AtSUC2 has a moderate affinity for sucrose ($K_{0.5} = 1.4$ mM) and transports a wide range of glucosides. All other type I SUTs have a similarly wide substrate specificity. We determined that AtSUC9 has a high substrate affinity ($K_{0.5} = 0.066$ mM), approximately 20-fold higher than AtSUC2 (2). AtSUC9 shares 67% aa identity with AtSUC2 and is therefore a good candidate to determine structural changes that affect substrate affinity.

AtSUC1 (type I transporter) from *Arabidopsis* has similar transport activity as AtSUC2 but has distinct function in the plant. AtSUC1 is expressed in the plasma membrane in roots, pollen and trichomes. Analysis of *atsuc1* mutants revealed defective pollen germination and reduced sucrose-induced anthocyanin accumulation in seedlings (3). This indicated that sucrose uptake is required for sucrose sensing in this pathway and that no other SUT can compensate for defects in AtSUC1.

In contrast to eudicot species, monocots lack type I SUTs and utilize type II transporters for sucrose uptake across the plasma membrane and in particular for phloem loading. Type II SUTs have a moderate to low affinity for sucrose ($K_{0.5} = 2-10$ mM) but are much more specific for sucrose (1) compared to type I SUTs. We are testing the hypothesis that in eudicots, glucosides in addition to sucrose are loaded into the phloem by SUTs. The implication is that in monocots phloem loading is more specific for sucrose.

We identified the function of type III SUTs. Each monocot and eudicot plant has one type III SUT gene. We analyzed LjSUT4 (type III) from the model legume *Lotus japonicus*. LjSUT4 is induced during nodulation and is expressed in nodules. LjSUT4 is a H^+ -coupled symporter and has a wide substrate specificity similar to type I SUTs and a low affinity for sucrose ($K_{0.5} = 12.9$ mM). In the cortex of nodules we localized LjSUT4 to the vacuole membrane (4). The results indicate type III SUTs transport sucrose and other glucosides into the cytoplasm from the vacuole lumen.

Science objectives for 2008-2009:

- Determine whether phloem loading in eudicots via type I SUTs is less selective than phloem loading in monocot species. We are utilizing *Arabidopsis atsuc2* mutants complemented with sucrose transporters from monocot species. *atsuc2* mutants are severely stunted due to a lack of phloem loading of sucrose and the plants accumulate starch and soluble sugars in leaves. In collaboration with Dr. Adrian Hegeman, we are comparing sugar content in phloem sap from *atsuc2* mutants complemented with wild-type AtSUC2 vs. mutants complemented with monocot SUTs.
- Analysis of the five rice sucrose transporters by heterologous expression and two-electrode voltage clamping.
- Determine if SUT4 is required for nodulation and nitrogen fixation in *Medicago truncatula*. This is in collaboration with Dr. J. Stephen Gantt, University of Minnesota.
- PsSUF4 is a sucrose transporter homolog from *Pisum* that has been reported to transport sucrose by facilitated diffusion. In collaboration with Drs. Grof and Patrick (University of Newcastle), we will determine whether PsSUF4 is H⁺ coupled or is a facilitator.

References to work supported by this project 2006-2008:

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Session 8
Plant Biochemistry - II

Functions of HKT Transporters in Protecting Plant Leaves from Salinity Stress and in Sodium Uptake into Roots.

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Overall Research Goals:

As desertification and water limitation progressively develop worldwide, increasing basic knowledge in 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 HKT transporters mediate a major mechanism for protection of plants from salinity stress. Our DOE research focuses on the biophysical, physiological and genetic mechanisms through which a class of HKT sodium cation transporters plays central roles in mediating salt tolerance in plants.

