CPBR Final Report

Project Title: Energy from Biomass Research and Technology Transfer Program
Award Number: DE-FG36-02GO12026-08
Recipient: The Consortium for Plant Biotechnology Research, Inc.
Project Locations: St. Simons Island, GA, and subaward universities (see below)
Reporting Period: October 1, 2001 – December 31, 2015

All Subawardees and Subaward Locations, All Years:

Alabama A&M University	Normal, AL
Albany State University	Albany, GA
Arkansas State University	Jonesboro, AK
Clemson University	Clemson, SC
Dartmouth College	Hanover, NH
Donald Danforth Plant Science Center	Columbia, MO
Florida A&M University	Tallahassee, FL
Florida State University	Tallahassee, FL
Georgia Institute of Technology	Atlanta, GA
Hampton University	Hampton, VA
Indiana University	Bloomington, IN
Iowa State University	Ames, IA
Kansas State University	Manhattan, KS
Kentucky State University	Frankfort, KY
Louisiana State University	Baton Rouge, LA
Michigan State University	East Lansing, MI
Michigan Technological University	Houghton, MI
Montana State University	Bozeman, MT
New Mexico State University	Las Cruces, NM
North Carolina A&T State University	Greensboro, NC
North Carolina State University	Raleigh, NC
North Dakota State University	Fargo, ND
Northwestern University	Evanston, IL
Ohio State University	Columbus, OH
Oregon State University	Corvallis, OR
Pennsylvania State University	University Park, PA
Purdue University	West Lafayette, IN
Rutgers University	Newark, NJ
Savannah State University	Savannah, GA
South Dakota State University	Brookings, SD
Southern Illinois University	Carbondale, IL
State University of New York	New York, NY
Syracuse University	Syracuse, NY
Texas A&M University	College Station, TX
University of Chicago	Chicago, IL
University of Colorado	Denver, CO
University of Connecticut	Storrs, CT
University of Florida	Gainesville, FL
University of Georgia	Athens, GA
University of Hawaii	Manoa, HI

University of Illinois	Urbana, IL
University of Iowa	Coralville, IA
University of Kentucky	Lexington, KY
University of Massachusetts	Amherst, MA
University of Michigan	Ann Arbor, MI
University of Minnesota	St. Paul, MN
University of Missouri	Columbia, MO
University of Nebraska	Lincoln, NE
University of Tennessee	Knoxville, TN
University of Toledo	Toledo, OH
University of Washington	Seattle, WA
University of Wisconsin	Madison, WI

All Cost-Sharing Partners, All Years:

Advanced Manufacturing Institute
Agrivida, Inc.
AgroFresh, Inc. (Rohm and Haas)
Alltech
American Chestnut Foundation
ArborGen LLC
Archer Daniels Midland Company
Ashland Specialty Chemical Company
Bailey Nurseries
BASF Plant Science, LLC
Bayer CropScience
Boise Cascade
BRDC
ButylFuel, LLC
C-Far Biomass Energy Special Research Institute
Cargill
Cellectis Plant Sciences
Ceres Inc.
Corn Marketing Program of Michigan
Dow AgroSciences LLC
DuPont Agricultural Biotechnology
Edenspace Systems Corporation
EnerGenetics International
Environmental Energy
FuturaGene Inc.
Georgia Food Processing Advisory Council
Hawaii Agriculture Research Center
Iowa Soybean Association
Kentucky Small Grain Growers Association
Kentucky Soybean Board
Landscape Plant Development Center
Martek Biosciences
MeadWestvaco Corporation
Mendel Biotechnology, Inc.
Minnesota Soybean Research & Promotion Council
Monsanto Company
North Carolina Natural Resources Foundation
Pacific Carbon & Graphite
Peanut Foundation, The
Phenotype Screening Corporation
PolyOne Corporation
Procter & Gamble Company, The
Rennessen
SuGanit Systems, Inc.
SunEthanol
Syngenta
Technology Crops International
Tree Free Technologies
UniSouth Genetics, Inc.
United AgriProducts
United Soybean Board
United States Golf Association
Verdia
ViaLactia/Key Ingredients
Weyerhaeuser Company
Wood-based Compopsite Center
WorldWide BioEnergy
XL TechGroup
Zolaris

Alabama A&M University
Albany State University
Arkansas State University
Clemson University
Dartmouth College
Donald Danforth Plant Science Center
Florida A&M University
Florida State University
Georgia Institute of Technology
Hampton University
Indiana University
Iowa State University
Kansas State University
Kentucky State University
Louisiana State University
Michigan State University
Michigan Tech University
Montana State University
New Mexico State University
North Carolina A&T State University
North Carolina State University
North Dakota State University
Northwestern University
Ohio State University
Oregon State University
Pennsylvania State University
Purdue University
Rutgers University
Savannah State University
South Dakota State University
Southern Illinois University
State University of New York
Syracuse University
Texas A&M University
University of Chicago
University of Colorado
University of Connecticut
University of Florida
University of Georgia
University of Hawaii
University of Illinois
University of Iowa
University of Kentucky
University of Massachusetts
University of Michigan
University of Minnesota
University of Missouri
University of Nebraska
University of Tennessee
University of Toledo
University of Washington
University of Wisconsin

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Project Monitor: William Schrode
Financial Assistance Specialist: Lalida Crawford
Contracting Officer: Jon Olsen

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Semi-Annual Progress Report
Energy from Biomass Research and Technology Transfer Program

Project Objective

The purpose of CPBR is to foster and facilitate research that will lead to commercial applications. The goals of CPBR's Energy from Biomass Research and Technology Transfer Program are to bring together industry, academe, and federal resources to conduct research in plant biotechnology and other bio-based technologies and to facilitate the commercialization of the research results to: (1) improve the utilization of plants as energy sources; (2) reduce the cost of renewable energy production; (3) facilitate the replacement of petroleum by plant-based materials; (4) create an energy supply that is safer in its effect on the environment, and (5) contribute to U.S. energy independence.

CPBR brings together the resources of member organizations to address the problems and the future of U.S. plant-derived energy resources and plant-based energy industries through a program of industrially relevant, scientifically meritorious basic, applied, and scale-up research.

The process of selecting projects is highly competitive. It insures that the work carried out through CPBR has technical merit, commercial relevance, and high potential for the transfer of new technologies from the academic research laboratory to the marketplace.

Status

1. Planned Activities

- CPBR has no further planned activities or funding under this Grant.

2. Progress in Past Six Months

- During the past six months, CPBR has proceeded as planned with all tasks.
- Under grant GO12026, eleven (11) projects were funded from the FY2002 funds, sixteen (16) from the FY2003 modification, nineteen (19) from the FY2004 modification, twenty one (21) from the FY2005 modification, twenty three (23) from the FY2006 modification, twenty three (23) from the FY2008 modification, twenty one (21) from the FY2009 modification, and fourteen (14) from the FY2010 modification, for a total of 148 projects.
- One hundred thirty-three (148) projects of the 148 have been completed, final reports provided to DOE, and closed.
- No, (0) projects of the 148 are active and are currently being managed by CPBR.

3. Variances

Grant was scheduled to end December 31, 2013 a No-Cost Extension was requested and approved to extend the grant to December 31, 2015.

4. Plans for Next Six Months

- This is the Final Report for this Grant.

5. Results from Commercialization Study

A 2013 survey of intellectual property and commercialization results of the 444 CPBR-funded university research projects showed the following: 94 US patents, 23 foreign patents, 108 patent applications pending, 44 active disclosures, 253 licenses (196 currently active), and at least 6 start-up companies. (Licensing fees and royalties are paid by the licensor to the universities that hold the patent rights.)

Patents: The cumulative average rate of US patents from CPBR-funded research projects is **1.96** patents per one million dollars ($1,000,000) of federal funding. The cumulative average university rate of US patents is **0.12** patents per one million dollars ($1,000,000) of federal funding (from the 191 US universities responding to 2011 AUTM survey). **The CPBR patent rate is 1670 percent higher than the average university rate.**

Active licenses: The cumulative rate of active licenses for technologies developed through CPBR-funded research projects is **4.09** licenses per one million dollars ($1,000,000) of federal funding. The cumulative average rate of active licenses from university research is **0.97** licenses per one million ($1,000,000) dollars of federal funding (AUTM survey). **The CPBR active license rate is 424 percent higher than the university average rate.** CPBR's superior commercialization results reflect its innovative, organized, competitive research and technology transfer process.

Publications: CPBR funding for university research has resulted in over **52** peer reviewed publications per one million dollars ($1,000,000) of federal funding.

CPBR's cumulative federal funding is **$47.9 million ($47,900,000).** The cumulative university federal funding is $**40 billion ($40,000,000,000)** (AUTM survey). The cumulative National Renewable Energy Laboratory federal funding is **$2.1 billion ($2,152,900,000)** (www.nrel.gov).

CPBR's federal funding is **0.12** percent of the university federal funding and **2.22** percent of NREL federal funding.

CPBR-funded research has focused on improved energy crops, advanced biofuels and other industrial biochemicals and bioproducts, protection of the environment, and other social and economic benefits. The reported university research results include all fields and all disciplines.

Comparative Percentages

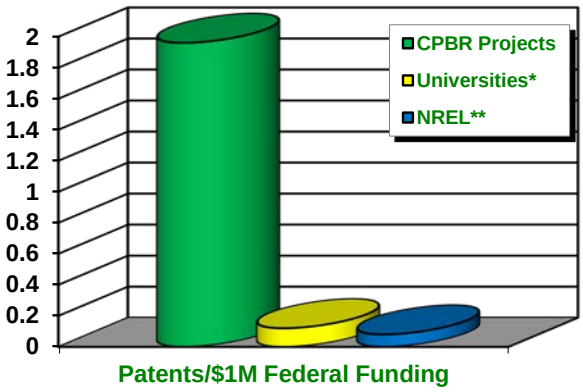
- CPBR-funded research projects' average US patent/federal dollar rate is 1670 percent higher than the average US patent/federal $ rate of the 191 US universities reporting.
- CPBR-funded research projects' average US patent/federal dollar rate is 2515 percent higher than DOE's National Renewable Energy Laboratory's patent/federal $ rate.
- CPBR-funded projects' cumulative active license rate is 424 percent higher than US universities' cumulative active license rate.
- CPBR-funded projects' publication/federal dollar rate is 110 percent higher than the 0.0004 percent reported by NSF.

IP and Commercialization Results as of 5/9/2013

(444 CPBR-funded University Research Projects)

US Patents Granted	94
Foreign Patents Granted	23
Patents Pending	108
Additional Disclosures	44
Start-Up Companies	6
Total Licenses	253
Total Licenses Currently Active	196

US Patents



CPBR funding for university research has produced over **1.96 US patents per one million dollars ($1M) of federal funding.** This shows the effectiveness of the CPBR technology transfer process compared with the technology transfer processes used by US universities and the U.S. Department of Energy's National Renewable Energy Laboratory ("NREL").

***From latest AUTM Survey (2011)**

AUTM Association of University Technology Managers. U.S. Licensing Activity Survey Highlights. Retrieved May 8, 2013, from www.autm.net

****All patents available for license over 5 years**

U.S. Department of Energy. Energy Innovation Portal, National Renewable Energy Laboratory Technologies Available for Licensing. Retrieved May 8, 2013, from http://techportal.eere.energy.gov/lab/NREL

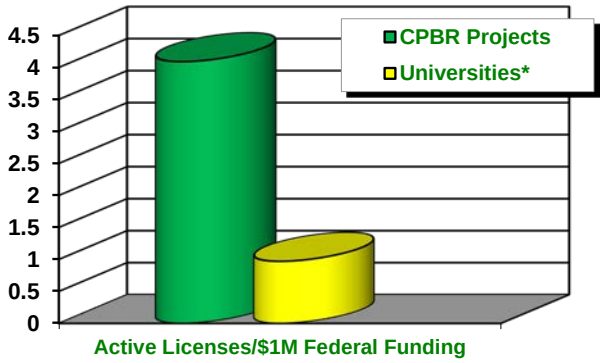
*CPBR's cumulative federal funding is **$47.9 million ($47,900,000).** The cumulative university federal funding is $**40 billion ($40,000,000,000)** (AUTM survey). The cumulative National Renewable Energy Laboratory federal funding is **$2.1 billion ($2,152,900,000)** (www.NREL.gov).*

*CPBR's federal funding is **0.12** percent of the university federal funding reported and **2.22** percent of NREL federal funding reported.*

Active Licenses Prove Commercialized Inventions

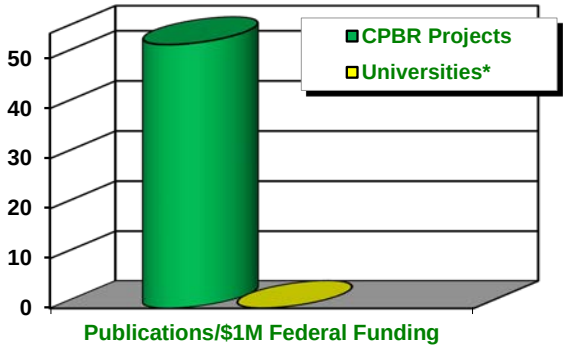
CPBR funding for university research has resulted in over **4.09 active US licenses per one million dollars ($1M) of Federal funding.** This figure shows CPBR-funded projects' commercialization results as compared with US universities' commercialization results.

***From 2011 AUTM Survey**



Peer Reviewed Scientific Publications

CPBR funding for university research has resulted in over **52 peer reviewed publications per one million dollars ($1M) of Federal funding.** This figure shows CPBR-funded projects' publication results as compared with US universities' publication results.



***5 year average of publications/$1M federal funding to universities (NSF)**
NSF National Science Foundation. Publications. Retrieved May 8, 2013, from http://www.nsf.gov/publications/

*CPBR's cumulative federal funding is **$47.9 million ($47,900,000).** The cumulative university federal funding is $**40 billion ($40,000,000,000)** (AUTM survey). The cumulative National Renewable Energy Laboratory federal funding is **$2.1 billion ($2,152,900,000)** (www.NREL.gov).*

*CPBR's federal funding is **0.12** percent of the university federal funding reported and **2.22** percent of NREL federal funding reported.*

6. Updated Gantt Chart/Roadmap

ID	Task	Planned Completion	Actual Completion	Comments
1	**CPBR 2011 Bioenergy Research Competition**			
1.1	Update Industry Research Needs list	August 2009	August 2009	
1.2	Issue Request for Preproposals	September 2009	September 2009	
1.3	Receive preproposals	December 2009	December 2009	
1.4	Send preproposals to member companies	January 2010	January 2010	
1.5	Preproposal posters presented to member company representatives at CPBR Symposium	February 9-10, 2010	February 9-10, 2010	
1.6	Compile company reviews of preproposal relevance	March 2010	March 2010	
1.7	Invite full proposals	April 2010	April 2010	
1.8	Receive full proposals	June 18, 2010	June 18, 2010	
1.9	Assist PIs to identify potential corporate sponsors and obtain matching commitments	September 2010	December 2010	
1.10	Peer reviewer matching and peer review	June-July 2010	July 2010	
1.11	Receive matching commitments on full proposals	September 2010	December 2010	
1.12	Coordinate meeting of Project Recommendation Committee to recommend projects for funding	December 2010	January 2011	
1.13	Board of Directors votes on Committee's recommendations	Competition was not Funded	Competition was not Funded	
1.14	Obtain and have NEPA consultant approve GO-EF1s, submit to DOE	Competition was not Funded	Competition was not Funded	
1.15	DOE Responses	Competition was not Funded	Competition was not Funded	
1.16	NEPA Approvals by DOE	Competition was not Funded	Competition was not Funded	
1.17	Make 2011 project awards	Competition was not Funded	Competition was not Funded	
2	**CPBR 2012 Bioenergy Research Competition**			
2.1	Issue Request for Preproposals	September 2010	October 2010	
2.2	Receive preproposals	December 2010	December 2010	
2.3	Send preproposals to member companies	January 2011	January 2011	
2.4	Preproposal posters presented to member company representatives at CPBR Symposium	March 1-2, 2011	March 1-2, 2011	
2.5	Compile company reviews of preproposal relevance	March 2011	March 2011	

2.6	Invite full proposals	April 2011	May 2011	
2.7	Receive full proposals	June 2011	July 2011	
2.8	Assist PIs to identify potential corporate sponsors and obtain matching commitments	September 2011	September 2011	
2.9	Peer reviewer matching and peer review	June-July 2011	July 2011	
2.10	Receive matching commitments on full proposals	September 2011	September 2011	
2.11	Coordinate meeting of Project Recommendation Committee to recommend projects for funding	January 2012	January 2012	
2.12	Board of Directors votes on Committee's recommendations	Competition was not Funded	Competition was not Funded	
2.13	Obtain and have NEPA consultant approve GO-EF1s, submit to DOE	Competition was not Funded	Competition was not Funded	
2.14	DOE Responses	Competition was not Funded	Competition was not Funded	
2.15	NEPA Approvals by DOE	Competition was not Funded	Competition was not Funded	
2.16	Make 2012 project awards	Competition was not Funded	Competition was not Funded	
3	**CPBR 2013 Bioenergy Research Competition**			
3.1	Issue Request for Preproposals	October 2011	December 2011	
3.2	Receive preproposals	December 2011	December 2011	
3.3	Send preproposals to member companies	January 2012	January 2012	
3.4	Preproposal posters presented to member company representatives at CPBR Symposium	March 6-7, 2012	March 6-7, 2012	
3.5	Compile company reviews of preproposal relevance	March 2012	April 2012	
3.6	Invite full proposals	May 2012	May 2012	
3.7	Receive full proposals	July 2012	July 2012	
3.8	Assist PIs to identify potential corporate sponsors and obtain matching commitments	September 2012	Ongoing	
3.9	Peer reviewer matching and peer review	July-August 2012	August 2012	
3.10	Receive matching commitments on full proposals	September 2012	Ongoing	
3.11	Coordinate meeting of Project Recommendation Committee to recommend projects for funding	December 2012	January 2013	
3.12	Board of Directors votes on Committee's recommendations	Competition was not Funded	Competition was not Funded	
3.13	Obtain and have NEPA consultant approve GO-EF1s, submit to DOE	Competition was not Funded	Competition was not Funded	

3.14	DOE Responses	Competition was not Funded	Competition was not Funded	
3.15	NEPA Approvals by DOE	Competition was not Funded	Competition was not Funded	
3.16	Make 2013 project awards	Competition was not Funded	Competition was not Funded	

4	**CPBR 2014 Bioenergy Research Competition**			
4.1	Issue Request for Preproposals	October 2012	October 2012	
4.2	Receive preproposals	December 2012	December 2012	
4.3	Send preproposals to member companies	January 2013	January 2013	
4.4	Preproposal posters presented to member company representatives at CPBR Symposium	March 2013	March 2013	
4.5	Compile company reviews of preproposal relevance	March 2013	March 2013	
4.6	Invite full proposals	May 2013	May 2013	
4.7	Receive full proposals	July 2013	July 2013	
4.8	Assist PIs to identify potential corporate sponsors and obtain matching commitments	September 2013	September 2013	
4.9	Peer reviewer matching and peer review	July-August 2013	August 2013	
4.10	Receive matching commitments on full proposals	September 2013	September 2013	
4.11	Coordinate meeting of Project Recommendation Committee to recommend projects for funding	December 2013	TBD	
4.12	Board of Directors votes on Committee's recommendations	Competition was not Funded	Competition was not Funded	
4.13	Obtain and have NEPA consultant approve GO-EF1s, submit to DOE	Competition was not Funded	Competition was not Funded	
4.14	DOE Responses	Competition was not Funded	Competition was not Funded	
4.15	NEPA Approvals by DOE	Competition was not Funded	Competition was not Funded	
4.16	Make 2013 project awards	Competition was not Funded	Competition was not Funded	
5	**CPBR 2015 Bioenergy Research Competition**			
5.1	Issue Request for Preproposals	October 2013	October 2013	
5.2	Receive preproposals	December 2013	December 2013	
5.3	Send preproposals to member companies	January 2014	January 2014	

5.4	Preproposal posters presented to member company representatives at CPBR Symposium	March 2014	March 2014	
5.5	Compile company reviews of preproposal relevance	March 2014	March 2014	
5.6	Invite full proposals	May 2014	June 2014	
5.7	Receive full proposals	July 2014	July 2014	
5.8	Assist PIs to identify potential corporate sponsors and obtain matching commitments	September 2014	Competition was not Funded	
5.9	Peer reviewer matching and peer review	July-August 2014	Competition was not Funded	
5.10	Receive matching commitments on full proposals	September 2014	Competition was not Funded	
5.11	Coordinate meeting of Project Recommendation Committee to recommend projects for funding	December 2014	Competition was not Funded	
5.12	Board of Directors votes on Committee's recommendations	Competition was not Funded	Competition was not Funded	
5.13	Obtain and have NEPA consultant approve GO-EF1s, submit to DOE	Competition was not Funded	Competition was not Funded	
5.14	DOE Responses	Competition was not Funded	Competition was not Funded	
5.15	NEPA Approvals by DOE	Competition was not Funded	Competition was not Funded	
5.16	Make 2013 project awards	Competition was not Funded	Competition was not Funded	
6	**CPBR 2010 and Previous Research Projects (Subgrant) Management**			
6.1	Perform Stage Gate analysis and reviews	Completed 12/31/2015	Completed 12/31/2015	
6.2	Obtain required documentation plus any revisions for amendments	Completed 12/31/2015	Completed 12/31/2015	
6.3	Prepare and process awards and subagreements	Completed 12/31/2015	Completed 12/31/2015	
6.4	Coordinate matching funds	Completed 12/31/2015	Completed 12/31/2015	
6.5	Coordinate and administer 2010 research projects and ongoing projects from previous years	Completed 12/31/2015	Completed 12/31/2015	
6.6	Review and approve second year requests after Scientific Consultant review and approval.	Completed 12/31/2015	Completed 12/31/2015	
6.7	Receive, review and approve university requests for payment	Completed 12/31/2015	Completed 12/31/2015	
6.8	Obtain required scientific progress reports and metrics reports	Completed 12/31/2015	Completed 12/31/2015	
6.9	Summarize interim and final scientific progress reports for updating *Funded Projects Report*	Completed 12/31/2015	Completed 12/31/2015	

6.10	Maintain list and file of publications resulting from CPBR-funded projects	Completed 12/31/2015	Completed 12/31/2015	
6.11	Disseminate results information	Completed 12/31/2015	Completed 12/31/2015	
7	**Symposia**			
7.1	Hold CPBR 2014 Symposium	March 2014	March 2014	
8	**Information Dissemination and Outreach**			
8.1	Accept into membership new university and company members	Completed 12/31/2015	Completed 12/31/2015	
8.2	Develop and disseminate information about projects	Completed 12/31/2015	Completed 12/31/2015	
8.3	Make presentations at relevant energy from biomass conferences and other events	Completed 12/31/2015	Completed 12/31/2015	
8.4	Participate in relevant energy from biomass conferences, workshops, and meetings	Completed 12/31/2015	Completed 12/31/2015	
8.5	Disseminate information to the CPBR constituent community through email newsletters, the website, print materials, reports, etc.	Completed 12/31/2015	Completed 12/31/2015	
8.6	Maintain liaison with affiliated minority institutions.	Completed 12/31/2015	Completed 12/31/2015	
9	**Commercialization**			
9.1	Update study of the commercialization of IP developed through CPBR funding	Completed 12/31/2015	Completed 12/31/2015	
10	**Program Evaluation and Metrics**			
10.1	Solicit feedback from program participants and interested parties	Completed 12/31/2015	Completed 12/31/2015	
10.2	Review interim and final scientific progress reports	Completed 12/31/2015	Completed 12/31/2015	
10.3	Review company sponsor metrics reports	Completed 12/31/2015	Completed 12/31/2015	

7. Patents and Licenses Applied for and Awarded

158-Nikolau

A patent entitled "Materials and methods for the alteration of enzyme and acetyl-CoA levels in plants" (US Patent number 6,764,851) issued July, 2004.

162-Qu

A patent application was filed in May, 2004: R. Qu and E. Sivamani: *Polyubiquitin rubi3 promoter and 5' regulatory sequences*

171-Qu

A disclosure entitled "promoter of rice polyubiquitin gene rubi3" was filed in February, 2003, with NCSU File No. 03-81.

A disclosure titled "Enhancement of rice polyubiquitin promoter (*rubi3*) with translational fusion of polyubiquitin amino acid coding sequences" was filed on Oct 6, 2003, with NCSU file No. 04-040.

A disclosure titled: "Enhancement of a rice polyubiquitin gene promoter *rubi3* with the first nine nucleotides (original or mutated) of its coding sequence, and identification of a downstream factor that enhances transcriptional activity of the *rubi3* promoter" was filed in March, 2004

A disclosure titled: "Enhancement of rice polyubiquitin promoter (rubi3) with modified poly ubiquitin gene sequences to allow authentic protein expression and identification of 69% dispensable fragment inside rubi3 5'UTR intron" was filed in May 2004\

A US patent was filed on May 14, 2004 (PCT Application No. PCT/US2004/015286, publication No. WO 2004/104174). Polyubiquitin rubi3 promoter and 5' regulatory sequences

175-Widholm

"Use of Tryptophan Analogs (other than 5- methyltryptophan) and Indole Analogs as selective agents for the Anthranilate Synthase Gene (ASA2) for Plastid Transformation" *filed February 21st 2006 to the* Office of Technology Management; University of Illinois - Urbana/Champaign *and provisional patent filed "USE OF TRYPTOPHAN, INDOLE AND ANTHRANILATE ANALOGS AS PLANT TRANSFORMATION SELECTION AGENTS " on June 30, 2006.*

193-Hildebrand

A patent application is pending entitled "RECOMBINANT *STOKESIA* EPOXYGENASE GENE", David Hildebrand and Tomoko Hatanaka inventors, filed 2003, still pending.

A provisional patent on *Vernonia* DGAT2 and uses thereof has been filed.

229-Yang

Yang Y, Reddy, N. Natural cellulosic fiber bundles from corn husks with length, fineness, strength and elongation suitable for textile applications and extraction of natural cellulose fiber bundles from corn husks therefor and textiles comprising corn fibers there from. U.S. Patent Application 20070199669.

245-Cheng

Zong-Ming Cheng and Xia Ye: A broad environmental stress-inducible promoter and its application in crops. Formal patent application is in progress. Some discussions and contacts have been made with ArborGen and Monsanto Company. (Co-funded with DOE-BioEnergy Science Center)

256-Preston

U.S. Provisional Application SN 61/115, 722 UF #12617 "Biocatalyst for complete conversion of hemicellulose to biobased products". Preston, J.F., C. Bi, and J.D. Rice. Filed 11/18/2008

U.S. Provisional Application SN 60/982,623. UF# 12619. Xylan-Utilization Regulon for Efficient Bioprocessing of Hemicellulose and Uses Thereof. Preston, J.F., V. Chow, G. Nong, J.D. Rice, and F.J. St. John. Filed 10/22/2008

258-Schall

"Pretreatment of Biomass", S. Varanasi, C. Schall, Anatharam, P. Dadi, J. Anderson, K.Rao, P. Paripati, and G. Kumar, US provisional patent application filed 03/ 14/2007; Filing # 60/894708

"Saccharifying Cellulose", S. Varanasi, C. Schall and Anatharam P. Dadi, US utility patent application filed 02/2007; Application Filing # 11/710,357.

259-Sticklen

European Patent Application. Production of beta-glucosidase, hemicellulase and ligninase in E1 and FLC-transgenic plants. Tracking No. 8707 0910 0707

261-Triplett

"*Klebsiella pneumoniae* inoculants for enhancing plant growth", issued July 1, 2008, no. 7,393,678. "Materials and methods for enhancing nitrogen fixation in plants", US Patent Application, 20090137390, May 28, 2009.

264-Yang

S.T. Yang, "Methods and Processes for Producing Esters" US provisional patent application Serial No. 61/116,108 filed on November 19, 2008; US Patent Application Serial No. 12/621,982 filed on November 19, 2009.

265-Yang

Y. Yang, N. Reddy, L. Ying, Production of high quality fibers from wheat proteins, kit, and products made from wheat protein fibers. U.S. Pat. Appl. Publ. (2006), 15 pp. CODEN: USXXCO US 2006282958 A1 20061221

268-Hildebrand

Hildebrand, D.F., R. Li and T. Hatanaka. Method for Increasing Renewable Oil Production. Provisional patent filed July, 2010.

Hildebrand, D.F., S. Rao and J.R. Thoguru. FUNGAL DESATURASES AND RELATED METHODS. U.S. Patent Appl No 12/346,234 Patent filed in Jan. 2009.

Hildebrand, D.F., R. Li and T. Hatanaka. Diacylglycerol Acyltransferase Sequences and Related Methods. Patent Appl No. 12/622,045 filed Nov. 19, 2009.

Hildebrand, D.F., W. Jamboonsri and T. Phillips. Early Flowering Chia and Uses Thereof. Patent filed October 30, 2009. 12/609,946.

Additional action on U.S. Patent Appl No 12/622,045, Vernonia DGATs Increase Epoxy Fatty Acid Accumulation including supplying all references.

Hildebrand, D.F. and T. Hatanaka. "Recombinant Stokesia epoxygenase gene". Issued April 29, 2008, US Patent # 7,364,901.

275-Boucias

Title: *Termite enzymes and uses thereof for* in vitro *conversion of lignin-containing materials to fermentable products*. Document serial No. *61/168,275*, International Publication Number WO 2010 / 117843 A2. No active licenses.

Title: *Use of RNA interference to validate new termiticide target sites and as a method of termite control*. Document serial No. *60/991,959*. No active licenses.

Title: *Carbohydrate-based cellulase inhibitors as feeding stimulants in termites*. Document serial No. *11/975,314*. No active licenses.

278-Doty

Patent pending on the use of the poplar yeast isolates for ethanol and xylitol production.

297-Sticklen

A U.S. Patent and an International Patent have been filed by MSU. Both are published.

310-Han

Han, K.-H., Kim, W.-C., Kim., J.-Y., and Ko, J.-H. 2013. Drought indicuble utility promoters. US Provisional Patent (MSUT File No. TEC2013-0115).

315-Rathinasabapathi

Rathinasabapathi, B., Sundaram, S (2013) Increased stress tolerance, yield, and quality via glutaredoxin overexpression. Patent No. 8,519,226.

323-Finer

Highly active soybean promoter from the SUBI-3 polyubiquitin gene and uses thereof, John J. Finer and Robert A. Bouchard. US Patent # 8,395,021, issued March 12, 2013.

A divisional filing with additional claims was made as an amendment to the above patent on February 7, 2013. “Highly active soybean promoters and uses thereof”

325-Hildebrand

Hildebrand, D.F. Triacylglycerol with very high oxidative stability & low temperature fluidity. IP Disclosure 2012

Hildebrand, D.F., W. Jamboonsri and T. Phillips. Early flowering mutant chia and uses thereof. Patent issued November 19, 2013. 8,586,831.

329-Larock

“Grafted Latexes from a Vegetable Oil-Based Aqueous Polyurethane Dispersion and Acrylics,” patent application filed with Archer Daniels Midland 10/12/10.

“Vegetable Oil-Based, Waterborne Polyurethane Dispersions,” patent application filed with Archer Daniels Midland 10/18/10.

“Surfactant-Free Core-Shell Hybrid Latexes,” patent application filed April 22, 2011 through the Iowa State University Research Foundation.

331-Qu

An invention disclosure was filed at NCSU Technology Transfer Office in November, 2011, regarding “*Transgenic switchgrass plants with altered S/G ratio and improved sugar release”* and was accepted (No. NCSU2011-12078).

A disclosure was filed with the Office of Technology Transfer, NCSU.

Qu, R., R. Li, J. Cheng, Z. Wang, J. Xu: Transgenic switchgrass plants with altered S/G ratio and improved sugar release (NCSU Disclosure No. 12212)

7. Publications /Presentations this Period

302-Meilan

Meilan, R. Improving water relations of *Populus* for use as a bio-energy crop. CPBR Symposium; Washington, D.C., 6 March 2013.

304-Gibson

Lin R, Glazebrook J, Katagiri F, Orf JH, Gibson SI (2014) Identification of genes differentially expressed between developing seeds of different soybean cultivars. Under revision

315-Rathinasabapathi

Rathinasabapathi, B. Engineering glutaredoxins for stress tolerance and yield in rice. Invited lecture, Nanjing University, Nanjing, China. June 4, 2013. (oral presentation).

Rathinasabapathi, B. Fern Glutaredoxins and Stress Tolerance. Invited lecture, Nanjing Agricultural University, Nanjing, China, June 2013. (oral presentation).

Krishnamurthy A, Rathinasabapathi, B. Constitutive overexpression of glutaredoxins in transgenic rice slowed early seedling development and increased high temperature stress tolerance. Annual meeting, Plant Molecular and Cellular Biology Program, Daytona Beach, FL, April, 2013. (oral presentation)

Krishnamurthy, A., Kilasi, N., Rathinasabapathi, B. Improving crop productivity under high temperature stress conditions in rice. Innovations for International Development Seminar, University of Florida, Gainesville, FL June 3, 2013. (oral presentation)

Auxins could help crops beat heat and high salinity, UF study suggests. May 21, 2013. http://news.ifas.ufl.edu/2013/05/auxins-could-help-crops-beat-heat-and-high-salinity-uf-study-suggests/

Krishnamurthy, A., Rathinasabapathi, B (2013) Oxidative stress tolerance in plants: Novel interplay between auxin and reactive oxygen species signaling. Plant Signaling and Behavior 8 (in press).

Krishnamurthy, A., Rathinasabapathi, B (2013) Auxin and its transport play a role in plant tolerance to oxidative stress from arsenite, high temperature stress and salinity in Arabidopsis thaliana. Plant Cell, & Environment 36: 1838-1849. DOI 10.1111/pce 12093.

Krishnamurthy, A., Rathinasabapathi, B (2013) Overexpression of fern glutaredoxin PvGrx5 improved thermotolerance of transgenic rice at the vegetative stage and reduced protein carbonylation in leaves during stress. (Plant Biotechnology Journal, MS in preparation as of Sep 2013).

319-Gibson

Gibson SI (2014) Use of LUCI1 to manipulate seed composition. Presented at the "CPBR Annual Symposium", Washington, DC

322-Fei

Jingjie Hao, Ying Feng, Chonglie Ma, Shui-zhang Fei. Construction of A High-Throughput Salt-Specific RNAi Library for Creeping Bentgrass (*Agrostis stolonifera* L) XXII Plant and Animal Genome Meeting, San Diego, CA

Jingjie Hao, Jiangli Dong, Shui-zhang Fei. Knockdown of CBF1 and CBF3 genes reduces the capacity for cold acclimation and freezing tolerance in Brachypodium distachyon. XXII Plant and Animal Genome Meeting, San Diego, CA

Ying Feng, Yanhai Yin, Shuizhang Fei. Down-regulation of BdBRI1, a putative brassinosteroid co-receptor gene enhances drought tolerance in *Brachypodium distachyon*

Ying Feng, Yanhai Yin, Shuizhang Fei. *BdVRN1* gene is key to the vernalization requirement and expression of *BdCBF* genes in *Brachypodium distachyon*

324-Giroux

Schlosser, A.J., P. Hofer, J.M. Martin, B. Beecher, and M.J. Giroux. Enhanced rice growth is conferred by increased leaf ADP-glucose pyrophosphorylase activity. In prep, submission February 2014.

327-Juvik

Sacks, Erik, K. Głowacka, L. Clark, S. Adhikari, J. Peng, J. Stewart, A. Nishiwaki, T. Yamada, U. Jorgensen, T. Hodkinson, J. Gifford, and J. Juvik. 2013. Genetic variation in Miscanthus ×giganteus and the importance of estimating genetic distance thresholds for differentiating clones. Global Change Biology: Bioenergy: In press.

Chae, Won Byoung, Sae Jin Hong, Justin M. Gifford, A. Lane Rayburn, Erik J. Sacks, and John A. Juvik. 2013. Plant morphology, genome size and SSR markers differentiate five distinct taxonomic groups among accessions in the genus *Miscanthus*. Global Change Biology: Bioenergy: doi: 10.1111/gcbb.12101.

333-Siegfried

Valencia, A. A.P. Alves, and B.D. Siegfried. 2013. Molecular cloning and functional characterization of an endocglucanase belonging to GHF45 from the western corn rootworm, *Diabrotica virgifera virgifera*. GENE 513:260-267.

Valencia, A., H. Wang, and B.D. Siegfried. Expression and characterization of a recombinant endoglucanase from the western corn rootworm, *Diabrotica virgifera virgifera* in *Pichia pastoris*. Submitted to *J. Insect Science*. (*Submitted*)

Euyn, S., H. Wang, Y. Pauchet, R.H. ffrench-Constant, E.N. Moriyama, and B.D. Siegfried. Molecular evolution of glycoside hydrolase genes in the western corn rootworm. PLoS ONE (*Submitted*)

Khajuria, C., Narva, K., and B.D. Siegfried. Functional analysis of four RNAi pathway genes in an economically important corn pest, Western corn rootworm (*Diabrotica virgifera virgifera* Le Conte). PLoS One (*In revision*)

334-Vadlani

D- lactic acid biosynthesis from biomass sugars using engineered *Lactobacillus delbrueckii* GSGSO symposium. Sep 21st 2013. IGP. Manhattan. KS.

Lactic acid production from xylose using Lactobacillus brevis. Sustainable BioEnergy REU poster section. August 1st, 2013, Manhattan, KS.

Zhang Y, Vadlani PV (2013) D-lactic acid biosynthesis from biomass-derived sugars via Lactobacillus delbrueckii fermentation. Bioprocess and Biosystems Engineering DOI 10.1007/s00449-013-0965-8

335-Venditti

Conversion of Industrial Paper Sludge to Ethanol: Fractionation of Sludge and Its Impact, Applied Biochem and Biotechnology, Hui Chen, Kevin Daniel, Qiang Han, Richard Venditti *, Hasan Jameel, submitted 2014.

Economic evaluation of the conversion of industrial paper sludge to ethanol, Energy Economics, Hui Chen, Richard Venditti *, Ronalds Gonzalez, Richard Phillips, Hasan Jameel, Sunkyu Park, submitted 2013.

