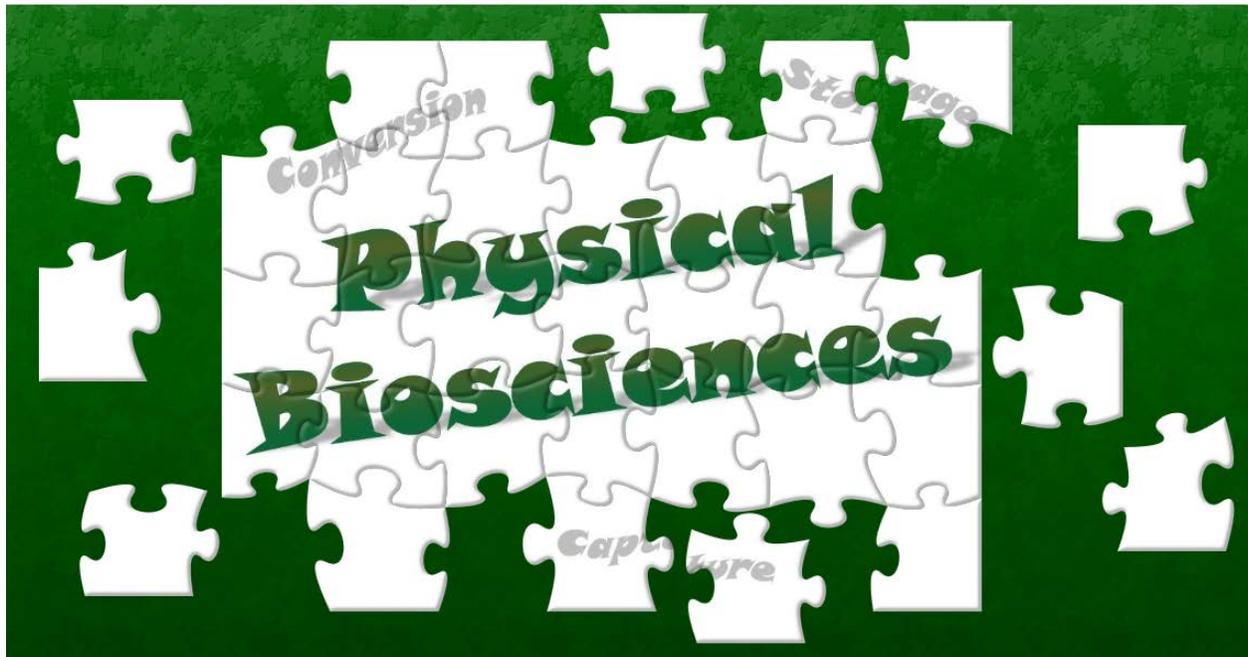


2014



Research Meeting

Westin Annapolis
Annapolis, MD
September 21-24, 2014



U.S. DEPARTMENT OF
ENERGY

Office of
Science

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

2014 Physical Biosciences Research Meeting

Program and Abstracts

Westin Annapolis
Annapolis, MD
September 21-24, 2014

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

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Foreword

This volume provides a record of the 4th biennial meeting of the Principal Investigators (PIs) funded by the Physical Biosciences program, and is sponsored by the Chemical Sciences, Geosciences, and Biosciences Division of the Office of Basic Energy Sciences (BES) in the U.S. Department of Energy (DOE). Physical Biosciences and Photosynthetic Systems are the two complementary programs within DOE-BES that fund basic research in energy-relevant biological sciences. These two programs, along with Solar Photochemistry, comprise the Photochemistry and Biochemistry Team within DOE-BES.

While we continue to believe in the power of the single PI grant, this year in particular we want to encourage you to consider enhancing your cooperation with other PIs from within our community having complementary expertise. This is why we asked you to address on your abstract submission: **“To take my project to the next level, my ideal collaborator would have expertise in...”**. We also asked you to identify your own scientific strengths, or what you in turn might offer as a potential collaborator to others. Hence the **“My scientific areas of expertise are...”** question posed to you in the abstract. While it is not our intent or desire to become “matchmakers”, we do believe that this exercise will help stimulate dialog and generate new ideas, and where appropriate, may lead to collaborative proposals with scientific and technical merit that surpasses what you might otherwise put forward on your own.

Some other things that are different this year: 1) Sunday night is “on your own”, in part to accommodate our colleagues from the west coast who have a difficult time getting here by 6PM. Plus, most of you know Annapolis well enough by now to know decent nearby restaurants. *Bon appetit!* 2) To provide additional time to explain important changes within DOE and the continuing evolution of the Physical Biosciences program, there will not be a keynote speaker to kick off our Monday morning session. 3) Our usual session on “Tools”, however, will remain with Simone Raugei from PNNL providing you with a primer on computational methodologies and Carsten Krebs from Penn State speaking to you about an innovative, immersive workshop on biophysical methodologies that is held biennially in University Park, PA. 4) You will hear about a newly-funded BES Energy Frontier Research Center (EFRC) called the Center for Biological Electron Transfer and Catalysis (BETCy), and receive updates from the two existing programmatically-related EFRCs renewed this year: Center for Lignocellulose Structure and Function (CLSF) and Center for Direct Catalytic Conversion of Biomass to Biofuels (C3Bio). 5) The BES Biosciences-funded Plant Research Lab (PRL) at Michigan State has undergone a transformation over the past 6-7 years, and they will be telling you all about the exciting changes happening there on Tuesday afternoon. 6) And finally, it is a pleasure as well for us to present talks from two outstanding recipients of BES Biosciences-funded Fellowships from the Life Sciences Research Foundation (LSRF), Anthony Studer and Aaron Stephan, to be introduced by former LSRF Fellow David Savage.

They say the only constant in life is change, and that is true as well in DOE Land. Our former Division Director (DD), Eric Rohlfing, is now the Deputy Director for Technology of the Advanced Research Projects Agency - Energy (ARPA-E), responsible for oversight of all technology issues relating to ARPA-E’s programs. We are delighted to welcome Tanja Pietraß as our new DD, who joins BES after six years of holding various leadership positions in NSF’s Division of Chemistry. Rich Greene, our Team Lead, retired after 30 years of federal service only to resurface as the Director of the Biosciences Center at NREL. My “new” Team Lead, in case you didn’t know, is the person who needs no introduction: Gail McLean. Which means we have a new Program Manager for Photosynthetic Systems to introduce...right?

In closing, everyone is aware that ongoing budgetary pressures force all of us to do more with less, but we can minimize impacts by pulling together as a community to meet these challenges. Thank you for doing your part, and thanks as well to Diane Marceau and Gail McLean from DOE-BES, and Connie Lansdon and Tim Ledford from Oak Ridge Institute for Science and Education (ORISE) for their help in planning and executing the many tasks associated with putting on a meeting of this type. Dr. Dawn Adin gets a special call-out in this regard...for this meeting would not be happening without her invaluable assistance at every step in the process!

Robert J. Stack, Program Manager, Physical Biosciences, DOE-BES

Agenda

AGENDA
4th Biennial Physical Biosciences Research Meeting
Westin-Annapolis Hotel, Annapolis, MD
September 21-24, 2014

Sunday, September 21, 2014

- 3:00 – 6:00 p.m. Meeting Registration (Diane Marceau)
6:00 – 9:00 Dinner On Your Own in Beautiful Annapolis, MD
8:00 – 11:00 Informal/Optional No-Host Reception at the Westin Lounge (Hotel Lobby)

Monday, September 22, 2014

- 7:15 – 8:00 a.m. Breakfast and Ongoing Registration (Diane Marceau)

Session I:

- 8:00 – 8:15 a.m. Welcome, DOE Updates
Gail McLean, Lead, Photochemistry and Biochemistry, DOE-BES
8:15 – 9:15 Physical Biosciences Program Notes
Robert Stack, Program Manager, Physical Biosciences, DOE-BES
9:15 – 9:45 Break

Session II:

Anne Katherine Jones, Moderator

- 9:45 – 10:15 [Molecular Mechanisms of Plant Cell Wall Loosening: Expansin Action](#)
Dan Cosgrove, Pennsylvania State University
10:15 – 10:45 [Secondary Wall Formation in Fibers](#)
Zheng-Hua Ye, University of Georgia
10:45 – 11:15 [Cell-type Specific Hemicellulose Structures in Plant Cell Walls](#)
Will York, University of Georgia
11:15 – 11:45 [Electron Flow and Energy Conservation in a Hydrogenotrophic Methanogen](#)
John Leigh, University of Washington
11:45 – 1:00 Lunch
1:00 – 3:00 **Free/Discussion Time** (Put up all posters)
3:00 – 3:30 Break

Session III:

David Knaff, Moderator

- 3:30 – 4:00 [Mechanism and Function of the Chaperonin from *Methanococcus maripaludis*: Implications for Archaeal Protein Homeostasis and Energy Production](#)
Judith Frydman, Stanford University
4:00 – 4:30 [Unraveling the Regulation of Terpenoid Oil and Resin Biosynthesis for the Development of Biocrude Feedstocks](#)
Markus Lange, Washington State University
4:30 – 5:00 [Molecular and Genetic Bases of Sodium and Potassium Transport and Distribution in Plants](#)
Julian Schroeder, University of California, San Diego

5:00 – 6:00 Update on the Physical Biosciences Related EFRCs
[Biological Electron Transfer and Catalysis EFRC \(BETCy\)](#)
John Peters, Montana State University
[Center for Lignocellulose Structure and Formation \(CLSF\)](#)
Dan Cosgrove, Pennsylvania State University
[Center for Direct Catalytic Conversion of Biomass to Biofuels \(C3Bio\)](#)
Joe Bozell, University of Tennessee

6:00 – 8:00 Dinner

Poster Session I

8:00 – 10:00 [Poster Session I](#), Odd Numbered Posters (No-Host)

Tuesday, September 23, 2014

7:15 – 8:00 a.m. Breakfast

Session IV:

Sean Elliott, Moderator

8:00 – 8:30 [Small Molecule Endosidin2 Targets Evolutionary Conserved Exo70 Proteins to Affect Exocytosis](#)

Natasha Raikhel, University of California, Riverside

8:30 – 9:00 [CSLD Proteins Are Essential During Polarized Cell Expansion and Cytokinesis](#)

Erik Nielsen, University of Michigan

9:00 – 9:30 [CHX Transporters at Dynamic Endomembranes: Roles in pH Homeostasis Critical for Vegetative and Reproductive Success of Land Plants](#)

Heven Sze, University of Maryland

9:30 – 10:00 [Extracellular Charge Transport in Microbial Redox Chains: Linking the Living and Non-Living Worlds](#)

Moh El-Naggar, University of Southern California

10:00 – 10:30 Break

Session V:

Moh El-Naggar, Moderator

10:30 – 11:00 [Genetic Analysis of Hydrogenotrophic Methanogenesis in *Methanosarcina* Species](#)

Bill Metcalf, University of Illinois at Urbana-Champaign

11:00 – 11:30 [Enzymology of Methanogenesis: Mechanism of Methyl-Coenzyme M Reductase](#)

Steve Ragsdale, University of Michigan Medical School

Session VI:

Bob Stack, Moderator

11:30 – 12:00 [Computational Capabilities for Understanding Enzymatic Energy Conversion](#)

Simone Raugei, Pacific Northwest National Lab (PNNL)

12:00 – 12:30 [The Penn State Bioinorganic Workshops](#)

Carsten Krebs, Pennsylvania State University

12:30 – 1:30 Lunch

1:30 – 3:30 **Free/Discussion Time**

3:30 – 4:00 Break

Session VII: *Zheng-Hua Ye, Moderator*

4:00 – 4:30 [Resolving Protein-Semiquinone Interactions by Advanced EPR Spectroscopy](#)
Sergei Dikanov, University of Illinois at Urbana-Champaign

4:30 – 5:00 [The Biosynthesis and Tissue Distribution of the Plant Cell Wall Pectic Polysaccharide Rhamnogalacturonan II](#)
Malcolm O’Neill, University of Georgia

5:00 – 5:30 [Structure, Function and Reactivity of CO Dehydrogenase from *Oligotropha carboxidovorans*](#)
Russ Hille, University of California, Riverside

5:30 – 6:30 [New Directions for the DOE-MSU-PRL](#)
David Kramer/Cheryl Kerfeld/Gregg Howe, Michigan State University

6:30 – 8:00 Dinner

Poster Session II

8:00 – 10:00 [Poster Session II](#), Even Numbered Posters (No-Host)

Wednesday, September 24, 2014

7:15 – 8:00 a.m. Breakfast

Session VIII: *Dave Savage, Moderator*

8:00 – 8:30 [The Role of Carbonic Anhydrase in C₄ Photosynthesis](#)
Anthony Studer, Donald Danforth Plant Science Center

8:30 – 9:00 [Genetic and Functional Identification of Sensory Mechanisms in Plants that Initiate Responses to Water Stress](#)
Aaron Stephan, University of California, San Diego

9:00 – 9:30 [Jasmonate Hormone: Regulating Synthesis of Reduced Carbon Compounds in Plants](#)
John Browse, Washington State University

9:30 – 10:00 [Mass Spectrometric Imaging of Plant Metabolites](#)
Basil Nikolau, Ames Laboratory, Iowa State University

10:00 – 10:30 Break

Session IX: *John Peters, Moderator*

10:30 – 11:15 [Nitrogenase Reduction of CO₂ to Hydrocarbons](#)
Dennis Dean, Virginia Tech
Lance Seefeldt, Utah State University

11:15 – 11:45 [Nanotube-Supported Phospholipid Bilayers](#)
Alex Smirnov, North Carolina State University

11:45 – 12:15 [Regulation of Plant Cells, Cell Walls, and Development by Mechanical Signals](#)
Elliot Meyerowitz, California Institute of Technology

12:15 – 1:15 Lunch (**Reminder: Make sure all posters are down**)

Session X: Discussion with DOE Program Staff *Bob Stack, Moderator*

1:15 – 3:00 **Robert Stack**, Program Manager, Physical Biosciences, DOE-BES
PS PM Intro, Program Manager, Photosynthetic Systems, DOE-BES
Gail McLean, Lead, Photochemistry and Biochemistry, DOE-BES

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Session II

Molecular Mechanisms of Plant Cell Wall Loosening: Expansin Action

Daniel J. Cosgrove, Principal Investigator

Yuning Chen (Nickolas Georgelis, former) Postdoctoral Research Associate

Department of Biology, Penn State University, University Park, PA 16802

Email: dcosgrove@psu.edu; Website: <http://bio.psu.edu/directory/fsl>

Overall research goals:

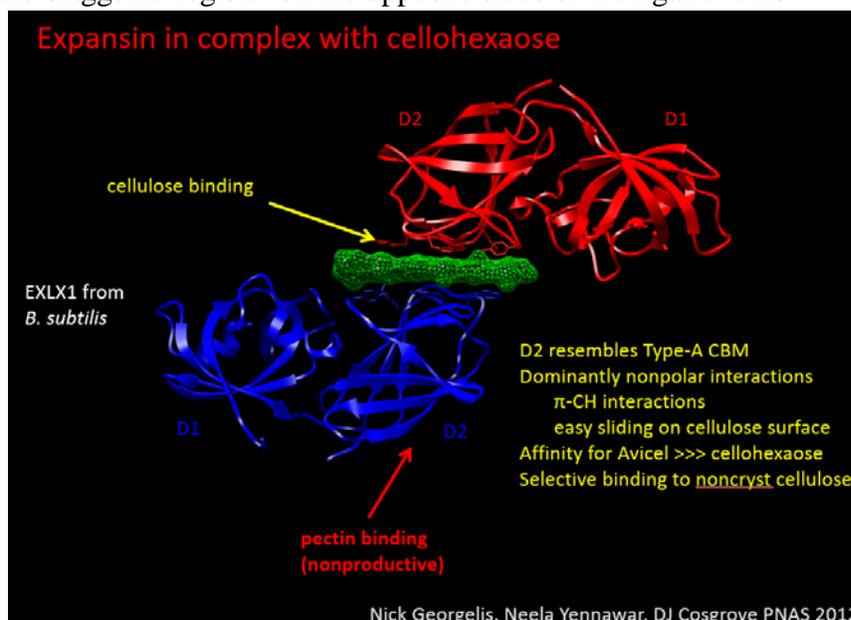
To elucidate the molecular biophysics of irreversible expansion of plant cell wall during cell growth. Our focus is on expansin structure and its wall-loosening action that results in wall stress relaxation and cell wall creep. Expansins are the mediators of “acid-induced growth” by auxin and participate in a growing list of developmental events that involve cell wall modification. In this project we have discovered three groups of expansins: plants have α - and β -expansins (EXPA and EXPB), and some bacteria have expansins (EXLX) as well. Expansins do not have wall lytic activity, yet they induce wall relaxation and cell wall creep. Their mechanism is enigmatic in part because our understanding of cell wall structure is woefully incomplete.

We have made use of bacterial expansins for structure/function analyses by X-ray crystallography and site-directed mutagenesis combined with binding assays, wall creep assays and dynamic mechanical analyses of cell walls. We are also pioneering the use of sensitivity-enhanced solid-state NMR to identify the target of bacterial and plant expansins (a collaboration with Dr. Mei Hong).

Current work will continue analysis of bacterial expansins as a pragmatic approach for studying expansin action, but will put renewed focus on the action of β -expansins and their target of action.

Significant achievements (2012-2014):

1. Elucidated the mechanism of glycan binding by the expansin (BsEXLX1) from *Bacillus subtilis* (*Publication #1 below*). This involved solving the structure of multiple protein:ligand complexes as well as use of site-directed mutagenesis and a variety of activity assays. In the crystal the protein:ligand packs in an exceptional configuration in which two proteins bind to a single ligand, with the proteins in opposite orientation and staggered register on the opposite sides of the ligand. This work also established expansin domain 2 (the C-terminal domain) as the founding member of Carbohydrate Binding Module family 63 (CBM63 in the CaZY database). Moreover we found that BsEXLX1 binding to whole cell walls was complex: the majority of binding was relatively nonspecific binding to pectins, driven by electrostatic interactions, and was nonproductive (meaning it reduced wall loosening activity of the protein); whereas the productive binding was between a β 1,4-glucan chain and a set of three aromatic residues set in line on the surface of domain 2.



2. We characterized the molecular target of the expansin BsEXLX1 by use of sensitivity-enhanced solid-state NMR in combination with a variety of sited-directed mutants and C13/N15 labeling. This was a collaboration with Dr. Mei Hong (currently at MIT). The result shows that expansin binds to cellulose with a conformation somewhat different (less organized) than that of bulk cellulose and that xyloglucan is in close proximity. This structure is very similar to the structure envisioned for the

limited 'biomechanical hotspots' that control cell wall extensibility (Park and Cosgrove, Plant Physiology 2012).

3. Solved the crystal structure of the expansin in *Clavibacter michiganensis*, both in apo-form and in complex with cello-oligosaccharides. The results confirm and extend the analysis of the *B. subtilis* expansin and indicate a cleavage of cellohexaose when it is trapped between the active sites of two expansin proteins. Cleavage did not occur in solution, however. Publication in preparation.

4. Discovered that maize EXPB1 dissolves the middle lamella of growing maize coleoptiles and facilitates separation of the cuticle from the epidermal cell walls. Biochemical mechanism is uncertain.

Science objectives for 2014-2016:

- Elucidate the mechanism of release of highly substituted glucuronoarabinoxylan (hsGAX) by EXPB1
 - Test for new reducing ends during hsGAX release from grass cell walls by EXPB1;
 - Test for GAX transglycosylase activity
 - Test for release of GAX and tissue weakening by EXPB1 with other cell wall materials (developmental state; species diversity)
 - Test for similar effects by other grass pollen β -expansins
 - Does EXPB1 treatment result in a change in accessible cellulose surface?
 - Identify the components of the gel-like material released by EXPB1 treatment of coleoptiles
- Identify the target of EXPB1 action by
 - Binding isotherms with defined ligands
 - Confocal microscopy of plant tissue cross sections with fluorescently tagged EXPB1
 - Nano-gold labeling of EXPB1 and tissue labelling by TEM
- Develop a bacterial pellicle compression assay as a model system to test expansin action
- Are cellulose junctions the site of expansin targeting? Assessment by AFM with nano-gold EXPB1

My scientific area(s) of expertise is/are: plant cell growth; cell wall biophysics and structure; biomechanics; plant molecular biology; recombinant protein expression; structural biology.

To take my project to the next level, my ideal collaborator would have expertise in: enzymology and biochemistry of polysaccharide modifications...looking for new mechanisms of expansin action; polysaccharide physical chemistry; coarse-grain model of plant cell walls; polymer biophysics;

Publications supported by this project 2012-2014:

- 1 Georgelis N., Yennawar N., and Cosgrove D.J. Structural basis for entropy-driven cellulose binding by a type-A cellulose-binding module (CBM) and bacterial expansin Proc. Nat. Acad. Sci. USA 109:14830-14835 (2012)
- 2 Carey, R.E. Hepler N.K. & Cosgrove D.J. Selaginella moellendorffii has a reduced and highly conserved expansin superfamily with genes more closely related to angiosperms than to bryophytes. BMC Plant Biology 13:4 doi:10.1186/1471-2229-13-4 (2013)
- 3 Wang T., Park Y.B., Caporini M.A., Rosay M., Zhong L., Cosgrove D.J., Hong, M. Sensitivity-enhanced solid-state NMR detection of expansin's target in plant cell walls. Proc. Nat. Acad. Sci. USA 110: 16444-16449 (2013)
- 4 Georgelis, N., Nikolaidis, N., & Cosgrove, D. J. Biochemical analysis of expansin-like proteins from microbes. Carbohydrate Polymers 100: 17-23 (2014)
- 5 Nikolaidis N., Doran N., & Cosgrove D.J. Plant expansins in bacteria and fungi: evolution by horizontal gene transfer and independent domain fusion. Mol Biol Evol 31: 376-386 (2014)
- 6 White, P. B.; Wang, T.; Park, Y. B.; Cosgrove, D. J.; Hong, M. Water – polysaccharide interactions in the primary cell wall of Arabidopsis thaliana from polarization transfer solid-state NMR. Journal of the American Chemical Society 136: 10399-10409 (2014)

Secondary Wall Formation in Fibers

Zheng-Hua Ye, Principal Investigator

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Email: zhye@plantbio.uga.edu; Website: <http://research.franklin.uga.edu/zhye/>

Overall research goals:

The overall goal of the DOE-funded project is to study the functional roles of genes involved in xylan biosynthesis during secondary wall formation. Xylan in dicots (typically called glucuronoxylan) is composed of a linear backbone of β -1,4-linked xylosyl residues, about 10% of which are substituted with α -1,2-linked glucuronic acid and/or 4-*O*-methylglucuronic acid residues. In addition, about 60% of xylosyl residues are acetylated at *O*-2 and/or *O*-3. Although a number of genes have been implicated in xylan backbone elongation and substitution of glucuronic acid residues, genes and their encoded enzymes involved in methylation of glucuronic acid residues and acetylation of xylosyl residues are not well studied. Because the presence of xylan in cellulosic biomass has been shown to hinder the efficiency of conversion of biomass into bioethanol, further understanding of how xylan is made will not only contribute to our knowledge of cell wall biosynthesis in general but also have important economic and agronomic implications, such as providing genetic tools for custom-designing cell wall composition tailored for biofuel production.

Significant achievements (2012-2014):

Xylan, a major polysaccharide in plant lignocellulosic biomass, is acetylated at *O*-2 and/or *O*-3 and its acetylation impedes the use of biomass for biofuel production. In *Arabidopsis*, about 60% of xylosyl residues in xylan are acetylated and the biochemical mechanisms controlling xylan acetylation are largely unknown. We have found the essential role of a DUF231 domain-containing protein, ESKIMO1 (ESK1), in xylan acetylation in *Arabidopsis* as the *esk1* mutation caused specific reductions in the degree of xylan 2-*O* or 3-*O*-monoacetylation and in the activity of xylan acetyltransferase. Our finding indicates that ESK1 is a putative xylan acetyltransferase required for 2-*O*- and 3-*O*-monoacetylation of xylosyl residues in xylan.

We have demonstrated that three *Arabidopsis* DUF579 domain-containing proteins are methyltransferases catalyzing the methylation of glucuronic acid residues in xylan. In *Arabidopsis*, about 10% of xylosyl residues in xylan are substituted with glucuronic acid, of which 60% are methylated at *O*-4. By contrast, all of the glucuronic acid substituents in *Populus* xylan are methylated at *O*-4. It is not known how the degree of glucuronic acid methylation in xylan is controlled. We found that simultaneous T-DNA knockout mutations of the three glucuronoxylan methyltransferase genes led to a complete loss of glucuronic acid methylation in xylan in *Arabidopsis* stems (Fig. 1). Overexpression of the methyltransferases in wild-type *Arabidopsis* resulted in an up to 5-fold increase in glucuronoxylan methyltransferase activity and as a result, up to 90% of the glucuronic acid side chains in xylan were methylated as opposed to 60% seen in the wild type. Our findings suggest that the degree of glucuronic acid methylation in xylan is largely controlled by the level of methyltransferase activities. We have also performed biochemical characterization of four wood-associated glucuronoxylan methyltransferases in poplar and found that two of them exhibit much higher substrate affinities than *Arabidopsis* ones, which may account for the complete methylation of glucuronic acid residues in poplar xylan.

We have revealed that *Arabidopsis* xylan contains galactose-glucuronic acid disaccharide side chains in addition to glucuronic acid, methylglucuronic acid and acetyl substitutions, which enriches our understanding of xylan structure in *Arabidopsis*.

We have found that three *Arabidopsis* glycosyltransferases belonging to family GT8 are glucuronyltransferases involved in the addition of glucuronic acid side chains onto the xylan backbone.

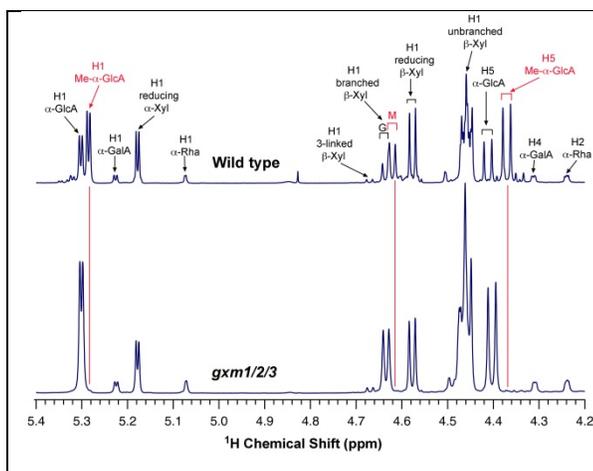


Fig. 1 NMR spectroscopy of xylans from the wild type and the triple mutant defective in three glucuronoxylan methyltransferase genes (*gxm1/2/3*). Xylans were digested by xylanase to generate xylooligosaccharides, which were further subjected to ^1H -NMR analysis. Resonances are labeled with the position of the assigned proton and the identity of the residue containing that proton. Note the absence of the Me- α -GlcA resonances (highlighted in red) in *gxm1/2/3* compared with the wild type.

Science objectives for 2014-2015:

The main objective for the following year will be to further investigate genes involved in xylan acetylation. We have recently identified several additional genes that are expressed during secondary wall biosynthesis and we propose that they are involved in xylan acetylation. We will generate mutants mutated in these genes, and examine effects of the mutations on xylan acetylation. The study of xylan acetylation will provide knowledge foundation for genetic modification of biomass with altered wall acetylation.

My scientific areas of expertise are: plant secondary walls; molecular biology; genetics.

To take my project to the next level, my ideal collaborator would have expertise in: biochemistry; enzymology.

Publications supported by this project [2012-2014]:

- 1 C. Lee, R. Zhong, Z.-H. Ye, "Arabidopsis family GT43 members are xylan xylosyltransferases required for the elongation of the xylan backbone." *Plant Cell Physiol.* **53**, 135-143 (2012).
- 2 C. Lee, R. Zhong, Z.-H. Ye, "Biochemical characterization of xylan xylosyltransferases involved in wood formation in poplar." *Plant Signal. Behav.* **7**, 332-337 (2012).
- 3 C. Lee, Q. Teng, R. Zhong, Z.-H. Ye, "Arabidopsis GUX proteins are glucuronyltransferases responsible for the addition of glucuronic acid side chains onto xylan." *Plant Cell Physiol.* **53**, 1204-1216 (2012).
- 4 C. Lee, Q. Teng, R. Zhong, Y. Yuan, M. Haghghat, Z.-H. Ye, "Three Arabidopsis DUF579 domain-containing GXM proteins are methyltransferases catalyzing 4-*O*-methylation of glucuronic acid on xylan." *Plant Cell Physiol.* **53**, 1934-1949 (2012).
- 5 Y. Yuan, Q. Teng, R. Zhong, Z.-H. Ye, "The Arabidopsis DUF231 domain-containing protein ESK1 is required for *O*-2 and *O*-3 acetylation of xylan." *Plant Cell Physiol.* **54**, 1186-1199 (2013).
- 6 C. Lee, Q. Teng, R. Zhong, Z.-H. Ye, "Alterations of the degree of xylan acetylation in Arabidopsis xylan mutants." *Plant Signal. Behav.* **9**, e27797 (2014).
- 7 Y. Yuan, Q. Teng, R. Zhong, Z.-H. Ye, "Identification and biochemical characterization of three wood-associated glucuronoxylan methyltransferase genes in *Populus*." *PLoS ONE* **9**, e87370 (2014).
- 8 Y. Yuan, Q. Teng, C. Lee, R. Zhong, Z.-H. Ye, "Modification of the degree of 4-*O*-methylation of secondary wall glucuronoxylan." *Plant Sci.* **219**, 42-50 (2014).
- 9 C. Lee, Q. Teng, R. Zhong, Z.-H. Ye, "Functional roles of rice glycosyltransferase family GT43 genes in xylan biosynthesis." *Plant Signal. Behav.* **9**, e27809 (2014).
- 10 R. Zhong, Q. Teng, C. Lee, Z.-H. Ye, "Identification of a disaccharide side chain 2-*O*- α -D-galactopyranosyl- α -D-glucuronic acid in Arabidopsis xylan." *Plant Signal. Behav.* **9**, e27933 (2014).

Cell-type specific hemicellulose structures in plant cell walls

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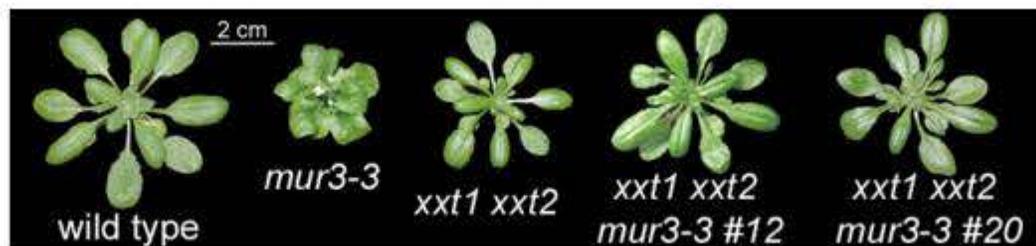
Email: will@ccrc.uga.edu; Website: <http://www.ccrcc.uga.edu/~mao/cellwall/main.htm>

Overall research goals:

Hemicelluloses including xyloglucan are ubiquitous components of the primary walls of growing vascular plant cells. Although many hypotheses have been proposed concerning the biological functions of hemicellulosic polysaccharides, their roles in plant growth and development have remained enigmatic. The conservation of many of the structural features of xyloglucans attests to their importance for the survival of plants in the wild, although plants completely lacking xyloglucan can thrive in a controlled laboratory environment. Our approach to understanding this apparent contradiction is to study mechanisms leading to the biosynthesis of xyloglucans with cell- and tissue-specific structural features and examining the phenotypes and chemotypes of plants in which these structures are genetically altered or suppressed. This includes the development of highly sensitive, cell-specific methods for the isolation and structural characterization of hemicelluloses and for the heterologous expression of catalytically active enzymes that are involved in xyloglucan biosynthesis.

Significant achievements (2012-2014):

- Using a multidisciplinary approach, we have identified and characterized root hair-specific xyloglucan structures. These include the a β -galacturonic acid residue at the terminus of xyloglucan sidechains, which we have not detected in other cell types. The absence of this acidic sidechain results in root hairs that do not extend normally.
- We have identified and biochemically characterized the root hair-specific galacturonosyl transferase that catalyzes the addition of galacturonic acid residues to xyloglucan.
- We have heterologously expressed and biochemically characterized several additional glycosyltransferases involved xyloglucan sidechain biosynthesis.
- To determine whether acidic xyloglucan in root hairs occurs in other plants or is unique to Arabidopsis, we have characterized the xyloglucan in the root hairs of soybeans and shown that it also has sidechains terminated with galacturonic acid residues.
- Studies with soybean also revealed root-hair-specific differences in xylan structure and the presence of larger amounts of mannan in the root hairs than in other root cells.
- We have found substantial differences as well as similarities in the structures of the xyloglucan and glucuronoxylan present in the walls of four different duckweeds (Spirodela, Lemna, Wolffia, Wolffia). Duckweeds are aquatic monocots in the subfamily Lemnaceae.
- Studies with Arabidopsis mutants affected in xyloglucan biosynthesis have allowed us to show that xyloglucan lacking a sufficient number of galactose-containing sidechains is dysfunctional. and that the presence of such incompletely-formed xyloglucan is more deleterious to the plant than the complete absence of the polysaccharide (see Fig. 1).



- Fig.1. Eliminating xyloglucan in the *mur3-3* mutant restores near-normal growth. *mur3-3* has a dwarf phenotype and synthesizes galactose-depleted xyloglucan. The *xxt1 xxt2* mutant forms no discernible amounts of xyloglucan but has near-normal growth. The triple mutant *xxt1 xxt2 mur3-3*

also forms no xyloglucan. Thus, eliminating xyloglucan restores growth. We conclude that dysfunctional xyloglucan is responsible for the dwarf phenotype of *mur3-3* plants.

- Our studies provide the basis for understanding fundamental relationships between xyloglucan structure and its functions in the primary cell wall.

Science objectives for 2014-2015:

- Demonstrate the ubiquity of acidic xyloglucan in the walls of tip growing cells.
- Continue studies to elucidate the role of acidic and fucosylated xyloglucan in the tip growing cells (root hairs and pollen). Use this data to include xyloglucan in current models of the wall in root hairs.
- Complete studies of the acceptor preferences, regiospecificity and catalytic properties of recombinant xyloglucan-specific glycosyltransferases involved in sidechain synthesis.
- Complete structural characterization of hemicelluloses in the cell walls of duckweeds.
- Initiate studies of the intracellular aggregates/vesicles containing xyloglucan and pectin that accumulate in the etiolated hypocotyls of the *mur3-3* mutant and determine if these aggregates exist in other cell wall mutants.

My scientific areas of expertise are: Plant cell wall structure and biosynthesis - Structural characterization of complex carbohydrates - NMR spectroscopy - Mass spectrometry – Biochemistry of enzymes involved in the synthesis and degradation of polysaccharides.

To take my project to the next level, my ideal collaborator would have expertise in: micro-printing technology capable of depositing various polysaccharide-specific enzymes or enzyme complexes on discrete regions of a plant tissue section that correspond to specific cell types.

Publications supported by this project (2012-2014):

- 1 Peña, M.J., Y. Kong, W.S. York, and M.A. O'Neill. 2012. A galacturonic acid-containing xyloglucan is involved in Arabidopsis root hair tip growth. *Plant Cell* **24**: 4511-4524.
- 2 Muszyński et al. The Structures of Rhamnogalacturonan I, Xyloglucan, Galactomannan, and Glucuronoxylan are not Identical in the Cell Walls of Soybean Root Hairs and Roots Stripped of Hairs (Glycobiology submitted - in revision).
- 3 Kong et al. Dysfunctional xyloglucan structure leads to dwarfism in Arabidopsis (submitted).

Direct electron input from formate implies that formate dehydrogenase (Fdh) as well as hydrogenase (Vhu) associates directly with the heterodisulfide reductase (Hdr) complex. By purifying subunits of this complex, we showed that the subunit VhuD interacts with Hdr and directly with either Fdh or Vhu. Hence, VhuD is a general an electron donor interface (Figure 2).

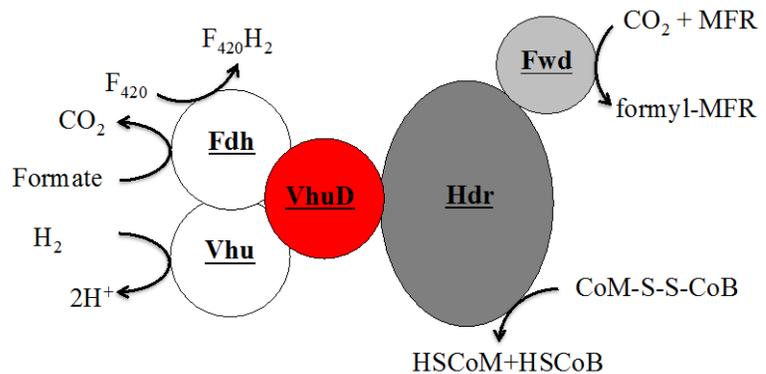


Figure 2. VhuD as the electron donor interface.

We documented that growth yield (grams cell dry weight per mole CH₄ produced) was three-fold higher when H₂ or formate was limiting than when H₂ or formate was in excess. The mechanism of this “energy spilling” remains unknown.

Science objectives for 2014-2015:

- Details of electron flow in the Hdr complex remain to be determined. We plan to initiate a cross-linking approach to identify the subunit interactions that mediate electron flow.
- Fdh plays a dual role, reduction of coenzyme F₄₂₀ and reduction of the Hdr complex. We plan to test the hypothesis that this occurs through a second electron bifurcation center contained within Fdh.
- Eha is encoded in an operon that contains 20 genes. We will initiate a genetic approach to test the functionality of each of these genes.

My scientific areas of expertise are: prokaryotic physiology and genetics.

To take my project to the next level, my ideal collaborator would have expertise in: enzyme biochemistry.

Publications supported by this project 2012-2014:

1. Lie, T. J., K. C. Costa, B. Lupa, S. Korpole, W. B. Whitman, and J. A. Leigh. 2012. Essential anaplerotic role for the energy-converting hydrogenase Eha in hydrogenotrophic methanogenesis. *Proc. Natl. Acad. Sci. USA* 109:15473-15478.
2. Costa, K. C., T. J. Lie, M. A. Jacobs, and J. A. Leigh. 2103. H₂-independent growth of the hydrogenotrophic methanogen *Methanococcus maripaludis*. *mBio* 4(2):e00062-13. doi: [10.1128/mBio.00062-13](https://doi.org/10.1128/mBio.00062-13).
3. Costa, K. C., T. J. Lie, Q. Xia, and J. A. Leigh. 2013. VhuD facilitates electron flow from H₂ or formate to heterodisulfide reductase in *Methanococcus maripaludis*. *J. Bacteriol.* 195: 5160-5165.
4. Costa, K. C., S. H. Yoon, M. Pan, J. A. Burn, N. S. Baliga, and J. A. Leigh. 2013. Effects of H₂ and formate on growth yield and regulation of methanogenesis in *Methanococcus maripaludis*. *J. Bacteriol.* 195:1456-1462
5. Lie T. J., K. C. Costa, D. Pak, V. Sakesan, and J. A. Leigh. 2013. Phenotypic evidence that the function of the [Fe]-hydrogenase Hmd in *Methanococcus maripaludis* requires seven *hcg* (*hmd* co-occurring genes) but not *hmdII*. *FEMS Microbiol. Lett.* 343: 156–160.

Session III

Mechanism and function of the chaperonin from *Methanococcus maripaludis*: implications for archaeal protein homeostasis and energy production

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Overall research goals: Archaea offer a potentially cost effective and renewable source of energy. This proposal is focused on understanding the cellular substrates and mechanism of action of the group II chaperonin complex Mm-Cpn, the central chaperone of the methanogen *M. maripaludis*, which obtains energy by sequestering H₂ and reducing CO₂ to methane by the methanogenic pathway. More recently, it has also been suggested that the methanogenesis pathway could be run in reverse, to produce H₂ growing the organism in formate. We envision our efforts as a first step in obtaining a multi-level understanding of archaeal protein homeostasis, which will be instrumental for improving the functionality and design of the enzyme pathways and complexes involved in energy production and storage. One additional importance consequence of a better understanding of archaeal protein homeostasis will be to increase their stress resistance, since their utilization for the efficient large-scale production of methane (and eventually also of H₂) requires that the organisms are resistance to a range of growth conditions. Through a combination of biochemistry, systems biology, biophysics and structural biology, our work aims to: (i) identify the archaeal substrate repertoire of Mm-Cpn, and (ii) define mechanistic and structural principles of Mm-Cpn mediated protein folding.

Significant achievements ([Click to Enter Years of Current Grant/FWP, e.g. 2012-2014]):

(1) Identification of the archaeal substrate repertoire of Mm-Cpn.

Proteomic analyses of chaperone interactions. During year 1 we carried out a mass spectrometry analysis of Mm-Cpn substrates, which identified 154 proteins that associate preferentially with the endogenous chaperonin, as expected from bona fide substrates. During our analyses of these substrates in the second year we found that Cpn interactome is highly enriched for highly connected proteins, with many interactions (not shown). While a number of substrates are involved in the ribosome, tRNA charging, DNA replication and repair (similar to the eukaryotic chaperonin); approximately a third are enzymes involved in various metabolic pathways, including methanogenesis and nucleotide and amino acid metabolism.

Flux through the chaperonin under different growth conditions: Hydrogenotrophic methanogenic Archaea are defined by an H₂ requirement for growth. Despite this requirement, *M. maripaludis* can also grow with formate as an electron donor for methanogenesis. Because several enzymes in the methanogenesis pathway were substrates of the chaperonin, we decided to examine the substrate flux through the chaperonin when the cells are grown in the presence of either electron donor. To this end, we established a system to grow *M. maripaludis* in the lab under conditions where we can carry out stable isotope labeling by amino acids in cell culture (SILAC). Briefly, *M. maripaludis* strain S2 was grown in pressurized flasks in ¹⁴N- or ¹⁵N- containing media that provided either H₂ or formate as electron donors for methanogenesis. Isolation of Chaperonin-containing complexes by immunoprecipitation showed clear differences from the GAPDH control. The mass spectrometry analysis of chaperonin substrates has already been carried out and we are awaiting analysis of the SILAC data.

(2) Define mechanistic and structural principles of Mm-Cpn mediated folding.

Mm-Cpn uses ATP cycling to drive a conformational cycle that promotes polypeptide folding. We are combining biochemical, biophysical and structural approaches to reveal how Mm-Cpn interacts with its substrates and what are the mechanics of Mm-Cpn folding. A number of exciting findings and approaches have been developed in this first year, including a novel observation hinting that the Chaperonin C-terminal tails play a central role in the chaperonin folding cycle. Our major efforts are briefly summarized below:

Mapping the conformational dynamics of Mm-Cpn along the folding cycle: We collaborate with Matthias Mayer in the University of Heidelberg to use HD-exchange in combination with mass spectrometry to map the conformational dynamics of the chaperonin in the different states of the folding cycle. Preliminary experiments show that the unliganded state is extremely dynamic, whereas the ATP-bound closed state is significantly more rigid. This approach will be applied to chaperonin-substrate complexes to gain insights into the nature of folding intermediates.

Elucidating the Functional Role of archaeal chaperonin Residues via Statistical Coupling Analysis: Statistical coupling analysis (SCA) can identify clusters of co-evolving residues within a protein family which form spatially contiguous functional networks. Coupling analyses have heretofore largely been restricted to small domains and monomeric proteins. The archaeal group II chaperonins pose a particular problem for established coupling workflows inasmuch as they form a complex oligomer composed of highly conserved multidomain monomers for which few (less than one thousand) sequences are available. To meet these challenges, we have devised and implemented a novel statistical coupling metric which renders analysis of very conserved, sparsely sequenced protein families. The metric constitutes an agnostic information theoretical measure which quantifies the degree of coupling between two columns in a multiple sequence alignment. Application of the MISC metric to an alignment of 1186 archaeal group II sequences successfully predicted a network of coevolving residues in MmCpn. The network contains the catalytic aspartate-386 responsible for nucleotide hydrolysis as well as lysine-161, which is implicated in sensing the departure of the γ -phosphate of ATP. This network is spatially compact and spans the lateral interface between MmCpn monomers in the chaperonin complex.

Role of the chaperonin tails in the folding cycle: Chaperonins fold substrates within their central chamber. Flexible C-termini extend from the base of their equatorial domains into the central cavity. In previous experiments, carried out in year 1, we obtained biochemical evidence that these tails are intimately involved in the function of the chaperonin. To examine the function and conformation of the termini during the folding cycle, we have developed an NMR labeling scheme using ^{13}C -methyl methionine which will allow the spectroscopic characterization of the termini. This system will also allow us to probe the C-termini spectroscopically during the ATPase cycle and in the presence and absence of substrates.

Science objectives for 2014-2015:

- Confirm the chaperonin substrates identified by proteomics
- Examine the effects of chaperonin mutants in stress resistance and pathway remodeling in archaea
- Structurally and mechanistically characterize Mm-Cpn and how it folds proteins
- Design chaperonin variants with altered folding characteristics

My scientific areas of expertise are: biochemistry, biophysics, cell biology.

To take my project to the next level, my ideal collaborator would have expertise in: archaeal physiology and metabolism; synthetic biology for energy production; structural biology

Unraveling the regulation of terpenoid oil and resin biosynthesis for the development of biocrude feedstocks

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Overall research goals:

Terpenoid essential oils and oleoresins are characterized by a high volumetric energy density and high degree of reduction, and are thus viable biocrude feedstocks for sustainably produced specialty chemicals. This project is aimed at developing single cell approaches to investigate plant cell types that are specialized for oil and resin biosynthesis. We are currently using three experimental model systems that accumulate valuable terpenoid feedstock chemicals: (1) peppermint glandular trichomes (l-menthol), (2) *Citrus* secretory cavities (d-limonene), and (3) loblolly pine resin ducts (α/β -pinene and abietic acid).

Significant achievements (2012-2014):

- **Peppermint.** We have been very successful in modulating the endogenous essential oil pathway of peppermint transgenics to accumulate different intermediates, in particular l-limonene, (+)-pulegone and (+)-menthofuran. The production of novel terpenoids (not found in wild-type peppermint oil) has also been achieved. We have further characterized the determinants for differences in essential oil composition in various mint cultivars: (1) fine tuning of gene expression and enzyme activity, (2) single nucleotide polymorphisms underlying the regiospecificity of terpenoid hydroxylases, and (3) DNA hypermethylation leading to gene silencing.
- **Citrus.** We have completed a comprehensive study of the ultrastructural changes associated with the development of secretory cavities in *Citrus* peel. We have been particularly interested in understanding the role of membrane contact sites between endoplasmic reticulum and leucoplasts, as these might be characteristic of epithelial cell types with high terpenoid secretory activity. We have also localized (+)-limonene synthase, the enzyme responsible for >90% of carbon flux into terpenoids in epithelial cells of secretory cavities, to specific areas of leucoplasts.
- **Loblolly pine.** We have been investigating the capacity of loblolly pine trees to accumulate oleoresin (composed mostly of monoterpenoid volatiles and diterpenoid acids) in resin ducts. This project included the measurement of volumes of resin ducts and their distribution for three entire trees. More recently, we have acquired cell type-specific transcriptome data for epithelial cells that surround resin ducts in loblolly pine needles. These data sets provide a first glimpse of the developmental and biosynthetic factors that play important roles in the initiation and filling of resin ducts.
- **Enzyme structure-function.** Terpene synthases convert a prenyl diphosphate of a specific chain length to the first pathway-specific (often cyclic) intermediate in the biosynthesis of a specific class of terpenoids. These enzymes are critical determinants of terpenoid chemical diversity, which is an important issue for the development of sustainable specialty chemicals. We have acquired the first comprehensive data set to map the active site of a model monoterpene synthase ((-)-limonene synthase) and evaluated residues with a potential function as catalytic base in the hallmark deprotonation reaction of this class of enzymes.

Science objectives for 2014-2015:

- **Peppermint.** Testing of glandular trichome-specific promoters for the expression of transgenes.
- **Citrus.** Experiments are completed. Finish manuscript (see below).
- **Loblolly pine.** Final experiments are running. Finish manuscript (see below).
- **Enzyme structure-function.** Evaluate the relationship between active site architecture, interactions of specific amino acids with transition state intermediates and product distribution in (-)-limonene synthase mutants.
- **Manuscripts** are at an advanced stage to report on the following topics:
 - Lange B.M. (2015) Evolution of plant secretory structures and the emergence of terpenoid chemical diversity. *Annu. Rev. Plant Biol.*, submission by August 31, 2014.
 - Turner G.W., Lange B.M. (2015) Development of *Citrus* essential oil secretory cavities and immunolocalization of (+)-limonene synthase, submission planned for September, 2014.
 - Turner G.W., Parrish A.N., Fishedick J.T., Lange B.M. (2015) Distribution, development and biosynthetic capabilities of resin ducts in loblolly pine (*Pinus taeda* L.), submission planned for October, 2014.

My scientific areas of expertise are:

- Single cell transcriptomics, metabolomics, functional genomics, and mathematical modeling for an improved understanding of metabolism in specialized plant cell types.
- Development of web-based resources for genomics, transcriptomics and metabolomics research related to the biosynthesis of specialized/secondary plant metabolites.

To take my project to the next level, my ideal collaborator would have expertise in:

- Synthetic biology – development of microbial platform strains for generating diverse terpenoids.
- Enzyme structure-function – quantum mechanical modeling of catalysis involving carbocationic transition state analogues in terpene synthases.

