Report Period:
September 30, 1999
through March 31, 2000

Energy Consortium for Future
Technology Research

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Semi-Annual Technical Report

The Department of Energy

Cooperative Agreement

DE-FCO5-92OR22072

Submitted By

Dorin Schumacher, Ph.D.

President

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THE CONSORTIUM FOR PLANT BIOTECHNOLOGY RESEARCH, INC.

SEMI-ANNUAL TECHNICAL REPORT
SEPTEMBER 30, 1999 THROUGH MARCH 31, 2000
FOR
DE-FCO5-92OR22072
TO
UNITED STATES DEPARTMENT OF ENERGY

OBJECTIVES:

The Consortium for Plant Biotechnology Research, Inc. ("CPBR") continues to operate according to objectives outlined in the proposal funded through the cooperative agreement. The italicized objectives below are addressed in this report, which covers the period September 30, 1999 through March 31, 2000.

1. Update the research agenda using information obtained from member companies.
2. *Identify and implement research projects that are deemed by industrial, scientific, and sponsoring agency evaluation to address significantly the problems and future of U.S. energy resources and that are relevant to the Department of Energy's mission.*

Specifically,

- *Announce research grants competition through a Request for Preproposals.*
- *Conduct a dual-stage review process:*
 - Stage one: industrial and DOE review of preproposals.*
 - Stage two: peer review, scientific consultants' review, DOE review of full proposals and Project Recommendation Committee evaluation and recommendation for funding.*
- *Board of Directors approval of recommended awards.*
- *Conduct ongoing project management.*
- *Obtain semiannual, annual and final reports for evaluation of research goals and technology transfer.*
- *Present reports to DOE.*

MAJOR ACCOMPLISHMENTS:

Governance:

No changes to report.

Administrative matters:

Ms. Helen Alexander left the position of Grants Fiscal Manager.

Federal Sponsors:

U.S. Department of Energy.

Energy from Biomass Competitions:

Awards in the CPBR 2000 Energy From Biomass Competition have not yet been made pending release of the FY 2000 CPBR appropriation.

The CPBR 2001 Energy From Biomass Competition was initiated with the Request for Preproposals issued in December 1999. A total of 51 new preproposals were received, most of them transmitted electronically. An additional nine preproposals representing fundable proposals from the 2000 competition were added to the new preproposals sent for industrial review.

In order to conserve financial resources for research, the symposium scheduled for March, 2000, was postponed. Evaluation of preproposals by industry was conducted by mail with the preproposals sent on a CD in PDF format. DOE was given the opportunity to evaluate the preproposals.

Invitations were issued in March inviting submission of 74 full proposals in the 2001 competition. Of the preproposals continuing in the competition, 44 are new; the remaining invitations represent top proposals considered fundable in the 2000 competition, but not yet funded.

New member program:

- Several universities requested and were provided membership information.
- Membership information was provided upon request to several companies.
New corporate members include:
Common Ground Group USA, LLC, and
Renessen LLC.
- Due to reduced funding, CPBR staff was unable to exhibit and/or attend national conferences to disseminate research information.

Evaluation of project results:

Thirty-three semi-annual or final scientific progress reports (copies attached) were received during the reporting period. In addition, the following information was reported.

Technology transfer (e.g., inventions, patents, disclosures or licensing agreements) was reported by the following investigators during this reporting period:

Invention disclosures:

INVENTORS	TITLE
Eric Triplett University of Wisconsin-Madison	<i>Eukaryotic nitrogen fixation by molybdopterin-containing enzymes</i>
Richard Vierstra, Joseph Walker University of Wisconsin-Madison	<i>Vector for the simultaneous and stoichiometric expression two proteins from one gene in plants. The coding regions for each gene is connected by the coding region aor a ubiquitin gene. Following expression of the fusion protein, the individual polypeptides are released by endogenous proteases.</i>

Patent disclosures:

INVENTORS	TITLE
Sandra Austin-Phillips, Richard R. Burgess, Thomas L. German, Thomas Ziegelhoffer University of Wisconsin-Madison	Patent Number 5,981,835 <i>Transgenic plants as an alternative source of lignocellulosic-degrading enzymes</i>
J. Jiang and F. Dong University of Wisconsin-Madison	Patent Application <i>DNA sequences specific to rice centromeres.</i>
A.S. Kelley, N.B. Mantzasris, F. Srienc University of Minnesota	US Patent Application 09/392,465 <i>Novel biopolymers and biopolymer blends and methods for producing same</i>
Basil Nikolau Iowa State University	US Patent Application 60/090,717 <i>Materials and methods for the alteration of acetyl-CoA levels in plants</i>
Basil Nikolau Iowa State University	International Patent Application PCT/US99/14382 <i>Materials and methods for the alteration of acetyl-CoA levels in plants</i>
Hee Sook Song, Jeffrey E. Brotherton, Jack Widholm University of Illinois at Urbana-Champaign	Patent Number 5,965,727 <i>Selectable marker and promoter for plant tissue culture transformation (10/12/99)</i>
Friedrich Srienc, David A. Somers University of Minnesota	Patent Application 09/392,465 <i>Biodegradable Plastics from Yeast and Plants</i>

INVENTORS	TITLE
Eric Triplett University of Wisconsin-Madison	Patent Number 5,858,762 <i>Plasmid for transformation of root nodule bacteria (1/12/99)</i>
Eric Triplett University of Wisconsin-Madison	Patent Number 5,906,929 <i>Enhanced inoculant for soybean cultivation (5/25/99)</i>

Metrics Reports

Thirteen Metrics Reports from sponsoring companies were received (copies attached).

Cumulative Technology Transfer Activities to Date:

53 inventions disclosed.

43 patents awarded or applied for.

23 inventions reported to be licensed to multiple licensees or under negotiation.

IMPLEMENTATION AND EVALUATION OF PROGRESS:

2000 PROJECTS

Energy from Biomass/Biofuels – Transportation and Power

DOE funding not released. Awards not yet made.

1999 PROJECTS

Energy from Biomass/Biofuels – Transportation and Power

Harvey D. Bradshaw , University of Washington <i>Map-Based Cloning of Genes To Increase Poplar Biomass</i>	IN PROGRESS <i>Report Enclosed</i>
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Stanton S. Gelvin , Purdue Research Foundation <i>Plant Genes Involved in T-DNA Integration and Radiation Sensivity</i>	IN PROGRESS <i>Report Enclosed</i>
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Jean T. Greenberg , The University of Chicago <i>Engineering Artificial Immunity to Plant Pathogens</i>	IN PROGRESS
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Robert Haselkorn , The University of Chicago <i>Increasing the Energy of Plants: Molecular Genetics of Acetyl-COA Carboxylase</i>	IN PROGRESS <i>Report Enclosed</i>
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David Hildebrand , University of Kentucky <i>Engineering Oilseeds for Epoxy Fatty Acid Accumulation</i>	IN PROGRESS <i>Report Enclosed</i>
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Lonnie O. Ingram , University of Florida <i>Ethanol Production from Uronic Acid-Substituted Xylose Residues in Hemicellulose Hydrolysates</i>	IN PROGRESS <i>Report Enclosed</i>
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Jiming Jiang , University of Wisconsin <i>Toward Cloning a Functional Rice Centromere</i>	IN PROGRESS <i>Report Enclosed</i>
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Gayle Lamppa , The University of Chicago <i>Accumulation of Products Within the Plastid for Biomass Conversion: Test System with Cellulase</i>	IN PROGRESS <i>Report Enclosed</i>
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Yi Li , The University of Connecticut <i>Genetic Improvement of Seed Productivity for Bioenergy Crops</i>	IN PROGRESS <i>Report Enclosed</i>
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John B. Ohlrogge , Michigan State University Christoph Benning <i>DNA Microarray Discovery of Gene and Networks Which Control Plant Seed Storage Products</i>	IN PROGRESS <i>Report Enclosed</i>
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Brenda Oppert , Kansas State University <i>Evaluation of Insect Serpins as Biopesticides</i>	IN PROGRESS <i>Report Enclosed</i>
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Eric W. Triplett , University of Wisconsin-Madison <i>Associative Nitrogen Fixation by Diazotrophic Endophytes In Switchgrass</i>	IN PROGRESS <i>Report Enclosed</i>
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Richard Vierstra , University of Wisconsin-Madison <i>Development of Vectors for the Stoichiometric Accumulation of Multiple Proteins in Transgenic Crops</i>	IN PROGRESS
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1998 PROJECTS