8. Cost Performance Report

Table 1: General Spending Information

			Approved Spending Plan			Actual Spent to Date		
Phase / Budget Period	**From**	**To**	**DOE Amount**	**Cost Share**	**Total**	**DOE Amount**	**Cost Share**	**Total**
Year 1	10/1/2002	9/30/2003	940,000	235,000	1,175,000	685,365	218,444	903,809
Year 2	10/1/2003	9/30/2004	1,935,475	481,750	2,417,225	750,896	215,035	965,931
Year 3	10/1/2004	9/30/2005	2,946,000	736,500	3,682,500	1,616,882	447,898	2,064,780
Year 4	10/1/2005	9/30/2006	1,952,665	488,164	2,440,829	3,169,441	1,144,396	4,313,837
Year 5	10/1/2006	9/30/2007	2,582,668	645,664	3,228,332	2,514,473	633,232	3,147,705
Year 6	10/1/2007	9/30/2008	1,590,667	397,672	1,988,339	2,166,056	66,773	2,232,829
Year 7	10/1/2008	9/30/2009	1,791,000	447,750	2,238,750	2,765,303	218,586	2,983,889
Year 8	10/1/2009	9/30/2010	2,460,000	615,000	3,075,000	1,426,626	829,397	2,256,023
Year 9	10/1/2010	9/30/2011	2,265,842	567,166	2,833,008	3,124,231	781,058	3,905,289
Year 10	10/1/2011	9/30/2012	2,265,842	567,167	2,833,009	2,459,235	242,708	2,701,943
Year 11	10/1/2012	9/30/2013	2,265,841	567,167	2,833,008	1,380,450	407,483	1,787,933
Year 12	10/1/2013	9/30/2014	-	-	-	352,190	132,130	484,320
Year 13	10/1/2014	9/30/2015	-	-	-	79,715	29,965	109,680
		Totals	$ 22,996,000	$ 5,749,000	$ 28,745,000	$ 22,490,863	$ 5,367,105	$ 27,857,968

Table 2: Detailed Cost Share Reporting Table for Subawardees.

Subawardee Information	Approved Spending Plan			Actual Invoiced to Date		
Award No. & Subawardee Name	DOE Amount	Cost Share	Total	DOE Amount	Cost Share	Total
166-Caula Beyl	20,000	5,237	25,237	20,000	8,880	28,880
151-Kathleen Danna	20,000	20,350	40,350	20,000	20,350	40,350
152-Chudwudi Obi Emeh	30,000	30,600	60,600	27,971	30,600	58,571
153-Scott Erdman	20,000	26,464	46,464	20,000	26,464	46,464
155-Yi Li	62,816	16,462	79,278	57,123	12,819	69,942
157-Steven Strauss/Meilan	81,661	21,399	103,060	81,661	21,399	103,060
158-Basil Nikolau	78,520	20,576	99,096	78,520	20,576	99,096
160-John Ohlrogge	78,520	30,726	109,246	78,520	30,726	109,246
161-James Preston	58,890	38,982	97,872	58,890	38,982	97,872
162-Rongda Qu	20,000	8,771	28,771	20,000	8,770	28,770
164-Steven Strauss	58,890	15,434	74,324	58,801	15,434	74,235
FY02 Subtotal	**$ 529,297**	**$ 235,000**	**$ 764,297**	**$ 521,486**	**$ 235,000**	**$ 756,486**
168-Bruce Dale	266,006	85,950	351,956	265,503	85,950	351,453
169-Stan Gelvin	195,194	63,070	258,264	195,194	63,070	258,264
177-David Hildebrand	20,000	6,462	26,462	19,889	6,462	26,351
178-Andrew Paterson	20,000	6,462	26,462	20,000	6,462	26,462
170-Andrew Paterson	200,000	64,623	264,623	200,000	64,623	264,623
171-Rongda Qu	140,000	45,236	185,236	139,982	45,236	185,218
172-Bryon Sosinski	20,000	6,462	26,462	19,998	6,462	26,460
173-Susan Sun	90,000	29,080	119,080	90,000	29,080	119,080
179-Christopher Taylor	20,000	6,462	26,462	20,000	6,462	26,462
174-Wilfred Vermerris	100,288	32,405	132,693	100,258	32,405	132,663

180-Donald Weeks	20,000	6,462	26,462	20,000	6,462	26,462
186-Donald Weeks	20,000	6,462	26,462	20,000	6,462	26,462
175-Jack Widholm	228,598	73,863	302,461	228,598	73,863	302,461
176-S.T. Yang	110,878	35,827	146,705	110,878	35,827	146,705
181-Yuanhui Zhang	20,000	6,462	26,462	20,000	6,462	26,462
185-Ravi Malik	20,000	6,462	26,462	20,000	6,462	26,462
FY03 Subtotal	**$ 1,490,964**	**$ 481,750**	**$ 1,972,714**	**$ 1,490,300**	**$ 481,750**	**$ 1,972,050**
191-Fredy Altpeter	282,000	78,972	360,972	282,000	78,972	360,972
190-Elliot Altman	212,000	59,369	271,369	155,461	59,369	214,830
203B-Victor Busov	38,631	10,818	49,449	38,358	10,818	49,176
192-Kyung-Hwan Han	20,000	5,601	25,601	20,000	5,601	25,601
193-David Hildebrand	219,110	61,360	280,470	219,100	61,360	280,460
194-Vera Lozovaya	212,000	59,369	271,369	211,599	59,369	270,968
195-Scott Merkle	150,554	42,162	192,716	149,210	42,162	191,372
196-Dennis Miller	192,000	53,768	245,768	191,888	53,768	245,656
197-Basil Nikolau	331,550	92,848	424,398	163,389	92,848	256,237
198-James Preston	279,946	78,397	358,343	279,917	78,397	358,314
211-Umesh Reddy	20,000	5,601	25,601	20,000	5,601	25,601
199-Ronald Sederoff	106,803	29,909	136,712	104,565	29,909	134,474
200-Bryon Sosinski	56,000	15,682	71,682	51,993	15,682	67,675
201-Neal Stewart	20,000	5,601	25,601	19,267	5,601	24,868
202-Mariam Sticklen	172,000	48,167	220,167	171,834	48,167	220,001
203A-Steven Strauss	45,359	12,702	58,061	45,358	12,702	58,060
204-Christopher Taylor	212,000	59,369	271,369	211,968	59,369	271,337
205-Jerrold Winandy	20,000	5,601	25,601	19,929	5,601	25,530
206-Howard Woodard	20,000	5,601	25,601	20,000	5,601	25,601
207-Yuanhui Zhang	20,000	5,601	25,601	19,999	5,601	25,600

FY04 Subtotal	**$ 2,629,953**	**$ 736,500**	**$ 3,366,453**	**$ 2,395,835**	**$ 736,500**	**$ 3,132,335**
212-Michael Antal, Jr.	105,000	30,415	135,415	105,000	30,415	135,415
213-Dimitri Argyropoulos	110,000	31,863	141,863	103,203	29,895	133,098
214-David Dai	20,000	5,793	25,793	20,000	5,793	25,793
215-Mark Eiteman	195,000	56,485	251,485	195,000	56,485	251,485
216-Susan Gibson	140,000	40,553	180,553	140,000	40,554	180,554
235-Michael Giroux	20,000	5,793	25,793	19,998	5,793	25,791
217-Terrence Graham	140,000	40,553	180,553	139,877	40,518	180,395
218-Eric Grulke	90,000	26,070	116,070	44,794	12,975	57,769
220-Schuyler Korban	300,000	86,900	386,900	299,868	86,862	386,730
221-Yi Li	95,000	27,518	122,518	91,896	26,619	118,515
222-Scott Merkle	100,000	28,967	128,967	49,511	14,342	63,853
223-Susan Nokes	90,000	26,070	116,070	89,644	25,967	115,611
224-Kelly Rusch	160,000	46,347	206,347	160,000	46,347	206,347
225-Ron Sederoff	145,000	42,002	187,002	72,500	21,001	93,501
226-Steven Strauss	80,000	23,173	103,173	80,000	23,173	103,173
227-Galen Suppes	140,000	40,553	180,553	140,000	40,554	180,554
228-Chung-Jui Tsai	20,000	5,793	25,793	20,000	5,793	25,793
219-Jack Widholm	180,478	52,279	232,757	180,478	52,279	232,757
229-S.T. Yang	138,000	39,974	177,974	124,671	36,113	160,784
230-Yiqi Yang	129,976	37,653	167,629	129,969	37,648	167,617
231-Yuanhui Zhang	170,000	49,244	219,244	127,582	36,956	164,538
FY05 Subtotal	**$ 2,568,454**	**$ 744,000**	**$ 3,312,454**	**$ 2,333,991**	**$ 676,083**	**$ 3,010,074**
242-Fredy Altpeter	100,000	30,720	130,720	99,991	30,717	130,708
243-Caula Beyl	20,000	6,144	26,144	20,000	6,144	26,144
244-Madan Bhattacharyya	100,000	30,720	130,720	100,000	30,720	130,720

245-Zong-Ming Cheng	70,000	21,504	91,504	69,388	21,316	90,704
246- W. David Dai	100,000	30,720	130,720	100,000	30,720	130,720
247-Michael Giroux	83,136	25,539	108,675	83,136	25,539	108,675
248-Kyung-Hwan Han	101,070	31,049	132,119	101,039	31,039	132,078
249-Chandrashekar Joshi	87,136	26,768	113,904	87,121	26,764	113,885
250-Vera Lozovaya	120,000	36,864	156,864	119,992	36,861	156,853
251-Ravindra Malik	20,000	6,144	26,144	20,500	6,298	26,798
253-Peggy Ozias-Akins	184,000	56,525	240,525	183,997	56,524	240,521
254-Andrew Paterson	366,440	112,570	479,010	366,354	112,544	478,898
255-Jim Peter	233,540	71,743	305,283	104,075	31,972	136,047
256-Jim Preston	140,000	43,008	183,008	70,000	21,504	91,504
257-Gerald Pullman	252,509	77,571	330,080	253,018	77,727	330,745
258-Constance Schall	20,000	6,144	26,144	20,000	6,144	26,144
259-Mariam Sticklen	160,000	49,152	209,152	155,750	47,846	203,596
260-X. Susan Sun	80,000	24,576	104,576	80,000	24,576	104,576
261-Eric Triplett	160,000	49,152	209,152	79,855	24,531	104,386
262-Chung-Jui Tsai	128,000	39,322	167,322	127,861	39,279	167,140
263-Wilfred Vermerris	104,000	31,949	135,949	103,996	31,947	135,943
264-S.T. Yang	90,000	27,648	117,648	89,882	27,612	117,494
265-Yiqi Yang	100,000	30,720	130,720	100,005	30,721	130,726
FY06 Subtotal	**$ 2,819,831**	**$ 866,250**	**$ 3,686,081**	**$ 2,535,960**	**$ 779,045**	**$ 3,315,005**
266-Roger Beachy	200,000	61,586	261,586	99,223	30,554	129,159
267-Jay Cheng	180,000	55,428	235,428	180,000	55,428	234,306
268-David Hildebrand	180,000	55,428	235,428	180,000	55,428	234,306
269-Chandrashekhar Joshi	168,885	52,005	220,890	168,885	52,005	219,838
270-John Juvik	200,000	61,586	261,586	200,000	61,586	260,340
271-Steven Knapp	180,000	55,428	235,428	179,661	55,323	233,864
273-Om Parkash	130,624	40,223	170,847	130,263	40,112	169,564

274-Gerald Pullman	200,000	61,586	261,586	200,000	61,586	260,340
275-Drion Boucias	119,999	36,952	156,951	119,941	36,934	156,128
276-Mark Eiteman	140,000	43,111	183,111	140,000	43,111	148,359
277-Fredy Altpeter	290,000	89,300	379,300	281,813	86,779	366,836
278-Sharon Doty	134,000	41,263	175,263	121,755	37,492	157,843
279-Stanton Gelvin	240,000	73,904	313,904	240,000	73,904	312,408
280-William Gurley	220,000	67,745	287,745	220,000	67,745	235,807
281-Tzvi Tzfira	177,000	54,504	231,504	175,409	54,014	228,330
285-Susan Gibson	48,000	14,781	62,781	48,000	14,781	62,482
286-Richard Larock	54,000	16,628	70,628	54,000	16,628	70,292
287-Joel Pawlak	54,000	16,628	70,628	53,829	16,576	70,069
288-William Powell	60,000	18,476	78,476	59,782	18,409	77,819
289-Rongda Qu	48,000	14,781	62,781	47,779	14,713	62,194
290-Constance Schall	78,000	24,019	102,019	78,000	24,019	101,533
291-Herman Scholthof	45,000	13,857	58,857	45,000	13,857	58,577
292-Jian Yu	48,000	14,781	62,781	47,962	14,769	62,433
FY08 Subtotal	**$ 3,195,508**	**$ 984,000**	**$ 4,179,508**	**$ 3,071,303**	**$ 945,753**	**$ 3,912,825**
295-Zong Ming Cheng	75,000	26,670	101,670	75,000	26,670	101,670
296-Chung Jui Tsai	150,000	53,341	203,341	150,000	53,341	203,341
297-Mariam Sticklen	100,000	35,560	135,560	95,599	33,995	129,594
299-Madan Bhattacharrya	128,552	45,714	174,266	128,552	45,714	174,266
300-Renata Bura	154,208	54,837	209,045	77,104	27,419	104,523
301-Shang Tian Yang	110,000	39,117	149,117	110,000	39,117	149,117
302-Richard Meilan	107,760	38,320	146,080	107,409	38,195	145,604
304-Susan Gibson	80,000	28,448	108,448	40,872	14,534	55,406
305-Scott Merkle	74,000	26,315	100,315	36,638	13,029	49,666
306-Ring Ming	166,365	59,160	225,525	166,365	59,160	225,525
307-Andrew Paterson	240,000	85,345	325,345	240,000	85,345	325,345

310-Kyung-Hwan Han	90,520	32,189	122,709	90,520	32,189	122,709
311-Clifford Weil	200,000	71,121	271,121	199,819	71,057	270,876
312-Dilip Shah	135,990	48,359	184,349	135,971	48,352	184,322
313-Gerald Pullman	160,000	56,897	216,897	160,000	56,897	216,897
314-Edward Kirby	227,610	80,939	308,549	227,610	80,939	308,549
315-Bala Rathinasabapathi	95,200	33,854	129,054	94,800	33,711	128,511
316-Hong Luo	100,518	35,745	136,263	50,259	17,872	68,131
317-William Powell	80,000	28,448	108,448	67,000	23,826	90,826
318-Stanton Gelvin	120,000	42,673	162,673	120,000	42,673	162,673
319-Susan Gibson	80,000	28,448	108,448	48,536	17,259	65,795
FY09 Subtotal	**$ 2,675,723**	**$ 951,500**	**$ 3,627,223**	**$ 2,422,052**	**$ 861,293**	**$ 3,283,346**
322-Shui-zhang Fei	77,202	29,420	106,622	76,546	29,170	105,717
323-John Finer	149,740	57,063	206,803	98,553	37,557	136,110
324-Michael Giroux	111,110	42,342	153,452	111,110	42,342	153,452
325-David Hildebrand	220,456	84,011	304,467	220,456	84,011	304,467
326-Stephen Howell	110,000	41,919	151,919	110,000	41,919	151,919
327-John Juvik	159,253	60,688	219,941	159,253	60,688	219,941
328-Kris Lambert	176,068	67,096	243,164	176,068	67,096	243,164
329-Richard Larock	175,076	66,718	241,794	175,076	66,718	241,794
330-Joel Pawlak	123,796	47,176	170,972	59,897	22,826	82,723
331-Rongda Qu	162,000	61,735	223,735	162,000	61,735	223,735
332-William Rooney	136,520	52,025	188,545	66,610	25,384	91,993
333-Blair Siegfried	181,500	69,166	250,666	181,500	69,166	250,666
334-Praveen Vadlani	80,000	30,486	110,486	80,000	30,486	110,486
335-Richard Venditti	105,376	40,157	145,533	33,023	12,585	45,608
FY10 Subtotal	**$ 1,968,097**	**$ 750,000**	**$ 2,718,097**	**$ 1,710,093**	**$ 651,680**	**$ 2,361,773**

Totals	**$ 17,877,827**	**$ 5,749,000**	**$ 23,626,827**	**$ 16,481,020**	**$ 5,367,105**	**$ 21,743,894**

9. Subaward Status

2002 Competition

Caula Beyl, Alabama A&M University *CPBR Fellowship: Characterization of transgenic aspen for wood productivity and quality*	**CLOSED**
Kathleen Danna, University of Colorado *CPBR Fellowship: Development of bioprocesses using highly active enzymes of anaerobic fungi*	**CLOSED**
Chukwudi Obi Emeh, Savannah State University *CPBR Fellowship: Mycotoxins, antibiotics and molecular genetics*	**CLOSED**
Scott Erdman, Syracuse University *CPBR Fellowship: Targeted inhibition of fungal adhesins*	**CLOSED**
Jiming Jiang, University of Wisconsin *CPBR Fellowship: Toward cloning a functional rice centromere*	**WITHDRAWN COULD NOT KEEP MATCH BECAUSE OF AWARD DELAY**
Yi Li, University of Connecticut **Zong-Ming Cheng**, University of Tennessee *Characterization of transgenic aspen for wood productivity and quality*	**CLOSED**
Lars Ljungdahl, University of Georgia *Development of bioprocesses using highly active enzymes of anaerobic fungi*	**WITHDRAWN COULD NOT KEEP MATCH BECAUSE OF AWARD DELAY**
Richard Meilan*, Oregon State University *Genetic engineering to maintain high biomass productivity in non-flowering transgenic trees* *(PI **Stephen Strauss** as of September 10, 2003)	**CLOSED**
Basil Nikolau, Iowa State University *Biotechnology of acetyl-CoA metabolism in plants*	**CLOSED**
Sue N. Nokes, University of Kentucky *Solid state culture of bacteria for enzyme production*	**WITHDRAWN COULD NOT KEEP MATCH BECAUSE OF AWARD DELAY**
John Ohlrogge, Michigan State University *DNA microarray discovery of genes and networks which control plant seed storage products*	**CLOSED**
James Preston, University of Florida *Utilization of hemicellulose-derived glucuronoxylose for ethanol*	**CLOSED**

Rongda Qu, North Carolina State University *Mechanisims of the enhanced transgene expression by 5' UTR intron in two monocot genes: rice act1 and maize ubi1*	**CLOSED**

Hassan Sreenath, University of Wisconsin *CPBR Fellowship: Production of low-cost cellulolytic enzymes by solid state cultivation*	**WITHDRAWN COULD NOT KEEP MATCH BECAUSE OF AWARD DELAY**

Steven Strauss, Oregon State University *Identification of major genes via activation tagging in Populus*	**CLOSED**

Charles Wyman, Dartmouth College *Improved cellulose hydrolysis technology for ethanol from biomass*	**WITHDRAWN COULD NOT KEEP MATCH BECAUSE OF AWARD DELAY**

2003 Competition

Bruce Dale, Michigan State University *Producing valuable proteins in plants for alcohol fuels*	**CLOSED**
Stanton Gelvin, Purdue University *Plant genes and Agrobacterium T-DNA integration*	**CLOSED**
David Hildebrand, University of Kentucky *CPBR Fellowship: Epoxy fatty acid accumulation in seed oil*	**CLOSED**
Ravindra Malik, Albany State University *CPBR Fellowship: Initial genetic evaluation of napiergrass germplasm*	**CLOSED**
Andrew Paterson, University of Georgia *Discovery and evaluation of SNP variation in cotton toward enhanced quality and efficiency of biobased fiber production*	**CLOSED**
Andrew Paterson, University of Georgia *CPBR Fellowship: Discovery and evaluation of SNP variation in resistance-gene analogs in peanut, toward more efficient oilseed production*	**CLOSED**
Rongda Qu, North Carolina State University *Mechanisms of enhanced transgene expression by 5' UTR intron sequence in two monocot genes, rice Act1 and maize ubi1*	**CLOSED**
Bryon Sosinski, North Carolina State University *CPBR Fellowship: Sweetpotatoes for ethanol diversification*	**CLOSED**
Susan Sun, Kansas State University *High strength degradable plastics from starch and poly(lactic acid)*	**CLOSED**
Christopher Taylor, University of Missouri / Danforth Plant Science Center *CPBR Fellowship: The isolation and characterization of microbial derived nematicidal proteins and peptides*	**CLOSED**
Wilfred Vermerris, Purdue University *Modifying lignin composition to enhance ethanol production*	**CLOSED**
Donald Weeks, University of Nebraska *CPBR Fellowship: Development of herbicide resistant biomass and energy crops*	**CLOSED**
Donald Weeks, University of Nebraska *CPBR Fellowship: Student and faculty research collaborations*	**CLOSED**

Jack Widholm, University of Illinois *Plastid transformation using a new selectable marker gene*	**CLOSED**
S. T. Yang, Ohio State University *Production of L(+)-lactic acid from plant biomass by filamentous fungi immobilized in fibrous bed bioreactor*	**CLOSED**
Yuanhui Zhang University of Illinois *CPBR Fellowship: Continuous thermochemical conversion (TCC) process to produce oil from livestock manure*	**CLOSED**

2004 Competition

Fredy Altpeter, University of Florida *Characterizing the ryegrass drought stress regulon*	**CLOSED**
Elliot Altman, University of Georgia *Novel peptide antibiotics*	**CLOSED**
Kyung-Hwan Han, Michigan State University *CPBR Fellowship: Novel gene discovery for value-added forest products*	**CLOSED**
David Hildebrand, University of Kentucky *Epoxy fatty acid accumulation in seed oil*	**CLOSED**
Vera V. Lozovaya, University of Illinois *Enhancing soybean root resistance to soilborne pathogens*	**CLOSED**
Scott Merkle, University of Georgia *In vitro propagation of American chestnut: a first step to an alternative biomass energy crop*	**CLOSED**
Dennis Miller, Michigan State University *Hydrogenation of amides and oligopeptides to amino and polyamino alcohols*	**CLOSED**
Basil Nikolau, Iowa State University *Functional genomics of soybean seed composition*	**CLOSED**
James F. Preston, University of Florida *Bacterial conversion of hemicellulose to ethanol*	**CLOSED**
Umesh Reddy, West Virginia State University *Microsatellite markers for sweetpotatoe molecular characterization*	**CLOSED**
Ronald R. Sederoff, North Carolina State University *The formation of dihydroconiferyl alcohol(DHCA) subunits in lignin of a cinnamyl alcohol dehydrogenase (CAD) deficient mutant of loblolly pine*	**CLOSED**
Bryon Sosinski, North Carolina State University *Sweetpotatoes for ethanol diversification*	**CLOSED**
C. Neal Stewart, University of Tennessee *CPBR Fellowship: Insecticidal genes in ferns*	**CLOSED**

Mariam Sticklen, Michigan State University *Maize chloroplast transgenesis development for bioenergy, health and environment*	**CLOSED**
Steven H. Strauss, Oregon State University **Victor Busov**, Michigan Technological University *Modification of gibberellin metabolism to enhance productivity, wood quality, and biosafety*	**CLOSED**
Christopher G. Taylor, University of Missouri & Danforth Center *Isolation and characterization of microbial derived nematicidal proteins and peptides*	**CLOSED**
Jerrold E. Winandy, University of Minnesota *CPBR Fellowship: Using fire-killed trees for wood composites*	**CLOSED**
Howard J. Woodard, South Dakota State University *CPBR Fellowship: The influence of agricultural production practices and processing factors to enhance corn grain fermentation characteristics*	**CLOSED**
Yuanhui Zhang, University of Illinois *CPBR Fellowship: Continuous thermochemical conversion (TCC) process to produce oil from livestock manure*	**CLOSED**

2005 Competition

Michael Antal, Jr., University of Hawai'i-Manoa *Biocarbon fuel cell demonstration*	**CLOSED**
Dimitris Argyropoulos, North Carolina State University *Supercritical fluid extraction systems to obtain high value products from thermally labile agricultural materials*	**CLOSED**
David Dai, North Dakota State University *CPBR Fellowship for A genetic engineering approach to solve chlorosis problems of trees*	**CLOSED**
Mark Eiteman, University of Georgia *A metabolic engineering approach to improve protein production*	**CLOSED**
Susan Gibson, University of Minnesota *Using genomics to increase soybean biodiesel yield*	**CLOSED**
Michael Giroux, Montana State University *Dissection of yield enhancement in cereals*	**CLOSED**
Terrence Graham, The Ohio State University *Novel pharmaceutical and crop protection chemicals from plants*	**CLOSED**
Eric Grulke, University of Kentucky *Ultrasonication for improved biodiesel production*	**CLOSED**
Schuyler Korban, University of Illinois Urbana-Champaign *Transgenic plants for production and delivery of an oral vaccine*	**CLOSED**
Yi Li, University of Connecticut *Removal of GM-genes from pollen and seed of GM aspen and maize*	**CLOSED**
Scott Merkle, University of Georgia *Capturing elite southern pine genotypes for clonal forestry*	**CLOSED**
Sue Nokes, University of Kentucky *Development of novel technology for in situ saccharification and biomass conversion*	**CLOSED**
Kelly Rusch, Louisiana State University *Degradation behavior characteristics of bioplastics*	**CLOSED**
Ronald Sederoff, North Carolina State University *Genomic regulation of growth and lignin in Eucalyptus*	**CLOSED**

Steven Strauss, Oregon State University *Activation tagging in the reference Nisqually-1 poplar genome*	**CLOSED**
Galen Suppes, University of Missouri-Columbia *Highly functional polyols for polyurethanes derived from vegetable oil*	**CLOSED**
Chung-Jui Tsai, Michigan Technological University *CPBR Fellowship for Protein engineering for manipulation of phenylpropanoid metabolism*	**CLOSED**
Jack M. Widholm, University of Illinois Urbana-Champaign *New selectable marker for plant transformation*	**CLOSED**
Shang-Tian Yang, The Ohio State University *A spouted bed bioreactor for solid state fermentation to produce enzymes and chemicals*	**CLOSED**
Yiqi Yang, University of Nebraska-Lincoln *Long cellulose fibers from corn stover for textiles and composites*	**CLOSED**
Yuanhui Zhang, University of Illinois Urbana-Champaign *Devise a continuous thermochemical conversion process for swine manure and optimize processing of the bio-fuel and liquid by-products*	**CLOSED**

2006 Competition

Fredy Altpeter, University of Florida *Chloroplast engineering for production of cell wall degrading enzymes*	**CLOSED**
Caula Beyl, Alabama A&M University *Characterization of root systems and stress proteins of aspen transformed for enhanced woody productivity/quality*	**CLOSED**
Madan Bhattacharyya, Iowa State University *Arabidopsis nonhost resistence for creating novel soybean germplasms with durable and broad-spectrum Phytophthora resistance*	**CLOSED**
Zong-Ming Cheng, University of Tennessee *Down-regulating the DHS Gene and translation initiation factor 5A to increase poplar biomass*	**CLOSED**
W. David Dai, North Dakota State University *Genetic improvement of woody species tolerant to iron chlorosis*	**CLOSED**
Michael Giroux, Montana State University *Modification of cereal grain texture, plant fungal resistance, and starch digestibility*	**CLOSED**
Kyung-Hwan Han, Michigan State University *Functional roles of selected regulatory genes in wood formation*	**CLOSED**
Chandrashekhar Joshi, Michigan Technological University *Modulation of cellulose crystallinity in transgenic trees*	**CLOSED**
Vera Lozovaya, University of Illinois Urbana-Champaign *Identifying the health promoting composition of soy seeds*	**CLOSED**
Ravindra Malik, Albany State University *Napeirgrass biomass yield and quality affected by use of poultry litter*	**CLOSED**
Peggy Ozias-Akins, University of Georgia *Peanut allergen reduction*	**CLOSED**
Andrew Paterson, University of Georgia *Physical mapping of the gene-rich euchromatin of sugarcane*	**CLOSED**
Gary Peter, University of Florida *Genes for improved wood quality*	**CLOSED**

James Preston, University of Florida *Direct conversion of hemicellulose to biobased products*	**CLOSED**
Gerald Pullman, Georgia Institute of Technology *Characterization of a somatic embryo maturation stimulator present in loblolly pine*	**CLOSED**
Constance Schall, University of Toledo *An efficient approach for saccharification of cellulose from biomass for ethanol production*	**CLOSED**
Mariam Sticklen, Michigan State University *Production of hemicellulase and ligninase in E1 cellulase-transgenic maize for alcohol fuel and a clean environment*	**CLOSED**
Xiuzhi Susan Sun, Kansas State University *Bio-Nanocomposites Derived From Renewable Materials*	**CLOSED**
Eric Triplett, University of Florida *Analysis of a novel mechanism of plant growth promotion*	**CLOSED**
Chung-Jui Tsai, Michigan Technological University *Protein engineering for manipulation of phenylpropanoid metabolism*	**CLOSED**
Wilfred Vermerris, University of Florida *Whole plant bioprocessing for ethanol production*	**CLOSED**
Shang-Tian Yang, The Ohio State University *Production of Organic Acids and Esters from Plant Biomass by Extractive Fermentation and Enzymatic Esterification*	**CLOSED**
Yiqi Yang, University of Nebraska-Lincoln *High quality protein fibers from wheat gluten for industrial applications*	**CLOSED**

2008 Competition

Fredy Altpeter, University of Florida *Development of a chloroplast transformation protocol for energycane*	**CLOSED**
Roger Beachy, Donald Danforth Plant Science Center *Developing a controlled gene switch system for plants*	**CLOSED**
Drion Boucias, University of Florida *Genomic dissection of cellulose utilization in termites*	**CLOSED**
John Burke, University of Georgia *Identifying novel exotic alleles for enhancing seed, oil, and biomass yield, drought tolerance, and hybrid performance in sunflower*	**CLOSED**
Jay Cheng, North Carolina State University *Genetically engineer switchgrass for ethanol production*	**CLOSED**
Sharon Doty, University of Washington *Enhanced efficiency of bioethanol production with novel yeast.*	**CLOSED**
Mark Eiteman, University of Georgia *A co-fermentation strategy for lignocellulosic hydrolysates to remove the key inhibitor acetic acid and more efficiently utilize mixed sugars*	**CLOSED**
Stanton Gelvin, Purdue University *Plant global regulator of Agrobacterium transformation*	**CLOSED**
Sharon Gibson, University of Minnesota *Using Genomics to Increase Soybean Biodiesel Yield*	**CLOSED**
William Gurley, University of Florida *Zinc finger-based regulation of plant gene expression*	**CLOSED**
David Hildebrand, University of Kentucky *Oilseeds as a renewable source of epoxy fatty acids*	**CLOSED**
Chandrashekhar Joshi, Michigan Technological University *Genetic master switches controlling cellulose biosynthesis in plants*	**CLOSED**
John Juvik, University of Illinois Urbana-Champaign *Improvement of Miscanthus as an energy and biofuel crop*	**CLOSED**

Richard Larock, Iowa State University *Development and commercialization of soy/corn/linseed oil bioplastics*	**CLOSED**
Peggy Ozias-Akins, University of Georgia *Lignin reduction in Giant Reed for biofuels and fiber*	**WITHDRAWN, COULD NOT KEEP MATCH BECAUSE OF AWARD DELAY**
Om Parkash, University of Massachusetts, Amherst *Developing Agrobacterium-mediated transformation system for Crambe abyssinica for phytoremediation of arsenic and production of biomaterials*	**CLOSED**
Joel Pawlak, North Carolina State University *The conversion of starch-hemicellulose-chitosan into high value gels with superior absorption, strength and antimicrobial activit*	**CLOSED**
William Powell, State University of New York *Developing blight resistance in transgenic American chestnut for agroforestry and restoration*	**CLOSED**
Gerald Pullman, Georgia Institute of Technology *MicroRNAs: tools to advance somatic embryo development in pine*	**CLOSED**
Rongda Qu, North Carolina State University *Individual and combined effects of transgenes conferring drought and salt tolerance in rice*	**CLOSED**
Constance Schall, University of Toledo *An efficient approach for saccharification of cellulose from biomass for fuel/chemical production*	**CLOSED**
Herman Scholthof, Texas AgriLife Research *Direct delivery of viral gene vectors into plant suspension cells*	**CLOSED**
Tzvi Tzfira, University of Michigan *Zinc-finger-mediated gene targeting in plant cells*	**CLOSED**
Jian Yu, University of Hawaii *Energy efficient biomass refining into fuel ethanol and bioplastics*	**CLOSED**

2009 Competition

Madan Bhattacharyya, Iowa State University *Nonhost resistance for engineering disease resistance*	**CLOSED**
Renata Bura, University of Washington *A biomass flexible bioconversion of lignocellulose to ethanol: a robust pretreatment method for processing mixed feedstocks*	**CLOSED**
Zong Ming Cheng, University of Tennessee *Enhance poplar growth under low-nitrogen conditions by metabolic engineering with Dof1 transcription factor*	**CLOSED**
Stanton Gelvin, Purdue University *Aptamers to determine protein function in plants*	**CLOSED** *Final report enclosed*
Susan Gibson, University of Minnesota *Exploiting genetic variation in soybean to increase oil*	**CLOSED** *Fianl report enclosed*
Susan Gibson, University of Minnesota *Using genomics to increase soybean biodiesel yield*	**CLOSED** *Final report enclosed*
Kyung-Hwan Han, Michigan State University *Biotechnology for improving drought-tolerance in plant*	**CLOSED** *Final report enclosed*
Edward Kirby, Rutgers University *Mechanisms for growth and stress resistance in GS poplar*	**CLOSED**
Hong Luo, Clemson University *Development of environmentally friendly transgenic turfgrass with enhanced drought and salt tolerance.*	**CLOSED**
Richard Meilan, Purdue University *Improving water relations of Populus for use as a bio-energy crop*	**CLOSED**
Scott Merkle, University of Georgia *Bioreactor-based scale-up of American chestnut for agroforestry and restoration*	**CLOSED**
Ray Ming, University of Illinois *Constructing a saturated genetic map for sugarcane improvement*	**CLOSED**
Andrew Paterson, University of Georgia *Improved genomics tools and resources for* Miscanthus.	**CLOSED**

William Powell, State University of New York *Developing blight resistance in transgenic American chestnut for agroforesry and restoration*	**CLOSED**
Gerald Pullman, Georgia Institute of Technology *Reprogramming conifer tissue for embryogenesis: can studies of gene expression during cell fate change in Norway spruce hypocotyl lead the way to induction of SE in mature tissue?*	**CLOSED**
Bala Rathinasabapathi, University of Florida *Engineering glutaredoxins for stress tolerance & yield*	**CLOSED**
Dilip Shah, Donald Danforth Plant Science Center *Sudden death syndrome and Asian rust resistant soybean*	**CLOSED**
Mariam Sticklen, Michigan State University *The promises of studies of down-regulation of maize lignin for biofuels and the environment*	**CLOSED**
Chung-Jui Tsai, University of Georgia *Post-translational regulation and transgenic manipulation of Populus alpha and beta-tubulins during wood formation*	**CLOSED**
Clifford Weil, Purdue University *Genetic control of starch digestion: Better food & fuel*	**CLOSED** *Final report enclosed*
Shang Tian Yang, The Ohio State University *Production of fumaric acid from sugars and starch by filamentous fungal fermentation*	**CLOSED**

2010 Competition

Shui-zhang Fei, Iowa State University *Discovery of novel genes in creeping bentgrass by high throughput gene silencing with specialized inverted repeat cDNA libraries*	**CLOSED** *Final report enclosed*
John Finer, The Ohio State University *Novel soybean promoters that are stronger than CaMV35S*	**CLOSED** *Final report enclosed*
Mike Giroux, Montana State University *Improving rice photosynthesis and yield*	**CLOSED**
David Hildebrand, University of Kentucky *Branch-chain fatty acid production in plants*	**CLOSED** *Final report enclosed*
Stephen Howell, Iowa State University *New genes for stress tolerance in bioenergy crops*	**CLOSED** *Final report enclosed*
John Juvik, University of Illinois-Urbana-Champaign *Manipulation of ploidy levels in Miscanthus species*	**CLOSED**
Kris Lambert, University of Illinois-Urbana-Champaign *Development of durable resistance to soybean nematodes*	**CLOSED**
Richard Larock, Iowa State University *Practical waterborne agricultural oil-based coatings*	**CLOSED**
Joel Pawlak, North Carolina State University *Sustainable wood-based barrier films for liquid packaging applications*	**CLOSED**
Rongda Qu, North Carolina State University *Lignin-reduced and cellulase-expressing switchgrass*	**CLOSED**
William Rooney, Texas A&M University *Molecular characterization of composition traits in sweet and energy sorghum germplasm for bioenergy production*	**CLOSED**
Blair Siegfried, University of Nebraska-Lincoln *Cellulose degrading enzymes from western corn rootworm larvae*	**CLOSED** *Final report enclosed*
Praveen Vadlani, Kansas State University *Paper mill waste bioconversion to high-value products*	**CLOSED**
Richard Venditti, North Carolina State University *Low cost conversion of industrial papermaking sludges to ethanol*	**Closed** *Final report ecnclosed*

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator Name(s) and Contact Information: **(Title, Mailing Address, Phone, Fax, Email)**	Stanton Gelvin Distinguished Professor of Biological Sciences Department of Biological Sciences 1393 Lilly Hall of Life Sciences West Lafayette, IN. 47907-1393 Phone: 765-494-4940 Fax: 765-496-1497 Email: gelvin@bilbo.bio.purdue.edu	
University:	Purdue University	
DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026-318**	
Project Title:	*Peptide aptarners to determine protein function in plants*	
Reporting Period:	**From: 10/1/11**	**To: 3/31/12**
Report Type:	**Check one:** [ ] Semiannual Report [X] Final Report	

Project Objectives

List each objective of the Project and the progress made toward each one during the reporting period.

We started funding for this grant in January, 2010. My post-doc Soyon Park arrived in February, 2010. Funding for the first year of this project terminated at the end of March, 2011, and second year funding has not been available.

1. We shall develop expression cassettes, vector systems, and BiFC-aptamer libraries for identification of aptamers that specifically interact with target proteins of interest *in vivo*. These aptamers can then be used for dissecting functional domains of target proteins, identification of native interactors of target proteins, and for aptamer-mediated "knockout" of target protein functions *in planta*.

With the help of the Citovsky laboratory, we constructed a first-generation novel aptamer expression vector. This vector is similar to the one described in the proposal, but has a Gateway site for insertion of the random aptamers instead of the I-*Sce*I site originally proposed. We made a random aptamer library in the Gateway entry vector pDONR221. This library consists of $2x10^8$ aptamers. These aptamers have been moved into the aptamer Gateway expression destination vector, with a total final library of ~$1x10^9$ primary colonies.