Publications supported by this project (2012-2014):

1. Srividya N., Davis E.E., Croteau R.B., Lange B.M. (2015) Functional analysis of spearmint (-)-limonene synthase mutants reveals determinants of catalytic outcome in a model monoterpene synthase. *Proc. Natl. Acad. Sci. USA*, submitted.
2. Ahkami A., Srividya N., Johnson S.R., Lange B.M. (2015) Multiple levels of regulation determine monoterpene essential oil compositional variation in the mint family. *Molecular Plant*, in revision.
3. Lange B.M. (2015) Biosynthesis and biotechnology of p-menthane monoterpenes, including menthol, carvone and limonene. *Adv. Biochem. Eng. Biotechnol.*, in press.
4. Voo S.S., Lange B.M. (2014) Sample preparation for single cell transcriptomics - essential oil glands in *Citrus* fruit peel as an example. *Methods Mol. Biol.* **1153**, 203-212.
5. Lange B.M., Rios-Estepa R. (2014) Kinetic modeling of plant metabolism and its predictive power – peppermint essential oil biosynthesis as an example. *Methods Mol. Biol.* **1083**, 287-311.
6. Lange B.M., Lange I., Turner G.W., Herron B.K. (2013) Utility of aromatic plants for the biotechnological production of sustainable chemical and pharmaceutical feedstocks. *Med. Aromat. Plants* **2**, 5.
7. Lange B.M., Ahkami A. (2013) Metabolic engineering of plant monoterpenes, sesquiterpenes and diterpenes - current status and future opportunities. *Plant Biotechnol. J.* **11**, 169-196.
8. Lange B.M., Turner G.W. (2013) Terpenoid biosynthesis in secretory glandular trichomes - current status and future opportunities. *Plant Biotechnol. J.* **11**, 2-22.

Molecular and Genetic Bases of Sodium and Potassium Transport and Distribution in Plants.

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Overall research goals:

Salinity stress is progressively causing reduced plant growth in irrigated lands and in natural saline soils and is detrimental to photosynthetic activity. Our DOE research focuses on the basic biophysical, physiological, and regulatory mechanisms through which HKT sodium cation transporters and chloroplast K⁺ transporters play central roles in mediating Na⁺ and K⁺ transport and salt tolerance in plants. Our DOE-funded research has shown that the AtHKT1;1 transporter mediates a key mechanism for protection of plants from salinity stress by excluding toxic Na⁺ ions from leaves [1]. Recent mapping of salinity resistance quantitative trait loci (QTL) in grasses in several labs suggest that the same *HKT* genes that the P.I.'s lab has identified in *Arabidopsis* are key salinity resistance mechanisms in grasses. However, the molecular basis for *HKT*-mediated salt resistance in grasses remains unknown, although this is of key relevance for basic energy sciences. The molecular mechanisms by which Na⁺ is toxic to photosynthetic activity and the protective K⁺ transport mechanisms across chloroplast membranes remain unknown and molecular and genetic analyses are being pursued.

Significant achievements (2012-2014):

Plastidial transporters KEA1, -2, and -3 are essential for chloroplast osmoregulation, integrity and pH regulation. Our recent research has identified the chloroplast-targeted KEA K⁺ transporters and their key roles in chloroplast function and an unexpected amelioration by Na⁺ of *kealkea2* double mutant plants [2]. KEA1 and KEA2 proteins are targeted to the inner envelope membrane of chloroplasts, whereas KEA3 is targeted to the thylakoid membrane. The pH component of the proton motive force across the thylakoid membrane was decreased in *kealkea2* mutants, indicating an altered chloroplast pH homeostasis. Furthermore, the *kealkea2* background enabled genetic analyses of other chloroplast transporters. Together, our findings demonstrate a fundamental role of inner envelope KEA1 and KEA2 and thylakoid KEA3 transporters in chloroplast osmoregulation, integrity, and ion and pH homeostasis [2]. We further localized the Na⁺/H⁺ antiporter1 NHD1 to the inner envelope of chloroplasts. *nhd1* mutant plants show reduced quantum yield of photosystem II in response to salinity stress [3]. With partial funding from this DOE grant, we have further identified four genes encoding three distinct mechanisms that provide a frame work for a newly recognized pathway through which elevated CO₂ down-regulates carbon influx into leaves [4].

AtHKT1;1 is essential for plant salt tolerance but transcriptional activators remain to be determined. Membrane transporters play important roles in abiotic stress tolerance and are crucial for plant growth [5]. Our initial research showed that *athkt1;1* mutations cause Na⁺ over-accumulation in leaves of *Arabidopsis* and that the AtHKT1;1 transporter protects photosynthetic activity in leaves from Na⁺ stress [1]. Research in the grasses rice and wheat suggests that this key function of class-1 HKT transporters in leaf Na⁺ exclusion is conserved in the grasses (see: [1;5]). Our present research focuses on the yet unknown regulators of *HKT* gene expression in response to Na⁺ stress. In a genome wide transcription factor (TF) screen we identified candidates that bind to the *AtHKT1;1* promoter. Knockout lines of some candidate TFs show promising salt stress phenotypes (unpublished data) and screening with new amiRNA libraries we have recently developed [6] will help identify gene families important for *AtHKT1;1* regulation.

Salinity stress signal transduction network in plants. We reviewed salt stress-induced propagating Ca²⁺ waves in roots [7]. Genes that encode specific plasma membrane Ca²⁺-permeable channels remain poorly defined in plants. Using biophysical approaches together with expression microarray data and knock out of candidate genes in *Arabidopsis*, we identified the CNGC5 & CNGC6 genes as encoding plasma membrane Ca²⁺ permeable channels [8].

The plant hormone abscisic acid (ABA) mediates resistance to abiotic stresses, including salinity stress. With partial support from this DOE grant, we successfully reconstituted the ABA

signaling pathway from ABA to ion channel activation via two distinct types of reconstituted ABA signaling cores [9]. In addition we developed first real-time FRET sensors for ABA, and could resolve ABA elevation in roots in response to salinity stress [10].

K⁺/Na⁺ transporter structure function. Transporters for K⁺ and Na⁺ play key roles in determining salinity tolerance of plants. In collaboration with former postdoctoral associate, Nobuyuki Uozumi, the transmembrane topology of the major class of high-affinity KUP/HAK/KT K⁺ uptake transporters was determined, which provides evidence for a new structural/biophysical mechanism for K⁺ selectivity [11]. In an additional study we found that K⁺ uptake channel activity is differentially regulated by Ca²⁺ in signaling mutants, providing evidence for a newly recognized compensatory feedback regulation [12].

Science objectives for 2014-2015:

- Isolate candidate regulatory genes that function in controlling *AtHKT1;1* expression and Na⁺ tolerance.
- Identify the molecular mechanisms underlying natural variation in salinity tolerance in grasses.
- Characterize the roles of plastid-localized KEA transporters in salinity stress responses.

My scientific areas of expertise are: membrane biophysics; molecular mechanisms of salinity resistance and transport.

To take my project to the next level, my ideal collaborators would have expertise in: quantitative genetics of important traits for salinity tolerance in grasses. Structure/function analyses of plant K⁺/Na⁺ transporters.

Publications supported by this project 2012–2014:

1. Deinlein, U., et al. & Schroeder JI. "[Plant salt-tolerance mechanisms.](#)" *Trends Plant Sci.* **19**: 371–379 (2014).
2. Kunz, H. H., et al. & Schroeder JI. "[The Plastidial Transporters KEA1, 2 and 3 are essential for chloroplast osmoregulation, integrity and pH regulation in Arabidopsis.](#)" *PNAS* **111**: 7480–7485 (2014).
3. Müller, M., Kunz, H.H., Schroeder JI., Kemp G., Young H.S., Neuhaus, H.E. "[Decreased capacity of sodium export out of Arabidopsis chloroplasts impairs salt tolerance, photosynthesis and plant performance.](#)" *Plant J.* **78**: 646–658 (2014).
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12. Laanemets K, Brandt, B., et al. & Kollist H., Schroeder JI. "[Calcium-Dependent and -Independent Stomatal Signaling Network and Compensatory Feedback Control of Stomatal Opening via Ca²⁺ Sensitivity Priming.](#)" *Plant Physiol.* **163**: 504–13 doi: [10.1104/pp.113.220343](#) (2013).

BETCy-EFRC Biological Electron Transfer and Catalysis Energy Frontier Research Center

John Peters, Montana State University; Principal Investigator, Montana State University
Michael Adams, University of Georgia; Brian Bother, Eric Boyd, & Ross Carlson, Montana State University; Caroline Harwood, University of Washington; Paul King & Pin-Ching Maness, National Renewable Energy Laboratory; Anne Jones, Arizona State University; Anne-Frances Miller, University of Kentucky; & Lance Seefeldt, Utah State University; Co-PIs
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Overall research goals:

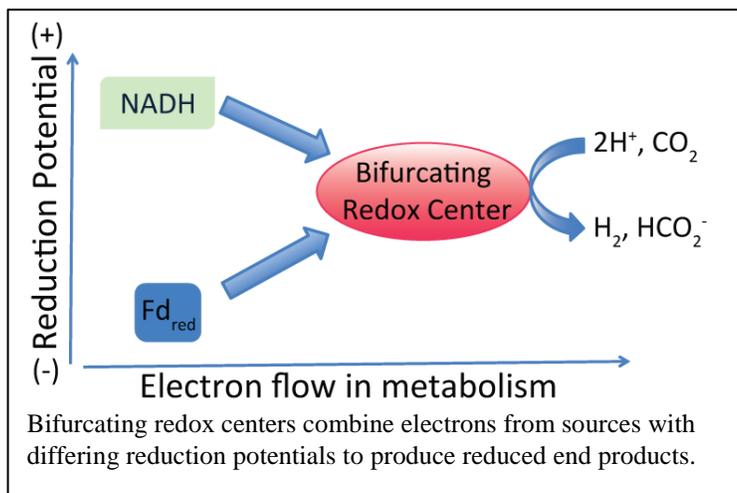
The mission of the newly established BETCy-EFRC is to expand on recent discoveries in the mechanism of microbial electron transfer and energy conservation, with the ultimate goal of streamlining the production of biofuels to help build the 21st century energy economy. A limitation with current production is that microbial metabolism is not optimized for maximal production of reduced biofuel end products. The Center's aim is to gain in-depth understanding of fundamental mechanisms that allow biology to overcome key thermodynamic barriers that limit the production of reduced products. To address this, the BETCy-EFRC will examine the molecular mechanisms and structural basis for controlling electron transfer in key enzymes for the production of hydrocarbon and hydrogen biofuels. The research is divided into three integrated thrust areas focused on 1) Energy Conservation and Electron Bifurcation, 2) ATP Coupled Electron Transfer, and 3) Thermodynamic Control of Proton Coupled Electron Transfer Reactions.

The Center's research could lead to dramatic advances in engineering microbes that can optimally direct electron flow to reduced products that we utilize as biofuels.

(1) Energy Conservation and Electron Bifurcation

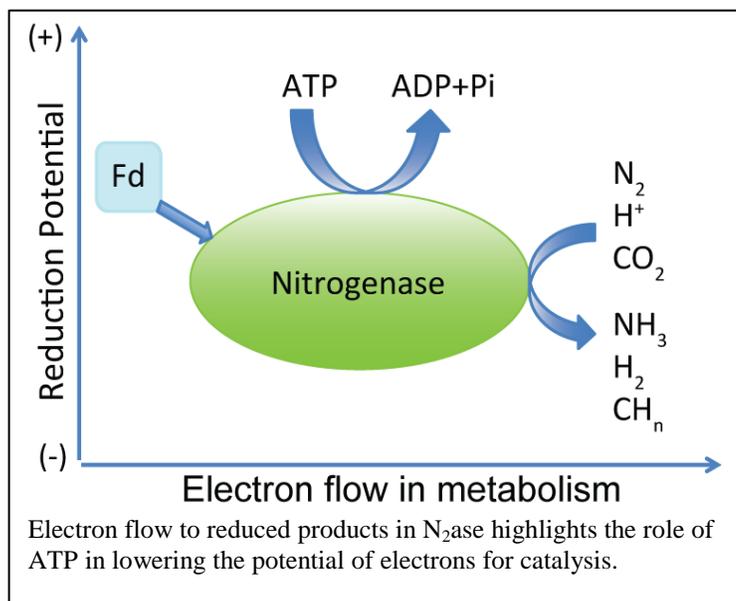
A significant portion of the metabolic electron flow in microbial metabolism is directed towards reduction of pyridine nucleotides. Thermodynamically, the reduced pyridine nucleotides are not sufficient in energy for the production of the desired highly reduced end products. In the past decade, it has become apparent that the biochemical mechanism termed "electron bifurcation" potentially overcomes this thermodynamic challenge by combining electrons from the reduced pyridine nucleotides with other lower potential electrons from reduced ferredoxins. The Center will focus on 5 tasks in this area:

1) Determine the electron bifurcation mechanisms in transhydrogenases. 2) Determine the electron bifurcation mechanisms in hydrogenases. 3) Determine the bifurcating mechanism in a flavoenzyme lacking FeS clusters. 4) Determine the effects of electron bifurcation integration into electron transfer networks. 5) Elucidate the mechanisms of electron transfer-linked ion-pumping in H₂-evolving membrane complexes.



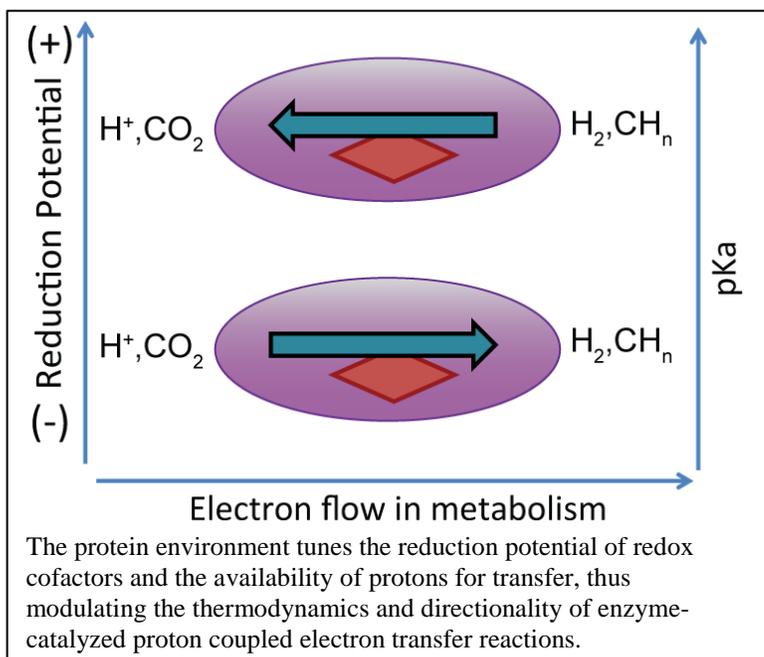
(2) ATP Coupled Electron Transfer.

Nitrogenase (N_2ase) is capable of utilizing ATP and ferredoxin to reduce CO_2 to various hydrocarbon products. A defining feature of N_2ase is the absolute coupling of ATP binding and hydrolysis to electron transfer reactions for substrate reduction. Recent studies have provided insights into the order of electron transport and ATP hydrolysis, however very little is known about how ATP binding and hydrolysis is thermodynamically coupled to electron transfer. The Center will focus on 3 tasks in this area. 1) Elucidate a comprehensive understanding of how ATP controls electron flow through N_2ase directed toward CO_2 reduction to hydrocarbons. 2) Extrapolate the *in vitro* results to photosynthetic cells. 3) Bridge knowledge of electron flow from metabolism to production of value added products. Electron flow in whole cells will be computationally modeled with the goal of developing a framework to predict conditions that will result in maximum synthesis of biofuels by bacteria.



(3) Thermodynamic Control of Proton Coupled Electron Transfer Reactions.

The energy barriers for transferring electrons through proteins and for coupling the reduction/oxidation of substrates have a fundamental role in controlling the overall reaction. Enzymes that catalyze these reactions control the local thermodynamics with mechanisms including the nature and availability of proton donor/acceptor groups at the active site and modulating the midpoint potential of cofactors, such as flavins and metal clusters. To better understand these mechanisms, the Center will focus on 3 tasks in this area. 1) Define structural features that control reduction potentials and coupled proton uptake at multielectron redox sites and correlate with catalytic directionality. 2) Determine how active site and accessory redox cluster thermodynamic properties are modulated by interactions with one another to impact catalytic bias. 3) Engineer new synthetic proteins for electrocatalysis. These studies, coupled with the insights gained in subdivisions 1 and 2 above, will underpin the long-term goal of controlling reaction thermodynamics at the level of design. Design principles will be put into practice by developing bio-inspired mimetics and re-designed enzymes.



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

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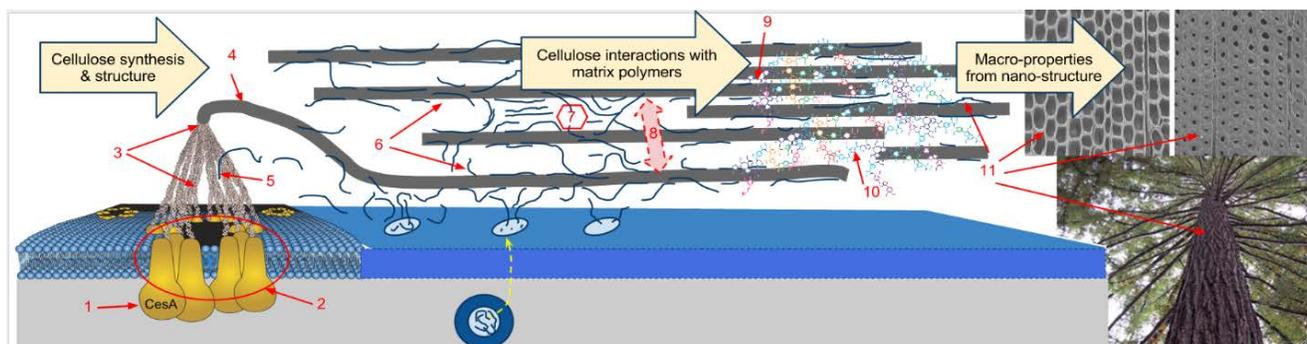
Other senior personnel: **Lead Institution: Pennsylvania State University:** Charles Anderson, Gong Chen, Ying Gu, Seong Han Kim, James Kubicki, Manish Kumar, Janna Maranas, Karl Mueller, B. Tracy Nixon, Ming Tien; **Other Institutions: North Carolina State University:** Candace Haigler, Yaroslava Yingling; **Massachusetts Institute of Technology:** Mei Hong; **Oak Ridge National Lab:** Paul Langan; **University of Rhode Island:** Alison Roberts; **University of Virginia:** Jochen Zimmer;

Overall research goals:

to develop a nano- to meso-scale understanding of plant cell walls, the main structural material in plants, and the mechanisms of their assembly, forming the foundation for significant advances in sustainable energy and novel biomaterials.

Cellulosic biomass (lignocellulose) holds great promise as a large-scale, renewable and sustainable source of liquid biofuels for transportation, if we could overcome technical obstacles stemming from its complex, hierarchical structure. Despite its huge economic importance, many aspects of lignocellulose structure and formation remain shrouded in mystery. For instance, little is known of the details of how the cellulose-synthesizing nano-machine at the cell surface links simple sugar molecules into long strands and extrudes them at the cell surface in such a way that they make a strong, insoluble and highly inert crystalline fibril. Likewise, the processes by which simple polymers are transformed into a strong and recalcitrant biomaterial are not well understood.

CLSF goals are to develop a detailed nano- to meso-scale understanding of plant cell wall structure and its mechanism of assembly, from the molecular mysteries of how glucose is assembled by cellulose synthase complexes to form cellulose microfibrils to the orderly, hierarchical interaction of cellulose with other components to form cell walls with diverse properties. The diagram below sketches some of the key points for lignocellulose formation, starting with cellulose synthesis by cellulose synthases (CESA) organized into a complex embedded in the plasma membrane (left, points 1-2), followed by cellulose crystallization and interactions with hemicelluloses to form a cohesive wall (points 3-8) and then changes that accompany lignin polymerization within the wall (points 9-11).



New understanding of these processes will form the scientific foundation for designing rational, science-based pretreatments to deconstruct cell walls and for using genetic techniques to coax plants

into making modified walls for significant advances in sustainable energy and novel materials. It will also yield insights into biomimetic ways to transform simple molecules into complex polymeric materials with diverse physical and chemical properties.

Science objectives for 2014-2018:

CLSF Theme 1 probes the mechanism of cellulose microfibril (CMF) formation. Specific objectives include:

- Determination of the structure and in-vitro activity of plant cellulose synthase (CESA) by biochemical means, X-ray crystallography and electron microscopy, as well as by computational modeling of the dynamics of glucan synthesis by a CESA protein.
- Assessing the roles of parts of the CESA protein for cellulose synthesis by genetic modification and analysis of CESA activity and cell wall phenotypes.
- Analysis of the contributions of CESA isoform to the activity of the Cellulose Synthesizing Complex (CSC) and properties of the microfibril and macrofibril through microscopic and genetic experiments.
- In-depth characterization of the plant CSC by isolating an active CSC, modeling CMF formation in silico, and identifying proteins that interact with CSCs that synthesize cellulose in secondary cell walls.
- Reconstitution of a functional CSC from purified plant components by assembling the essential components of the plant CSC.

CLSF Theme 2 investigates the nano- and meso-scale structure and assembly of cell walls and the basis for their important physical and biological properties. Research objectives include:

- Analysis of mesoscale wall architecture and dynamics by Atomic Force Microscopy (AFM), small-angle neutron scattering, and solid-state Nuclear Magnetic Resonance (ssNMR) in combination with enzymatic and genetic modifications of the wall.
- Testing of the current model of the grass cell wall using enzymatic, biomechanical and physical approaches.
- Testing how matrix polymer delivery relates to cellulose biosynthesis and order using click-labeling of matrix polysaccharides combined with analysis by AFM and transmission electron microscopy.
- Analysis of the mobility of water, polysaccharides and proteins by neutron scattering and ssNMR to assess influence of wall components and wall charge on wall dynamics.
- Incorporation of new as well as existing data into computational models of primary and secondary cell wall architecture and material properties.
- Analysis of the physical effects of lignin polymerization in cell wall explants and analogs, including mobility of water and polysaccharides.
- Development and use of sum frequency generation (SFG) spectroscopy (a) to analyze meso-scale organization of cellulose in single cell walls and (b) to refine the interpretation of SFG spectra.

Our scientific areas of expertise include: plant cell wall structure; cell and molecular biology; plant and bacterial genetics; structural biology including protein crystallography, cryo-EM, neutron scattering and solid-state NMR; biomechanics; computational methods for structural biology and chemistry including quantum mechanical modeling, molecular dynamics simulation and coarse-grain models; atomic force microscopy, electron microscopy, confocal microscopy; carbohydrate chemistry and synthesis; spectroscopy.

Center for direct catalytic conversion of biomass to biofuels (C3Bio)

Maureen C. McCann, Principal Investigator

Mahdi M Abu-Omar¹, Rakesh Agrawal¹, Craig E Barnes², Gregg T Beckham³, Joseph J Bozell², Alison Buchan², Nicholas C Carpita¹, Clint C S Chapple¹, Kari L Clase¹, Michael F Crowley³, W Nicholas Delgass¹, Shi-You Ding³, Bryon S Donohoe³, Michael E Himmel³, Hilkkka I Kenttamaa¹, Lee Makowski⁴, Richard Meilan¹, Nathan S Mosier¹, Angus S Murphy¹, Wendy A Peer¹, Fabio H Ribeiro¹, Garth J Simpson¹, Christopher J Staiger¹, Daniel B Szymanski¹, and Melvin P Tucker³: ¹Purdue University; ²University of Tennessee; ³National Renewable Energy Laboratory; ⁴Northeastern University, Co-PI(s)

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Overall research goals:

New capabilities to predict, design and control the chemistries of carbon could answer a global imperative to transition from fossil-based to sustainable transportation fuels. Lignocellulosic biomass has only one-third the energy density of crude oil and lacks petroleum's versatility as a feedstock for fuels and chemicals. These limitations keep biomass conversion below the efficiency level needed for strategic impact while the scientific challenge of routing carbon from one molecular context to another remains unmet.

The polysaccharides and lignins of the plant cell wall form a complex, cross-linked polymeric structure of substantial physical and chemical resilience that impedes access of catalysts to targeted chemical bonds. Its complexity results in heterogeneous product streams after catalytic or pyrolytic processing. Cross-links among plant cell wall biopolymers generate nanoscale architectures and distinct mesoscale domains that have dramatically different properties than those observed in mixtures of biopolymers. C3Bio research demonstrated that the disparity between theoretical and actual yields of liquid hydrocarbons and high-value chemicals is a consequence of this structural complexity. C3Bio aims to develop critical systems-level understanding of how biomass structural complexity at molecular, nanoscale, and mesoscale levels impacts the yields and selectivities of desired reaction products from catalytic and pyrolytic transformations. We aim to modulate cell wall complexity and catalytically transform intact biomass in order to gain unprecedented control of effective routing of carbon: we will specify both the structures within, and the reaction products from, lignocellulosic biomass.

Significant achievements 2009-2014

To convert biomass, including lignin, to hydrocarbon fuels and low molecular weight aromatic compounds, hydrogenation and hydrogenolysis catalysts were needed that are highly robust, efficient and with low toxicity. We developed a bimetallic Palladium/Zinc/Carbon catalytic system for the disassembly of lignin via β -O-4 ether bond cleavages and characterized the reaction mechanism. Dimeric lignin model compounds and synthetic lignin polymers are cleaved with near quantitative conversions and yields of 80–90%. Disassembly of about half of the lignin even from intact woody biomass into two methoxy-substituted propylphenols as major products is the technology basis of the C3Bio start-up company, Spero Energy. Removing lignin first is compatible with subsequent xylan conversion to furfural and cellulose conversion to hydroxymethylfurfural. The ability to deconstruct lignin first from intact biomass revolutionizes the conventional concept of the biorefinery.

A partnership of chemical engineers and chemists led to the development of a lab-scale, high-pressure, continuous-flow, fast-hydrolysis and vapor-phase catalytic hydrodeoxygenation reactor system, new analytical methods and a novel tandem mass spectrometer (International Patent Application

PCT/US2012/056909) with exceptional versatility to improve the fundamental understanding of fast-pyrolysis. Identification of the reaction mechanism by which cellobiose is converted to levoglucosan, was determined using a combination of computational modeling and experimental validation. By capturing the very first reaction products, we can reduce the complexity of bio-oils from thousands of molecular species to just a few, making the discovery and design of hydrodeoxygenation catalysts feasible.

C3Bio-developed maleic acid catalysis converts xylan in intact biomass in a one-pot, two-step reaction to the useful platform chemical furfural with high yield and selectivity. Chemists and biologists revealed an impact of maleic acid catalysis on cell-cell adhesion when applied to biomass with modified (high syringyl) lignin composition. By gaining control over lignin composition and structure via modifying expression of enzymes in the lignin biosynthetic pathway, the synergistic impact is to disrupt the middle lamella between cells upon treatment with maleic acid. This has the potential to dramatically reduce energy inputs for biomass comminution.

Science objectives for 2014-2018:

Goal 1. Develop catalytic and pyrolytic processes specifically designed for the structural complexity of biomass. We focus on generating streams of monomers and oligomers via biomass fractionation, transforming these substrates to useful products, and identifying catalytic pathways that act on molecules embedded in intact biomass.

Goal 2. Redesign the structure of biomass for carbon- and energy-efficient catalytic and pyrolytic transformations. We reduce the complexity of biomass structure complexity in ways that enable conversion processes to preserve every carbon atom and minimize energy inputs. We use synthetic biology to redesign lignin and cellulose microfibrils. Site-specific delivery of catalysts to cell walls and modifications of cell wall composition from the molecular to mesoscale will generate new “fit-for-catalysis” substrates and architectures beyond what is found in Nature.

Goal 3. Deliver innovative pathways for targeted product portfolios from tailored biomass. We will apply our imaging and analytical toolkits to materials generated in Goals 1 and 2 to understand how the native system of molecular interactions is changed to a new, more effective state, and how we can use computational modeling to predict and select optimal transition pathways from tailored biomass to desired reaction products.

Our scientific areas of expertise are: Plant cell wall architecture and biosynthesis, heterogeneous and homogeneous chemical catalyses, fast-pyrolysis, mass spectrometry, multi-scale imaging, computational modeling.

To take our project to the next level, our ideal collaborators would have expertise in: Plant synthetic biology, organic chemistry, materials science.

Publications supported by this project 2009-2014:

Our 99 publications can be found in the list of all EFRC publications at <http://science.energy.gov/bes/efrc/publications/>. To find C3Bio publications, please note that publications are ordered first by year, then alphabetically by EFRC acronym, and finally alphabetically by first author.

Poster Session I

Hyperthermophilic Multiprotein Complexes and Pathways for Energy Conservation and Catalysis

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

Significant achievements 2013-2014: The model organism for these studies is *Pyrococcus furiosus* (Pf), an archaeon that grows optimally at 100°C. Pf obtains carbon and energy for growth by fermenting carbohydrates and producing H₂ and by reducing elemental sulfur (S⁰) to H₂S. It has a respiratory metabolism in which it couples H₂ production by a ferredoxin-dependent, membrane-bound hydrogenase (MBH) to ion translocation and formation of a membrane potential that Pf utilizes to synthesize ATP (Figure 1). Pf also contains a cytoplasmic hydrogenase (SHI) that has the rare property of evolving H₂ from NADPH, a reaction of utility in H₂ production systems (Figure 1). Addition of S⁰ to Pf prevents the synthesis of MBH and SHI, and induces the synthesis of a highly homologous membrane complex which we term MBX. MBX is proposed to oxidize ferredoxin, reduce S⁰ and conserve energy by an as yet unknown mechanism. The specific aims of the proposed research are to characterize the novel energy-conserving complexes MBH and MBX, and to structurally characterize native SHI and minimal forms of four-subunit SHI and fourteen-subunit MBH. Over the past two years we have taken advantage of the recently developed genetic system in Pf. An affinity-tagged version of SHI has been over-expressed ~10-fold, purified by a one-step procedure, and has been used in *in vitro* enzyme pathways to produce H₂. An affinity tagged-version of MBH has also been solubilized and purified, and characterized using biochemical and SAXS analyses. An extensive phylogenetic and bioinformatic analysis of MBH and MBX revealed that they are modular in nature and represent ancestral respiratory complexes. Hence another hyperthermophilic archaeon contains a respiratory complex termed formate hydrogen lyase (FHL) that is similar to MBH but contains an additional formate dehydrogenase module. The 18-gene operon that encodes FHL was successfully expressed in Pf and it enabled the organism to convert formate to H₂ and CO₂ in an energy-yielding reaction (Figure 1). The results of this research will provide a fundamental understanding of how the metabolism of S⁰ and H₂ leads to energy conservation (MBH and MBX) in Pf and the structure and function of H₂- and H₂S-metabolizing enzymes.

Science objectives for 2014-2015:

To optimize production of detergent-solubilized affinity-tagged forms of fourteen-subunit MBH and thirteen-subunit MBX for structural and biochemical characterization.

To engineer the production of five-subunit forms of cytoplasmic portions of both MBH and MBX for structural and biochemical characterization.

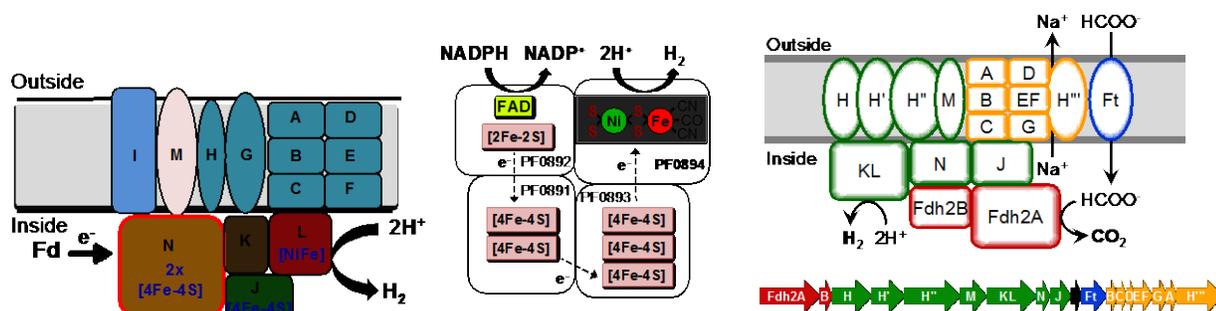


Figure 1. Proposed subunit organization, cofactor content and electron flow pathway in fourteen-subunit MBH (*left*), four-subunit SHI (*center*) and eighteen-subunit FHL (*right*).

My scientific areas of expertise are: anaerobic biochemistry and metalloenzymes.

To take my project to the next level, my ideal collaborator would have expertise in: cryo-EM.

Publications supported by this project 2013-2014:

Schut, G. J., Boyd, E. S., Peters, J. W and Adams, M. W. W. (2013) “The modular respiratory complexes involved in hydrogen and sulfur metabolism by heterotrophic hyperthermophilic archaea and their evolutionary implications” *FEMS Microbiol. Rev.* 37, 182-203.

Zhu, Y., Kumar, S., Menon, A. L., Scott, R. A. and Adams, M. W. W. (2013) “Regulation of iron metabolism by *Pyrococcus furiosus*” *J. Bacteriol.* 185, 2400-2407.

del Campo, J. S. M., Rollin, J., Myung, S., Chun, Y., Chandrayan, S., Patiño, R. Adams, M. W. W. and Zhang, Y.-H. (2013) ”High-yield dihydrogen production from xylose through cell-free synthetic cascade enzymes” *Angew. Chemie* 55, 4587-4590

Hawkins, A. S., McTernan, P., Lian, H., Kelly, R. M. and Adams, M. W.W. (2013) “Biological conversion of carbon dioxide and hydrogen into liquid fuels and industrial chemicals” *Curr. Opin. Biotechnol.* 24, 376-384.

Kamali, S., Wang, H., Mitra, D., Ogata, H., Lubitz, H., Manor, B. C., Rauchfuss, T. B., Byrne, D., Bonnefoy, V., Jenney, F. E., Adams, M. W. W., Yoda, Y., Alp, E., Zhao, J. and Cramer, S. P. (2013) “Observation of the Fe-CN and Fe-CO vibrations in the active site of [NiFe] hydrogenase by nuclear resonance vibrational spectroscopy” *Angew. Chemie* 52, 724-728.

Myung, S., Rollin, J., You, C., Sun, F., Chandrayan, S., Adams, M. W. W. and Zhang, P. (2014) “*In vitro* metabolic engineering of hydrogen production at theoretical yield from sucrose” *Metab. Engineering* 13,70-77

Scott, J. W., Poole, F. L., and Adams, M. W. W. (2014) “Characterization of ten heterotetrameric NDP-dependent acyl-CoA synthetases of the hyperthermophilic archaeon *Pyrococcus furiosus*” *Archaea* 176863, 1-9

Lipscomb, G. L., Schut, G. J., Thorgersen, M. P., Nixon, W.J., Kelly, R. M. and Adams, M. W. W. (2014) “Engineering hydrogen gas production from formate in a hyperthermophile by heterologous production of an 18-subunit membrane-bound complex” *J. Biol. Chem.* 289, 2873-2879

Esteves, A. M., Chandrayan, S. K., McTernan, P. M., Borges, N., Adams, M. W. W. and Santos, H. (2014) “Mannosylglycerate and di-myo-inositol phosphate have interchangeable roles during adaptation of *Pyrococcus furiosus* to heat stress” *Appl. Environ. Microbiol.* 80, 4226-4233

McTernan, P. M., Chandrayan, S. K., Wu, C.-H., Vaccaro, B. J., Yang, Q., Fu, D., Hura, G. L., Tainer, J. A. and Adams, M. W. W. (2014) “Intact functional fourteen-subunit respiratory membrane bound [NiFe]-hydrogenase complex of the hyperthermophilic archaeon *Pyrococcus furiosus*” *J. Biol. Chem.* 289, 19364-19372

Amidase Mediated Modulation of N-Acylethanolamine (NAE) Signaling

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Overall research goals:

Research in our labs, supported since 2005 by Basic Energy Sciences, has led to the discovery of a new lipid mediator pathway that influences phytohormone regulation of plant growth and development—the so-called *N*-acylethanolamine (NAE) regulatory pathway. This pathway in plants shares conserved metabolic machinery with the endocannabinoid signaling system of vertebrates that regulates a plethora of physiological and behavioral processes in mammals, suggesting that the metabolism of NAEs is an important regulatory feature of eukaryotic biology. The current evidence in plants points to interaction between NAE metabolism and ABA signaling, and our overall goal is to understand the role of NAE metabolism in the transition from embryonic development to seedling establishment and the acquisition of photoautotrophic growth. Our results suggest that NAE metabolism influences chloroplast development and root elongation during seedling establishment and may operate as part of a process to synchronize early plant growth and the assembly of photosynthetic systems. The long term impact of this work will shed light on the fundamental biochemical and molecular mechanisms that control the assembly of photosynthetic machinery in plant systems.

Significant achievements (2012-2014):

1. Analytical methodology was developed to allow for the identification and quantification of two important and unknown metabolite pools—the *N*-acylphosphatidylethanolamines (precursors of NAEs) and the oxylipin metabolites of polyunsaturated NAEs.
2. These technological advances facilitated the discovery and description of NAE metabolism during seedling establishment with unprecedented detail. Further, they have provided a new appreciation for the bioactivity of lipoxygenase-derived NAE metabolites, and have supported a bifurcating, complex action of NAEs with some species affecting only chloroplast assembly and others regulating root elongation. Transcriptional profiling suggests the involvement of both ABA and light signaling pathways in the action of NAE oxylipins.
3. Studies on the biochemical properties of fatty acid amide hydrolase (FAAH) have led to the discovery of a product-feedback regulation by ethanolamine and offers insights into potential regulation *in vivo*. In addition, a domain responsible for the oligomeric association of FAAH was identified and this domain influences the interaction of FAAH with other Arabidopsis proteins.
4. Genetics approaches have identified mutants with altered NAE sensitivity, and chemical genetics strategies have identified several candidate small molecules that interfere with NAE-mediated seedling development.

Science objectives for 2014-2015:

- Characterization of mutants identified with tolerance and hypersensitivity toward NAEs.
- Elucidate details of the interactions of NAE oxylipins with ABA and light signaling pathways.
- Map the spatial distribution of NAEs and metabolites in Arabidopsis seeds and seedlings *in situ*.

My scientific areas of expertise are: lipid biochemistry, enzymology, cell and molecular biology.

To take my project to the next level, my ideal collaborator would have expertise in: structural biology, especially with expertise with membrane associated proteins.

Publications supported by this project 2012-2014:

Primary, peer-reviewed research papers

1. **Horn PJ, Korte AR, Neogi PB, Love E, Fuchs J, Strupat K, Borisjuk L, Shulaev V, Lee Y-J, Chapman KD** (2012) Spatial mapping of lipids at cellular resolution in embryos of *Gossypium hirsutum*, L. *Plant Cell*, 24(2):622-36. doi: 10.1105/tpc.111.094581. PMID: [22337917](#). **Featured article for “In-Brief” by J. Mach, cited as recommended by Faculty 1000.** Partial funding by BES - important to goals outlined for BES proposal.
2. **Kilaru A, Tamura P, Isaac G, Welti R, Venables BJ, Seier E, Chapman KD.** (2012) Lipidomic analysis of *N*-acylphosphatidylethanolamine molecular species in Arabidopsis suggests feedback regulation by *N*-acylethanolamines. *Planta* 236(3): 809-824. PMID: [22673881](#). **Special issue in plant metabolic regulation.** Supported entirely by BES
3. **Teaster ND, Keereetaweep J, Kilaru A, Wang Y-S, Tang Y, Tran C, Chapman KD and Blancaflor EB** (2012) Overexpression of fatty acid amide hydrolase induces early flowering in Arabidopsis thaliana. *Frontiers in Plant Science* vol 3: article 32. doi: [10.3389/fpls.2012.00032](#). Epub 2012 Feb 20, PMID: [22645580](#). **In a Special Edited Collection of Articles in Plant Lipid Signaling.** Supported entirely by BES.
4. **Keereetaweep K, Blancaflor EB, Hornung E, Feussner I, Chapman KD** (2013) Ethanolamide oxylipins of linolenic acid can negatively regulate Arabidopsis seedling development. *Plant Cell*, 2013 Oct 22. [Epub ahead of print] PMID: [24151297](#). **Article featured with “In-brief” by N. Hoffmann.** Supported entirely by BES.
5. **Faure L, Nagarajan S, Hwang H, Montgomery CL, Khan BR, John G, Koulen P, Blancaflor EB, Chapman KD** (2014) Synthesis of Phenoxyacyl-Ethanolamides and their Effects on Fatty Acid Amide Hydrolase Activity. *Journal of Biological Chemistry*, 2014 Feb 20. [Epub ahead of print] PMID: [24558037](#). Supported entirely by BES.

Invited Reviews (peer-reviewed)

1. **Faure L, Chapman KD** (2012) *N*-Acylphosphatidylethanolamines (NAPEs), *N*-Acylethanolamides and other Acylamides: Metabolism, Occurrence and Function in Plants. American Oil Chemists Society Lipid Library- On-line review articles in Plant Biochemistry. <http://lipidlibrary.aocs.org/plantbio/nape/index.htm>.
2. **Blancaflor EB, Kilaru A, Keereetaweep J, Khan BR, Faure L, Chapman KD** (2014) *N*-Acylethanolamines: lipid metabolites with functions in the regulation of plant growth and development. *Plant Journal*, 79(4):568-83. doi: [10.1111/tbj.12427](#). [Epub ahead of print] PMID: [24397856](#) for **Special Issue Small Molecules. Cover, August Issue.** Credits work supported by BES.

Invited book chapters/ editorials/ commentaries

1. **Kim SC, Faure L, Chapman KD** (2013) Analysis of Fatty Acid Amide Hydrolase (FAAH) Activity in Plants. In T. Munnik and I. Heilmann, eds, *Plant Signaling Protocols, Methods Molecular Biology* vol 1009:115-27. doi: [10.1007/978-1-62703-401-2_12](#). PMID: [23681529](#), Humana Press, Springer, NY. Supported by entirely by BES
2. **Wang X, Chapman KD** (2013) Lipid signaling in plants. *Frontiers in Plant Science* 27;4:216. doi: [10.3389/fpls.2013.00216](#). Introduction to Research Topic E-Book on Plant Lipid Signaling. <http://flashbook.frontiersin.org//clients/Frontiers/Lipid%20signaling%20in%20plants/EBook.html> Supported entirely by BES
3. **Kilaru A, Chapman, KD** (2012) *N*-Acylated phospholipid metabolism and seedling growth: insights from lipidomics studies in Arabidopsis. *Plant Signaling and Behavior* 7(9) 1200-2. doi: [10.4161/psb.21314](#). PMID: [22899060](#). Supported entirely by BES.

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

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

Analytical Services and Collaboration Studies: The DOE Center enables several types of collaboration and services to be offered to researchers. Scientists who request analytical services (see below) receive a written report containing a description of (i) the analytical procedures used, (ii) publishable quality results (data) of the analyses of their samples, and (iii) an authoritative interpretation of the results. CCRC personnel also provide collaborative service by becoming involved in "in depth" scientific research projects with individuals from other laboratories. One hallmark of the collaboration and services offered is the continued addition of new technologies originally developed in the CCRC research laboratories. For example, several new services have recently been offered, including glycome profiling and lignin and/or lignin/carbohydrate analysis. Over the past four years, the DOE Center Grant at CCRC has processed over 520 projects in analytical service or collaborative services to outside investigators. CCRC personnel consult with external scientists via e-mail and telephone, helping the scientists address specific analytical problems or interpretation of data. The CCRC's website (www.ccrc.uga.edu) provides freely accessible, internet-searchable databases in aspects of carbohydrate science, a scheduler for shared use of instrumentation, and descriptions of the CCRC's various research projects.

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

1. Purification and analysis of plant and microbial polysaccharides
2. Purification and characterization of plant and microbial glycoproteins
3. Molecular weight determination by SEC, MALDI-MS, or ESI-MS
4. Glycosyl composition analysis: GC-MS and HPAEC
5. Glycosyl linkage analysis
6. Determination of absolute configuration

7. Structural characterization by mass spectrometry
 - a. ESI-MS and ESI-MS/MS
 - b. MALDI-MS and MALDI-MS/MS
 - c. Online liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS/MS)
8. 1-D and 2-D NMR spectroscopy
9. Lignin analysis using Py-MBMS and Py-GC-MS
10. CarboSource Services: production and distribution of rare nucleotide-sugars, acceptors required for polysaccharide and glycoconjugate biosynthesis, and of monoclonal antibodies reactive against plant cell wall epitopes
11. Lignin and tannin analysis by Py-MBMS and Py-GC-MS
12. Glycome profile analysis

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

Training courses currently offered at the CCRC include:

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

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

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

Course 4. *Mass Spectrometry of Glycoproteins*

References

1. National Research Council. [*Transforming Glycoscience: A roadmap for the future.*](#) National Academies Press (2012).

Structure biology of membrane proteins

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Overall research goals:

The overall goal of the proposed research is to understand the membrane-associated active processes catalyzed by an alkane ω -hydroxylase (AlkB) from eubacterium *Pseudomonase oleovorans*. AlkB performs oxygenation of unactivated hydrocarbons found in crude oils. The enzymatic reaction involves energy-demanding steps in the membrane with the uses of structurally unknown metal active sites featuring a diiron [FeFe] center. At present, a critical barrier to understanding the membrane-associated reaction mechanism is the lack of structural information. The structural biology efforts have been challenged by technical difficulties commonly encountered in crystallization and structural determination of membrane proteins. The specific aims of the current budget cycle are to crystallize AlkB and initiate X-ray analysis to set the stage for structural determination. The long-term goals of our structural biology efforts are to provide an atomic description of AlkB structure, and to uncover the mechanisms of selective modification of hydrocarbons. The structural information will help elucidating how the unactivated C-H bonds of saturated hydrocarbons are oxidized to initiate biodegradation and biotransformation processes. The knowledge gained will be fundamental to biotechnological applications to biofuel transformation of non-edible oil feedstock. Renewable biodiesel is a promising energy carry that can be used to reduce fossil fuel dependency. The proposed research capitalizes on prior BES-supported efforts on over-expression and purification of AlkB to explore the inner workings of a bioenergy-relevant membrane-bound enzyme.

Significant achievements (2008-2014):

- Determine the crystal structure of a zinc transporter at 2.9 angstrom resolution
- Determine the structural dynamics of a zinc transport reaction at millisecond time resolution

Science objectives for 2014-2015:

- Purify AlkB in crystallographic quantity and quality
- Identify initial crystallization conditions

My scientific areas of expertise are: Membrane biochemistry, biophysics and structural biology

To take my project to the next level, my ideal collaborator would have expertise in: biochemistry and catalytic mechanism of bio-energy related enzymes.

Publications supported by this project 2008-2014:

Lu M, Chai J, **Fu D.**, Structural basis for autoregulation of the zinc transporter YiiP. *Nat Struct Mol Biol.* 16(10):1063-7 (2009)

Lin, W., Chai, J., Love, J., **Fu, D.** Selective electrodiffusion of zinc ions in a Zrt-, Irt-like protein, ZIPB. *J. Biol. Chem.* 285(50): 39013-20 (2010). *This paper was selected as a paper-of-the-week and featured on the journal cover.*

Fu, D. Zinc transporter YiiP from *Escherichia coli.*, book chapter in Handbook of Metalloprotein, volume 4&5 (2011) (edited by Albrecht Messerschmidt, published by Wiley-Balchwell)

Hoch E, Lin W, Chai J, Hershinkel M, ***Fu D**, *Sekler I. Histidine pairing at the metal transport site of mammalian ZnT transporter controls Zn²⁺ over Cd²⁺ selectivity. *Proc Natl Acad Sci U S A.* 8;109(19):7202-7 (2012).

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McTernan PM, Chandrayan SK, Wu CH, Vaccaro BJ, Lancaster WA, Yang Q, **Fu D**, Hura GL, Tainer JA, Adams MW. Intact Functional Fourteen-subunit Respiratory Membrane-bound [NiFe]-Hydrogenase Complex of the Hyperthermophilic Archaeon *Pyrococcus furiosus* *J Biol Chem.* 289(28):19364-19372 (2014)

Fu, D. and Finney, L., Metalloproteomics: challenges and prospective for clinical research applications. *Expert Rev Proteomics* **11** (1), 13 (2014)

Gupta, S., Chai, J., Cheng, J., D'Mello, R., *Chance, MC, ***Fu, D.**, Visualizing the kinetic power stroke that drives proton-coupled Zn(II) transport, *Nature*, (2014) in press (doi:[10.1038/nature13382](https://doi.org/10.1038/nature13382)), *Corresponding authors

Hogstrand, C. and **Fu, D.**, Chapter 22, Zinc. RSC metallobiology series No. 2: binding, transport and storage of metal ions in biological cells, edited by Maret, W, and Wedd, A., *The Royal Society of Chemistry* (2014), in press

Fluctuation Detector for Photosynthesis Efficiency

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

Significant Achievement of the 2012-2014 Period. AtRGS1 is a 7 transmembrane Regulator of G Signaling (RGS) protein that exerts GTPase activation (GAP) on its substrate protein, the G α subunit of the heterotrimeric G protein complex. This is the nexus for G protein coupling between extracellular signals to cytoplasmic targets called effectors. Understanding how AtRGS1 functions is the central goal of this project. AtRGS1 is involved in glucose sensing. Given that glucose is critical for normal growth and development, it is surprising that loss-of-function mutations in *RGS1* confer subtle phenotypes in development when grown under normal lights on-lights off growth chambers. Therefore we tested *rgs1* mutant plants under conditions that mimic the natural environment and found that the mutants are dramatically different than wild type. As shown in Figure 1, only under fluctuating light, *rgs1* mutants are dramatically less efficient at photosynthesis II.