Significant Achievements:

AtHKT1 Mediates 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 is a sodium (Na^+) transporter that functions in mediating tolerance to salt stress. To investigate the membrane targeting of AtHKT1 and its expression at the translational level, antibodies were generated against peptides corresponding to the first pore of AtHKT1. Immunoelectron microscopy studies using anti-AtHKT1 antibodies demonstrated that AtHKT1 is targeted to the plasma membrane in xylem parenchyma cells in leaves. AtHKT1 expression in xylem parenchyma cells was also confirmed by *AtHKT1* promoter GUS reporter gene analyses. Interestingly, *AtHKT1* disruption alleles caused large increases in the Na^+ content of the xylem sap (Sunarpi et al., 2005; Horie et al., 2006). Our findings show that AtHKT1 selectively unloads sodium directly from xylem vessels to xylem parenchyma cells. Thus AtHKT1 reduces the sodium content in xylem vessels and leaves, thereby playing a central role in protecting plant leaves from salinity stress (Sunarpi et al., 2005; Horie et al., 2006; Horie et al., 2008). Recent research suggests that the close orthologs of AtHKT1 in the grasses rice and wheat also mediate salinity stress protection via an analogous mechanism, indicating that this subclass of HKT transporters provide a central salinity resistance mechanism in plants.

OsHKT2;1 Mediates a Major Sodium Influx Mechanism in Rice Roots: Excessive accumulation of sodium in plants causes salt toxicity. No mutation that greatly diminishes sodium (Na^+) influx into plant roots had been isolated. The OsHKT2;1 (previously named OsHKT1) transporter from rice functions as a relatively Na^+ -selective transporter/channel in heterologous expression systems, but the *in vivo* function of OsHKT2;1 remained unknown. We isolated and analyzed transposon-insertion rice lines disrupted in *OsHKT2;1*. Interestingly, three independent *oshkt2;1* null alleles exhibited significantly reduced growth compared to wildtype plants under low Na^+ and K^+ starvation conditions (Horie et al., 2007). The mutant alleles accumulated less Na^+ , but not less K^+ , in roots and shoots. *OsHKT2;1* was mainly expressed in the cortex and endodermis of roots. $^{22}\text{Na}^+$ tracer influx experiments

revealed that Na⁺ influx into *oshkt2;1* null roots was dramatically reduced compared to wildtype plants (Horie et al., 2007). A rapid repression of OsHKT2;1-mediated Na⁺ influx was found when wildtype plants were exposed to 30 mM NaCl or a protein kinase inhibitor, suggesting post translational regulation of OsHKT2;1. These analyses demonstrate that Na⁺ can enhance growth of rice under K⁺ starvation conditions, and that OsHKT2;1 is the central transporter for nutritional Na⁺ uptake into K⁺-starved rice roots (Horie et al., 2007). This research was supported by a grant from the Department of Energy to J.I.S.

Science objectives for 2008-2009:

- Biophysical transport analyses of two highly homologous rice HKT family members, OsHKT1 and OsHKT2, expressed in animal and yeast cells have previously pointed to differential ion selectivities of these transporters. These two transporters will be expressed in plant cells to examine their cation selectivities and transport kinetics directly in plants.
- Two families of HKT transporters from plants have been characterized which differ in their ion selectivities. But plant disruption mutants have not yet been isolated and analyzed in the second HKT2 class of HKT transporters. Two highly homologous HKT2 class transporters are expressed in rice. In the following year, we will isolate rice double mutant alleles in these two homologous genes, for future functional genomic and physiological transport analyses.
- Our research on HKT transporters provides evidence for rapid regulation of Na⁺ transport activities *in planta* (Horie et al., 2007; Horie et al., 2006; Horie et al., 2008). However, no HKT protein interactors or regulators of plant HKT transporters are known. Strategies are being developed, similar to those we have recently applied with collaborators (Bregante et al., 2008), to identify HKT protein interactors.