Since the last report, we have re-designed our aptamer expression vector, with the expression cassette consisting of the CaMV 35S promoter, followed by an aptamer polyprotein (cCFP-aptamer-mCherry), followed by a polyA addition sequence. We have moved the Gateway aptamer library from pDONR221 into this new vector, generating a library of ~$1x10^9$ primary colonies. This library has subsequently been amplified and DNA isolated to use in transfection experiments.

2. As proof of concept, we shall screen our BiFC aptamer libraries in tobacco BY-2 protoplasts for interactions with specific *Agrobacterium* and *Arabidopsis* target proteins known to participate in fundamental cellular processes important for *Agrobacterium*-mediated transformation. We shall subsequently test these aptamers in plants for inhibition of the target protein function and *Agrobacterium*-mediated plant genetic transformation. We have done some screening using VirE2 and VIP1 as "bait" proteins. However, as we started screening, we noted a high background of "non-specific" interaction of the bait and prey plasmids. We have therefore gone back to examine a number of nYFP/nVenus derivatives which would lower the non-specific background. A mutant form of nYFP (I152L) appears to give no background, and we shall use this in the future.

3. We shall validate of the use of random peptide aptamers to identify interacting protein partners for proteomic studies. No progress yet.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

We are developing peptide aptamer methodology to investigate protein function and protein-protein interactions *in planta*. Peptide aptamers are small synthetic peptides of random amino acid sequence. When expressed in plant cells, aptamers can specifically bind to target proteins and inhibit function. They can thus be used to define the functional roles of proteins in various cellular processes, as well as defining specific peptide motifs within these proteins that are important for the proteins to mediate these roles. Furthermore, the aptamer sequences can be used as functional identification "marks" of plant proteins that naturally interact with the target proteins. Finally, by inhibiting protein function, they can provide an alternative to traditional mutagenesis approaches for defining gene/protein function and developing novel phenotypes.

We have developed expression vectors and libraries that express peptide aptamers both for use in transient transformation experiments and for *Agrobacterium*-mediated transformation. The constructions incorporate BiFC technology which we have developed to detect and visualize aptamer-protein interactions, and Gateway technology for mobilization of aptamer expression cassettes among the various vectors. As proof of concept, we shall test tagged plant proteins, known to interact with *Agrobacterium* virulence proteins, for interaction with aptamers, and the effects of these aptamers on various cell biological processes and *Agrobacterium*-mediated transformation.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

See attached.

Publications and Presentations

List all talks, posters, scientific publications, news releases, etc., about this Project during the reporting period. Provide one copy of each publication, report, or news article resulting from activities supported under the grant as well as any announcements, press releases, statements or photographs depicting Project activities.

"Thirty-two years of *Agrobacterium* research, from the plant's perspective". Retirement symposium for Dr. Eugene W. Nester, Seattle, WA. · October 1-2, 2010.

"Plant genes involved in *Agrobacterium*-mediated plant genetic transformation". Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, London, Ontario, Canada. ·October 5-7, 2010.

"Technology development: Peptide aptamer mutagenesis". Department of Biological Sciences, Purdue University. October 14, 2010.

"Direct screening of a plant cDNA library against a bait protein *in planta*: An application of bimolecular fluorescence complementation" · November 5-7, 2010.

"Bimolecular fluorescence complementation to image protein-protein interactions in plants." Bimolecular Fluorescence Complementation Workshop, Purdue University, West Lafayette, IN. December 7, 2010.

"Manipulating the plant genome to enhance *Agrobacterium*-mediated transformation."

Advancement in Crop Transformation Technology Workshop, 19th Annual Plant and Animal Genome meeting, San Diego, CA. January 15-17, 2011.

"Manipulating the plant to enhance *Agrobacterium*-mediated transformation." Plant Transformation Technology II Meeting, Vienna, Austria. February 19-22, 2011.

"Direct screening of a plant cDNA library against a bait protein *in planta*: A novel plant two-hybrid system." Plant Gene Identification Technologies Meeting, Vienna, Austria. February 23-26, 2011.

"Improving *Agrobacterium*-mediated plant genetic transformation." Dow AgroSciences, Zionsville, IN. April 13, 2011.

"Manipulating the plant genome to increase susceptibility to *Agrobacterium*-mediated genetic transformation". Plants for the Future Symposium, University of Missouri, Columbia, MO. May 24-27, 2011.

"Manipulating the plant genome to increase susceptibility to *Agrobacterium*-mediated transformation". Mini-symposium on Recent Advances in Plant and Microbial Biology, Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan. June 8, 2011.

"Manipulating the plant genome to increase susceptibility to *Agrobacterium*-mediated transformation". NSF EPSCOR P3 conference, Heber Springs, Arkansas. July 26-28, 2011.

Technology Transfer

Describe all technology transfer (inventions, disclosures, patents, licensing agreements, etc.) resulting from the Project during the reporting period.

Purdue University filed a patent covering this peptide aptamer technology on January 14, 2011.

Commercial Accomplishments

Describe the most significant accomplishments resulting from the Project during the reporting period.

1. We have generated two different Gateway®-compatible destination vectors to express aptamers in plants.
2. We have generated a peptide aptamer library, consisting of $2x10^8$ aptamers, in the Gateway® donor vector pDONR221.
3. We have transferred the peptide aptamer library into each of the destination aptamer expression vectors. The libraries consists of ~$1x10^9$ primary colonies.
4. We have cloned several *Agrobacterium* virulence proteins into BiFC vectors to use as "baits" for aptamer interaction experiments.

Educational Accomplishments

Describe the most significant educational accomplishments resulting from the Project during the reporting period. (Number of students, post-docs, etc. trained)

During the course of this grant, we have trained:

One post-doctoral research associate, Soyon Park

One research assistant, Yanjun Yu

In addition, during the summer of 2011, two minority undergraduate students from Brooklyn College, NY (Janaye Dolly and Krystan Burnett) worked in our laboratory, learning techniques useful for this aptamer project.

Additional Funding

List any additional funding generated as a result of the Project during the reporting period.

We obtained a small "starter" grant from the Purdue University Cancer Center ($2500) to adapt the peptide aptamer technology for use in animal systems.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

After the end of the first year, and when funds were discontinued, both the people working on this project (Soyon Park, Post-doctoral Research Associate, and Yanjun Yu, Research Assistant) left the laboratory.

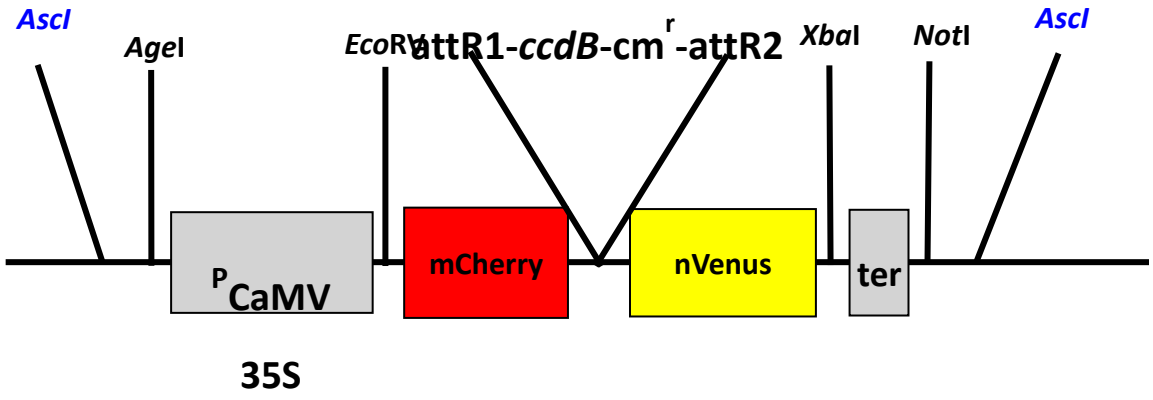
Scientific Accomplishments

(Through October, 2010)

1. Generation of a Gateway®-compatible peptide aptamer expression vector

In collaboration with the Citovsky laboratory, we converted our former peptide aptamer expression vector (containing a multiple cloning site for insertion of aptamer coding sequences) into a Gateway-compatible vector. This was done by exchanging the multiple cloning site with a *ccdB*-cm^r cassette flanked by *attR1* and *attR2* sites. The final destination vector, shown in Figure 1, will facilitate generation of an aptamer expression library. Aptamers, cloned into the vector pDONR221, can be exchanged into this destination vector using the Invitrogen L-R clonase reaction. This vector will express aptamers as a polyprotein flanked on their N-termini by mCherry and on their C-termini by nVenus.

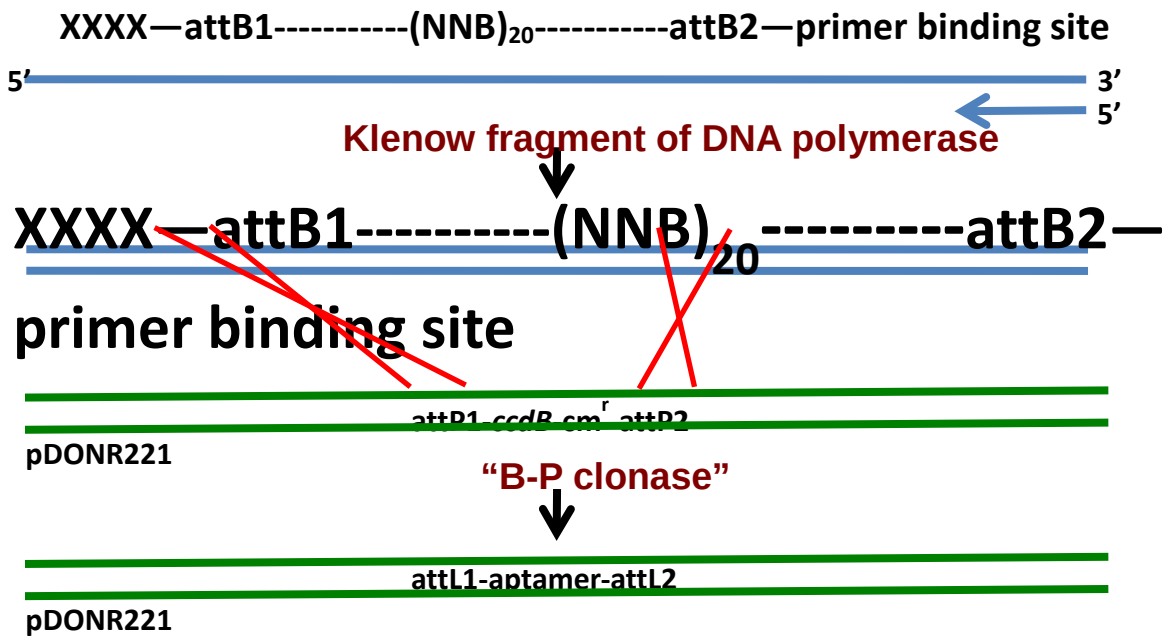
Figure 1. Gateway®-compatible aptamer expression vector



2. Generation of a peptide aptamer library in pDONR221

We purchased a "mixed" oligomer consisting of the Gateway® *attB1* site, followed by a mixed 20-mer of NNB (N=A,C,G,T; B=C,G,T), followed by an *attB2* site, followed by a T7 primer binding site. We converted this into a double-strand molecule using Klenow fragment of DNA polymerase I. The derived double-strand mixed oligonucleotides were recombined into the vector pDONR221 using the Invitrogen B-P clonase reaction (Figure 2). The resulting library contains $2x10^8$ primary aptamer members.

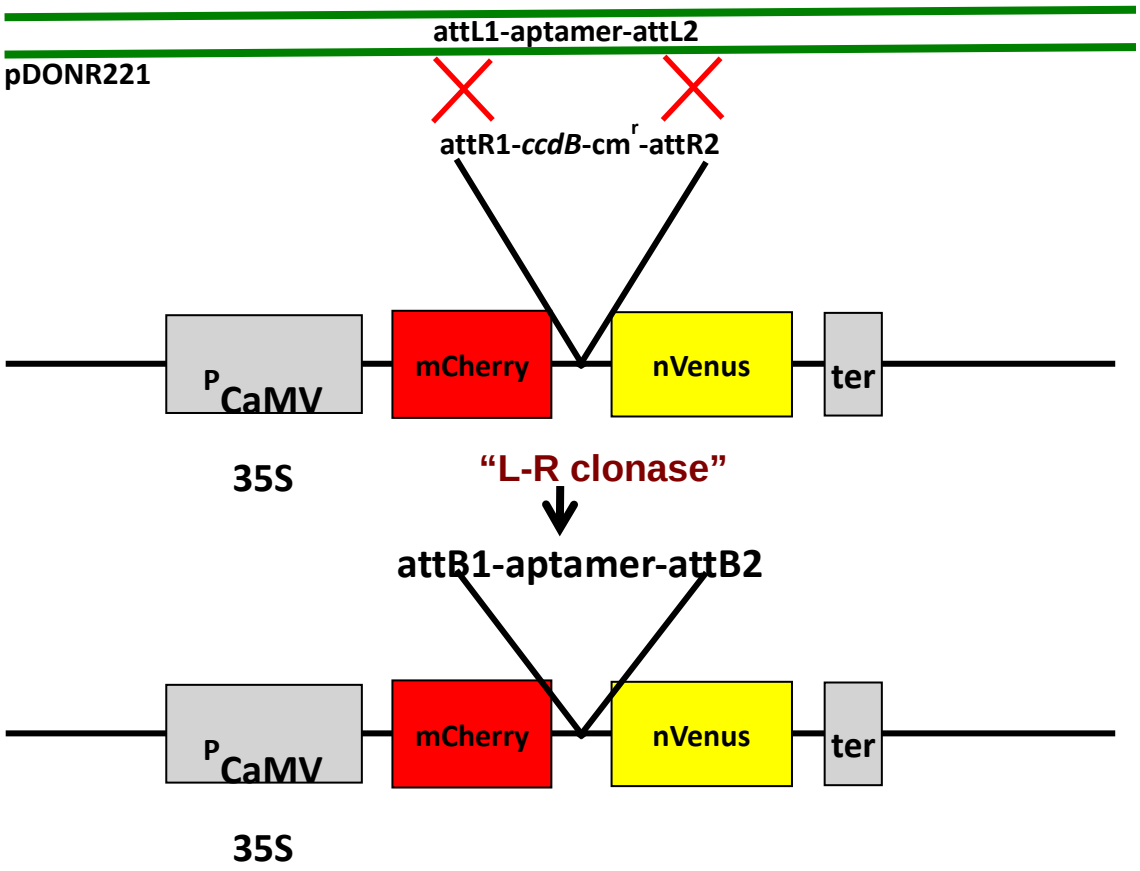
Figure 2. Generation of the peptide aptamer library in pDONR221



3. Generation of a peptide aptamer library in the aptamer destination expression vector

We next used the Invitrogen L-R Clonase reaction to move the aptamer expression library from pDONR221 to the aptamer destination expression vector (Figure 3). The resulting library contains ~$1x10^9$ primary colonies.

Figure 3. Generation of a peptide aptamer library in the aptamer destination expression vector



4. Generation of "bait" constructs for BiFC

We have generated "bait" constructs for use with the aptamer library. These consist of several proteins important for *Agrobacterium*-mediated transformation (VirD2, VirE2, VIP1) tagged with cCFP. Interaction of these proteins with aptamers will generate yellow fluorescence as the nVenus portion of the aptamer polyprotein interacts and correctly folds with the cCFP tag on the bait proteins.

Scientific Accomplishments

(October, 2010-March, 2011)

After we constructed the peptide aptamer expression library, we started screening the library against *Agrobacterium* VirE2-cCFP. We found many aptamer clones that did not interact with VirE2-cCFP, but DNA sequence analysis of these clones indicated that in almost every instance, the aptamer coding sequence was out of frame with either mCherry or nVenus. Those aptamer clones that gave a yellow fluorescence signal when matched with VirE2-cCFP (>80% of the sequenced clones) all contained aptamers in-frame with both mCherry and nVenus. These results indicated that the yellow fluorescence signal was most likely a "false positive" resulting from "self-assembly" of the nVenus (linked to the aptamer) and cCFP (linked to VirE2). In other words, the two parts of the yellow fluorescent protein were assembling without the necessity of

the attached bait protein and peptide aptamer interacting. This was confirmed when we incubated the aptamer with an "empty" (lacking an interacting protein) cCFP vector.

We had previously seen this "background" problem, but were able to mitigate it using lower-strength promoters (such as that from the nopaline synthase [*nos*] gene). However, we could not completely eliminate the aptamer polyprotein interacting with an "empty" cCFP vector by using the *nos* promoter on the bait construction (see Table 1). We therefore initiated a study to find a nYFP/nVenus derivative that would not "self-assemble" with cCFP.

We tested four different versions of nYFP/nVenus against an empty cCFP expression vector. These included nYFP, nVenus, nYFP I152L, and nVenus I152L. The mutant I152L version of the protein was suggested to us by Dr. Chang-Deng Hu (Purdue University), who had published that this mutation inhibited "self-assembly" of nVenus with cCFP in animal cells. However, he suggested to us that this may not work in plant cells.

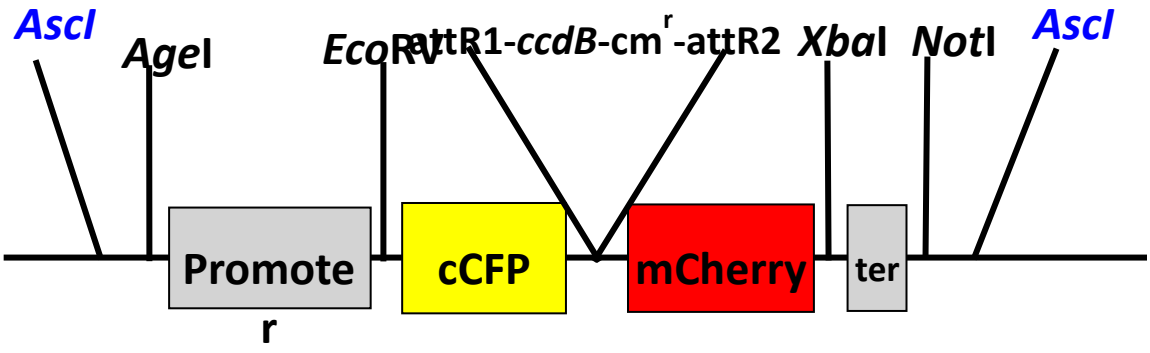
Table 1 shows that incubation of VirE2-cCFP (using either a CaMV double 35S promoter or a *nos* promoter) with an empty nVenus or nVenus I152L vector did NOT eliminate the "self-assembly" problem. However, the use of nYFP I152L completely prevented "self-assembly", but not interaction of VirE2-nYFP I152L with VirE2-cCFP or VirE2-nYFP I152L with VIP1-cCFP. Thus, we shall use the nYFP I152L mutant in all further constructions.

Table 1. Interaction of various forms of nYFP/nVenus with empty cCFP vector or VirE2/VIP1-cCFP

	35S::E2-cCFP	**Nos::E2-cCFP**	**35S::VIP1-cCFP**	**Nos::VIP1-cCFP**	**35S::cCFP**	**Nos::cCFP**
35S::nVenus 155-N	Yes (very strong)	Yes (less strong)	Yes (very strong)	No	Yes	Yes
35S::nVenus I152L-N	Yes (strong)	Yes (less strong)	Yes (strong)	No	Yes	Yes
35S::nYFP 155-N	Yes	Yes (few cells)	Yes		No	No
35S::nYFP I152L-N	No	No	No	No	No	No
35S::E2-nYFP-155	Yes (strong)	Yes (moderate)	Yes		No	No
35S::E2-nYFP I152L	Yes(weak)	Yes (weak)	Yes (weak)		No	No

Because we shall rebuild the aptamer expression library in a different expression vector, we have reconsidered our entire strategy. It would be better to link the aptamer to cCFP (rather than to nYFP), since it is easier to change the nYFP moiety on a bait construct rather than rebuild an aptatmer-nYFP library many times. This new aptamer expression vector would link the aptamer with cCFP. In addition, we reasoned that if cCFP preceded the aptamer, and mCherry followed it, we could screen for those aptamers that were in-frame, since only these would express mCherry and generate red fluorescent cells. Thus, we re-designed the aptamer expression cassette as described in Figure 4. Further libraries were generated and tested using this system.

Figure 4. New peptide aptamer expression cassette



Scientific Accomplishments
(April, 2011-September, 2011)

Funding for this project ended in the Spring of 2011. However, using funds from other projects, we were able to continue work on this project. Our additional progress includes:

1. We constructed the new aptamer expression vector described in Figure 4 above.
2. We exchanged the aptamer library ($2x10^8$ members in pDONR221) into this new aptamer expression library. We were able to obtain ~10^9 primary colonies. Thus, with 5x coverage, it is likely that all aptamers in the pDONR221 library are represented in the new aptamer expression library.
2. We amplified the new aptamer expression library and extracted DNA. This DNA will be used to co-transfect tobacco BY-2 cells with bait constructs to search for aptamers that specifically interact with bait proteins.

Scientific Accomplishments
October, 2011-March, 2012

Because we did not receive funding for the second year of this project, there has been no additional progress

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator Name(s) and Contact Information: **(Title, Mailing Address, Phone, Fax, Email)**	Susan Gibson Associate Professor Department of Plant Biology 1501 Gortner Ave. St. Paul, MN. 55108-1024 Phone: 612-624-7408 Fax: 612-624-6264 Email: gibso043@umn.edu	
University:	University of Minnesota	
DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026-304**	
Project Title:	*Exploiting genetic variation in soybean to increase oil*	
Reporting Period:	**From: 4/30/2015**	**To: 12/31/2015**
Report Type:	**Check one:** [ ] Semiannual Report [x] Final Report	

Project Objectives

List each objective of the Project and the progress made toward each one during the reporting period.

Objective 1. Determine chromosomal locations of expression level polymorphism (ELP) genes.

Progress: Work on this objective is now complete. The unique chromosomal locations of 135 expression level polymorphism (ELP) genes have been determined. These ELP genes were shown previously by us to exhibit significant differences in transcript levels between developing seeds of the Minsoy and Archer soybean varieties.

Objective 2. Use BeadChips to measure ELP gene transcript levels from additional recombinant inbred lines. To improve the resolution with which eQTL are mapped, developing seeds from 48 additional recombinant inbred lines and both parental lines will be measured in triplicate at 3 developmental stages.

Progress: Work on this objective is now complete. First, BeadChips were used to measure mRNA levels for all of the 433 ELP genes in a total of 67 recombinant inbred lines. Based on analyses of these results, 51 ELP genes were tentatively identified as having eQTL. The transcript levels of these 51 genes were measured in all of the 107 available recombinant inbred lines.

Objective 3. Identify *cis* and *trans* eQTL.

Progress: Work on this objective is complete. We obtained nCounter data for the mRNA levels of the 51 ELP genes found by our preliminary analyses to be most likely to have eQTL associated with them. The mRNA levels of these 51 ELP genes were determined in all of the 107 available recombinant inbred lines. Putative eQTL were identified for 43 of these 51 ELP genes. In addition, eQTL identified for 28 ELP genes are likely to represent *cis* eQTL and eQTL identified for an additional 7 ELP genes may also represent *cis* eQTL.

Objective 4. Compare eQTL and QTL map locations to determine which eQTL may correspond to QTL for seed composition (oil and protein content), size or yield.

Progress: Work on this objective is complete. The results of these analyses indicate that potentially *cis* eQTL associated with nine ELP genes map to the same region of the genome as a QTL of interest. We exceeded this objective by initiating experiments designed to test whether any of these ELP genes do in fact correspond to the QTL with which they co-localize. Towards that end, we generated plant transformation constructs designed to overexpress six of the ELP genes. Seeds were obtained from transgenic plants transformed with each of the six ELP constructs. Future work (outside the scope of this project) will focus on characterizing these transgenic lines to determine ELP gene expression levels and to identify plants that are homozygous for different transgene insertion events.

Please complete.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

The focus of this project is on seed composition (protein and lipid contents) and yield traits, which have a major effect on soybean profitability. To be able to breed new varieties with the best possible seed composition and yield characteristics, it is necessary to have more information regarding the genes that control these traits. Towards this end, we exploited new information and resources for soybean to carry out eQTL analyses. These eQTL analyses allowed us to identify genes that are candidates to control seed composition and/or yield. These candidate genes are being called "ELP" genes. Based on our results, increased activity of six of these ELP genes is predicted to result in alterations in seed composition and/or yield. To test these predictions, transgenic soybean lines with increased activity levels for each of these six genes were generated and will be characterized as part of a future project.

Please complete.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

The focus of this project was on seed composition (protein and lipid contents) and yield traits, which have a major effect on soybean profitability. To be able to breed new varieties with the best possible seed composition and yield characteristics, it is necessary to have more information regarding the genes that control these traits. Regulation of gene activity levels plays a critical role in determination of desirable traits. In brief, a major part of the reason that two varieties differ in these traits is that the varieties differ in the activity levels of important genes. So, by identifying expression quantitative trait loci, or eQTL, that control variations in gene activities, it should be possible to identify many of the loci that control variations in seed composition and yield. Once these eQTL are identified, molecular markers that map to the same regions of the genome can be identified. The availability of these molecular markers will significantly decrease the amount of time and money needed to develop new soybean varieties with desirable seed composition and yield traits by facilitating marker-assisted breeding efforts.

The Minsoy and Archer varieties were chosen for this project because they are Minnesota adapted varieties that differ with respect to seed composition and yield. Also, several laboratories, including the one headed by Dr. Jim Orf, had already invested a lot of time and effort into characterizing these varieties. The results of their past research greatly facilitated our research. The first step in identifying the genetic factors that control variations in gene activity levels, and seed composition and yield, was to identify genes that have different activity levels in the two varieties of interest. This goal was accomplished by collecting tissue from Minsoy and Archer seeds at three critical developmental stages and measuring gene activity levels in these samples using Affymetrix Soybean GeneChips. Based on our analyses, a total of 433 genes were

found to have significantly different activity levels between the two varieties. These genes were thus designated as "expression level polymorphism", or "ELP", genes. A manuscript on this work was recently published, making all the information obtained from these studies available to all researchers on a publicly accessible database. Thus, this information will benefit other researchers' work in addition to our own.

Our next specific aim was to measure the activity levels of these 433 ELP genes in developing seeds of recombinant inbred lines derived from a cross between Minsoy and Archer. To accomplish this goal we first needed to develop and test a method for analyzing the activity levels of the 433 ELP genes. Although it would have been possible to use Affymetrix Soybean Genechips for these analyses, we chose not to do that because these Genechips are very expensive. Instead, we worked with Illumina to develop Beadarrays that allow measurement of activity levels of the 433 ELP genes of interest plus an additional 77 control genes that, based on our earlier results, have similar activity levels in Minsoy and Archer. These control genes are important for normalizing the data so that the expression levels of the ELP genes can be normalized and thus determined accurately between different experiments. Testing of these Beadarrays revealed that they do allow us to obtain the information we need and cost significantly less than GeneChips.

The post-doctoral research associate working on the project then performed over 600 RNA isolations from tissue samples collected from developing soybean seeds from different recombinant inbred lines derived from a cross between the Minsoy and Archer varieties. Next, the RNA preparations from a total of 67 different recombinant inbred lines were analyzed using the Beadarrays. As three different developmental stages were analyzed and three samples were examined for each line at each developmental stage, a total of 67 X 3 X 3 = 603 Beadarray experiments were successfully performed.

To improve the resolution with which we can map eQTL, we worked with Dr. David Hyten to determine the segregation patterns of approximately 740 markers in the 238 recombinant inbred lines being used by this project. The availability of this information about the segregation patterns of such a large number of markers has significantly improved the resolution with which we can determine the chromosomal locations of eQTL. This improved resolution is also allowing us to predict more accurately which eQTL are most likely to correspond to *cis* versus *trans* eQTL and which eQTL are most likely to correspond to QTL for one of the traits of interest (seed yield, composition and size).

We also worked with a bioinformaticist to use information from the soybean genome project to determine the chromosomal locations of as many of the ELP genes as possible, using the currently available information. Information about the chromosomal locations of the ELP genes allowed us to compare the chromosomal locations of each ELP gene with the chromosomal locations of the putative eQTL we identified. This information thus allowed us to determine which eQTL are most likely to correspond to *cis* eQTL and which are most likely to correspond to *trans* eQTL. We were able to identify unique chromosomal locations for 135 of the ELP genes.

We next used all of the transcriptional profiling data that was available at the time, as well as the improved genotyping information, to identify eQTL associated with one or more of the 135 ELP genes for which we had identified unique chromosomal locations. These analyses resulted in identification of potential eQTL for 51 ELP genes. To further improve the resolution with which we were able to map eQTL, we next analyzed the expression of the 51 ELP genes for which we had tentatively identified an eQTL in additional recombinant inbred lines, so that we

now have expression profiling data for these ELP genes from a total of 107 recombinant inbred lines. These experiments were done on developing seed samples isolated from each of the 51 recombinant inbred lines and both parental lines. All of the recombinant inbred lines and both parental lines were grown in three plots, with two rows of each line planted in each plot. Duplicate tubes of tissue were collected from each plant line in each plot. By collecting seeds from three different plots and from several plants from the duplicate rows in each plot, the effects of local environmental variations were minimized. RNA was isolated from each of these tissue samples and checked to ensure that all the RNA samples were of sufficient quality and quantity for transcriptional profiling experiments. We then measured the expression levels of each of the 51 ELP genes in the recombinant inbred lines using the new nCounter system by NanoString, rather than using BeadChips, as nCounter is a far more cost effective method for analyzing expression of 59 genes (51 experimental genes plus eight control genes) in large numbers of samples than is the BeadChip method.

We also completed an analysis of all of our transcriptional profiling data to identify ELP genes that may correspond to eQTL that are likely or possibly *cis* eQTL and that may co-localize with one of the QTL of interest. The results of these analyses are summarized in Table 1. These analyses resulted in the identification of nine ELP genes that correspond to eQTL that are likely or possibly *cis* eQTL and that may co-localize with a QTL of interest. An indication of the mapping resolutions of the eQTL is provided by Figure 1. *The identification of these nine ELP genes is encouraging, as they are candidates to correspond to QTL for seed composition, size or yield.*

We then began experiments designed to test the effects of overexpressing six of the ELP genes (ELP genes 1, 2, 3, 7, 8, and 11) that are candidates to correspond to QTL of interest. These six ELP genes were chosen because, based on the available data, if one of these six ELP genes does correspond to the QTL with which it co-localizes, overexpression of that ELP gene is predicted to result in an improvement in the trait partially regulated by that QTL. For example, ELP1 co-localizes with a QTL for seed yield. Seed yield is higher in recombinant inbred lines carrying the Archer allele of that QTL than in recombinant inbred lines carrying the Minsoy allele of that QTL. As ELP1 is expressed at higher levels in Archer than in Minsoy, these results suggest that, if ELP1 does correspond to that QTL, increased expression of ELP1 will promote increased seed yield (Table 1). In contrast, overexpression of ELP genes 9 and 10 is predicted by the available QTL data to result in a worsening in one of the agronomic traits of interest, if ELP genes 9 or 10 do correspond to the QTL that map near ELP genes 9 and 10 (Table 1). It should be noted that overexpression of ELP4 is also predicted to result in improvement in seed protein content, if ELP4 does correspond with the seed protein QTL located on around cM 65-73 on chromosome 4. However, we did not continue research on ELP4 because we were unable to determine which soybean gene corresponds to ELP4. This is because it is not always possible to determine which Affymetrix probe set correspond to which soybean gene.

Consequently, we generated plant transformation constructs to overexpress ELP genes 1, 2, 3, 7, 8 and 11. Each of these six constructs contains the entire coding region plus ~ 1 kb of upstream sequences and ~ 750 bp of downstream sequences from one of these six ELP genes. Each ELP gene was isolated from genomic DNA harvested from whichever parental line (Minsoy or Archer) expresses that particular ELP gene at the higher level (Table 1). These genomic DNA sequences were then cloned into the pTF101.1gw1 plant transformation vector. After cloning was complete, the ELP gene inserts were sequenced to ensure that no mutations were inadvertently introduced during the pcr amplification or cloning processes.

The plant transformation constructs were submitted to the Plant Transformation Facility at Iowa State University. Each of the plant transformation constructs was introduced into *Agrobacterium tumefaciens* strain EHA101. The transgenic EHA101 cultures were then used to infect soybean cultivar Williams 82, as this cultivar has been transformed with great success. Transgenic soybean lines were successfully generated for all six ELP genes (Table 2) and seeds were isolated from the putative transgenic lines for all six of the ELP genes. Progeny testing was also completed for the majority of the transgenic lines. The results of this testing indicated that the putative transgenic lines do carry a plant transformation construct. In future experiments that are outside the scope of this project, these transgenic lines will be characterized to determine the expression levels of the ELP gene with which they were transformed. Transgenic lines with increased ELP gene transcript levels will be analyzed to determine whether they have altered seed composition and/or yields.

Table 1. QTL for seed yield, composition and size identified from a Minsoy X Archer cross. Seven chromosomal regions that control one or more of the traits of interest have been identified using recombinant inbred lines derived from a Minsoy X Archer cross. The ELP genes that map near these QTL and the types of eQTL (likely *cis*, possibly *cis* or likely *trans*) are indicated. Note that in some cases (e.g. on chromosome 19), two ELP genes and the *cis* eQTL that regulate those two ELP genes map to the same region of the genome as a QTL. In these cases, either or both of the ELP genes may be responsible for the QTL. Whether under or overexpressing an ELP gene might result in an improvement in the trait regulated by the indicated QTL is indicated.

QTL				ELP gene			
Chromosome	~ Location	Trait(s) regulated	Trait higher in	ELP gene	eQTL type	ELP gene expression higher in	Under or over express ELP gene
1	64 cM	seed yield	Archer	1	*cis?*	Archer	over
				9	*cis?*	Minsoy	under
4	10 cM	seed oil	Minsoy	2	*cis*	Archer	under
4	10 cM	seed oil 8-2	Archer	2	*cis*	Archer	over
4	10 cM	seed volume	Archer	2	*cis*	Archer	over
4	65-73 cM	seed protein	Minsoy	3	*cis?*	Minsoy	over
				4	*cis*	Minsoy	over
4	65-73 cM	seed volume, seed weight	Archer	3	*cis?*	Minsoy	under
				4	*cis*	Minsoy	under
5	89 cM	seed oil	Archer	none	none	N/A	N/A
7	19 cM	seed volume	Archer	none	none	N/A	N/A
7	39 cM	seed protein	Minsoy	10	*cis?*	Archer	under
				11	*cis?*	Minsoy	over
19	70 cM	seed yield, seed weight	Archer	7	*cis*	Archer	over
				8	*cis*	Archer	over

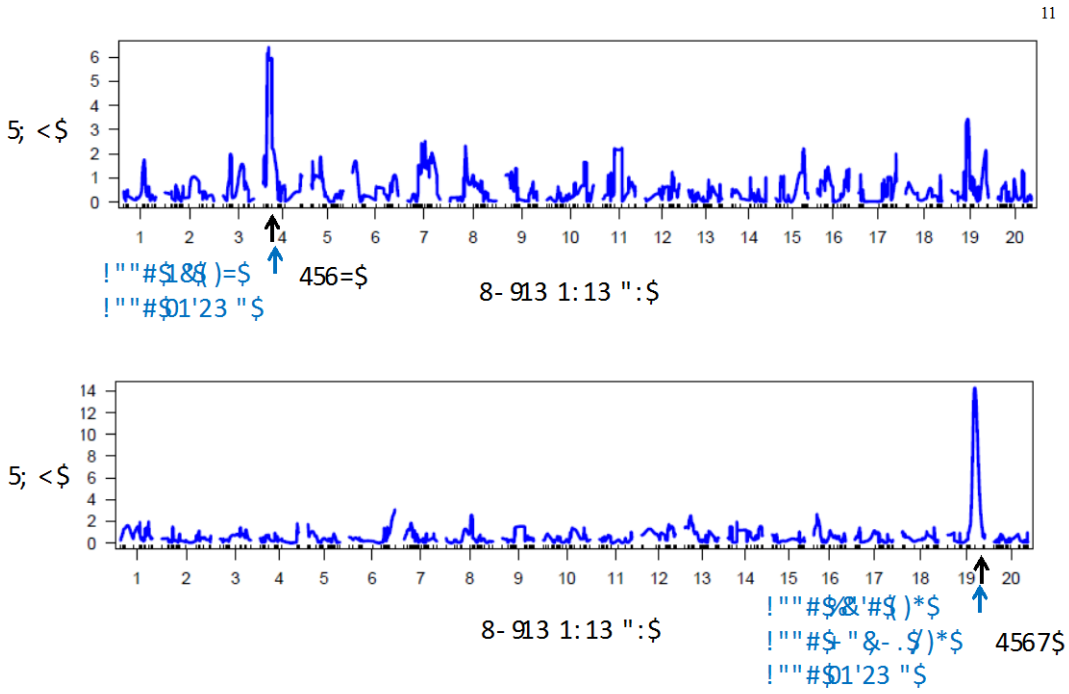


Figure 1. Mapping of eQTL for ELP2 and ELP7. eQTL mapping results for ELP2 and ELP7 are shown by the dark blue lines. The black arrow underneath each eQTL peak indicates the map position of the ELP gene. The light blue arrow underneath each eQTL peak indicates the map positions of the centers of the peaks for the indicated QTL.

Table 2. Summary of progress in generating transgenic soybean lines overexpressing six ELP genes. Plant transformation constructs were generated for ELP genes 1, 2, 3, 7, 8 and 11. These plant transformation constructs were used to generate transgenic lines of the Williams 82 soybean cultivar. The status of these transgenic soybean lines is indicated.

ELP gene	Lines for which seeds obtained	Lines tested for plant transformation construct	Total R1 seeds obtained
1	7	7	1,262
2	8	4	733
3	9	9	1,289
7	3	2	270
8	6	6	837
11	5	5	548

Please complete.

Publications and Presentations

List all talks, posters, scientific publications, news releases, etc., about this Project during the reporting period. Provide one copy of each publication, report, or news article resulting from activities supported under the grant as well as any announcements, press releases, statements or photographs depicting Project activities.

Manuscript

Lin R, Glazebrook J, Katagiri F, Orf JH, Gibson SI (2015) Identification of differentially expressed genes between developing seeds of different soybean cultivars. Genomics Data 6:92-98.

Talk

"Nutrient response networks, at the heart of plant development and metabolism?". Presented by Sue Gibson at the University of Minnesota, September 2015.