Science Objectives for 2014-2015. Prepare full length AtRGS1 by *in vitro* translation and test for glucose binding and its ability to GAP the G α subunit.

My scientific areas of expertise are: cell biology, signal transduction.

To take my project to the next level, my ideal collaborator would have expertise in: 2-D crystallography and solution NMR.

DOE Publications Since 2012.

Colaneri, AC, Tunc-Ozdemir, M, Huang, JP, Jones, AM (2014) G protein couples saline stress and shoot growth in Arabidopsis. *BMC Plant Biology* **14**:129

Montgomery, ER, Temple, BRS, Booker, BK, Martin, JW, Smolski, WC, Rogers, SL, Jones, AM and Meigs, TE (2014) Class-distinctive residues of G α 12 necessary for Hsp90-dependent mitogenic signaling. *Molecular Pharmacology* **85**:586-597

Fu, Y, Lim, S, Urano, D, Phan, NG, Elston, TC, Jones, AM Reciprocal encoding of signal intensity and duration in the glucose-sensing circuit *Cell* **156**: 1084-1095

Xu, T., Dai, N., Nagawa, S., Chen, J., Cao, M., Zhou, Z., Li, H., Jones, AM, Patterson, S, Bleecker, AB, and Yang, Z The ABP1-TMK complex perceives auxin that activates ROP GTPase signaling pathways. *Science* **343**: 1025-1029

Urano, D, Fu, Y, Jones, AM (2013) Activation of an unusual G-protein in the simple protest *Trichomonas vaginalis*. *Cell Cycle* **12**: 19,1-2

Effendi, Y., Jones, AM, and Scherer, GFE (2013) AUXIN-BINDING-PROTEIN1 (ABP1) in phytochrome-B-controlled responses. *J. Expt Bot.* **64**(16):5065-74

- Lorek, J, Griebel, T, Jones, AM, Panstruga, R (2013) The role of Arabidopsis heterotrimeric G-protein subunits in MLO2 function and MAMP-triggered immunity. *Molecular Plant-Microbe Interactions*. **25**: 991-1003.
- Mudgil, Y. and Jones, AM (2013) NDL protein regulation of meristem initiation and shoot branching. *PLoS One*. 2013 Nov 4;8(11):e77863. doi: 10.1371/journal.pone.0077863
- Colaneri, A, Jones AM (2013) Genome-wide quantitative identification of DNA differentially methylated sites in Arabidopsis seedlings grown at different water potential. *PLoS One* **8**(4):e59878.
- Bradford, W., Buckholz, A., Morton, J., Price, C., Jones, AM, Urano D. (2013) Ancestral regulation of eukaryotic G protein signaling. *Science Signaling* **6**: ra37
- Thung, L., Chakravorty, D., Trusov, Y., Jones, AM, Botella JR (2013) Signaling specificity provided by the *Arabidopsis thaliana* heterotrimeric G-protein gamma subunits AGG1 and AGG2 is partially but not exclusively provided through transcriptional regulation. *PLoS One* **8**(3):e58503.

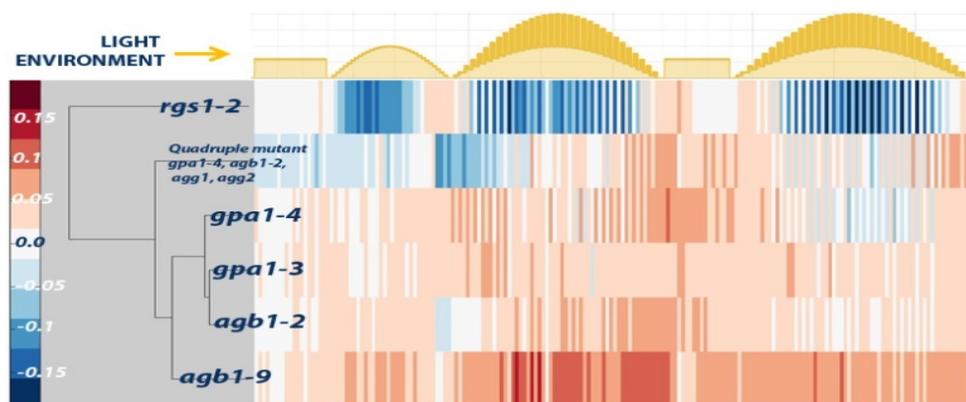


Figure 1 Seedlings lacking AtRGS1 have less efficient PSII *only* when grown in a fluctuating light environment. Heat map of the photosynthesis II efficiency difference of the indicated mutants from wildtype. Cool colors are less than

wildtype and hot colors are greater than wildtype (scale is far left). The results are clustered according to genotype based on similarities. All alleles are null. AtRGS1 (*rgs1-1* and *rgs1-2*); AtGPA1 (*gpa1-3* and *gpa1-4*); AGB1 (*agb1-2* and *agb1-9*). Also included is the quadruple mutant with null alleles of AtGPA1, AGB1, and G γ subunits AGG1 and AGG2. TOP, the light environment in which the 6 week old Arabidopsis plants are grown. The 1st environment is “lights on” in the morning then “lights off” 18 hours later. The 2nd environment is 18 hours of light that increases to midday then decreases. This mimics a cloudless day. The 3rd environment is a 18-h day increasing to midday then decreasing but with fluctuation in irradiance throughout the day. This environment mimics dynamic cloud cover. The 4th environment repeats the 1st and the last environment repeats the third. The frequency of measurements in the experiment depended on the intensity profile (flat day, 2 per hour; sinusoidal day, 4 per hour; and fluctuating day, 8 per hour) and were optimized to obtain sufficient time resolution but minimize perturbations to the physiological state of the plant by saturation pulses and dark adaptation periods required for obtaining photosynthetic parameters

Unbalancing Symbiotic Nitrogen Fixation: Can We Make Effectiveness More Effective?

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Overall research goals:

One of the most critical and least flexible uses of energy is in generating nitrogen fertilizers to increase crop growth. Ammonia fertilizer is made by reducing N_2 using the Haber-Bosch process, a process which uses about 4% of the world's natural gas production. This fertilizer is essential—without it there would only be enough fixed nitrogen entering agriculture to feed about 3 billion of the world's ~7 billion people. There are problems associated with using nitrogen fertilizers and the best chance to secure substantial and sustainable amounts of N for the future without using Haber-Bosch is likely to be greater use of symbiotic nitrogen fixation (SNF), a term that describes mutualistic interactions where nitrogen-fixing bacteria provide fixed nitrogen to their plant hosts.

Using SNF to generate nitrogen has motivated the incorporation of crops that can support SNF into agriculture for thousands of years, even though understanding the fundamental basis for yield increase is relatively recent. More progress in using SNF is likely to depend on having a deeper understanding of how SNF works—knowledge that has been elusive. For example, nitrogen fixation in free-living bacteria is organized to relieve nitrogen stress. In this case, nitrogen fixation is regulated by proteins that regulate other components of the nitrogen stress response (NSR) and is activated when, despite bacterial efforts to obtain fixed nitrogen through scavenging, the bacteria reduce nitrogen gas as an expensive last resort. The situation for bacteroids carrying out SNF in association with a legume is clearly different—they are fixing nitrogen even while they are making an excess of fixed nitrogen and exporting this to the plant. How this makes evolutionary and ecological sense must be understood to manipulate the system and increase crop productivity.

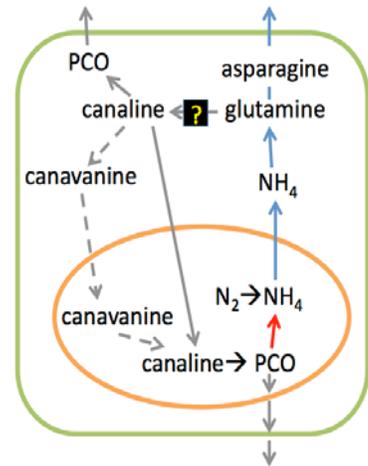
Significant achievements (2011-2014; 2014-2016):

During the current grant we are investigating unusual mutants of *Sinorhizobium meliloti*, a symbiotic partner of forage crops like alfalfa and sweet clover. These mutants fix dinitrogen at a normal rate (e.g. they are Fix⁺) but they are not effective in supporting improved plant growth on nitrogen-free media (e.g. they are Eff⁻). A few others have reported Fix⁺Eff⁻ mutants, but our mutants appear to have the tightest and most reproducible phenotype. The mutations in our strains are located in the N-terminus of *glnD*, the major NSR sensor in the bacteria and they inactivate a domain that uridylylates the PII proteins in response to nitrogen stress. Modifying PII proteins is thought to be the only mechanism by which GlnD regulates metabolism in *E. coli* but we have shown that the situation in *S. meliloti* is more complex. Both the free-living and symbiotic phenotypes of *S. meliloti* mutants that lack the PII proteins or contain a PII protein that cannot be modified are influenced by the *glnD* allele, indicating that GlnD can affect *S. meliloti* by another mechanism.

The Fix⁺Eff⁻ phenotype contains a puzzle—how is it possible for bacteroids to fix nitrogen at a normal rate without benefiting the plants? We have shown that the fixed nitrogen leaves the nodules and that nodulated plants can assimilate other nitrogen sources, like urea, so the problem is not in general nitrogen assimilation. We proposed that the bacteria were synthesizing a nitrogen-containing compound that the plant could not catabolize so this compound would not provide the plant with nitrogen. We have identified a candidate compound, pyruvate canaline oxime (PCO), which is abundant only in mutant nodules. PCO is very likely made from canaline, an ornithine analog with an aminoxy group at the end of the R moiety of the amino acid. Canaline and canavanine, its arginine analog derivative, are non-protein amino acids that are important feeding deterrents made only by a large subclass of legumes. A model that integrates canaline into nodule metabolism is shown below.

A model for canaline-coupled nitrogen flow.

After nitrogen is fixed in the bacteroids (orange oval), it is exported and assimilated via GS-GOGAT-AS into glutamine and asparagine (blue arrows) then released from plant cells (green line). In the $\Delta glnD$ -sm2 nodules it could be channeled to make the non-protein amino acids canaline and canavanine (gray arrows) by an unknown mechanism (black box). Canaline is toxic and would normally be converted to canavanine in the plant compartment. In the mutant nodules canaline may be converted to PCO or other oximes in the plant or be transferred into the bacteroids. The dashed gray line indicates that while canaline is involved, canavanine may not be. If canaline is transferred to the bacteroids (as canaline or canavanine), it is converted to PCO as a detoxification or storage mechanism. In one form of the hypothesis, PCO would be degraded to alanine and homoserine, returning the carbon and nitrogen to the bacteroid metabolic pool (red arrow) but, like degradation of most N-containing compounds, PCO is probably blocked in the $\Delta glnD$ -sm2 mutant. Significant C and N must be converted to PCO for this to have impact.



Our hypothesis is that if we can divert a limited amount of nitrogen to a compound like PCO in a way that alfalfa was not overcommitting to this synthesis, the plant would compensate for the sink by fixing more nitrogen to restore nitrogen to normal levels, increasing total nitrogen fixation by the plants. Other plants might assimilate this excess nitrogen in crop rotation or co-cultivation.

Science objectives for 2014-2015:

- Quantify the level of PCO and related compounds produced in nodules formed by *glnD* mutants and determine its fate. Understand if PCO is part of normal metabolite flow in *Medicago* SNF.
- Determine changes in nodule and plant proteomes as a result of nodulation by *glnD* mutants.
- Determine if producing non-metabolizable compounds in symbiosis stimulates nitrogen fixation

My scientific areas of expertise are: Bacterial genetics, physiology and metabolism. Biology of nitrogen fixation.

To take my project to the next level, my ideal collaborator would have expertise in: *Medicago* genetics and biochemistry. Biosynthesis of aminoxy compounds.

Publications supported by this project [Click to Enter Years of Current Grant/FWP, e.g. 2012-2014]:

1. Yurgel SN, Rice J, Kahn M. 2012. Nitrogen metabolism in *S. meliloti*-alfalfa symbiosis: Dissecting the role of GlnD and PII proteins. *Mol Plant Microbe Interact.* 25(3):355-62
2. Yurgel SN, Rice J, Kahn ML. 2013. Transcriptome Analysis of the Role of GlnD/GlnBK in Nitrogen Stress Adaptation by *Sinorhizobium meliloti* Rm1021. *PLoS One.* 2013; 8(3):e58028.
3. Yurgel SN, Mortimer MW, Rice JT, Humann JL, Kahn ML. 2013. Directed construction and analysis of a *Sinorhizobium meliloti* pSymA deletion mutant library. *Appl Environ Microbiol.* 79:2081-7.
4. Yurgel SN, Rice JT, Mulder M, Kahn ML, Belova VS, Roumiantseva ML. 2013. Truncated betB2-144 plays a critical role in *Sinorhizobium meliloti* Rm2011 osmoprotection and glycine-betaine catabolism. *European Journal of Soil Biology* 54:48-55.
5. Humann JL, Kahn ML. 2014. Genes involved in desiccation resistance of rhizobia and other bacteria. in "Biological Nitrogen Fixation" F. deBruijn, ed. John Wiley and Sons. (invited review, in press)
6. Long SR, Kahn ML, Seefeldt L, Tsay Y-F, Kopriva S. 2014 "Nitrogen and Sulfur" Chapter 16, *Biochemistry and Molecular Biology of Plants*. Ed: BB Buchanan, W Gruissem, RL Jones. Am Soc Plant Biol., Rockville MD (in press)
7. Yurgel SN, Qu Y, Rice JT, Brown JN, Lipton MS, Kahn ML. Metabolic specialization in a nitrogen-fixing symbiosis: proteome differences between *Sinorhizobium medicae* bacteria and bacteroids (in revision)
8. Yurgel SN, Rice JT, Domreis E, Lynch J, Sa N, Qamar Z, Sathish Rajamani S, Gao M, Roje S, Bauer WD. 2014 *Sinorhizobium meliloti* flavin secretion and bacteria-host interaction: Role of the bifunctional RibBA protein. *Mol Plant Microbe Interact.* 27(5):437-45. DOI: [10.1094/MPMI-11-13-0338-R](https://doi.org/10.1094/MPMI-11-13-0338-R).

Nano-to-Meso-scale Structural Understanding of Cellulose Microfibrils in Plant Cell Walls

Seong H. Kim, Principal Investigator

Daniel J. Cosgrove, Director, Center for Lignocellulose Structure and Formation (EFRC-CLSF)

Christopher Lee, Kabindra Kafle, Graduate Students

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Overall research goals: As part of the Center for Lignocellulose Structure and Formation, a DOE-funded Energy Frontiers Research Center, this project seeks a detailed understanding of nano- to- meso-scale hierarchical structuring of cellulose and other matrix polymers inside plant cell walls. With the previous EFRC funding, we have demonstrated that vibrational sum-frequency-generation (SFG) spectroscopy can selectively detect cellulose in plant cell walls without interferences from hemicellulose, pectin, and lignin. The noncentrosymmetry and phase synchronization requirements of the SFG process make this method sensitive to the crystal structure and amount of cellulose as well as the hierarchical assembly of cellulose microfibrils dispersed in amorphous matrix polymers. Using SFG and other complementary analytical techniques, this research aims to find the correlations between the cellulose-matrix interactions and the mesoscale arrangement of cellulose microfibrils in plant cell walls.

Achievements in 2011-2014:

It was found that the total intensity of SFG signals can be used for quantification of crystalline cellulose amount in plant cell walls upon construction of a calibration curve with proper reference samples. The average orientation of cellulose microfibrils in plant cell walls can be determined from the relative intensity changes of SFG peaks upon rotation of the sample with respect to the laser incidence plane. Due to the phase synchronization requirement of the SFG process, the CH₂/OH intensity ratio in the cellulose SFG spectra vary with the spatial distribution of cellulose microfibrils in the mesoscale (between nm and μm). The differences in SFG spectra of chemically-identical celluloses from various biological sources shown in Figure 1 illustrate the sensitivity of the method to cellulose microfibril ordering at the meso-scale. The cellulose microfibril assembly is closely related to the function of cell walls at different growth stage as well as environmental stresses during the cell growth. This is an important breakthrough in cellulose analysis since it will allow non-invasive spectroscopic investigation of cellulose microfibril packing in plant cell walls.

Scientific objectives in 2014 and beyond:

- Develop methodology to determine the cellulose content, crystallinity, and packing in cotton fibers at various growth stages using IR, Raman, XRD, and SFG (in collaboration with C. Haigler)
- Find how genetic mutations of cellulose and other wall polymer synthesis affect the crystallinity and meso-scale assembly of cellulose microfibrils in Arabidopsis cell walls (in collaboration with C. Anderson, D. Cosgrove, Y. Gu, and M. Tien)
- Monitor changes in cellulose microfibril assembly upon transition from primary to secondary cell walls.
- Study cellulose synthesis and crystallization by isolated membrane protein (in collaboration with T. Nixon, M. Tien, and J. Zimmer).
- Combine SFG spectroscopy with an optical microscope for non-invasive spectroscopy analysis with a μm-scale spatial resolution. Once completed, this technique will be used to study nano-to-mesoscale structures of cellulose microfibrils at the cellular level.

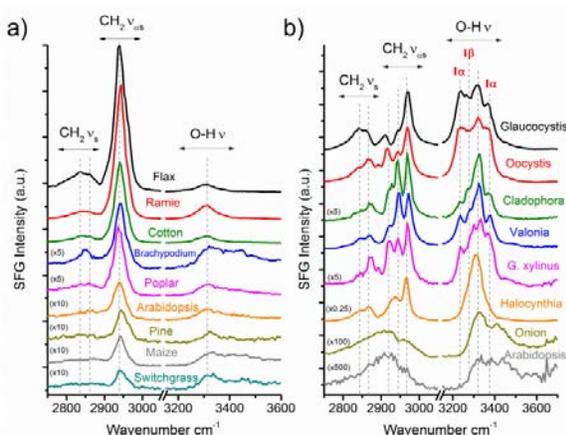


Figure 1. SFG spectra of cellulose in biological tissues. (a) Mature secondary cell walls of land plants. (b) Algal cell walls, bacterial biofilm, tunicin mantle, and primary plant cell walls.

Publications supported by this project:

1. L. Lei, T. Zhang, R. Strasser, C. M. Lee, M. Gonneau, L. Mach, S. Vernhettes, S. H. Kim, D. Cosgrove, S. Li, and Y. Gu “The jiaoyao1 mutant is an allele of korrigan1 that abolishes endoglucanase activity and affects the organization of both cellulose microfibrils and microtubules” *Plant Cell* **2014**, 26, 2601-2616.
2. K. Kafle, R. Shi, C. M. Lee, A. Mittal, Y. B. Park, Y.-H. Sun, S. Park, V. Chiang, S. H. Kim “Vibrational sum-frequency-generation (SFG) spectroscopy study of the structural assembly of cellulose microfibrils in reaction woods” *Cellulose* **2014**, 21, 2219-2231
3. Y. B. Park, C. M. Lee, K. Kafle, J. Park, S. Park, D. J. Cosgrove, and S. H. Kim “Effects of matrix wall polymers on crystalline structure of bacterial cellulose using sum frequency generation (SFG) vibration spectroscopy and x-ray diffraction” *Biomacromolecules* **2014**, 15, 2718–2724.
4. C. Lee, K. Kafle, Y.-B. Park, S. H. Kim, “Probing Crystal Structure and Mesoscale Assembly of Cellulose Microfibrils in Plant Cell Walls, Tunicate Tests, and Bacterial Films Using Vibrational Sum Frequency Generation (SFG) Spectroscopy” *Phys. Chem. Chem. Phys.* **2014**, 16 (22), 10844 - 10853.
5. K. Kafle, K. Greeson, C. M. Lee, and S. H. Kim “Detecting cellulose polymorphs in cotton fabrics processed with commercial textile mills for mercerization and liquid ammonia treatments” *Textile Res. J.* **2014**, (in press; DOI: 10.1177/0040517514527379).
6. L. Kong, C. M. Lee, S. H. Kim, and G R. Ziegler “Characterization of starch polymorphic structures using vibrational sum frequency generation (SFG) spectroscopy” *J. Phys. Chem. B*, **2014**, 118, 1775–1783.
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Ferredoxin-Dependent Plant Metabolic Pathways (DE-FG03-99ER20346)

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Overall research goals:

(1) To elucidate the detailed mechanisms of the complex, multi-electron transfer reactions catalyzed by several enzymes involved in nitrogen, carbon and sulfur assimilation, all of which use reduced ferredoxin as the physiological electron donor. (2) To characterize the complexes formed between ferredoxin and its target enzymes, by identifying the domains on the proteins that are involved in ferredoxin/enzyme complex formation and by measuring the thermodynamic parameters for complex formation.

Significant achievements:

- (1) *Nitrate Reductase*: The roles of four absolutely conserved, basic amino acids in the ferredoxin-dependent nitrate reductase from the cyanobacterium *Synechococcus* sp. PCC 7942 have been investigated by site-directed mutagenic replacement. Replacement of either Lys58 or Arg70 by glutamine results in a complete loss of activity, when either reduced ferredoxin or a non-physiological substitute, reduced methyl viologen, serves as the electron donor. The fact that K58R and R70K variants are also inactive indicates that the requirement for these two amino acids is quite specific and that simply having a positively-charged side chain does not suffice. Replacement of Lys130 by glutamine causes substantial, but not total loss of activity, while replacement by arginine at this position has less effect. Replacement of Arg146 has no significant effect on activity. Replacement of Lys58 causes large losses of both the Mo and iron-sulfur cluster prosthetic groups and makes the cluster much more susceptible to oxidative damage. With the exception of the large decrease in the affinity of ferredoxin binding exhibited by the K58Q variant, these mutagenic replacements have relatively small effects on substrate-binding affinities. An *in silico* three-dimensional model of the enzyme has been used to provide a rationalization for these results. A flash photolysis study of the kinetics of electron transfer from reduced ferredoxin to the enzyme has been carried out. In the presence of nitrate, under conditions where only a single electron is transferred to nitrate reductase, the rate of reduction of nitrate reductase shows a biphasic dependence on enzyme concentration. At low enzyme concentrations the dependence is approximately linear, with an estimated second-order rate constant of $7.4 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. At enzyme concentrations above $2 \mu\text{M}$, the rate increases in a non-linear fashion to an asymptotic value of approximately 300 s^{-1} , indicating the presence of a rate-limiting step in the process. Difference spectra suggest that the electron gained by the enzyme passes transiently through the enzyme's [4Fe-4S] cluster and subsequently reduces the enzyme's Mo center.
- (2) *Arsenate Reductase*: Cyanobacteria contain an arsenate detoxification system that involves an initial 2-electron reduction of arsenate to arsenite. The arsenate reductase from the cyanobacterium *Synechocystis* sp. PCC 6803 uses one of three glutaredoxins present in cells, glutaredoxin A, as its preferred electron donor and we have used X-ray crystallography to obtain a three-dimensional structure of the glutaredoxin A protein at 1.8 Å resolution. We have used site-directed mutagenesis to demonstrate that only one of the two cysteines at the active site of glutaredoxin A, Cys15, is required for its activity as an electron donor to arsenate reductase. We have also used site-directed mutagenesis to demonstrate that only three of the five cysteine residues present in the reductase (Cys8, Cys80 and Cys82) are essential for activity, while the other two (Cys13 and Cys35) are not. We have combined these mutagenic studies with *in silico* structural modeling and kinetic studies to provide support for a proposed mechanism and to identify a key enzyme intermediate.
- (3) *Glutamate Synthase*: A loop, 27 amino acids in length, which is in close proximity to the enzyme's FMN and [3Fe-4S] cluster prosthetic groups, is present only in ferredoxin-dependent forms of the enzyme. A variant in which the loop has been deleted retains wild-type activity with reduced

ferredoxin as the donor, suggesting that the loop is not essential for ferredoxin-dependent activity. *In silico* modeling has identified a likely ferredoxin-binding site that does not involve this loop.

- (4) *Cysteine Chemistry*: The attachment of heme to apo-cytochromes *c* requires two reductive steps: reducing a disulfide at the CXXCH heme-binding site of the protein; and reducing the Fe³⁺ of the heme to Fe²⁺. We have shown that the yeast flavoprotein, Cyc2p, catalyzes reduction of the heme iron, using NADPH as the electron donor and we characterized the redox properties of Cyc2p's flavin and of the disulfide/dithiol couple of yeast mitochondrial apo-cytochrome *c*. A fusion protein, combining peroxiredoxin (Prx) and nitroreductase (Ntr) domains, occurs naturally in the marine thermophile *Thermatoga maritima*. We have characterized the redox properties of the two domains and shown that the Prx domain can reductively detoxify hydroperoxides using reduced glutaredoxin as an electron donor. The Ntr domain is able to reduce hydroperoxides, using NAD(P)H as the electron donor. An *Arabidopsis* chloroplast protein, Nfu2, has been shown to assemble both 2-Fe and 4-Fe clusters and to be specifically involved in cluster insertion into an *Arabidopsis* APS reductase.

Science objectives for 2014-2015:

- To use site-directed mutagenesis, combined with kinetic, binding and spectroscopic measurements, to identify additional amino acids involved in substrate binding and catalysis in a ferredoxin-dependent cyanobacterial nitrate reductase.
- To use isothermal titration calorimetry to determine the enthalpies and entropies of substrate binding to ferredoxin-dependent enzymes and examine cooperativity in substrate binding.
- To characterize the ferredoxin-binding site of glutamate synthase.

My scientific area of expertise is: Biochemistry.

To take my project to the next level, my ideal collaborator would have expertise in: Enzyme kinetics; EPR spectroscopy; and protein structure determination by either X-ray crystallography or NMR spectroscopy.

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1. S. G. Kim, J.-S. Chung, R. B. Sutton, J.-S. Lee, L. López-Maury, S. Y. Lee, F. J. Florencio, T. Lin, M. Zabet-Moghaddam, M. J. Wood, K. Nayak, V. Madem, J. N. Tripathy, S.-K. Kim, and D. B. Knaff, "Redox, mutagenic and structural studies of the glutaredoxin/arsenate reductase couple from the cyanobacterium *Synechocystis* sp. PCC 6803". *Biochim. Biophys. Acta* **1824**: 392-403 (2012).
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Technical Advances in Mass Spectrometric Imaging of Plant Metabolites

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Overall research goals: The overarching goal of our research is to understand plant metabolic biology at the level of a single cell, and eventually at the subcellular and possibly the sub-organelle levels using mass spectrometric (MS) imaging techniques. Such information provides unprecedented details about the distribution of metabolites from cell to cell, and enables the visualization of cooperative and antagonistic effects among the metabolites that are programmed by the genetics of the organisms and modified by environmental influences. These details will ultimately lead to a predictive understanding of the mechanisms that multicellular organisms use to regulate metabolic processes.

Significant achievements in 2012-2014: As the technology matures, a critical limitation of MS imaging for practical applications is identifying compounds directly on biological tissues. We have previously developed 'multiplex MS imaging' to acquire both high-resolution MS and MS/MS in a single data acquisition and demonstrated this capability for flavonoids of Arabidopsis flowers. Recently, we have advanced this methodology to include polarity switching, so that we can visualize both positive and negative ions with confident molecular identity (Figure 1).

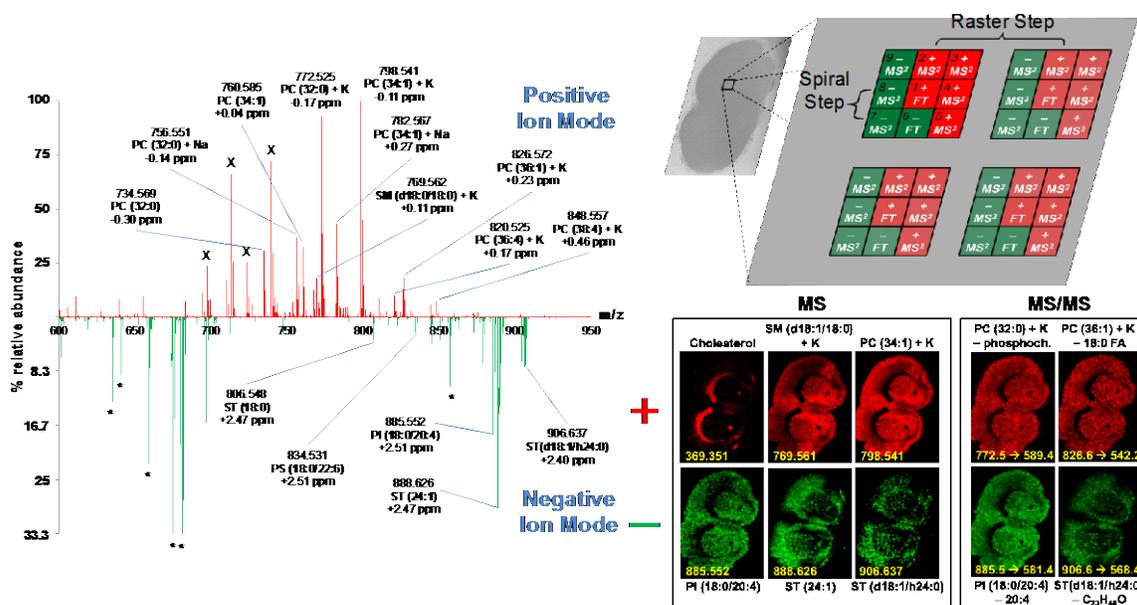


Figure 1. Multiplex MS Imaging with polarity switching. (Left) MALDI-MS spectra in positive and negative ion mode, demonstrating different classes of lipids detected in each polarity. (Top Right) Data acquisition scheme for multiplex MS imaging with polarity switching. Each raster step is split into nine spiral steps: Orbitrap MS (FT; #1, #6) and ion trap MS/MS (#2, 3, 4, 5 or #7, 8, 9) for each polarity. (Bottom Right) Various lipid images obtained from a mouse brain section for both positive and negative ion mode, and in high-resolution MS and MS/MS (ref 5).

Because of wide range of chemical functionalities, ionization of small metabolites has strong matrix dependence and it is challenging to visualize certain metabolites in MALDI MS imaging. To overcome this limitation, we have developed diaminonaphthalene as a new matrix for small molecules in negative ion mode and screened fifteen different nanoparticles for their ability to ionize over thirty different plant metabolites. Previously we had achieved 10 μm spatial resolution in MS imaging, and currently this has been enhanced to a resolution of 5 μm . Such high spatial resolution will enable the investigation of metabolite trafficking at the cellular and subcellular levels. We also have developed new sample

preparation methods, imprinting and fracturing, to visualize internal plant metabolites in lateral resolution and applied these methods to study chemical interfaces in plant-pathogen interactions.

Science objectives for 2014-2016:

- Study metabolite trafficking in cellular and subcellular level with 5 μm spatial resolution.
- Visualize dynamic metabolic changes occurring in corn seed germination in 2D and 3D chemical images at various germination time points.
- Understand NALDI (nanoparticle-assisted laser desorption ionization) mechanism.
- Expanding mass spectrometric imaging to metabolomics scales by incorporating multiplex MS imaging scheme, data acquisition with various matrices, and several bioinformatics tools.
- Developing and applying atmospheric pressure LDI for the imaging of plant metabolites, which will allow real time imaging on living plants and measurements of volatile metabolites.
- Evaluation of a 100 fs laser for laser desorption and ablation in 3D imaging of plant materials

My primary expertise is in: mass spectrometry.

To take my project to the next level, my ideal collaborator would have expertise in: plant metabolic biology.

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Proteolytic Regulation and Macromolecular Organization of Phenylpropanoid-Lignin Biosynthesis

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Overall research goals:

Phenylpropanoid biosynthesis in plant engenders myriad phenolics with diverse biological functions. In particular, as one of the structural components of the cell wall, lignin imparts rigidity and strength to vascular tissues, while lowering the cell wall's digestibility. The long-term goals of this project are to elucidate the molecular- and biochemical- mechanisms underlying the synthesis and deposition of lignin, and to elucidate the concurrent regulatory mechanisms of phenylpropanoid-lignin biosynthesis. Specifically, we have two objectives: 1) To clarify the post-translational regulations employed in lignin biosynthesis, and define their biological significance; and 2) Evolve monoglignol 4-*O*-methyltransferases to modify the conventional lignin precursors, or to produce novel monomeric precursors, and use them to explore the chemical- and biochemical-mechanisms of lignin polymerization and to modulate lignin content and structure.

Significant achievements (2012-2014):

- Phenylalanine ammonia-lyase (PAL) is the first enzyme in the pathway directing carbon flux into the phenylpropanoid branch. By Yeast-Two-Hybrid (Y2H) screening and pair-wise validation, we identified four Arabidopsis Kelch repeat F-Box (KFB) proteins, AtKFB01, 20, 39, and 50, that physically interact with four PAL isozymes, and mediate PALs' turnover via the ubiquitination-26S proteasome pathway. These *KFB* genes are differentially expressed in Arabidopsis tissues and respond in different ways to developmental cues and environmental stresses, such as a high concentration of carbon and intense UV-radiation. The change of *KFB* gene expression reciprocally affects the stability of PAL enzymes and the production and accumulation of phenylpropanoids. Our data suggest that KFB-mediated ubiquitination and degradation of PALs act as a post-translational regulation mechanism, negatively controlling phenylpropanoid metabolism.
- Using Co-Immunoprecipitation coupled with LC-MS analysis, and the mating-based split ubiquitin Y2H system, we also explored potential enzyme complexes associated with the membrane-bound P450 enzymes involved in lignin biosynthesis. In contrast to recent reports, our study does not support the direct physical interaction of C4H and C3H, the two hydroxylases for monoglignol synthesis; instead, we found that two membrane-bound enzymes localize on the ER in close proximity, and strongly interact with a common scaffold protein.

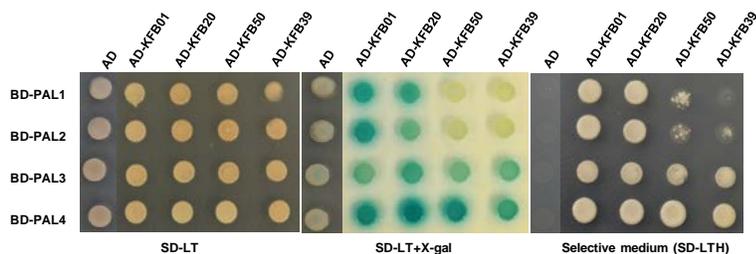


Fig.1 Kelch repeat domain-containing F-Box proteins, the KFB01, 20, 39, and 50, interact with Arabidopsis four PAL isozymes, thereby mediating PALs' ubiquitination and turnover.

- Previously, we evolved a set of monoglignol 4-*O*-methyltransferases (M4OMTs) via structure-based iterative-saturation mutagenesis. To further explore their effects on plant lignification, we expressed a M4OMT variant in poplar, which results in a moderate (~16%) reduction of total lignin content, but more than a 50% decrease in the thioacidolytically cleaved S-lignin monomers and the considerable increase in condensed cross-linkages (e.g., β -5), pointing to a substantial alteration in lignin

composition and structure in the transgenic poplar plant. Nevertheless, the saccharification efficiency of wood biomass of the transgenic poplar increased by up to 62%, compared to control plants. These data suggest that condensation of lignin is not necessarily correlated with the digestibility of wood biomass in poplar, and that M4OMT-mediated alteration of lignin composition might globally affect the cell wall's structure and the interactions between the cell wall's polymers.

Science objectives for 2014-2015:

- The KFB proteins we identified are regulated with sugar signals or the availability of carbon source. We further will explore the regulatory- and coordinating- roles of KFBs in balancing the distribution of carbon flux into cell-wall lignin and other biopolymers.
- We will continue to explore the biochemical and biological functions of the scaffold protein and its organized P450 enzyme complexes in hydroxylation reactions and in lignin synthesis
- We will adopt biophysical and spectroscopic approaches to explore how alteration of the composition of lignin affects cell wall's ultrastructural and biomechanical properties in the M4OMT transgenic poplar that we generated.

My scientific areas of expertise are: Cell wall biogenesis and modification; Plant secondary metabolism; (Plant Biochemistry; Protein structure and engineering).

To take my project to the next level, my ideal collaborator would have the following expertise: Elucidation of cell-wall structure; NMR spectroscopy for establishing the composition of cell walls.

Publications supported by this project (2012-2014):

1. Zhang K, Bhuiya MW, Pazo JR, Miao Y, Kim H, Ralph J, and **Liu C-J** (2012). An engineered monoglignol 4-*O*-methyltransferase (MOMT4) represses lignin polymerization and confers novel metabolic capability in Arabidopsis. *Plant Cell* 24 (7), 3135-52, doi/10.1105/tpc.112.101287.
2. Gou J-Y, Miller LA, Hou G, Yu X-H, Chen X-Y, **Liu C-J** (2012) Acetyltransferase-mediated deacetylation of pectin impairs cell elongation, pollen germination, and plant reproduction. *Plant Cell* 24: 50-65
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9. Zhang X, **Liu C-J** (2014) Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. *Mol Plant* (accepted)
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Molecular Dissection of the Arabidopsis 26S Proteasome

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Overall research goals:

Our project is directed toward continued genetic and biochemical characterizations of the plant 26S proteasome, using the *Arabidopsis* complex as a model. This 2.5-MDa particle is the main protease in the ubiquitin system where it plays a central role in controlling the abundance of many important cell regulators, and in general housekeeping and recycling by removing aberrant and unwanted proteins. During this project, we aim to: (i) define the functions of many of its core regulatory subunits, (ii) identify various accessory proteins that aid its assembly and regulate its substrate specificity, (iii) study alternative proteasomes with different subunit isoforms that may have unique activities, (iv) confirm our hypothesis that the 26S proteasome is recycled by autophagy, and (v) define the transcriptional regulon that co-ordinately adjusts proteasome synthesis. Ultimately, we hope to devise strategies to manipulate 26S proteasome functions/abundance when its activity limits agricultural yield, growth of crops under adverse environments, and/or the production of renewable biofuels.

Significant achievements (2010-2015):

The *Arabidopsis* 26S proteasome complex is highly dynamic, caused in part by the use of paired genes to encode many subunits, the binding of an array of cofactors, and the presence of various post-translational modifications, including acetylation, phosphorylation and ubiquitylation. Mass spectrometric analyses of 26S proteasomes affinity purified via tagged core protease (CP) and regulatory particle (RP) subunits revealed substantial modification with ubiquitin but no significant bias in the assembly of different isoforms, suggestion random incorporation of isoforms into the particle. Orthologs of the suite of yeast chaperons that help 26S proteasome assembly were also detected. As in yeast, the CP-dedicated chaperones PBAC1-3 and UMP1 and the alternative cap PA200 were found associated with the CP, and the RP-dedicated chaperones ECM29, HSM3, NAS2 and NAS6 were found associated with the RP. We also detected a plant-specific protein that strongly associates with the *Arabidopsis* CP and might represent an additional chaperone for assembling plant CP sub-particles. This protein binds PBAC1 by Y2H and BiFC *in planta* and co-purifies with the CP, possibly via a C-terminal HbYX motif known to help dock the RP to the CP α -subunit ring.

Transcriptomic analyses of *Arabidopsis* seedlings compromised in 26S proteasome activity either genetically or through inhibitors uncovered a large transcriptional regulon that up-regulates the expression of most proteasome-associated genes and other stress-related loci under growth conditions when proteasome demand is high. This up-regulation is at least partially controlled by a pair of NAC family transcription factors that bind to a *cis*-acting DNA element common to many members of the regulon. Following assembly of the 26S proteasome, we also discovered its abundance is strongly regulated by turnover through the autophagy system, which involves the vesicular transport of particles to the vacuole for breakdown. Such turnover can occur both non-selectively e.g. upon nutrient starvation, or selectively upon chemical or genetic inhibition. Selective proteasome turnover might involve the RP subunit RPN10, which is one of the main ubiquitin receptors within the core 26S proteasome. As well as harboring motifs that bind ubiquitin and ubiquitin-related domains, the C-terminal half of *Arabidopsis* RPN10 also contains an ATG8-interacting motif that strongly associates with the autophagic cargo receptor ATG8, as detected by Y2H, BiFC and *in vitro* binding assays. As this autophagic turnover is stimulated by proteasome inhibitors, we propose that it may be a major route for clearing damaged 26S proteasome complexes. How RPN10 delivers proteasomes to the expanding autophagic vesicles is unclear, but this could follow ubiquitylation of compromised particles.

Science objectives for 2014-2015:

- Continued MS Analysis of the 26S Proteasome.
- Analyse Potential Variations in Core 26S Proteasome Composition
- Reverse Genetic Analysis of the RP.
- Analysis of PIPs as Possible Proteasome Assembly Chaperones
- Defining the Link between 26S Proteasome Turnover and Autophagy.
- Defining the *Cis* and *Trans*-acting Elements in the Proteasome Stress Regulon.

My scientific areas of expertise are: Plant Genetics, Biochemistry, and Structural Biology.

To take my project to the next level, my ideal collaborator would have expertise in: Electron Tomography, Systems Biology and Network Analysis.

Publications supported by this project 2010-2015:

1. **Marshall, R. S., Lee, K. H., Kim, D. Y., and Vierstra, R. D.** (2015). The regulatory particle AAA-ATPase subunit RPT4 regulates ABA signaling by promoting the stability of the positive effector ABI5. *Plant Cell*. In preparation.
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6. **Book, A. J., Gladman, N. P., Lee, S. S., Scalf, M., Smith, L. M., and Vierstra, R. D.** (2010). Affinity purification of the *Arabidopsis* 26S proteasome reveals a diverse array of plant proteolytic complexes. *J. Biol. Chem.* **285**, 25554-25569.

Bioenergetic Aspects of Syntrophic Fatty and Aromatic Acid Metabolism

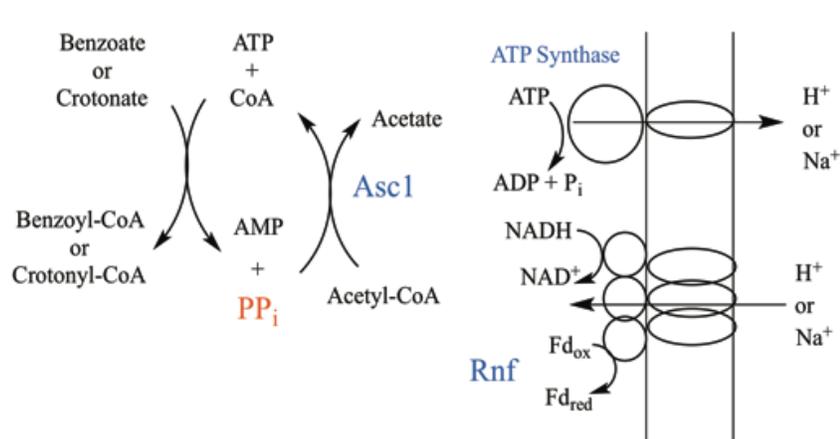
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Overall research goals:

Syntrophy is essential in the global cycling of organic matter to methane and carbon dioxide. A distinctive feature of syntrophy is the need for reverse electron transfer. The objectives of my project are: (1) to detect the membrane complexes involved in reverse electron transfer, (2) to conduct gene expression and operon analyses to determine if key gene systems are induced under growth conditions that require reverse electron transfer, (3) to determine the functions of a butyrate-induced, membrane complex in *Syntrophomonas wolfei* and a NADH:ferricyanide oxidoreductase activity in *Syntrophus aciditrophicus*, and (4) to determine if *S. wolfei* and *S. aciditrophicus* have electron-bifurcating hydrogenases and formate dehydrogenases that could be used for reverse electron transfer of electrons from NADH to H₂ or formate.



Significant achievements:

Figure 1. Pyrophosphate cycling in *S. aciditrophicus*. Pyrophosphate made during substrate activation is used by Acs1 to make ATP. Rnf uses an ion gradient to make reduced ferredoxin from NADH.

We found that *S. aciditrophicus* uses a novel mechanism for ATP from acetyl-CoA. This specialized

bacterium uses pyrophosphate, an important prebiotic energy source, and the AMP-forming, acetyl-CoA synthetase (Acs1) to produce ATP. *S. aciditrophicus* uses AMP-forming, acyl-CoA synthetases to activate benzoate, cyclohexane-1-carboxylate, and crotonate to their respective coenzyme A (CoA) derivatives (Fig. 1). The pyrophosphate formed during substrate activation can then be used by Acs1 to produce ATP, indicating the importance of pyrophosphate cycling in *S. aciditrophicus*.

We used a combination of genomic, transcriptional and enzymatic analyses to determine the mechanism of interspecies electron transfer by two model syntrophic microorganisms, *S. wolfei* and *S. aciditrophicus*. Butyrate metabolism and CH₄ production by washed cell suspensions of *S. wolfei* and *Methanospirillum hungatei* were inhibited by hydrogenase inhibitors (cyanide and carbon monoxide), but not by a formate dehydrogenase inhibitor (hypophosphite). Syntrophic benzoate oxidation and CH₄ production by washed cell suspensions of *S. aciditrophicus* and *M. hungatei* were inhibited by hypophosphite, but not cyanide and carbon monoxide. All three inhibitors halted syntrophic cyclohexane-1-carboxylate metabolism. Thus, these syntrophic microorganisms have flexible metabolisms that allow them to use either H₂ or formate transfer depending on the substrate involved.

We delineated the major conduit for electron flow from butyryl-CoA to hydrogen and formate in *S. wolfei*, which involves a membrane-bound FeS oxidoreductase and a hydrogenase (Hyd2). In *S.*

aciditrophicus, we showed that Rnf catalyzes the energetically unfavorable reduction of viologen dyes with NADH (Fig. 1). Quantitative real time-polymerase chain reaction and proteomic studies provided strong evidence for the involvement of these enzyme systems in syntrophic metabolism.

Science objectives for 2014-2015:

- Determine whether the production of cyclohexane-1-carboxylate by *S. aciditrophicus* is coupled to ATP formation by an AMP-forming, cyclohexane-1-carboxyl-CoA synthetase.
- Determine whether other anaerobes that lack acetate kinase or phosphate phosphotransacetylase use AMP-forming, acetyl-CoA synthetases to synthesize ATP from acetyl-CoA, AMP and pyrophosphate.

My scientific areas of expertise are: Microbial physiology and enzyme analyses.

To take my project to the next level, my ideal collaborator would have expertise in: Protein chemistry/structural biology, especially with expertise in membrane protein complexes.

Publications supported by this project (2012-2014):

1. J. R. Sieber, M. J. McInerney, and R. P. Gunsalus. 2012. Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. *Ann. Rev. Microbiol.* 66:429–452.
2. E. Hotze, H. Le, J. Sieber, C. Bruxvoort, M. J. McInerney, and R. Tweten. 2012. Identification and characterization of the first cholesterol-dependent cytolysins from Gram-negative bacteria. *Infection and Immunity* 81: 216–225.
3. M. Kim, H. Le, M. J. McInerney, and W. Buckel. 2013. Identification and characterization of *Re*-citrate synthase in *Syntrophus aciditrophicus*. *J. Bacteriol.* 195:1689-1696.
4. J. R. Sieber, H. M. Le and M. J. McInerney. 2014. The importance of hydrogen and formate transfer for syntrophic fatty, aromatic and alicyclic metabolism. *Environ. Microbiol.* 16:177-188.
5. K. L. James, L. A. Ríos-Hernández, N. Q. Wofford H. Mouttaki, J. R. Sieber, C. S. Sheik, Y. Yang, Y. Xie, L. Rohlin, E. A. Karr, J. A. Loo, R. R. O. Loo, G. B. Hurst, R. P. Gunsalus, and M. J. McInerney. A new twist on ATP formation in bacteria: pyrophosphate-dependent ATP synthesis. In preparation.
6. J. R. Sieber, B. R. Crable, C. S. Sheik, G. B. Hurst, L. Rohlin, R. P. Gunsalus and M. J. McInerney. Proteomic analysis reveals metabolic and regulatory systems involved the syntrophic and axenic lifestyle of *Syntrophomonas wolfei*. Preparing a MBio submission. In preparation.
7. B. R. Crable, J. R. Sieber, H. M. Le, C. Sheik, A. Lykidis, A. L. Lapidus, E. Goltsman, E. Kim, L. Rohlin, G. Hurst, D. E. Culley, R. P. Gunsalus, M. J. McInerney. The genome and proteome of *Methanospirillum hungatei*: new insights into syntrophic metabolism and biological methane production. *Frontiers of Microbiology* submission. In preparation.

Conformational and Chemical Dynamics for Single Proteins in Solution by Suppression of Brownian Motion

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Overall research goals:

The primary objective of this research is to study and understand the behavior of individual photosynthetic proteins and redox electron-transfer enzymes in a physiologically relevant solution environment. Single-molecule measurements have been firmly established as a cutting-edge technique for elucidating mechanistic details of numerous biological processes. These measurements are most powerful when they are applied over a long enough time to watch a process undergo multiple, statistically meaningful state transitions. At the same time, because such measurements should not be perturbed by surface attachment or encapsulation, we make use of the ABEL trap (Anti-Brownian ELectrokinetic trap) to suppress the usual Brownian motion of the single biomolecule. By measuring multiple observables for each single molecule, such as emission brightness, excited state lifetime, emission spectrum, emission anisotropy, we aim to infer mechanisms for observed photodynamics of both antenna proteins and energy-related enzymes. Using the newest incarnation of the trap which senses the diffusion coefficient and electrokinetic mobility of the trapped molecule, we will infer the size and charge state of the biomolecule which enables assessment of protein-protein interactions and oligomerization state.