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Interdisciplinary Research and Training Program in the Plant Sciences

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Overall research goals: Plants are the major energy transducers in the biosphere, using solar energy to convert carbon dioxide and water into chemical energy and biomass that humans use for food, fiber, pharmaceuticals and fuel. The mission of the PRL is to conduct basic plant biology research to better understand this energy conversion and to train graduate students and postdoctoral associates in this area of science. The research currently conducted by the PRL faculty focuses on three areas germane to energy capture, flow, and deposition: the cell biology of chloroplasts and other organelles involved in energy flow; understanding how plants cope with environmental factors that affect plant growth and development; and the synthesis and functions of plant cell walls.

Significant achievements in 2006-2008: A major finding was the identification of the jasmonic acid receptor and a family of proteins involved in the regulation of the jasmonic acid signaling pathway. The F-box protein COI1 was identified as a component of the jasmonate receptor; proteins that repress the jasmonic acid signaling pathway were discovered, the JAZ proteins; and the coronatine- and jasmonate-dependent formation of the COI1-JAZ receptor complexes was demonstrated (studies in collaboration with John Browse, Washington State University). In addition, novel peroxisomal enzymes involved in the biosynthesis of the plant hormone jasmonate were discovered.

In regard to energy capture, results indicate that the plastid is a major regulator of a light signaling network that controls both photosynthesis-related gene expression and photomorphogenic development. Also, phytochrome A was found to have distinct and opposing roles in the photoregulation of cell expansion in response to far-red light exposure in *Arabidopsis* and the phytochrome-class photoreceptor RcaE was shown to regulate cell elongation and cell shape in the cyanobacterium *Fremyella diplosiphon*. These results imply evolutionarily conserved roles for phytochrome-class proteins in the photoregulation of cell elongation or expansion and provide fundamental new information about spatial-specific roles for phytochromes in higher plant development.

Several proteins of the plant peroxisome proliferation machinery were identified and revealed a signaling pathway that induces peroxisome proliferation in the light. In an in-depth analysis of the *Arabidopsis* leaf peroxisomal proteome, low-abundance proteins were identified that have novel functions in plant peroxisomes. It was demonstrated that a key component of the retrograde transport machinery that operates at the Golgi/ER interface, the small GTPase ARF1, is also involved in post-Golgi protein transport and that it recruits specific effectors on post-Golgi organelles which are different from those recruited on the Golgi. This is a novel finding that has not been previously reported in any biological system.

Through various biochemical and genetic approaches, genes were identified that have important roles in the synthesis of nucleotide sugars and cell wall polysaccharides. The *Arabidopsis* CBF transcription factors which control freezing were demonstrated to increase freezing tolerance in potato and a family of transcription factors were identified that potentially provide the long sought after link between calcium signaling and low temperature gene regulation. By screening transposon mutants unable to fix N₂ in air, a series of signal transduction genes required for heterocyst maturation in *Anabaena* were identified as were genes involved in the synthesis of glycolipids, molecules that are critical in establishing and maintaining the micro-oxic character of the interior of

heterocysts, a process essential for *Anabaena* to fix N₂ in air. It was also discovered that Amanita toxins are synthesized by a novel pathway. Unlike all other known cyclic peptides from fungi, the Amanita toxic peptides are synthesized on ribosomes as proproteins. α -amanitin, for example, is synthesized on ribosomes as a 35-amino acid proprotein from which the eight amino acids of the final peptide are subsequently proteolytically excised, cyclized, and hydroxylated. The amino acid sequences surrounding the eight amino acid α -amanitin peptide are present in many fungal genes suggesting that these might serve as cassettes into which many different peptide sequences are inserted and processed to become cyclic peptides, some with toxic activities.

Science objectives for 2008-2009:

- Efforts will continue to identify genes involved in the biogenesis and function of intracellular organelles and determine the mechanisms of signaling between organelles.
- Biochemical and genetic approaches will be used to continue study of the synthesis and function of cell wall polysaccharides.
- Plant signaling pathways related to light and both abiotic and biotic stress will continue with the long term goal of building a map of the transcriptional regulatory networks involved in plant responses to the environment.
- A new line of research, project PlaNet (Plant Energy Bionetwork) will be initiated to build the transcriptional regulatory network for genes involved in photosynthesis.