Please complete.

Technology Transfer

Describe all technology transfer (inventions, disclosures, patents, licensing agreements, etc.) resulting from the Project during the reporting period.

None during this reporting period.

Please complete.

Commercial Accomplishments

Describe the most significant accomplishments resulting from the Project during the reporting period.

None during this reporting period.

Please complete.

Educational Accomplishments

Describe the most significant educational accomplishments resulting from the Project during the reporting period. (Number of students, post-docs, etc. trained)

One post-doc and one undergraduate student received training and experience by participating in this project. The post-doc participating in the project came into the project with a strong background in molecular biology. Participation in the project has allowed him to significantly broaden his research background by learning about modern plant breeding strategies. The undergraduate student gained experience with molecular biology as well as with plant biology.

Please complete.

Additional Funding

List any additional funding generated as a result of the Project during the reporting period.

None during the current reporting period.

Please complete.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

None during the current reporting period.

Please complete.

Scientific Progress Report

Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.

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University:	University of Minnesota

DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026-319**	
Project Title:	*Using genomics to increase soybean biodiesel yield*	
Reporting Period:	**From: 4/1/2015**	**To: 12/31/2015**
Report Type:	**Check one:** [] Semiannual Report [x] Final Report	

Project Objectives

Objective 1: Identify additional homozygous mutant lines for *S4*.
Progress: This objective has been completed as we have identified seedlings homozygous for a second mutant allele of *S4* (= *s4-2*).

Objective 2: Re-screen greater numbers of *s4* mutant plants for alterations in seed yield.
Progress: This aim was exceeded as seed yield was assayed not only for *s4*, but also for *hac1* and *z* mutants. Please see below for an analysis of the data from these experiments.

Objective 3: Re-screen greater numbers of *z* mutant plants for alterations in seed size.
Progress: Mutations in *Z* were found to cause a slight decrease in average seed size.

Objective 4: Characterize the effects of *sov3*, and possibly *s4*, mutations on: seed size, number of siliques/plant, number of seeds/silique and seed composition.
Progress: Seed size and composition were analyzed in one *s4* and five *sov3* mutants. Please see below for analyses of the data from these experiments. Potential soybean homologs of *S4* and *SOV3* were also identified. In addition, soybean QTL for seed composition that map near these potential homologs were identified.

Objective 5: Characterize the effects of the *hac1*, and possibly *z*, mutations on: seed composition.
Progress: Seed composition was analyzed in three *hac1* and three *z* mutants. Please see below for analyses of the data from these experiments.
In addition, although not within the scope of the original proposal, seed composition was also analyzed in the *sis8* and *sis3* mutants. Please see below for an analysis of data from these experiments.
Potential soybean homologs of *HAC1, SIS8* and *SIS3* were identified. In addition, soybean QTL for seed composition that map near these potential homologs were identified. As the *SIS8* gene is a particularly strong candidate to play an important role in regulating seed composition, transgenic soybean carrying a *SIS8* expression construct were generated.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

Additional sources of renewable energy are needed to help reduce usage of fossil fuels. Soybean is particularly attractive as a source of renewable energy. A primary need in developing soybean as a viable source of renewable energy is that the costs of producing soydiesel must be reduced. This goal may be achieved by increasing the amount of oil produced by soybean or by increasing the value of co-products, such as soy protein meal. A promising approach to improving soybean is to identify genes that help control seed composition or yield and to use those genes in breeding programs to develop improved soybean varieties. However, identifying the correct genes in the soybean genome is difficult for technical reasons. Therefore, we are making use of the resources available in the plant *Arabidopsis thaliana*, which is much easier to work with than soybean. In previous work we identified genes (*HAC1, S4, Z, SOV3, SIS8* and *SIS3*) that are promising candidates to play important roles in the regulation of seed composition. We screened Arabidopsis carrying mutations in all six of these genes for alterations in seed yield. The results of these experiments indicate that plants that are homozygous for mutations in *Z* tend

to produce a greater weight of seeds per plant than wild-type plants grown and harvested under identical conditions. In addition, plants homozygous for mutations in *HAC1* produce, on average, 20% fewer mg of seeds per plant than do wild-type plants grown and harvested under the same conditions. Measurements of protein content in seeds from *s4*, *hac1*, *sov3* and *z* mutants indicate that a mutation in *S4* causes a significant reduction in seed protein levels. Mutations in the *SIS3* gene cause significant alterations in the transcript levels of six genes that encode oleosins (components of seed oil bodies) and five genes encoding seed storage proteins, suggesting that *SIS3* also plays a role in regulating seed composition. We also found that a mutation in the *SIS3* gene causes significant alterations in seed carbon/nitrogen (C/N) ratios, providing further evidence that SIS3 plays an important role in seed composition. Altered expression of *SIS8* also affects seed composition. In particular, overexpressing *SIS8* under the control of its own promoter causes a significant increase in seed oil levels and a corresponding decrease in seed protein levels in transgenic Arabidopsis. We also identified potential soybean homologs for *S4*, *SOV3*, *HAC1*, *SIS8* and *SIS3*. In addition, we identified soybean QTL for seed composition that map near some of these potential soybean homologs, and may thus correspond to the same genetic loci.

Several lines of evidence suggest that the *SIS8* gene is a particularly promising candidate to help control seed composition. Lines of Arabidopsis carrying mutations in *SIS8* have altered seed protein and oil levels and altered C/N ratios. Also, the Arabidopsis *SIS8* gene maps to the same region of the genome as an Arabidopsis QTL for seed composition. Similarly, a putative *SIS8* homolog from soybean maps to the same region of the soybean genome as a soybean QTL for seed composition. Therefore, a plant transformation construct designed to express the Arabidopsis *SIS8* gene at high levels was generated. Seeds were obtained from ten independent transgenic soybean lines that were generated using a *SIS8* overexpression construct. Although not within the scope of this project, these transgenic soybean lines will be characterized to determine whether altered expression of *SIS8* can alter seed composition in soybean.

Please complete.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

The overall goal of this project was to identify genes that play important roles in controlling seed yield, composition and/or size. Our approach for identifying such genes was based on the following rationale. First, seed yield, and possibly seed size, are believed to be partially controlled by the amount of fixed carbon (e.g. sugars) partitioned to developing seeds at the whole plant level. In addition, seed composition is believed to be partially controlled by the amount of fixed carbon partitioned to lipid versus starch versus protein at the molecular level. Carbon partitioning, in turn, is thought to be partially regulated by the levels of soluble sugars, such as glucose and sucrose. In source tissues, such as mature leaves, high levels of soluble sugars have been shown to feedback inhibit expression of certain photosynthetic genes. According to one model, if a plant is producing sugars at a higher rate than it is able to utilize those sugars, soluble sugar levels rise in the leaves and inhibit expression of specific photosynthetic genes. The rate of photosynthesis than decreases, bringing the rate of production of soluble sugars by source tissues (such as mature leaves) more in line with the rate of utilization of those sugars by sink tissue (such as immature leaves, roots and seeds). Therefore, sugar levels are believed to help control the strength of the source tissues that produce sugars. Sugar levels are also believed to help

control the strength of the sink tissues. For example, experiments on transgenic potato plants have shown that increasing the levels of soluble sugars in the stolons leads to formation of increased numbers of tubers per stolon (Müller-Röber et al., 1992), increasing the size of the sink tissues and thus the ability of the plant to utilize sugars. In both of these example, the plant needs to be able to sense and response to available sugar levels. Thus, sugar response is considered to be a key factor in regulating carbon partitioning and, consequently, seed yield, size and composition. Therefore, our hypothesis is that, by manipulating sugar response, we can alter carbon partitioning. To be able to manipulate sugar response, we need to identify some of the genes that control sugar response. We identified genes that are candidates to be involved in controlling sugar response by using Affymetrix GeneChips to identify Arabidopsis genes that are themselves sugar-regulated at the steady-state mRNA level. We next screened the list of sugar-regulated genes to identify those genes that are predicted to encode proteins with functions that are often found in response pathways, such as protein kinases, protein phosphatases and transcription factors. In this manner we narrowed the list of candidate genes to 189.

In previous work we used a reverse genetics approach to identify 189 genes that were promising candidates to affect seed size, seed composition, yield and/or sugar response. We obtained lines carrying mutations in over 130 of these genes and screened them for defects in one or more of these traits. Based on these analyses, identified four genes, *HAC1, Z, SOV3* and *S4*, as being particularly promising candidates to help regulate one or more of the traits of interest. *HAC1* encodes a histone acetylase, *SOV3* and *Z* both encode leucine-rich repeat transmembrane protein kinases and *S4* encodes a zinc finger protein. Fortunately we were able to identify plants homozygous for multiple independent mutant alleles of each of these four genes. We obtained plants homozygous for three different mutant alleles of *HAC1*, three different mutant alleles of *Z*, five different mutant alleles of *SOV3* and two different mutant alleles of S*4*. The availability of multiple independent mutant lines for each of these genes was important, because it allowed us to determine whether observed phenotypes were likely to be caused by a mutation in the gene of interest by checking whether that same mutant phenotype was observed in lines carrying independent mutant alleles of the same gene.

The primary goals for this project were to screen plants homozygous for mutation in *HAC1, Z, S4* and *SOV3* for defects in seed yield, composition (protein or oil amounts) and seed size. Towards this end, we performed two independent biological replicates of a seed yield experiment. For each of these experiments, we grew eight pots each of plants homozygous for three different *HAC1* mutant alleles, three different *Z* mutant alleles, four different *SOV3* mutant alleles and one mutant *S4* allele, as well as 16 pots of wild-type plants. The pots were approximately 5” on a side and plants were grown at a density of 5 plants/pot. Mutant and wild-type plants were sown and grown at the same time in the same growth space, with pots containing mutant and wild-type plants placed in a random pattern. Plants were grown to full maturity under a 16-hr photoperiod and seeds harvested from each pot. After drying in a desiccator, the total weight of seeds harvested from each pot was determined and divided by the number of plants in the pot to determine the total weight of seeds produced per plant in each pot.

The results of these seed yield experiments indicate that plants homozygous for mutations in *SOV3* and *S4* produce weights of seeds per plant that are similar to those produced by wild-type plants grown in parallel (Figure 1). In contrast, plants that are homozygous for mutations in *HAC1* produce, on average, 20% fewer mg of seeds per plant than do wild-type plants grown and harvested under the same conditions. Interestingly, plants homozygous for mutations in *Z* produce, on average, 5% more mg of seeds per plant than do wild-type plants grown and harvested at the same time, under the same conditions.

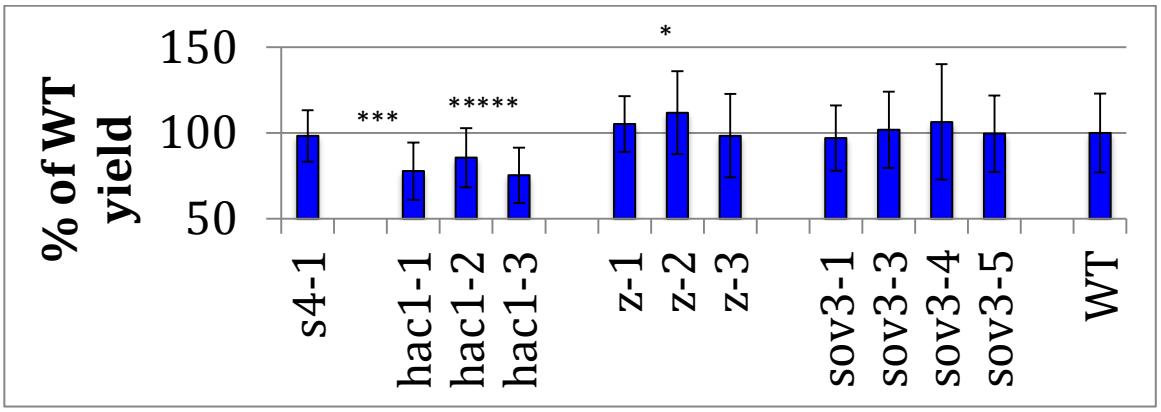

Figure 1. Effects of mutations on seed yield. Mutant and wild-type (WT) lines were grown in multiple, randomized, pots under a 12-h photoperiod and the total weight of seeds produced per plant determined. Columns represent averages and error bars represent STD DEV. Mutant and wild-type yields differed with a *p* value of < 0.1 (*), < 0.05 (**), or < 0.01 (***), according to a Student's T-test. N = 16-32.

Aliquots of the seeds collected for one of the seed yield experiments were weighed to determine the average weight (size) per seed for plants grown in each pot. The results of these experiments indicate that mutations in *SOV3* tend to cause slight increases in seed size whereas mutations in *S4* and *Z* tend to cause slight decreases in seed size.

Seed composition, particularly seed storage lipid and seed storage protein contents, is a critical determinant of economic value for seed crops. Consequently, it was of interest to determine whether mutations in the genes of interest affect seed lipid or seed protein production. Seed protein levels were assayed for one allele of *S4*, two alleles of *HAC1*, five alleles of *SOV3* and three alleles of *Z* (Figure 2). Seed protein levels were also measured in lines carrying two different mutant alleles of the *SIS8* gene. The *SIS8* gene was not included in the original proposal, but subsequent experiments indicated that *SIS8*, and possibly also *SIS3*, may affect seed composition as mutations in these genes affect the expression of many of the major oleosin (components of seed oil bodies) and seed storage protein genes (see Figure 3 for results from a *sis3* mutant). The results of the seed protein experiments indicate that mutations in *S4* and *SIS8* cause a significant reduction in seed protein content. In contrast, mutations in *HAC1*, *SOV3* and *Z* do not cause significant alterations in seed protein content.

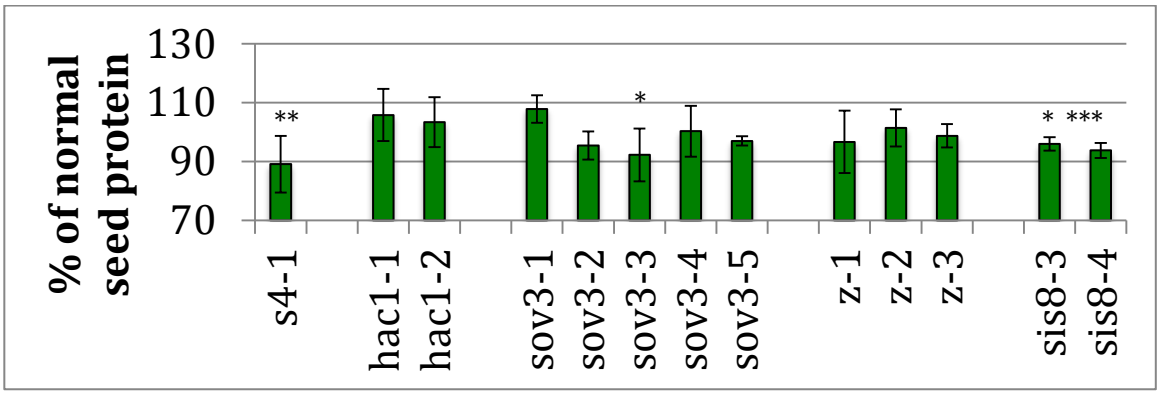

Figure 2. Effects of mutations on seed protein levels. Protein levels were analyzed for seeds harvested from multiple pots. Results are shown as the percentage of normal seed protein levels. Columns indicate averages and error bars represent standard deviations. Mutant and wild-type seed protein levels differed with a *p* value of < 0.2 (*), < 0.06 (**), or < 0.01 (***), according to a Student's T-test. N = 2-15.

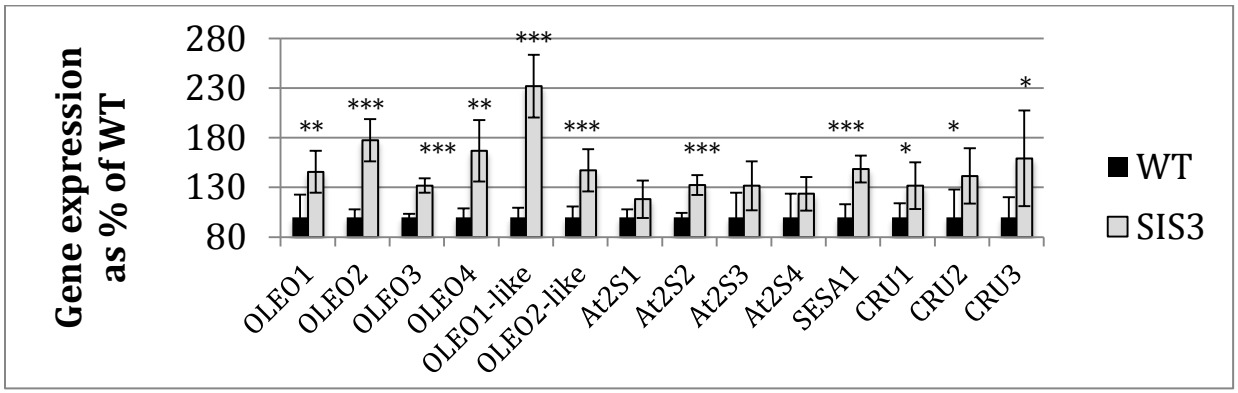

Figure 3. Transcript levels of seed storage protein and oleosin genes are increased in *sis3*. Steady-state mRNA levels of oleosin and seed storage protein genes in wild-type (WT) and *sis3* mutant germinating seeds are shown. Columns indicate averages and error bars indicate STD DEV. Mutant and wild-type mRNA levels differed with a *p* value of < 0.15 (*), < 0.08 (**), or < 0.04 (***), according to a Student's T-test. N = 3.

The preliminary experiments described above resulted in the identification of six genes that are strong candidates to help regulate seed composition. Subsequently, measurements of seed protein levels showed that at least some of these genes do affect seed protein levels (Figure 2), and other mutations are likely to affect seed composition (Figure 3). Although mutations in *HAC1, SOV3* and *Z* were found not to affect seed protein levels (Figure 2), the possibility remained that mutations in these genes affect seed lipid levels as it is possible for loci to affect seed lipid levels without affecting seed protein levels. Therefore, lines carrying mutations in all six genes were screened for alterations in carbon/nitrogen (C/N) ratios. Mutations that affect seed lipid or protein levels affect C/N ratios as proteins, but not lipids, contain large amounts of N. C/N ratios were thus used to examine seed composition rather than direct measurements of seed lipid levels as measurements of C/N ratios are easier to perform and have been found to be particularly reproducible (Li *et al*., 2006).

For the first set of experiments measuring C/N ratios, mutant and wild-type lines were grown in eight pots each in a randomized pattern under controlled growth conditions in a greenhouse. Plants were grown to maturity and seeds harvested. Aliquots of seeds from each pot were dried, weighed on an analytical balance and placed in tin sample capsules. The capsules/seeds were then sent to the Duke Environmental Stable Isotope Laboratory (DEVIL) at Duke University for measurement of C/N ratios using a Carlo Erba Instruments NC 2100 elemental analyzer. Seeds from wild-type plants and plants carrying loss-of-function mutations in *S4, HAC1* or *SIS8* were analyzed in the first set of experiments. A line that carries a construct that causes overexpression of SIS8 was also tested. The results of the first set of experiments measuring C/N ratios indicate that mutations in *S4*, *HAC1* and *SIS8* cause significant reductions in the percentage of the seed that is composed of carbon, but have much lesser effects on the percentage of the seed that is composed of nitrogen (Figure 4). These results are consistent with mutations in these genes causing a reduction in the percentage of the seed that is composed of lipid, as reductions in lipid content would be expected to lower carbon content without significantly affecting nitrogen content.

Three additional sets of experiments examining C/N ratios were completed. The results of these experiments indicate that mutations in some of the genes being studied do affect seed composition. Most notably, the *sis3-2* mutation results in a significant increase in C/N ratios (Figure 5). Seeds carrying the *sis3-2* mutation were compared with seeds harvested from plants

(grown and harvested in parallel) that carry the *sis3-2* mutation as well as a wild-type copy of the *SIS3* gene (i.e. a line where the *sis3-2* mutation has been complemented). The seeds from the uncomplemented *sis3-2* line exhibit a significantly higher average C/N ratio than the seeds from the complemented *sis3-2* line. This result is consistent with the *sis3-2* mutation causing either an increase in the percentage of the seed that is comprised of lipid or a decrease in the percentage of the seed that is comprised of protein.

In our most recent set of experiments, we used NMR to measure seed lipid and seed protein content in wild-type seeds and seeds with alterations in SIS8 activity. The most striking finding from these experiments is that an Arabidopsis line that was transformed with a copy of the wild-type *SIS8* gene under the control of its own promoter produces seeds with significantly increased seed oil and decreased seed protein. This line has increased levels of *SIS8* activity due to having multiple copies of the *SIS8* gene. Interestingly, two transgenic lines of Arabidopsis that overexpress the wild-type *SIS8* gene under the control of the CaMV 35S promoter, rather than under the control of the *SIS8* promoter, do not exhibit significant alterations in seed composition. These results suggest that increased expression of *SIS8* in particular cell or tissue types and/or at certain stages of development may be necessary for significant alterations in seed composition (Figure 6).

To further determine which of the above genes are the most promising candidates to affect seed composition, it was of interest to determine whether any of these genes might correspond to QTL for seed composition. Although only limited research has been done to identify QTL for seed composition in Arabidopsis, four QTL for seed oil content have been identified (Hobbs et al., 2004). Interestingly, *S4, SOV3* and *SIS8* map to the same regions of the genome as two of the Arabidopsis QTL for seed oil content (Figure 7). *HAC1, Z* and *SIS3* do not map within the chromosomal regions defined by these four seed oil QTL. However, that does not rule out the possibility that, as more Arabidopsis QTL for seed composition and/or yield are identified, one or more of these genes might be found in the future to map near such a QTL.

In addition to determining which of the above genes co-localize with Arabidopsis QTL for seed oil content, potential soybean homologs were identified for these genes and the chromosomal locations of these potential soybean homologs compared with the known locations of soybean QTL for seed protein content. One potential soybean homolog was identified for *S4*. The chromosomal location of the *S4* soybean homolog is near the chromosomal location of soybean QTL for seed protein content. Three potential soybean homologs were identified for *SOV3* and two of these potential homologs were found to map on or near the locations of soybean QTL for seed protein content. Two potential soybean homologs were identified for *HAC1*. One of these potential homologs was found to map on or near the locations of soybean QTL for seed protein content and the second potential *HAC1* homolog was found to lie approximately 9 cM from another soybean QTL for seed protein content. No potential soybean homologs were found for *Z*. BLASTP searches comparing the Arabidopsis *Z* amino acid sequence with predicted amino acid sequences of proteins encoded by the soybean genome revealed that the most similar soybean proteins are 64-69% identical to the Arabidopsis Z protein sequence. Two potential soybean homologs were identified for *SIS8*. One of these potential soybean *SIS8* homologs was found to lie approximately 3 cM from a soybean QTL for seed protein content. The other of these potential soybean *SIS8* homologs was found to lie approximately 9 cM from a soybean QTL for seed protein content. Four potential soybean homologs were identified for *SIS3*. Three of these potential soybean *SIS3* homologs lie near soybean QTL for seed protein composition, with one of the homologs mapping to almost the exact location of the center of a QTL peak.

Although outside the scope of this project, we have initiated experiments to determine the effects of expressing *SIS8* in soybean. Towards this end, we cloned the Arabidopsis *SIS8* gene into the pTF101.1 plant transformation vector, under the control of a strong promoter (the CaMV 35S promoter). We sequenced the *SIS8* sequences in the construct and determined that no mutations were inadvertently introduced during the amplification and cloning processes. We then sent this plant transformation construct to the Plant Transformation Facility at Iowa State University. The 35S-SIS8 plant transformation construct was introduced into *Agrobacterium tumefaciens* strain EHA101. These transgenic *Agrobacterium tumefaciens* cells were then used to infect soybean cultivar Williams 82. Williams 82 was chosen for these experiments because it has previously been used with great success in soybean transformation experiments. A total of ten independently-generated soybean lines were generated using the SIS8 plant transformation construct. These transgenic soybean lines will be used to test the effects of expressing the Arabidopsis *SIS8* gene on soybean seed composition, size and yield.

Scientific Progress Report

Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.

Principal Investigator Name(s) and Contact Information: **(Title, Mailing Address, Phone, Fax, Email)**	Shui-zhang Fei, PhD Associate Professor, Grass Genetics and Breeding Department of Horticulture Iowa State University Ames, IA 50011 Phone: 515-294-5119 Fax:515-294-0730 Email: sfei@iastate.edu	
University:	Iowa State University	
DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026-**	
Project Title:	*Discovery of Novel Genes in Creeping Bentgrass by High Throughput Gene Silencing with Specialized Inverted Repeat cDNA Libraries*	
Reporting Period:	**From: April 1, 2015**	**To: December 31, 2015**
Report Type:	**Check one:** [ ] Semiannual Report [x] Final Report	

Project Objectives

List each objective of the Project and the progress made toward each one during the reporting period.

Objective 1: construct RNAi libraries from a conventional creeping bentgrass cDNA library with mRNA from salt-treated tissue.

Progress: Two hairpin RNAi libraries enriched for genes responsive to cold (brachypodium) and salinity (creeping bentgrass) have been made.

No progress to be reported for this period.

Objective 2. transform the RNAi libraries en masse into creeping bentgrass to create a mutant population.

Progress: A subset of creeping bentgrass RNAi library consisting of 12 randomly selected genes was used for transformation of creeping bentgrass. Insertion of the transgene was confirmed in 23 putative transgenic plants by PCR.

Objective 3 screen mutant phenotypes and isolate novel genes causing altered phenotypes.

Progress: The identity of the inverted repeat in each of the transgenic plants is being examined. No progress to be reported for the period.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

Using primers designed for the *bar* gene, which confers resistance to the herbicide glufosinate, we confirmed that 23 of the putative transgenic creeping bentgrass plants contain the *bar* transgene. We are currently determining the number of insertions and the identity of the inverted repeat in each of the confirmed transgenic plants. We also plan to evaluate the salinity tolerance of those transgenic plants and determine the function of the gene for which the inverted repeat was introduced.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

A total of 66 putative transgenic plants derived from 9 independent events were produced. To confirm the presence of a transgene, we designed a forward primer 5' GGATCTACCATGAGCCCAGA 3' and a reverse primer 5'GAAGTCCAGCTGCCAGAAAC 3' for the *bar* gene and performed PCR reactions. PCR was performed with the following program: 5 minutes at 94ºC, 35 cycles of 20 seconds at 94ºC, 30 seconds at 58ºC and 50 seconds at 72ºC with a final extension of 5 minutes at 72 ºC. PCR products were visualized in a 0.7% agarose gel. Out of the 66 plants, 23 were confirmed to have the *bar* gene. These transgenic plants were derived from three independent events. Fig. 1 below is an example showing the presence or absence of the PCR amplicons in putative transgenic plants.

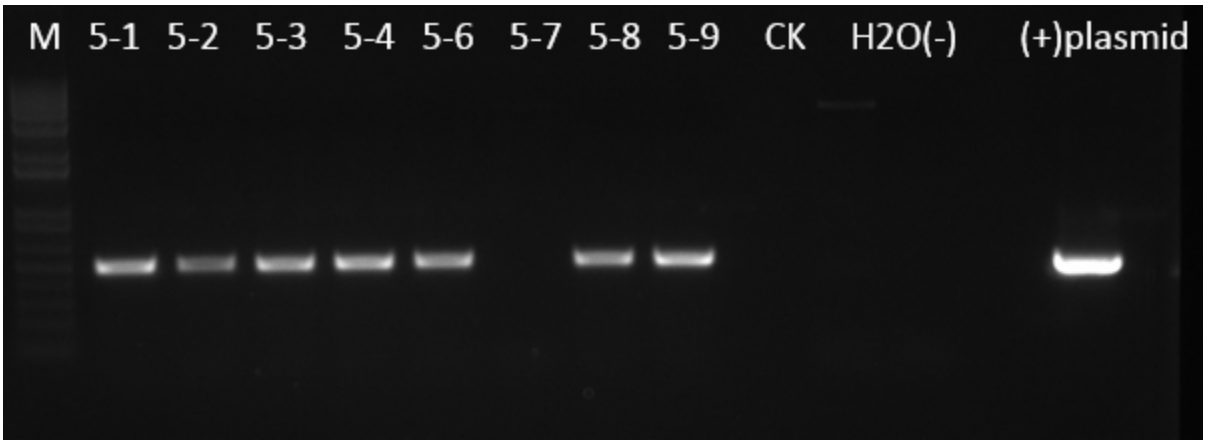

Fig. 1 The presence of the *bar* gene was confirmed by PCR. M-molecular ladder, 5-1, 5-2, 5-3, 5-4, 5-6, 5-7, 5-8, 5-9 are putative transgenic plants derived from the transgenic event 5. CK-Non-transformed negative plant control. H_2O is the water only negative control and (+) plasmid is the positive control.

The number of insertions and the identity of the inverted repeat that accompany the *bar* gene are currently being determined by using the primer pair from the spacer (which separates the inverted repeats) and upstream of the insert. Once the identity of the inverted repeated is identified for each transgenic plant, we will attempt to evaluate the salinity tolerance of those transgenic plants and determine the function of the gene for which the inverted repeat was introduced.

Publications and Presentations

List all talks, posters, scientific publications, news releases, etc., about this Project during the reporting period. Provide one copy of each publication, report, or news article resulting from activities supported under the grant as well as any announcements, press releases, statements or photographs depicting Project activities.

Published Abstracts:

Shui-zhang Fei, Ying Feng, Jiangli Dong, Conglie Ma and Yanhai Yin 2015 High throughput RNAi for rapid gene discovery in grasses. Invited presentation made during the Crop Science Society of America annual meeting in Minneapolis, MN.

Shui-zhang Fei, Jingjie Hao and Yanhai Yin 2015. Down-regulation of the brassinosteroid receptor gene, BRI1 in creeping bentgrass results in a dwarf phenotype and increased drought tolerance. Presentation made during the Crop Science Society of America annual meeting in Minneapolis, MN. Presentations

Additional presentations:
High throughput RNAi in polyploid perennial grasses, Interdepartmental Graduate Major in Plant Biology, Iowa State University, Ames, IA

Establishment of a high throughput RNAi platform for rapid gene discovery in creeping bentgrass. Invited to make an oral presentation for the 9th International Symposium on Molecular Breeding of Forage and Turf in Lanzhou, China in August.

Technology Transfer

Describe all technology transfer (inventions, disclosures, patents, licensing agreements, etc.) resulting from the Project during the reporting period.

None.

Commercial Accomplishments

Describe the most significant accomplishments resulting from the Project during the reporting period.

None.

Educational Accomplishments

Describe the most significant educational accomplishments resulting from the Project during the reporting period. (Number of students, post-docs, etc. trained)

N/A

Additional Funding

List any additional funding generated as a result of the Project during the reporting period.

None.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

N/A

Scientific Progress Report

Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.

Principal Investigator Name(s) and Contact Information: **(Title, Mailing Address, Phone, Fax, Email)**	**David Hildebrand** **Professor** **403 Plant Science Bldg.** **1405 Veterans Dr.** **Univ. Kentucky** **Lexington, KY 40546-0312** **Office: 859/218-0760** **Fax: 859/257-7874** **Lab: 859/218-0798** **dhild@uky.edu**	
University:	**University of Kentucky**	
DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026-325**	
Project Title:	***Branch-chain fatty acid production in plants***	
Reporting Period:	**From: 04/01/2015**	**To: 03/15/2016**
Report Type:	**Check one:** [x] Semiannual Report [ ] Final Report	

Project Objectives

List each objective of the Project and the progress made toward each one during the reporting period.

Objective 1. Cloning and characterization of genes encoding branch-chain fatty acid (BCFA) biosynthetic enzymes from source organisms.

Objective 1a. Cloning of BCFA synthases from plants and bacteria.

- Obtained a putative CFS gene from *Achromobacter* sp.

Objective 1b. Comparison of BCFA synthases from plants and from *Escherichia coli* expressed in yeast and petunia leaves

- See the progress for Objective 3a.

Objective 2. Cloning and characterization of enzymes involved in the accumulation of BCFA from source organisms

Objective 2a. Cloning of diacylglycerol acyltransferases (DGATs) from plants

- A pair of degenerate PCR primers for DGAT1 was obtained and RACE was attempted.

Objective 2b. Characterization of diacylglycerol acyltransferases expressed in yeast and petunia leaves

- See the progress in Objective 3b.

Objective 3. Characterization of model plants and oilseeds engineered with enzymes involved in the formation and accumulation of BCFAs

Objective 3a. Transformation of plants with cyclopropane fatty acid synthases

- SSE transformation was attempted with a full-length longan CFS (DlCFS) cDNA along with LcDGAT2 or DlDGAT2. No SSEs with either DlCFS, LcDGAT2 and/or DlDGAT2 gene were obtained so far.

Objective 3b. Transformation of plants with diacylglycerol acyltransferases

- Some soybean lines transformed with a very active DGAT1 have a 4% increase in oil levels with no reduction in seed protein levels. Also see the progress for Objective 3a.
- Additional SSEs were transformed with *Vernonia* DGAT1A (VgDGAT1A) and a high oil transcription factor (RcWRI1) and Twenty one lines of hygromycin resistant SSEs were matured, but none of them contained either RcWRI1 or VgDGAT1A.

Objective 3c. Characterization of triacylglycerols and membrane lipids from transgenic plants

- Work on this objective will further proceed after the other above tasks that this builds from are mostly complete.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

We have isolated and cloned additional genes encoding enzymes responsible for transferring branched-chain fatty acids (BCFAs) into final seed oil triacylglycerol. Constructs were made with full-length genes for metabolic engineering of oilseeds such as soybeans with genes encoding enzymes for BCFA biosynthesis and accumulation in seed oil. We also created additional gene constructs and an expression system for increasing seed oil content and production along with BCFA accumulation. Soybean somatic embryos have been transformed with these gene constructs for both BCFA biosynthesis and accumulation and embryos positive for both genes were confirmed and regenerated into whole plants which were grown out in the greenhouse. Progeny seeds from these plants were screened for the presence of both gene(s) and several confirmed to also have genes for both aspects of BCFA production. Three different BCFA synthesis genes and two BCFA accumulation genes are being tested in several combinations for determining the optimum approach for high renewable BCFA levels in oilseeds. Lipids are being analyzed from the seeds of regenerated plants with gene insertions confirmed to verify the increased accumulation of BCFAs as a basis of economical and renewable BCFA production. Soybeans are also being engineered for increased renewable oil production without reducing seed protein levels. We have developed stable lines with a 4% increase seed oil and total oil + protein levels.

This work shows significant progress toward the goal of metabolically engineering plant oils as a source of renewable lubricants such as motor oils. As a `proof of principle' of this approach we have chemically converted most of the double bonds of several plant oils that can be grown and produced on a commercial scale into BCFA derivatives and filled in any remaining double bonds by hydrogenation. All initial unsaturated fatty acids were converted in one small-scale experiment. Preliminary experiments with our industrial partner, Valvoline, indicate that such plant oil derivatives can have the needed oxidative stability and low temperature fluidity to meet demanding lubricant applications such as for motor oils. Further work to scale-up and refine the process is in progress including complete conversion of double bonds into BCFA derivatives. This includes testing the reaction progress with higher sonication power assisted catalysis.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

Cloning of BCFA synthases from plants and bacteria

Initial attempts to clone a CFS gene from *Enterobacter ludwigii* resulted in obtaining non-CFS genes. However, the sequences of these genes indicated that the bacteria isolate we have is *Achromobacter* sp instead of *E. ludwigii* as originally informed. A pair of cloning primers were designed from the multiple alignment of the published CFS genes from *Achromobacter* spp. and a putative cDNA was obtained. The sequencing of the gene revealed that it is a CFS gene from *Achromobacter* sp.

Comparison of BCFA synthases from plants and from bacteria expressed in yeast and petunia leaves

We previously reported the detection of both truncated LcCFS and truncated DlCFS activity in agro-infiltrated leaves of Petunia and *Nicotiana benthamiana* leaves and in transgenic *N. tabacum* plants.

The quantitative comparison of these recombinant gene products was found to be impractical by agroinfiltration or stable leaf transformation due to low expression/accumulation as well as overlapping peaks in GC analysis. Hence, we opted to skip the functional analysis of full length LcCFS and or DlCFS using agroinfiltration. However, we have not been able to obtain DlCFS-engineered soybeans to date.

As mentioned above, we have cloned *Achromobacter* CFS (AcCFS) gene and created a seed expression construct of AcCFS. Soybeans will be transformed with this AcCFS construct.

Cloning of diacylglycerol acyltransferases from plants

The cDNA of a longan DGAT2 (DlDGAT2) as well as lychee DGAT2 (LcDGAT2) were obtained previously and were inserted into a seed-specific expression vector. Unfortunately, no clear increase in the DGAT activity has been observed in engineered soybeans. See the details of soybean transformation in the section below.

Degenerate primers were designed from multiple alignment of plant DGAT1 peptides and obtained. Initial attempts to clone DGAT1 fragments from previously prepared 1st-strand cDNAs from longan or lychee have failed. Fresh cDNAs from RNA prep will be prepared for RACE and cloning of DGAT1 fragment will be attempted.

Characterization of diacylglycerol acyltransferases expressed in yeast and petunia leaves

We opted to express the LcDGAT2 and DlDGAT2 with a seed-specific expression vector along with the truncated longan BCFA synthase genes (trDlCFS) and/or other BCFA synthase genes (see the next section).

Transformation of plants with cyclopropane fatty acid synthases and diacylglycerol acyltransferases

We have attempted 36 particle delivery sessions of SSEs with DlCFS with a combination of putative LcDGAT2 or DlDGAT2 with no success of obtaining DlCFS positive SSEs (overall 83 Hyg-resistant SSEs). Since DlCFS were inserted into a pGOP soybean oleosin promoter driven expression vector, we will attempt inserting DlCFS in a pPHI phaseolin promoter driven expression vector for the next transformation. Unfortunately, no restriction site is available for easy transfer of the current cDNA. Cloning with the updated primers has been done and the clones are being sequenced.

As mentioned in the previous report, a transformation attempt of SSE with LcDGAT2 with truncated DlCFS (trDlCFS) resulted in four independent lines positive with LcDGAT2 and trDlCFS. Due to weak or no activity of trDlCFS, the effect of LcDGAT2 has not been assessed. Crossing of one of these lines and an EcCFS soybean was attempted, but so far no hybrid has been obtained. More crossing will be attempted.