Significant achievements (2012-2014):

- **Single-molecule spectroscopy reveals LH2 complexes switch between emissive states:** Photosynthetic organisms flourish under low light intensities by converting photoenergy to chemical energy with near unity quantum efficiency and under high light intensities by safely dissipating excess photoenergy and deleterious photoproducts. The molecular mechanisms balancing these two functions remain incompletely described. One critical barrier to characterizing the mechanisms responsible for these processes is that they occur within proteins whose excited-state properties vary drastically between individual proteins and even within a single protein over time. In ensemble measurements, these excited-state properties appear only as the average value. To overcome this averaging, we have investigated the purple bacterial antenna protein, light harvesting complex 2 (LH2) from *Rhodospseudomonas acidophila* at the single-protein level. We utilize a novel single-molecule technique, the Anti-Brownian ELectrokinetic trap, to study LH2 in a solution-phase (non-perturbative) environment. By performing the first simultaneous measurements of fluorescence intensity, lifetime and spectra of single LH2 complexes, we identify three distinct states, and observe transitions occurring between them on a timescale of seconds. Our results reveal LH2 complexes undergo photoactivated switching to a quenched state, likely by a conformational change, and thermally revert to the ground state. This is a previously unknown, reversible quenching pathway, and is one mechanism through which photosynthetic organisms are able to adapt to changes in light intensities.
- **Monitoring biomolecular interactions in the ABEL trap by time-dependent single-molecule transport coefficients:** Most single-molecule studies infer the behavior of the system from changes in the emission properties of a fluorescent label, such as brightness, excited state lifetime, or spectrum. At the same time, biomolecular interactions are generally accompanied by modifications in the size and charge of biomolecules at the nanometer scale, but these do not in general lead to changes in the emission of a fluorescent label. We have developed a new and advanced ABEL trap method to sense these changes in real time based on statistical learning of diffusive and electric field-induced motion parameters of a trapped molecule in solution. Essentially, the analysis system measures the residual motion of the trapped single molecule, which

contains information about its diffusion and its response to the trapping fields. In this way, new single-molecule variables are made available, which provide new ways to distinguish different molecules in solution, as well as to observe their dynamical changes. We have demonstrated the approach by resolving a monomer-trimer mixture for allophycocyanin along a protein dissociation pathway and by visualizing the binding-unbinding kinetics of a single DNA molecule.

Science objectives for 2014-2015:

- We will explore the excited states of light-harvesting complex II (LHCII), the primary antenna from green plants (in collaboration with Rienk van Grondelle and Roberta Croce). The pathways by which plants dissipate excess energy, known as non-photochemical quenching (NPQ), are critical to prevent the generation of deleterious photoproducts, but the mechanisms of these pathways remain notoriously mysterious. To determine the contribution of LHCII to these processes, we will characterize and identify new quenched states of individual LHCII complexes under NPQ conditions.
- We will merge our previous work on trimeric allophycocyanin (APC) with studies of the state transitions in monomeric APC, and by measuring brightness, lifetime, anisotropy, and spectrum we will develop a model for the light-driven transformations.
- We will determine the feasibility of measuring electron transfer dynamics in single redox enzymes undergoing electron flow, specifically Fet3p, a multi-copper oxidase capable of dioxygen reduction (in collaboration with Dan Kosman). The complex catalytic cycle of Fet3p makes it a fascinating target for our ability to construct of a microscopic picture of electron transfer.
- We will apply the dramatic new capability of the ABEL trap, direct measurement of transport properties of the trapped particle in real-time which sense molecular size and charge. This will allow protein-protein interactions, aggregation, and oligomerization to be explored.
- We will complete ABEL trap studies of the crucial energy-processing enzyme, F₀F₁ ATPase via single-molecule FRET (in collaboration with M. Boersch, Univ. Jena).

My scientific areas of expertise are: physical chemistry and chemical physics of single molecules, single-molecule biophysics, photodynamics of photosynthetic protein and redox enzymes, super-resolution imaging and tracking in cells, and trapping of single molecules in solution.

To take my project to the next level, my ideal collaborator would have expertise in: expression and purification of photosynthetic antenna proteins, biochemistry of oligomeric protein complexes, site-specific mutation and fluorescent reporter labeling of energy-related enzymes.

Publications supported by this project 2012-2014:

1. Quan Wang and W. E. Moerner, "Lifetime and spectrally resolved characterization of the photodynamics of single fluorophores in solution using the Anti-Brownian Electrokinetic trap," *J. Phys. Chem. B* **117**, 4641-4648 (2013).
2. G.S. Schlau-Cohen, Q. Wang, J. Southall, R.J. Cogdell, W.E. Moerner, "Single-molecule spectroscopy reveals LH2 complexes switch between emissive states," *Proc. Nat. Acad. Sci. (USA)* **110**, 10899-10903 (2013).
3. Samuel D. Bockenhauer, Thomas M. Duncan, W. E. Moerner and Michael Börsch, "The regulatory switch of F₁-ATPase studied by single-molecule FRET in the ABEL Trap," *Proc. SPIE* **8950**, 89500H 1-14 (2014).
4. Quan Wang and W. E. Moerner, "Spectroscopic and transport measurements of single molecules in solution using an electrokinetic trap," *Proc. SPIE* **8950**, 895004 1-10 (2014).
5. Quan Wang and W. E. Moerner, "Single-molecule motions enable direct visualization of biomolecular interactions in solution," *Nature Methods* **11**, 555-558 (2014).
6. Gabriela S. Schlau-Cohen, Samuel Bockenhauer, Quan Wang, and W. E. Moerner, "Single-molecule spectroscopy of photosynthetic proteins in solution: exploration of structure-function relationships," Minireview, *Chem. Sci.* **5**, 2933-2939 (2014).

Two-Dimensional Electronic Spectroscopies for Probing Coherence and Charge Separation in Photosystem II

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Overall research goals:

Photosystem II (PSII) is the only known natural enzyme that uses solar energy to split water, making the elucidation of its design principles critical for our fundamental understanding of photosynthesis and for our ability to mimic PSII's remarkable properties. This project focuses on key deficits in our current understanding of the PSII reaction center (PSII RC). The project involves the development of new spectroscopic methods, and their application to address the following open questions:

- 1) What is the electronic structure of the PSII RC?
- 2) What are the charge separation pathways in the PSII RC?
- 3) Does coherence facilitate charge separation in the PSII RC?

The proposed experiments build on our previous work in which we developed simple and high signal-to-noise approaches to two-dimensional electronic spectroscopy (2DES) that enable a direct view of electronic couplings and energy transfer and charge separation processes over a broad range of frequencies with ultrafast time resolution.

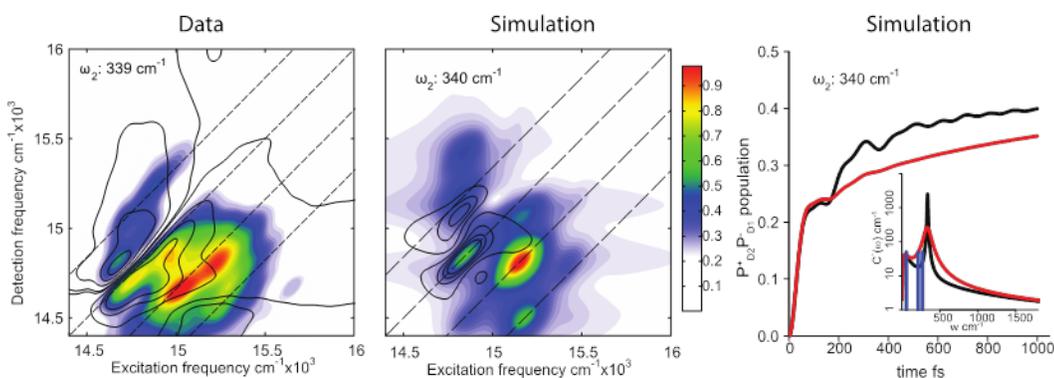


Figure 1: Left: Coherence amplitude map (filled contours) derived from the real rephasing 2D spectra at $\omega_2 = 339 \text{ cm}^{-1}$. These maps show the distribution of observed coherences throughout the entire 2D spectrum. Middle: Simulated coherence amplitude maps (filled contours) derived from the simulated real rephasing 2D spectra for the special pair dimer model with a 339 cm^{-1} mode, showing good agreement between theory and experiment. The dashed black lines indicate the diagonal and parallel lines offset from the diagonal by $\pm\omega_2$ and $-\omega_2$. Overlaid open contours show the real rephasing 2D spectrum, averaged over waiting time t_2 . Right: Simulated population of the charge transfer state for coherent and incoherent cases of the 340 cm^{-1} vibrational mode. The bath spectral densities for the coherent (black line) and incoherent (red line) mode are shown in the inset. Blue lines mark optical excitonic splittings.

Significant achievements (2014):

We have developed a novel implementation of 2DES with improved sensitivity. We published this in a methods paper (1), and applied the new approach to studies of the PSII RC. These studies clearly revealed the presence of coherent dynamics. There is currently considerable debate in the field about the physical origin of coherent dynamics and their potential importance for photosynthetic function. Supported by simulation work and extensive characterization of the coherences we believe that they have mixed electronic and vibrational character and that they are functionally important for charge separation. To assign the origin of the experimentally observed coherences and determine their influence on charge separation, we performed theoretical simulations of the dimeric special pair of the PSII RC including explicit coupling to discrete vibrations, obtaining good agreement with the

experimental data, as shown in Figure 1. Further simulations using an exact hierarchical equations of motion (HEOM) method demonstrated that resonant vibrations speed up the initial step of charge separation in the PSII RC. This is shown in Figure 1, where we consider the effect of one of the observed vibronic coherences on the charge transfer (CT) state population evolution. Here we consider the 339 cm^{-1} vibronic coherence and compare the *coherent* (weakly damped) and *incoherent* (strongly damped) cases. These cases correspond to different protein spectral densities as shown in the inset in Figure 1. We see that the coherent 339 cm^{-1} vibration dramatically increases the growth of the CT state population compared to the damped case. With the exception of the 251 cm^{-1} vibration, the other vibrational frequencies affect the CT state population weakly. The origin of the CT enhancement effect is related to the optical excitonic splittings: when the vibrational frequency is close to excitonic resonance, the vibrational coherence effectively drives the charge separation. Such vibrational/excitonic resonance also results in the speed-up of energy relaxation, i.e., exciton transport or charge separation. *This may represent an important design principle for enabling the high quantum efficiency of charge separation in oxygenic photosynthesis.* In the next funding period we aim to further examine both the physical origin of the observed coherences and their importance for charge separation. We recently published this work in Nature Chemistry (2).

In the past year we have also obtained our first data on the PSII RC where we have combined Stark spectroscopy with time-resolved transient-grating spectroscopy. We have seen that we can clearly resolve charge transfer processes with this method. In addition we have recently studied PSII cores (RC with CP43 and CP47) by 2DES.

Science objectives for 2014-2015:

- Having obtained transient-grating Stark spectroscopy data we will extend the method to also perform 2DES combined with Stark spectroscopy on the PSII RC. This data will allow us to better test models of the charge separation pathways in wild-type and mutant PSII RCs.
- With the data that we have collected on PSII cores we are testing existing exciton models, aiming to understand the energy landscape and energy transfer pathways in this system.
- We plan further 2DES studies to probe the importance of vibronic coherence to charge separation in the PSII RC and in PSII cores.

My scientific areas of expertise are: Nonlinear spectroscopy and microscopy, photosynthetic systems

To take my project to the next level, my ideal collaborator would have expertise in: Electronic structure calculations, simulations of spectroscopic signals.

Publications supported by this project (2014 only):

1. Fuller FD, Wilcox DE, Ogilvie JP. 2014. Pulse shaping based two-dimensional electronic spectroscopy in a background free geometry. *Optics Express* 22:1018-27
2. Fuller FD, Pan J, Butkus V, Gelzinis A, Senlik S, et al. 2014. Vibronic coherence in oxygenic photosynthesis. *Nature Chemistry* 6:706-11

Quantitative Analysis of Central Metabolism and Seed Storage Synthesis

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Overall research goals:

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

Significant achievements (2012-2014):

- The large scale metabolic network of *Brassica napus* developing seeds (*bna572*) was extended by seed additional relevant metabolic pathways and adapted to comply with modeling and annotation standards and for full compatibility with most powerful and widely used modeling software (COBRA).
- The photosynthetic performance of *Brassica napus* embryos growing *in planta* was modeled by use of our stoichiometric model (*bna572*) and integrating photosynthetic measurements and *in-planta* growth kinetics.
- We analyzed multiple *Brassica napus* transgenics with modifications in central carbon metabolism. Transgenics and wild types were compared by parallel analysis of metabolic flux, metabolite- and transcript levels.
- We analyzed 9 *Brassica napus* varieties (genotypes) with differences in composition of seed biomass (seed oil content). Characterization of *in-vitro* grown embryos by ^{13}C metabolic flux analysis, metabolite analysis, transcriptomics and proteomics.

Science objectives for 2014-2015:

- Analysis of new transgenics in *Brassica napus* with modifications in central carbon metabolism.
- Expression in Pennycress of bifunctional fusion of enzymes that catalyze sequential biochemical conversions.
- Adapt large scale metabolic model *bna572* to pennycress.

My scientific areas of expertise are: Plant primary metabolism, ^{13}C -metabolic flux analysis, genome-scale metabolic models.

To take my project to the next level, my ideal collaborator would have expertise in: I could use a collaborator experienced in techniques and strategies used in genetic transformation of multiple genes in plants (gene stacking).

Publications supported by this project :

1. J. Fan, C. Yan, C. Andre, J. Shanklin, J. Schwender, C.Xu, "Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in *Chlamydomonas reinhardtii*". *Plant Cell Physiol.* 53:1380 (2012).
2. O'Grady, J., Schwender, J., Shachar-Hill, Y., and Morgan, J. A. Metabolic cartography: Experimental quantification of metabolic fluxes from isotopic labeling studies. *J. Exptl. Botany* 63(6), 2293-308 (2012).
3. Lohr M., Schwender J., and Polle J.E.W. Isoprenoid biosynthesis in eukaryotic phototrophs: A spotlight on algae (Review). *Plant Science*, 185-186:9-22 (2012).
4. Schwender J. and Hay J.O. Predictive modeling of biomass component tradeoffs in *Brassica napus* developing oilseeds based on in silico manipulation of storage metabolism. *Plant Physiol.* 160(3),1218-36 (2012).
5. Borisjuk L, Neuberger T, Schwender J, Heinzl N, Sunderhaus S, Fuchs J, Hay JO, Tschiersch H, Braun HP, Denolf P, Lambert B, Jakob PM, Rolletschek H (2013) Seed Architecture Shapes Embryo Metabolism in Oilseed Rape. *The Plant Cell* 25: 1625-1640
6. Yan Y, Candrea J, Shi H, Ernst E, Martienssen R, Schwender J, Shanklin J (2013) Survey of the total fatty acid and triacylglycerol composition and content of 30 duckweed species and cloning of a Delta6-desaturase responsible for the production of gamma-linolenic and stearidonic acids in *Lemna gibba*. *BMC Plant Biol* 13: 201
7. Lou Y, Schwender J, Shanklin J (2014) FAD2 and FAD3 Desaturases Form Heterodimers That Facilitate Metabolic Channeling in Vivo. *J Biol Chem.* 289:17996-18007.

Regulation of Actin Stochastic Dynamics: Single Actin Filaments as Tracks for Delivery of Materials to the Cell Wall

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Overall research goals:

Plant cell walls represent the largest renewable source of carbon for use in biofuel production. Maximizing extraction of wall-stored carbon for industrial purposes requires a detailed understanding of polysaccharide composition, biophysical properties, and assembly mechanisms. Although primary cell wall composition varies depending on plant species, developmental status, and organ or tissue type, these walls are generally comprised of cellulose, crosslinking glycans, and pectin. It is well established that cellulose microfibrils are synthesized at the plasma membrane (PM) from rosette complexes containing the integral-membrane protein CESA. Conversely, noncellulosic polysaccharides like xyloglucans are synthesized in the cell's endomembrane system and delivered to the PM by secretory vesicle trafficking. When integrated into the wall, xyloglucans and glucoarabinoxylans form a fibrous network that coats and interconnects cellulose microfibrils via hydrogen bonds. A second gel-like matrix is comprised of pectins and other polysaccharides that are trafficked to and secreted at the PM. Understanding how, how much, and where noncellulosic polysaccharides are delivered and incorporated into walls is a major knowledge gap which currently impedes efficient use of biomass for biofuels. In plants, a cytoplasmic network of dynamic filamentous structures, the actin cytoskeleton, functions as tracks or highways for long-distance transport. Although it is widely believed that actin provides the transportation infrastructure to deliver matrix polysaccharides and biosynthetic enzymes to the PM and cell wall, there is little direct evidence to this effect.

Groundbreaking work from our lab has uncovered some of the molecular mechanisms that underpin actin filament construction and turnover. Using state-of-the-art fluorescence imaging methods, we have described the dynamic remodeling of actin filament arrays in live cells and quantified key properties of individual filament turnover. Moreover, we have established genetic control over actin filament dynamics by overexpressing and downregulating several actin-binding proteins. One such player, the heterodimeric capping protein (CP), modulates availability of filament ends and therefore controls filament length and lifetime. We predict that the single filaments serve as highways for secretory vesicle traffic; thus, when these tracks are long and long-lived, cargo delivery is most efficient. Therefore, we will test the **central hypothesis** that single actin filament lengths and lifetimes regulate exocytosis, thereby controlling the rate of deposition of matrix polysaccharides and the delivery of cell wall biosynthetic enzymes to the PM.

The **specific aims** of this proposal include:

- 1) establishing whether single actin filaments serve as tracks for trafficking of matrix polysaccharides and cell wall biosynthetic enzymes;
- 2) testing whether filament length and lifetime alter exo- and endocytosis; and

Significant achievements (2013-2014):

- Demonstrated that overexpression of *Arabidopsis* capping protein (*CP OX*) has the opposite cell expansion and cytoskeletal phenotypes compared to *cp* knockdown lines. Specifically, *CP OX* lines have reduced axial cell expansion in etiolated hypocotyls as well as less dense

cytoskeletal arrays. Changes to single filament turnover in *CP OX* lines results in actin filaments with reduced annealing frequency, shorter maximum length and decreased lifetime.

- Performed subcellular fractionation, sucrose-density gradient separations and immunocytochemistry to establish that CP is a membrane-associated protein and associates with endomembranes such as Golgi.
- Established collaborations to determine cell wall composition in *cp* mutants and showed that crystalline cellulose content was significantly reduced compared to WT, in preliminary experiments. Compared monosaccharide content in *cp* to WT and found an increase in xylose and fucose, whereas total glucose was essentially unchanged. This is consistent with an increased xylan content in *cpb-1* walls. In contrast, arabinose and galactose content decreased significantly, suggesting a reduction in pectin or arabinogalactan proteins.

Science objectives for 2014-2015:

- Develop *cp* knockdown and *CP OX* lines expressing fluorescent reporters for CesA and noncellulosic polysaccharides (e.g. CBMs).
- Analyze composition of cell walls in *cp* mutants in collaboration with the glycomics facility at CCRC.
- Determine monosaccharide content of *cp* mutant cell walls with assistance from the Carpita Lab.
- Confirm that amorphous and crystalline cellulose content are reduced in *cp* mutants and examine cytological features of the cell wall.
- Establish assays and metrics for monitoring vesicle trafficking along dynamic actin filaments. Quantify rates of exo- and endocytosis in *cp* mutant lines with established reporters.

My scientific areas of expertise are: Quantitative Cell Biology; Live-cell Imaging; Cytoskeletal Dynamics; Microscopy; Biochemistry.

To take my project to the next level, my ideal collaborator would have expertise in: The perfect collaborator, in the short term, would bring expertise in labeling and developing probes for cell wall polysaccharides and secretory vesicle components that could be used for high spatial resolution fluorescence microscopy. In the long run, a collaborator would have skills in generating computational simulations/models of cytoskeletal network dynamics.

Publications supported by this project 2013-2015: (*corresponding author)

1. Pleskot, R., Li, J., Žárský, V., Potocký, M., and **Staiger, C.J.*** (2013) Regulation of cytoskeletal dynamics by phospholipase D and phosphatidic acid. *Trends Plant Sci.* **18**, 496-504
2. Li, J., Arieti, R., and **Staiger, C.J.*** (2014). Actin filament dynamics and their role in plant cell expansion. In "Plant Cell Wall Patterning and Cell Shape", H. Fukuda, ed. John Wiley & Sons, NJ, pp. 127-162
3. Li, J., Staiger, B.H., Henty-Ridilla, J.L., Abu-Abied, M., Sadot, E., Blanchoin, L., and **Staiger, C.J.*** (2014) The availability of filament ends modulates actin stochastic dynamics in live plant cells. *Mol. Biol. Cell*, **25**, 1263-1275
4. Jiménez-López, J.C., Wang, X., Kotchoni, S.O., Huang, S., Szymanski, D.B., and **Staiger, C.J.*** (2014) Heterodimeric capping protein is a membrane-associated, actin-binding protein in *Arabidopsis*. *Plant Physiol.* revision submitted 08-14
5. Li, J., Blanchoin, L., and **Staiger, C.J.*** (2015) Signaling to actin stochastic dynamics. *Annu Rev. Plant Biol.* **66**, in press

Functional Analysis of Plant Sucrose Transporters

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Anke Reinders, Postdoctoral Research Associate

Margaret Taylor and Prapapan Lasin, Graduate Students

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Overall research goals: The main research goal is to understand the transport activity and structure/function relation of plant sucrose transporters (SUTs, also called SUCs). These are proton coupled sucrose uptake transporters in the plasma and vacuole membranes in plants. SUTs are essential for the long distance transport of carbohydrate in vascular tissue and for uptake into sink tissues such as seeds. We are studying structural basis for substrate affinity and specificity using mutagenesis, heterologous expression and electrophysiology. Sucrose transporters with modified activity are tested using complementation of Arabidopsis mutants as a model. The long-term goal is to use sucrose transporters to engineer changes in carbon partitioning.

Significant achievements 2012-2014:

- The rice OsSUT1 structure was modeled using the known structures of LacY, GlpT, and EmrD, distantly related prokaryotic transporters, as templates. This provided a revised transmembrane model and allowed us to select six conserved charged amino acid positions within transmembrane spans for mutagenesis. We found three positions that appear to be essential for transport activity [1].
- The rice OsSUT1 R188K mutant does not transport ^{14}C -sucrose but produces a H^+ leak that is blocked by sucrose. Some non-transported glucosides also block H^+ transport through the R188K mutant indicating that binding and translocation may be independent. Fluorination at the glucosyl 3 and 4 positions of α -phenyl glucoside greatly decreased transport by wild type OsSUT1 but did not affect the ability to block H^+ leak in the OsSUT1 R188K mutant. Therefore, OsSUT1 R188 appears to be essential for sucrose translocation but not for substrate interaction that blocks H^+ leak which may occur at a different binding site. Replacing the corresponding Arg in type I and type III SUTs, Arabidopsis AtSUC1(R163K) and *Lotus japonicus* LjSUT4(R169K), respectively, also resulted in loss of sucrose transport activity so the corresponding Arg in type I and III SUTs are equally important. We propose that this Arg in sucrose transporters interacts with glucosyl 3-OH and 4-OH during translocation [2].
- We developed a novel fluorescent assay for sucrose transporters. Yeast expressing type I sucrose transporters accumulate the fluorescent coumarin glucoside esculin and can be detected using several methods including fluorescence-activated cell sorting (FACS) [3].
- Gene shuffling and selection by FACS was used to identify positions in sucrose transporter proteins that contribute to substrate specificity [4]. We identified 4 amino acid changes that convert selective type II sucrose transporters to type I selectivity.
- We collaborated with Dr. Jong-Seong Jeon (Kyung Hee University, Korea) to study the activity of OsSUT2, a vacuolar sucrose transporter from rice, and the phenotype of *ossut2* mutants. The results led to the hypothesis that vacuolar storage of sucrose contributes to phloem loading capacity in rice [5]. Phenotypes of the *ossut2* mutants included higher sugar concentration in leaves and reduced growth indicating that in the mutant phloem loading was reduced.
- Completed genome sequence for the lycophyte *Selaginella*, the bryophyte *Physcomitrella*, several species of red algae and RNAseq data for several green algae enabled our analysis of the origins of higher plant SUTs. We conclude that type I SUTs, present only in eudicots, evolved from type III SUTs that are localized to the vacuole [6]. It is of particular interest for this project that monocots use a type II SUT for phloem loading while eudicots use a type I SUT and that these two types of SUTs differ strongly in substrate specificity. We demonstrated that the type II SUT from barley, HvSUT1, reverses the growth phenotype of an Arabidopsis *atsuc2* mutant [6].

Science objectives for 2014-2015:

- To make significant progress in understanding the structure/function relation of sucrose transporter, a protein crystal structure is necessary. We expressed three His-tagged sucrose transporters in yeast, all were tagged at the C-terminal end and all were functional. StSUT1 from potato had the highest activity and the highest amount 6-His-tagged protein was purified on Talon NTA. We are working to increase the yield and purity of this preparation and will test other sucrose transporters. A main objective is to demonstrate that the purified protein is functional by reconstituting it into proteoliposomes.
- To confirm the structural model of OsSUT1 [1], the membrane topology of the protein needs to be determined experimentally. We intended to use cysteine scanning accessibility mutagenesis to investigate topology. We found that two of nine Cys are required for activity; no substitutions appear to be tolerated. C216 and C220 are on an extracellular loop between TMS5 and TMS6. We are exploring other methods to study topology.
- We are testing the effect of further mutagenesis at each of the four positions identified as important for substrate specificity [4]. To begin, we replaced OsSUT1 A86 with all 19 amino acids and tested activity in yeast, all are functional and we are testing substrate specificity by expression in oocytes.
- Arabidopsis *atsuc2* mutants cannot load sucrose into the phloem and have severe growth defects. Complementation with type II monocot sucrose transporters appears to be complete but a careful study has not been done yet. We are analyzing these complemented lines for differences in phloem loading, growth and yield.

My scientific areas of expertise are: Heterologous expression in yeast and *Xenopus* oocytes, transmembrane transport assays, electrophysiology.

To take my project to the next level, my ideal collaborator would have expertise in: Membrane protein structural analysis.

References to work supported by this project 2012-2014:

1. Sun Y, Lin Z, Reinders A, Ward JM (2012) Functionally important amino acids in rice sucrose transporter OsSUT1. *Biochemistry* 51: 3284-3291.
2. Sun Y, Ward JM (2012) Arg188 in rice sucrose transporter OsSUT1 is crucial for substrate transport. *BMC Biochem* 13: 26.
3. Gora PJ, Reinders A, Ward JM (2012) A novel fluorescent assay for sucrose transporters. *Plant Methods* 8: 13.
4. Reinders A, Sun Y, Karvonen KL, Ward JM (2012) Identification of amino acids important for substrate specificity in sucrose transporters using gene shuffling. *J Biol Chem* 287 30296-30304.
5. Eom JS, Choi SB, Ward JM, Jeon JS (2012) The mechanism of phloem loading in rice (*Oryza sativa*). *Mol Cells* 33: 431-438.
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Asparagine synthetase gene regulatory networks and plant nitrogen metabolism

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Overall research goals: The goal of this DOE project is to model and alter gene regulatory networks affecting Nitrogen (N) assimilation into Asparagine (Asn), a Carbon- (C) and N- efficient amino acid used to transport and store N in seeds. Altering transcription of *ASN1*, the major gene controlling Asn synthesis in Arabidopsis, effects increases in seed-N, and this technology is in field trials of corn and other crops. Using a combined genetic, genomic and systems biology approach, we have uncovered components regulating Asn synthesis and metabolism in response to C, Light (L) and N signals. In a positive genetic selection, we uncovered the role of SDG8 - a histone methyltransferase - in the C and L repression of *ASN1* expression. Our goal is to use the SDG8 mutant to explore the epigenetic and transcriptional control of gene expression in response to nutritional and environmental stimuli, with a combination of epigenetic (Aim1), transcriptional (Aim2) and metabolic approaches (Aim3).

Significant achievements (2010-2014):

AIM 1. CHROMATIN: Role of histone methylation in the *ASN1* metabolic regulatory network.

To uncover components involved in the regulation of *ASN1*, the *ASN1* promoter was used in a positive genetic selection to identify an Arabidopsis mutant *cli186* impaired in *ASN1* repression by C and L signals. We showed by Gene-chip mapping and complementation that the *cli186* mutant (now renamed *sdg8-5*) carries a complete deletion of SDG8, a gene encoding a Histone 3 lysine 36 (H3K36) methyltransferase. We identified 728 genome-wide targets of SDG8, and probed how histone methylation by SDG8 affected regulation of these target genes. Remarkably, >50% of the 728 SDG8 targets are light-regulated genes that span energy generation (*e.g.* light harvesting and oxidative phosphorylation) and energy utilization (*e.g.* N-assimilation). The H3K36me3 marks on SDG8 target genes correlates with their elevated gene expression level, which is disrupted in the *sdg8-5* mutant. We thus propose a model in which SDG8 targets a specific subset of light and energy-related genes for permissive H3K36me3 histone marks in the gene body, to allow high-level expression of its target genes. We also tested the hypothesis that the unique zf-CW domain of SDG8 serves to “read” H3K4 methylation (at promoters), which directs SDG8 to “write” H3K36 methylation of the gene bodies.

AIM 2. TRANSCRIPTION: Role of Transcription Factors (TFs) in the *ASN1* metabolic regulatory network. We focused on testing TFs that are epigenetic targets of SDG8, *and* are predicted to regulate genes in the *ASN1* metabolic network. Our proof-of-principle for this aim is GLK1, a TF which is predicted to regulate *ASN1*, and has been previously associated with chloroplast development. Thus, GLK1 coordinates the regulation of genes involved in energy generation (photosynthesis), with energy-use (N-assimilation). We validated that *ASN1* is indeed a target of GLK1 using GLK1 transgenic lines. We also identified 196 global direct targets of GLK1 using *TARGET*, a rapid cell-based TF perturbation assay we developed. These GLK1 target genes are enriched with GO-terms “photosynthesis”, “chlorophyll binding”, and “light-harvesting complex”. This finding confirmed and extended the previously reported roles for GLK1 based on *in planta* studies.

AIM 3. METABOLITES: Role of metabolic control of transcription in the *ASN1* metabolic regulatory network. In this aim, we explored whether and how metabolic signaling is mediated via dynamic histone methylation. To do this, we assayed the global H3K36me3 profiles of plants treated with a 5hr inorganic N metabolic signal, comparing *sdg8-5* mutant to WT. Our results indicate that *sdg8-5* shows an impaired epigenetic response to the supply of N-nutrient signals. The list of affected genes in *sdg8-5* includes N-assimilation genes *ASN2* and *NIR1*, and several known N-response regulators which have reported roles in fine-tuning the N-signaling response. This suggests that dynamic methylation of histones associated with N-regulated genes are part of the mechanism of N metabolic signaling.

Science objectives for 2014-2015:

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

My scientific area of expertise is: Plant systems biology

To take my project to the next level, my ideal collaborator would have expertise in: epigenetics, energy metabolism.

Publications and patents supported by this project 2004-2014:

1. Li Y, Mukherjee I, Thum KE, Tanurdzic M, Katari MS, Obertello M, McCombie WR, Martienssen RA, Coruzzi GM (2014) Global targets of the histone methyltransferase SDG8 uncover a role for histone modification in environmental sensing and metabolism tuning. *Genome Biology* (under revision).
2. Krouk G, Ruffel S, Gutiérrez RA, Gojon A, Crawford NM, Coruzzi GM and Lacombe B. (2011). A framework integrating plant growth with hormones and nutrients. *Trends in Plant Sci.*16(4):178-182.
3. Obertello M, Krouk G, Katari M, Runko S, and Coruzzi G (2010). Modeling the global effect of the basic-leucine zipper transcription factor 1 (bZIP1) on nitrogen and light regulation in Arabidopsis *BMC Systems Biology* 4:111
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6. Coruzzi GM, Burga A, Katari MS, and Gutierrez RA (2009). Systems Biology: Principles and Applications in Plant Research. In *Plant Systems Biology*, Annual Plant Reviews; Blackwell Publishing: Oxford, UK. Vol. 35. Pgs 3-31.
7. Thum KE, Shin, MJ Gutierrez RA, Mukherjee I, Katari MS, Nero D, Shasha D, and Coruzzi GM (2008). An integrated genetic, genomic and systems approach defines gene networks regulated by interaction of light and carbon signaling pathways in Arabidopsis. *BMC Syst Biol* 2, 31.
8. Gutierrez R, Stokes T, Thum K, Xu X, Obertello M, Katari MS, Tanurdzic M, Dean A, Nero D, McClung CR, and Coruzzi GM (2008). Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene CCA1. *Proc Natl Acad Sci USA* 105, 4939-4944.
9. Gutierrez R, Shasha S, and Coruzzi G. (2005) Systems Biology for the Virtual Plant. *Plant Phys.* 138: 550-554.
10. Thum K, Shin M, Palenchar P, Kouranov A, Coruzzi G (2004) Genome-wide investigation of light & carbon signaling interactions in Arabidopsis. *Genome Biology* 5:R10.1- R10.20. (Faculty of 1000 & Review by Reyes & Chua (2004) *Genome Biology* 5:213)
11. Wong HK, Chan HK, Coruzzi GM, Lam HM (2004) Correlation of ASN2 gene expression with ammonium metabolism in Arabidopsis. *Plant Physiology* 134: 332-338.

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

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

Session IV

Small Molecule Endosidin2 Targets Evolutionary Conserved Exo70 Proteins to Affect Exocytosis

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Overall research goals:

Our research program over the years has been focused toward understanding the cellular processes involved in the mobilization of vacuolar cargoes and improving the efficiency of these processes. For this purpose, we have established a set of long term goals:

1. Mechanisms. Define the cellular mechanism(s) involved in the uptake, compartmentalization and mobilization of energy-relevant nutrients and storage proteins including soluble and membrane proteins that may serve to regulate or optimize these processes developmentally or in response to environmental cues.
2. Components. Define the genetic elements required for the accumulation and mobilization of reserves in vacuole(s) of both vegetative and seed tissues in Arabidopsis.
3. Regulation. Understand how reserves and other cellular proteins and metabolites are partitioned dynamically between sites of accumulation (vacuoles) and sites of intracellular utilization or extracellular utilization via secretory pathways.
4. Applications. Design experimental approaches to improve biomass, production yield, and fitness through the efficient storage and utilization of energy-rich stores in vacuoles.

Significant achievements (2013-2014)

The plant endomembrane system is composed of multiple organelles with distinct morphology and functions. Endomembrane trafficking processes are continuous and highly regulated. However, the phenotypes observed by constitutive disruption of gene functions are usually the consequence of equilibrated feedback which can obscure real gene functions. Synthetic chemicals that target specific proteins involved in membrane trafficking are valuable tools in studying dynamic processes because small molecule effects are transient permitting direct cellular responses to be observed. Conditional mutant screening can identify the gene targets of small molecules. Through large-scale chemical library screening, we have identified groups of small molecules that affect the endomembrane trafficking in Arabidopsis. We have used biochemical approaches combined with genetic screening to identify the target proteins of these bioactive compounds. One of the compounds we recently characterized is Endosidin2. We found that Endosidin2 targets Exo70 proteins in both Arabidopsis and mammalian cells to affect the process of exocytosis. We expect to use Endosidin2 as a tool to identify genes that are involved in the regulation of exocytosis.

Science objectives for 2014 -2015

- Understand the role of the exocyst in regulating vacuole trafficking. *We hypothesize that the exocyst functions as part of a second regulatory mechanism controlling the dynamics of vacuole trafficking and exocytosis.*

An excellent opportunity to understand the regulation of vacuole trafficking is through the use of chemical biology. We have completed high-content screening of chemicals for those

perturbing endomembrane trafficking, including aberrant targeting to the vacuole. One such compound, ES2, acts by targeting the exocyst, an essential complex involved in exocytosis of soluble and membrane-bound cargoes. This results in inhibition of exocytosis and accumulation of cargoes in the vacuole. Our preliminary data indicates that a cognate target of ES2 is the major exocyst complex protein EXO70A1 which may function in regulating the dynamics between vacuole targeting and exocytosis. This presents a second excellent opportunity to understand the potential regulation of vacuole trafficking by dissecting the role of EXO70s and the exocyst.

- Determine the structure of Arabidopsis EXO70A1 and map the ES2 binding sites and functional domains. We hypothesize that identification of the binding site for ES2 will reveal the functional regions of EXO70A that are responsible for regulation of vacuole trafficking and exocytosis.
This will provide a new insight into mechanisms and regulation permitting applications in biotechnology to enhance the efficiency of protein and carbohydrate storage in vacuoles. We believe that our results will provide insight into potentially novel regulatory mechanisms and specific machineries controlling the fate (vacuole accumulation or exocytosis) and extent of accumulation of soluble and membrane cargoes destined for the vacuole.

My scientific area of expertise is: cell biology

To take my project to the next level, my ideal collaborator would have expertise in: crystallography.

Publications supported by this project (2013-2014)

1. C. Zhang, G.R. Hicks, N.V. Raikhel, "Plant vacuole morphology and vacuolar trafficking." *Frontiers in Plant Sciences* (in press) (2014).
2. C. Zhang, N.V. Raikhel, G.R. Hicks, "CLASping microtubules and auxin transport." *Dev Cell*. 24(6):569-71 (2013) [PMID: [23537629](#)].
3. C. Zhang, G.R. Hicks, N.V. Raikhel, "Chemical biology in plants: Finding new connections between pathways using the small molecule Sortin1. *Concepts and Case Studies in Chemical Biology* (Chapter 19). Published 2014; Wiley-VCH Verlag GmbH & Co. KGaA. Pages 285-294.

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

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Overall research goals:

The overall goal of this research proposal is to characterize the molecular machinery responsible for polarized secretion of cell wall components in *Arabidopsis thaliana*. We have used the polarized expansion that occurs during root hair cell growth to identify membrane trafficking pathways involved in polarized secretion of cell wall components to the expanding tips of these cells, and we have recently shown that CSLD3 is preferentially targeted to the apical plasma membranes in root hair cells, where it plays essential roles during cell wall deposition in these cells.

Significant achievements (2012-2014):

1). Cyclin B turnover precedes CSLD5 accumulation during cell division. Earlier results indicated that several CSLD proteins appear to play important roles during cell division, with CSLD2, CSLD3, and CSLD5 all selectively localizing to newly-forming cell plates in dividing cells. Examination of a fluorescent CSLD5 fusion protein driven by its endogenous promoter sequences revealed an expression pattern was highly reminiscent of the expression pattern of the mitotic cyclin, CYCB1. However, when plants expressing fluorescent versions of both CYCB1 and CSLD5 were examined few cells expressing both these cells were observed. We interpreted these results to indicate that CSLD5 is likely expressed and selectively accumulates after CYCB1 does in dividing cells. To examine this question directly, we performed time-lapse confocal fluorescence microscopy of root cortex cells in the meristematic zone of plants stably expressing fluorescently-tagged versions of both Cyclin B (Fig1; GFP-CYCB1) and CSLD5 (Fig1; CFP-CSLD5). These results confirm our earlier observations that 1) CSLD5 accumulates and functions in a cell cycle-dependent manner, and 2) that CSLD5 only accumulates during late stages of mitosis (anaphase and telophase), after Cyclin B is removed from the cell. To our knowledge CSLD5 represents the first cell wall biosynthesis component whose accumulation and function is intimately linked to the cell division cycle in plants, and will likely provide important insight into the regulation of cell wall biosynthesis during phragmoplast-driven cytokinesis events in plants.

2). CSLD5 is rapidly degraded upon completion of cytokinesis. We previously observed that CFP-CSLD5 accumulated in dividing cells. This was particularly apparent during the differentiation of stomatal guard cells, in which asymmetric and symmetric cell divisions occur rapidly during stomatal guard cell development. Interestingly, relative levels of accumulation of CFP-CSLD5 protein within these cells correlated with the presumed timing of these divisions. This, taken together with our time-lapse microscopy results (see Fig1 above), supported the possibility that CSLD5 was quickly lost from cells upon completion of cytokinesis. We therefore examined the protein turnover characteristics of CFP-CSLD5 and showed that unlike CSLD3 and CESA6, CSLD5 is a rapidly destabilized protein. Rapid turnover of CFP-CSLD5 appeared to be dependent upon ubiquitin-mediated processes.

Science objectives for 2014-2015:

- Examine how CSLD5 function is integrated with regard to other members of the CSLD family during cell wall synthesis in non-dividing cells in *A. thaliana*.
- Determine if CSLD proteins assemble into multi-subunit complexes.
- Characterize CSLD synthetic activity and examine the nature of the polysaccharides synthesized by these enzymes.

My scientific areas of expertise are:

Plant Cell Biology, Polarized Membrane Trafficking, Plant Lipid Signalling, Plant Cell Wall Biogenesis.

Figure 1

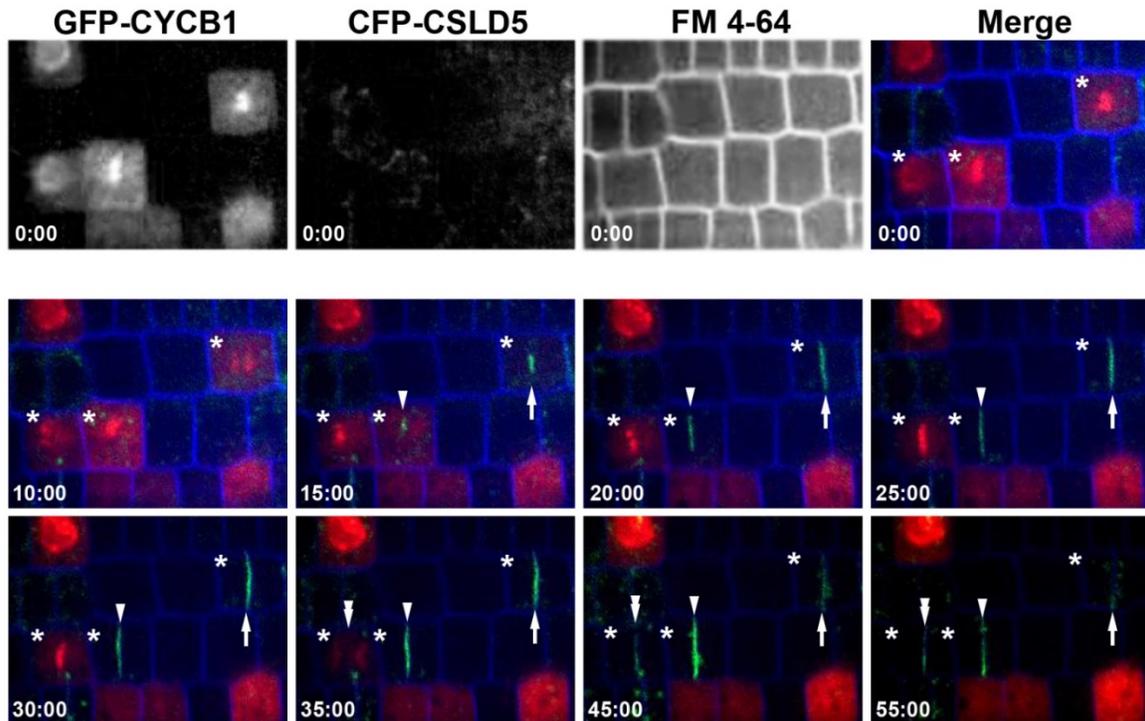


Figure 1. Temporal dynamics of Cyclin B and CSLD5 accumulation during cell division. Seven day-old *Arabidopsis* seedlings stably expressing GFP-CYCB1 (red) and CFP-CSLD5 (green) under endogenous promoters were placed in a live-imaging microscopy perfusion chamber and incubated with 30 μ M FM 4-64 (blue) in 0.25 X MS media for five minutes. FM 4-64 was washed out and confocal optical sections were collected from cortical root tissues at 30 second time intervals. Representative images from 0, 10, 15, 20, 25, 30, 35, 45, and 55 minutes are presented. In this image montage, three cells (asterisks) expressing high levels of GFP-CYCB1 fluorescence initiate mitosis. Transition from metaphase to anaphase is accompanied by rapid loss of GFP-CYCB1 signal at 10 minutes (right-most cell marked with asterisk), 15 minutes (center cell marked with asterisk), and 35 minutes (left-most cell marked with asterisk), respectively. Only after loss of GFP-CYCB1, can CFP-CSLD5 be observed at the newly-forming cell plate as observed (arrow at 15 mins, arrowhead at 15 min, and double arrowhead at 35 mins). Upon completion of cytokinesis CFP-CSLD5 signal is rapidly lost (compare 35 and 45 minute time points for right-most cell marked with asterisk; compare 45 and 55 minute time points for center cell marked with asterisk).

To take my project to the next level, my ideal collaborator would have expertise in: I believe my research aims would be significantly enhanced by collaboration with groups with expertise in two main areas: 1) biochemical analysis of cell wall composition and/or measurement of *in vitro* activities of cell wall biosynthetic enzymes. 2) protein expression and purification of integral membrane proteins for structural analysis of protein complexes either by cryoelectronmicroscopy or x-ray crystallography methods

Publications supported by this project 2012-2014:

- 1) Bak, G., Gu, F., and **Nielsen, E.** (2014) Polarity in Plants. Cellular Logistics, *submitted*.
- 2) Antignani*, V., Klocko*, A.L., Bak, G., Chandrasekaran, S.D., and **Nielsen, E.** (2014) RabA4B-mediated Recruitment of PUB13 and the PI4Kb1/b2 Phosphatidylinositol-4 Kinases Play Important Roles During SA-mediated Plant Defense Signaling in *Arabidopsis thaliana*. *Plant Cell*, *In revision*. (* co-first authors)
- 3) Gu, F., and **Nielsen, E.** (2013) Targeting and Regulation of Cell Wall Synthesis during Tip Growth in Plants. *J. Integr. Plant Biol.*, *55*, 835-46.
- 4) Marella H., **Nielsen, E.**, Schachtman, D.P., and Taylor, C.G. (2013) The amino acid permeases, AAP3 and AAP6, are involved in root-knot nematode parasitism of *Arabidopsis*. *Mol. Plant-Microbe Interact.*, *26*, 44-54.
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- 6) Park, S., Szumlanski, A.L., Gu, F., Guo, F., and **Nielsen, E.** (2011) A role for CSLD3 during cell wall synthesis in apical plasma membranes of tip-growing root hair cells. *Nature Cell Biol.*, *13*, 973-80.

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

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Recent Collaborators: Rajini Rao (*J. Hopkins U.*); Alice Cheung (*U. Mass.*)

Overall research goals

All organisms have evolved mechanisms to regulate ion and pH homeostasis in order to respond to developmental cues and adapt to a constantly changing environment. The central goal of my laboratory has been to identify transporters that regulate cation (e.g. Ca^{2+} , K^+) and pH homeostasis, and understand their biological roles. The dynamic endomembrane system of eukaryotes is emerging as a critical and central coordinator of processes, including signaling, cell wall modeling and stress tolerance in plants. One hypothesis is that mechanisms regulating the internal and external environment of endomembrane compartments provide the conditions for spatial and temporal membrane trafficking and cargo sorting. Yet many molecular players involved in cation and pH homeostasis remain unidentified. We focus on a *novel* gene family predicted as Cation/H⁺ eXchanger (CHX). Though under-studied, CHX proteins are emerging as important players for plant survival on land. We want to determine the mode of transport, and the biological roles of selected CHX proteins in flowering plants.

Significant achievements in 2011-14

- a) ***Provided evidence of CHX17 and related homologs in pH homeostasis, K⁺ transport, and cargo sorting:***
 - Arabidopsis thaliana* CHX17-CHX20 proteins are implicated in pH homeostasis because their expression restored growth of alkaline-sensitive yeast strain at pH 7.5.
 - CHX17 mediated $^{86}\text{Rb}(\text{K}^+)$ transport when expressed in an *E. coli* strain deficient in K^+ -uptake pathways.
 - CHX17 affects membrane trafficking and cargo sorting, as its expression in yeast confers hygromycin-resistance. As aminoglycosides enter cells by endocytosis, and resistance is thought to be mediated by its sorting to vacuoles for degradation, the results suggest CHX17 affects the pH homeostasis of endosomes thus affecting membrane trafficking and protein/cargo sorting. (Chanroj et al. 2011. *J. Biol. Chem*)
- b) Showed CHX17 and related proteins localize to endomembranes in plants. GFP-tagged CHX17 is an active protein and was localized to prevacuolar compartment (PVC) and to the plasma membrane in transient and stable transgenic plants indicating they are associated with dynamic endomembranes. Furthermore, the long hydrophilic C tail characteristic and unique to this gene family has a role in sorting CHX17 to its intended destination (Chanroj et al. 2013 *Mol Plant*).
- c) Less quadruple mutants (chx16-17-18-19) are recovered than expected, and pods show reduced seed set suggesting a role of these genes in reproduction. (Chanroj et al. 2013)

Science Objectives for 2014-15

-To understand the mode of transport, we are using homology modeling to identify critical residues in CHX17 using the crystallized structure of E. coli NhaA. Site-directed mutagenesis of AtCHX17 and activity assays in yeast in progress supports a model of a K^+/H^+ antiporter.

-To complete analysis of CHX function in reproduction and seed set using a chx triple mutant. Male fertility is compromised in triple chx mutant, so we are determining if pollen tube growth, guidance, tube rupture or gamete fertilization are compromised. CHXs are expressed in pollen tube and sperm. Our results will show how perturbing pH and cation homeostasis would impact male fertility and seed development.

My primary expertise is in: plant physiology, membrane transport, biochemistry, cell biology

To take my project to the next level, my ideal collaborator would have expertise in:

X-Ray crystallography and transport protein structure-function relationship

Biophysics and reconstituting channel or transporter activity using nanotechnology, super sensitive or electrical methods

References to work supported by DOE

Aranda-Sicilia MN, Cagnac O, Chanroj S, Sze H, Rodríguez-Rosales MP, Venema K. (2012). Arabidopsis KEA2 a homolog of bacterial KefC encodes a K^+/H^+ antiporter with a chloroplast transit peptide.