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The structure and regulation of the acetyl-CoA metabolic network: the fundamental underpinning of high-energy biorenewable metabolites

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Overall research goals: The research objectives are to elucidate the structure and regulation of the acetyl-CoA metabolic network that generates high-energy metabolites, such as oils, hydrocarbons, waxes, and terpenoids; integrate genetic, biochemical and physical methods to characterize metabolic flux and single molecule dynamics, which regulate the metabolism of acetyl-CoA; optimize the genetic network for enhancing the synthesis of high-energy, acetyl-CoA-derived biorenewable metabolites.

Significant achievements in 2006-2008: Using *Arabidopsis* as the model genetic organism we have identified three metabolic pathways that generate distinct acetyl-CoA pools (Fig. 1) (1, 2, 5-10, 13). Plants utilize this metabolic network to biosynthesize metabolites that serve a number of

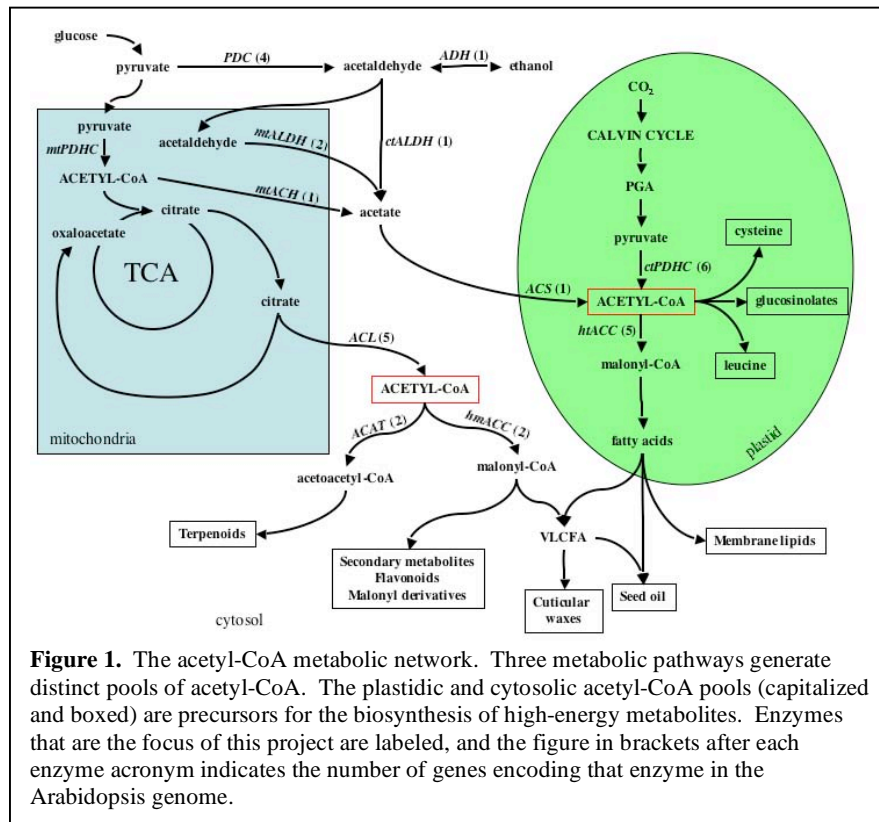


Figure 1. The acetyl-CoA metabolic network. Three metabolic pathways generate distinct pools of acetyl-CoA. The plastidic and cytosolic acetyl-CoA pools (capitalized and boxed) are precursors for the biosynthesis of high-energy metabolites. Enzymes that are the focus of this project are labeled, and the figure in brackets after each enzyme acronym indicates the number of genes encoding that enzyme in the *Arabidopsis* genome.

distinct processes, including growth regulation and carbon and energy storage. Therefore, it is essential to understand the regulation and interactions of this metabolic network if we are to rationally modify plant systems to maximize the biosynthesis of the high-energy containing metabolites, while minimally affecting plant growth and development.