As for DlDGAT2 transformation, like DlCFS, the cDNA was inserted into a pGOP vector and 36 particle delivery sessions resulted in few Hyg-resistant SSEs but none positive with DlDGAT2. We will try to insert the DlDGAT2 into pPHI as mentioned for DlCFS.

As mentioned in the previous report, we are including not only BCFA biosynthesis and accumulation genes per se but are combining these with a high oil accumulation gene, *Vernonia* DGAT1A (VgDGAT1A), and a high fatty acid biosynthesis and an oil transcription factor, a WRI1 ortholog, we cloned from developing seeds of a very high oil plant source. Initially, these genes were on separate plasmids independent of the Hyg-resistance gene. Unfortunately, very few plants with any of these genes were obtained after four transformation attempts.

We attempted examining the function of WRI1 ortholog using agroinfiltrated *N. benthamiana* leaves. Unfortunately, no difference was found in lipid concentration or compositions between UKWRI1-

infiltrated leaves and vector-infiltrated leaves. The expression levels of UKWRI1 is being examined. It is possible the *Agrobacterium* strain GV3850 might have failed to infect leaf tissues effectively. Another aggressive strain GV3101 was recently obtained and the agroinfiltration will be repeated using the new *Agrobacterium* strain.

Model system renewable lubricant studies with Valvoline

For our model system renewable lubricant studies with Valvoline we have been able to convert most of the double bonds of common plant oils into BCFA derivatives. We find that the process works much better with oils highest in fatty acids with many double bonds. We can often convert tri-unsaturated fatty acids completely into BCFA derivatives and have now achieved > 90% conversion of mono- unsaturated fatty acids. Even small amounts of mono-unsaturated fatty acids render a renewable lubricant insufficiently oxidatively stable for motor oil applications. However removal of any remaining double bonds via hydrogenation can result in a renewable lubricant with sufficient oxidative stability and low temperature fluidity for motor oil applications. A major drawback to our renewable lubricant products previously was the excessively dark color but we modified our reaction conditions resulting in a much lighter BCFA oil derivative.

So far when we subject such oils to complete hydrogenation to remove any remaining double bounds the resulting oil derivative is again fairly dark. In further work to fully hydrogenate BCFA derivatized oils we have had difficulty in full hydrogenation. It is possible to fully hydrogenate plant oils without any darkening of the color on an industrial scale so that this problem should not be an impediment to our approach to produce renewable oils with both excellent oxidative stability and low temperature fluidity. The catalyst we are using for the hydrogenation is palladium on fine carbon and it is surmised that we are having difficulty removing the carbon after the reaction. Again industrial hydrogenation can be readily achieved without such problems. However if we can convert all double bonds into BCFA derivatives no hydrogenation would be needed. This has been achieved in several reactions except for the mono-unsaturated fatty acid oleate. In a variation of our cyclopropanation protocol we have achieved complete conversion of all double bonds including oleate (18:1) in a small scale reaction eliminating the need for hydrogenation. We are refining the optimum reaction conditions for most efficient and economical complete cyclopropanation of plant oils. This optimized protocol will be repeated on a larger scale. We find conversion of plant oil double bonds into BCFA is greatly facilitated by sonication assisted catalysis. We find higher conversion with higher sonication power. We recently were able to procure a much more powerful sonicator and are testing this process with 5 – 10 fold or greater sonication power than our previous reactions.

Further analysis of the products of our BCFA conversion process indicates most if not all of the starting unsaturated fatty acids have been converted into mono-, di- and tri-cyclopropanated derivatives, CP1-18:0, CP2-18:0 and CP3-18:0 in a 60 g reaction. Preliminary tests at Valvoline indicate one of the most completely cyclopropanated plant oil having equal or greater properties as the best synthetic petroleum-based lubricants.

The development of higher oil oilseeds such as soybeans without reduction in protein levels will make such chemical conversions of renewable oils into lubricants more economical.

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Verner, O.M., and K.M. Sinyak. 1988. Composition of fatty acids of *Bacillus anthracis* and other soil bacilli. Biology Bulletin of the Academy of the Sciences of the USSR 15:25-30.

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. 164:455-465.

Publications and Presentations

List all talks, posters, scientific publications, news releases, etc., about this Project during the reporting period. Provide one copy of each publication, report, or news article resulting from activities supported under the grant as well as any announcements, press releases, statements or photographs depicting Project activities.

Oral Presentations:

David Hildebrand, Jessime Kirk, Maythem Al-Amery, Kim Gyeong-Ok, Carloalberto Petti, Will Serson, Robert Geneve Seth DeBolt & Tim Phillips presented an oral presentation titled "Physical & Chemical Properties of *Salvia hispanica* Seeds in Comparison with very High Fiber & ω3 Sources" at the 104th American Oil Chemists' Society Annual Meeting & Expo held at Montreal, CA on May 5 to 8, 2013.

Tomoko Hatanaka, William Serson, Runzhi Li, Paul Armstrong, Keshun Yu & David Hildebrand. "VgDGAT1A increases oil content by 3-4% in *Glycine max*" at the American Society of Plant Biology Annual Meeting held at Providence, RI on Aug. 3 to 6, 2013.

C-4 Division, Seed physiology Production and technology. "Drying Seeds High in Polyunsaturated Fatty Acids to Constant Weight Can Lead to Erroneous Results," ASA/CSSA/SSSA Tri-Society Meeting. Minneapolis, MN. November 16, 2015.

Publications:

Maythem AL-Amery, Watchareewan Jamboonsri, Chad Lee, James Hammond, Tim Phillips and David Hildebrand. 2016. Evaluation of flax as a viable crop again in the south central U.S. J. KY Academy of Sci. (in press).

Al-Amery, M., H. Fukushige, W. Serson and D. Hildebrand. 2016. DNA analysis for single seed genotyping in soybeans. Journal of Crop Improvement (in press).

Hayes, D.G. and D. Hildebrand. 2016. Perilla (*Perilla frutescens*). Chapter 11.9 In: Industrial Oilseed Crops, McKeon et al. editors, Elsevier.

Fukushige, H. and D. Hildebrand. 2016. Hemp (*Cannabis sativa* L.). Chapter 11.3 In: Industrial Oilseed Crops, McKeon et al. editors, Elsevier.

Hatanaka, T., W. Serson, R. Li, P. Armstrong, K. Yu, T. Pfeiffer, X. Li and D. Hildebrand. 2016. Increasing Renewable Oil Production. Theoretical and Applied Genetics (submitted).

Obendorf, R., H. Moon, D. Hildebrand, R. Torisky and G. Collins. 2016. Deficiency of pinitol and galactopinitols in maturing soybean somatic embryos. Crop Science (submitted).

Afitlhile, M., K. Duffield; M. Fry and D. Hildebrand. 2015. The toc132toc120 heterozygote mutant of *Arabidopsis thaliana* accumulates reduced levels of hexadecatrienoic acid. Plant Physiology and Biochemistry 96:426-435.

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Zou, Y., S. Chintamanani, P. He, H. Fukushige, L. Zhu, D. Hildebrand, X. Tang and J.-M. Zhou. 2016. A gain-of-function mutation in a mechanosensitive ion channel triggers cell death and wound-induced hyperaccumulation of JA in Arabidopsis. Journal of Integrative Plant Biology (JIPB) (in press).

Al-Amery, M., H. Fukushige and D. Hildebrand. 2015. Single seed selection for low phytate soybeans. JAOCS 92:1119-1123.

Schmidt, M., W. Parrott, D. Hildebrand, D.F., R. Berg, A. Cooksey, K. Pendrvis, Y. He, F. McCarthy and E. Herman E.M. 2015. Transgenic soybean seeds accumulating β-carotene exhibit collateral enhancements of high oleate and high-protein content traits. Plant Biotechnology J. 13: 590-600.

Li, R., K. Yu, Y. Wu, T. Hatanaka, H. Fukushige and D. Hildebrand. 2013. Soybean oil biosynthesis, characterization of two diacylglycerol acyltransferases. Functional & Integrative Genomics 13:99-113.

Technology Transfer

Describe all technology transfer (inventions, disclosures, patents, licensing agreements, etc.) resulting from the Project during the reporting period.

Preliminary experiments with our industrial partner, Valvoline, indicate that the cyclopropanated plant oil derivatives we have produced can have the needed oxidative stability and low temperature fluidity to meet demanding lubricant applications such as for motor oils. This process is being improved and refined and a patent of our improved process is being drafted together with our industrial partner.

PATENTS:

Hildebrand, D.F., R. Li and T. Hatanaka. Method for Increasing Renewable Oil Production. US patent # 9,133,469 issued September 15, 2015.
Hildebrand, D.F., W. Jamboonsri and T. Phillips. Early Flowering Chia and Uses Thereof. US Patent No. 8,586,831 issued November 19, 2013.
Hildebrand, D.F., R. Li and T. Hatanaka. Diacylglycerol Acyltransferase Sequences and Related Methods. US Patent No. 8,431,772 issued April 30, 2013.

Hildebrand, D.F. Triacylglycerol with very high oxidative stability & low temperature fluidity. IP Disclosure 1/19/2012

Hildebrand, D.F., S. Rao and J.R. Thoguru. FUNGAL DESATURASES AND RELATED METHODS. U.S. Patent # 8,053,633 Patent issued Nov. 8, 2011.

Commercial Accomplishments

Describe the most significant accomplishments resulting from the Project during the reporting period.

We have successfully isolated and sequenced genes for enzymes responsible for transferring branched-chain fatty acids (BCFAs) into seed oils in addition to several genes encoding branched-chain fatty acid synthase enzymes. Constructs were made for metabolic engineering of oilseeds such as soybeans with such genes encoding enzymes for BCFA biosynthesis and accumulation in seed oil. We also cloned additional genes and made additional metabolic engineering constructs for increasing seed oil content and production along with BCFA accumulation. Soybean somatic embryos have been transformed with these gene constructs and the plant progenies are being screened for the presence of the gene(s). Some transgenics have been shown to have introduced genes for both BCFA biosynthesis and accumulation. Lipids will be analyzed from the somatic embryos with gene insertion confirmed to verify the increased accumulation of BCFAs as a basis of economical and renewable BCFA production. This work shows significant progress toward the goal of metabolically engineering plant oils as a source of renewable lubricants such as motor oils. As a `proof of principle' of this approach we have chemically converted most of the double bonds of several plant oils that can be grown and produced on a commercial scale into BCFA derivatives and filled in any remaining double bonds by hydrogenation. Preliminary experiments with our industrial partner, Valvoline, indicate that such plant oil derivatives can have the needed oxidative stability and low temperature fluidity to meet demanding lubricant applications such as for motor oils. This process is being improved and refined and a patent of our improved process is being drafted together with our industrial partner.

Educational Accomplishments

Describe the most significant educational accomplishments resulting from the Project during the reporting period. (Number of students, post-docs, etc. trained)

Two graduate students, a technician and one postdoctoral research scholar are being trained.

Additional Funding

List any additional funding generated as a result of the Project during the reporting period.

Kentucky Small Grains Assoc., D. Hildebrand and T. Phillips, Development of chia as a sustainable crop. $81,000, Sept. 1, 2011 to Dec. 31, 2016.

Kentucky Soybean Promotion Board, D. Hildebrand, Soybean Oil as a High Value Fuel Cell Energy Source & Lubricant. $27,841, April 1, 2014 to March 31, 2015.

Cono SA, D. Hildebrand and T. Phillips, Development of chia as a sustainable crop. Development of Chia, *Salvia hispanica* L., as a sustainable oil source. $24,000, Sept. 1, 2014 to Dec. 31, 2016.

Valvoline, D. Hildebrand, Optimization of cyclopropanation of renewable oils with novel functionality. $42,000, Feb. 1, 2016 to Jan. 31, 2017.

The graduate students involved with this project, Will Serson and Maythem Alamery have been awarded fellowships.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

Two graduate students, Will Serson and Maythem Alamery continued with this project during the reporting period. A research associate, Hirotada Fukushige, worked on the project for a significant % of his time.

Scientific Progress Report

Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.

Principal Investigator Name(s) and Contact Information: **(Title, Mailing Address, Phone, Fax, Email)**	Stephen H. Howell Professor of Genetics, Development and Cell Biology Iowa State University Ames, Iowa 50011 Phone: 515 294-5737 Fax: 515 294-5256 Email: shh@iastate.edu
University:	Iowa State University

DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026-326**	
Project Title:	*New genes for stress tolerance in bioenergy crops*	
Reporting Period:	**From: 10/1/2014**	**To: 3/31/2015**
Report Type:	**Check one:** [ ] Semiannual Report [X] Final Report	

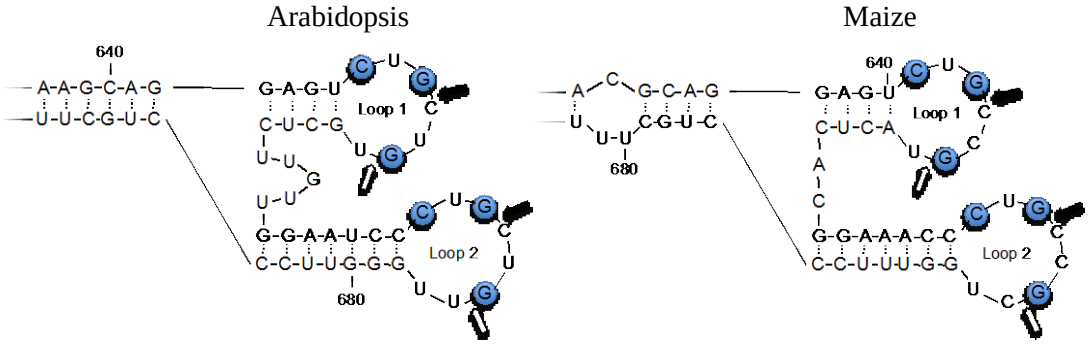
Project Objectives

The goals for this project were:

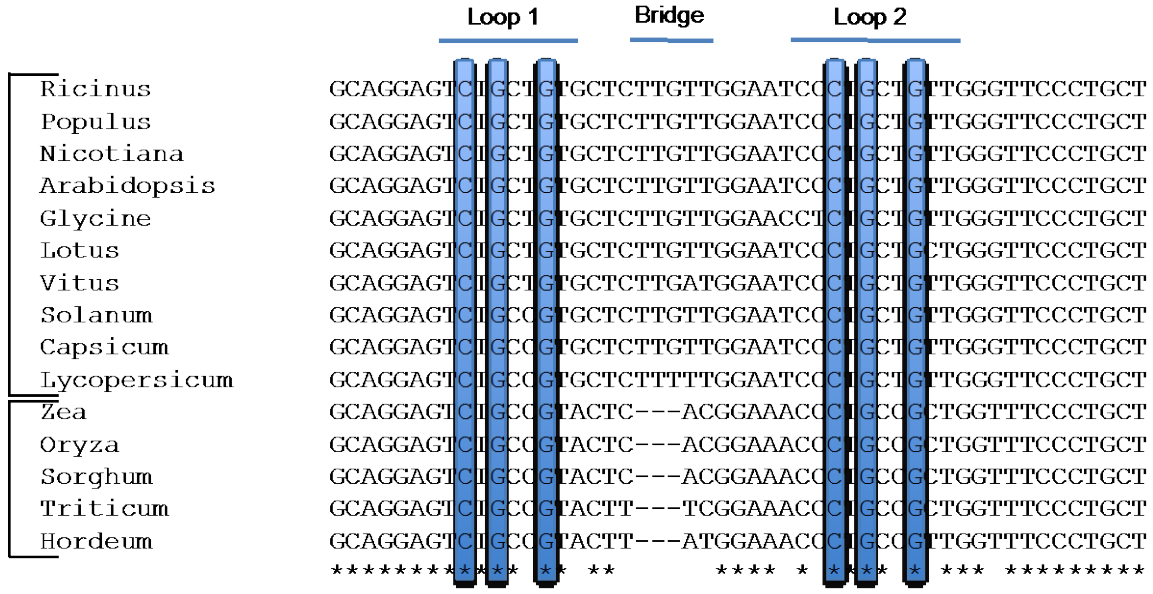
1) Establish a PCR reaction to detect the splicing of ZmbZIP60 mRNA in corn.
2) Determine what environmental conditions elicit the RNA splicing response in corn seedlings.
3) Determine the molecular signature of the ER stress response in maize and identify genes that can be used to monitor ER stress.
4) Determine the time course of the RNA splicing reaction in response to ER stress.
5) Determine the transition temperature for elicitation of ER stress responses in the lab.
6) Analyze the ER stress response under field conditions.
7) Demonstrate the role of ZmbZIP60 in upregulating maize BiP genes.

Most major crop losses are caused by adverse environmental conditions that produce plant stress. Adverse conditions, such as hot weather, can produce a form of plant stress called endoplasmic reticulum stress (ER stress) (Howell, 2013). ER stress results from an accumulation of misfolded proteins in the ER. The accumulation of misfolded protein elicits the "unfolded protein response" (UPR). The UPR upregulates a constellation of genes, which mitigates the damage caused by ER stress and brings the capacity of the protein folding machinery in line with demands.

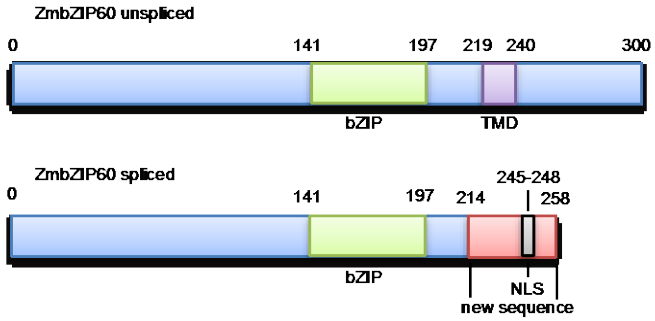
The preliminary work by the PI on plant ER stress was done on Arabidopsis. We found that there were two arms of the ER stress pathway in Arabidopsis (Howell, 2013). One arm involving membrane-associated transcription factors and another arm involving the RNA splicing enzyme IRE1. For this project, we focused on the activities of IRE1, one of the major players in plant UPR. In Arabidopsis, IRE1 splices the messenger RNA for bZIP60, a stress response transcription factor (Deng et al., 2011). We discovered a homolog of bZIP60 in maize, which we called ZmbZIP60 (Li et al., 2012). ZmbZIP60 was considered to be a likely target for IRE1's splicing activity because its messenger RNA had a region that folded into a structure characteristic of a IRE1 splice recognition site (see below). The IRE1 recognition site is composed of twin kissing hairpin loops with three conserved bases in each loop.



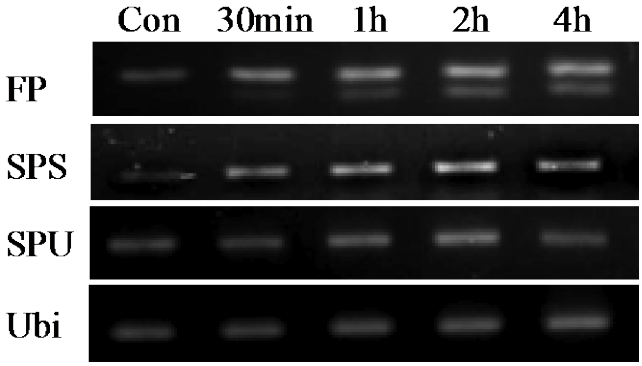
Arabidopsis and maize structures did have a small difference in that the bridge region between the two loops in maize was three bases shorter than in Arabidopsis and, therefore, the size of the excised intron is three bases smaller. However, in comparing the Arabidopsis and corn IRE1 recognition sites to those in bZIP60 homologs in other plants, we found that the sequence difference reflected a difference in dicots and monocots.



Despite the slight differences between IRE1 recognition sites between Arabidopsis and maize, the consequences are the same. Splicing produces a frame shift that converts a mRNA, which encodes a bZIP transcription factor with a transmembrane domain and predicted to be an ER-membrane protein, to a factor without a transmembrane domain, but having a acquired a nuclear targeting sequence and predicted to located in the nucleus.

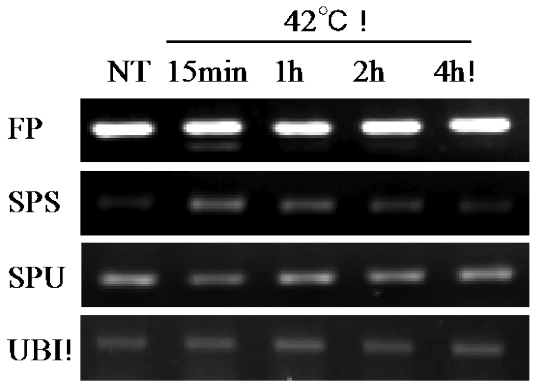


Given these predictions about the possible role of ZmbZIP60 in ER stress responses in corn, we tested corn seedlings in the lab for the splicing ZmbZIP60 mRNA in response to treatment by ER stress agents, such dithiothreitol (DTT) or tunicamycin (TM). These agents are effective in eliciting ER stress in the laboratory, because they interfere with protein folding. To test for ZmbZIP60 mRNA splicing, we set up RT-PCR assays to detect the production of the spliced RNA. One assay was called the flanking primer (FP) assay in which we amplified both the spliced and unspliced forms of ZmbZIP60 mRNA. Because the FP assay amplified both the unspliced and spliced forms of ZmbZIP60 mRNA, it was not particularly sensitive for detecting the spliced form. Therefore we developed additional RT-PCR assays that were specific for the spliced form (SPS assay) or the unspliced form (SPU). We treated young corn seedlings with DTT and found, indeed, that treatment with the ER stress agents elicited an RNA splicing response.



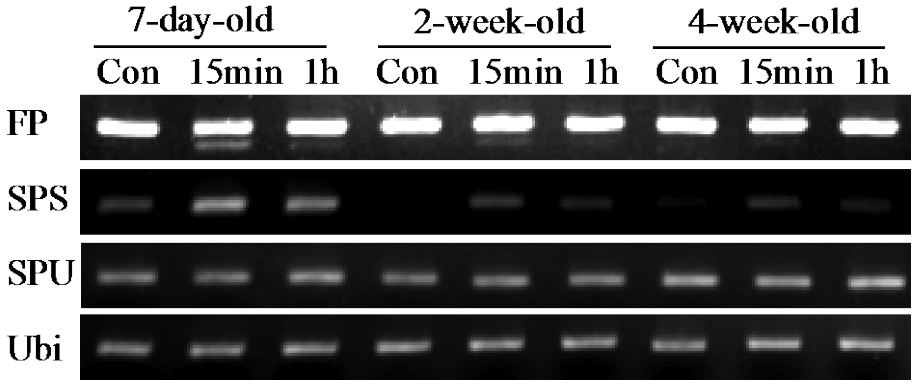
ZmbZIP60 mRNA splicing in response to treatment of seedlings with DTT. 7-day-old B73 seedlings were treated with 2.5 mM DTT, and samples of roots were taken at the times indicated. RNA was extracted from the root samples and RT-PCR splicing assays were conducted to detect the accumulation of spliced ZmbZIP60 mRNA. FP is a flanking primer assay that amplifies both unspliced (upper band) or spliced (lower band) ZmbZIP60 mRNA. SPS is an assay specific for the spliced mRNA and SPU is specific for the unspliced form. Ubi is a ubiquitin RNA loading control.

In addition to ER stress agents, we found that heat shock also induces ZmbZIP60 mRNA splicing in corn seedlings. The RNA splicing response is somewhat different in response to heat treatment as opposed to ER stress agents in that response to heat is transient, peaking somewhere between 15 min to an hour after shifting seedlings from 23°C to 42°C.



ZmbZIP60 mRNA splicing in response to heat shock. 7-day-old-B73 seedlings were grown at room temperature (23°C) in laboratory and shifted to 42°C. Root samples were taken at the times indicated, RNA was extracted and subjected to RNA splicing assays as above.

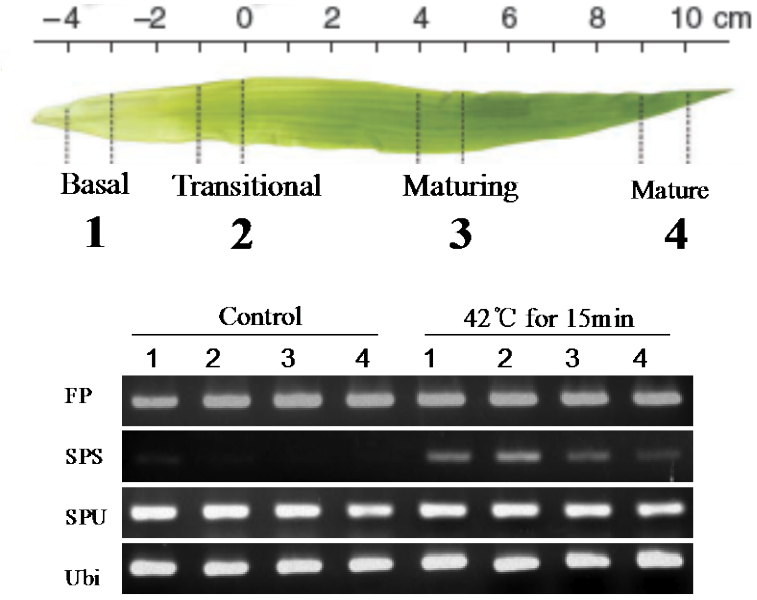
We have observed that the efficiency of splicing in response to heat stress declines during seedling development. We tested seedlings at 7 days, 2 weeks and 4 weeks and found that the RNA splicing response to ER stress declines with plant age.



ZmbZIP60 mRNA splicing during development in corn seedlings. Seedlings were heat treated (42°C) for times as indicated. Splicing was analyzed using flanking primer (FP), specific primer spliced (SPS) or specific primer unspliced (SPU) RT-PCR assays.

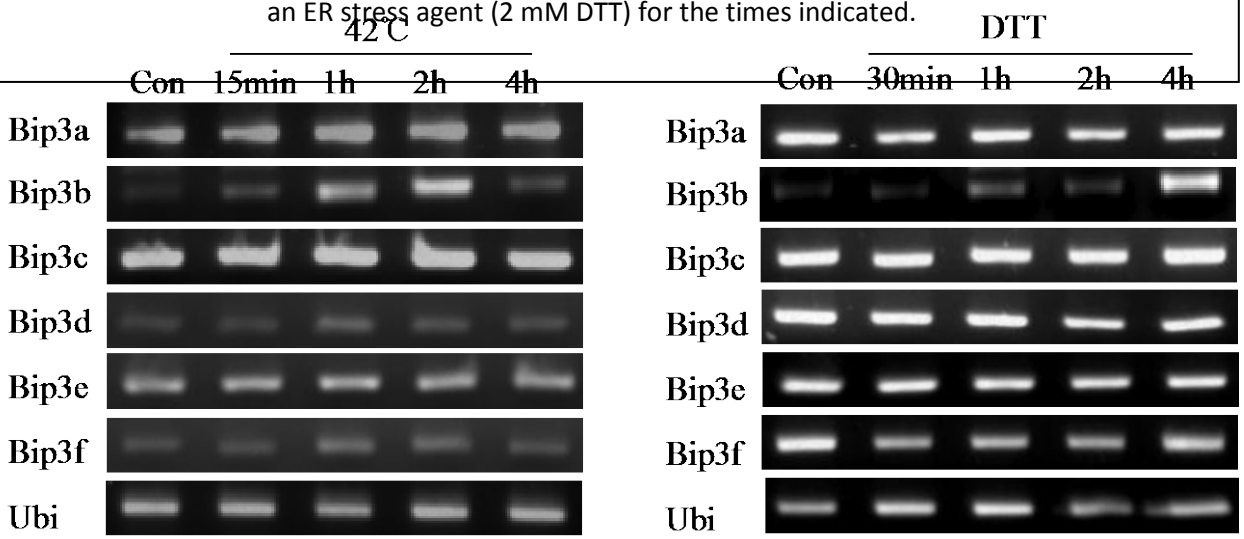
Likewise, we found that the efficiency of splicing declines from the base of the corn leaf to the tip. Maize leaves grow from their base and so there is a developmental gradient from the base of the leaf to the tip. We sampled various regions of the leaf as defined by Li et al. (2010) and found that the most robust splicing occurred in the basal and transitional regions (regions 1 and 2 in the figure below) of the leaf, where active leaf growth occurs.

Efficiency of ZmbZIP60 along the proximal distal axis of a maize leaf. Seven day-old corn seedlings were either heated treated at 42°C for 15 min or not heat treated. Segments of leaves were sampled as indicated representing different stages of leaf development. RNA from the samples were analyzed in the flanking primer or splicing specific assay for ZmbZIP60 splicing.

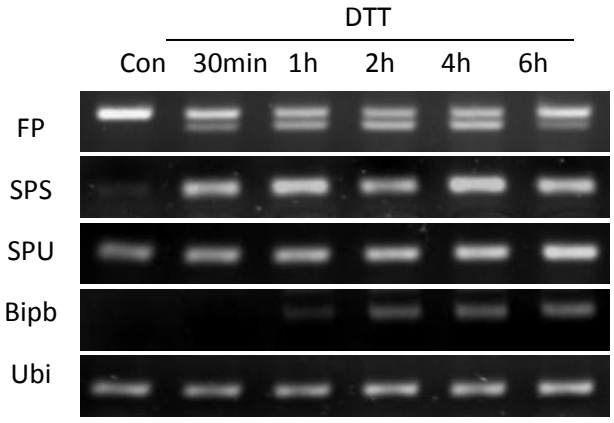


One of the key biomarkers for UPR in Arabidopsis is the upregulation of Binding Protein (BIP) gene expression. In Arabidopsis, there are three BIP genes, and BIP3, in particular, is upregulated by ER stress in manner that is largely dependent on AtbZIP60. In maize there are 22 BIP-like genes, and it is difficult to pinpoint the one(s) most similar to AtBIP3 because the Arabidopsis BIP genes are more closely related in sequence to each other than to the maize BIP-like genes. Therefore, we identified six maize BIP genes that scored highest on BLAST searches using AtBIP3 as a query sequence and tested each of those for response to ER stress. (For simplicity, we identified those genes as ZmBIPa-f.) It is clear that ZmBIPb is most highly upregulated in response to ER stress (see figure below). This is an important finding because it represents another way that we can easily monitor ER stress in corn – by assessing the level of ZmBIPb expression.

RT-PCR assay for the expression of various BIP genes. Seedlings were heat treated (42°C) or treated with an ER stress agent (2 mM DTT) for the times indicated.

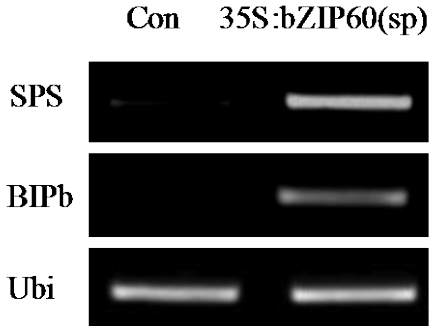


We have also observed that ZmbZIP60 mRNA is spliced when corn cell suspension cultures are subject to ER stress (see below). In this case, we used a Black Mexican Sweet cell culture line to test ER stress response.



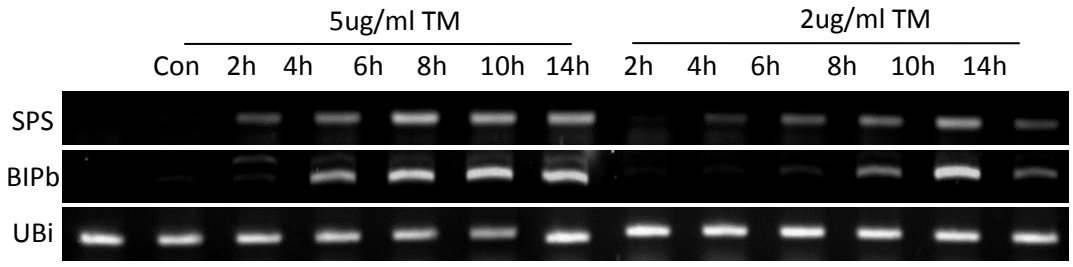
ER stress response in a maize Black Mexican Sweet cell suspension culture. 2 mM DTT was added at zero time and cell samples were taken at the times indicated. ZmbZIP60 mRNA slicing was assayed as above in FP and SPS assays. SPU detects the unspliced form. BiPb is an RT-PCR assay for an ER stress response gene. Ubi is ubiquitin, a loading control.

An advantage of the cell suspension culture system is that we can transfect protoplasts with various constructs and test them for their role in UPR signaling. In this case, we wanted to know whether BiPb was a direct target of ZmbZIP60. To test this, we transfected protoplasts with an activated form of ZmbZIP60 and showed that BiPb was upregulated under unstressed conditions.



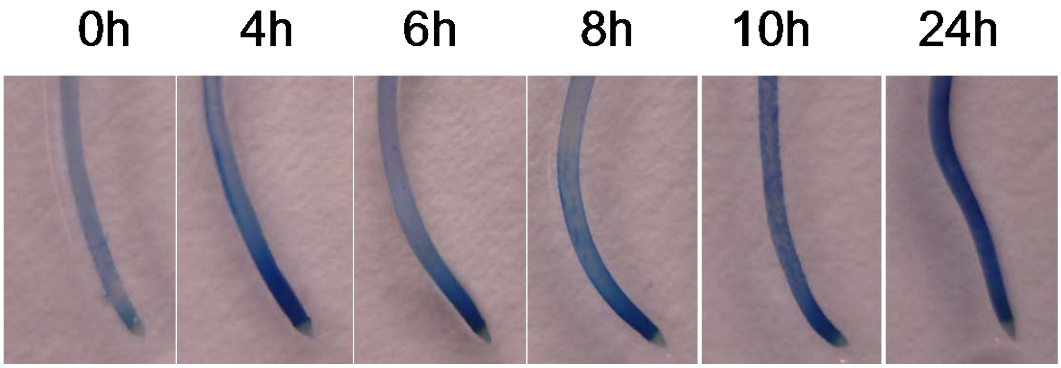
A constitutively expressed, activated form of bZIP60 (35S:bZIP60(sp)) upregulates the expression of BIPb in maize protoplasts. Mock control (Con) transfected with vector alone. RT-PCR of SPS=spliced form of bZIP60 mRNA, BiPb=RNA transcript from stress activated BiP gene in corn, Ubi=ubiquitin mRNA used as a loading control.

We have been concerned about the role of ER stress and cell survival or cell death in corn. We observed in Arabidopsis that mild ER stress induces autophagy (Liu et al., 2012), a cell sparing response, while more severe or persistent ER stress leads to cell death. We set up conditions for assessing cell survival versus cell death response to subjecting maize seedlings to different concentrations of ER stress agents, such as tunicamycin (TM). As seen the figure below, higher concentrations of TM lead to more robust ZMbZIP60 splicing activity.



Treatment of seedlings with different concentrations of tunicamycin (TM) for the purpose of eliciting cell sparing responses versus cell death. Samples were taken at the times indicated and assayed for ZmbZIP60 splicing or or Bipb upregulation.

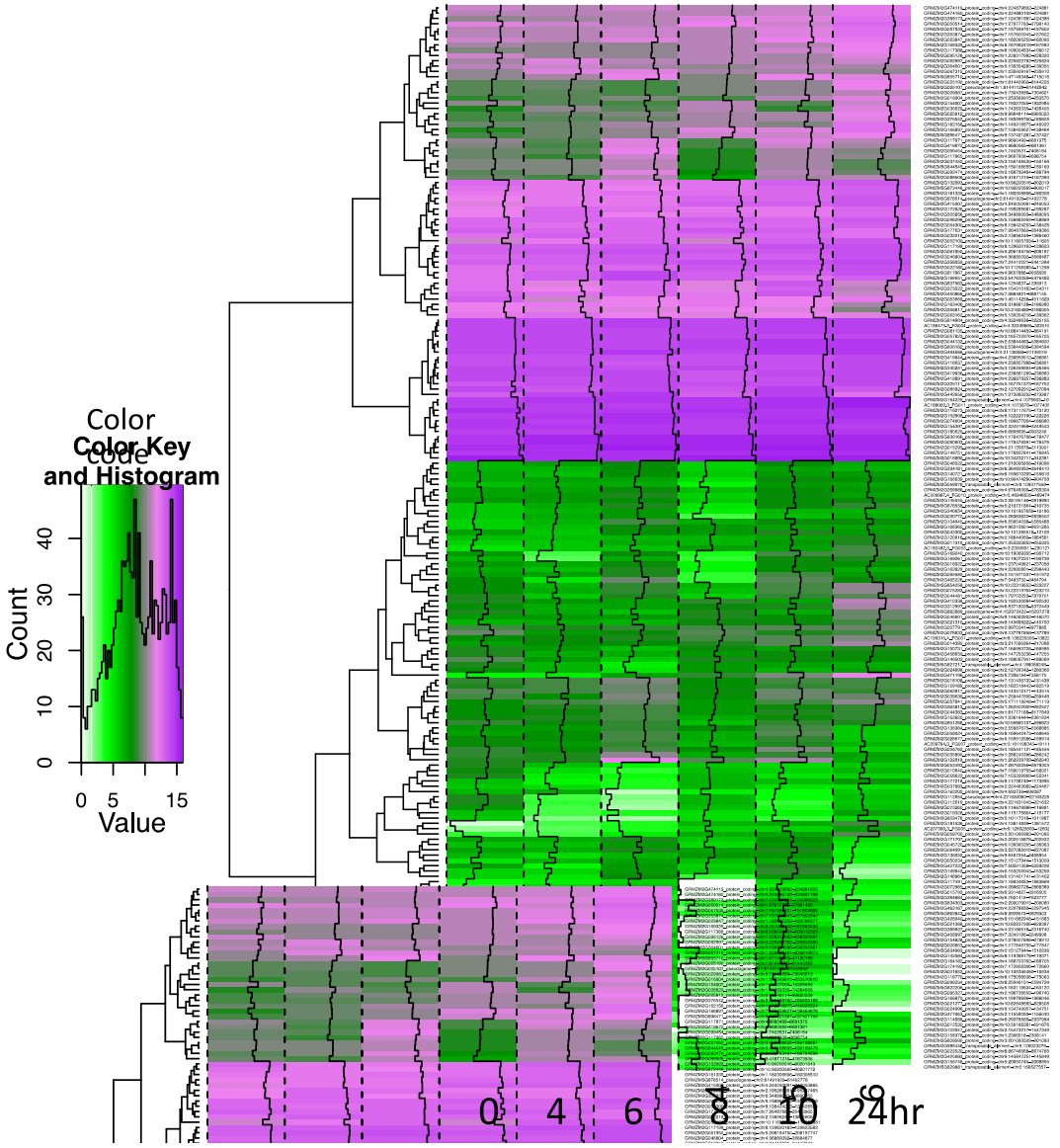
To determine whether autophagy or cell death is the fate of seedlings subjected to ER stress, we established assays for autophagy and cell death. For the cell death assay, we stained roots of stress-treated seedlings with a vital stain Evans blue (see below).