Biochim Biophys Acta – Biomembranes 1818(9):2362-71

Chanroj S, Lu Y, Padmanaban S, Nanatani K, Uozumi N, Rao R, Sze H. (2011) Plant-specific cation/ H^+ exchanger 17 and its homologs are endomembrane K^+ transporters with roles in protein sorting. **J Biol Chem.** 286(39):33931-41

Chanroj S, Wang GY, Venema K, Zhang MW, Delwiche CF, H Sze (2012). Conserved and diversified gene families of monovalent cation/proton antiporters from algae to flowering plants. *Frontiers in Plant Science* 3, 25 PMID: [22639643](#)

Chanroj S, Padmanaban S, Czerny DD, Jauh GY, Sze H (2013) K^+ transporter AtCHX17 with its hydrophilic C tail localizes to membranes of the secretory/endocytic system: role in reproduction and seed set. *Mol. Plant* 6(4):1226-46

Lu Yongxian, S Chanroj, L Zulkifli, M Johnson, N Uozumi, AY Cheung, H Sze (2011). Pollen tubes lacking a pair of cation/ H^+ exchangers fail to target ovules in Arabidopsis. **Plant Cell** 23(1):81-93] PMID: [21239645](#). Highlighted in Faculty of 1000 (Plant Biology) Jan 2011

Extracellular Charge Transport in Microbial Redox Chains: Linking the Living and Non-Living Worlds

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Overall research goals:

This project focuses on understanding the *in vivo* assembly, biophysical charge transport mechanism, and energetic consequences of extracellular redox chains that are proposed to mediate long-range (many cell-lengths) electron transport between microbial biofilms and inorganic surfaces ranging from natural environmental minerals to engineered electrodes. To gain a physics-based understanding, the research plan is designed to achieve three specific objectives:

- (1) Test whether electron transport measurements in bacterial nanowires and individual multiheme cytochromes agree with the theoretical predictions of a multistep redox hopping mechanism.
- (2) Quantify interfacial microbe-surface electron transfer at the level of single *Shewanella oneidensis* MR-1 cells, and discover how this fundamental single-cell respiration rate is impacted by the surface redox potential and selected mutations for membrane cytochromes and bacterial nanowires.
- (3) Monitor, *in situ*, the assembly of bacterial nanowires from individual cells, thereby identifying their composition, formation mechanism, and directly measure the impact of these structures on the extracellular respiration activity of live cells.

Significant achievements (2013-2014):

- Using *in vivo* fluorescence measurements, immunolabeling, and quantitative gene expression analysis, we found that *S. oneidensis* MR-1 nanowires are extensions of the outer membrane and periplasm that include the multiheme cytochromes responsible for electron transport, rather than pilin-based structures as previously thought. These bacterial nanowires are associated with outer membrane vesicles and vesicle chains, structures ubiquitous in gram-negative bacteria. The localization of multiheme cytochromes MtrC and OmcA to these membrane extensions directly supports one of the two intensely debated models of electron transport through the nanowires (multistep hopping). We also found, for the first time, that the production of bacterial nanowires is correlated with an increase in cellular reductase activity.

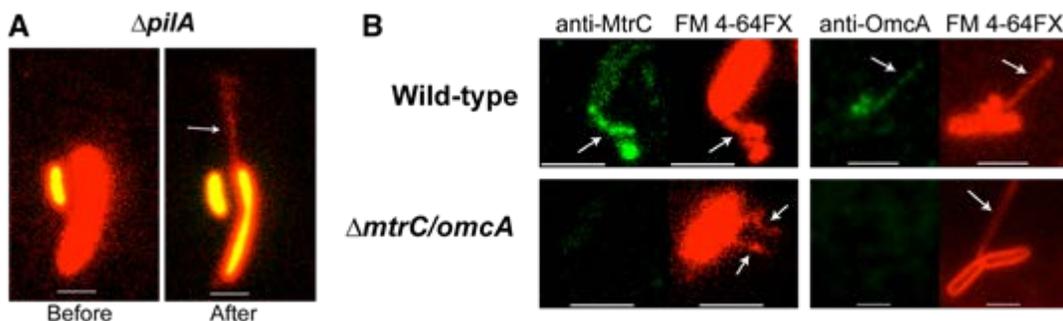


Fig. 1. The molecular basis of bacterial nanowires. (A) Combined respiration (RedoxSensor Green) and membrane (FM 4-64FX, red) fluorescence of the $\Delta pilA$ strain, lacking the type IV pilin major subunit PilA, before and after the production of a bacterial nanowire. $\Delta pilA$ is capable of producing bacterial nanowires with a similar respiratory impact as wild-type *S. oneidensis* MR-1, evidenced by the increase in reductase activity (green fluorescence) after nanowire production. (B) Labeling with antibodies against MtrC (left panel) or OmcA (right panel) and membrane fluorescence (FM 4-64FX) images of wild-type (top) compared to the $\Delta mtrC/omcA$ control strain (bottom). Nanowire-localized MtrC/OmcA are observed in the wild-type strain. Scale bars, 2 μ m.

- We performed scanning tunneling microscopy, single-molecule tunneling spectroscopy, and Kinetic Monte Carlo (KMC) simulations of multistep electron hopping in the multiheme cytochrome (MtrF) from the dissimilatory metal-reducer *S. oneidensis* MR-1. These are the first KMC simulations and single molecule measurements in MtrF (manuscript submitted).

Science objectives for 2014-2015:

- Motivated by our experimental localization of multiheme cytochromes over micrometer-long nanowires, we will extend the multistep charge hopping simulations (currently treating heme chains within single molecules) to entire multi-cytochrome assemblies. These simulations will help set limits on a currently unresolved question: what is the *inter*-cytochrome electron exchange mechanism, and can it sustain microbial respiration currents?
- Over the past year, we have been working to perfect a combined optical trapping and electrochemical approach designed to measure *in vivo* electron transfer at the level of individual cells. During the next year, we will assess how this fundamental single-cell respiration rate is impacted by the surface redox potential and selected mutations for membrane cytochromes. We will also develop microfluidic tools to simultaneously monitor the formation and electrochemical activity of bacterial nanowires at electrode surfaces.

My scientific areas of expertise are: Biological electron transport, *in vivo* microscopy, scanning probe measurements, nanoscience.

To take my project to the next level, my ideal collaborator would have expertise in: Synthetic Biology.

Publications supported by this project 2013-2014:

1. S. Pirbadian, S. E. Barchinger, K.M. Leung, H.S. Byun, Y. Jangir, R.A. Bouhenni, S.B. Reed, M.F. Romine, D.A. Saffarini, L. Shi, Y.A. Gorby, J. H. Golbeck, and M.Y. El-Naggar, “*Shewanella oneidensis* MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components.” Proceedings of the National Academy of Sciences of the United States of America (2014). [DOI: [10.1073/pnas.1410551111](https://doi.org/10.1073/pnas.1410551111)].
2. N. Michelusi, S. Pirbadian, M.Y. El-Naggar, U. Mitra, “A stochastic model for electron transfer in bacterial cables.” IEEE Journal on Selected Areas in Communication (2014). [In press].

Session V

Genetic Analysis of Hydrogenotrophic Methanogenesis in *Methanosarcina* Species

William W. Metcalf, Principal Investigator

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Overall research goals:

The long-term goal of our research is to expand our knowledge regarding hydrogen-dependent (hydrogenotrophic) methanogenesis by members of the genus *Methanosarcina*. A 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. **The specific goals are; (1) characterization of energy-conserving electron transport in *M. barkeri* via a proposed hydrogen-cycling mechanism, (2) characterization of hydrogen-independent energy-conserving electron transport in *M. acetivorans*, and (3) assessment of the roles of the multiple heterodisulfide reductase (Hdr) isozymes in *M. barkeri* and *M. acetivorans*.**

Significant achievements (2011-2015):

Our most significant finding is that *Methanosarcina barkeri* utilizes a “hydrogen cycling” electron transport chain during growth on all methanogenic substrates, including methanol, methylamines and acetate. During this process, H₂ produced in the cytoplasm diffuses out of the cell where it is re-oxidized with transfer of electrons into the energy conserving electron transport chain. During this process, protons are consumed to generate H₂ in the cytoplasm and produced in the periplasm during H₂ oxidation. Thus “hydrogen cycling” leads directly to production of a proton motive force that can be used by the cell for ATP synthesis. In contrast, other *Methanosarcina* species, exemplified by *Methanosarcina acetivorans*, have evolved a hydrogen-independent energy-conserving electron transport chain. Genetic experiments in both species have revealed the major membrane-bound and cytoplasmic components of the two distinct energy-conserving electron transport chains. Moreover, examination of numerous genome sequences suggests that hydrogen cycling predominates in freshwater *Methanosarcina* species, while the hydrogen-independent electron transport chain predominates in marine species.

Science objectives for 2014-2015:

- Our data suggest that H₂-cycling requires direct interaction of cytoplasmic and periplasmic components. To assess this we are currently performing a series of protein-tagging and “pull-down” experiments to identify interacting protein partners
- Our data indicate that *M. barkeri* responds to mutations in genes encoding the H₂-cycling pathway by inducing synthesis of an alternate electron transport chain. We are currently using RNA-seq technology to identify the players in this alternate energy-conserving pathway.
- We are currently attempting to develop 3-D mathematical models that capture the rates and physical arrangement of the hydrogen-cycling electron-transport components to better understand the constraints of this unusual energy conserving process.

My scientific areas of expertise are: Physiology, metabolism, biochemistry genetics, molecular biology, and genomics of diverse microorganisms.

To take my project to the next level, my ideal collaborator would have expertise in: Single molecule imaging of fluorescently labeled proteins, and 3-D modeling of diffusion mediated cellular metabolism.

Publications supported by this project 2011-2014:

1. G. Kulkarni, Guss, A.M., Buan, N.R. and W.W. Metcalf. 2014. Discovery of latent alternative electron transport pathways in the methane-producing archaeon *Methanosarcina acetivorans*. *In preparation*.
2. G. Kulkarni and W.W. Metcalf. 2014. Experimental demonstration of hydrogen cycling as a mechanism of energy conservation in methanogenic Archaea. *In preparation*.
3. G. Kulkarni, T. Mand and W.W. Metcalf. 2014. Characterization of mutants lacking each of the three distinct hydrogenases in *Methanosarcina barkeri*. *In preparation*.

Enzymology of Methanogenesis: Mechanism of Methyl-Coenzyme M Reductase

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Overall research goals: Methyl-coenzyme M reductase (MCR) from methanogenic archaea catalyzes the terminal step in the biological synthesis of methane. Using coenzyme B (HSCoB) as the two-electron donor, MCR reduces methyl-coenzyme M to methane. Two catalytic mechanisms have been proposed for methane synthesis (Figure 1): one involves an organometallic methyl-Ni(III) intermediate, while the other includes a methyl radical. Our research goals are (a) to elucidate the mechanism of methane formation by MCR, (b) to determine the mechanism of activation of MCR and (c) determine the crystal structure of the Ni(I) state of MCR and the structures of catalytic intermediates.

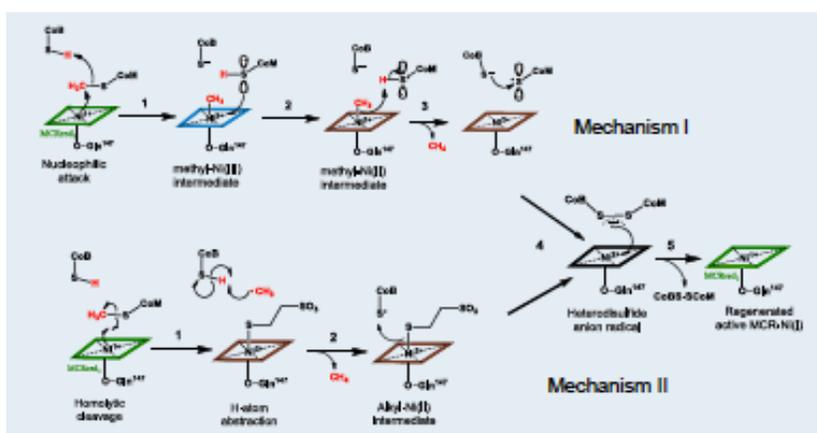


Figure 1

Significant achievements 2012-2014:

We performed steady-state and transient kinetic studies of the MCR mechanism with the native substrates and with CoBSH analog (CoB6SH) in which heptanoyl moiety was replaced with a hexanoyl group. These studies allow us to observe for the first time intermediate states of the Ni-F430 cofactor during the reaction cycle. Based on these results, we revised the MCR mechanism and assigned rate constants for the intermediate steps in the MCR reaction.

We also have used transient kinetic experiments to study the reverse MCR reaction, which is the initial step in the pathway for anaerobic methane oxidation. These studies involve monitoring changes in the redox and ligation state of the nickel-F430 cofactor by enzyme-monitored turnover.

Science objectives for 2014-2015:

- In collaboration with John Leigh (U. Washington), use mutagenesis studies to test the proposed mechanism for MCR.
- Perform transient kinetic studies of the reverse methanogenesis reaction on MCR.
- In collaboration with Dayle Smith (PNNL), perform computational studies on the MCR mechanism.
- Complete activation energy profiles for various steps in the MCR mechanism and compare the experimental data with theoretical energy profiles, obtained by computational chemistry experiments.

To take my project to the next level, my ideal collaborator would have expertise in:

Computational Chemistry, Dayle Smith, PNNL. We wish to use computational methods to test proposed intermediates in the MCR mechanism.

Methanogen genetics: John Leigh, Univ. Washington. We wish to generate mutations in MCR to test mechanistic proposals.

References to work supported by this project 2010-2014:

1. Cedervall, P. E., Dey, M., Li, X., Sarangi, R., Hedman, B., Ragsdale, S. W., and Wilmot, C. M. (2011) Structural analysis of a Ni-methyl species in methyl-coenzyme M reductase from *Methanothermobacter marburgensis*, *J. Am. Chem. Soc.* *133*, 5626-5628. [PMC3086036](#).
2. Zhou, Y., Dorchak, A.E., and Ragsdale, S.W. (2013) In vivo activation of methyl-coenzyme M reductase by carbon monoxide, *Frontiers in Microbiology* *4*, 69. [PMC3612591](#).

Session VI

Computational capabilities for understanding enzymatic energy conversion

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Understanding principles of how enzymes catalyze the activation of energy-relevant small molecules (e.g., H₂, CO₂, N₂) under mild conditions represents a critical step toward the design of highly selective, efficient and sustainable bio-inspired catalysts. Despite notable advances, questions remain about how these enzymes achieve these characteristics. To this end it is critical to have a detailed knowledge of the catalytic functionalities that are at the core of the precise energy and mass flow and reactivity in enzymes. Their characterization and understanding in these complex molecular systems require reliable structural, spectroscopic, thermodynamic, kinetic, and electrochemical information that can only be obtained through multidisciplinary efforts. Critical to successfully linking all of this information is the application of theory and computation, which can provide unprecedented insights and knowledge.

The breadth of the computational and theoretical tools necessary to characterize the principles that underpin enzymatic catalysis and to translate them into synthetic platforms extends beyond the methodologies commonly employed in small molecule chemistry. It encompasses both traditional electronic structure methods (density functional theory (DFT) and post Hartree-Fock methods) and advanced methodologies, such as *ab initio* molecular dynamics based on linear scaling DFT, mixed quantum mechanical/molecular mechanical (QM/MM) simulations, and state-of-the-art statistical mechanics approaches for free energy calculations in complex systems. A distinguishing element of enzymatic reactivity often lies in functional large-scale motions that occur on timescale not currently accessible by direct atomistic simulations. Their description requires the use of coarse-grained approaches based on simplified interaction potential functions and/or accelerated dynamics schemes, which eliminate fine details unessential for the long-timescale of the system. All these simulations schemes can be combined with Marcus theory to efficiently investigate the multi-proton, multi-electron processes that are the core of energy-relevant enzymatic transformations. Computational efforts must also include microkinetic modeling of the complex multistep reaction pathways for calculations of catalytic turnover frequencies. As a whole, these techniques are at the forefront of theory and computation and yield quantities that directly assist the interpretation of the experimental data and prediction of new catalysts.

In this talk we will illustrate how modern computation and simulation are able to provide critical insights that link enzyme structure and function, and complement experimental approaches. We will take examples from recent studies carried out in our group and dealing with proton delivery in hydrogenases, electron delivery and dinitrogen (N₂) activation in nitrogenase, and dihydrogen (H₂) production and oxidation by bio-inspired molecular catalysts.

References:

1. D. M. A. Smith, K. Danyal, S. Raugei, and L. C. Seefeldt. *Substrate Channel in Nitrogenase Revealed by a Molecular Dynamics Approach*. *Biochemistry* **53**, 2278 (2014).
2. M. L. Reback, G. W. Buchko, B. L. Kier, B. Ginovska-Pangovska, Y. Xiong, S. Lense, J. Hou, J. A. S. Roberts, C. M. Sorensen, S. Raugei, T. C. Squier, and W. J. Shaw. *Enzyme Design from the Bottom Up: An Active Nickel Electrocatalyst with a Structured Peptide Outer Coordination Sphere*. *Chem. Eur. J.* **20**, 1510 (2014).
3. S. Chen, M.-H. Ho, R. M. Bullock, D. L. DuBois, M. Dupuis, R. Rousseau, and S. Raugei. *Computing Free Energy Landscapes: Application to Ni-based Electrocatalysts with Pendant Amines for H₂ Production and Oxidation*. *ACS Catal.*, **4**, 229 (2014).
4. B. Ginovska-Pangovska, M.-H. Ho, Y. Cheng, M. Dupuis, S. Raugei, Wendy J. Shaw. *Molecular Dynamics Study of the Proposed Proton Transport Channels in [FeFe]-Hydrogenase*. *BBA-Bioenergetics*, **1837**, 137 (2014).
5. M. L. Reback, B. Ginovska-Pangovska, M.-H. Ho, A. Jain, M. P. Stewart, T. C. Squier, S. Raugei, J. A. S. Roberts, W. J. Shaw. *The Role of a Dipeptide Outer-Coordination Sphere on H₂ Production Catalysts: Influence on Catalytic Rates and Electron Transfer*. *Chem. Eur. J.*, **19**, 1928-1941 (2013). Paper designed as VIP (Very Important Paper) and selected as the journal cover.
6. M. O'Hagan, M.-H. Ho, J. Yang, A. Appel, M. DuBois, S. Raugei, W. J. Shaw, D. L. DuBois, R. M. Bullock. *Proton Delivery and Removal in [Ni(P^RN^{R'})₂]²⁺ Hydrogen Production and Oxidation Catalysts*. *J. Am. Chem. Soc.*, **134**, 19409 (2012).



The Penn State Bioinorganic Workshops

Carsten Krebs, Principal Investigator

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J. Martin Bollinger, Jr., Co-PI(s)

Squire J. Booker, Co-PI(s)

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Overall research goals:

The field of bioinorganic chemistry is diverse, and includes a wide variety of scientific disciplines, including genetics, molecular biology, biochemistry, bioinformatics, analytical chemistry, and physical chemistry. Many different experimental techniques (e.g. reaction kinetics, a wide variety of spectroscopies, X-ray crystallography, and mass spectrometry) and theoretical methods (e.g. electronic structure calculations and bioinformatics methods) are combined in this area of research to understand the formation, function, and regulation of the many metallo-cofactors found in Nature as well as to identify novel metallo-cofactors. Many metalloenzyme-catalyzed reactions are extremely complex, but of fundamental importance to science and society.

Because the methods used in bioinorganic chemistry are so diverse and require sophisticated technical expertise, it is particularly important that students and postdocs receive broad training. The biennial Penn State Bioinorganic Workshops provide a unique training opportunity of the next generation of scientists working in the area of bioinorganic chemistry.

Significant achievements:

The 2014 Penn State Bioinorganic Workshop provided training for a total of 162 participants. The workshop consisted of 16 90-min lectures by experts in the field on relevant topics and hands-on training in small groups (typically not more than 6 “students”) in 21 different methods offered in 2-h time blocks. The various hands-on methods were offered up to 12 times, allowing each participant to learn up to 12 new methods. 75 of the 162 participants (i.e. nearly half) served as teachers in the hands-on part. The “teachers” included undergraduate and graduate students, postdoctoral associates, research technicians, and faculty. A total of ca. 1200 2-h training units were offered to the participants in the hands-on section. The workshop was combined with the 3rd Frontiers in Metallobiochemistry Symposium, which most of the workshop participants attended. The latter meeting featured 12 shorter talks by students and postdocs (selected from abstracts), in addition to 21 faculty talks. At both meetings, there were 3 2-h poster sessions, allowing for additional networking amongst participants.

Science objectives for 2014-2015:

We will evaluate the feedback provided by the participants of the 2014 workshop and begin with the planning of the 2016 Penn State Bioinorganic Workshop

Session VII

Resolving protein-semiquinone interactions by advanced EPR spectroscopy

Sergei Dikanov, Principal Investigator

Antony R. Crofts, Robert B. Gennis, Colin A. Wraight, Co-PIs

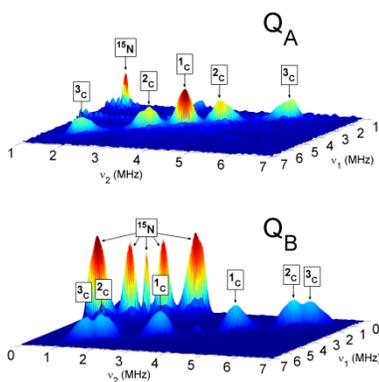
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Overall research goals: Our focus is on use of modern, high-resolution EPR spectroscopy to explore the catalytic domains trapped in states with semiquinone (SQ) as an intermediate. The catalytic sites we study, - the Q_A and Q_B sites of the bacterial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

Significant achievements 2013-2014:

Methoxy orientation modulates the redox potential of ubiquinone in reaction centers. The methoxy group dihedral angle has been suggested to have a strong influence on the redox midpoint potential (E_m) of ubiquinones. To address this we carried out 2D ESEEM studies of the SQ_A and SQ_B in RCs where natural UQ_{10} was extracted and was replaced with the ($^{13}CH_3$, $^{13}CH_3O$) UQ_8 . ^{13}C 2D ESEEM spectra are shown in Figure. In each case three ^{13}C features attributable to CH_3 and two CH_3O are observed, giving us ^{13}C hfi tensors for these groups. Comparison of experimental isotropic hfi constants of the 2-methoxy groups in Q_A (1.4 MHz) and Q_B (5.7 MHz) with QM calculated values as a function of the 2-methoxy dihedral angle θ defines four possible combinations for the dihedral angle θ ($C_mO_mC_2C_1$) in the two SQs. The angles determined were then compared to the computed relationship between the dihedral angle and the resulting electron affinity. X-ray structures support dihe- dral angle difference $\Delta\theta = -80^\circ$ corresponding to a redox potential gap (ΔE_m) between Q_A and Q_B of 180 mV that is significantly larger than the experimental difference in redox potentials (60–75 mV) of the two quinones. Additional confirmation of the larger contribution to the E_m gap between Q_A and Q_B comes from mutants of the Q_A site that lower the E_m of Q_A . Mutation of isoleu- cine M265 to threonine (mutant M265IT) decreases the E_m of Q_A by 100-120 mV, substantially increasing ΔE_m , the driving force for electron transfer from Q_A^- to Q_B . In this and similarly polar mutants 3-MeO-UQ is completely inactive as Q_B . Taking into account the 60-75 mV favorable ΔE_m for UQ in wild type RCs, the failure of 3-MeO-UQ in M265IT mutant RCs indicates that its E_m in the Q_B site is more than 160-195 mV lower than that of ubiquinone. This is consistent with a contribution of ≥ 180 mV from a correctly oriented 2-methoxy group. These data clearly indicate a role for the 2-methoxy group in setting the functional redox potential gap between Q_A and Q_B through different dihedral angles for Q_A and Q_B .



Nuclear tensors of nitrogen H-bond donors of SQ_B . Our studies of SQ_B by X-band (~ 9.7 GHz) 2D ESEEM revealed interactions with N_δ of His-L190 and N_p of Gly-L225 H-bonded to the Q_B carbonyls, based on both estimated quadrupole coupling constants (qcc) and a comparison of the experimental and calculated isotropic hfi couplings. However, complete hfi and nqi tensors were not determined. S-band (3.6 GHz) ESEEM was used with the aim of obtaining the nqi tensors for both nitrogens by approaching the cancellation condition $|v_N - a/2| \sim 0$ at lower microwave frequencies. By performing measurements at S-band we found a dominating contribution of the N_δ only. The hfi tensors for N_δ and N_p were obtained from simultaneous simulations of 1D and 2D $^{14,15}N$ X-band ESEEM and three-pulse ^{14}N S-band spectra with all nuclear tensors defined in the SQ_B g-tensor coordinate system. This allowed us to conclude that the contribution of N_p to the S-band spectrum is suppressed by its large qcc and weak isotropic hfi, comparable to the level of hfi anisotropy, despite the near-cancellation condition of N_p at S-band. This effect was not reported previously. The agreement between our ESEEM data and DFT calculations of the nitrogen hfi and nqi tensors for SQ_B justifies their use in theoretical investigations for an understanding of the relationship between tensor characteristics and the strength and geometry of H-bonds.

Science objectives for 2014-2015:

- ^{13}C couplings provide insight into the SQ binding to the protein. We will apply a biochemical approach for selective ^{13}C labeling of ring carbons in the SQ_H . The quinones biochemically labeled in bo_3 enzyme will also be used in studies of bacterial reaction center and bc_1 complex.
- We will exploit Q-band ^1H and ^2H ENDOR to address specific questions about the *orientation* of the H-bonds around SQ in Q_B and Q_H sites based on the hfi (and nqi) tensors of exchangeable protons (deuterons).

My scientific areas of expertise are: Continuous-wave and pulsed Electron Paramagnetic Resonance; magnetic resonance as a structural tool and its application in inorganic biochemistry, photosynthesis, structure-function relations in metalloproteins and quinone processing sites studies.

To take my project to the next level, my ideal collaborator would have expertise in: Pulsed EPR spectroscopy and methods of selective isotope labeling employed in this work will be beneficial for structural studies of metal cofactors and radical species in other areas of bioenergetics, photosynthesis, and bioinorganic chemistry. This technique can reveal otherwise invisible structural features, and possible dynamics of active-site residues. The approach might also contribute to the development of strategies for design and engineering of new metalloenzymes and devices for biotechnological applications. It has already been extended towards $[\text{2Fe-2S}] (\text{Cys})_{4-n}(\text{His})_n$ ($n=0,1,2$) clusters in thermophile metalloenzymes and the clusters of the more complex structures in nitrogenase and $[\text{Fe-Fe}]$ hydrogenase. The collaboration on hydrogenase was initiated during the DOE 2013 Photosynthetic Systems meeting in discussions with Dr. Paul King (NREL).

Publications supported by this project 2012-2014:

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2. Dikanov, S.A. (2013) Resolving protein-semiquinone interactions by two-dimensional ESEEM spectroscopy. *Electron Paramagnetic Resonance* **23**, 103–179.
3. Maklashina, E., Cecchini, G., Dikanov, S.A. (2013) Defining a direction: electron transfer and catalysis in *Escherichia coli* complex II enzymes. *Biochim. Biophys. Acta* **1827**, 668–678.
4. Taguchi, A.T., O'Malley, P.J., Wraight, C.A., Dikanov, S.A. (2013) Conformational differences between the methoxy groups of Q_A and Q_B site ubisemiquinones in bacterial reaction centers: a key role for methoxy group orientation in modulating ubiquinone redox potential. *Biochemistry* **52**, 4648–4655.
5. Taguchi, A.T., Mattis, A.J., O'Malley, P.J., Dikanov, S.A., Wraight, C.A. (2013) Tuning cofactor redox potentials: The 2-methoxy dihedral angle generates a redox potential difference of >160 mV between the primary (Q_A) and secondary (Q_B) quinones of the bacterial photosynthetic reaction center. *Biochemistry* **52**, 7164–7166.
6. Taguchi, A.T., O'Malley, P.J., Wraight, C.A., Dikanov, S.A. (2014) Nuclear hyperfine and quadrupole tensor characterization of the nitrogen hydrogen bond donors to the semiquinone of the Q_B site in bacterial reaction centers: A combined X- and S-band $^{14,15}\text{N}$ ESEEM and DFT study. *J. Phys. Chem. B* **118**, 1501 – 1509.
7. de Almeida, W.B., Taguchi, A.T., Dikanov, S.A., Wraight, C.A., O'Malley, P.J. (2014) The 2-methoxy group orientation regulates the redox potential difference between the primary (Q_A) and secondary (Q_B) quinones of Type II bacterial photosynthetic reaction centers. *J. Phys. Chem. Lett.* **5**, 2506–2509.
8. Taguchi, A.T., O'Malley, P.J., Wraight, C.A., Dikanov, S.A. (2014) Hyperfine and nuclear quadrupole tensors of nitrogen donors in the Q_A site of bacterial reaction centers: Correlation of the histidine $\text{N}\delta$ tensors with hydrogen bond strength. *J. Phys. Chem. B* **118**, 9225–9237.

been unable to detect apiose in the moss gametophore cell walls nor did we detect the "UAS" transcript in *P. patens* protonema by RT-PCR. We obtained a synthetic ORF for the loci but the recombinant protein had no discernible UAS activity, although small amounts of UDP-Xyl were formed. A KY amino acid sequence present in vascular plant UAS is replaced by QQ in the *P. patens* and other moss "UAS" proteins. Converting QQ to KY led to the formation of small amounts of UDP-Api (<1% of that formed by SpUAS). Initial protein domain swapping studies indicate that the the N-terminal region is responsible for the lack of substantial UDP-Api activity.

RG-II in the walls of different plant tissue and cells. We have shown that RG-II exists as the borate ester cross-linked dimer in the walls of soybean roots and root hairs (with R. Carlson CCRC and G. Stacey, U. Missouri) and in the walls of duckweeds (*Spirodella*, *Lemna*, *Wolffia* and *Wolffiella*). In collaboration with Paula McSteen (U. Missouri), we have shown that only 38% of the RG-II is cross-linked in the tassels of the maize *tassel-less1* (*tls1*) mutant whereas ~80% of the RG-II is cross-linked in wild type tassels. *tls1* encodes a borate channel protein and the mutant has defects in inflorescence development similar to the effects of boron deficiency. Our data suggests that growth defects of the *tls1* mutant are due in part to changes in the cell wall (Durbak et al 2014).

Material enriched in RG-II oxidizes 3,3',5,5',tetramethylbenzidine. We made the unexpected discovery that RG-II (or a molecule that cochromatographs with RG-II) in the presence of hydrogen peroxide oxidizes 3,3',5,5',tetramethylbenzidine to its blue-colored derivative. This reaction occurs with RG-II isolated from red wine, from the walls of suspension-cultured sycamore and tobacco cells, from Arabidopsis leaves and an RG-II-containing fraction *Spirodella*.

Science objectives for 2014-2015:

- Use UDP-Api produced by SpUAS to identify Apiosyltransferases in Arabidopsis and Spirodela.
- Over-express SpUAS in Arabidopsis to determine if UDP-Api availability limits RG-II formation.
- Over-express SpUAS in *P. patens* and determine the fate of the UDP-Api.
- Complete domain swapping studies to map the region(s) that impair UAS activity in the *P. patens* protein and to gain insight into the evolution of functional UAS in land plants.
- Complete structural characterization of RG-II from maize vegetative and inflorescence tissues.
- Determine if RG-II itself oxidizes tetramethylbenzidine or if oxidation results from a specific or non-specific association of RG-II with cell wall peroxidases.

My scientific areas of expertise are: Plant cell walls, Structural analyses of polysaccharides, Polysaccharide biosynthesis, Enzymology.

To take my project to the next level, my ideal collaborator would have expertise in: techniques including NMR spectroscopy and molecular imaging to gain insight into RG-II 3-D structure and how this structure controls the interaction between RG-II and borate.

Publications supported by this project 2012-2014:

1. AR. Durbak, KA. Phillips, S. Pike, MA. O'Neill, J. Mares, A. Gallavotti, ST. Malcomber, W. Gassmann, P. McSteen (2014). Transport of boron by the *tassel-less1* aquaporin is critical for vegetative and reproductive development in maize. *Plant Cell* Published online July 17, doi:[10.1105/tpc.114.125898](https://doi.org/10.1105/tpc.114.125898).

Structure, Function and Reactivity of CO Dehydrogenase from *Oligotropha carboxidovorans*

DE-FG02-13ER16411

Russ Hille, Principal Investigator

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Overall research goals:

We propose to examine the reaction mechanism of the CO dehydrogenase from *Oligotropha carboxidovorans*, an enzyme with a unique molybdenum- and copper-containing enzyme that is responsible for the removal of some 10^8 metric tons of CO from the atmosphere annually. The overall goal is to gain a deeper understanding of the chemical mechanism by which this incredible amount of naturally-occurring bioremediation occurs. The approach involves the application of a combination of mechanistic and spectroscopic studies to understand the chemistry that is catalyzed, which includes H_2 oxidation as well as that of CO. Through a combination of rapid reaction kinetic and spectroscopic studies, the proposed work will lead to a better understanding of how the structure (both physical and electronic) of the active site molybdenum centers relate to reactivity. We intend to:

1. Understand the manner in which CO binds to the binuclear center of CO dehydrogenase using a combination of spectroscopic approaches. We will examine the active site molybdenum center of CO dehydrogenase using EPR, ENDOR and magnetic circular dichroism to identify how CO binds to the enzyme's unique binuclear active site.
2. Gain better insight into the mechanistic relevance spectral intermediates seen with CO dehydrogenase. We will examine in detail the kinetics of enzyme in studies that include the use of FT-IR and enzyme-monitored turnover experiments to identify the spectral signature of the EPR-active intermediate that accumulates with either substrate in the course of enzyme-monitored turnover experiments.
3. Use silver-substituted CO dehydrogenase as a probe of electronic structure and mechanism. We will further examine the reactivity of a functional, silver-substituted form of the enzyme with CO by EPR, ENDOR and MCD in order to identify those features of the binuclear center that are responsible for its reactivity.
4. Understand the mechanism by which CO dehydrogenase oxidizes H_2 . We will continue our investigation of the newly discovered reactivity of CODH toward H_2 with a detailed kinetic analysis of the reaction. We will examine its kinetic behavior in detail and in isotope exchange experiments specifically test the possibility that a metal hydride is formed in the course of the reaction.

Significant achievements (2013-2014):

- We have performed an ENDOR study of an $S = 1/2$ intermediate state trapped during reduction of CO dehydrogenase by CO. ENDOR spectra of this state confirm that the $^{63,65}\text{Cu}$ of the enzyme's Mo/Cu binuclear active site exhibits strong and almost entirely isotropic coupling. When the intermediate is generated using ^{13}CO , coupling to the ^{13}C is observed, with $a_{\text{iso}} = +17.3$ MHz. These results indicate that the intermediate contains a partially reduced, Mo(V)/Cu(I), center with CO bound at the copper and provide strong experimental support for a reaction mechanism that proceeds from a comparable complex of CO with fully oxidized, Mo(VI)/Cu(I), enzyme, in which the Cu-complexed CO is activated for nucleophilic attack.
- The reaction of CO dehydrogenase with H_2 has also been examined. The enzyme is reduced by H_2 with a limiting rate constant of 5.3 s^{-1} and a dissociation constant K_d of $525 \text{ }\mu\text{M}$; the kinetics are

largely pH-independent. During the reaction with H₂, a new EPR signal arising from the Mo/Cu-containing active site of the enzyme is observed which is distinct from the signal seen when the enzyme is reduced by CO, with greater g anisotropy and larger hyperfine coupling to the active site ^{63,65}Cu. The signal also exhibits hyperfine coupling to at least two solvent-exchangeable protons of bound substrate that are rapidly exchanged with solvent. Proton coupling is also evident in the EPR signal seen with the dithionite-reduced native enzyme, and this coupling is lost in the presence of bicarbonate. We attribute the coupled protons in the dithionite-reduced enzyme to coordinated water at the copper site in the native enzyme and conclude that bicarbonate is able to displace this water from the copper coordination sphere. On the basis of our results, a mechanism for H₂ oxidation is proposed which involves initial binding of H₂ to the copper of the binuclear center, displacing the bound water, followed by sequential deprotonation through a copper-hydride intermediate to reduce the binuclear center.

Science objectives for 2014-2015:

- To complete an isotope exchange study of the reaction of CO dehydrogenase with H₂ testing a specific mechanistic hypothesis.
- Conduct ENDOR studies of the bicarbonate complex of the enzyme, using ¹³C-labeled bicarbonate.
- Initiate FT-IR studies of the CO-complexed reduced enzyme.
- Improve the reactivation protocol for the enzyme.

My scientific areas of expertise are: rapid reaction enzyme kinetics; enzyme reaction mechanism; electron paramagnetic resonance spectroscopy.

To take my project to the next level, my ideal collaborator would have expertise in: Cloning and recombinant expression of metalloproteins; Expertise in spectroscopic methods such as ENDOR, MCD and FT-IR.

Publications supported by this project (2013-2014):

Shanmugam, M., Wilcoxon, J., Habel-Rodriguez, D., Kirk, M.L., Hoffman, B.M. & Hille, R. (2013) ¹³C and ^{63,65}Cu ENDOR studies of CO dehydrogenase from *Oligotropha carboxidovorans*. Experimental evidence in support of a copper-carbonyl intermediate. *J. Am. Chem. Soc.* **135**, 17775-17782.

Reschke, S., Nicks, D., Wilson, H., Sigfridsson, K.G.V., Haumann, M., Rajagopalan, K.V., Hille, R. & Leimkühler, S. (2013) Impact of the cysteine molybdenum ligand by selenocysteine on structure and function of the active site in human sulfite oxidase. *Biochemistry* **52**, 8295-8303.

Wilcoxon, J., & Hille, R. (2013) The hydrogenase activity of CO dehydrogenase from *Oligotropha carboxidovorans*. *J. Biol. Chem.* **288**, 36052-36060.

Cao, H., Hall, J., & Hille, R. (2014) Crystal structures of xanthine oxidoreductase complexed with guanine and indole-3-aldehyde. *Biochemistry*, 533-541.

Anderson, R.F., Shinde, S.S, Hille, R, Rothery, R.A., Weiner, J.H., Rajagukguk, S., Maklashina, E. & Cecchini, G. (2014) Efficient electron transfer to the heme in complex II involves the [3Fe-4S] and quinone sites of the enzyme. *Biochemistry* **53**, 1637-1646.

Photosynthetic Energy Capture, Conversion and Storage: From Fundamental Mechanisms to Modular Engineering

Michael Thomashow, Principal Investigator

Federica Brandizzi, Jin Chen, Jeff Cruz, Daniel Ducat, John Froehlich, Sheng Yang He, Gregg Howe, Jianping Hu, Cheryl Kerfeld, David Kramer, Beronda Montgomery, Tom Sharkey, and Peter Wolk, Co-Investigators

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The Michigan State University-Department of Energy Plant Research Lab (PRL) was established in 1965. For more than 45 years, the mission of the PRL was to conduct high quality basic research on photosynthetic organisms and to train graduate students and postdocs in this area of science. The PRL faculty and associates, including more than 260 graduate students and 550 postdoctoral researchers, successfully fulfilled this mission, making fundamental discoveries in the fields of hormone biology, biosynthesis of energy-rich molecules, plant interactions with the environment, photosynthesis and related aspects of energy metabolism, organelle function and biogenesis, and inter-organelle communication.

The PRL entered a new era in 2014. Formerly, PRL faculty conducted research as individual labs that collaborated when interests overlapped. The new PRL model is to conduct research as teams addressing interrelated research themes that fall along the continuum of carbon and energy capture, conversion and deposition. The issues tackled are complex in nature, ranging from addressing long-

standing and emerging questions in photobiology, to developing first principles enabling rational design of cellular and subcellular modules for enhanced carbon fixation and high-energy redox reactions.

PHOTOSYNTHETIC ENERGY CAPTURE, CONVERSION AND STORAGE: FROM FUNDAMENTAL MECHANISMS TO MODULAR ENGINEERING

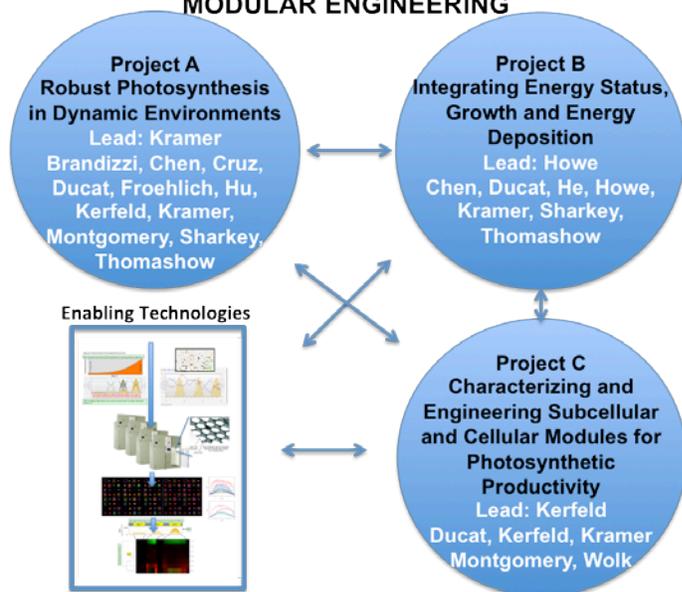


Figure 1. Research Program

Current research is organized around three major themes—“Robust Photosynthesis in Dynamic Environments,” “Integrating Energy Status, Growth, and Energy Deposition,” and “Characterizing and Engineering Subcellular and Cellular Modules for Photosynthetic Productivity” (see Figure 1). The core research group includes 14 faculty members who bring a broad spectrum of formal training, basic research interests, and experimental expertise to

the program, and a team of talented postdoctoral research associates, graduate research assistants, and research technicians. The program includes the development and use of novel high-throughput, non-invasive phenotyping technologies—the Dynamic Environmental Phenotyping Imager (DEPI), the Environmental Photobioreactor (ePBR), and associated data analysis and visualization software—that enable investigators to monitor an array of fundamental photosynthetic properties and growth in

dynamic environments. In addition, the program includes a series of forward-looking lines of investigation to understand the structural and biochemical bases of energy-storing modules using rational, engineering-based approaches. Examples of specific lines of research and recent progress will be presented in a talk at the 2014 Contractors Meeting.

Poster Session II

Role of HydF in Hydrogenase Maturation

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Overall research goals:

The overall goal of this project is to advance our understanding of the mechanism of H-cluster assembly through the use of physical biochemical approaches to characterize key intermediates on the scaffold protein HydF. We will achieve this overall goal through a multi-pronged approach that involves detailed biochemical and biophysical characterization of HydF, characterization of H-cluster biosynthetic intermediates on HydF, and expression of physiologically relevant intermediate states of HydF in a hyperthermophilic host.

Significant achievements (2013-2014):

- We have utilized analytical gel filtration together with a combination of UV-visible, circular dichroism, and electron paramagnetic resonance spectroscopic techniques to show that the iron-sulfur cluster states of HydF change with sample handling and oligomeric state of the protein; our results suggest that the dimeric state of HydF is the most physiologically relevant state during H-cluster precursor assembly.
- We have found that HydF^{EAG} is capable of activating HydA to a small extent, while HydF^{GAE} does not. The nature of the cluster precursor on HydF^{EAG} has not yet been determined.
- We have optimized the overexpression of E302A and D312A variant forms of HydF and have carried out initial spectroscopic (UV-Vis, CD, EPR) analysis. Both variant proteins have also been expressed in a background of HydE and HydG; the purified variant proteins do effect hydrogenase maturation, but it is to a lesser extent than wild type protein.
- We have optimized expression of HydF^{EG} in *E. coli* and have obtained good quality FTIR data on this protein.
- We have shown that HydG synthesizes the CO and CN⁻ ligands of the H-cluster via a radical SAM mechanism involving reversible H-atom abstraction from tyrosine.
- We have succeeded in expressing *Thermotoga maritima* HydF in *Pyrococcus furiosus*, and have obtained an FTIR spectrum of the purified protein showing that it contains an H-cluster precursor.
- We have demonstrated the incorporation of deuterium from a solvent exchangeable thiol into 5'-deoxyadenosine for various molecules during HydE turnover, suggesting that the substrate for HydE contains a thiol functional group. This information coupled to HydE's sequence similarity and genome context network suggest a pathway in which HydE catalyzes the formation of thioformaldehyde which could be joined with ammonia to synthesize a dithiomethylamine (DTMA)-like fragment. Our data suggest that the source of the sulfur atoms in the DTMA bridge of the H-cluster are likely derived from the thiol substrate of HydE.

Science objectives for 2014-2015:

- Examine the effects of HydE and HydG on the oligomeric state of HydF.
- Complete the spectroscopic/functional characterization of dimeric and tetrameric states of HydF.
- Use FTIR, EPR, and Mössbauer to characterize the cluster biosynthetic intermediates present on HydF^{EAG} and HydF^{GAE}.
- Continue our biochemical and spectroscopic analysis of HydF variant proteins and determine the role of the amino acid substitutions in FeS cluster coordination and in the maturation step of hydrogenase.

- Express and purify HydF^{ΔEG}, HydF^{EAG}, HydF^{GAE}, and HydF^{EG} from *Pyrococcus* in larger quantities and begin spectroscopic and functional characterization.
- Examine DTMA bridge formation by HydE and transfer to HydF. Probe the role of HydF in this process.

My scientific areas of expertise are: Bioinorganic chemistry, iron-sulfur clusters in biology, biological radical reactions, radical SAM enzymes.

To take my project to the next level, my ideal collaborator would have expertise in: Mössbauer spectroscopy.

Publications supported by this project 2013-2016:

1. Adam V. Crain, Kaitlin S. Duschene, John W. Peters, and Joan B. Broderick, "Iron-Sulfur Cluster / SAM Enzymes and their Role in Hydrogenase Maturation," *Encyclopedia of Metalloproteins*, **2013**, 1034-1044.
2. Eric M. Shepard, Amanda S. Byer, Kevin D. Swanson, Eric S. Boyd, John W. Peters, and Joan B. Broderick, [FeFe]-Hydrogenase Cofactor Assembly," in *Encyclopedia of Inorganic and Bioinorganic Chemistry*, edited by R.A. Scott. John Wiley & Sons, Ltd: Chichester, UK (**2013**). DOI: [10.1002/9781119951438.eibc2153](https://doi.org/10.1002/9781119951438.eibc2153).
3. David W. Mulder, Michael W. Ratzloff, Eric M. Shepard, Amanda S. Byer, Seth M. Noone, John W. Peters, Joan B. Broderick, and Paul W. King, "EPR and FTIR Analysis of the Mechanism of H₂ Activation by [FeFe]-Hydrogenase HydA1 from *Chlamydomonas reinhardtii*," *J. Am. Chem. Soc.* **2013**, *135*(18), 6921-6929.
4. Rebecca C. Driesener, Benjamin R. Duffus, Ian R. Bruzas, Eric M. Shepard, Natalie Coleman, Alexander P. G. Marrison, Enrico Salvadori, Christopher W. M. Kay, John W. Peters, Joan B. Broderick and Peter L. Roach, "Biochemical and kinetic characterization of radical AdoMet enzyme HydG," *Biochemistry*, **2013**, *52* (48), 8696-8707.
5. Eric S. Boyd, Gerrit J. Schut, Eric M. Shepard, Joan B. Broderick, Michael W. W. Adams, and John W. Peters, "Origin and evolution of Fe-S proteins and enzymes," in *Iron-Sulfur Clusters in Chemistry and Biology*, edited by Tracey Roualt, de Gruyter, **2014**.
6. Jeremiah Betz, Eric M. Shepard, and Joan B. Broderick, "Radical SAM enzymes and their roles in complex cluster assembly," in *Metalloproteins: New insights from theory and experiment with implications for experiments and Challenges to the Theory*, edited by William Goddard and Art Cho, CRC Press, **2014**, *in press*.
7. Joan B. Broderick, Benjamin R. Duffus, Kaitlin S. Duschene, and Eric M. Shepard, "Radical SAM Enzymes," *Chem. Rev.*, **2014**, *114*(8), 4229-4317 (*invited contribution to special issue on Bioinorganic Enzymology*).
8. Joan B. Broderick, Amanda S. Byer, Kaitlin S. Duschene, Benjamin R. Duffus, Jeremiah N. Betz, Eric M. Shepard, John W. Peters, "H-cluster Assembly During the Maturation of [FeFe]-Hydrogenase," *J. Biol. Inorg. Chem.* **2014**, *19*, 747-757.
9. Eric M. Shepard, Florence Mus, Jeremiah N. Betz, Amanda S. Byer, Benjamin R. Duffus, John W. Peters, and Joan B. Broderick, "[FeFe]-Hydrogenase Maturation," *Biochemistry* **2014**, *53*(25), 4090-4104.
10. Amanda S. Byer, Eric M. Shepard, John W. Peters, and Joan B. Broderick, "Radical S-Adenosyl-L-methionine Chemistry in the Synthesis of Hydrogenase and Nitrogenase Metal Cofactors," *J. Biol. Chem.* **2014**, *in press*.
11. Benjamin R. Duffus, Shourjo Ghose, John W. Peters, and Joan B. Broderick, "Reversible H Atom Abstraction by the Radical SAM Enzyme HydG," *J. Am. Chem. Soc.* **2014**, *in press*.