Our biochemical and genetic research has established how these distinct pools of acetyl-CoA are generated. The plastidic pyruvate dehydrogenase complex (ptPDHC) is primarily

responsible for the generation of plastidic acetyl-CoA, and ATP-citrate lyase (ACL) generates the cytosolic pool. The role of acetyl-CoA synthetase (ACS) in this metabolic network is one of the issues that we are now elucidating (7). We propose to build on our previous studies by

focusing on the coordination between the acetyl-CoA-generation and acetyl-CoA-utilizing portions of the network. This research will utilize the extensive mutant collection we have generated in the genes that encompass the acetyl-CoA network. We envision this research will generate new physical and chemical analytical methods for measuring metabolic flux *in vivo* (4, 11) and for visualizing dynamics of single metabolite and protein molecules in cells (3, 12) as they respond to changes in metabolic flux. The resulting data should be the basis for the formulation of a rational design approach to improve the biosynthesis of high-energy acetyl-CoA-derived metabolites.

Science objectives for 2008-2009:

- Continue the molecular genetic and biochemical characterizations of Arabidopsis mutants in the acetyl-CoA network.
- Develop new NMR- and mass-spec-based methods for analyzing metabolic flux in Arabidopsis mutants for deciphering the acetyl-CoA network
- Develop new physical methods for visualizing the dynamics of single molecules (proteins and metabolites) as they regulate the acetyl-CoA network.

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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, 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 N-transport/storage amino acid (e.g. in seeds). Studies performed under this DOE grant, have shown that N-assimilation into asparagine, mediated via changes in transcription of the asparagine synthetase (ASN1) gene, can be used to effect increases in N-content of seed in Arabidopsis and also in field trials of corn. Our goal is to now alter gene regulatory networks affecting Asn metabolism to effect changes in N-use efficiency, an energy issue whose importance has grown in the era of biofuels. Thus, this renewal application relates to the DOE mission in two main areas, energy-use efficiency & systems biology predictive modeling.

Significant achievements from 2004-2008: Using a combination of genetics, genomics and systems biology, we identified components of a putative “energy conservation” mechanism that regulates N-assimilation into asparagine (Asn), a “carbon-efficient” amino acid (C4:N2) used to transport & store nitrogen (N) when carbon-skeletons are limiting or when levels of assimilated N are high. In previous studies, we showed that N-assimilation into Asn is controlled by the asparagine synthetase (ASN1) gene in Arabidopsis, and using transgenic plants overexpressing ASN1 we demonstrated increased N-content of seed. This finding resulted in patents and commercial licensing agreements with two major US companies, whose field tests have demonstrated increases in grain protein and N-use efficiency in crops such as corn. In the most recent cycle of this DOE grant, we developed genetic, genomic and systems approaches to identify and manipulate the regulatory components that control N-assimilation into Asn via ASN1 in response to changes in Carbon (C), Light (L) and Nitrogen (N) “energy” status. We have shown that these identified regulatory hubs act at the level of histone modification and transcription to coordinate ASN1 expression with related metabolic processes including C-metabolism, energy & photosynthesis. Thus, this systems approach has enabled us to take a step up in the hierarchy to modify regulatory hubs to coordinate changes in ASN1 regulation to be coupled to changes in related metabolic and developmental pathways including seed development, to increase N-use efficiency.