Maize seedlings were treated with 5 μg/ml of tunicamycin (TM) and stained with a vital stain (Evan trypan blue) at various times after the beginning of treatment. Living cells exclude the stain, while dead cells are stained.

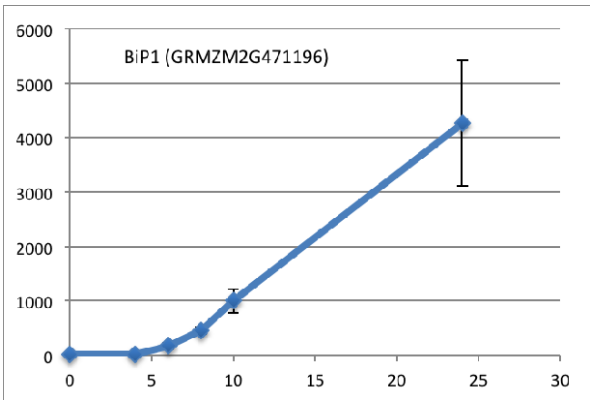
We are interested in determining how plants switch from adaptation to cell death. What program of gene expression do the seedlings follow that leads to these very different cell fates? In particular, what gene expression program unfolds during cell death? Therefore, we conducted RNA-seq analysis on seedlings at various times after treating with high levels (5 μg/ml) of tunicamycin

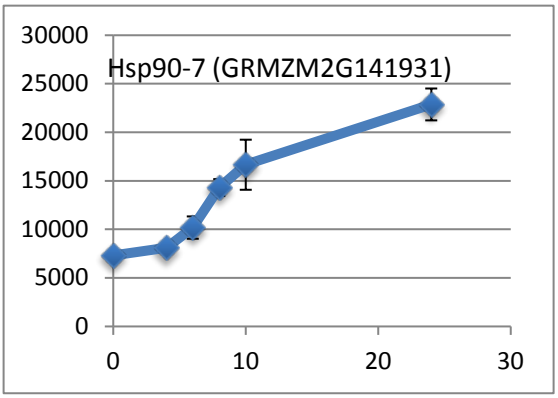
RNA was extracted from seedlings at the times indicated . cDNA libraries were generated from the RNAs and subjected to sequencing analysis on the Illumina system. The data from the time points were normalized and the 549 differentially expressed genes with a p-value cutoff of 0.2 were plotted in heat map.



Heat map of differentially expressed genes in maize seedling subjected to 5 µg/ml tunicamycin. The columns numbered 1-6 represent the time points of 0, 4, 6, 8,10 and 24hr and the rows represent genes clustered by similar patterns of expression.

The patterns of expression from the RNA seq analysis of two genes characteristic of the UPR in other organisms are shown below.





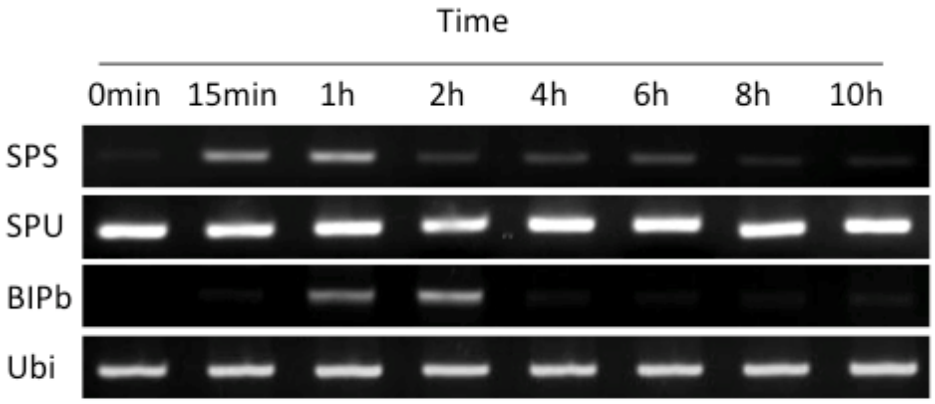
Data from the RNA seq analysis representing differentially expressed genes that are that are characteristic of the UPR in other organisms. Horizontal axis indicates time points in hours and vertical axis represents the normalized numbers of RNA transcripts derived from the indicated genes.

The RNAseq data will be further mined for other information that will help us shed light on the events that transpire during various stages of the UPR in corn seedlings. Hopefully, we will also find biomarker to that can be used as indicator of cell death.

Field studies of ER stress responses in corn

Iowa experienced one of the hottest summers on record in 2012, and we set out to measure plant responses to abiotic stress in the field during one of the hottest days. The work described above had been conducted in the laboratory usually using ER stress agents, rather than the abiotic conditions that elicit ER stress in the field.

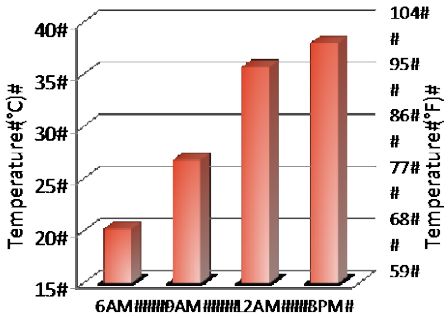
We were not sure what to expect in the field because UPR induced by heat shock in the laboratory is a transient response. When corn seedlings were subjected to heat shock (42°C) in the laboratory, both indicators of the UPR, the splicing of ZmbZIP60 mRNA (SPS) and BIPb expression, were transient. ZmbZIP60 splicing peaked at about an hour after the beginning of heat shock treatment and then subsided (see below). BIPb was induced at 1-2 hrs after the beginning of heat shock, and it too subsided thereafter. The seedlings did not die during the heat shock treatment nor was there a large-scale destruction of RNA transcripts as demonstrated by the fact that he levels of unspliced ZmbZIP60 mRNA and ubiquitin (Ubi) mRNA did not decline during this period.



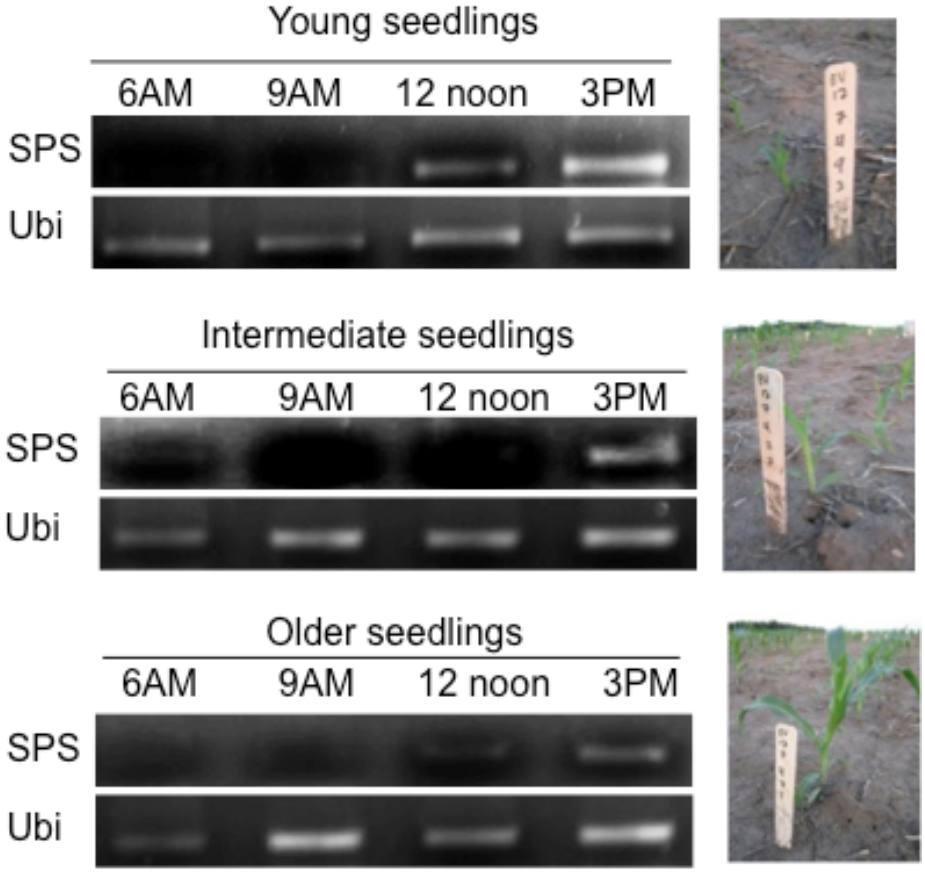
Heat shock induction of bZIP60 mRNA splicing in the laboratory. Seven-day old maize seedlings were grown at room temperature (23°C) and shifted to 42°C at zero time. RNA was extracted from seedling roots at times indicated and subjected to RT-PCR analysis. Spliced bZIP60 mRNA was detected using a specific primer spliced (SPS) assay and unspliced bZIP60 mRNA using a specific primer unspliced (SPU) assay. Binding Protein b (BIPb) and ubiquitin 5 (Ubi) were detected using conventional RT-PCR assays.

The transient pattern of the ZmbZIP60 mRNA splicing and BIPb mRNA accumulation seen in heat shock experiments in the lab would seemingly have profound effects on the UPR under field conditions. One might expect to see splicing in the field when the temperature reached a critical level, but then the levels of spliced ZmbZIP60 mRNA would rapidly diminish. In the laboratory, we had observed and previously published that the induction of ZmbZIP60 mRNA splicing and BIPb mRNA accumulation was developmental-stage dependent, in which the younger seedlings were more responsive. For the field studies, B73 corn was planted in three waves about 150 heat units apart, such that three different developmental stages could be tested at a single time. The field was irrigated with a sprinkler boom, so that drought conditions did not prevail.

A day was chosen in late June for these studies when the temperature was predicted to reach or exceed 37°C. Our strategy was to sample plants as the temperature rose during the day. Samples in triplicate were taken every three hours starting at 6AM until the temperature peaked out during the day at 3 PM. The third leaf was sampled from each of three seedlings at the three different developmental stages. In doing so, the age of the leaf reflected the age of the plant. The leaf samples were immediately frozen in liquid nitrogen in the field, and RNA was extracted from the samples in the lab.



Air temperature record in the corn field on June 27, 2012. Temperature was measured 30 cm above ground.

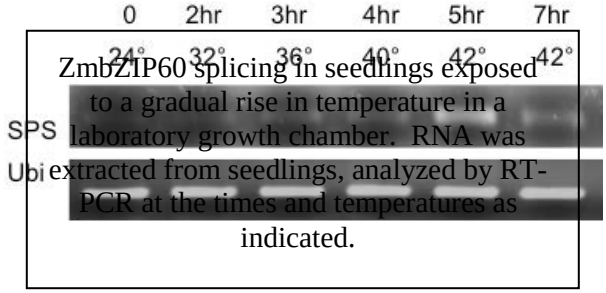


ZmbZIP60 splicing in the maize seedlings in the field. (A) The third leaves on seedlings were sampled at the times indicated, and RNA was extracted. Spliced ZmbZIP60 mRNA (SPS) and ubiquitin 5 (Ubi) were analyzed by RT-PCR assays. Young seedlings were 9 days old (234 HUs), intermediate seedlings were 16 days old (377 HUs) and older seedlings were 23 days old (527 HUs).

In the youngest seedlings (9 day-old, 234 heat units (HUs)), spliced bZIP60 was not detected in the 6AM or 9AM samples (see above). At those times, the air temperatures (measured 30 cm above the ground) were 19 and 26°C, respectively. However, at 12 noon when the air temperature reached 35°C spliced bZIP60 mRNA began to accumulate in the young seedlings. At 3 PM when the air temperature exceeded 37°C, even more spliced bZIP60 mRNA accumulated. In the intermediate age seedlings (16 days, 366 HUs), spliced bZIP60 mRNA was not detected until the hottest time of day, 3 PM. At that time, spliced bZIP60 mRNA reached about half the level as the young seedlings. Similarly, spliced bZIP60 mRNA was not observed until 3 PM in the oldest seedlings (23 days, 527 HUs). At that time the levels of spliced bZIP60 mRNA were even lower than in the intermediate seedlings. Therefore, UPR is elicited in maize seedlings in the field during the hottest times of day on days when temperatures reach or exceed 35°C. Furthermore, the youngest leaves (i.e., the third leaf on the youngest plants) showed the greatest response.

This study demonstrates for the first time that the UPR in plants which, to date, had only been studied in the laboratory, occurs in field conditions. In the lab, the UPR is typically induced by ER stress agents, such as tunicamycin and DTT. However, these agents are only proxies for more natural environmental conditions in the field. In these experiments, the UPR was induced in young seedlings starting around noon when air temperature reached 35°C, and extended into mid afternoon, the hottest time of the day when the temperatures measured at 12 in above the ground reached 38.5°C.

So, why was it that that the ER stress response to heat shock was so brief in the lab, but sustained during the afternoon in the field? Of course the heat shock treatment in the lab differs from the conditions in the field. On a hot summer day, temperatures rise slowly during the day, while in the laboratory seedlings tested for ER stress responses were subjected to a sudden heat shock. To determine whether the gradual rise in temperature during the day helped to sustain the UPR, we simulated a gradual rise in temperature for seedlings in growth chamber conditions. Temperatures were ramped up at 1.5°C per ½ hr starting at the beginning of the light period in a 16 hr light:8 hr dark cycle and reaching 42°C 4 ½ hr later. We began to observe bZIP60 splicing starting at about 3 hr (36°C) and reaching a peak at 5 hr at maximum temperature (42°C) (see below). Spliced bZIP60 mRNA levels did decline somewhat at 7 hr and beyond while holding the seedlings at 42°C. Nonetheless, we concluded that bZIP60 mRNA splicing did not quickly attenuate after spliced RNA began to appear at about 3 hr. Instead, the levels of spliced RNA keep rising until the maximum temperature (42°) was reached. The levels of spliced RNA were not sustained at the highest temperature, nonetheless, the temperature ramping experiment does suggest that the gradual rise in field temperature may suppress the rapid attenuation of bZIP60 mRNA accumulation seen in the laboratory in response to heat shock. Thus, the acquisition of thermotolerance by gradually ramping up the temperature may explain why maize seedlings continue to accumulate more bZIP60 mRNA until midafternoon on a hot day.



ZmbZIP60 splicing in seedlings exposed to a gradual rise in temperature in a laboratory growth chamber. RNA was extracted from seedlings, analyzed by RT-PCR at the times and temperatures as indicated.

We conclude from this analysis that the ER stress response is a real phenomenon that happens to corn seedlings in the field. We do not yet have data in corn on the extent to which this stress response protects plants from heat stress. However, we do have data from Arabidopsis in which we can disable the UPR with mutants, and there we have found that the ER stress response has profound effects on conferring ER stress tolerance in seedlings.

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Deng, Y., Humbert, S., Liu, J.X., Srivastava, R., Rothstein, S.J., and Howell, S.H. (2011). Heat induces the splicing by IRE1 of a mRNA encoding a transcription factor involved in the unfolded protein response in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **108,** 7247-7252.

Howell, S.H. (2013). ER stress responses in plants. Ann. Rev. Plant Biol. **64**.

Li, P., Ponnala, L., Gandotra, N., Wang, L., Si, Y., Tausta, S.L., Kebrom, T.H., Provart, N., Patel, R., Myers, C.R., Reidel, E.J., Turgeon, R., Liu, P., Sun, Q., Nelson, T., and Brutnell, T.P. (2010). The developmental dynamics of the maize leaf transcriptome. Nat Genet **42,** 1060-1067.

Li, Y., Humbert, S., and Howell, S.H. (2012). ZmbZIP60 mRNA is spliced in maize in response to ER stress. BMC Res Notes **5,** 144.

Liu, Y., Burgos, J.S., Deng, Y., Srivastava, R., Howell, S.H., and Bassham, D.C. (2012). Degradation of the endoplasmic reticulum by autophagy during endoplasmic reticulum stress in Arabidopsis. The Plant cell.

Scientific Progress Report

Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.

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University:	North Carolina State University

DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026**--335	
Project Title:	*"Low cost conversion of industrial papermaking sludges to ethanol"*, CPBR Agreement GO12026-335	
Reporting Period:	**From: 1/1/11**	**To: 3/31/2015**
Report Type:	**Check one:** [ ] Semiannual Report [x] Final Report	

Project Objectives

List each objective of the Project and the progress made toward each one during the reporting period.

Objectives:

1. Characterize the local sources of sludge available. Milestone: full report on the virgin paper mill and recycled paper mill sludges.
2. Determine the efficiency of several operations to produce MFC material free from inorganic filler. Milestone: full report on the performance of each of the identified separation mechanisms. Identification of a process with greater than 80% separation efficiency of inorganics from carbohydrates.
3. Determine the effect of inorganic loading and microgrinding on production of films from sludge materials. Milestone: full report on the effect of inorganic loading on properties of film material. Identification of a separation process, grinding process to make a film that has at least 80% of the performance of a MFC film from virgin micro grinded pulp.
4. Determine the effect of additives on the barrier and physical properties of the MFC materials developed from sludge. Milestone: full report on the effect of additive loading on film performance. Identification of film with 50% of the moisture barrier properties of a suitable polyolefin film.
5. Determine the effect of inorganic material loading levels on the ethanol production process. Milestone: full report on the effect of inorganic loading on ethanol production efficiency. Ability to show that an identified fractionation process is capable of producing an ethanol conversion of 90% of theoretical.
6. Define the economic benefit of using sludge to produce films, ethanol and soil conditioner. Excel spreadsheet produced. Report identifying the net profit based on a three dimensional set of coordinates including % ethanol, % film, and % fertilizer.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

The objective of this project is to develop an effective process that takes papermaking sludge and separates the carbohydrate fraction from the inorganic fraction in order to use the carbohydrate fraction for biofuel or biomaterials and the inorganic fraction as a soil amendment. We have determined that the sludge to ethanol process using fractionation to remove ash produces a better economic return when compared to a non- fractionated process for several sludges. The project has been completed and this is the final report. Several peer reviewed manuscripts have been published documenting the research.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

Different types of waste paper materials were studied in this research in terms of their feasibility for fermentable sugar production via enzymatic hydrolysis. This was done as a simple way to understand factors that will affect papermaking sludge conversion to sugars. Low enzyme dose on recovered office paper was investigated. Ash removal was identified to be necessary since both acid soluble and acid insoluble ash adsorbed enzyme during enzymatic hydrolysis. This ash-enzyme interaction was proven to have higher affinity than cellulose-enzyme interaction. The effect of hornification - irreversible pore collapse in lignocellulosic fibers was also studied. Mechanical refining by a PFI mill of previously dried fibers improved sugar recovery to similar or higher levels as never dried fibers.

Paper sludge is another attractive biomass source for the conversion to ethanol. A mechanical fractionation process was proposed in order to remove ash from sludge prior to enzymatic hydrolysis. This process removed 82-98% of the ash with fiber yields from 39-69%. Fractionation efficiency was also evaluated by testing different size mesh screen openings, aiming to optimize this fractionation process. The ash rich streams had a lower C:N ratio than the original sludge, which improved its suitability as soil amendment.

Process simulation using engineering process simulation software WinGEMS and financial analysis on the feasibility of the process developed in Chapter 3 were conducted. The financial impact of the addition of the sludge fractionation step was discussed based on using sludge from virgin and recycled paper mills. The most profitable case was fractionated virgin sludge (from a virgin paper mill) to ethanol (F-VK1) with a net present value (NPV) of US$ 11.4 million, internal rate of return (IRR) of 28%, payback period of 4.4 years and minimum ethanol revenue (MER) of US$ 0.32 per liter. Risk analysis showed that the F-VK1 case obtained a near 100% probability of business success with both optimistic and pessimistic assumptions.

Newspaper contains high lignin content among various waste paper materials. As a model of wastepaper and deinking sludges, research focused on developing a pretreatment process ideal for newspaper saccharification was conducted. The effects of non-ionic surfactant and flexo link were also studied. Tween 80 improved sugar conversion of newspaper and flexo ink was proven to have no inhibition effects on enzymatic hydrolysis. Pretreatment including autohydrolysis, mechanical refining, oxygen, alkaline and green liquor (GL) pretreatments were evaluated on newspaper. Except mechanical refining and oxygen pretreatment, all the other pretreatment methods adversely affected enzymatic hydrolysis of newspaper. It was presumably due to the pore collapse in the fibers during the pretreatment process.

Publications and Presentations

List all talks, posters, scientific publications, news releases, etc., about this Project during the reporting period. Provide one copy of each publication, report, or news article resulting from activities supported under the grant as well as any announcements, press releases, statements or photographs depicting Project activities.

Enzymatic Hydrolysis of Pretreated Newspaper Having High Lignin Content for Bioethanol Production, Hui Chen, Qiang Han, Richard A. Venditti,* and Hasan Jameel, BioResources, 10(3), 4077-4098, 2015.

Conversion of Industrial Paper Sludge to Ethanol: Fractionation of Sludge and Its Impact, Hui Chen, Qiang Han, Kevin Daniel, Richard Venditti, Hasan Jameel, Appl Biochem Biotechnol 174, 2096-2113, 2014.

Economic evaluation of the conversion of industrial paper sludge to ethanol, H. Chen, R. Venditti, R. Gonzalez, R. Phillips, H. Jameel, S. Park, Energy Economics, 44, 281-290, 2014.

Enzymatic hydrolysis of recovered office printing paper with low enzyme dosages to produce fermentable sugars, Hui Chen, Richard A. Venditti, Hasan Jameel, Sunkyu Park, Appl. Biochem. Biotechnol, 166, 1121-1136, 2012.

Process Development and Fundamental Study on Enzymatic Hydrolysis of Cellulosic Biomass to Fermentable Sugars for Ethanol Production by Hui Chen. Doctoral Thesis, NCSU, 2014.

EXPLORING THE POTENTIAL OF PAPER INDUSTRY SLUDGES FOR ETHANOL PRODUCTION
Hui Chen, Ronalds Gonzalez, Richard Phillips, Richard Venditti *, Hasan Jameel, Sunkyu Park, ICPPB 2012.

Technology Transfer

Describe all technology transfer (inventions, disclosures, patents, licensing agreements, etc.) resulting from the Project during the reporting period.

None.

Commercial Accomplishments

Describe the most significant accomplishments resulting from the Project during the reporting period.

None.

Educational Accomplishments

Describe the most significant educational accomplishments resulting from the Project during the reporting period. (Number of students, post-docs, etc. trained)

We have trained two graduate students and one undergraduate student.

Additional Funding

List any additional funding generated as a result of the Project during the reporting period.

We received a $5000 grant from NCSU to do research in the same area, in 2014.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

None.

Scientific Progress Report

Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.

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University:	University of Nebraska-Lincoln	
DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026-**	
Project Title:	*Cellulose Degrading Enzymes from Western Corn Rootworm Larvae*	
Reporting Period:	**From: Sept. 1, 2010**	**To: June 30, 2015**
Report Type:	**Check one:** [X] Semiannual [ ] Final Report	

Project Objectives

List each objective of the Project and the progress made toward each one during the reporting period.

1. *Characterize the transcriptome of the rootworm larval midgut.* Analysis of the larval rootworm midgut transcriptome to identify potential biological and molecular functions of midgut specific genes and specifically to identify genes important to digestion of plant cell walls has been completed. We have completed analysis of all glycosyl hydrolase enzymes and a manuscript has been published that summarizes the work.

   We have also tested an *E. coli*-based cell-free extract to perform in vitro translation of an endogenous β-1, 4-endoglucanase (Dvv-ENGase I) gene from the western corn rootworm beetle, *Diabrotica virgifera virgifera*, belonging to the glycoside hydrolase family (GHF) 45. The cDNA encoding Dvv-ENGase I was expressed as a 27-kDa polypeptide. Recombinant Dvv-ENGase I protein tagged with 6 poly Histidines was purified in one simple and single step via magnetocapture using Ni-based magnetic beads. The recombinant Dvv-ENGase I protein exhibited enzymatic activity not only on hydroxyethyl-cellulose **(**HEC), a nonionic and water-soluble cellulose polymer but also toward the fluorogenic 4-methylumbelliferyl-beta-D-glucopyranoside (4-MU-glucopyranoside). The applicability of *E. coli*-based cell-free expression system to the assembly of some insect genes indicates that it is possible obtain efficient and coupled transcription and translation of recombinant protein in a short period of time providing enough functionally active proteins for an array of downstream applications. A manuscript describing this work has been submitted for publication and is still under review.

   In addition to the genes involved in cell wall metabolism, we have been actively searching the transcriptome sequences for other gene classes that may provide novel target sites for rootworm pest management. Specifically we have identified a number of genes that are involved with chemical perception including both odorant receptors and gustatory receptors. Thus far, we have identified 11 unique odorant receptors (OR) and 53 gustatory receptors (GR). Disruption of these genes through RNA interference (RNAi) or design of chemicals that mimic specific chemical ligands may provide a novel and specific pest management approach. Our initial efforts have been with the gustatory receptors (GR) involved in perception of carbon dioxide. Importantly, CO_2 perception has been identified as an important host-finding cue for neonate rootworm larvae. We have also been optimizing methods for RNAi knockdown of these receptor genes and developing reliable bioassay methods to measure response to CO_2 in order to functionally validate the function of these receptors. Initial attempts have involved injection of gravid adult females to achieve silencing in neonates as they hatch. We have also been exposing neonate larvae to dsRNA treated artificial diet. Both methods have achieved gene knockdown for other genes and we anticipate that one of these methods will be suitable for these receptor genes. A manuscript identifying three gustatory receptor genes and their relative expression among different tissues and developmental stages has been submitted for publication.

   To further increase coverage and the potential to identify of genes involved with chemosensing, we have completed sequencing of different tissues from both larvae and adults. A transcriptome of the heads of neonate larvae has recently been completed. Sensory organs from the heads of neonate larvae are likely to express those genes associated with perception of environmental cues. In addition, we have sequenced transcriptomes from both male and female adult antennae, which should be specifically adapted to chemical perceptions and will provide an opportunity to compare

the expression with neonates so that we can more accurately identify those genes associated with host-finding. Summary statistics for the additional transcriptome sequencing follows.

Table 1. Summary statistics for Illumina HiSeq 2500, paired end transcriptome sequencing to identify genes associated with chemosensory receptors.

Statistic	Larval Head Capsule	Adult Male Antennae	Adult Female Antennae
Total Reads After Filtering	416194544	507246422	492960278
Total base pairs	$4.1x10^{10}$	$5.0x10^{10}$	$4.9x10^{10}$
Total Contigs after assembly	139096	141258	125675
Average Length	1057	1025	1261

Additional bioinformatics work is ongoing to provide annotation and to identify additional genes that are important to corn rootworm in host find and that will provide potential target for developing sustainable pest management approaches.

A second transcriptome from the closely related southern corn rootworm *Diabrotica undecimpuntata howardi* is also currently being analyzed. This species is important in that it shares many biological attributes of the western corn rootworm but has a much broader larval host range. Because rootworms are considered a possible threat to crops grown as potential biomass feedstocks, we believe that comparisons of the transcriptomes from the two species will provide important insight into the potential risks posed to biomass crops. We have completed sequencing of transcriptome representing all relevant life stages of this species. The sequences were assembled using previously developed methods and the final assembly of 191.2×10^6 total reads resulted in 148,923 contigs with an N50 of 4248 bp. We are currently attempting to determine the differences between the two species in their ability to identify and develop on alternative host plants and the effects on gene expression.

The transcriptome data for western corn rootworms generated from our project has been made publically available and has been used by a variety of collaborators including:

University of Illinois; Differential gene expression an adult rotation-resistant and wild-type western corn rootworm larvae.
University of Tennessee; Proteomic analysis of Bt toxin receptors in western corn rootworm larvae.
University of Nebraska and Iowa State University; RNASeq analysis of Cry3Bb1 resitance in western corn rootworms.
University of Illinois and University of Nebraska; Sequencing a potential underground pest of biofuel crops.

Through an Industry Partnership Grant from the University of Nebraska Lincoln we have established a project with a corporate sponsor in which we have been using our transcriptome to identify potential target sequences for silencing by RNA interference. This project has resulted in a number of possible target sequences that result in mortality of both larval and adults when dsRNA is ingested and that may be exploited for developing transgenic maize plants for corn rootworm pest management

2. *Characterize the gut microbial communities associated with western corn rootworm larvae.*
Microorganisms that inhabit insect guts play important roles in host nutrition, development, reproduction, and resistance to pathogens. Moreover, changes or loss of these microorganisms can dramatically impact fitness of the host, its pest status and susceptibility to toxins that specifically target the insect. Digestive symbioses appear to be common among insect herbivores, especially those that feed on highly lignified plant materials such as the western corn rootworm. In spite of their

apparent importance and evolutionary significance, symbiotic associations remain unexplored for most insects, including many of the largest and economically important families. A better understanding of insect-microbe interactions may lead to new strategies to reduce the effects of these pest species and as a resource for identifying enzymes of commercial significance.

We have successfully completed sequencing the metagenome of western corn rootworm larvae that have been either starved or fed with corn roots as well as similar comparisons for the closely related southern corn rootworms. Approximately, 100 3rd instars of non-diapause western corn rootworms were purchased from a commercial vendor and held in 100x15 mm Petri dish (Fisher) with moisturized filter paper in the absence of food. An additional treatment involved providing larvae with fresh corn roots. The petri dishes were kept in lab at room temperature. After 24 hrs, the midgut was dissected under magnification and midgut contents were collected with borosilicate glass capillaries (World precision instrument) and saved in a 1.5 ml centrifuge tube on dry ice. Approximately 100 3rd instar larvae were dissected for contents from both treatments.

Total DNA was extracted from midgut contents and the quality and quantity was evaluated on 1% agros gel and NanoDrop-1000. The DNA samples were submitted to the Center for Biotechnology-University of Nebraska-Lincoln for NGS analysis. The shotgun library for each DNA sample was prepared with Ion Torrent library prep kit (Life Tech) and next generation sequencing conducted for 400 bp runs using Ion torrent (Life Tech). The read data generated from the Ion Torrent sequencing were first used for assembling with Ray Meta (version 2.3) software using default settings. The assembled data (fasta) were further annotated and analyzed with a web-based metagenomics analysis tool (http://metagenomics.anl.gov/) using default settings.

Initial analysis of the WCR metagenomes indicates that the majority of species identified were bacteria (>80% for both fed and starved treatments) Fig. 1.

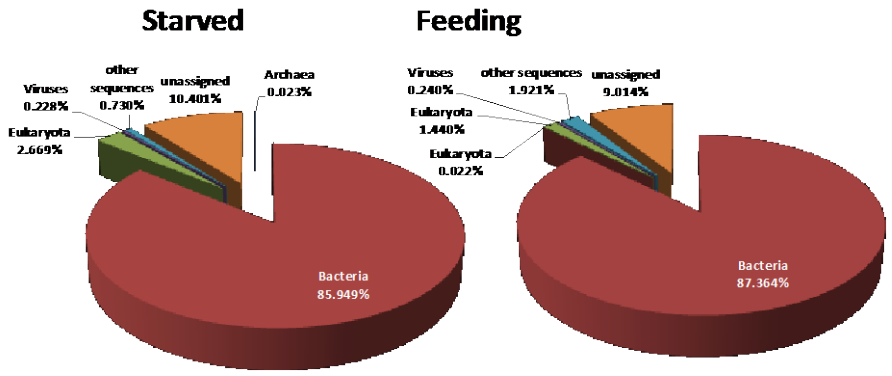


Figure 1. Distribution of western corn rootworm gut metagenome sequences among different phyla from both starved and feeding 3rd instars.

Additional analyses of the gut metagenome from both western and western corn rootworms are currently in progress to better understand the role that these organisms play in digestive physiology and as possible tools that be exploited for sustainable pest management systems.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

The most significant accomplishments of the project involve completing the sequencing of the western corn rootworm midgut transcriptome. We have increased significantly the number of genes that that have been identified, and have uncovered whole families of plant cell wall degrading enzymes not previously identified. From this effort, over 30,000 unique sequences have been identified from which we hope to identify enzymes with unique properties that are specifically adapted to digest biomass with the potential for increased efficiency of biomass conversion to ethanol and may provide insight into novel control strategies for this important pest species. These sequences also provide important information that allow identification of novel mechanisms to control this economically important pest species.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

This project was initiated in 2010 with matching resources from our corporate partner, Pioneer Hi-Bred International. Significant progress has been made with this funding on both objectives. Please see progress on objectives (above) for specific information on accomplishments.

Publications and Presentations

List all talks, posters, scientific publications, news releases, etc., about this Project during the reporting period. Provide one copy of each publication, report, or news article resulting from activities supported under the grant as well as any announcements, press releases, statements or photographs depicting Project activities.

1. *A manuscript summarizing the identification and phylogenetic analysis of glycolsyl hydrolases has been published in PLoS One.*

Euyn, S., H. Wang, Y. Pauchet, R.H. ffrench-Constant, E.N. Moriyama, and B.D. Siegfried. 2014. Molecular evolution of glycoside hydrolase genes in the western corn rootworm. PLoS ONE 9: e94052. doi: 10.1371/journal.pone.0094052.

2. A second manuscript describing the recombinant expression of GHF45 has been published in Journal of Insect Science.

Valencia, A., H. Wang, and B.D. Siegfried. *2014.* Expression and characterization of a recombinant endoglucanase from the western corn rootworm, *Diabrotica virgifera virgifera* in *Pichia pastoris*. *J. Insect Science*. (*In press*)

3. A manuscript describing the role of horizontal gene transfer in coleopterans has been published.

Kirsch, R., L. Gramzow, G. Thiessen, B.D. Siegfried, R.H. ffrench-Constant, D.G. Heckel, Y. Pauchet. Horizontal gene transfer and functional diversification of plant cell wall degrading polygalacturonases: Key Events in the Evolution of Herbivory in Beetles. Insect Biochem. Molec. Biol. 52: 33-50.

4. A manuscript describing the cell-free expression and characterization of a western corn rootworm endoglucanase has been submitted for publication.

Valencia, A. H. Wang, and B.D. Siegfried. Expression and purification of an insect endoglucanase using an *E. coli*-based cell-free system. Protein Express. Purif. (*Submitted*).

5. A manuscript describing genes involved in the RNA interference pathway have been submitted for publication.

Khajuria, C., K. Narva, and B.D. Siegfried. Functional analysis of four RNAi pathway genes in an economically important corn pest, Western corn rootworm (*Diabrotica virgifera virgifera* Le Conte). PLoS One (In revision).

6. A manuscript has been submitted describing two gene involved in embryonic development that can be effected by parental RNAi.

Khajuria, C., A.M. Velez, H. Wang, E.Fishilevich, M.L. Frey, N. Carneiro, P. Gandra, K.P. Narva. and **B.D. Siegfried**. Parental RNA interference of genes involved in embryonic development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Insect Biochem. Molec. Biol. (In revision)

7. A manuscript describing housekeeping genes for quantitative RT-PCR standardization has been published.

Rodrigues, T.B., C. Kahjuria, H. Wang, N. Matz, D.C. Cardoso, and B.D. Siegfried. 2014. Validation of reference housekeeping genes for gene expression studies in the western corn rootworm (*Diabrotica virgifera virgifera*). PLoS One 9(10):e109825. doi: 10.1371/journal.pone.0109825.

8. A manuscript describing the identification and expression of three carbon dioxide receptor genes has been submitted for publication.

Rodrigues, T.B., E.N. Moriyama, H. Wang, C. Khajuria, and B.D. Siegfried. Carbon dioxide receptor genes and their expression profile in *Diabrotica virgifera virgifera.* BMC Genomics (*Submitted*)

PDF copies of published manuscripts are attached

Invited Presentations

DATE	AUDIENCE	TITLE	LOCATION
December 2011	Entomological Society of America; Section Symposium, State of the art Molecular Research of Global Interest	Developing RNA interference as a tool for target site discovery in western corn rootworms	Reno, NV
March 2012	Colorado State University, Department of Crop and Soil Sciences	Developing sustainable approaches to corn insect pest management	Fort Collins, CO
October 2012	Monsanto Corn Rootworm Academic Summit	Developing genomic tools for sustainable rootworm pest management	St. Louis, MO
November 2012	University of Illinois	New technologies for management of corn rootworms	Champaign-Urbana, IL
November 2012	Entomological Society of America, Annual Meeting	Developing methods for RNAi interference in western corn rootworms	Knoxville, TN
April 2013	Bayer Crop Sciences	Developing sustainable approaches to corn insect pest management	Raleigh, NC

DATE	AUDIENCE	TITLE	LOCATION
June 2013	IOBC/WPRS Working Group; 6th Meeting on Ecological Impact of GMO's	Keynote Address: RNA interference in pest management: Opportunities and potential risks	Berlin, Germany
November 2013	Entomological Society of America, Annual Meeting	Rootworm transcriptomes and RNAi: Understanding Gene Function and Identifying Novel Target Sites	Austin, TX
March 2015	Department of Entomology, University of Georgia	A 30 year partnership with Western corn rootworms: Updates on new technologies and resistance evolution	Athens, GA
April 2015	Department of Plant Sciences, University of Missouri	A 30 year partnership with Western corn rootworms: Updates on new technologies and resistance evolution	Columbia, MO

Technology Transfer

Describe all technology transfer (inventions, disclosures, patents, licensing agreements, etc.) resulting from the Project during the reporting period.

A number of disclosures have been submitted to the University of Nebraska Office of Research and Economic Development related to RNAi interference and rootworm pest management.

Commercial Accomplishments

Describe the most significant accomplishments resulting from the Project during the reporting period.

While commercial outcomes related to identification of enzymes important to cellulosic ethanol production have yet to be realized, we have made significant accomplishments related to the potential for RNAi as a pest management tool. These accomplishments would not have been feasible without the genomic resources that were made available through CPBR funding.

Educational Accomplishments

Describe the most significant educational accomplishments resulting from the Project during the reporting period. (Number of students, post-docs, etc. trained)

Dr. Arnubio Valencia has made several visits to Nebraska to work on characterization of cellulosed degrading enzymes. He also received advanced training in bioinformatics that he has been able to use in his own research program at his home institution in Colombia.