The Molecular Origins of Twist in Cellulose I

Lintao Bu, James Matthews, Michael E. Himmel, and Michael F. Crowley

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Abstract:

The observation of twisted microfibrils in cellulose I both in imaging and in molecular simulations has been reported and studied for years. Other crystalline forms of cellulose do not show evidence of twisting at the microfibril scale. This article reports a computational modeling study of cellulose I twist showing its strong dependence on fibril diameter and no dependence on fibril length or DP. We report the cause of the twist in the model empirically and analytically as the hydrogen bonding that spans the glycosidic linkage. The lack of twist in other forms of cellulose has been explained in other reports and is strengthened by the need for the TG orientation of primary alcohols to form the twist-causing hydrogen bonds. My scientific areas of expertise are computational modeling and biopolymer structural biology. Collaboration with researchers working to improve protein and polysaccharide force fields would significantly enhance my research progress.

Integration of sugar transport, metabolism and sensing in Arabidopsis

Wolf B. Frommer, Principal Investigator

Li-Qing Chen, Postdoctoral Research Associate

Xiao-Qing Qu, Grad. Student; now: Postdoctoral Research Associate

Yuanhu Xuan, Postdoctoral Research Associate, now Professor in China

I W. Lin, Grad. Student, Stanford University

Dep. Plant Biology, Carnegie Institution for Science, 260 Panama St., Stanford CA 94305

Email: wfrommer@carnegiescience.edu; Website: <https://dpb.carnegiescience.edu>

Overall research goals:

Our 10-year goal is to identify the key mechanisms that control carbon allocation in plants and to develop a cellular-resolution map and mathematical model for sugar fluxes between organs that can be used to reliably predict the outcome of genetic modifications in key functions and key regulatory components. To this end, we have created diagnostic tools that enable us to quantify sugar levels and dynamics with cellular and subcellular resolution in a minimally invasive manner. We expanded our tool kit for sensing sugars and created a novel set of sucrose sensors. We will use these sensors *in planta* to provide data for modeling, identify the key players and use them to get at the regulatory networks. We succeeded in identifying the key transporters involved in phloem loading in leaves, nectar secretion, vacuolar sugar accumulation, and seed filling, i.e. SUT sucrose proton cotransporters and SWEET sugar uniporters. Over the past three years, we made massive progress by identifying characterizing and assigning specific roles to SWEETs at specific steps in carbon allocation.

Significant achievements (2010-2014):

- Engineering of fluorescent sensors for glucose and sucrose and optimization of signal-to-noise
- Generation of plant lines expressing the sensors in the cytosol and apoplasm
- Development of an imaging platform based on microfluidics for sensor output
- Identification of a new family of sugar transporters in plants and humans: SWEETs
- Assignment of functions of subsets of SWEETs in phloem loading, seed filling, nectar secretion as well as fructose accumulation in the vacuole
- Identification of bacterial ‘half transporters’ for sugars: SemiSWEETs
- Determination of an oligomeric quaternary structure of the SWEETs and SemiSWEETs
- Crystal structures for two SemiSWEETs in ‘open to outside’ and ‘occluded’ states

Science objectives for 2014-2015:

- Deploy fluorescent sugar sensors for quantifying sugar levels and dynamics in live plants
- Improve imaging modalities for optimal resolution and signal to noise analysis of sugar flux in plants
- Expand analysis of SWEET family and use to redirect fluxes
- Expand biochemical analyses of SWEETs, including analysis of carbon sequestration into the rhizosphere

My scientific areas of expertise are: Plant Science, cell biology, physiology, biochemistry.

To take my project to the next level, my ideal collaborator would have expertise in: ^{11}C flux analysis; photosynthesis imaging, remote imaging, modeling

Publications supported by this project [Click to Enter Years of Current Grant/FWP, e.g. 2012-2014]:

1. Frommer W.B., Cheung L., Chen L.Q & Tanner W. (2014) "Sugar transporters". *Ann. Rev. Biochem.* invited
2. Chen L.Q., Lin I W., Qu XQ, Sosso D., McFarlane H.E., Londoño A., Samuels A.L. & Frommer W.B. (2014) "A cascade three sequentially expressed SWEET sucrose efflux transporters in maternal tissues of the seed coat necessary for embryo nutrition". *Plant Cell*, under review.
3. Xu Y, Tao Y, Cheung L., Fan C., Chen LQ, Xu S., Perry K., Frommer W.B. & Feng L. (2014) "Open and occluded structures of small triple-helical homo-dimeric SWEET transporter homologs". *Nature* in press.
4. Lin I W., Sosso D., Chen L.Q., Gase K., Kim S.G., Kessler D., Klinkenberg P., Qu X.Q., Hou B.H., Carter C., Baldwin I.T. & Frommer W.B. (2014) "The conserved sucrose transporter SWEET9 is key for nectar secretion in Eudicot flowers. *Nature* 508, 546-9." (PMID: [24670640](#))
5. Guo W.J., Nagy R., Chen S.Y., Frommer W.B. & Martinoia E. (2014) "SWEET17, a facilitative fructose transporter in the tonoplast of Arabidopsis roots". *Plant Physiol.* 164, 777-89 (PMID: [24381066](#)).
6. Xuan Y.H., Hu, Y.B., Chen L.Q., Sosso D., Ducat, D.C., Hou B.H. & Frommer W.B. (2013) "Functional role of oligomerization for bacterial and plant sugar transporters of the SWEET family". *Proc. Natl. Acad Sci USA* 110, E3685-94.
7. Schroeder J.I., Delhaize M., Frommer W.B., Guerinot M.L., Harrison M., Herrera-Estrella L., Horie, T., Kochian L., Munns R., Nishizawa N., Tsay Y.F. & Sanders D. (2013) "Using membrane transporters to improve crops for sustainable food production". *Nature* 497, 60-66.
8. Jones A., Grossmann G., Danielson, J.A.H., Sosso D., Chen L. Q., Ho, C.H. & Frommer W.B. (2013) "In vivo biochemistry: Applications for small molecule biosensors in plant biology". *Curr. Opin. Plant Biol.* 16, 389-95.
9. Sosso D., Chen L.Q. & Frommer W.B. (2013) "The SWEET glucoside transporter family: In: *Encycl. Biophysics*, (Roberts G., Ed.), Springer. 5, 2556-2558.
10. Xue W., Ruprecht C., Street N., Hematy K., Chang C., Frommer W.B., Persson T. & Niittylä T. (2012) "Paramutation-like Interaction of T-DNA loci in Arabidopsis". *PLoS One* 7(12), e51651. doi: [10.1371/journal.pone.0051651](#).
11. Grossmann G., Meier M., Cartwright H.N., Sosso D., Quake S.R., Ehrhardt D.W. & Frommer W.B. (2012) "Time-lapse fluorescence imaging of Arabidopsis root growth with rapid manipulation of the root environment using the RootChip". *J Vis Exp.* 65, 4290. doi: [10.3791/4290](#).
12. Chen L.Q., Qu X.Q., Hou B.H., Osorio S., Fernie A.R. & Frommer W.B. (2012) "Sucrose efflux mediated by SWEET proteins as a key step for phloem transport". *Science* 335, 207-211.

Bioenergy Production by a Photosynthetic Bacterium

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Kathryn Fixen, Postdoctoral Research Associate

University of Washington, HSC Rm K340, 1705 NE Pacific St, Box 357735, Seattle, WA 98195-7735

Email: csh5@uw.edu; Website: <https://depts.washington.edu/cshlab/>

Overall research goals:

The development of devices that can capture sunlight and convert it to readily consumable forms of energy is an area of intense interest in the alternative energy arena. Ideally, effective light harvesting devices should be able to 1) capture light with maximum efficiency; 2) maintain this efficiency even when subjected to changes in light intensity caused by shading or the diurnal cycle; 3) be composed of abundant and inexpensive materials; and 4) self-repair or limit damage. Photosynthetic organisms meet these criteria and are ideal models for understanding/developing light harvesting devices and as a platform for biofuel production.

Our research goal is to understand how the synthesis of light harvesting (LH) complexes is regulated in the purple non-sulfur photosynthetic bacterium *Rhodospseudomonas palustris* (Rpal). This species carries out cyclic photophosphorylation under anaerobic conditions and does not generate oxygen. Light harvesting complexes are circular pigment (bacteriochlorophyll/carotenoids)-protein complexes that absorb light and transfer it to a reaction center. The smallest unit is composed of alpha and beta polypeptides (encoded by *puc* genes). Between 8 and 9 of these units form a circular structure that binds photosynthetic pigments in a very specific orientation to promote light energy absorption and transfer to a reaction center. In the reaction center, electrons are energized for use in the production of ATP or of value added products like hydrogen.

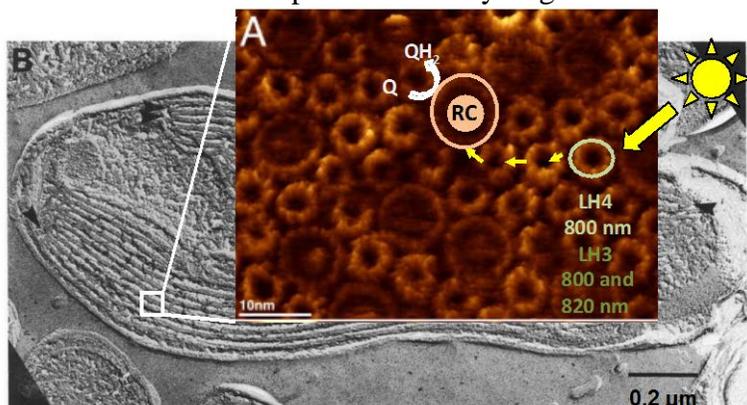


Fig. 1. Freeze fracture EM image of an Rpal cell showing its laminated photosynthetic membranes. Overlain on top (A) is an atomic force microscopy image of light harvesting and reaction center complexes. Light absorption and the flow of energy are shown. A reduced quinone (QH₂) pool is used to generate a protonmotive force for ATP synthesis. [from: Scheuring *et al.* (2006). *J Mol Biol.* 358(1): 83-96. Varga and Staehelin. (1983). *J Bact.* 154(3): 1414-30.]

Significant achievements (2012-2014):

- An analysis of 16 closely related strains of Rpal revealed that most strains have five sets of light harvesting genes. These are designed LH2a, LH2b, LH2e, LH3 and LH4. Two of these gene sets, LH2a and LH4 are expressed at much higher levels under low light conditions.
- Under extremely low light conditions (< 1 μE/m²/s; equivalent to the amount of light coming in through the crack at the bottom of a closed closet door), the LH4 genes are required for optimal growth.
- However, expression of other LH complexes compensates to a certain extent for the lack of LH4.
- We carried out experiments that lead to a model for regulation of LH4 gene (*pucBAD*) expression (Fig 2A).

- We studied in detail two bacteriophytochromes (Bph2/Bph3) that are an essential component of the LH4 signal transduction cascade. These proteins sense light quality to fine-tune LH4 expression and are part of a phosphorelay leading to LH4 gene expression.
- We determined that the protein Rpa3018 is responsible for sensing light intensity and we have evidence that it does so by sensing light intensity as a redox signal (Fig. 2B). We determined that it has two redox sensitive cysteines.

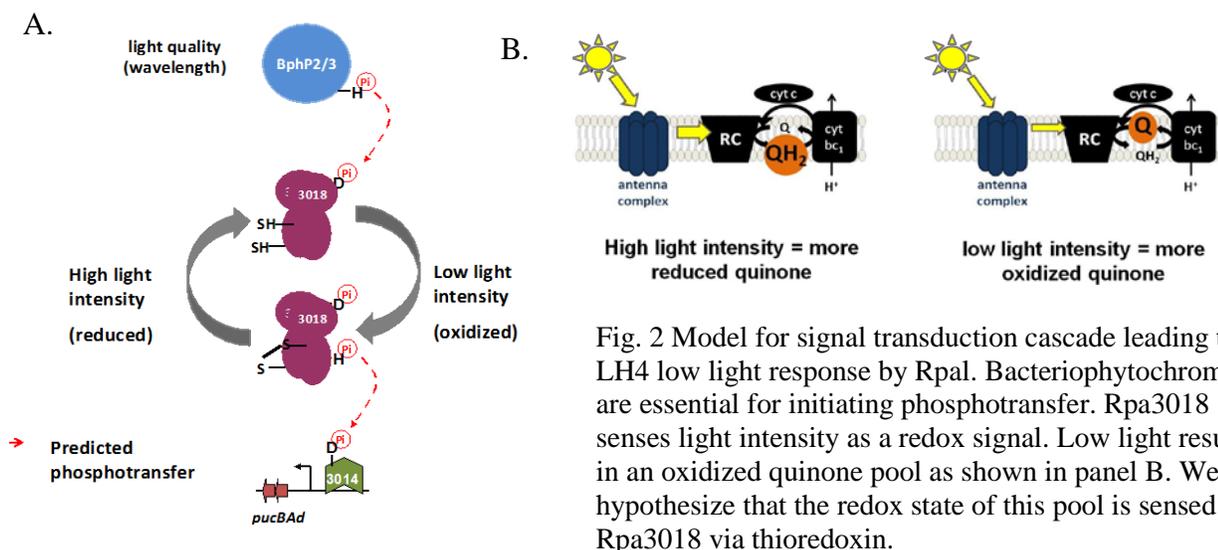


Fig. 2 Model for signal transduction cascade leading to LH4 low light response by Rpa1. Bacteriophytochromes are essential for initiating phosphotransfer. Rpa3018 senses light intensity as a redox signal. Low light results in an oxidized quinone pool as shown in panel B. We hypothesize that the redox state of this pool is sensed by Rpa3018 via thioredoxin.

Science objectives for 2014-2015:

- To show that Rpa3018 senses redox changes *in vivo* as well as *in vitro*. This will allow us to establish a direct link between light intensity, intracellular redox and transcription of the LH4 *puc* operon.
- To investigate the possible role of thioredoxin in reducing Rpa3018.
- To determine if the stability of light harvesting mRNAs is an important contributor to their apparent extremely high levels of expression.

My scientific areas of expertise are: Microbial physiology and molecular biology.

To take my project to the next level, my ideal collaborator would have expertise in: Proteomics.

Publications supported by this project 2013-2014:

1. Fixen KR, A. W. Baker, E. A. Stojković, J. T. Beatty, and C.S. Harwood. 2014. Apo-bacteriophytochromes modulate bacterial photosynthesis in response to low light. *Proc. Natl. Acad. Sci. USA.* 111: E237-44.
2. McKinlay JB, Y. Oda, M. Ruhl, AL Posto, U. Sauer and CS Harwood. 2014. Non-growing *Rhodospseudomonas palustris* increases the hydrogen gas yield from acetate by shifting from the glyoxylate shunt to the tricarboxylic acid cycle. *J Biol Chem.* 289:1960-70.

Utilization of protein film electrochemistry to characterize the mechanisms imparting aerotolerance and bidirectionality in soluble, multimeric [NiFe]-hydrogenases

Anne Katherine Jones, Principal Investigator

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Email: jonesak@asu.edu; Website: <http://bioenergy.asu.edu/faculty/jones/index.html>

Overall research goals:

The goal of this project is to characterize structure/function relationships controlling the reactivity of soluble [NiFe]-hydrogenases (SH) as a model for energetically relevant multielectron redox catalysis. Specific aims are to understand the factors that determine (1) catalytic bias, i.e. ratio of oxidative to reductive catalytic activity and (2) reactions with oxygen and oxygen-tolerance. This fundamental knowledge should prove beneficial to bioengineering efforts in which redox enzymes may be manipulated to perform non-physiological reactions.

Significant achievements (2012-2014):

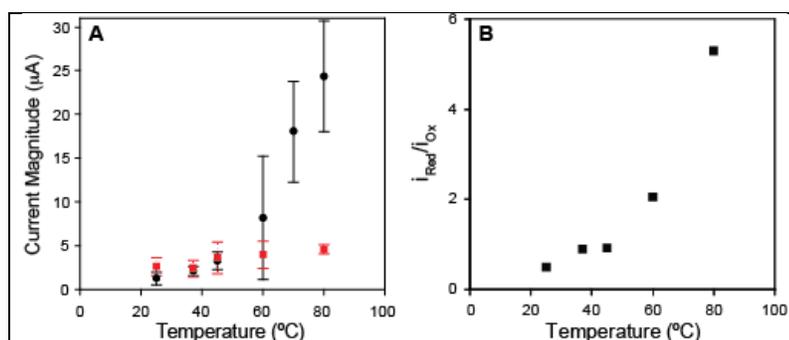


Figure 1: Temperature dependence of (A) H⁺ reduction (black) and H₂ oxidation (red) currents from *PfSHI* adsorbed to a graphite electrode and (B) the ratio of maximal reductive activity to oxidative activity for *PfSHI*. Experiments were performed in a mixed buffer with pH=6.5 at the indicated temperatures.

The electrocatalytic activity of the SHI from *Pyrococcus furiosus*, *PfSHI*, has been characterized under a number of conditions with Prof. Michael Adams (UGA). This showed: (1) the proton reduction activity is highly dependent on temperature such that the bias of the enzyme shifts to strongly favor proton reduction under physiological conditions. This shift arises primarily from an increase in proton reduction activity since H₂ oxidation activity is unchanged; (2) *PfSHI* retains nearly 100% of its proton reduction activity in the presence of 1% oxygen over the entire temperature range investigated; (3) aerobic, oxidative inactivation

results in the formation of two states with distinct redox properties; (4) *PfSHI* retains some hydrogen oxidation activity in the presence of brief bursts of oxygen. This oxygen-tolerance is more pronounced at higher temperatures; (5) hydrogen oxidation activity is also maintained during long (15 minute) exposures to oxygen, but the inactive state requires longer reduction to reactivate.

Science objectives for 2014-2015:

- Characterize the electrocatalytic properties of the hydrogenase subdimer of *PfSHI*. In particular, catalytic bias as a function of temperature and reactivity in the presence of oxygen will be investigated and compared to the holo-enzyme.
- Determine the proton reduction activity of *PfSHI* at high temperature in atmospheres with oxygen content greater than 1 %.
- Purify and electrochemically characterize the SH from *Arthrospira maxima* and compare its properties to *PfSHI* and the more standard, uptake [NiFe]-hydrogenases.

My scientific areas of expertise are: redox chemistry, bioinorganic chemistry, metalloprotein design

To take my project to the next level, my ideal collaborator would have expertise in: Synthetic biology/expression of large gene clusters; culture of unusual microorganisms; surface spectroscopy.

Photobiohybrid Solar Fuels

Paul W. King, Principal Investigator

Katherine A. Brown, Staff Scientist, NREL

David W. Mulder, Staff Scientist, NREL

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Overall research goals:

A long-term objective of this BES-Photosynthetic Systems Program project is to understand energy transduction in photochemical systems that combine the light-harvesting, charge-separation of nanoparticles with redox active enzymes as model complexes for solar energy conversion. Semiconducting nanomaterials exhibit wide spectral responses, high saturation intensities and are promising materials for solar harvesting in artificial photosynthetic schemes. The fundamental mechanisms essential to charge-transfer between nanomaterials and biocatalysts are being addressed to understand energy transduction processes across molecular junctions. A reaction of particular interest for this project has been the photochemical production of H₂ involving hydrogenase enzymes. In photosynthetic microbes hydrogenases couple to low potential reductant pools to help maintain electron flow under anaerobic-aerobic transitions. The ubiquitous role of H₂ as an energy carrier in microbial systems is underscored by significant structural-functional diversity among the different hydrogenase enzyme classes. We are investigating active site coordination environment, substrate transfer pathways and cofactor compositions of hydrogenases towards understanding enzyme catalytic function, and integration with different charge-transfer reactions and molecules.

Significant achievements (2012-2014):

Biophysical analysis of [FeFe]-hydrogenase and the catalytic mechanism. The [FeFe]-hydrogenase from the green alga *Chlamydomonas reinhardtii*, consisting of only the catalytic H-cluster, was analyzed using EPR and FTIR spectroscopy of enzymes poised under reducing and oxidizing conditions. The spectra revealed new paramagnetic signals and IR bands under various reductive treatments. Collectively these results have provided new insights on the electronic structure of the H-cluster, and the basis for a revised catalytic scheme (summarized in Figure 1) for [FeFe]-hydrogenases. The model incorporates electron exchange steps between the two subclusters ([4Fe-4S]_H and 2Fe_H) during enzymatic turnover. We propose that oxidation of the 2Fe_H sub-site by [4Fe-4S]_H helps drive the H₂ binding and activation steps, and initiates the intermolecular electron-transfer reactions to soluble electron carriers.

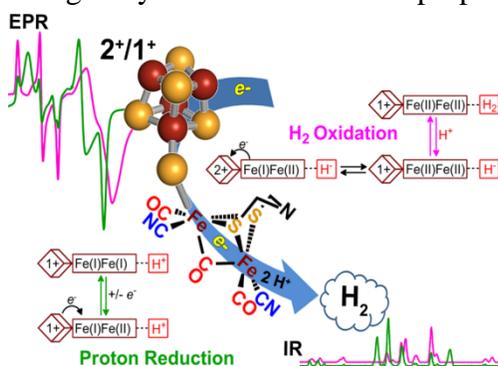


Figure 1. EPR and FTIR spectra of the reduced (green) and H₂ activated (magenta) H-cluster (center) of [FeFe]-hydrogenase; along with the proposed models for reversible catalysis (bottom-left, top-right).

Computational modeling of proton-transfer in [FeFe]-hydrogenase. The free energies along proton-transfer (PT) pathways in [FeFe]-hydrogenase were investigated using QM/MM and umbrella sampling techniques. Key residues, including a H-cluster proximal Cys were identified, along with pK_a estimations from a thermodynamics integration method and used to model the PT profiles to the H-cluster.

Solar energy conversion and catalysis in photobiohybrid complexes. We have shown that the [FeFe]-hydrogenase CaI from *Clostridium acetobutylicum* self-assembles with

nanoparticles (NP) to form photocatalytic complexes. Under illumination, NP light adsorption and charge-separation leads to interfacial electron-injection into the bound CaI via a surface localized [4Fe-4S]-cluster adjacent to the ferredoxin-binding site. Ultrafast measurements showed photoexcited, interfacial electron-transfer from NPs occurs at rates of $\sim 10^7 \text{ s}^{-1}$. We investigated how altering NP diameter, and thus changing charge recombination kinetics and the free-energy (ΔG_{ET}) of electron-transfer (Figure 2), affects electron-transfer and H_2 production in NP-CaI complexes. Surprisingly, the NP \rightarrow CaI electron-transfer step was minimally affected by a lowering of ΔG_{ET} , suggesting a gated, or chemically coupled, interfacial electron-transfer process. As a result, H_2 production rates were largely controlled by competition with rates of recombination, which are slower in larger NPs allowing for higher yields.

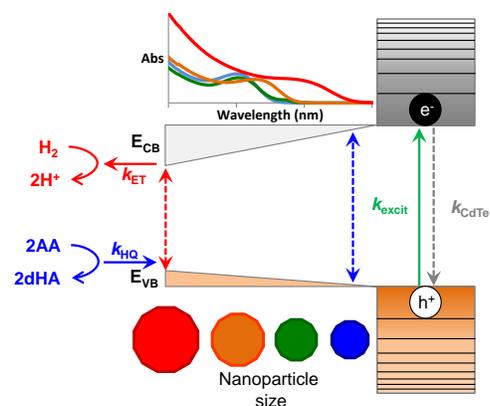


Figure 2. Energy level diagram showing the size dependent processes in CdTe-CaI

Science objectives for 2014-2015:

- Use time-resolved cryo-FTIR to measure the photoreduction of [FeFe]-hydrogenase in NP complexes, and investigate how FeS-cluster properties affect the interfacial electron transfer kinetics.
- Determine the mechanism of electron injection from NP \rightarrow CaI, and the possible role of site-differentiation in the electron-transfer properties of the distal [4Fe-4S] cluster in CaI.
- A Cys \rightarrow Ser substitution of an H-cluster proximal conserved Cys disrupts proton-transfer, and selectively enriches for catalytic states. Future work is aimed at using Mössbauer and pulsed EPR techniques to correlate Fe oxidation levels with subcluster spin-states and coordination spheres towards elucidating the catalytic mechanism of these enzymes.

My scientific areas of expertise are: Spectroscopy, molecular biology, mechanisms of hydrogenases, photochemical biocatalysis.

To take my project to the next level, my ideal collaborator would have expertise in: multi-dimensional spectroscopy of metalloenzymes.

Publications supported by this project 2012-2014:

1. K.A. Brown, M.B. Wilker, M. Boehm, G. Dukovic, P.W. King. "Characterization of photochemical processes of H_2 production by CdS nanorod-[FeFe] hydrogenase complexes." *J. Am. Chem. Soc.* 134(12):5627 (2012).
2. Yacoby, L.T. Tegler, S. Pochekailov, Z. Zhang, P.W. King. "Optimized expression and purification for high-activity preparations of algal [FeFe]-hydrogenase." *PLoS ONE.* e0035886 (2012).
3. D.W. Mulder, M.W. Ratzloff, E.M. Shepard, A.S. Byer, S.M. Noone, J.W. Peters, J.B. Broderick, P.W. King. "EPR and FTIR analysis on the mechanism of H_2 activation by [FeFe]-hydrogenase HydA1 from *Chlamydomonas reinhardtii*." *J. Am. Chem. Soc.* 135(8):6921 (2013).
4. P.W. King "Designing interfaces of hydrogenase-nanomaterial hybrids for efficient solar conversion." *Biochim. Biophys. ACTA-Bioenergetics.* 1827(8-9):949 (2013).
5. H. Long, P.W. King, C.H. Chang. "Proton Transport in *Clostridium pasteurianum* [FeFe] Hydrogenase I: A Computational Study." *J. Phys. Chem. B.* 118(4):890 (2104).
6. M.B. Wilker, K.E. Shinopoulos, K.A. Brown, D.W. Mulder, P.W. King, G. Dukovic. "Electron transfer kinetics in CdS nanorod-[FeFe] hydrogenase complexes and implications for photochemical H_2 generation." *J. Am. Chem. Soc.* 136(11):4316 (2014).
7. K.D. Swanson, M.W. Ratzloff, D.W. Mulder, J. Artz, S. Ghose, A. Hoffman, S. White, O.A. Zadovnyy, J.B. Broderick, B. Bothner, P.W. King, J.W. Peters. "[FeFe]-hydrogenase oxygen inactivation is initiated by the modification and degradation of the H cluster 2Fe subcluster." *J. Am. Chem. Soc.* Submitted.
8. K.A. Brown, Q. Song, D.W. Mulder, P.W. King. 2014. "Diameter dependent electron transfer kinetics in semiconductor-enzyme complexes." *ACS Nano.* Submitted.

Transmethylation reactions during methylotrophic methanogenesis in methanogenic Archaea

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Overall research goals:

Most methane produced from biological sources comes from methanogenic Archaea. Of these organisms, *Methanosarcina* spp. and their relatives have the most diversified substrate range. Our overall goal is to understand the enzymes and molecular biology underlying these methanogenic pathways. We have primarily focused on methanogenesis from monomethylamine (MMA), dimethylamine, and trimethylamine (TMA). Methanogenesis from these substrates is began by three methyltransferases (respectively, MtmB, MtbB, and MttB) that methylate cognate corrinoid proteins, which are then used to methylate coenzyme M, forming the direct precursor of methane. The methylamine methyltransferases are non-homologous, yet each of their encoding genes contains an in-frame UAG codon. Metabolism of methylamines obligately depends on translation of an amber codon as the 22nd amino acid, pyrrolysine (Pyl). In previous periods, we have made inroads into understanding how pyrrolysine is biosynthesized, and how it is genetically encoded. Our current focus is to examine the function of pyrrolysine in the methylamine methyltransferases. Our operating hypothesis has been that this amino acid acts to bind and orient methylamines for methyl transfer to the Co(I)-corrinoid protein. Our approaches include site directed mutagenesis of Pyl-containing methyltransferases, study of naturally occurring homologs lacking Pyl, and physical methods to detect pyrrolysine interaction with methylamines.

Significant achievements (2013-2014):

Function of non-Pyl MttB homologs: A major conundrum has been the presence of a large number of homologs of MttB (the TMA methyltransferase) in the microbial genome databases that lack pyrrolysine. In the previous period we, in collaboration with the Ferguson laboratory, began work with DSY3156, a non-Pyl MttB homolog in *Desulfitobacterium hafniense*. Work from my lab has in this period shown DSY3156 demethylates glycine betaine with cob(I)alamin at near unit stoichiometry. We also demonstrated that *D. hafniense* cells use glycine betaine to produces one CO₂ and one dimethylglycine, dependent on the presence of an external electron acceptor. We have now shown that DSY3157, an adjacently encoded protein to DSY3156, is a methylcobalamin:tetrahydrofolate methyltransferase, adding further support to our hypothesis that this non-Pyl MttB homolog acts to move a methyl group into the THF oxidation pathway to generate reducing power for growth at the expense of an external electron acceptor. A revised manuscript was recently sent back to consideration at PNAS.

DSY3156 lacks the ability to use TMA, unlike methanogen Pyl MttB enzymes. We have proposed that the active site of the glycine betaine methyltransferase could be remodeled to that of the TMA methyltransferase. If so, this would demonstrate pyrrolysine essentiality for TMA dependent methylation and uniquely reveal the functional relationship between members of this large family of proteins. As a pre-requisite to this, we predicted residues of the glycine betaine methyltransferase that would bind glycine betaine. We have now performed site directed mutagenesis with these residues, and found that these mutations drastically reduce activity of the protein. Furthermore, we collaborated with Dr. Bing Hao who crystallized the protein in complex with glycine betaine. The preliminary structure indicates that each of our predicted binding site residues can be seen in contact with the substrate, and that these do form the binding site for glycine betaine. An overlay of the structures of the pyl-MttB and the glycine betaine methyltransferase reveals that pyrrolysine occupies a position overlapping the glycine betaine binding residues of DSY3156

Site directed mutagenesis of pyrrolysine in the Pyl-MttB active site. Traditionally, we have assayed MttB activity indirectly as part of a three component assay in which coenzyme M consumption was monitored. A much more direct and sensitive assay is the methylation of the cognate corrinoid protein, which we had not previously achieved with the TMA methyltransferase. This assay requires sources of purified MttB and MttC (the TMA cognate corrinoid protein), as well as an activation protein termed RamA which reduces the corrinoid protein to the Co(I) state required for methyl transfer. We have now succeeded in obtaining purified recombinant MttC and MttB by expression of the *Methanosarcina barkeri* genes in *Methanosarcina acetivorans*. Additionally, we now have a robust tagged version of RamA made in *E. coli* which is very stable. We have now assayed MttB and obtained a production rate of 4 micromoles methyl-MttC min⁻¹•mg, an apparent turnover rate of 200 min⁻¹.

We currently have in hand five stable MttB variants in which pyrrolysine is substituted with other residues. We have now measured the activity of two of these and found they possess approximately 1000-fold activity relative to wild type MttB. This is consistent with an important catalytic role for pyrrolysine in the TMA methyltransferase reaction. We are currently investigating the residual activity in the variant enzymes, although slow, the rate is 10-fold higher than our lower limit of detection in this assay. The small amount of residual activity in the MttB without pyrrolysine may be due to the presence of trace amounts of co-purifying wild type MttB enzyme from the *M. acetivorans* host, but if due to the mutant enzyme, might dictate re-evaluation of the current model for pyrrolysine function.

We have in hand an unpublished crystal structure of MttB solved by the Michael Chan group in collaboration with us. The structure reveals no residue functional groups within H-bonding distance of pyrrolysine in the TMA methyltransferase, quite unlike the MMA methyltransferase that is in H-bonding distance of several residues. However, a water molecule is within H-bonding distance to the imine nitrogen of pyrrolysine. The water is further H-bonded to the hydroxyl of a tyrosine residue. The Tyr-water combination could serve to protonate the imine nitrogen upon formation of a TMA adduct with the imine carbon of pyrrolysine. If so we predict a drop in activity should the tyrosine be mutagenized to phenylalanine. We made this mutant, and found the Y:F substitution results in an enzyme with six percent of wild type activity, consistent with a role in enabling protonation of the imine nitrogen.

Science objectives for 2014-2015:

- Further testing of pyl mutations in MttB. Identification of source of trace residual activity.
- Active site remodeling of the glycine betaine methyltransferase to include pyrrolysine.
- Recombinant expression of MtmC for MtmB assays and effects of active site mutagenesis.
- Completion of isothermal calorimetry tests for binding of MMA to wild type MtmB and variants lacking pyrrolysine.

My scientific areas of expertise are: Metabolism and biochemistry of anaerobic Archaea and Bacteria. Enzyme identification, isolation, and characterization. Methanogenesis. Corrinoid dependent methyltransferases. Microbial methylamine metabolism. Genetic encoding and biosynthesis of pyrrolysine. Practical genetics in methanogens.

To take my project to the next level, my ideal collaborator would have expertise in: NMR, mass spectrometry, crystallography.

Publications supported by this project [2013-2014]:

1. Ticak, T., D. J. Kountz, K.E. Girosky, J.A. Krzycki, and D. J. Ferguson. 2014. A non-pyrrolysine member of the widely distributed trimethylamine methyltransferase family is a glycine betaine methyltransferase. in review.
2. Krzycki, J.A. 2013. The path of lysine to pyrrolysine. *Curr. Opin. Chem. Biol.* 17:619-625.

A Holistic Approach to Lignification, Lignin Primary Structures and Associated Metabolism (DE-FG-0397ER20259)

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Laurence B. Davin, Co-PI(s)

Doralyn S. Dalisay, Assistant Research Professor

Syed G. A. Moinuddin, Postdoctoral Research Associate

Joaquim V. Marques, Postdoctoral Research Associate

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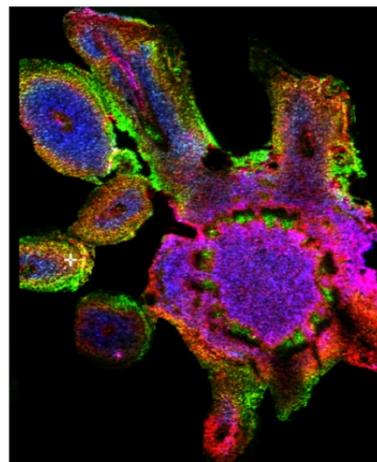
Overall research goals:

There are three specific objectives in this proposal, with only the first two objectives being the subject of this reporting cycle:

- i. To further develop cell specific metabolomic, proteomic, and transcriptomic approaches for a holistic determination of distinct cell wall forming processes involved in lignin biosynthesis and related metabolism in both wild type and mutant/genetically modified plant lines.
- ii. To further enable determination of lignin primary structures/assembly mechanisms via comparison of cell-wall specific, wild type (native), and genetically modified lignins.
- iii. To further correlate lignin primary structures with biophysical properties conferred in distinct vascular plant cell wall types.

Significant achievements (2012-2014):

1. Developed MALDI TOF metabolite imaging approaches *in situ* to optimize the study of lignin/lignan/allyl-propenyl phenol, phenyl ethanol, and alkaloid forming biochemical processes at the individual cell type/tissue level in various plant species, with the initial focus being on detection and characterization of distinct metabolite classes and pathway intermediates in specific cell types/tissues. For example, the MALDI-TOF metabolite imaging approach was developed to successfully map out spatial localization of biochemical pathway intermediate metabolites in formation and deposition of oligomeric lignan-secoisolariciresinol diglucoside hydroxymethyl glutaryl ester complexes in developing flax seed coats.
2. Discovered hitherto unknown relationships between photosynthesis and lignin reduction in Arabidopsis wild type and arogenate dehydratase mutant lines, in terms of both effects on photosynthetic efficiency and overall metabolism.
3. Next generation sequencing, metabolomics, laser micro-dissection together with RT-PCR, and bioinformatics approaches in study of lignin/lignan biochemical pathways resulted in deduction of hitherto unknown but biochemically related pathways and their evolutionary significance. Investigated and correlated gene expression patterns with sites of metabolite accumulation determined by metabolite imaging.
4. Developed targeted and untargeted metabolomics approaches to comprehensively establish effects of manipulating lignin and related biochemical pathways on both phenylpropanoid and overall metabolism in greenhouse and field grown poplar.
5. Applied proteomics/metabolomics/transcriptomics to compare and contrast “big data” from same through modulating lignin contents in poplar and Arabidopsis.
6. Mass spectrometry approach being developed for sequencing lignins and other oligomeric lignans, with polystyrene utilized for methods development. Together with using the Kendrick mass



Overlaid MALDI images of podophyllotoxin (m/z 453.0969, red), podophyllotoxin glucoside (m/z 615.1496, blue) and kaempferol 3-*O*-(6''-*O*-malonyl)-glucoside (m/z 573.0660, green) in *P. hexandrum* rhizome (15 μ m thickness)

defect analysis approach, we were able to identify conditions for sequential “peeling” of the polymer, one monomer at a time, and to identify the various modes of fragmentation.

7. Graduate student Ms. Kim Hixson was awarded the Withycombe-Charalambous Graduate Student Symposium Award from the Agricultural and Food Chemistry Division at the American Chemical Society national meeting in Dallas for her “integrated omics” approaches.
8. N.G. Lewis was selected as a 2014 Fellow of the American Society of Plant Biologists.
9. N.G. Lewis was selected as a 2014/2015 Fulbright Professor (Science without Borders, Brasil), with an emphasis on biofuels and specialty chemicals.

Science objectives for 2014-2015:

The three specific objectives, remain unchanged:

1. To further develop cell specific metabolomic, proteomic, and transcriptomic approaches for a holistic determination of distinct cell wall forming processes involved in lignin biosynthesis and related metabolism in both wild type and mutant/genetically modified plant lines.
2. To further enable determination of lignin primary structures/assembly mechanisms via comparison of cell-wall specific, wild type (native), and genetically modified lignins.
3. To further correlate lignin primary structures with biophysical properties conferred in distinct vascular plant cell wall types.

My scientific areas of expertise are: Phenylpropanoid pathway metabolism; cell wall deposition; lignification; lignan biosynthesis; dirigent proteins; metabolite imaging *in situ* in plants; transgenic plant lines for biofuels/specialty chemicals; phytochemistry and natural products (all fields); metabolomics; proteomics; transcriptomics.

To take my project to the next level, my ideal collaborator would have expertise in: My laboratory currently has a number of collaborations underway at three National labs. At PNNL, we have two EMSL supported projects to study dirigent protein structure and mechanism (with J. Cort) and proteomics/transcriptomics (with K. Hixson and M. Lipton) of lignin and related biochemical pathways in *Arabidopsis* and poplar. In addition, metabolite imaging collaborations are underway (with Ben Bowen) at LBNL using their OpenMSI interactive web-based imaging system in order to enhance data resolution and have in place a user-friendly interactive system to probe metabolic processes in intact plant tissues in real time.

My ideal collaborator currently would be in linking photosynthetic approaches to our “downstream” metabolism (e.g. to plant cell wall metabolism, production of biofuels/specialty chemicals), in order to identify fully the chemistry and biochemistry impacting carbon allocation/re-allocation. This is currently being pursued (with no funding with Richard Sayre at NMC/LANL and Helmut Kirchoff, WSU).

Publications supported by this project 2012-2014:

1. Corea, O.R.A., Ki, C., Cardenas, C.L., Kim, S.J., Brewer, S.E., Patten, A.M., Davin, L.B., and Lewis, N.G. 2012. Arogenate dehydratase isoenzymes profoundly and differentially modulate carbon flux into lignins. *J. Biol. Chem.* **287**: 11446-11459. <http://dx.doi.org/10.1074/jbc.M111.322164>
2. Corea, O.R.A., Ki, C., Cardenas, C.L., Davin, L.B., and Lewis, N.G. 2012. Laser microdissection and genetic manipulation technologies to probe lignin heterogeneity and configuration in plant cell walls. *Methods Mol. Biol.* **908**: 229-250. http://dx.doi.org/10.1007/978-1-61779-956-3_20
3. Corea, O.R.A., Bedgar, D.L., Davin, L.B., and Lewis, N.G. 2012. The arogenate dehydratase gene family: Towards understanding differential regulation of carbon flux through phenylalanine into primary *versus* secondary metabolic pathways. *Phytochemistry* **82**: 22-37. <http://dx.doi.org/10.1016/j.phytochem.2012.05.026>
4. Lee, C., Bedgar, D.L., Davin, L.B., and Lewis, N.G. 2013. Assessment of a putative proton relay in *Arabidopsis* cinnamyl alcohol dehydrogenase catalysis. *Org. Biomol. Chem.* **11**: 1127-1134. <http://dx.doi.org/10.1039/C2OB27189C>
5. Marques, J.V., Dalisay, D.S., Yang, H., Lee, C., Davin, L.B., and Lewis, N.G. 2014. A multi-omics strategy resolves the elusive nature of alkaloids in *Podophyllum* species. *Molecular BioSystems*: (Manuscript in press). <http://dx.doi.org/10.1039/c4mb00403e>
6. Seneviratne, H.K., Dalisay, D.S., Kim, K.-W., Moinuddin, S.G.A., Yang, H., Davin, L.B., and Lewis, N.G. 2014. Non-host disease resistance response in pea (*Pisum sativum*) pods: Biochemical function of DRR-206 and phytoalexin pathway localization. *Phytochemistry*: (Manuscript submitted).

Chemo/Mechanical Energy Conversion via Supramolecular Self-Assembly

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Emory University, Departments of Chemistry and Biology

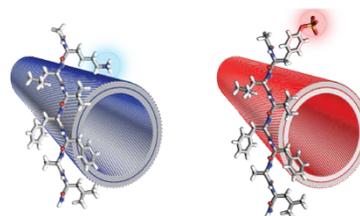
1515 Dickey Drive, Atlanta, GA 30322

<http://www.chemistry.emory.edu/faculty/lynn/index.html>; <http://conticellolab.wordpress.com/>

Overall Goals: A desirable design for the creation of synthetic meso-scale devices would retain the favorable properties of self-association that characterize biological systems, while allowing expansion of its functional capacity. This fruitful collaboration between **Emory University** and the **Argonne/Oak Ridge/Brookhaven National Laboratories** has been to development methods for directing the assembly of synthetic oligopeptides into nano-scale objects with a degree of order approaching that observed in biological systems. We have designed and engineered diverse functionality within and along the contour length of a series of peptide-based supramolecular assemblies. Two distinct yet complementary self-assembling peptide nanostructures based on cross- β and α -helical nanotubes are employed. We are using these frameworks for developing catalytic templates for oligomerization and polymerization reactions, sequestering specific proteins for array functionality, organizing antenna complexes for light harvesting and energy transfer, and defining nanoscale actuators that mirror the complex biochemical cycle of physical and chemical energy inter-conversion. These achievements are fundamental to creating simple bio-inspired supramolecular assemblies capable of emulating and extending critical components for physical/chemical/mechanical energy inter-conversion.

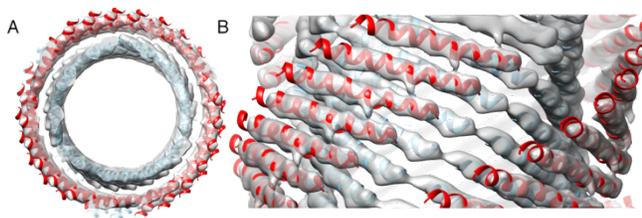
Scientific Achievements (2014-2014). Our overriding hypothesis has been that organized arrays of functionalized nano-scale structures created from peptide self-assembly can serve as platforms for the creation of unique nano-scale devices. We have made great progress in extending the range of structures available (1-3) and accomplished a critical step of emulating natural photosynthesis by directing our peptide assemblies to organize molecules into both covalent and non-covalent arrays and used them to functionally transfer light energy. Fluorescence lifetime image micrograph (FLIM) reveals remarkable control over distinct surface domains, and we remain interested in information storage and readout inherent in the lifetime transitions encoded on the nanotube surface (4).

We have established methods for associating proteins specifically to the surfaces of these nanotubes (5) and are now positioned to extend these associations selectively to the internal and external surfaces selectively for nanoscale resolution of positioning of functional elements.



to

Science Objectives: Our specific aims then attempt to reach a balance with hybrid systems based on native biological assembly motifs that incorporate functional capacity beyond that observed in nature, and yet whose principle functions may be re-introduced into biology. Building from the bottom up on the resources acquired through previous funding cycles, we propose to:



Aim 1. Construct functional surfaces: Prepare α -helical and cross- β peptide nanotubes with precisely patterned registries and functional internal and external surfaces,

Aim 2. Extend surface reactions that optimize diverse surface reactions capable of creating chemical gradients across the surfaces,

Aim 3. Demonstrate chemo/mechanical movement: Combine smaller nanotube assemblies capable of templating the assembly of larger tube assemblies with the specifically patterned and functional surfaces for directional control.

Our structural models have drawn inspiration from the assemblies associated with functional protein-based nanotubes of bacterial organisms, such as the flagellar filament, type IV pilus, filamentous phage capsids, and type III secretion system needle. It has not escaped our attention that the use of peptide materials and the availability of high-resolution structural data affords the unprecedented opportunity to create protein-based nanomaterials of precisely defined structure and controllable function that can be engineered into cellular networks.

Scientific Areas of Expertise: Biophysical Chemistry, Structural Biology, Molecular Biology, Chemical Evolution

Ideal Collaborator: Microbial / Plant Molecular Biologist, Synthetic Biology

Publications completed by this project in 2014.

1. Q Guo, AK Mehta, MA Grover, W Chen, DG Lynn, Z Chen **2014**. Shape selection and multi-stability in helical ribbons, *Applied Physics Letters* 104, 211901. <http://dx.doi.org/10.1063/1.4878941>

2. T Jiang, C Xu, Y Liu, Z Liu, JS. Wall, X Zuo, T Lian, K Salaita, C Ni, D Pochan, and VP. Conticello 2014 Structurally Defined Nanoscale Sheets from Self-Assembly of Collagen-Mimetic Peptides *J. Am. Chem. Soc.* 136, 4300–4308. DOI: 10.1021/ja412867z

3. T Jiang, C Xu, X Zuo, VP. Conticello 2014 Structurally Homogeneous Nanosheets from Self-Assembly of a Collagen-Mimetic Peptide, *Angew Chem Inter Edit*, 53, 8367-8371 DOI: 10.1002/anie.201403780

4. NR Anthony, AK Mehta, DG Lynn, KM Berland 2014 Mapping amyloid- β (16-22) nucleation pathways using fluorescence lifetime imaging microscopy, *Soft Matter*, 2014, 4162-4172. DOI: 10.1039/C4SM00361F

5. S Li, AN Sidorov, AK Mehta, AJ Bisignano, D Das, WS Childers, E. Schuler, Z Jiang, TM Orlando, K Berland, DG Lynn, **2014**. Neurofibrillar Tangle Surrogates: Histone H1 Binding to Patterned Phosphotyrosine Peptide Nanotubes, *Biochemistry* 53, 4225–4227. DOI: [10.1021/bi500599a](http://dx.doi.org/10.1021/bi500599a)

Multifunctional Ubiquitin-fold Proteins of Archaea

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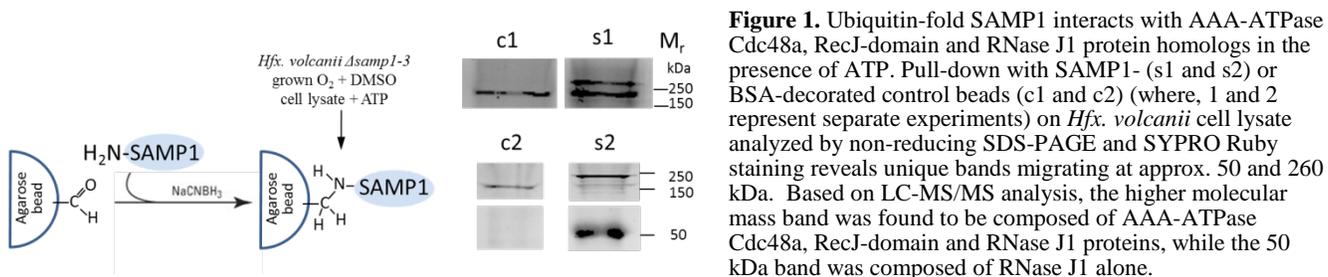
Overall research goals:

The overall research objective is to study ubiquitin-proteasome system (UPS) homologs with the long term goal to use these systems to control metabolic function for optimal production of renewable fuels and chemicals in extremophilic biocatalysts. This research project is focused on advancing fundamental knowledge related to the molecular mechanisms of UPS homologs that modulate protein abundance, enzyme activity and the biosynthesis of sulfur-containing biomolecules in Archaea, a domain with a richness of extremophile microbial diversity. The specific objectives of this project are to: (1) determine the molecular factors that regulate the switch between sulfur mobilization and protein modification for the UPS homologs including the multifunctional ubiquitin-activating E1-like enzyme UbaA and ubiquitin-fold SAMPs of the halophilic archaeon *Haloferax volcanii* and (2) ascertain the biological roles for isopeptide linkage of SAMP2 to its protein target TATA-binding protein 2 (Tbp2), which appears linked to modulating the levels of this protein in the cell through an ancient N-end rule pathway. Extending study of the UPS to the archaeal homologs should reveal novel insights into post-translational mechanisms that modulate metabolic pathways in extremophilic biocatalysts. These findings may perhaps lead to use of these organisms as platforms for higher rates of energy-related bioconversion processes with enhanced stabilities compared to current technologies.

Significant achievements (2014-2016):

Initial investigations utilized pull-down experiments with the ubiquitin-like SAMP1 as bait to ascertain cellular interaction partners which may regulate function of the UPS homologs of Archaea. For this purpose, SAMP1 was purified from recombinant *Escherichia coli*, immobilized on beads by amide linkages, and incubated with cleared cell lysate of *Hfx. volcanii* in the presence of ATP. Analysis of the proteins retained by the SAMP1-decorated beads revealed a prominent protein band of ~250 kDa that was detected by SYPRO Ruby staining of non-reducing SDS-PAGE gels (Fig. 1). Subsequent analysis by LC-MS/MS of tryptic peptides derived from this band identified four proteins that were unique to the samples including a Cdc48-type AAA ATPase, RNase J1 and RecJ-domain protein homologs. This finding was experimentally reproducible and reveals novel proteins that interact with UPS homologs of Archaea in the presence of ATP.