Science objectives for 2008-2011: This plan employs a combined genetic, genomic and systems approach to model and investigate the regulatory mechanisms coordinating the response of gene networks linking regulation of N-assimilation into the N-transport/storage amino acid Asn with related metabolic, energy and developmental processes in response to changes in light, carbon and nitrogen status. These studies will uncover the mechanisms underlying basic regulatory processes including mediation of C+L and/or energy signaling via histone modifications and TFs hubs controlling seed development which may have practical aspects for agriculture. As this regulatory network affects the ASN1 gene shown to affect N-content in seed, this work should have practical implications for coordinating increases in C/N-use efficiency and seed nitrogen content with related biological processes required for seed development.

The specific aims are as follows: **Aim 1. Identify mechanisms by which histone modification and TFs mediate the C+L regulation of an Asn gene regulatory network.** Studies of cli186, an Arabidopsis mutant impaired in C+L regulation of ASN1, indicate that histone methylation (H3K4/36) is involved in coordinating the C+L responses of a network of genes involved in N and C metabolism, energy, and photosynthesis with developmental processes including flowering. Our planned studies will identify how this histone modification integrates C+L signals and controls C-regulation of flowering and also test whether an “energy” sensing component is involved in C+L sensing. Genetic and reverse-genetic

studies will test transcription factor (TF) hubs in this network (including an HD-Zip TF) and identify additional components of this C+L signaling pathway. **Aim 2. Define mechanisms regulating Asn by C and N status during seed development.** Manipulation of ASN1 expression in transgenic plants leads to increased N-content of seed [1]. Here, transcription factor (TF) hubs predicted to mediate the 140-fold induction of ASN1 during seed development and in response to CN signaling, will be tested in vivo. A prioritized set of 8 putative TF hubs in this seed regulatory network will be tested in vivo using mutants and transgenics. Of special interest is a bZip TF pair for their postulated role in mediating transcription of ASN1 during seed development and in response to changes in C and N status. **Aim 3. Improving N-use, seed N and yield via manipulations of Asn regulatory network in transgenic plants.** Mutants and transgenic lines overexpressing TF hubs regulating the ASN1 seed network defined in Aim 2 will be tested for alterations in N-use, seed-N and yield. Combined, these three aims will uncover the mechanisms underlying basic regulatory processes for chromatin level mediation of C+L signaling via histone modifications and identify mechanisms and TFs that coordinate C+L and C+N regulation of Asn synthesis with related metabolic and developmental processes. As these regulatory mechanisms coordinate the regulation of gene networks involved in N-assimilation into Asn for N-storage in response to C, L and N status, these regulatory hubs will be targeted for tests in transgenic plants to manipulate increases in C/N-use efficiency, seed-N content and yield in concert with related biological processes required for seed development.

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

The patents related to this grant include a new one on TFs regulating the CN response of the ASN1 network. Studies covered in these patents have shown that alterations in the ASN1 gene, can be used to effect increases in N-content of seed in Arabidopsis and in field trials of corn. The technology covered by these patents has been commercially licensed by two major US agricultural companies for crops including corn, a major biofuel crop.