Dr. Seong-il Eyun received his Ph.D. in bioinformatics using research funded by this project.

Dr. Thais Rodrigues was a visiting graduate student who worked in our laboratory for 12 months and was supported through a Brazilian fellowship. Her research involved identifying and characterizing CO_2 receptor genes identified from our transcriptome.

Numerous undergraduate and graduate students working on this and related projects received advance training in entomology, molecular biology, and bioinformatics.

Additional Funding

List any additional funding generated as a result of the Project during the reporting period.

A proposal to the USDA Biotechnology Risk Assessment Grant Program has been funded that utilizes the transcriptome resources generated from this project to conduct expression profiling of Bt resistant and susceptible strains of western corn rootworms and to potentially identify molecular markers for resistance. We have also received additional support the Nebraska Corn Board to investigate the potential for CO_2 receptors to serve as viable targets for pest management. A proposal to the Monsanto Corn Rootworm Knowledge Program to investigate pyrethroid resistance in western corn rootworms that will utilize our transcriptome resources. We have also received funding through the USDA NIFA program on Bioenergy to identify changes in gene expression associated with alternative host plant usage.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

We currently have no personnel supported through CPBR funding. All remaining funding has been used for further sequencing efforts.

Scientific Progress Report

Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.

Principal Investigator Name(s) and Contact Information: **(Title, Mailing Address, Phone, Fax, Email)**	Richard Venditti, Professor - Dept of Forest Biomaterials, North Carolina State University, Biltmore Hall Rm 1204, 2820 Faucette Drive, Raleigh NC 27695-8005, (919) 515-6185, richard_venditti@ncsu.edu, http://www4.ncsu.edu/unity/users/r/richardv/www/ Dr. Hasan Jameel, Co-PI Professor Address same as above Office: 919-515-7739 Email: jameel@nsu.edu Dr. Sunkyu Park, Co-PI Assistant Professor, Address same as above Office: 919-515-0473 Email: sunkyu_park@ncsu.edu Dr. Joel Pawlak, Co-PI Associate Professor Address same as above Office: 919-513-0511 Email: joel_pawlak@ncsu.edu
University:	North Carolina State University
DOE Prime Agreement:	**DE-FG36-02GO12026**
Agreement Number:	**GO12026**--335
Project Title:	*"Low cost conversion of industrial papermaking sludges to ethanol"*, CPBR Agreement GO12026-335
Reporting Period:	**From: 1/1/11** **To: 3/31/2015**
Report Type:	**Check one:** [ ] Semiannual Report [x] Final Report

Project Objectives

List each objective of the Project and the progress made toward each one during the reporting period.

Objectives:

7. Characterize the local sources of sludge available. Milestone: full report on the virgin paper mill and recycled paper mill sludges.
8. Determine the efficiency of several operations to produce MFC material free from inorganic filler. Milestone: full report on the performance of each of the identified separation mechanisms. Identification of a process with greater than 80% separation efficiency of inorganics from carbohydrates.
9. Determine the effect of inorganic loading and microgrinding on production of films from sludge materials. Milestone: full report on the effect of inorganic loading on properties of film material. Identification of a separation process, grinding process to make a film that has at least 80% of the performance of a MFC film from virgin micro grinded pulp.
10. Determine the effect of additives on the barrier and physical properties of the MFC materials developed from sludge. Milestone: full report on the effect of additive loading on film performance. Identification of film with 50% of the moisture barrier properties of a suitable polyolefin film.
11. Determine the effect of inorganic material loading levels on the ethanol production process. Milestone: full report on the effect of inorganic loading on ethanol production efficiency. Ability to show that an identified fractionation process is capable of producing an ethanol conversion of 90% of theoretical.
12. Define the economic benefit of using sludge to produce films, ethanol and soil conditioner. Excel spreadsheet produced. Report identifying the net profit based on a three dimensional set of coordinates including % ethanol, % film, and % fertilizer.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

The objective of this project is to develop an effective process that takes papermaking sludge and separates the carbohydrate fraction from the inorganic fraction in order to use the carbohydrate fraction for biofuel or biomaterials and the inorganic fraction as a soil amendment. We have determined that the sludge to ethanol process using fractionation to remove ash produces a better economic return when compared to a non- fractionated process for several sludges. The project has been completed and this is the final report. Several peer reviewed manuscripts have been published documenting the research.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

Different types of waste paper materials were studied in this research in terms of their feasibility for fermentable sugar production via enzymatic hydrolysis. This was done as a simple way to understand factors that will affect papermaking sludge conversion to sugars. Low enzyme dose on recovered office paper was investigated. Ash removal was identified to be necessary since both acid soluble and acid insoluble ash adsorbed enzyme during enzymatic hydrolysis. This ash-enzyme interaction was proven to have higher affinity than cellulose-enzyme interaction. The effect of hornification - irreversible pore collapse in lignocellulosic fibers was also studied. Mechanical refining by a PFI mill of previously dried fibers improved sugar recovery to similar or higher levels as never dried fibers.

Paper sludge is another attractive biomass source for the conversion to ethanol. A mechanical fractionation process was proposed in order to remove ash from sludge prior to enzymatic hydrolysis. This process removed 82-98% of the ash with fiber yields from 39-69%. Fractionation efficiency was also evaluated by testing different size mesh screen openings, aiming to optimize this fractionation process. The ash rich streams had a lower C:N ratio than the original sludge, which improved its suitability as soil amendment.

Process simulation using engineering process simulation software WinGEMS and financial analysis on the feasibility of the process developed in Chapter 3 were conducted. The financial impact of the addition of the sludge fractionation step was discussed based on using sludge from virgin and recycled paper mills. The most profitable case was fractionated virgin sludge (from a virgin paper mill) to ethanol (F-VK1) with a net present value (NPV) of US$ 11.4 million, internal rate of return (IRR) of 28%, payback period of 4.4 years and minimum ethanol revenue (MER) of US$ 0.32 per liter. Risk analysis showed that the F-VK1 case obtained a near 100% probability of business success with both optimistic and pessimistic assumptions.

Newspaper contains high lignin content among various waste paper materials. As a model of wastepaper and deinking sludges, research focused on developing a pretreatment process ideal for newspaper saccharification was conducted. The effects of non-ionic surfactant and flexo link were also studied. Tween 80 improved sugar conversion of newspaper and flexo ink was proven to have no inhibition effects on enzymatic hydrolysis. Pretreatment including autohydrolysis, mechanical refining, oxygen, alkaline and green liquor (GL) pretreatments were evaluated on newspaper. Except mechanical refining and oxygen pretreatment, all the other pretreatment methods adversely affected enzymatic hydrolysis of newspaper. It was presumably due to the pore collapse in the fibers during the pretreatment process.

Publications and Presentations

List all talks, posters, scientific publications, news releases, etc., about this Project during the reporting period. Provide one copy of each publication, report, or news article resulting from activities supported under the grant as well as any announcements, press releases, statements or photographs depicting Project activities.

Enzymatic Hydrolysis of Pretreated Newspaper Having High Lignin Content for Bioethanol Production, Hui Chen, Qiang Han, Richard A. Venditti,* and Hasan Jameel, BioResources, 10(3), 4077-4098, 2015.

Conversion of Industrial Paper Sludge to Ethanol: Fractionation of Sludge and Its Impact, Hui Chen, Qiang Han, Kevin Daniel, Richard Venditti, Hasan Jameel, Appl Biochem Biotechnol 174, 2096-2113, 2014.

Economic evaluation of the conversion of industrial paper sludge to ethanol, H. Chen, R. Venditti, R. Gonzalez, R. Phillips, H. Jameel, S. Park, Energy Economics, 44, 281-290, 2014.

Enzymatic hydrolysis of recovered office printing paper with low enzyme dosages to produce fermentable sugars, Hui Chen, Richard A. Venditti, Hasan Jameel, Sunkyu Park, Appl. Biochem. Biotechnol, 166, 1121-1136, 2012.

Process Development and Fundamental Study on Enzymatic Hydrolysis of Cellulosic Biomass to Fermentable Sugars for Ethanol Production by Hui Chen. Doctoral Thesis, NCSU, 2014.

EXPLORING THE POTENTIAL OF PAPER INDUSTRY SLUDGES FOR ETHANOL PRODUCTION
Hui Chen, Ronalds Gonzalez, Richard Phillips, Richard Venditti *, Hasan Jameel, Sunkyu Park, ICPPB 2012.

Technology Transfer

Describe all technology transfer (inventions, disclosures, patents, licensing agreements, etc.) resulting from the Project during the reporting period.

None.

Commercial Accomplishments

Describe the most significant accomplishments resulting from the Project during the reporting period.

None.

Educational Accomplishments

Describe the most significant educational accomplishments resulting from the Project during the reporting period. (Number of students, post-docs, etc. trained)

We have trained two graduate students and one undergraduate student.

Additional Funding

List any additional funding generated as a result of the Project during the reporting period.

We received a $5000 grant from NCSU to do research in the same area, in 2014.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

Scientific Progress Report

Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.

Principal Investigator Name(s) and Contact Information: **(Title, Mailing Address, Phone, Fax, Email)**	Clifford Weil Professor Agronomy Dept 915 West State Street West Lafayette, IN. 47907-2054 Phone: 765-496-1917 Fax: 765-496-2926 Email: cweil@purdue.edu	
University:	Purdue University	
DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026-317**	
Project Title:	*Genetic control of starch digestion: better food & fuel*	
Reporting Period:	**From: 10/1/2013**	**To: 5/31/2013**
Report Type:	**Check one:** [] Semiannual Report [X] Final Report	

Project Objectives

List each objective of the Project and the progress made toward each one during the reporting period.

Aim 1. Establish homozygous mutant lines from rapidly digesting and slowly digesting mutants, analyze these using a combined amylase/glucoamylase digestion, then establish complementation groups within each category by intercrossing and re-analysis; initiate mapping and isolation of these mutant alleles and genes.

Progress: Putative homozygous mutants were intercrossed in Summer 2013 for allelism testing. Analysis of these progeny suggested that in progress.

Aim 2. Complete detailed chemical analyses of starch structure and composition, use dual labeling to compare the endosperm proteome and lipidome profiles in 20 lines to better understand mechanisms of altered digestion and inform further efforts to manipulate it genetically. These analyses will be coupled with ongoing NIR spectroscopy of all the mutant lines to begin developing calibration curves for predicting digestibility.

Progress: Proteomic analysis on these lines proved inadequate for determining the reasons for altered digestibility, primarily because assignment of different charge/mass peaks could not be completed reliably. In addition, NIR has failed to produce a usable calibration curve for prediction of digestibility except for amylose:amylopectin ratio: higher amylose content correlates with slower digestibility, but this correlation was already well-known. IN contrast other lines with altered digestibility were not showing significant shifts in their amylose:amylopectin ratios, suggesting other causes. Consistent with this result, we had initiated debranching profile analysis of the starch in our various mutant lines using High Performance Size Exclusion Chromatography (HP-SEC) and found two of the more slowly digesting mutants appear to have increased branch lengths in the amylopectin fraction, which is predicted to increase retrogradation and therefore slow digestion. However, this explanation has not accounted for all the lines tested. We have moved to transcript profiling in immature endosperm of induced mutants and natural variants with altered digestion, and are analyzing changes in the relatively well-characterized endosperm transcriptome by comparison with W22 and B73. These immature ears were produced in Summer 2013, however the RNA and sequencing libraries to do RNA-Seq analysis failed.

Aim 3. Assay the starch digestibility for NAM RILs developed from eight Maize Diversity Lines representing different subtypes of significantly altered digestibility, then correlate the digestibility of these RILs with publicly available genotyping data for the RILs and identify candidate regions and genes that impact digestibility.

Progress: After discussion with the corporate partner, Syngenta, our focus has shifted to uncooked starch and potential for improving animal feed. More rapidly digesting feed would reduce the time getting chickens to market weight, while decreasing digestibility has been suggested to increase feed use efficiency. Two lines, HP301 and CML322, with significantly more rapid digestion uncooked, and two lines (Mo17 and Tx303) with significantly slower digestion uncooked have been identified. Immature ears were harvested in Summer 2013 and are being analyzed for variation in their transcriptomes.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

We have identified genetic variation in how quickly or slowly starch is digested to glucose when it is either eaten by humans in its cooked form, eaten by livestock in its raw form or broken down to make ethanol. Some of this variation is in mutant ines we have made and some is naturally

occurring variation in existing corn varieties from around the world. Understanding this variation should allow breeders to develop lines of corn specifically suited to use as a food ingredient to combat obesity and diabetes, act as a more efficient and cost-effective animal feed, and, potentially, to make a better ethanol fermentation feedstock for biofuel production in the short term. While we had hoped to develop a fast, spectroscopic prediction tool for digestibility this did not prove possible. We are determining what factors are altered in these new varieties, to understand how best to recreate them and optimize their productivity.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

Starch from domesticated cereals is a fundamental part of most human activity. The starch is typically broken down to glucose for biochemical energy from food and, more recently, to serve as feedstock for fermented biofuels. However, these two uses demand opposite characteristics of cereal starch. The health crisis posed by obesity, diabetes, etc. calls for slow, steady glucose release from food, typically after cooking the starch to gelatinize it, and more efficient livestock gains from feed could be realized with more slowly digesting corn that is uncooked. In contrast, glucose release for biofuels from ground corn must be rapid to minimize energy inputs and costs, typically after gelatinizing the starch, while more rapidly-digesting, uncooked corn may reduce the time it takes to get poultry to market weights. An understanding of the genetic differences between corn diversity lines and their starch digestion rates will allow us to determine which genetic components significantly impact this important trait. Induced and natural variation has been identified in maize as impacting starch digestibility in corn flour. Mutagenized lines, as compared to their nonmutant siblings are being analyzed to understand what genes contribute to this complex trait. In addition, the extensive natural diversity of maize inbreds from one another provides another source of variation in starch digestibility.

Starch digestion can be impacted by the structure of the starch molecules (branching patterns, branch length, etc.) as well as by protein and lipid components in the flour produced when kernels are ground. All these factors are under genetic control and, thus, amenable to genetic manipulation. While many of the starch biosynthetic enzymes and the genes that encode them have been studied for decades, the genes encoding starch interacting factors are largely unknown.

We have identified 20 mutant lines in forward screens of the Maize TILLING Populations that show altered starch digestion (Groth et al, 2006). In addition, we have screened inbred lines of the Maize Association Panel, including founders of the maize NAM population. We are profiling the starch structure and chain length distribution, and of starch-associated proteins and lipids in these lines to better understand mechanisms of altered digestion. For two NAM founders with rapid digestion (HP301and two with slow digestion, we are also screening the NAM RILs and will utilize the extensive genotyping resources of this population to identify various components involved in digestion. This approach will let us quickly narrow down genomic regions responsible for the altered digestion, allowing a candidate gene approach for isolating the genes. Our use of NIR has been scaled back to establishing total starch and moisture content rather than attempting to predict components of digestibility. While accurate for predicting amylose:amylopectin ratios (which can impact digestibility), NIR has not proven effective in predicting digestibility directly in any other way.

We have harvested 14-day old ears of the four NAM founder lines with altered digestion to profile the endosperm transcriptomes in comparison to those of the reference inbreds W22 and B73.

Publications and Presentations

List all talks, posters, scientific publications, news releases, etc., about this Project during the reporting period. Provide one copy of each publication, report, or news article resulting from activities supported under the grant as well as any announcements, press releases, statements or photographs depicting Project activities.

None during this reporting period

Technology Transfer

Describe all technology transfer (inventions, disclosures, patents, licensing agreements, etc.) resulting from the Project during the reporting period.

None to date.

Commercial Accomplishments

Describe the most significant accomplishments resulting from the Project during the reporting period.

One of the lines that digests rapidly is a popcorn. We have initiated a proposal together with a member of the Animal Science Dept. at Purdue to test whether popcorns in general may act as a better poultry feed. Should this prove to be the case, it will greatly expand the popcorn market beyond snack food.

Educational Accomplishments

Describe the most significant educational accomplishments resulting from the Project during the reporting period. (Number of students, post-docs, etc. trained)

Four graduate students and four undergraduate students trained on this project to date. A rotation PhD candidate, Clara Assisi is working on the project currently and I am hoping she will return to the project fulltime at the end of her rotations. Another undergraduate is set to continue after her rotation is complete.

Additional Funding

List any additional funding generated as a result of the Project during the reporting period.

One grant proposal for the chicken feeding trial to AgSeed for $50,000

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

None during the reporting period

Scientific Progress Report

Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.

Principal Investigator Name(s) and Contact Information: **(Title, Mailing Address, Phone, Fax, Email)**	John J. Finer Department of Horticulture and Crop Science OARDC/The Ohio State University 1680 Madison Ave. Wooster, OH 44691 Tel: 330-263-3880 Fax: 330-263-3887 Finer.1@osu.edu	
University:	The Ohio State University	
DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026-323**	
Project Title:	*Novel soybean promoters that are stronger than CaMV35S*	
Reporting Period:	**From: Jan 1, 2010**	**To: Dec 31, 2015**
Report Type:	**Check one:** [ ] Semiannual Report [X] Final Report	

Project Objectives

List each objective of the Project and the progress made toward each one during the reporting period.

1. Characterize the ten Gmubi and ten GmERF promoters fully using our three tiered system of (i) transient expression analyses, (ii) expression in soybean hairy roots and (iii) transgenic soybean. Assay for strength and expression profile in tissues.

As this proposal was submitted in 2010 and approval/funding was delayed, parts i and ii of this objective were completed and published a year prior to project initiation. This portion of this work was primarily performed by a former graduate student, who was awarded a University graduate fellowship and the manuscript was published in 2010. Unfortunately, since this work was done prior to receipt of the award, CPBR was not acknowledged in this 2010 paper.

Hernandez-Garcia CM, Bouchard RA, Rushton PJ, Jones ML, Chen X, Timko MP, Finer JJ (2010) High level transgenic expression of soybean (*Glycine max*) GmERF and Gmubi gene promoters isolated by a novel promoter analysis pipeline. BMC Plant Biology 10:237, doi:10.1186/1471-2229-10-237

The other components of Objective 1 were also completed during the course of this research and publications describing the results from this research have been published. In order to assemble manuscripts that include sufficient information for a higher impact publication, parts of the results from part iii have been combined with some results from Objectives 2-4. In all of these publications, CPBR and Bayer CropScience have been acknowledged.

A manuscript describing modifications of the Gmubi3 promoter was published in early 2015 (De La Torre CM, Finer JJ (2015) The intron and 5' distal region of the soybean Gmubi promoter contribute to very high levels of gene expression in transiently and stably transformed tissues. Plant Cell Reports 34:111-120). This manuscript described the GmubiXL promoter (XL refers to extra-long, ~1500 bases longer than Gmubi3), which is a very strong soybean promoter. Using transient expression (part i), this promoter was 2.5X stronger than Gmubi, which was 5-7X stronger than the CaMV35S promoter. In stably-transformed hairy roots (part ii), GmubiXL was 2X stronger than Gmubi. In stably transformed soybean plants, the expression pattern of GmubiXL followed that of Gmubi and 35S but expression levels were much higher with GmubiXL. Since the Gmubi promoter was 5-7X stronger than the CaMV35S promoter, GmubiXL was roughly 10-25X stronger than this viral promoter. Movement of the Gmubi leading intron upstream of the promoter, in either orientation, resulted in an enhancement in expression, suggesting that the intron contains enhancer elements. Insertion of intronic sequences within the intron to create a "stuffed" intron, also resulted in an enhancement in expression, further supporting the idea of the enhancers within the intron.

A second manuscript describing additional modifications to the Gmubi promoter with specific emphasis on intronic sequences is close to submission (Grant TNL, De La Torre CM, Zhang N, Finer JJ Use of synthetic introns to identify sequences in the 5'UTR intron of the *Glycine max* polyubiquitin (Gmubi) promoter that give increased promoter activity). This manuscript describes the generation of a new cloning vector (pSINC; plasmid for Stuffed INtron Cassette), which was used extensively to identify important sequences within the intron that contribute to high levels of gene expression. As of this report date, the target journal for this submission is BMC Plant Biology and we have provided the final draft of the manuscript to CPBR and Bayer CropScience (Industrial sponsor) for approval.

A third manuscript describing extensive analysis of the GmERF3 promoter and a new cis-regulatory element was recently published, after the end of the grant term (Hernandez-Garcia CM, Finer JJ (2016) A novel cis-acting element in the GmERF3 promoter contributes to inducible gene expression in soybean and tobacco after wounding. Plant Cell Reports 35(2), 303-316). In that manuscript, we describe expression analysis of the various forms of the GmERF3 promoter in transgenic soybean plants. The GmERF3 promoter provides base levels of expression in most of the tissues of young plants, with good expression in the roots and the highest expression in root tips. Induction of the GmERF3 promoter is tissue specific, with no to minimal induction in roots and moderate induction in cotyledons, hypocotyls, and leaves following wounding. Physical wounding using either a scalpel blade or hemostat gave induction of the promoter within 24-48 hr. The timing of induction is not typical of wound induction, which

usually takes only a few hours. We classified this induction as “Wound Inducible and Delayed Expression” or “WIDE” and named the element in this way.

Finally, an additional family of soybean promoters, from highly expressing soybean genes, was identified and cloned. This family was not included in the original proposal but, since so much of the proposed work was completed, we decided to move forward with this new family of promoters, which has turned out to be extremely interesting and gave rise to a 4th paper from this research (Zhang N, McHale LK, Finer JJ (2015) Isolation and characterization of "GmScream" promoters that regulate highly expressing soybean (*Glycine max* Merr.) genes. Plant Science 241:189-198 DOI: 10.1016/j.plantsci.2015.10.010). Promoter selection for the “GmScream” family was based on bioinformatic analysis of RNAseq data from different sources, with emphasis on genes that expressed the highest in most but not necessarily all tissues. Our corporate sponsor for this research was specifically interested in new promoters that regulate high levels of gene expression and this new approach was taken following their encouragement. GmScream promoters displayed higher expression than the CaMV35S promoter using transient expression analysis and in stably-transformed hairy roots. Interestingly, the highest expressing soybean promoters in the GmScream family all contained leading introns, as does Gmubi. Unlike the Gmubi intron, which contains classical promoter enhancer elements within the intron, the sequences in the GmScreamM8 intron seem to be optimally functional within the intron. “Intron cis-regulatory elements” have been reported but little is known about how they function to increase or modulate gene expression.

2. Through computational analysis, identify and select elements within the Gmubi and GmERF promoter family.

Analysis of promoter regions using most of the element detection databases results in a large number of putative elements that must be analyzed using our validation tools. We have used the PLACE database; http://www.dna.affrc.go.jp/PLACE/, that recognizes previously-identified elements, and the Arabidopsis Promoter Element Discovery Tool; http://stan.cropsci.uiuc.edu/tools.php, which was designed for Arabidopsis but has some functionality with soybean promoter element ID. As stated previously, all of these databases reveal large numbers of potential elements. These potential elements are a starting point but must be validated using the tools that we have developed for our soybean promoter analysis pipeline. This can be especially time-consuming.

A G-box element was identified in the Gmubi3 promoter, which seems to contribute to the high levels of gene expression found when using this promoter. G-box elements have also been identified within the GmScreamM8, GmScreamM4, and GmScreamM1 promoters, which are the highest expressing GmScream promoters. Tetramers of the native and variant elements were generated and fused to either 35S core promoter or GmScreamM8 core promoter with or without the GmScreamM8 leader intron to regulate gene expression. The elements act coordinately with core sequences within the promoter and the leading intron. Some of the G-box elements were functional while others were not. Perhaps, one of the most exciting discoveries of this work was the conversion of one of the nonfunctional G-box elements into a functional element following mutation of specific flanking sequences.

3. Generate element tetramers for validation of function using transient expression analysis.

We have generated a large number of element tetramers from GmERF3, Gmubi and GmScream promoters. Tetramers of possible elements from the GmERF3 promoter revealed two regions that show significant activity (-184 through -233, -234 through -277). Other regions may also possess elements but this -184 through -277 region seemed to carry the highest activity and was initially emphasized. From this larger region, the -234 through -277 region showed the most activity as a tetramer and putative elements within this region seemed to contain a late wound-inducible element. The putative element region was selected for generation of mutations. This was described in the Hernandez-Garcia and Finer (2016) paper.

Elements have been identified and validated within the GmScreamM8 promoter, which is the highest expressing GmScream promoter. Rather than relying on bioinformatics tools, which have been marginally reliable and predictive, 25 bp regions from the GmScreamM8 promoter were selected directly to generate tetramers, which was based on the 5’ truncation analysis of GmScreamM8 promoter. The 25 bp tetramers were placed upstream of a minimal GmScreamM8 promoter along with its native intron. The resulting

synthetic promoter was substantially smaller than the full-length promoter. The high expression observed with the 25 bp tetramer, upstream of a minimal GmScreamM8 promoter along with its native intron, suggests that this 25 bp region contributes in a major way to gene expression. One of the 25 bp regions seems to contain a G-box element but this has not been validated using expression analysis while elements within the other 25 bp region has not been identified. More G-box-like elements (see 2 above) identified from the GmScreamM4 and GmScreamM1 promoters were also validated to better understand the interaction among promoter element tetramers, a core promoter, and a leading intron. Element tetramers upstream of a GmScream core promoter (GmSM8 core) showed very high activity, only if the native leading intron was included, indicating that interesting interactions may exist among intron-containing sequences, promoter element sequences and the core promoter.

4. Characterize the promoter elements using our three tiered system.

As reported in 3 above, we have identified a number of elements (some previously identified and some novel) from the GmERF3 promoter. Tetramers of these elements revealed a novel element that contributed to high levels of gene expression using transient expression and with hairy roots. The promoter was not inducible in hairy roots. Transgenic tobacco, containing the GmERF3 promoter element tetramers, showed inducibility with the wild type (not mutated) element tetramer, indicating the mutations to the either of the elements eliminated induction. Induction was observed after 24 hours, indicating Wound induction with delayed expression pattern.

5. Evaluate native promoters and hybrid component plant promoters for their utility in driving transgene expression.

We have generated large numbers of hybrid promoters during the reporting period and this has become standard practice in the laboratory. Promoters have been generated using novel combinations of the promoter core sequences, along with introns and tetramers of various element sequences. The introns themselves have contained tetrameric repeats of both intron and classical promoter-based elements. All of these native and synthetic promoters were evaluated using our first and second tier analyses. The synthetic promoters with the most interesting expression levels were introduced into soybean for stable expression studies.

6. Output: A toolbox of native and hybrid component plant soybean-based promoters for use by the soybean community.

We have identified elements from both native promoters and introns that contribute to gene expression. We have made the Gmubi promoter available to the biotechnology community and this promoter is receiving widespread use. The GmScream promoters have also recently been published and have very recently been distributed to a few different laboratories. The concept of promoter elements that contribute to gene expression is very straightforward and individual elements, when isolated and studied, yield predictable results. But, as we continue to generate synthetic promoters, the interaction between different elements increases and experimental outcomes become more interesting. It seems that the best approach for this work is to isolate the elements and perform separate analyses of each. As might be imagined with the number of native promoters that are in the genome [equivalent to the number of genes (>30,000 in soybean)], a limitless number of new synthetic promoters are possible. The constraint seems to be the generation and analysis of stably transformed soybean with even the most interesting promoter constructions.

Please complete.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

The agricultural landscape is dominated by genetically engineered soybean, which represents greater than 90% of the total soybean acreage in the United States. The first commercial transgenic soybean contained only a single gene for resistance to the herbicide Roundup. The expression of this gene was controlled by a DNA fragment in front of the gene called the "promoter". In the case of this first transgenic soybean, the promoter originated from a plant virus, which led to high levels of gene expression and excellent protection from Roundup application. The most recent generation of Roundup resistant soybean contains the same resistance gene, which is controlled by a different promoter, a hybrid promoter that contains both viral and plant components. Although strong promoters are useful when the introduced gene product needs to be present at high levels, other promoters may also be useful when the gene product should be expressed only in certain tissues or only during certain times in the life cycle of the plant. In addition, promoters that are native to plants may be more useful for certain applications. Today, transgenic plants contain larger numbers of gene that are assembled into stacks. Each gene should be individually controlled to express at defined levels, in specific tissues and only when needed. For this project, native soybean promoters have been cloned and studied. We are interested in promoters of different strengths and those promoters that cause gene expression under specific conditions. We wish to dissect and reconstruct the promoter region to determine which DNA sequences contribute to gene expression and develop unique promoters that have not previously been made. For this research, we will develop a soybean promoter toolbox that can be used for both basic and applied research for precise regulation of transgenes in soybean.

Three different families of soybean promoters were cloned and emphasized in this research. One to three promoters from each of these families were selected for detailed studies of the elements that contribute to gene expression. One of the promoters is inducible and we have identified the specific region of DNA that contributes to inducible gene expression. Induction was observed following cutting of tissues with a scalpel. The other promoters are not inducible but are very strong, leading to high levels of gene expression in many tissues. Some similarities exist in the promoter regions of the highly expressing genes and the DNA sequences within these regions have been isolated. We have generated unique combinations of these native DNA sequences and studied the components that modulate gene expression. We have also generated novel promoters, which consist of native plant sequences but do not currently exist in nature. The promoters that we have generated through the course of this research may show utility for fine regulation of transgenes in soybean.

Please complete.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

Overview:

For the proposed research, 3 different families of soybean promoters were isolated and characterized using a number of different validation tools. The first family was the *Glycine max* polyubiquitin (Gmubi) family, which generally drive constitutive levels of gene expression. We have extensive experience with the Gmubi3 promoter, which provides the highest level of expression of any of the 10 Gmubi promoters.

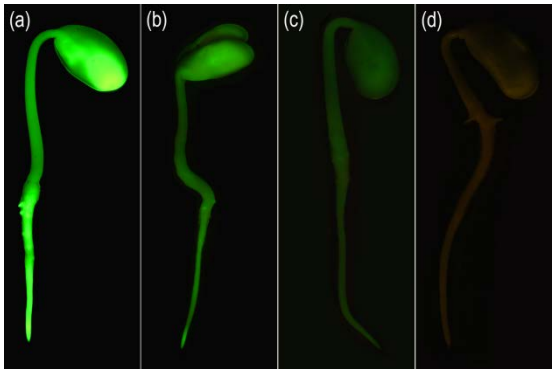
The second family was the *Glycine max* Ethylene Response Factor (GmERF), which are typically inducible. The GmERF promoters are typically induced by wounding, pathogen attack, ethylene exposure or application of methyl jasmonate. This family of inducible promoters brings some additional challenges to the research as induction can be challenging to control. The GmScream family of soybean promoters was identified based on analysis of RNAseq and microarray data, capturing the promoters for soybean genes that are expressed at the highest levels in the most tissues.

A tiered analysis of the promoters was used. Transient expression analysis was evaluated first, followed by stable expression analysis in soybean hairy roots. Finally, analysis in stably-transformed soybean plants has been performed for only those promoters, that show the most interesting or useful expression results from the first two rapid validation tests. As 5-9 months is required for production of transgenic soybean, tobacco was also been used for gene introduction in some cases to accelerate our efforts. Elements within some of these promoters have been identified and validated using transient expression and hairy roots. For the final stages of this project, promoter elements that seem to provide unique or useful expression have been and will be evaluated in stably-transformed tobacco and soybean plants. In addition, hybrid or synthetic promoters continue to be synthesized and validated using the same validation tools used previously.

Forty different soybean promoters (10 GmERF, 10 Gmubi, and 20 GmScream) were identified and isolated using the soybean genome database to provide starting sequences for primer design. Promoters were cloned using PCR, placed upstream of the *green fluorescent protein* (*gfp*) gene and introduced into lima bean cotyledons via particle bombardment, for transient expression analysis. Promoter constructs were also stably introduced into soybean using *Agrobacterium rhizogenes* for production of soybean hairy roots. Selected promoters were then introduced into either embryogenic soybean cultures for generation of stably-transformed soybean plants or tobacco leaves for production of transgenic tobacco plants.

Gmubi promoter

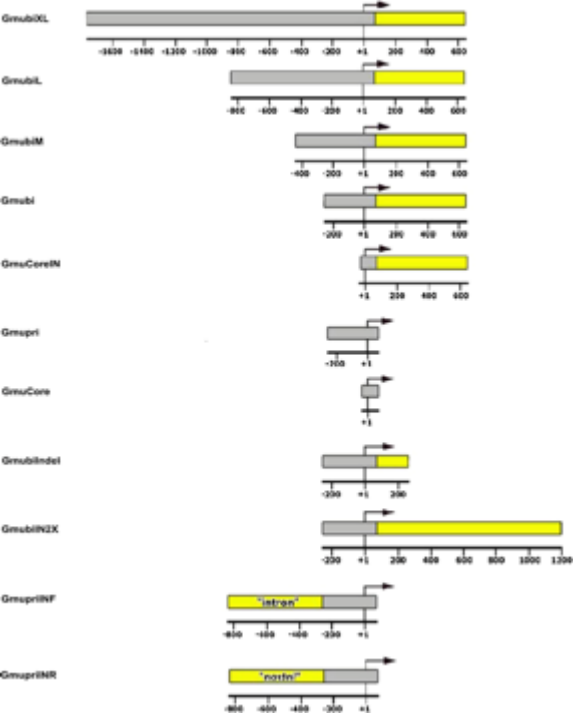
Gmubi promoters were not wound-inducible and studies on induction were therefore not pursued. The Gmubi1 and 3 promoters were the strongest promoters and Gmubi3 was selected for further characterization by analysis of the leading intron and regulatory elements. Numerous 5' extensions of Gmubi3 promoter resulted in the recovery of GmubiXL (~1500 bases longer than Gmubi3), which was one of the strongest soybean promoters that we have cloned and partially characterized. Using transient expression, this promoter is 2.5X stronger than Gmubi. In stably-transformed hairy roots, GmubiXL is 2X stronger than Gmubi.

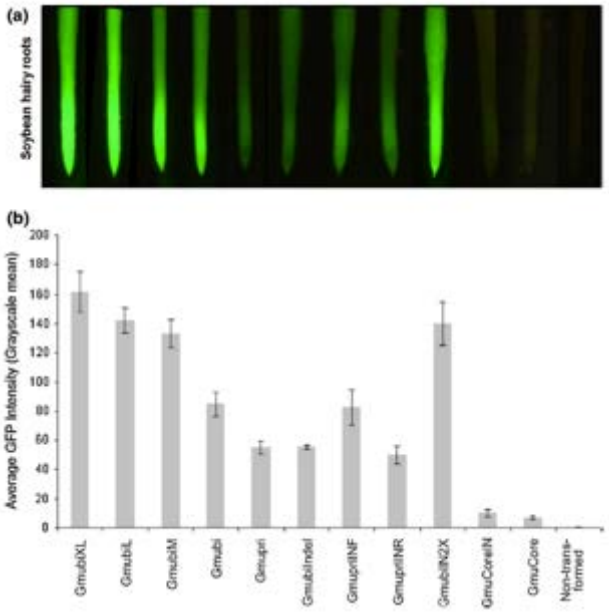


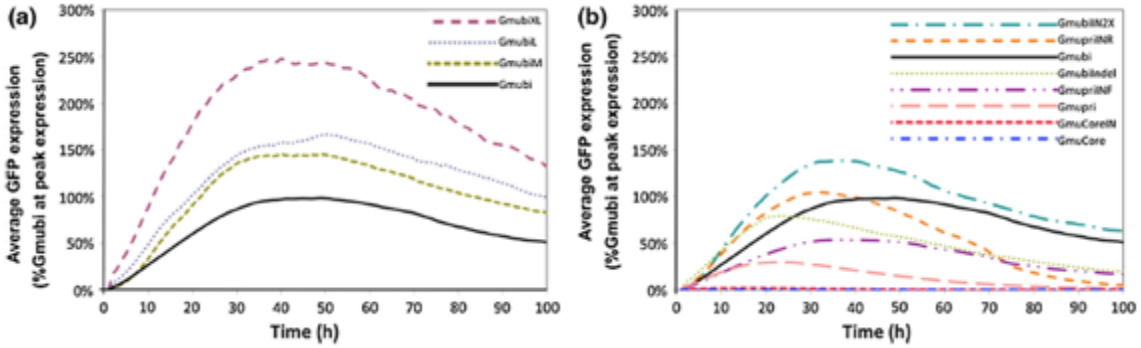
In stably transformed soybean plants (left; a – GmubiXL, b – Gmubi, c – CaMV35S, d - nonTransformed), the expression pattern of GmubiXL follows that of Gmubi and 35S but expression levels are much higher with GmubiXL that the other 2 "strong, constitutive" promoters. GmubiXL ranges from 10-25X stronger than the CaMV35S promoter.