In addition to interacting partners, our work provides evidence for an ancient N-end rule pathway of Archaea and that is related to eukaryotes in its incorporation of N-terminal and penultimate residue degrons that stimulate ubiquitin-like modification and subsequent degradation of proteins by proteasomes. This working model is based on our identification of post-translational factors that modulate the levels of Tbp2 in *Hfx. volcanii* including: (1) phosphorylation of the penultimate serine residue of Tbp2, (2) methionine aminopeptidase cleavage of Tbp2, and (2) acetylation of Tbp2 or another protein factor by the GNAT acetyltransferase encoded in genome synteny with SAMP2.



Science objectives for 2014-2015:

- Determine molecular factors that regulate the switch between sulfur mobilization and protein modification for the multifunctional ubiquitin-activating E1-like UbaA and ubiquitin-fold SAMPs of *Hfx. volcanii*. As discussed above, we have already identified four proteins that associate with the ubiquitin-fold SAMP1 through non-covalent linkages in the presence of ATP. Similar methods have also been used to identify protein factors that associate with the E1-like UbaA (data not shown). To further understand these findings, the interacting proteins will be epitope tagged, purified from *Hfx. volcanii*, and analyzed for non-covalent associations with other protein factors including the UPS homologs SAMP1 and UbaA. Mutant strains deficient in production of the interacting proteins will be generated and analyzed for function of SAMP-mediated sulfur mobilization and protein conjugation based on established phenotypic and biochemical assays.
- Ascertain biological roles for the isopeptide linkage of multifunctional ubiquitin-fold proteins to their protein targets using SAMP2 and the target substrate TATA-binding protein 2 (Tbp2) as a model system. To initiate this objective, we will determine whether Tbp2 is required for optimal growth under different culture conditions through generation of a conditional depletion strain (based on traditional gene deletion methods, Tbp2 appears essential for growth). Pulse-chase analysis of wild-type and mutant strains will be used to determine whether the archaeal UPS homolog SAMP2 influences the stability of Tbp2. Covalent modification(s) of Tbp2, which may alter sanylation and subsequent proteasome-mediated degradation, will also be analyzed by quantitative LC-MS/MS.

My scientific areas of expertise are: microbiology, molecular biology, genetics, biochemistry and proteomics with emphasis on study of microorganisms of the domain Archaea.

To take my project to the next level, my ideal collaborator would have expertise in: quantitative proteomics and physical biochemical techniques including isothermal titration calorimetry and x-ray crystallographic analysis of proteins and protein partners.

Publications supported by this project 2014-2016:

- [1] Chavarria, NE, S Hwang, S Cao, X. Fu, M Holman, D Elbanna, S Rodriguez, D Arrington, M Englert, S Uthandi, D Söll and JA Maupin-Furlow. 2014. Archaeal Tuc1/Ncs6 homolog required for wobble uridine tRNA thiolation is associated with ubiquitin-proteasome, translation, and RNA processing system homologs. *PLoS One* 9:e99104.
- [2] Prunetti, L, CJ Reuter, NL Hepowit, Y Wu, L Barrueto, HV Miranda, K Kelly and JA Maupin-Furlow. 2014. Structural and biochemical properties of an extreme ‘salt-loving’ proteasome activating nucleotidase from the archaeon *Haloflex volcanii*. *Extremophiles* 18:283-293.
- [3] Miranda, HV, H Antelmann, N Hepowit, NE Chavarria, DJ Krause, JR Pritz, K Bäsell, D Becher, MA Humbard, L Brocchieri and JA Maupin-Furlow. 2014. Archaeal ubiquitin-like SAMP3 is isopeptide-linked to proteins by a UbaA-dependent mechanism. *Mol Cell. Proteom.* 13:220-39.
- [4] Maupin-Furlow, J. 2014. Prokaryotic ubiquitin-like protein modifiers. *Annu Rev Microbiol* 68:155-175.
- [5] Shively JM, GC Cannon, S HeinHorst, JA Fuerst, DA Bryant, E Gantt, JA Maupin-Furlow, D Schüler, F Pfeifer, R Docampo, C Dahl, J Preiss, A Steinbüchel, BA Federici. 2014. Intracellular structures of prokaryotes: inclusions, compartments and assemblages. *Encyclopedia of Microbiology*. Elsevier Press, St. Louis, Mo.
- [6] Maupin-Furlow, J. 2014. Cell Regulation by Proteolytic Systems and Protein Conjugation. In A. Oren and R.T. Papke. (Eds.) *Evolution of Halophiles: Genomes and Genetics*, Horizon Press, Norwich, UK.

Functional Analysis and Genetic Manipulation of Plant ABCB Organic Ion Transporters

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Overall research goals:

The subject of this project is the elucidation of the structural determinants of substrate specificity of plant ABCB/PGP transporters. ABC transporters have been shown to be primary transporters of plant hydrophobic and phenylpropanoid compounds that are primary resource materials for biofuel conversion. The G subclass of these transporters exhibits broader substrate specificity similar to what is seen in the B subclass of xenobiotic exporters in animals. The plant B subclass transports a much more restricted range of substrates. Some isoforms, best represented by ABCB4 from Arabidopsis, function as conditional importers. The ultimate goal of the project is to be able to design custom transporters of plant compounds that have utility for biofuel development and to develop regulatory regimens that are direct and implementable in crop plants. The model transporters that have been analyzed in this project are the highly specific ABCB19 auxin transporter, the ABCB14 malate/citrate transporter, and the ABCB4 conditional auxin transporter.

The strategy has been to express mutated forms of these proteins in yeast and then express the mutant forms in knockout lines of Arabidopsis to verify altered function. Unexpectedly, expression of fusions of these models under the control of global, constitutive promoters results in distributions identical to those seen with native promoters. Another project in the lab directed at stress responses (not funded by DOE) had shown that some *ABCB* expression is positively regulated by abscisic acid (ABA) via the ABI4 transcription factor, but that *ABCB4* abundance was rapidly reduced after treatment with ABA. Exploration of that phenomenon in this project showed that ABA treatment did not reduce ABCB4 abundance in the *abi4* background, suggesting an ABI4-regulated post-translational regulatory mechanism. Further ABCB4 was shown to cycle at the plasma membrane via an endomembrane compartment marked by SYP61. ABI4 was subsequently found to directly regulate expression of *Aspartyl Protease A2 (APA2)*. Loss of *ABCB4* results in reduced lateral root formation and shootward transport from the root apex, while *apa2* and *abi4* mutants exhibit enhanced lateral root formation and increased shootward IAA transport. ABCB4 was found to be significantly more stable in *abi4* and *apa2* following ABA, cytokinin and auxin treatment. Recombinant APA2 specifically cleaves ABCB4 *in vivo* and *in vitro*. APA2 localizes to the plasma membrane and *trans* Golgi network, suggesting that these membranes are sites of ABCB4 cleavage by APA2. APA2 was shown to mediate sorting of ABCB4 the PVC. Inclusion of a discrete sequence in an otherwise conserved extracellular loop of ABCB transporters results in APA2 cleavage. These results indicate the presence of an unanticipated mechanism that functions in ABA and cytokinin-dependent regulation of plasma membrane proteins. As APA2 is highly conserved across plant species, these results provide a mechanism for engineered regulation of plasma membrane transporter abundance.

Science objectives for 2014-2015:

- Complete mutational and domain swapping analysis expression in Lactococcus, yeast, Arabidopsis
- Produce sufficient quantity of pure ABCB19 in insect cells to grow crystals using non-detergent solubilization methods

My scientific areas of expertise are: Cell Biology, Membrane Transporters, Hormone Biology

To take my project to the next level, my ideal collaborator would have expertise in: 1. Lipid analysis and use of labeled lipids. 2. Protein crystal growing and crystallography

Publications supported by this project (2012-2014):

1. Shkolnik-Inbar D, Murphy AS (2014) The Arabidopsis ABCB4 auxin transporter is regulated by ABA and cytokinin via ABI4 and an endosomal aspartyl protease. *in review*
Note: work on ABA mediation of ABCB4 transcription mediated by the ABI4 transcription factor was funded by a BARD fellowship to Dr. Doron Shkolnik. Work on posttranslational regulation of ABCB transporters by the APA2 aspartyl protease was funded by DOE-BES
2. Tapken W, Jenness M, Yang H, Reemmer J, Carraro N, Kim Y, Blakeslee JJ, Zhang J, Peer WA, Murphy AS (2014) Loss of Multiple ABCB Transporters Clarifies their Functional Relationship with FKBP42. *in review*
3. Spartz AK, Park M, Ren H, Grandt K, Lee SH, Murphy AS, Gray W (2014) SAUR inhibition of PP2C-D phosphatases activates plasma membrane H⁺-ATPases to promote cell expansion in Arabidopsis. *Plant Cell*, *in press*
4. Marhavý P, Duclercq J, Weller B, Feraru E, Offringa R, Friml J, Schwechheimer K, Murphy M, Benková E (2014) Cytokinin controls polarity of PIN1-dependent auxin transport during lateral root organogenesis. *Current Biology*, 24:1031-1037
5. Yang H, Murphy AS (2013) Membrane Preparation, Sucrose Density Gradients and Two-phase Separation Fractionation from Five-day-old *Arabidopsis* seedlings. *Bio-protocols*. 1.12.14
6. Sze H, Geisler M, Murphy AS (2013) Linking evolution of plant transporters to their functions. doi: [10.3389/fpls.2013.0054](https://doi.org/10.3389/fpls.2013.0054)
7. Yang H, Richter G, Wang X, Młodzińska E, Carraro N, Ma G, Jenness M, Chao D, Peer WA, Murphy AS (2013) Sterols and sphingolipids differentially function in trafficking of the Arabidopsis ABCB19 auxin transporter. *Plant J.* 74:37-47 *Note: funded partially by DE-FG02-06ER15804.*
8. Janková Drdová E; Synek L, Pečenková T; Hála M; Kulich I; Fower J, Murphy A, Zarsky V (2012) The exocyst complex contributes to PIN auxin efflux carrier recycling and polar auxin transport in Arabidopsis *Plant J* 73:709-19.
9. Spartz AK, Lee SH, Wenger JP, Gonzalez N, Itoh H, Inze D, Peer WA, Murphy AS, Overvoorde P, Gray WH (2012) The SAUR19 subfamily of SMALL AUXIN UP RNA genes promote cell expansion *Plant J.* 70: 978-990.
10. Kubeš M, Yang H, Richter GL, Cheng Y, Młodzińska E, Wang X, Blakeslee JJ, Carraro N, Petrášek J, Zažímalová E, Hoyerová K, Peer WA and Murphy AS (2012) The Arabidopsis concentration-dependent influx/efflux transporter ABCB4 regulates cellular auxin levels in the root epidermis. *Plant J.* 69: 640-654.

Novel microbial based enzymatic CO₂ fixation mechanisms

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Overall research goals:

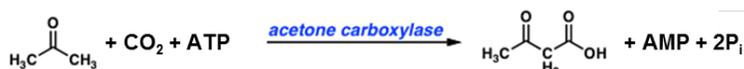
We are examining the catalytic mechanism of carboxylation enzymes in the microbial metabolism of propylene and acetone. Metabolic studies point to the key role of CO₂ as a substrate in these pathways. Two of the CO₂ fixing enzymes in these processes, AC and 2-KPCC, are distinct mechanistically and have unique cofactor requirements. Our research plan involves kinetic studies and structure/function experiments including x-ray structure determination to dissect the molecular mechanism of these CO₂ fixing enzymes.

Significant achievements:

The obligate aerobic *Xanthobacter autotrophicus* Py2 can utilize propylene and isopropanol as growth substrates. For each of these substrates, convergent pathways have been characterized in which the final step is a carboxylation reaction to produce acetoacetate. In one pathway, acetone carboxylase (AC) fixes CO₂ by addition to acetone, forming acetoacetate in an ATP dependent reaction. In the second pathway, 2-ketopropyl-coenzyme M carboxylase (2-KPCC), fixes CO₂ by addition to 2-ketopropyl-coenzyme M (CoM) to form acetoacetate and regenerate CoM. The requirement for CoM in this pathway was unexpected, as it was previously thought that CoM was strictly involved in methanogenesis. Putative genes for CoM biosynthesis have been identified in *X. autotrophicus*, and it appears that CoM is synthesized by a different biosynthetic pathway than present in methanogens.

A

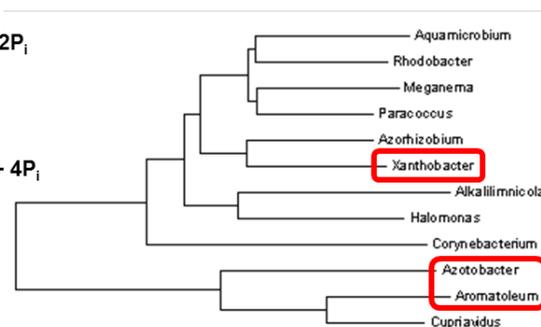
Xanthobacter autotrophicus



Aromatoleum aromaticum / *Azotobacter vinelandii*



B



C

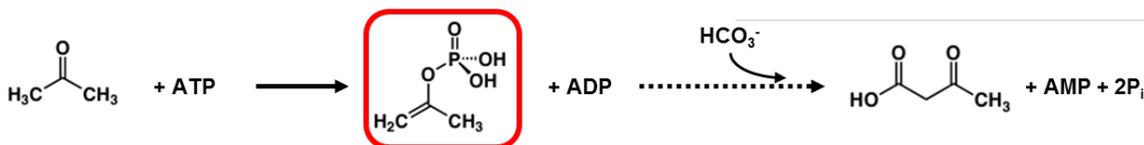


Figure 1. (A) Stoichiometry of the AC carboxylation reaction; (B) Phylogenetic analysis of the AC; (C) Hypothesized intermediate steps of the carboxylation reaction.

• AC. We have heterologously expressed in *E. coli*, the AC from *X. autotrophicus*, *Aromatoleum aromaticum*, and *Azotobacter vinelandii*. Our data shows that AC enzymes differ with regard to the stoichiometry of ATP used in the carboxylation reaction. For *X. autotrophicus*, one ATP was required per acetone carboxylated (Fig. 1A). In contrast, two ATP were consumed per acetone for *A. aromaticum* and *A. vinelandii*. Interestingly, phylogenetic analysis indicates that AC from *X. autotrophicus* lies on a different clade than AC from *A. aromaticum* and *A. vinelandii* (Fig. 1B).

Surprisingly, our data suggests that AC occurs in two different variants, which show similar K_m values for acetone and bicarbonate but differ in ATP stoichiometry. We have identified that the reaction proceeds through a phosphoenolacetone intermediate and defined that ATP γ phosphate cleavage is required for the production of this intermediate or acetone activation. Furthermore, ATP β is likely involved directly in carboxylation, potentially in the activation of bicarbonate (Fig. 1C). Structural characterization of *X. autotrophicus* and *A. vinelandii* ACs is ongoing; crystallization conditions have been identified and crystals are being optimized to improve diffraction quality.

- 2-KPCC. Work on 2-KPCC is focused on elucidating the mechanisms by which the enzyme promotes carboxylation by discriminating between CO_2 and proton as substrate electrophiles. The crystal structure of 2-KPCC revealed a unique *cis*-proline flanked loop adjacent to the active site and contributed to encapsulation of the active site cavity in the substrate bound state. Substitution of the two loop flanking prolines by alanine resulted in reduced rates of acetoacetate production, suggesting possible mechanistic importance of this loop. We are currently assessing the specific impact on the ability of the variants to promote carboxylation at different CO_2 concentrations to probe whether the loop region has a specific role in discriminating substrate electrophiles.

- CoM Biosynthesis. In *X. autotrophicus*, a gene cluster downstream from the epoxide carboxylase pathway is implicated as the putative CoM biosynthetic operon. With the goal of identifying the intermediates and defining the steps in the pathway, we have cloned the putative operon for expression in *E. coli*. Using synthetic CoM and extracts from propene induced *X. autotrophicus* as positive controls, we are able to detect nanomolar amounts of CoM by HPLC-FLD and HPLC-MS using the thiol reactive fluorescent probes N-pyrenyl-maleimide and *o*-phthalaldehyde. However, no CoM is detected in *E. coli* expressing our operon construct. We are currently overexpressing each of the putative operon open reading frames in *E. coli* in an attempt to characterize the individual activities of each gene product.

- Cysteine desulfhydrase. The fifth open reading frame of the putative operon, *xcbE1*, is homologous to D-cysteine desulfhydrases, a group of PLP-dependent enzymes that extract a sulfur from D-cysteine and produce pyruvate, H_2S and ammonium. We have expressed and purified the 37 kDa XcbE1 from *X. autotrophicus* in *E. coli*. Supplying XcbE1 with either L- or D-cysteine yields H_2S and pyruvate. XcbE1 homologs have been shown to exhibit specificity for D-cysteine, and will not catalyze a reaction in the presence of L-cysteine. However, our kinetic data suggests that XcbE1 does not have a strict preference for either L- or D-cysteine. Studies are underway to determine whether the H_2S liberated by XcbE1 are incorporated as the thiol moiety in CoM.

Science objectives for 2014-2015:

Optimize the crystallization conditions and determine the structure for *X. autotrophicus* and *A. vinelandii* ACs

Probe aspects of AC mechanism using synthetic intermediates and mechanism based inhibitors

Identify and characterize the contribution of key amino acid residues in 2-KPCC that function to discriminate between CO_2 or protons as electrophilic substrates

Elucidate the pathway of CoM biosynthesis in bacteria

My scientific areas of expertise are: Mechanistic enzymology and structure determination using x-ray diffraction methods.

To take my project to the next level, my ideal collaborator would have expertise in: Organic synthesis/bioorganic mechanism

Publications supported by this project:

Mus, F., Fugate, C.J., George G.H., and Peters J.W., Delineating the roles for ATP β and γ phosphate cleavage reactions in acetone carboxylase, *In preparation for Biochemistry*

Engineering self-assembled bioreactors from protein microcompartments

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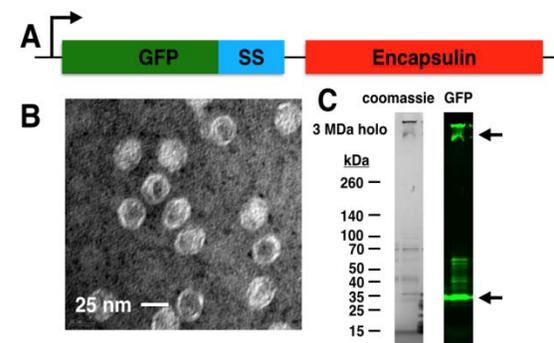
Overall research goals:

The research objectives are to investigate the mechanism and functioning of proteinaceous bacterial microcompartments using: (1) live-cell fluorescence imaging and genetics in the native cyanobacterial hosts, (2) synthetic construction of microcompartments in heterologous hosts, and (3) biochemical reconstitution of microcompartments and their underlying substructures *in vitro*. In the long-term our goal is to enable the modular construction of synthetic organelles and other novel types of protein-based materials.

Significant achievements (2011-2014):

- Developed a heterologous system for the expression of carboxysome microcompartments in *E. coli*. We have shown that intact, functional carboxysomes can be produced in *E. coli* in a manner reminiscent of the native chemoautotrophic host.
- Live-cell imaging of carboxysomes in cyanobacteria suggests that microcompartments may be synthesized in a template-like fashion.
- Have demonstrated the potential of other proteinaceous-like structures as novel materials. We have isolated a signal sequence for the so-called nanocompartments encapsulin complex and demonstrated heterologous expression and targeting of proteins in *E. coli*. Intriguingly, target proteins inside this structure appear to be stabilized from external chemical insults.

Figure 1. Expression and purification of engineered encapsulins. (A) GFP can be targeted to the inside by fusion to the C-terminus of ferritin-like protein (SS). (B) EM analysis of purified particles with GFP. (C) GFP is stabilized inside the capsid, even under denaturing conditions.



Science objectives for 2014-2015:

- We are currently attempting to biochemically reconstitute the entire carboxysome formation pathway *in vitro*. As part of this, we are using the novel technique microscale thermophoresis to completely map the scope of protein-protein interactions. In future work, we intend to identify the specific polypeptides critical for carboxysome formation.
- As described above, encapsulated proteins appear to possess increased stability. We are currently assaying the biophysical basis of this mechanism and exploring these complexes as materials for increasing the stability of industrially-relevant enzymes.

My scientific area(s) of expertise is/are: molecular biology, bacteriology, live-cell imaging, protein biophysics.

To take my project to the next level, my ideal collaborator would have expertise in: cryoelectron microscopy.

Publications supported by this project 2011-2014:

Bonacci W, Teng PK, Afonso B, Niederholtmeyer H, Grob P, Silver PA, Savage DF. 2012. Modularity of a carbon-fixing protein organelle. *Proc Natl Acad Sci USA* 109: 478–483.

Chen AH, Afonso B, Silver PA, Savage DF. 2012. Spatial and temporal organization of chromosome duplication and segregation in the cyanobacterium *Synechococcus elongatus* PCC 7942. eds. A.H. Chen, B. Afonso, P.A. Silver, and D.F. Savage. *PLoS ONE* 7: e47837.

Chen AH, Robinson-Mosher A, Savage DF, Silver PA, Polka JK. 2013. The Bacterial Carbon-Fixing Organelle Is Formed by Shell Envelopment of Preassembled Cargo ed. M.T. Laub. *PLoS ONE* 8: e76127.

Yokoo R, Hood RD, Savage DF. 2014. Live-cell imaging of cyanobacteria. *Photosynthesis Research*. In revision.

D. F. Savage. *Sequestered: Design and Construction of Synthetic Organelles*. Wiley-Blackwell Biotechnology Series, in revision (2014).

Modification of Plant Lipids

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Overall research goals:

This program is designed to provide fundamental understanding of chemical transformations and energy flow in systems relevant to DOE missions. Specifically to understand the molecular mechanisms involved in the capture of light energy and its conversion into chemical energy through biological pathways. Our overall goal is to create the knowledge base to improve plant and microbial oils as alternative and improved energy and chemical feedstocks; which will contribute to United States energy and fossil feedstock independence. Lipids and oils are energy-dense compounds that occur in a wide variety of forms, including the storage lipids of higher plants. The molecular mechanisms involved in chiral lipid-modification reactions using fatty acid desaturation as a model are being studied. X-ray crystallography, spectroscopy, molecular genetics, and biochemistry are employed to probe structure-function relationships within these enzymes. Understanding the factors that control the selectivity and specificity of these processes is allowing us to redesign lipid-modification enzymes with improved function. The ultimate goal of this program is to provide the knowledge base for optimizing the chemical transformations necessary for creating improved plant lipid accumulation of desired chiral lipids in non-food crops.

Significant achievements (2012-2014):

- Parsed the factors responsible for parallel and competitive pathways for substrate desaturation, hydroxylation, and radical rearrangement by the non-heme diiron hydroxylase AlkB with the use of radical clock probes.
- Identified feedback regulation of plastic acetyl-CoA carboxylase by 18:1-acyl carrier protein in *Brassica napus*.
- Determined that an alkane producing enzyme ADO was inhibited by a hydrogen peroxide produced by the interaction of reduced ferredoxin with oxygen. The enzyme was constitutively activated by fusing catalase to an alkane-producing enzyme by converting the inhibitory byproduct H₂O₂ to the cosubstrate O₂.
- Completed a survey of the total fatty acid and triacylglycerol composition and content of 30 duckweed species led to the identification of a Δ 6-desaturase responsible for the production of γ -linolenic and stearidonic acids in *Lemna gibba*.
- Characterized the interactions between the FAD family of desaturases and performed metabolic flux analysis to demonstrate a metabolic channel between 18:1 and 18:3 exists by FAD2 and FAD3 forming a functional heterodimer in which 18:2 product from FAD2 is directly converted to 18:3 by FAD3 without release of the 18:2 intermediate.
- Created a camelina line with seeds that contain 70% ω -7 fatty acids.

Science objectives for 2014-2015:

- Perform experiments employing heterodimers of wild type and mutant subunits purified by tandem affinity chromatography to determine the functional significance of the dimeric organization of the acyl-ACP desaturase class of enzymes. Test the hypothesis that acyl-ACP desaturases operate using a half-sites reactivity mechanism.

- Use the heterodimer approach to test the mechanism of electron transport to the diiron active site. Test the hypothesis that the two electrons required for desaturation are supplied by concerted one e⁻ reduction of each protomer followed by disproportionation to yield an Fe²⁺ Fe²⁺ and an Fe³⁺ Fe³⁺ diiron cluster.
- Achieve a crystal structure of the *Thunbergia* Δ6-18:0-desaturase either alone or with its acyl-ACP substrate and use HADDOCK docking software to evaluate ACP binding modes and thereby understand the mechanism of regioselective insertion of a double bond at the Δ6 position.
- Solve a crystal structure of the aldehyde deformylating oxygenase in the presence or absence of substrate or fatty acid ligand.

My scientific area(s) of expertise is/are: Enzyme structure-function analysis, biochemical regulation, enzyme and pathway engineering.

To take my project to the next level, my ideal collaborator would have expertise in: Electron transport within proteins; chloroplast transformation.

Publications supported by this project 2012-2014:

1. Rawat, R., X.-H. Yu, M. Sweet, and J. Shanklin. 2012. Conjugated fatty acid synthesis: Residues 111 and 115 influence product partitioning of the *Momordica charantia* conjugase. *J. Biol. Chem.* 287(20):16230-16237.
2. Andre, C., R. P. Haslam, and J. Shanklin. 2012. Feedback regulation of plastic acetyl-CoA carboxylase by 18:1-acyl carrier protein in *Brassica napus*. *Proc. Natl. Acad. Sci. USA* 109(25):10107-10112.
3. Fan, J., C. Yan, C. Andre, J. Shanklin, J. Schwender, and C. Xu. 2012. Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* 53(8):1380-1390.
4. Cooper, H. L. R., G. Mishra, X. Huang, M. Pender-Cudlip, R. N. Austin, J. Shanklin, and J. T. Groves. 2012. Parallel and competitive pathways for substrate desaturation, hydroxylation and radical rearrangement by the non-heme diiron hydroxylase AlkB. *J. Amer. Chem. Soc.* 134(50):20365-20375.
5. Andre, C., S.-W. Kim, X.-H. Yu, and J. Shanklin. 2013. Fusing catalase to an alkane-producing enzyme maintains enzymatic activity by converting the inhibitory byproduct H₂O₂ to the cosubstrate O₂. *Proc. Natl. Acad. Sci. USA* 110(8):3191-3196.
6. Yan, Y., J. Candreva, H. Shi, E. Ernst, R. Martienssen, J. Schwender, and J. Shanklin. 2013. Survey of the total fatty acid and triacylglycerol composition and content of 30 duckweed species and cloning of a Δ6-desaturase responsible for the production of γ-linolenic and stearidonic acids in *Lemna gibba*. *BMC Plant Biol.* 13:201.
7. Yu, X.-H., R. R. Prakash, M. Sweet, and J. Shanklin. 2014. Coexpressing *Escherichia coli* cyclopropane synthase with *Sterculia foetida* lysophosphatidic acid acyltransferase enhances cyclopropane fatty acid accumulation. *Plant Physiol.* 164(1):455-465.
8. Wang, W., G. Haberer, H. Gundlach, C. Gläßer, T. Nussbaumer, M.-C. Luo, A. Lomsadze, M. Borodovsky, R. Kerstetter, J. Shanklin, T. Mockler, D. Bryant, K. Appenroth, J. Grimwood, J. Jenkins, J. Chow, C. Choi, C. Adam, X.-H. Cao, J. Fuchs, I. Schubert, D. Rokhsar, J. Schmutz, T. Michael, K. Mayer, and J. Messing. 2014. The *Spirodela polyrhiza* genome reveals insights into its neotenus reduction fast growth and aquatic lifestyle. *Nature Communications* 5:3311.
9. Canto-Pastor, A., A. Molla-Morales, E. Ernst, W. Dahl, J. Zhai, Y. Yan, B. C. Meyers, J. Shanklin, and R. Martienssen. 2014. Efficient transformation and artificial miRNA gene silencing in *Lemna minor*. *Plant Biology* doi: [10.1111/plb.12215](https://doi.org/10.1111/plb.12215).
10. Lou, Y., J. Schwender, and J. Shanklin. 2014. FAD2 and FAD3 desaturases for hetero-dimers that facilitate metabolic channeling in vivo. *J. Biol. Chem.* 289:17996-8007.
11. Nguyen HT, Park H, Koster KL, Cahoon RE, Nguyen HT, Shanklin J, Clemente TE, Cahoon EB. 2014. Redirection of metabolic flux for high levels of omega-7 monounsaturated fatty acid accumulation in camelina seeds. *Plant Biotechnol J.* doi: [10.1111/pbi.12233](https://doi.org/10.1111/pbi.12233).

A proteomic study of steroid regulation of plant growth

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Overall research goals: The research objectives are to study brassinosteroid hormone regulation of plant growth and development by: (1) developing a quantitative proteomic methods to identify brassinosteroid-induced proteomic changes, (2) identify microtubule-associated proteins that respond to brassinosteroid treatment; (3) study brassinosteroid regulation of cellulose synthases (CESAs); (4) study brassinosteroid regulation of vesicle trafficking. Brassinosteroid (BR) is a major growth-promoting hormone in plants and is considered a major target for increasing biomass productivity. Brassinosteroid controls many major cellular growth processes including chloroplast development, cell wall synthesis, cytoskeleton organization, and vesicle secretion, which are all critical for biomass accumulation. We have identified proteins that are regulated by the BR signal transduction pathway. Our study not only generates fundamental knowledge of the molecular mechanisms underlying cellular growth, which is important for improving plant productivity.

Significant achievements 2013-2014:

Brassinosteroid has been shown to promote plant growth by increasing cell elongation, which is the major contributor to overall plant growth and biomass accumulation. Cell elongation involves orientation of the cytoskeleton, synthesis of cell wall materials, and secretion of biomaterials to the cell wall. Understanding how these processes are regulated during plant growth is important for engineering plant growth.

Microtubules, assembled from tubulins, are important for many cellular activities, including the movement of cells, cargo vesicles transport, cell morphogenesis, cell wall deposition. We performed proteomic analysis of tubulin/microtubule-associated proteins. To achieve high-confidence quantitation, we used the metabolic ^{15}N -labelling method in our LC-MS/MS analysis. To identify tubulin/microtubule-associated proteins, we grow plants expressing a tubulin6-green fluorescence protein (TUB6-GFP) fusion protein on ^{14}N medium, and the control plants expressing GFP alone on ^{15}N medium. After immunoprecipitation by an anti-GFP antibody, the proteins from TUB6-GFP and GFP samples were mixed and analyzed by LC-MS/MS. LC-MS/MS analyses detected 990 proteins. Among them, 549 proteins were enriched (>4 fold) in the TUB6-GFP sample compared to the ^{35}S -YFP control. These include 100 proteins annotated with functions related to cytoskeleton (Gene Ontology); these include many previously known TAPs, such as MAP65, Prefoldin2, 3, 4, 5 and 6, TCP-1/CPN60 chaperonin family proteins, tubulin folding cofactor D, and heat shock family proteins. Additional proteins include MAP kinases, calcium dependent protein kinases; several phosphatase, the 14-3-3 proteins, actin, and actin depolymerizing factors, and lipid transfer proteins, and cellulose synthase family proteins.

We have performed several BR treatment experiments followed by LC-MS/MS analysis, using ^{15}N -labeling. We purified phosphopeptides from total protein samples of BR treated and untreated seedlings, and LC-MS/MS analysis identified and quantified 11,047 phosphopeptides on 3,135 proteins. We found phosphorylation decreases at 80 sites and increases at 25 sites on 91 proteins upon BR treatment. These include 7 known proteins of the BR pathway, and the rest unknown previously for a function in BR response. These new BR-responsive phosphoproteins represent new links from BR signaling to various cellular processes, including signaling by other receptor kinases, RNA splicing, translation, and crosstalk with other signaling pathways, as well as cellulose synthesis, cation exchangers, microtubule-associated proteins, and vacuolar protein sorting factor.

Science objectives for 2014-2015:

- Functional analysis of the BR-regulated phosphorylation events using site-directed mutagenesis, including microtubule-associated protein (SPR1 and TPX2), cation exchangers (CAX1), and cellulose synthase (CESA5).
- Quantitative proteomic analysis of the BR-regulated proteins in vesicles and CESA5-associated proteins.

My scientific areas of expertise are: genetics, molecular biology, genomics, and proteomics.

To take my project to the next level, my ideal collaborator would have expertise in: mass spectrometry, computation biology.

References to work supported by this project 2013-2014:

1. Ni, W.M., Xu, S.L., Tepperman, J.M., Stanley, D.J., Maltby, D.A., Gross, J.D., Burlingame, A.L., Wang, Z.-Y., Quail, P.H. (2014). A mutually assured destruction mechanism attenuates light signaling in *Arabidopsis*. *Science*. 344, 1160-1164.
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4. Deng, Z., Osés-Prieto, J. A., Kutschera, U., Tseng, T.-S., Hao, L., Burlingame, A. L., Wang, Z.-Y., Briggs, W. R. (2014). Blue light-induced proteomic changes in etiolated *Arabidopsis* seedlings. **J. Proteome Res.** 13, 2524-2533.
5. Ni, W., Xu, S.L., Chalkley, R.J., Pham, T.N., Guan, S., Maltby, D.A., Burlingame, A.L., Wang, Z.-Y., Quail, P.H. (2013). Multisite Light-Induced Phosphorylation of the Transcription Factor PIF3 Is Necessary for Both Its Rapid Degradation and Concomitant Negative Feedback Modulation of Photoreceptor phyB Levels in *Arabidopsis*. *Plant Cell* 25, 2679-2698.
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Intracellular Lipid Transfer in the Biosynthesis of Photosynthetic Membranes Lipids and Storage Triacylglycerol

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Overall research goals:

The overall goal of this project is to understand the molecular mechanisms involved in the biosynthesis and intracellular trafficking of lipids in plants. The specific objectives are to: (1) identify the genes affected in two lipid-trafficking mutants isolated in a forward genetic screen, (2) determine the functional role of the encoded proteins at the molecular, biochemical, and physiological levels; (3) investigate oil biosynthesis and regulation in vegetative tissues of plants. The results from this study should create the knowledge base to enhance our ability to manipulate the processes that regulate photosynthetic membrane biogenesis and carbon storage in plants, and lay the intellectual foundation for future engineering endeavors aimed at the development of novel biomass crops for the production of nutrition-rich feed and renewable fuels.

Significant achievements (2012-2014):

Two mutant loci were identified through a map-based cloning approach. One encodes a putative membrane protein of unknown function and was designated *tgd5-1* because the mutant accumulates trigalactosyldiacylglycerol in leaves similar to previously described four *tgd* mutants. Additional analyses with two independent alleles containing T-DNA insertion mutations in the *TGD5* gene confirmed our findings with the *tgd5-1*. Genetic analysis showed that TGD5 functions in the same lipid trafficking pathway as TGD4 and is localized in envelope membranes of the chloroplast. The second mutant locus represents a new allele of *tgd2* mutants.

Additional work related to this project focused on analyzing the pathway of TAG biosynthesis and its physiological significance in vegetative tissues of plants. Taking advantage of Arabidopsis *tgd* mutants that accumulate oil in nonseed tissues, we found that phospholipid: diacylglycerol acyltransferase (PDAT) is critical in mediating oil biosynthesis in rapidly growing tissues such as young leaves. We show that disruption of PDAT1 in the *tgdl-1* mutant background causes serious growth retardation, gametophytic defects and premature cell death in developing leaves. Lipid analysis data indicated that knockout of PDAT1 results in increases in the levels of free fatty acids and diacylglycerol. In vivo radiotracer labeling experiments showed that, compared with wild-type, *tgdl-1* exhibits a 3.8-fold higher rate of fatty acid synthesis, which is unaffected by disruption or over-expression of PDAT1, indicating a lack of feedback regulation of fatty acid synthesis in *tgdl-1*. Overexpression of PDAT1 increases leaf TAG accumulation, leading to oil droplet overexpansion through fusion. Ectopic expression of oleosin promotes the clustering of small oil droplets. Coexpression of PDAT1 with oleosin boosts leaf TAG content by up to 6.4% of the dry weight without affecting membrane lipid composition and plant growth. PDAT1 overexpression stimulates fatty acid synthesis and increases fatty acid flux toward the prokaryotic glycerolipid pathway. The combined overexpression of PDAT1 with oleosin in *tgdl-1* increases leaf TAG content to 8.6% of the dry weight and total leaf lipid by fourfold. In the *act1* mutant, which is defective in the prokaryotic glycerolipid pathway, PDAT1 overexpression enhances TAG content at the expense of thylakoid membrane lipids, leading to defects in chloroplast division and thylakoid biogenesis. Collectively, these results reveal a dual role for

PDAT1 in enhancing fatty acid and TAG synthesis in leaves, thereby protecting against free fatty acid-induced cell death in fast-growing tissues of plants.

Science objectives for 2014-2015:

- Preliminary genetic analysis indicates that TGD4 is epistatic to TGD5 in the lipid trafficking pathway. We will test the genetic interactions between TGD4 and TGD1, TGD2 or TGD3 by double mutant analysis.
- Bimolecular fluorescence complementation and coimmunoprecipitation assays will be carried out to test the potential physical interactions between TGD5 and other TGD proteins.
- Our recently results show that disruption of TGD1 results marked increases in rates of both fatty acid synthesis and turnover. Constructing and characterizing the double mutants between *tgdl-1* and the mutant defective in SUGAR-DEPENDENT1 triacylglycerol lipase, PEROXISOMAL TRANSPORTER1 or Arabidopsis lipins will help to define the pathway of fatty acid β -oxidation in vegetative tissues of plants.

My primary expertise is in: molecular genetics and biochemistry of plant lipids

To take my project to the next level, my ideal collaborator would have expertise in: membrane protein crystallography

Publications supported by this project 2012-2014:

1. Fan J, Yan C, Andre C, Shanklin J, Schwender J, Xu C. Oil accumulation is controlled by the availability of carbon precursors for fatty acid synthesis in *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* 53:1380-90 (2012).
2. Wang Z, Xu C, Benning C. TGD4 involved in ER-to-chloroplast lipid trafficking is a phosphatidic acid binding protein. *Plant J.* 70: 614-23 (2012).
3. Fan J, Yan C, Xu C. Phospholipid:diacylglycerol acyltransferase-mediated triacylglycerol biosynthesis is crucial for protection against fatty acid-induced cell death in growing tissues of Arabidopsis. *Plant J.* 76: 930-942 (2013)
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5. Yan C, Fan J, C Xu. Analysis of oil droplets in microalgae. *Methods Cell Biol.* 116: 71-82 (2013).
6. Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M. X., Arondel, V., Bates, P. D., Baud, S., Bird, D., DeBono, A., Durrett, T. P., Franke, R. B., Graham, I. A., Katayama, K., Kelly, A. A., Larson, T., Markham, J. E., Miquel, M., Molina, I., Nishida, I., Rowland, O., Samuels, L., Schmid, K. M., Wada, H., Welti, R., Xu, C., Zallot, R., and Ohlrogge, J. Acyl-lipid metabolism. *The Arabidopsis Book*, Vol. 11: e0161, The American Society of Plant Biologists, Rockville, MD (January, 2013).

Structural model of plant cellulose synthase

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Overall research goals:

As part of the Center for Lignocellulose Structure and Formation, a DOE-funded Energy Frontier Research Center, this project seeks to explain structure-to-function relationship of plant cellulose synthase (CesA). The structure-to-function relationship can be directly addressed through three-dimensional (3D) structure exploration and comparison. However, without experimentally determined 3D models of these features of plant CesAs, our ability to postulate mechanisms of function and gain insights into how these might foster rosette formation/interactions and affect cellulose synthesis is limited. A major goal of our research is to provide a fundamental understanding of the mechanistic details of cellulose synthase (CesA) and cellulose synthase complex (CSC) structure, assembly, and operation to support cellulose polymerization and crystallization. To surmount this problem, we use a combination of ab-initio structure prediction, molecular dynamics and coarse-grained simulations to predict structural models of plant CesAs and the assembly of CesAs into cellulose synthase complex (CSC).

Significant achievements (2013-2014):

Project 1. *Prediction of cytosolic region of cotton Cesa*. Our group used de-novo protein structure prediction to generate all-atom 3D model of cytosolic region of CesAs protein structure of cotton. Due to complexity and scale of CesAs proteins we initially subdivided the prediction of transmembrane helices organization in a membrane from the prediction of a catalytic part of a CesA protein. Our model showed agreement in the catalytic mechanisms between bacteria and plants and the specific roles of other conserved parts of CesAs.

Project 2. *The role of plant conserved regions*. Plant CesA's possess distinct areas in their catalytic domain that differentiates them from their prokaryotic forebears: the Plant Conserved Region (CR-P) and a Class Specific Region (CSR). In the absence of an experimentally determined model of plant CESAs, we used structure prediction and molecular modeling techniques to survey the structural motifs of these domains in order to further understand their evolutionary relationships and their biochemical roles in controlling cellulose production. We show that while PCR structures are very similar, the CSR's distinguish paralogous classes from each other.

Project 3. *Putative transporter loop in the transmembrane region*. The transmembrane helices (TMH) 5 and TMH6 of all CesA are connected via a long chain of amino acids, the length of this chain is highly conserved. We used ab-initio prediction of TMH and found that this chain/loop can exist in two energetically favorable conformations. In general, the bimodal folding mechanisms of the loop between two TMHs are known to be responsible for reentrant behavior. Our results suggest that the transmembrane region in plant cellulose synthases is highly dynamic and may be a fruitful target of genetic manipulation.

Science objectives for 2014-2015:

- The structure-function relation and the modes of CSC assembly requires an identification and characterization of all of the protein parts and their modes of interaction and functional operation. We will apply protein structure prediction and molecular modeling methods to build a prototype computer model to investigate the differences and similarities of individual CesAs from plants and bacteria, CesA packing within the CSC, interactions of CesAs with other proteins and motion of CSC in the membrane.

- Our previous study did not include the trans-membrane part of the protein nor Z-finger and C-terminus. We will use a combination of ab-initio structure prediction and Monte Carlo algorithms to assemble transmembrane helices (TMH) in the membrane, which will be refined with explicit MD simulations with lipids and water. Then the effects of known single amino acid changes on the overall structure should be readily evaluated. Our model will help is to answer many questions: what is the role of individual helices on glycan chain transport, the role of mutations, lipid composition, the mechanisms of pore formation, interactions with other TMH helices from other CesAs. We will then dock the Z-finger into our model and investigate the contribution of Z-finger and C-terminus on the structure and its assembly into CSC. The comparison between BcsA and plant CesA will be made.

My scientific areas of expertise are: computational structure prediction, molecular modeling and molecular dynamics simulations.

To take my project to the next level, my ideal collaborator would have expertise in: structural and molecular biology.

Publications supported by this project 2009-2014:

1. E. Slabaugh, L. Sethaphong, C. Xiao, J. Amick, C. T. Anderson, C. H. Haigler and Y. G. Yingling, "Computational and genetic evidence that different structural conformations of a non-catalytic region in cellulose synthase (CESA) affect CESA function", *Journal of Experimental Botany* (2014), in press
2. E. Slabaugh, J. K. Davis, C. H. Haigler, Y. G. Yingling, J. Zimmer, Cellulose synthases: new insights from crystallography and modeling, *Trends in Plant Science* 19 (2013) 99-106.
3. L. Sethaphong, C. H. Haigler, J. D. Kubicki, J. Zimmer, D. Bonetta, S. DeBolt, Y. G. Yingling, "Tertiary model of a plant cellulose synthase", *Proc. Natl. Acad. Sci. USA* 110 (2013) 7512-7517.
4. H. Yi, S. Thakur, L. Sethaphong, Y. G. Yingling, "X3DBio2: A Visual Analysis Tool for Biomolecular Structure Exploration", *Proc. SPIE 8654 VDA* (2013) 86540W.
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Multi-scale imaging of plant cell walls to reveal properties of improved feedstocks for biofuels or biomaterials

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Abstract:

Economic, sustainable production of biofuels from lignocellulosic biomass will require learning to control the chemical and physical complexity that has evolved in plant cell walls. Using multi-scale microscopy and quantitative image analysis, we have shown how catalytic conversion treatments alter the micro- and nanostructure of wild-type and genetically modified cell walls. Specifically, we investigate several genetic variants of *Arabidopsis*: the wild type, which makes a lignin polymer of primarily guaiacyl (G) and syringyl (S) monomeric units, the *fah1* mutant, which makes lignin from almost exclusively G subunits, and a ferulate 5-hydroxylase (F5H) overexpressing line (C4H: F5H) that makes lignin from S subunits. Increased dislocation, surface roughness, delamination, and nanofibrillation revealed by direct observation confirms changes in cell wall architecture and explains the superior performance of some treatments. At the nano scale, we have used 3D electron tomography as a primary tool to investigate the changing architecture of plant cell walls during catalytic conversion. We are continuing to improve methods to discern and model cell wall polymers in situ by augmenting traditional analysis with advanced image processing. My scientific areas of expertise are plant and algal cell biology and cellular structural biology. Collaboration with researchers working to develop novel mesoscale structural biology tools for application to plant cell walls would significantly enhance my research progress.

Session VIII

The role of carbonic anhydrase in C₄ photosynthesis

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Overall research goals:

The primary goal of this research is to examine the role of carbonic anhydrase (CA) in photosynthesis of C₄ grasses, and identify strategies for optimizing the efficiency of carbon assimilation. Carbonic anhydrase catalyzes the first dedicated step of C₄ photosynthesis, and has been implicated in both water use and nitrogen use efficiency. Using genetics, molecular biology, and plant physiology approaches, the requirement of CA for C₄ photosynthesis in the crop plant *Zea mays* is being investigated. In addition, comparative genomics approaches are being employed to dissect the evolution of C₄ photosynthesis in the grass lineage, and elucidate the genes underlying this complex trait.

Significant achievements (2012-2014):

Insertional mutants of several CA genes were generated with the transposable element *Dissociation*. These mutants were characterized, and provide evidence for a limited role for CA in C₄ photosynthesis in maize under current atmospheric CO₂ levels. However, the CA mutants in maize have a higher stomatal conductance. Further experiments need to be conducted to determine if the CA mutants have reduced drought tolerance and water use efficiency.

To facilitate comparative genomics approaches to dissecting C₄ photosynthesis, a draft genome sequence of the C₃ panicoid grass *Dichanthelium oligosanthes* was completed along with a RNA-seq analysis of the leaf transcriptome. The primary advantage of *D. oligosanthes* as a C₃ comparator is that it is in the same phylogenetic clade as the most productive C₄ grasses such as maize, sorghum, sugarcane, switchgrass, and Miscanthus.

Science objectives for 2014-2015:

- We hypothesize that CA likely maintains high rates of photosynthesis when CO₂ availability is reduced due to stomatal closure under temperature or drought stress. Using the CA mutants in maize grow under drought stress, current experiments focus on the contribution of CA to stress tolerance.
- In the C₃ dicot *Arabidopsis*, CA has been implicated in stomatal sensing of CO₂ levels. Preliminary data suggests that CA may function as a sensor of CO₂ in the C₄ monocot maize. Current experiments that track stomatal movement and photosynthesis in response to changing environmental conditions are being used to analyze the effect of reduced CA in maize.
- Thus far, CA mutant plants have been grown under growth chamber and greenhouse conditions. While informative, it is important to observe the impact of reduced CA under field conditions. Thus there are multi-location replicated field trials of maize CA mutant growing in the summer of 2014, that will be repeated during the summer of 2015.
- The relocation of CA from the chloroplast to the cytosol is a necessary step for the evolution of C₄ photosynthesis. The genetic changes that facilitated this subcellular placement are not known. Several experiments are underway that address this question including transgenic fluorescently tagged C₃ and C₄ isoforms of CA and sequence analysis of CA in multiple independent evolutionary lineages of C₄ photosynthesis in the grasses.

My scientific areas of expertise are: Quantitative and Molecular Genetics

To take my project to the next level, my ideal collaborator would have expertise in: Plant physiology and high-throughput phenotyping capabilities in the field.

Publications supported by this project 2013-2014:

1. A. J. Studer, A. Gandin, A. R. Kolbe, L. Wang, A. B. Cousins, and T. P. Brutnell, “A limited role for carbonic anhydrase in C₄ photosynthesis as revealed by a *calca2* double mutant in maize.” *Plant Physiology* **165**:2 608-617 (2014). doi: [10.1104/pp.114.237602](https://doi.org/10.1104/pp.114.237602).

Genetic and Functional Identification of Sensory Mechanisms in Plants that Initiate Responses to Water Stress

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Overall research goals:

Abiotic stresses such as drought, soil salinity and low humidity reduce plant growth and quality. While many technologies have focused on enhancing downstream responses to drought, selection of biomass-producing plants for these traits often results in reduced growth under non-stressed or mild-stress conditions. Since the severity and duration of drought cannot be predicted, optimal plant growth under both stressed and non-stressed conditions requires the plant to activate responses at an appropriate level commensurate with the severity of the drought stress. However, the sensors and associated regulatory mechanisms that initiate drought- and osmotic-tolerance responses in plants are currently unknown. My overall research goal is to identify molecular mechanisms that initiate the responses to osmotic stress and water stress as well as to identify regulatory mechanisms involved in this process.