From the Soil to the Seed: Metal transport in Arabidopsis

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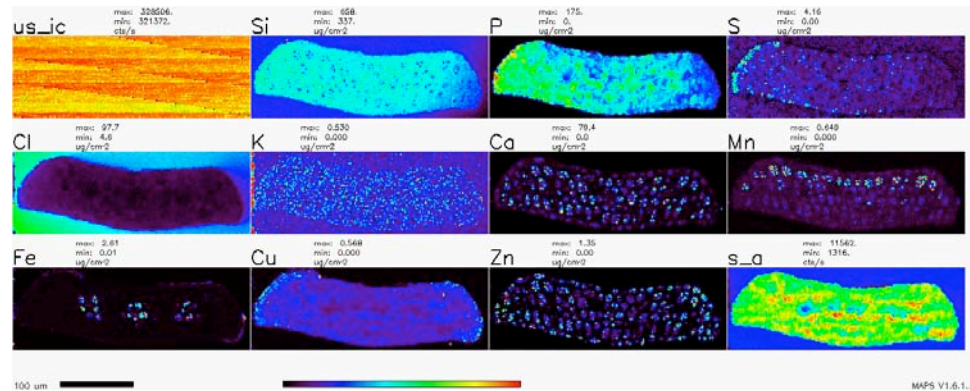
Overall research goals: Deficiencies of micronutrients such as Fe and Zn commonly limit plant growth and crop yields. The long-term goals of our program are to understand how plants acquire metal micronutrients from the soil and distribute them throughout while protecting themselves from the potential 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 2006-2008: At the start of our project, we had just used synchrotron x-ray fluorescence (XRF) microtomography to show that the vacuolar membrane transporter VIT1 determines the spatial distribution of iron in Arabidopsis seed, with most of the iron stored in the pro-vascular strands in the embryo (Kim et al., 2006). Such storage is critical for seedling development as *vit1-1* seedlings grow poorly compared to wild type seedlings when iron is limiting. Taken together, our results demonstrate that proper localization of iron as well as an ability to access this store play important roles in iron homeostasis. Our study also demonstrates the power of combining mutant analysis with a technique that can image the elemental composition of living plant material in 3-D. Most importantly, we have uncovered a fundamental aspect of seed biology that will ultimately aid the development of nutrient-rich seed, benefiting both human health and agricultural productivity. We are now examining what form of iron is stored in the vacuole. We have examined the localization of iron in seeds from plants that no longer have phytate in their seeds or no longer express the major seed ferritin, FER2. Neither mutant showed any changes in iron abundance or distribution when examined by XRF. We are also using ion exchange chromatography followed by ICP-MS to identify iron-containing fractions from embryos. We have also overexpressed *VIT1* using promoters that drive different patterns of gene expression and are in the process of characterizing these lines. We are continuing to examine metal distribution using XRF in seeds from a variety of metal transporter mutants. The *zip9* loss of function mutant has more zinc in the radicle than in the cotyledons, suggesting a role for ZIP9 in loading the cotyledons. 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 at Argonne National Lab and the Stanford Synchrotron Radiation Laboratory. We have just started to analyze the images from our first session using the APS. We can easily see individual cells and can actually image metals inside of cells. Use of this beam line will allow us to look at intracellular distribution of metals in various mutants. Shown in the figure is a

section of the embryonic cotyledon of a wild type plant. You can easily see that Fe is localized to the vasculature and that manganese is accumulated in a particular layer of cells near the abaxial surface.

Ca and Zn are uniformly distributed, allowing us to “see” all the cells.

We now believe that the main function of ZIP2 and ZIP5 is to take



up zinc from the soil, as we see strong expression of both in the epidermis. A *zip5zip2* double mutant does not grow well under zinc limiting conditions, whereas either of the single mutants grows as well as wild type. We have also continued to characterize ZIP3, a close paralog of ZIP5. ZIP3 has a unique pattern of expression among the 16 members of the ZIP family in that its mRNA accumulates in response to zinc deficiency predominantly in the roots. ZIP3 is mainly expressed in the pericycle and vasculature of the root elongation zone. *zip3* mutants have a growth defect when zinc is limited. They also accumulate less zinc than wild type plants both in the roots and shoots when zinc is sufficient. *zip3* mutants are more resistant compared to wild type plants when challenged with high levels of zinc. Consistent with its localization to the pericycle and vascular tissue, *zip3* mutants accumulate less zinc in the pericycle and vasculature as shown by staining with a zinc fluorophore. Additionally, *zip3* loss of function mutants showed up-regulation of many ZIP family members when plants were transferred to zinc limited conditions. Using grafting experiments, we can show that this response in the shoots is mediated by loss of ZIP3 expression in the roots.

Science objectives for 2008-2009:

- 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 *VIT1* overexpression lines including lines where VIT1 expression is being driven by a seed-specific promoter.

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