Gmubi Intron Variants: Twelve variants of the Gmubi promoter (below) were generated in order to better understand regions that may contribute to the high levels of gene expression obtained with this promoter. Promoter variants were evaluated using both transient expression and stable transformation in soybean hairy roots. Expression intensities were consistent between transient expression and stable transformation in hairy roots. With both validation systems, translocation of the Gmubi leading intron upstream of the promoter, in either orientation, resulted in an enhancement in expression, suggesting that the intron contains enhancer elements. Insertion of intronic sequences within the intron to create a "stuffed" intron, also results in an enhancement in expression, further supporting the idea of the enhancers within the intron. Deletion of a portion of the intron resulted in a reduction in the intensity of

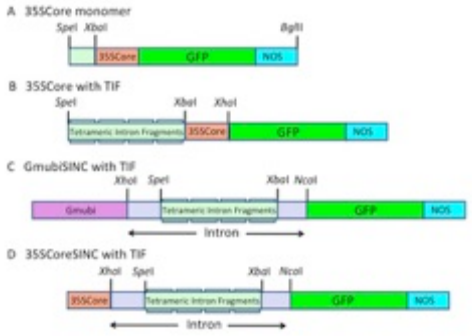
gene expression. Transient expression profiles for all of the Gmubi promoter variants were similarwith the exception of the two intron variants Gmupri (intron-less) and GmubiInDel (deletion of an internal intron fragment), which showed an earlier peak and more rapid decline in GFP expression. This expression pattern suggests a stabilizing effect of the intron on gene expression. All data below has been published in De La Torre CM, Finer JJ (2015) The intron and 5' distal region of the soybean Gmubi promoter contribute to very high levels of gene expression in transiently and stably transformed tissues. Plant Cell Reports 34:111-120





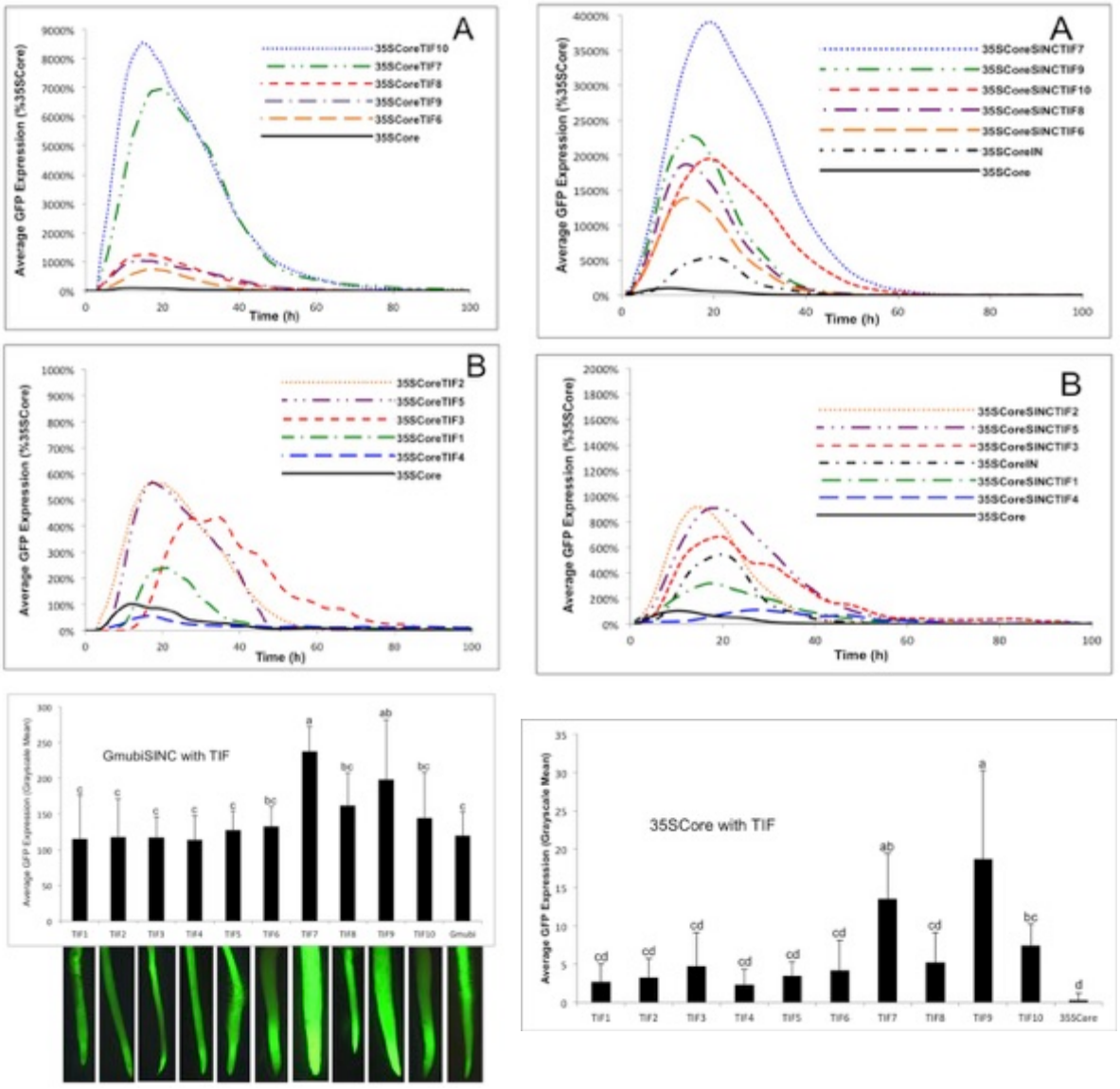


To further study and expand on the concept of a “stuffed intron” and intron manipulation, we have generated pSINC which is a plasmid which allows us to evaluate various Stuffed INtron Cassettes. A cassette system like this has not been previously constructed. Different pSINC plasmids forms were generated; one contains a minimal CaMV35S promoter while the other contains the GmUbi promoter. For intron element validation, 10 different regions of the Gmubi intron were cloned and tetramers were made. The tetramers were evaluated upstream of a 35S promoter (as other putative elements have been evaluated) and within the 35SSINC construct (within a synthetic intron, downstream of a minimal 35S promoter). Sections of the intron, as tetramers, were able to increase gene expression in both constructs but the intron fragments gave rise to higher expression when placed within the synthetic intron, compared to upstream of the minimal promoter. This work will be submitted soon for publication and will be the first report of the use of a synthetic intron to evaluate potential intron elements. The use of synthetic introns to carry regular promoter elements and intron elements may be very useful to the plant biotechnology community, for enhancement and stabilization of gene expression



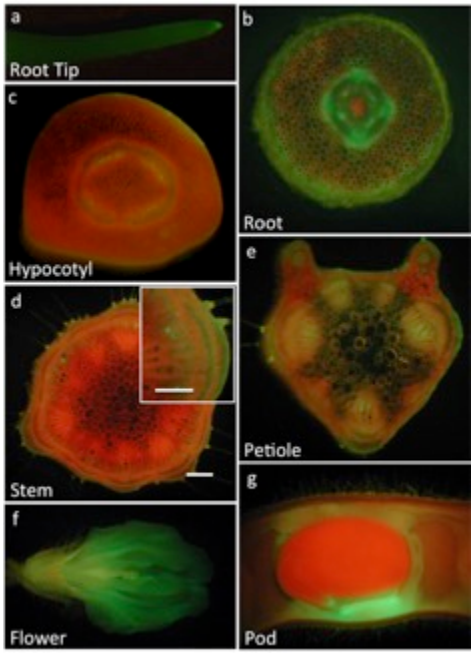
For identification of intron element, 10 different regions of the Gmubi intron were cloned and tetramers of these intron fragments were made (schematic above). The tetramers were evaluated upstream of a 35S promoter as other putative elements have been evaluated, within the 35SSINC construct within a synthetic intron, downstream of a minimal 35S promoter) and finally within the GmubiSINC construct (within a synthetic intron, downstream of the Gmubi promoter).

All constructs were evaluated using the first two tiers of promoter analysis; transient expression and then transgenic hairy roots. Regions from the intron, used as tetramers, were all able to increase gene expression in all constructs but the intron fragments gave rise to the highest expression when placed within the synthetic intron constructs, compared to upstream of the minimal promoter. This suggests that the intron elements can function as classical promoter elements but show a higher function when positioned within intronic sequences. This work represents the first report of the use of a synthetic intron to evaluate potential intron elements. Since the minimal 35S promoter shows very low expression levels, it is very sensitive to components that enhance gene expression. The 35S core promoter often shows increases in expression levels when fused with regulatory sequences. The most striking increase in gene expression was shown with the GmubiSINC construct containing tetramers of the different intron fragments (images below). The intron fragments that gave the highest expression for transient expression and in soybean hairy roots for all 3 forms of the intron element tetramer were 7, 8, 9 and 10. These intron fragments were all located at the 3’ end of the intron and they all share some common sequences. These sequences are potentially valuable and useful for regulating high expression levels.

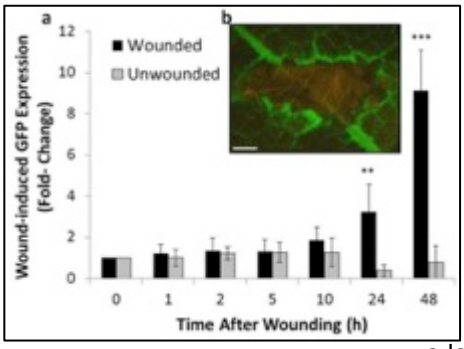


The use of synthetic introns to carry elements, which originate from both promoters and introns may be very useful to the plant biotechnology community, for enhancement and stabilization of gene expression.

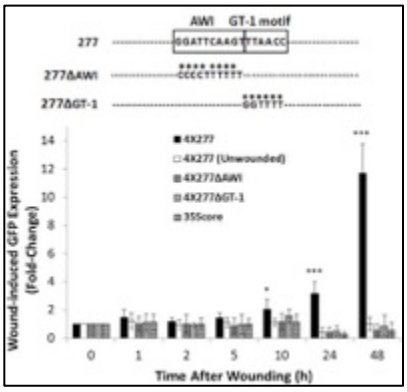
GmERF3 promoter



Of all of the GmERF promoters, the GmERF3 promoter was the strongest. The GmERF3 promoter was fused with gfp and introduced into soybean to generate stably-transformed soybean plants. This promoter displayed low levels of expression throughout young soybean plants, with higher expression in the roots and especially root tips (left). As plants matured, expression in the vegetative tissue declined but remained strong in the flowers and the point of attachment seed to the pod. Induction of the GmERF3 promoter was tissue specific, with no induction in roots, moderate induction in leaves and high induction in seedling cotyledons and hypocotyls after wounding. The timing of wound induction was not typical, showing an increase in expression at 24 and 48 h post-wounding (below, left). Wound induction usually requires on a few hours. It seems that this promoter is delayed, following wound indcution, which would not be unusual for a family of genes that is associated with ethylene responses.

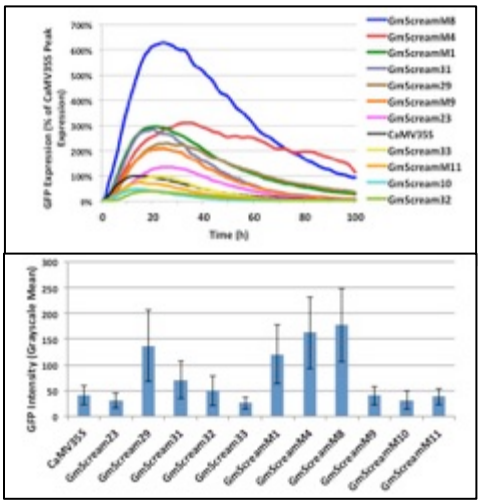


Truncation analysis of the GmERF3 promoter revealed one region from -138 through -277, which seemed to contribute to high levels of gene expression, using both transient expression analysis and expression in hairy roots. The regions from -185 through -233 and from -234 through -277 seem to contain regulatory elements as deletions of these areas resulted in large declines in expression intensity. Tetramers of just these regions, fused to a minimal CaMV35S promoter, result in moderate levels of expression, confirming that regulatory elements are located within these small regions of DNA. Mutations to some of the bases in these regions (below, left) and evaluation of these mutants as tetramers, gave either increased or reduced expression levels, further supporting the conclusion that these regions contain at least some of the regulatory elements in the GmERF3 promoter. Induction following wounding has been observed in transgenic tobacco using tetramers of a long element, identified within the -234 through -277 region. Mutations of the nucleic acids in either region of this long element eliminated inducibility, further confirming that this element is quite large. Induction was also observed in transgenic tobacco, containing element tetramers of only the wild type and not mutant forms (left).

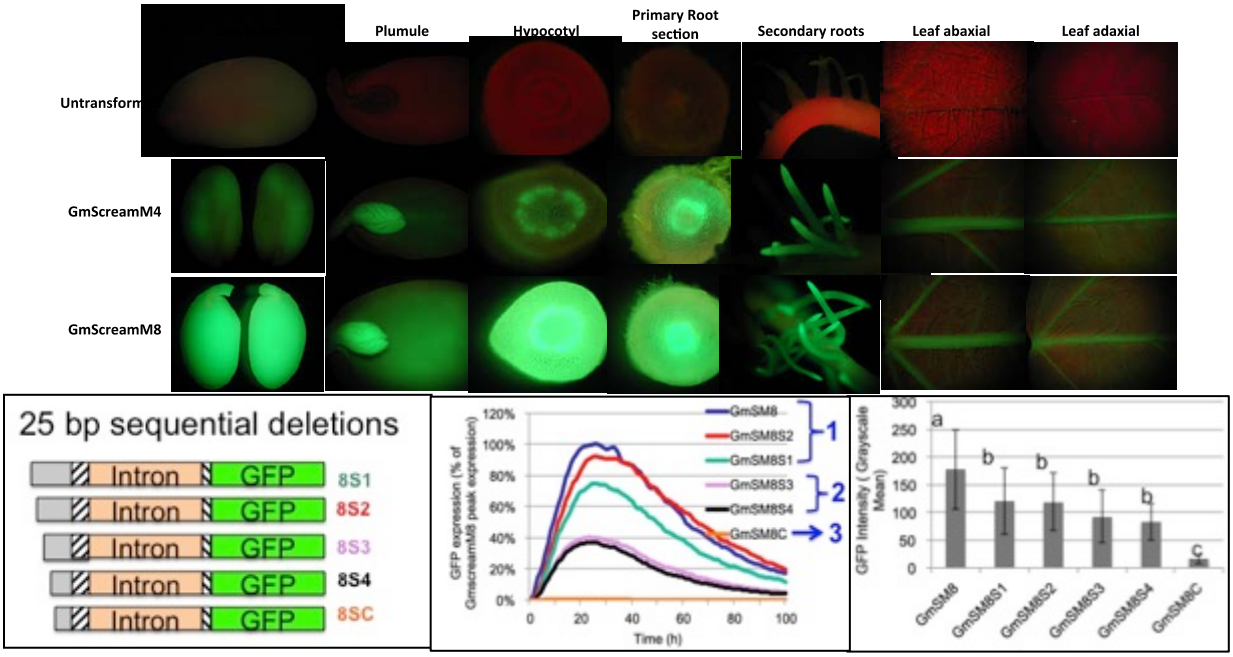


GmScream promoters

Twenty different GmScream promoters were identified, cloned and characterized using both transient expression and stable expression in soybean hairy roots. Of the 20 initially cloned promoters, the 11 highest expressing were selected for more detailed analysis. Using transient expression analysis, the strongest promoter from the GmScream group was GmScreamM8 (below, top curve, blue line) and the second strongest promoter with an unusual extended expression profile was GmScreamM4 (below, top curve, red line). GmScreamM8 showed 6x the expression of the 35S promoter while GmScreamM4 showed 3x the expression of the 35S promoter (black line, which peaks at the 100% activity mark on the y-axis). Using the hairy root system, these two promoters showed similar expression intensities and were about 4x stronger than the 35S promoter (right, histogram on bottom). Both of these promoters regulate elongation factor Tu proteins. These genes may express at high and consistent levels in many different tissues, similar to the ubiquitin genes as they are also involved as housekeeping genes. Both of these promoters also contain a leading intron. Deletion of the leading intron resulted in a reduction in expression. Movement of the intron upstream of the promoter did not increase expression indicating that the intron did not contain any classical promoter-type regulatory elements. However, further manipulation of the intron as part of a 5'UTR revealed the presence of intron-specific regulatory elements. These intron-specific elements seemed to interact with promoter elements identified in the proximal region of the GmScreamM8 promoter (explained in more detail below in the section on leading introns).



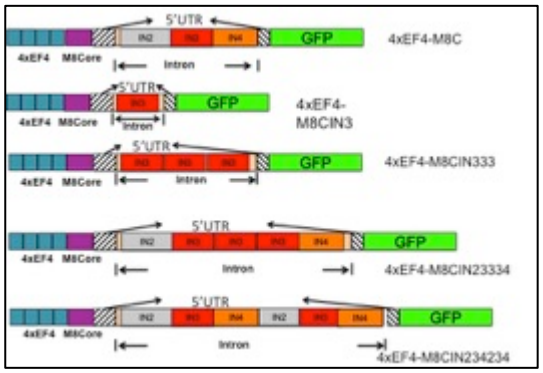
The GmScreamM4 and GmScreamM8 promoters, regulating GFP, were then introduced into soybean. Transgenic soybean plants containing either promoter displayed a high constitutive expression in most soybean tissues, with preferentially higher GFP expression in the procambium, vascular tissues, root tips and seeds (below).



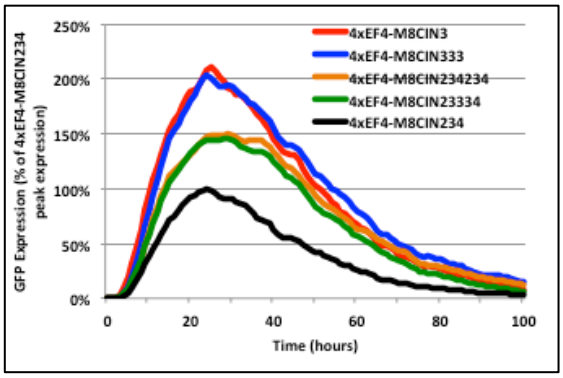
Truncation of the GmScreamM8 promoter (below) revealed two regions of 25 bp [when S4 was truncated to SC (minimal **c**ore promoter region) and when S2 was truncated to S3; all depicted in graphs below] that contained promoter regulatory elements.

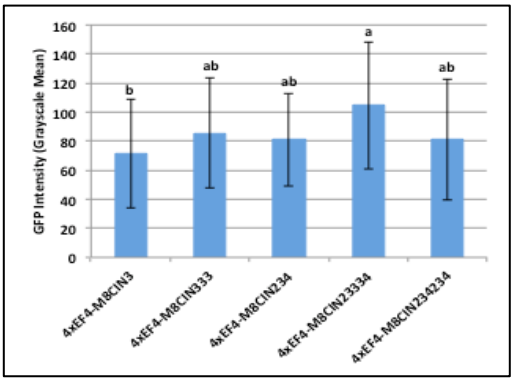
Tetramers of the most active two 25 bp regions showed very high activity, especially when used with the GmScreamM8 core promoter with the intron. Without the intron, tetrameric promoter element activity was difficult to detect, suggesting the contribution of the intron to promoter activity.

Leading Introns



GmScream Intron Variants: Intron variant constructions of the GmScream promoters were then generated (left) which contained the strongest promoter tetramer (blue boxes), upstream of the minimal GmScreamM8 core promoter (purple box) and various intron fragments in different combinations (gray, red and orange boxes). The middle portion of the intron in the 5'UTR (red box labeled as "IN3" in image) affected gene expression more than the other portions. Surprisingly, multiplication of the IN3 intronic fragment did not further enhance gene expression. But high activity was retained when the smallest intron, containing only the IN3 portion and other essential intron processing components, was used with the tetramer upstream of the GmScreamM8 core (4XEF4-M8CIN3, above). Use of this new compact promoter/intron combination gave extremely high expression using transient expression and in soybean hairy roots (bottom graphs).





Please complete.

Publications and Presentations

List all talks, posters, scientific publications, news releases, etc., about this Project during the reporting period. Provide one copy of each publication, report, or news article resulting from activities supported under the grant as well as any announcements, press releases, statements or photographs depicting Project activities.

De La Torre CM, Finer JJ (2015) The intron and 5' distal region of the soybean Gmubi promoter contribute to very high levels of gene expression in transiently and stably transformed tissues. Plant Cell Reports 34:111-120

Zhang N, McHale LK, Finer JJ (2015) Isolation and characterization of "GmScream" promoters that regulate highly expressing soybean (*Glycine max* Merr.) genes. Plant Science 241:189-198 DOI: 10.1016/j.plantsci.2015.10.010

Hernandez-Garcia CM, Finer JJ (2016) A novel cis-acting element in the GmERF3 promoter contributes to inducible gene expression in soybean and tobacco after wounding. Plant Cell Reports 35(2), 303-316 DOI: 10.1007/s00299-015-1885-7

Grant TNL, De La Torre CM, Zhang N, Finer JJ (To be submitted soon) Use of synthetic introns to identify sequences in the 5'UTR intron of the *Glycine max* polyubiquitin (Gmubi) promoter that give increased promoter activity. BMC Plant Biology

Please complete.

Technology Transfer

Describe all technology transfer (inventions, disclosures, patents, licensing agreements, etc.) resulting from the Project during the reporting period.

An internal disclosure with the Technology and Commercialization Office of The Ohio State University titled, "Use of soybean elongation factor 1-alpha promoters for high levels of gene expression" was submitted and "accepted". Based on comments from Bayer CropScience, a patent on this promoter was not pursued.

A patent titled "Highly active soybean promoter from the SUBI-3 polyubiquitin gene and uses thereof", John J. Finer and Robert A. Bouchard. US Patent # 8,395,021 was issued March 12, 2013. This patent was filed on May 8, 2008 and is unfortunately, not considered part of the funded research, which started in 2010.

Please complete.

Commercial Accomplishments

Describe the most significant accomplishments resulting from the Project during the reporting period.

Nothing to report at this time.

Please complete.

Educational Accomplishments

Describe the most significant educational accomplishments resulting from the Project during the reporting period. (Number of students, post-docs, etc. trained)

Two PhD students (Carlos Hernandez-Garcia and Ning Zhang) were supported on this project. They have both been extremely productive. Their publications on this project are listed in the publications section. A technician (Carola De La Torre) and a post-doc (Trudi Grant) were supported indirectly on the project as part of the matching support. Their publications are also listed in the publications section above.

Please complete.

Additional Funding

List any additional funding generated as a result of the Project during the reporting period.

Nothing to report at this time.

Please complete.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

Dr. Hernandez is currently working in the private sector for Epicrop Technologies in Lincoln, NE.
Dr. Grant is currently a post-doc at the University of Florida.
Ms. De La Torre is currently a PhD student at the University of Missouri
Ms. Zhang is finishing up her PhD in my laboratory.

Scientific Progress Report

Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.

Principal Investigator Name(s) and Contact Information: **(Title, Mailing Address, Phone, Fax, Email)**	Blair D. Siegfried Department of Entomology 202 Entomology Hall Lincoln, NE 68506 Phone: 402-472-8714 Fax: 402-472-4687 bsiegfried1@unl.edu	
University:	University of Nebraska-Lincoln	
DOE Prime Agreement:	**DE-FG36-02GO12026**	
Agreement Number:	**GO12026-**	
Project Title:	*Cellulose Degrading Enzymes from Western Corn Rootworm Larvae*	
Reporting Period:	**From: Sept. 1, 2010**	**To: June 30, 2015**
Report Type:	**Check one:** [X] Semiannual [ ] Final Report	

Project Objectives

List each objective of the Project and the progress made toward each one during the reporting period.

3. *Characterize the transcriptome of the rootworm larval midgut.* Analysis of the larval rootworm midgut transcriptome to identify potential biological and molecular functions of midgut specific genes and specifically to identify genes important to digestion of plant cell walls has been completed. We have completed analysis of all glycosyl hydrolase enzymes and a manuscript has been published that summarizes the work.

   We have also tested an *E. coli*-based cell-free extract to perform in vitro translation of an endogenous β-1, 4-endoglucanase (Dvv-ENGase I) gene from the western corn rootworm beetle, *Diabrotica virgifera virgifera*, belonging to the glycoside hydrolase family (GHF) 45. The cDNA encoding Dvv-ENGase I was expressed as a 27-kDa polypeptide. Recombinant Dvv-ENGase I protein tagged with 6 poly Histidines was purified in one simple and single step via magnetocapture using Ni-based magnetic beads. The recombinant Dvv-ENGase I protein exhibited enzymatic activity not only on hydroxyethyl-cellulose **(**HEC), a nonionic and water-soluble cellulose polymer but also toward the fluorogenic 4-methylumbelliferyl-beta-D-glucopyranoside (4-MU-glucopyranoside). The applicability of *E. coli*-based cell-free expression system to the assembly of some insect genes indicates that it is possible obtain efficient and coupled transcription and translation of recombinant protein in a short period of time providing enough functionally active proteins for an array of downstream applications. A manuscript describing this work has been submitted for publication and is still under review.

   In addition to the genes involved in cell wall metabolism, we have been actively searching the transcriptome sequences for other gene classes that may provide novel target sites for rootworm pest management. Specifically we have identified a number of genes that are involved with chemical perception including both odorant receptors and gustatory receptors. Thus far, we have identified 11 unique odorant receptors (OR) and 53 gustatory receptors (GR). Disruption of these genes through RNA interference (RNAi) or design of chemicals that mimic specific chemical ligands may provide a novel and specific pest management approach. Our initial efforts have been with the gustatory receptors (GR) involved in perception of carbon dioxide. Importantly, CO_2 perception has been identified as an important host-finding cue for neonate rootworm larvae. We have also been optimizing methods for RNAi knockdown of these receptor genes and developing reliable bioassay methods to measure response to CO_2 in order to functionally validate the function of these receptors. Initial attempts have involved injection of gravid adult females to achieve silencing in neonates as they hatch. We have also been exposing neonate larvae to dsRNA treated artificial diet. Both methods have achieved gene knockdown for other genes and we anticipate that one of these methods will be suitable for these receptor genes. A manuscript identifying three gustatory receptor genes and their relative expression among different tissues and developmental stages has been submitted for publication.

   To further increase coverage and the potential to identify of genes involved with chemosensing, we have completed sequencing of different tissues from both larvae and adults. A transcriptome of the heads of neonate larvae has recently been completed. Sensory organs from the heads of neonate larvae are likely to express those genes associated with perception of environmental cues. In addition, we have sequenced transcriptomes from both male and female adult antennae, which should be specifically adapted to chemical perceptions and will provide an opportunity to compare

the expression with neonates so that we can more accurately identify those genes associated with host-finding. Summary statistics for the additional transcriptome sequencing follows.

Table 1. Summary statistics for Illumina HiSeq 2500, paired end transcriptome sequencing to identify genes associated with chemosensory receptors.

Statistic	Larval Head Capsule	Adult Male Antennae	Adult Female Antennae
Total Reads After Filtering	416194544	507246422	492960278
Total base pairs	$4.1x10^{10}$	$5.0x10^{10}$	$4.9x10^{10}$
Total Contigs after assembly	139096	141258	125675
Average Length	1057	1025	1261

Additional bioinformatics work is ongoing to provide annotation and to identify additional genes that are important to corn rootworm in host find and that will provide potential target for developing sustainable pest management approaches.

A second transcriptome from the closely related southern corn rootworm *Diabrotica undecimpuntata howardi* is also currently being analyzed. This species is important in that it shares many biological attributes of the western corn rootworm but has a much broader larval host range. Because rootworms are considered a possible threat to crops grown as potential biomass feedstocks, we believe that comparisons of the transcriptomes from the two species will provide important insight into the potential risks posed to biomass crops. We have completed sequencing of transcriptome representing all relevant life stages of this species. The sequences were assembled using previously developed methods and the final assembly of 191.2×10^6 total reads resulted in 148,923 contigs with an N50 of 4248 bp. We are currently attempting to determine the differences between the two species in their ability to identify and develop on alternative host plants and the effects on gene expression.

The transcriptome data for western corn rootworms generated from our project has been made publically available and has been used by a variety of collaborators including:

University of Illinois; Differential gene expression an adult rotation-resistant and wild-type western corn rootworm larvae.
University of Tennessee; Proteomic analysis of Bt toxin receptors in western corn rootworm larvae.
University of Nebraska and Iowa State University; RNASeq analysis of Cry3Bb1 resitance in western corn rootworms.
University of Illinois and University of Nebraska; Sequencing a potential underground pest of biofuel crops.

Through an Industry Partnership Grant from the University of Nebraska Lincoln we have established a project with a corporate sponsor in which we have been using our transcriptome to identify potential target sequences for silencing by RNA interference. This project has resulted in a number of possible target sequences that result in mortality of both larval and adults when dsRNA is ingested and that may be exploited for developing transgenic maize plants for corn rootworm pest management

4. *Characterize the gut microbial communities associated with western corn rootworm larvae.*
Microorganisms that inhabit insect guts play important roles in host nutrition, development, reproduction, and resistance to pathogens. Moreover, changes or loss of these microorganisms can dramatically impact fitness of the host, its pest status and susceptibility to toxins that specifically target the insect. Digestive symbioses appear to be common among insect herbivores, especially those that feed on highly lignified plant materials such as the western corn rootworm. In spite of their

apparent importance and evolutionary significance, symbiotic associations remain unexplored for most insects, including many of the largest and economically important families. A better understanding of insect-microbe interactions may lead to new strategies to reduce the effects of these pest species and as a resource for identifying enzymes of commercial significance.

We have successfully completed sequencing the metagenome of western corn rootworm larvae that have been either starved or fed with corn roots as well as similar comparisons for the closely related southern corn rootworms. Approximately, 100 3rd instars of non-diapause western corn rootworms were purchased from a commercial vendor and held in 100x15 mm Petri dish (Fisher) with moisturized filter paper in the absence of food. An additional treatment involved providing larvae with fresh corn roots. The petri dishes were kept in lab at room temperature. After 24 hrs, the midgut was dissected under magnification and midgut contents were collected with borosilicate glass capillaries (World precision instrument) and saved in a 1.5 ml centrifuge tube on dry ice. Approximately 100 3rd instar larvae were dissected for contents from both treatments.

Total DNA was extracted from midgut contents and the quality and quantity was evaluated on 1% agros gel and NanoDrop-1000. The DNA samples were submitted to the Center for Biotechnology-University of Nebraska-Lincoln for NGS analysis. The shotgun library for each DNA sample was prepared with Ion Torrent library prep kit (Life Tech) and next generation sequencing conducted for 400 bp runs using Ion torrent (Life Tech). The read data generated from the Ion Torrent sequencing were first used for assembling with Ray Meta (version 2.3) software using default settings. The assembled data (fasta) were further annotated and analyzed with a web-based metagenomics analysis tool (http://metagenomics.anl.gov/) using default settings.

Initial analysis of the WCR metagenomes indicates that the majority of species identified were bacteria (>80% for both fed and starved treatments) Fig. 1.

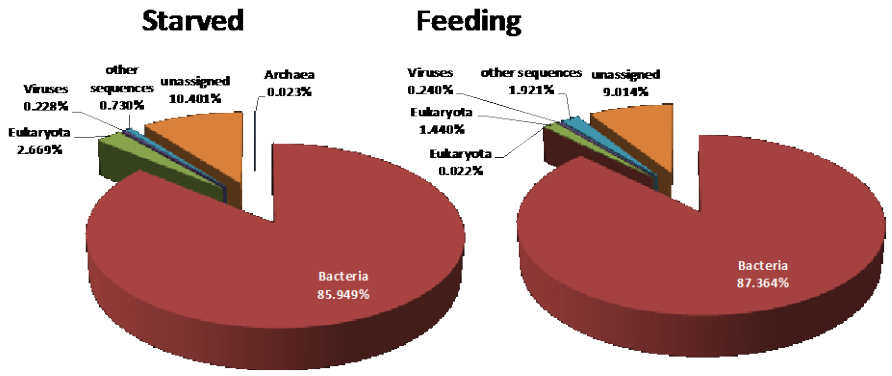


Figure 1. Distribution of western corn rootworm gut metagenome sequences among different phyla from both starved and feeding 3rd instars.

Additional analyses of the gut metagenome from both western and western corn rootworms are currently in progress to better understand the role that these organisms play in digestive physiology and as possible tools that be exploited for sustainable pest management systems.

Layperson's Summary

Summarize in one or two paragraphs the most significant scientific accomplishments of the Project during the reporting period. Use language that a non-scientist can understand.

The most significant accomplishments of the project involve completing the sequencing of the western corn rootworm midgut transcriptome. We have increased significantly the number of genes that that have been identified, and have uncovered whole families of plant cell wall degrading enzymes not previously identified. From this effort, over 30,000 unique sequences have been identified from which we hope to identify enzymes with unique properties that are specifically adapted to digest biomass with the potential for increased efficiency of biomass conversion to ethanol and may provide insight into novel control strategies for this important pest species. These sequences also provide important information that allow identification of novel mechanisms to control this economically important pest species.

Scientific Accomplishments

Describe in two to ten pages, excluding tables and figures, the most significant scientific accomplishments of the Project during the reporting period.

This project was initiated in 2010 with matching resources from our corporate partner, Pioneer Hi-Bred International. Significant progress has been made with this funding on both objectives. Please see progress on objectives (above) for specific information on accomplishments.

Publications and Presentations

List all talks, posters, scientific publications, news releases, etc., about this Project during the reporting period. Provide one copy of each publication, report, or news article resulting from activities supported under the grant as well as any announcements, press releases, statements or photographs depicting Project activities.

1. *A manuscript summarizing the identification and phylogenetic analysis of glycolsyl hydrolases has been published in PLoS One.*

Euyn, S., H. Wang, Y. Pauchet, R.H. ffrench-Constant, E.N. Moriyama, and B.D. Siegfried. 2014. Molecular evolution of glycoside hydrolase genes in the western corn rootworm. PLoS ONE 9: e94052. doi: 10.1371/journal.pone.0094052.

2. A second manuscript describing the recombinant expression of GHF45 has been published in Journal of Insect Science.

Valencia, A., H. Wang, and B.D. Siegfried. *2014.* Expression and characterization of a recombinant endoglucanase from the western corn rootworm, *Diabrotica virgifera virgifera* in *Pichia pastoris*. *J. Insect Science*. (*In press*)

3. A manuscript describing the role of horizontal gene transfer in coleopterans has been published.

Kirsch, R., L. Gramzow, G. Thiessen, B.D. Siegfried, R.H. ffrench-Constant, D.G. Heckel, Y. Pauchet. Horizontal gene transfer and functional diversification of plant cell wall degrading polygalacturonases: Key Events in the Evolution of Herbivory in Beetles. Insect Biochem. Molec. Biol. 52: 33-50.

4. A manuscript describing the cell-free expression and characterization of a western corn rootworm endoglucanase has been submitted for publication.

Valencia, A. H. Wang, and B.D. Siegfried. Expression and purification of an insect endoglucanase using an *E. coli*-based cell-free system. Protein Express. Purif. (*Submitted*).

5. A manuscript describing genes involved in the RNA interference pathway have been submitted for publication.

Khajuria, C., K. Narva, and B.D. Siegfried. Functional analysis of four RNAi pathway genes in an economically important corn pest, Western corn rootworm (*Diabrotica virgifera virgifera* Le Conte). PLoS One (In revision).

6. A manuscript has been submitted describing two gene involved in embryonic development that can be effected by parental RNAi.

Khajuria, C., A.M. Velez, H. Wang, E.Fishilevich, M.L. Frey, N. Carneiro, P. Gandra, K.P. Narva. and **B.D. Siegfried**. Parental RNA interference of genes involved in embryonic development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Insect Biochem. Molec. Biol. (In revision)

7. A manuscript describing housekeeping genes for quantitative RT-PCR standardization has been published.

Rodrigues, T.B., C. Kahjuria, H. Wang, N. Matz, D.C. Cardoso, and B.D. Siegfried. 2014. Validation of reference housekeeping genes for gene expression studies in the western corn rootworm (*Diabrotica virgifera virgifera*). PLoS One 9(10):e109825. doi: 10.1371/journal.pone.0109825.

8. A manuscript describing the identification and expression of three carbon dioxide receptor genes has been submitted for publication.

Rodrigues, T.B., E.N. Moriyama, H. Wang, C. Khajuria, and B.D. Siegfried. Carbon dioxide receptor genes and their expression profile in *Diabrotica virgifera virgifera.* BMC Genomics (*Submitted*)

PDF copies of published manuscripts are attached

Invited Presentations

DATE	AUDIENCE	TITLE	LOCATION
December 2011	Entomological Society of America; Section Symposium, State of the art Molecular Research of Global Interest	Developing RNA interference as a tool for target site discovery in western corn rootworms	Reno, NV
March 2012	Colorado State University, Department of Crop and Soil Sciences	Developing sustainable approaches to corn insect pest management	Fort Collins, CO
October 2012	Monsanto Corn Rootworm Academic Summit	Developing genomic tools for sustainable rootworm pest management	St. Louis, MO
November 2012	University of Illinois	New technologies for management of corn rootworms	Champaign-Urbana, IL
November 2012	Entomological Society of America, Annual Meeting	Developing methods for RNAi interference in western corn rootworms	Knoxville, TN
April 2013	Bayer Crop Sciences	Developing sustainable approaches to corn insect pest management	Raleigh, NC

DATE	AUDIENCE	TITLE	LOCATION
June 2013	IOBC/WPRS Working Group; 6th Meeting on Ecological Impact of GMO's	Keynote Address: RNA interference in pest management: Opportunities and potential risks	Berlin, Germany
November 2013	Entomological Society of America, Annual Meeting	Rootworm transcriptomes and RNAi: Understanding Gene Function and Identifying Novel Target Sites	Austin, TX
March 2015	Department of Entomology, University of Georgia	A 30 year partnership with Western corn rootworms: Updates on new technologies and resistance evolution	Athens, GA
April 2015	Department of Plant Sciences, University of Missouri	A 30 year partnership with Western corn rootworms: Updates on new technologies and resistance evolution	Columbia, MO

Technology Transfer

Describe all technology transfer (inventions, disclosures, patents, licensing agreements, etc.) resulting from the Project during the reporting period.

A number of disclosures have been submitted to the University of Nebraska Office of Research and Economic Development related to RNAi interference and rootworm pest management.

Commercial Accomplishments

Describe the most significant accomplishments resulting from the Project during the reporting period.

While commercial outcomes related to identification of enzymes important to cellulosic ethanol production have yet to be realized, we have made significant accomplishments related to the potential for RNAi as a pest management tool. These accomplishments would not have been feasible without the genomic resources that were made available through CPBR funding.

Educational Accomplishments

Describe the most significant educational accomplishments resulting from the Project during the reporting period. (Number of students, post-docs, etc. trained)

Dr. Arnubio Valencia has made several visits to Nebraska to work on characterization of cellulosed degrading enzymes. He also received advanced training in bioinformatics that he has been able to use in his own research program at his home institution in Colombia.

Dr. Seong-il Eyun received his Ph.D. in bioinformatics using research funded by this project.

Dr. Thais Rodrigues was a visiting graduate student who worked in our laboratory for 12 months and was supported through a Brazilian fellowship. Her research involved identifying and characterizing CO_2 receptor genes identified from our transcriptome.

Numerous undergraduate and graduate students working on this and related projects received advance training in entomology, molecular biology, and bioinformatics.

Additional Funding

List any additional funding generated as a result of the Project during the reporting period.

A proposal to the USDA Biotechnology Risk Assessment Grant Program has been funded that utilizes the transcriptome resources generated from this project to conduct expression profiling of Bt resistant and susceptible strains of western corn rootworms and to potentially identify molecular markers for resistance. We have also received additional support the Nebraska Corn Board to investigate the potential for CO_2 receptors to serve as viable targets for pest management. A proposal to the Monsanto Corn Rootworm Knowledge Program to investigate pyrethroid resistance in western corn rootworms that will utilize our transcriptome resources. We have also received funding through the USDA NIFA program on Bioenergy to identify changes in gene expression associated with alternative host plant usage.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

We currently have no personnel supported through CPBR funding. All remaining funding has been used for further sequencing efforts.