Significant achievements (2011-2014):

I have established a reporter assay in *Arabidopsis* to quantify rapid hyperosmotic- Ca^{2+} responses. This approach affords key insights into the earliest events occurring in abiotic stress sensory pathways. Using this system, I found that this response is greatly amplified in *Arabidopsis* depending on physiological conditions and is modulated by hormone pre-treatment. I tested mutations in candidate osmo-sensory genes, and found that members from two mechanosensitive channel families are not required. I found that mutations in genes affecting water relations and general ion homeostasis had a significant effect on this rapid Ca^{2+} response, and a transporter double mutant greatly dampens this response. I have conducted a genetic screen and identified 20 EMS-mutagenized *Arabidopsis* lines that display altered responses. Preliminary work to characterize downstream abiotic stress response phenotypes in these mutant lines has revealed effects on growth and transpiration rates.

Science objectives for 2014-2015:

A manuscript will be prepared concerning environmental factors “sensitizing” the osmotic-induced calcium response, as well as interaction with mutants that affect this response. Additionally, linkage-based mapping of select EMS mutant lines will be completed to identify novel genes involved in this response, as well as further screening for novel mutant lines. The genes that are identified will be characterized functionally, biochemically, and genetically to understand their functions in the sensory machinery.

My scientific areas of expertise are:

calcium signaling, sensory biology, bioinformatics, quantitative biology, plant physiology, ion channels/transporters, signal transduction

To take my project to the next level, my ideal collaborator would have expertise in:

Water relations and hydraulics in plants, sub-cellular microscopy of structural components

Publications supported by this project 2011-2014:

1. Brandt, B., Brodsky, D.E., Xue, S., Negi, J., Iba, K., Kangasjärvi, J., Ghassemian, M., Stephan, A.B., Hu, H., and Schroeder, J.I. (2012). Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. *PNAS* *109*, 10593–10598.
2. Saidu, S.P.*, Stephan, A.B.*, Talaga, A.K., Zhao, H., and Reiser, J. (2013). Channel properties of the splicing isoforms of the olfactory calcium-activated chloride channel Anoctamin 2. *J Gen Physiol* *141*, 691–703.
3. Deinlein, U., Stephan, A.B., Horie, T., Luo, W., Xu, G., and Schroeder, J.I. (2014). Plant salt-tolerance mechanisms. *Trends in Plant Science* *19*, 371–379.
4. Stephan, A.B., and Schroeder, J.I. (2014). Plant salt stress status is transmitted systemically via propagating calcium waves. (Commentary). *PNAS* *111*, 6126–6127.

Jasmonate Hormone: Regulating Synthesis of Reduced Carbon Compounds in Plants

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

Significant achievements (2012-2015): We have had a breakthrough year in advancing these original goals, and in progress towards understanding two opposing function of JA: inducing synthesis of valuable reduced carbon compounds and restriction of plant growth. Separating these two functions is key to exploiting JA signaling to enhance bioenergy outcomes. We are now in an excellent position to implement biochemical, biophysical and genetic approaches to separating these two actions of JA in plants. In the last year, we have published on the JA-Ile hydrolase, ILL6, as well as two protocol papers, to complete our work on degradation of JA-Ile (Refs 1-3).

1. Tissue-Specific Expression of COI1 Reveals Sites of JA Action. The COI1 F-Box protein is a JA-Ile coreceptor and *coi1* mutant plants lack JA responses. We have tested the possibility that sites of JA action can be probed by using tissue-specific promoters to drive expression of a COI1-YFP fusion protein in *coi1* mutant plants deficient in stamen and pollen function. When we expressed COI1-YFP behind a filament-specific promoter (from the *DAD1* gene), filament elongation was restored but not anther dehiscence or pollen function (Fig. 1A). Three tapetum specific promoters, all failed to restore any of these three functions (Fig. 1B) but, unexpectedly, a promoter active in the stomium and epidermal cells, restored both pollen function and anther dehiscence (Fig. 1C). Most importantly, our results demonstrate the power of *promoter::COI1-YFP* constructs in revealing the primary sites of JA-regulated gene expression that control developmental and other responses in neighboring tissues. We now plan to use this new tool to test current hypotheses about JA action in other organs of the plant, including proposals that: **1.** Induction of secondary-product pathways in stems and leaves requires JA perception in phloem parenchyma cells. **2.** JA perception in epidermal cells controls leaf growth processes. **3.** JA perception in meristems reduces cell division and organ growth.

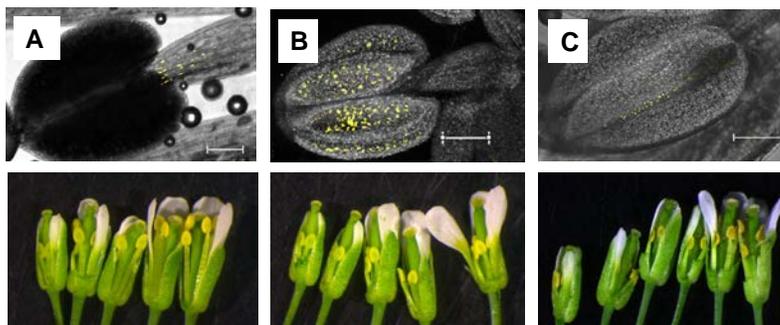


Figure 1. Tissue-Specific Action of JA-Ile Revealed by *Promoter::COI1* transgenes. **A.** Filament expression. **B.** Tapetum expression has no effect. **C.** Septum expression restores function, but not filament elongation. (Top, YFP reporter. Bottom, flower phenotype.)

We now plan to use this new tool to test current hypotheses about JA action in other organs of the plant, including proposals that: **1.** Induction of secondary-product pathways in stems and leaves requires JA perception in phloem parenchyma cells. **2.** JA perception in epidermal cells controls leaf growth processes. **3.** JA perception in meristems reduces cell division and organ growth.

2. The MYC5 Transcription Factor Controls Plant Growth Responses. The MYC2, MYC3, and MYC4 proteins are the primary transcription factors initiating defense and root growth responses to JA signaling. However, transgenic plants overexpressing these proteins do not show any substantial reduction in shoot growth, even though they have increased expression of many JA-responsive genes. MYC5 is closely related to MYC2, MYC3, and MYC4 but has not previously been considered a candidate in JA signaling, in part because *myc5* mutants exhibit no overt phenotype. However, when

we overexpressed the MYC5 protein in wild-type Arabidopsis we got a surprising result. Whereas plants overexpressing MYC2 were similar in size to wild type, as observed previously, MYC5-OE plants were much smaller, as shown in Fig. 2. This is an exciting discovery because it indicates that growth responses to JA may be controlled by a signaling pathway that is distinct from that pathway (or pathways) controlling secondary-product synthesis and defense. Further investigation of this phenotype, the MYC5 transcription factor and other JA response regulators will be incorporated into this grant.

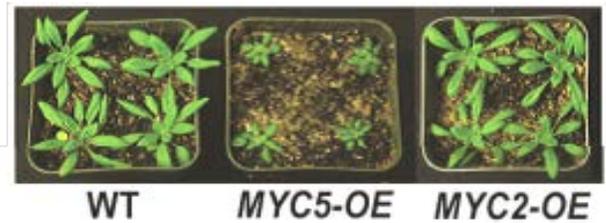


Figure 2. Overexpression of the MYC5 Transcription Factor (Center) Severely Restricts Plant Growth

Science Objectives for 2014-2015:

3. A Screen for Mutants Resistant to Growth Inhibition Upon Wounding. To complement the work described above, and to discover additional genes required for JA regulation of biomass growth we shall expand a screen for mutants that do not show reduced growth in response to wounding. A chemically mutagenized M2 population is not suitable for this screen because plant-to-plant variation in growth would mask responses. Instead we have ordered homozygous T-DNA mutant lines from the Arabidopsis Stock Center. We are growing three plants of each line (together with wild-type controls) in individual peat plugs under carefully controlled conditions for 14 days, and then wounding them each day for ten days while observing growth of the plants. Plant growth is very uniform and the replicates make it possible to readily identify putative mutants that maintain leaf growth following wounding. Because the gene disrupted by the T-DNA in each line is known, it is possible to order and test additional alleles, if they are available. So far, from 2,000 lines screened, we have found one mutants (Fig. 3) that has a T-DNA inserted in the gene encoding an F-box/Kelch protein. A second, independent allele shows the same resistance to wound-induced stunting, so it is likely that this F-Box/Kelch protein is a previously unknown contributor to JA-regulation of plant growth.

These new discoveries mean that my research program is now poised to develop a new and clearer understanding of how to separate the pathways of JA signaling that restrict plant growth from those that induce the synthesis of valuable reduced carbon compounds.

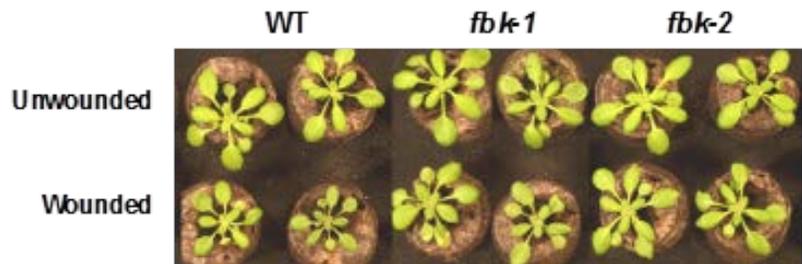


Figure 3. Compared with wild-type controls (WT), two allelic mutants deficient in an F-box/Kelch protein show no significant restriction of shoot growth following wounding.

My scientific areas of expertise are: Biochemistry and function of membrane lipids and lipid-derived regulators.

To take my project to the next level, my ideal collaborator would have expertise in: Analyzing fluxes and regulation of pathways for synthesis of reduced carbon compounds. Biophysical and proteomics approaches to investigating the effects of protein modification.

Publications supported by this project in 2013:

1. Bhosale R., Jewell J.B et al. 2013 Predicting Gene Function from Uncontrolled Expression Variation among Individual Wild-Type Arabidopsis Plants. *Plant Cell*. **25**:2865-77. DOI: <http://dx.doi.org/10.1105/tpc.113.112268>

2. Thines B., Mandaokar A. and Browse J. 2013 Characterizing jasmonate regulation of male fertility in Arabidopsis. *Methods Mol Biol*. **1011**:13-23. DOI: http://dx.doi.org/10.1007/978-1-62703-414-2_2

3. Bates P.D., Jewell J.B. and Browse J. 2013 Rapid separation of developing Arabidopsis seeds from siliques for RNA or metabolite analysis. *Plant Methods*. **9**:9. DOI: <http://dx.doi.org/10.1186/1746-4811-9-9>

Mass Spectrometric Imaging of Plant Metabolites

Basil J. Nikolau, Principal Investigator

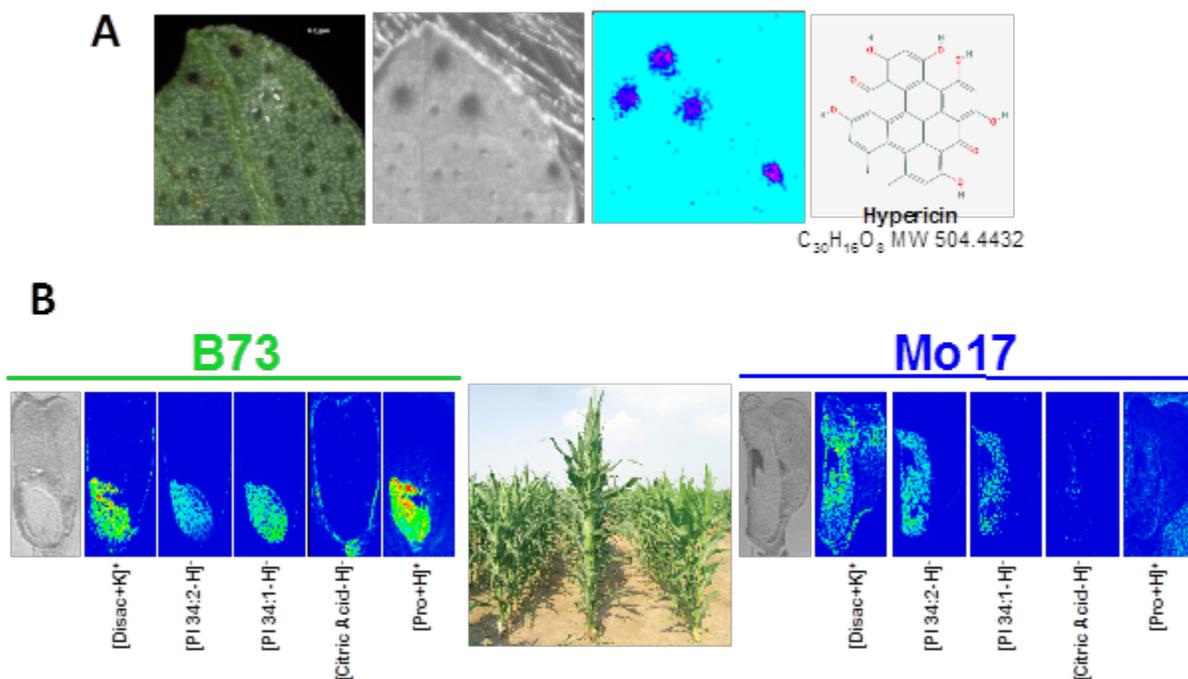
Young-Jin Lee, Robert S. Houk, Co-Principal Investigators

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Email: dimmas@iastate.edu; Web: <http://www.ameslab.gov/cbs/fwp/mass-spectrometric-imaging>

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

Significant achievements 2012-2014: The research focused on three significant metabolic systems: 1) germinating maize seeds; 2) C4 photosynthesis cells of maize leaves; 3) polyketide and flavonoid natural products in secretory structure (translucent glands or cavities, black nodules and secretory canals) of *Hypericum* leaves. These systems were specifically targeted because they offer the ability to decipher the asymmetric distribution of metabolites among discrete cellular-sized structures, and such data is highly revealing of the underlying regulatory mechanism(s) that control these complex metabolic processes. Integrating high-spatial resolution MSI data with global genome expression profiling (either at the level of the transcriptome or proteome) is providing testable hypothesis concerning gene-functions. Validating these hypotheses via functional genomics analysis is revealing more accurate predictive understanding of the mechanisms that multicellular organisms use to regulate metabolic processes.



Examples of MSI technology applied to solving functional genomics analysis of plant metabolism. A) Asymmetric distribution of hypericin in leaves *Hypericum*. B) Asymmetric distribution of various metabolites in seeds of two maize inbred (B73 and Mo17). When these two inbreds are intermated the phenomenon of hybrid-vigor is expressed (illustrated in middle image).

Science objectives for 2014-2015:

The project is developing unique genetic germplasm to further dissect the biochemical and genetic regulatory mechanisms that modulate specific metabolic processes, associated with acetyl-CoA metabolism. Specifically, the project will have access to over 1500 maize hybrid lines (developed by the inter-mating of two inbreds, B73 and Mo17), which express different degrees of hybrid vigor. These lines will be used to dissect the metabolic basis of hybrid vigor, a complex genetic trait that has been the basis for enhanced agricultural production of biomass in the US Midwest states. Additional, germplasm that is being developed are transgenic Arabidopsis lines that express novel genes isolated from maize that are involved in the biosynthesis of surface cuticle lipids, which is the most energy-rich biochemicals that biological systems produce.

The choice of these experimental systems is guided by three criteria: 1) focus on metabolism leading to the biosynthesis of energy-dense molecules (i.e., molecules composed primarily of carbon and hydrogen, and lacking oxygen); 2) use the high-spatial resolution MSI technology (~5 μm resolution) to visualize metabolites and metabolism to the level of subcellular compartments within a single plant cell; and 3) take advantage of our team's expertise in the biochemistry and genetics of acetyl-CoA metabolism.

My primary expertise is in: Plant biochemistry.

To take my project to the next level, my ideal collaborator would have expertise in: metabolic computational modeler.

References to work supported by this project 2012-2014:

1. L. W. Sumner, Z. Lei, B. J. Nikolau, K. Saito. "Modern Plant Metabolomics: Advanced Natural Product Gene Discoveries, Improved Technologies, Challenges and Future Prospects", *Natural Product Reports*. In press.
2. M. C. Crispin, Z. Song, H. L. Babka, A. R. Korte, Y-J. Lee, B. J. Nikolau, E. S. Wurtele, Acylphloroglucinol Localization and Biosynthesis in the Translucent Glands of *Hypericum gentianoides*. In review.
3. A. R. Korte, G. B. Yagnik, A. T. Feenstra, Y. J. Lee, "Multiplex MALDI-MS Imaging of Plant Metabolites Using a Hybrid MS System", an invited chapter to 'Mass Spectrometry Imaging of Small Molecules', Ed. Lin He, in a series of 'Methods in Molecular Biology', Humana Press, in press.
4. A. R. Korte, Y. J. Lee, "MALDI-MS Analysis and Imaging of Low Molecular Weight Metabolites with 1,5-Diaminonaphthalene (DAN)", *J. Mass Spectrom.*, **49**, 737-741 (2014). DOI: [10.1021/ac2033547](https://doi.org/10.1021/ac2033547)
5. A. R. Korte, Y. J. Lee, "Multiplex Mass Spectrometric Imaging with Polarity Switching for Concurrent Acquisition of Positive and Negative Ion Images", *J. Am. Soc. Mass Spectrom.* **24**, 949-955 (2013).
6. G. B. Yagnik, A. R. Korte, Y. J. Lee, "Multiplex Mass Spectrometry Imaging for Latent Fingerprints", *J. Mass Spectrom.*, **48**, 100-104 (2013). DOI: [10.1002/jms.3134](https://doi.org/10.1002/jms.3134)
7. E. A. Smith, S. Park, A. T. Klein, Y. J. Lee, "Bio-Oil Analysis Using Negative Electrospray Ionization: Comparative Study of High Resolution Mass Spectrometers and Phenolic vs. Sugarcic Components", *Energy & Fuels*, **26**, 3796-3802 (2012). DOI: [10.1021/ef3003558](https://doi.org/10.1021/ef3003558)
8. A. R. Korte, Z. Song, B. J. Nikolau, Y. J. Lee, "Mass Spectrometric Imaging as a High-Spatial Resolution Tool for Functional Genomics: Tissue-Specific Gene Expression of TT7 Inferred from Heterogeneous Distribution of Metabolites in Arabidopsis flowers", *Anal. Methods*, **4**, 474 -481 (2012). DOI: [10.1039/C2ay05618f](https://doi.org/10.1039/C2ay05618f)
9. P. J. Horn, A. R. Korte, P. B. Neogi, E. Love, J. Fuchs, K. Strupat, L. Borisjuk, V. Shulaev, Y. J. Lee, K. D. Chapman, "Spatial mapping of lipids at cellular resolution in embryos of *Gossypium hirsutum*, L.", *Plant Cell*, **24**, 622-636 (2012). DOI: [10.1105/TPC.111.094581](https://doi.org/10.1105/TPC.111.094581)
10. Y. J. Lee, D. C. Perdian, Z. Song, E. S. Yeung, B. J. Nikolau, "Use of mass-spectrometry for imaging metabolites in plants", *Plant J.*, **24**, 622-636 (2012). Doi [10.1111/J.1365-313x.2012.04899.X](https://doi.org/10.1111/J.1365-313x.2012.04899.X)

Session IX

Nitrogenase Reduction of CO₂ to Hydrocarbons

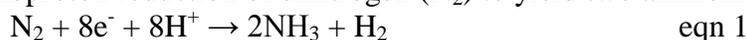
Dennis R. Dean, Principal Investigator

Fralin Hall, 101 West Campus Drive, Fralin Life Science Institute, Blacksburg, VA 24060

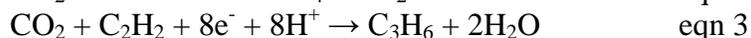
Email: deandr@vt.edu; Website: <http://www.fralin.vt.edu/>

Overall research goals:

This project represents an integrated collaborative project with the Seefeldt group. Our contribution includes genetic approaches and large-scale fermentation of mutant strains. The research objectives are to gain a molecular level understanding of the activation and reduction of CO₂ to hydrocarbons catalyzed by the bacterial enzyme nitrogenase. Nitrogenase normally catalyzes the multi-electron/proton reduction of dinitrogen (N₂) to yield two ammonia (NH₃).



This is one of the most demanding of the small molecule reduction reactions achieved in nature. We have discovered that the protein side-chains approaching the active site metal cluster, FeMo-cofactor, can be remodeled to permit reduction of larger compounds. In one such remodeled nitrogenase, we were able to demonstrate that nitrogenase could reduce CO₂ by 8 electrons/protons all the way to methane (CH₄) in a catalytic reaction unprecedented in biology.



We further demonstrated that it was possible to couple the reduction of two substrates at the same time. Acetylene (C₂H₂) and CO₂ could be activated, with observation of C₃ hydrocarbon products. Our goals are to understand these reactions and to improve the reactions through further remodeling of the active site of nitrogenase.

Significant achievements (2013-2014):

A major research effort has been to understand how multi-electron reduction of substrates by nitrogenase is achieved. We have made considerable progress in this direction by trapping and characterizing some key intermediates along the reaction pathway. One of the most significant observations from these studies is our characterization of two Fe-hydrides (Fe-H) bound to the active site metal cluster FeMo-co before N₂ binds. We have put forward a mechanism for how these hydrides might participate in N₂ reduction in a *Chemical Reviews* article. Namely, we have developed a mechanism that invokes the Fe-H⁻ as a way to accumulate electrons and H at the active site with subsequent utilization of these Fe-H⁻ in the stepwise reduction of N₂ through a metal bound diazene (HN=NH) and hydrazine (H₂N-NH₂) finally to 2NH₃.

We are now working to understand the mechanism of CO₂ activation at the active site FeMo-co and the possible roles of metal hydrides in the mechanism. Toward this end, we have recently made an insightful discovery. When the CO₂ reduction assay is conducted in D₂O rather than H₂O, a significant enhancement (>10x) of methane (CD₄) formation is achieved compared to our initial observation (**Fig 1**). This enhancement (an inverse isotope effect) is not observed for any other substrates we have examined thus far, indicating the critical role of a C-D/H bond making/breaking step as rate limiting in the reaction pathway. We will focus studies over the coming year to exploit this strong inverse isotope effect to develop a model for the mechanism for CO₂ reduction. We will be examining the isotope effect on a number of other

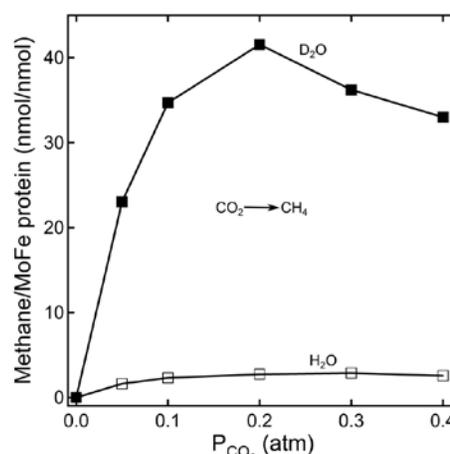


Fig 1: Nitrogenase reduction of CO₂ to CH₄/CD₄ in H₂O or D₂O.

reactions, including the $\text{CO}_2 + \text{C}_2\text{H}_2$ coupling reaction, as a way to refine our model. We anticipate from this line of study to be able to put forward a model for how nitrogenase activates and couples CO_2 , a key outcome in our efforts to understand activation of this recalcitrant molecule.

We have also initiated a collaboration with Drs. Dayle Smith and Simone Raugei (computational chemists) at the Pacific Northwest National Laboratory (PNNL) to apply high-level computations to better understand CO_2 reduction by nitrogenase. In an initial study, we have used molecular dynamics to investigate channels for substrate access to the active site. From these studies, a novel substrate channel has been observed and energy calculations indicate that this channel offers a low energy pathway for N_2 access to the active site (see **Fig 2**). We are now continuing these calculations to explore the energetics of CO_2 movement through the channel and possible binding sites and intermediates at FeMo-cofactor. Calculations at this level have not been applied to nitrogenase in the past. Our goal is to develop an energy profile for CO_2 access to the active site and CH_4 egress. Longer term, we will seek to apply QM/MM methods to define the energy favored reaction coordinate for multi-electron/proton reduction of CO_2 .

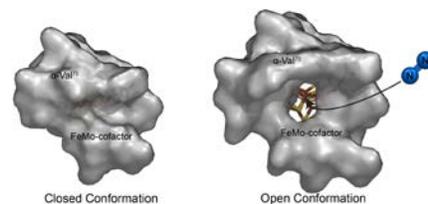


Fig 2: Substrate channel revealed by dynamic calculations.

Science objectives for 2014-2015:

- Gaining insights into the possible roles of metal hydrides in catalyzing the CO_2 reduction reaction. These efforts are focused primarily on isotope effects (D vs H) in the reaction rates and product distribution.
- Apply high level calculations in collaboration with Dayle Smith and Simone Raugei to develop an energy landscape for possible reaction pathways going from CO_2 to CH_4 . This will include calculations for early steps including CO_2 to CO and formate.
- Define reaction parameters and possible pathways for the reduction of nitrite (NO_2^-) and nitrate (NO_3^-) as analogs of CO_2 reduction. These studies are expected to provide greater insights into the roles of metal hydrides in these parallel reactions.
- Discover amino acid substitutions around FeMo-cofactor that alter and expand the reactivity toward CO_2 .
- Explore the reactivity of isolated FeMo-cofactor toward CO_2 activation. This is a much simpler system that would likely provide greater insights into mechanism if reactivity could be demonstrated. A collaboration with the Reek group at the University of Amsterdam has been initiated to explore the effects of different ligands bound to isolated FeMo-co on the reactions with N_2 and CO_2 .

My scientific areas of expertise are: Genetic analysis of biological nitrogen fixation and metallocluster assembly.

To take my project to the next level, my ideal collaborator would have expertise in: Rapid high-resolution crystallographic analysis of remodeled nitrogenases.

Publications supported by this project 2013-2014:

1. Hoffman, B. M., Lukoyanov, D., Yang, Z.-Y., Dean, D. R., and Seefeldt, L. C. (2014) Mechanism of nitrogen fixation by nitrogenase: the next stage. *Chem. Rev.* 114, 4041–4062.

Nitrogenase Reduction of CO₂ to Hydrocarbons

Lance C. Seefeldt, Principal Investigator

Dennis R. Dean, Co-PI(s)

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Email: lance.seefeldt@usu.edu; Website: <http://www.lancesefeldt.org/>

Overall research goals:

The research objectives are to gain a molecular level understanding of the activation and reduction of CO₂ to hydrocarbons catalyzed by the bacterial enzyme nitrogenase. Nitrogenase normally catalyzes the multi-electron/proton reduction of dinitrogen (N₂) to two ammonia (NH₃).



This is one of the most demanding of the small molecule reduction reactions achieved in nature. We have discovered that the protein surrounding the active site metal cluster, FeMo-cofactor, can be remodeled, allowing larger compounds to become substrates for nitrogenase. In one such remodeled nitrogenase, we were able to demonstrate that nitrogenase could reduce CO₂ by 8 electrons/protons all the way to methane (CH₄) in a catalytic reaction unprecedented in biology.



We went on to demonstrate that it was possible to couple the reduction of two substrates at the same time. Acetylene (C₂H₂) and CO₂ could be activated, with observation of C₃ hydrocarbon products. Our goals are to understand these reactions and to improve the reactions through further remodeling of the active site of nitrogenase.

Significant achievements (2013-2014):

A major research effort has been to understand how multi-electron reduction of substrates by nitrogenase is achieved. We have made considerable progress in this direction by trapping and characterizing some key intermediates along the reaction pathway. One of the most significant observations from these studies is our characterization of two Fe-hydrides (Fe-H⁻) bound to the active site metal cluster FeMo-co before N₂ binds. We have put forward a mechanism for how these hydrides would participate in N₂ reduction in a *Chemical Reviews* article. In a nutshell, we have developed a mechanism that invokes the Fe-H⁻ as a way to accumulate electrons and H at the active site and then utilization of these Fe-H⁻ in the stepwise reduction of the N₂ through a metal bound diazene (HN=NH) and hydrazine (H₂N-NH₂) finally to 2NH₃.

We are now working to understand the mechanism of CO₂ activation at the active site FeMo-co and the possible roles of metal hydrides in the mechanism. Toward this end, we have recently made an insightful discovery. When the CO₂ reduction assay is conducted in D₂O rather than H₂O, a significant enhancement (>10x) of methane (CD₄) formation is achieved compared to our initial observation (**Fig 1**). This enhancement (an inverse isotope effect) is not observed for any other substrates we have examined thus far, indicating the critical role of a C-D/H bond making/breaking step as rate limiting in the reaction pathway. We will focus studies over the coming year to exploit this strong inverse isotope effect to develop a model for the mechanism for CO₂ reduction. We will be examining the isotope effect on a number of other reactions, including the CO₂ + C₂H₂ coupling reaction, as a

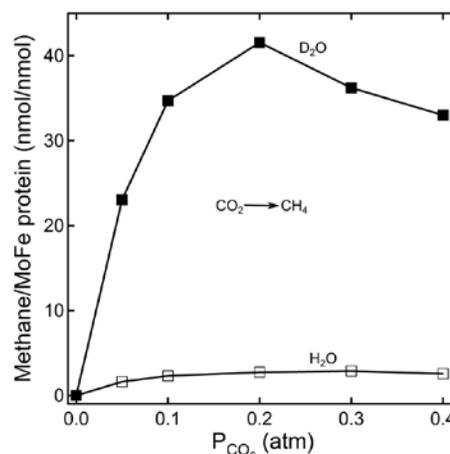


Fig 1: Nitrogenase reduction of CO₂ to CH₄/CD₄ in H₂O or D₂O.

way to refine our model. We anticipate from this line of study to be able to put forward a model for how nitrogenase activates and couples CO₂, a key outcome in our efforts to understand activation of this recalcitrant molecule.

We have also initiated a collaboration with Drs. Dayle Smith and Simone Raugei (computational chemists) at the Pacific Northwest National Laboratory (PNNL) to apply high-level computations to better understand CO₂ reduction by nitrogenase. In an initial study, we have used molecular dynamics to investigate channels for substrate access to the active site. From these studies, a novel substrate channel has been observed and energy calculations indicate that this channel offers a low energy pathway for N₂ access to the active site (see **Fig 2**). We are now continuing these calculations to explore the energetics of CO₂ movement through the channel and possible binding sites and intermediates at FeMo-cofactor. Calculations at this level have not been applied to nitrogenase in the past. Our goal is to develop an energy profile for CO₂ access to the active site and CH₄ egress. Longer term, we will seek to apply QM/MM methods to define the energy favored reaction coordinate for multi-electron/proton reduction of CO₂.

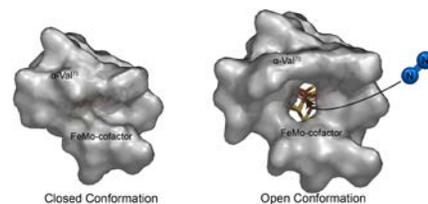


Fig 2: Substrate channel revealed by dynamic calculations.

Science objectives for 2014-2015:

- Gaining insights into the possible roles of metal hydrides in catalyzing the CO₂ reduction reaction. These efforts are focused primarily on isotope effects (D vs H) in the reaction rates and product distribution.
- Apply high level calculations in collaboration with Dayle Smith and Simone Raugei to develop an energy landscape for possible reaction pathways going from CO₂ to CH₄. This will include calculations for early steps including CO₂ to CO and formate.
- Define reaction parameters and possible pathways for the reduction of nitrite (NO₂⁻) and nitrate (NO₃⁻) as analogs of CO₂ reduction. These studies are expected to provide greater insights into the roles of metal hydrides in these parallel reactions.
- Discover amino acid substitutions around FeMo-cofactor that alter and expand the reactivity toward CO₂.
- Explore the reactivity of isolated FeMo-cofactor toward CO₂ activation. This is a much simpler system that would likely provide greater insights into mechanism if reactivity could be demonstrated. A collaboration with the Reek group at the University of Amsterdam has been initiated to explore the effects of different ligands bound to isolated FeMo-co on the reactions with N₂ and CO₂.

My scientific areas of expertise are: Mechanism of metalloenzymes; CO₂, N₂ and H₂ activation.

To take my project to the next level, my ideal collaborator would have expertise in: Calculations to define the energy profile for the reaction pathways.

Publications supported by this project 2013-2014:

1. Smith, D., Danyal, K., Raugei, S., and Seefeldt, L. C. (2014) Substrate channel in nitrogenase revealed by a molecular dynamics approach. *Biochemistry* 53, 2278–2285.
2. Hoffman, B. M., Lukoyanov, D., Yang, Z.-Y., Dean, D. R., and Seefeldt, L. C. (2014) Mechanism of nitrogen fixation by nitrogenase: the next stage. *Chem. Rev.* 114, 4041–4062.

Nanotube-Supported Phospholipid Bilayers

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Antonin Marek and Maxim A. Voynov, Research Associates

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Overall research goals:

The overall long term objective of this project is to develop a new class of nanoscale biotechnological objects - substrate-supported lipid nanotubes - and to utilize these nanoscale structural elements for building robust hybrid biological nanodevices that are based on functionally active membrane proteins. Specifically, we aim at: 1) developing efficient experimental protocols for loading and self-assembling bilayer membranes of various lipid compositions inside the nanochannels formed in anodic aluminum oxide (AAO) substrates; 2) improving technology for fabricating homogeneous AAO with desired pore dimensions and low light absorbance to enable biophysical studies of light-harvesting biomolecular systems; 3) investigating effects of surface chemistry on the lipid self-assembly and the properties of the lipid bilayers formed by surface modification of nanoporous substrates; 4) demonstrating initial feasibility of employing lipid nanotube technology for building hybrid nanostructures based on membrane proteins.

Significant achievements (2011-2014):

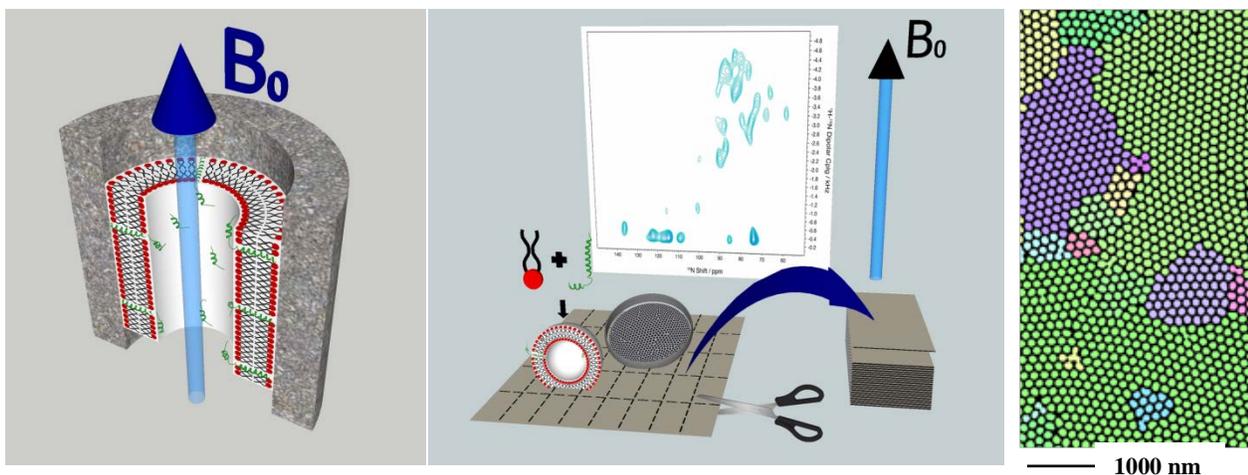


Figure 1. Graphical Concept: A schematic of nanotubular bilayer with a single transmembrane helix protein inserted into the bilayer and the orientation of the nanopore with respect to the external magnetic field for NMR and EPR studies. Middle Panel: A cartoon of preparation of lipid nanotube arrays containing functional membrane proteins for magnetic resonance or biophysical studies with following steps: (a) preparation of proteoliposomes by self-assembly, (b) incorporation of proteoliposomes into nanoporous substrates by one-step centrifugation, (c) computerized cutting of the substrates into the desired shapes, (d) stacking the strips to increase signal-to-noise ratio of NMR spectra and aligning the stack with respect to magnetic field, and (d) spectroscopic measurements by solid-state oriented sample NMR. Right: SEM of AAO with domains colored.

Our major accomplishments under the current DOE award are summarized as follows:

- Developed centrifugation-based method and accessories to increase loading of lipid bilayers into AAO nanochannels by at least twofold regardless the phospholipid composition.
- Developed and optimized a number of high throughput procedures for fabricating high quality nanoporous substrates with pore diameters ranging from 25 to 90 nm and exceptionally narrow (4 to 5 nm) pore diameter distributions.

- Developed nanoporous substrates that are transparent at wavelength above 260 nm for biophysical studies of light-harvesting biomolecular systems.
- Demonstrated hybrid lipid-AAO-QCM biochip for studying lipid self-assembly and interactions with nanoporous substrates and utilized those for studying lipid-protein interactions.
- By using lipid nanotube technology showed that the Pf1 helix tilt angles in two lipid systems are not entirely governed by the membrane thickness but could be rationalized by hydrophobic interactions of lysines with lipids at the bilayer interface.
- Carried out initial experiments on incorporating rhodopsins into lipid nanotubes formed inside macroscopically aligned ceramic nanoporous substrates for NMR and EPR structural studies as well as for biosensor applications.

Science objectives for 2014-2015:

- To employ newly developed nanoporous QCM crystal for studying lipid-protein interactions and self-assembly under conditions of nanoscale confinement including effects of surface charges.
- Carry out measurements of the lipid bilayer surface potential upon illuminating RC proteins and rhodopsins incorporated into lipid nanotube arrays.
- Develop new EPR methods for studying local electrostatic phenomena and apply those to study energy transduction in biological systems such bRC and PSII and develop new collaborations.

My scientific areas of expertise are: Magnetic resonance, multifrequency EPR, hybrid nanomaterials, biophysics of lipid bilayers and membrane proteins.

To take my project to the next level, my ideal collaborator would have expertise in: molecular biology and optical methods.

Publications supported by this project (2011-2014):

1. Maxim A. Voinov, Izarys Rivera, Alex I. Smirnov, "Surface Electrostatics of Anionic Bilayers of Mixed Lipid Composition by EPR of a pH-Sensitive Spin-Labeled Lipid", *Biophysical J.*, 104, 106-116 (2013).
2. Jens O. Lagerstedt, Jitka Petrlova, Silvia Hilt, Antonin Marek, Madhu Budamagunta, Renuka Sriram, Jean F. Desreux, David Thonon, Thomas Jue, Alex I. Smirnov, and John C. Voss, "EPR assessment of protein sites for incorporation of Gd(III) MRI contrast labels", *Contrast Media and Molecular Imaging*, 8 252–264 (2013).
3. H. Mebrahtu, I. Borzenets, D. Liu, H. Zheng, Yu. Bomze, Alex Smirnov, H. Baranger, G. Finkelstein "Quantum Phase Transition in a Resonant Level Coupled to Interacting Leads", *Nature*, 488, 61–64, (2012).
4. H. T. Mebrahtu, I. V. Borzenets, H. Zheng, Y. V. Bomze, Alex I. Smirnov, S. Florens, H. U. Baranger, and G. Finkelstein, "Observation of Majorana quantum critical behavior in a resonant level coupled to a dissipative environment", *Nature Physics*, 9: 732-737 (2013) DOI: [10.1038/NPHYS2735](https://doi.org/10.1038/NPHYS2735)
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Regulation of Plant Cells, Cell Walls, and Development by Mechanical Signals

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Overall research goals:

The goal of the project is to test and extend a new view of plant development, in which mechanical signals between cells in growing tissues play a role as critical as that of chemical signals. Our earlier work has shown at least two different effects of mechanical forces on the shoot apical meristem. One is that the microtubule cytoskeleton of the epidermal cells of the meristem aligns parallel to the principal direction of maximal stress when stress is anisotropic. The stresses in this case are largely supracellular, resulting from the fact that the epidermal cells are under tension (as the inner cells press out on them), and from the shape of the meristem – regions between newly forming leaves or flowers and the meristem are saddle-shaped, creating high anisotropic tension parallel to the saddle. The microtubules align across these saddles, and if this alignment is prevented, the valley between meristem and primordium fails to form – presumably because the direction of cellulose synthesis is directed by the microtubules, and the newly deposited cellulose fibrils reinforce the cells against stress.

A second feedback is on auxin transport. The PIN1 auxin transporter, responsible for auxin movement between shoot meristem L1 cells in the inflorescence stage (and therefore for the rate and pattern of appearance of flowers), is mechanically controlled, such that it accumulates preferentially in the most stressed (or strained) side wall of each L1 cell. As auxin makes cells expand (by weakening the cell wall and allowing turgor to force cell growth), and this expansion creates stress on neighboring cells, there is a general tendency for auxin to move from cells with lower auxin to those with higher. This has been shown by computational models to generate the known phyllotactic patterns.

Computational models of growing meristems that include these mechanical feedbacks show behavior very much like that of the meristems themselves. There are nonetheless many unanswered questions. The specific research goals of the current period of funding have been to answer several of these: first, can we show that the predicted mechanical effects are due to physical forces and not other forms of cell-cell communication? As most of the earlier tests of the auxin part of the model involved cell ablations, we need experiments to rule out (or in) wounding effects. Second, can we extend the model to new aspects of meristem growth, such as cell wall biosynthesis and cell division? Third, does mechanical signaling control growth in parts of the plant other than the meristem? And fourth, can we use the predicted feedbacks and computational models to further probe the mechanics and behavior of plant cells and plant cell walls?

Significant achievements (2012-2015):

We have created a set of reversible treatments that change the stress patterns in a shoot apex, such as changing turgor with mannitol solutions, flattening with a coverslip, and local treatment with cell wall loosening enzymes (for example cellulases), and they all cause the predicted changes in PIN1 localization. Thus, the effects seen earlier were due to mechanics, and not wounding.

We have developed a set of fluorescent reporter strains for live-imaging of cellulose synthase complexes, to see if as expected the cellulose synthases move parallel to microtubules (which they are thought to ride as tracks), and therefore, that reinforcement of cell walls follows stress patterns. As microtubules also may dictate plane of cell division, we have developed a mathematical approach to predicting cell wall division plane, and have shown that a model that does not include stress (but does include all parameters previously thought to influence division plane, such as strain and cell shape) does not predict division well in the meristem, especially in regions where stress is most anisotropic.

We performed extensive sets of experiments with pavement cells of developing cotyledons, and found that the stress pattern in these cells with complex shapes (as predicted by finite element method models

of the cells and of the tissue) correspond with their microtubule arrays, and with cell wall reinforcements detected by atomic force microscopy that are consistent with the expected properties of cellulose fibrils. As stresses in these cells are determined largely by cell shape, and vary in different cellular regions, we can add a third source of stress (tissue shape, local tissue expansion, and now cell shape) that feeds back to plant cells, and can infer that the mechanism by which the microtubules align to anisotropic stress are subcellular. Further experiments showed that stress changes induce microtubule severing, and that microtubule realignment to stress in the pavement cells requires the severing enzyme katanin.

One additional prediction of the stress model is that auxin flow patterns in the meristem will depend on the properties of the cell wall, as its viscoelasticity is regulated by stress (via microtubules and cellulose synthesis). Any mutation that changes the viscoelasticity or ability to grow of the cell wall could therefore change phyllotactic pattern, as could mutations that affect PIN1 localization. We have collected phyllotactic mutants and find that some do code for suspected cell wall biosynthetic enzymes, such as cellulose synthase family members CSLDs, which have unknown function. Gene expression analysis shows them to be expressed in meristems, and cell wall carbohydrate linkage analysis shows them strongly to affect heteromannan and homogalacturonan levels. We thus have a new way of probing for cell wall mutants. We have also found mutants (affecting sterol biosynthesis) that change PIN1 patterns, giving a clue to the PIN1 relocalization mechanism.

Science objectives for 2014-2015:

- Use cellulose synthase reporters to track relation of microtubule array and cell wall biosynthesis
- Add stress to cell division model to see if it then allows prediction of cell division plane
- Continue analysis of CSLD mutants, analyze CESA (cellulose synthase) mutants for phyllotaxis, cytoskeletal and auxin transport effects
- Associate changes in cell wall linkages in *cslD* mutants to cell wall biosynthetic enzymes by RNAseq of mutant meristems, to see if feedback from wall alters wall biosynthetic gene expression

My scientific area(s) of expertise is/are: Genetics and molecular biology of plants, plant genomics, plant developmental biology.

To take my project to the next level, my ideal collaborator would have expertise in: Mechanical engineering and the development of physical models of plant cells and tissues, especially by finite element methods. We also need (but have) collaborators with expertise in computational modeling, in plant cell wall biosynthesis and analysis, and in plant cytoskeletal dynamics.

Publications supported by this project 2012-2015:

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*Principal Investigator
Awards and Recognition*

Principal Investigator Awards and Recognition

This is a new (and recurring) addition to our Physical Biosciences meeting book – we want to add our best wishes and heartfelt congratulations to every one below for your excellence in research as recognized by your peers. If you have received a significant award from other than your home institution that does not appear below, or wish to update any of this information, please email Robert.Stack@science.doe.gov as this list will be updated periodically as appropriate. Thank you!

Elected Members of the National Academy of Science (Year Elected in Parentheses)

Natasha Raikhel, University of California, Riverside (2012)
Caroline Harwood, University of Washington (2009)
W.E. Moerner, Stanford University (2007)
Daniel Cosgrove, Penn State University (2005)
Michael Thomashow, Michigan State University/PRL (2003)
Dieter Soll, Yale University (1997)
Elliot Meyerowitz, Cal Tech (1995)

American Association for the Advancement of Science (AAAS) Fellows (Year Elected)

Alan Jones, University of North Carolina (2012)
Lance Seefeldt, Utah State University (2012)
Alan Davill, University of Georgia/CCRC (2010)
David Lynn, Emory University (2010)
Heven Sze, University of Maryland (2010)
Michael Thomashow, Michigan State University/PRL (2010)
Stephen Ragsdale, University of Michigan (2009)
Caroline Harwood, University of Washington (2008)
Gary Stacey, University of Missouri (2008)
John Shanklin, Brookhaven National Laboratory (2007)
Julian Schroeder, University of California San Diego (2006)
Gloria Coruzzi, New York University (2005)
Michael Sussman, University of Wisconsin (2005)
Russ Hille, University of California, Riverside (2004)
Wolf Frommer, Carnegie Institute/Stanford University (2003)
W.E. Moerner, Stanford University (2003)
Natasha Raikhel, University of California, Riverside (2003)
Clint Chapple, Purdue University (2002)
Richard Vierstra, University of Wisconsin (2002)
Daniel Cosgrove, Penn State University (1993)
Elliot Meyerowitz, California Institute of Technology (1990)
David Knaff, Texas Tech University (1984)

American Society of Plant Biologists (ASPB) Fellows (Year Elected)

Norman Lewis, Washington State University (2014)
Chris Staiger, Purdue University (2014)
Gloria Coruzzi, New York University (2010)
Elliot Meyerowitz, California Institute of Technology (2010)
Gary Stacey, University of Missouri (2010)
Michael Thomashow, Michigan State University/PRL (2009)
Alan Jones, University of North Carolina (2009)
Daniel Cosgrove, Penn State University (2007)
Natasha Raikhel, University of California Riverside (2007)
Heven Sze, University of Maryland (2007)

American Academy of Microbiology (AAM/ASM) Fellows (Year Elected)

William Metcalf, University of Illinois (2010)
Gary Stacey, University of Missouri (2010)
Michael McInerney, University of Oklahoma (??)
Joseph Krzycki, The Ohio State University (2007)
Stephen Ragsdale, University of Michigan (2006)
Michael Adams, University of Georgia (2003)
Caroline Harwood, University of Washington (2000)

Thomson Reuters “Most Highly Cited Researchers” (2014 Only)

Angus Murphy, University of Maryland
Julian Schroeder, University of California San Diego
Michael Thomashow, Michigan State University/PRL
Richard Vierstra, University of Wisconsin
Zheng-Hua Ye, University of Georgia

Other Notable Awards and Recognition (Year Awarded)

Michael Adams, University of Georgia
International Society of Extremophiles (ISE) Lifetime Achievement Award (2014)

Mohammed El Naggari, University of Southern California
Presidential Early Career Award for Scientists and Engineers (PECASE) Award (2014)

Judith Frydman, Stanford University
The Dorothy Crowfoot Hodgkin Award, sponsored by Genentech, for exceptional contributions in protein science (2014)

Wolf Frommer, Carnegie Institute/Stanford University
American Society of Plant Biologists Laurence Bogorad Award (2013)

Alan Jones, University of North Carolina
Elected President of the American Society of Plant Biologists (2013)

David Lynn, Emory University
American Chemical Society Charles Herty Award (2013)

Elliot Meyerowitz, California Institute of Technology
Awarded the Dawson Prize in Genetics by the University of Dublin (2013)

W.E. Moerner, Stanford University
American Chemical Society Peter Debye Award in Physical Chemistry (2013)
Washington University Engineering & Applied Science Alumni Achievement Award (2013)
John Gamble Kirkwood Medal for Outstanding Achievement in Science from Yale University and the New Haven Section of the American Chemical Society (2013)

Natasha Raikhel, University of California Riverside
American Society of Plant Biologists (ASPB) Adolph E. Gude, Jr. Award (2013)
American Society of Plant Biologists (ASPB) Stephen Hales Prize (2004)
The Guggenheim Fellowship (1996)

David Savage, University of California Berkeley
NIH Director’s New Innovator Award (2013)

Julian Schroeder, University of California, San Diego
Churchill International Fellow, Cambridge University, England (2012)

Michael Thomashow, Michigan State University/PRL
Elected Chair, AAAS Section on Agriculture, Food, and Renewable Resources (2014)
American Society of Plant Biologists (ASPB) Stephen Hales Prize (2014)

Richard Vierstra, University of Wisconsin
Elected to the governing board of the American Society of Plant Biologists (2012)