Energy from Biomass

Richard Amasino , University of Wisconsin <i>Regulation of Plant Senescence</i>	IN PROGRESS <i>Report Enclosed</i>
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Zong-Ming Cheng , North Dakota State University <i>Evaluation and Characterization of Rooting Capability of Hybrid Aspens Transformed With Rooting Genes</i>	IN PROGRESS <i>Report Enclosed</i>
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Richard B. Meagher , University of Georgia <i>Phytoremediation and Phytomining of Marginal Lands: Direct Evolution of Mercuric Ion Reductions to Reduce a Wide Repertoire of Heavy Metal Ions</i>	TERMINATED
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Scott A. Merkle , University of Georgia <i>Clonal Propagation of Hybrid Southern Hardwoods</i>	IN PROGRESS
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Basil J. Nikolau , Iowa State University <i>How Do Plants Generate Acetyl-CoA</i>	IN PROGRESS <i>Reports Enclosed</i>
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Peter J. Reilly , Iowa State University <i>Glucoamylase Mutagenesis to Increase Glucose Yield and Reduce Energy Use</i>	CLOSED <i>Final Report Enclosed</i>
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Friedrich Srienc , University of Minnesota <i>Biodegradable Plastics from Yeast & Plants</i>	IN PROGRESS <i>Report Enclosed</i>
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Steven H. Strauss , Oregon State University <i>Genes Controlling the Transition Between Vegetative and Reproductive Phases in Forest Trees</i>	IN PROGRESS <i>Report Enclosed</i>
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Eric Triplett , University of Wisconsin—Madison <i>Engineering Sinorhizobium for Increased Alfalfa Biomass</i>	IN PROGRESS <i>Report Enclosed</i>
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Jack M. Widholm , University of Illinois <i>A New Selectable Marker and Promoters Of Plant Origin</i>	IN PROGRESS <i>Report Enclosed</i>
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1997 PROJECTS

Energy from Biomass

Harvey Bradshaw , University of Washington <i>Microsatellite (SSR) Marker Map for Populus</i>	CLOSED <i>Final Report Enclosed</i>
Lars Ljungdahl , University of Georgia <i>Novel Hydrolytic Enzymes from Anaerobic Fungi Degrading Biomass</i>	CLOSED <i>Final Report Enclosed</i>
Lee Lynd , Dartmouth College <i>Pretreatment Process for Cellulosic Biomass</i>	IN PROGRESS <i>Report Enclosed</i>
John Ohlrogge , Michigan State University <i>Increasing Plant Oil Synthesis via Genetic Engineering of Acetyl-CoA Carboxylase</i>	IN PROGRESS
Martin Spalding , Iowa State University <i>Coordinated Expression of Multiple Anti-pest Proteins</i>	CLOSED <i>Final Report Enclosed</i>
David Somers , University of Minnesota <i>Incorporation of Value-Added Traits into Alfalfa for Biomass Energy</i>	IN PROGRESS <i>Report Enclosed</i>
John Davis , University of Florida <i>Molecular Biology of Defense Responses in Populus</i>	IN PROGRESS
John W. Dudley , University of Illinois <i>High Starch Adds Value to Corn for Production</i>	IN PROGRESS
Richard Vierstra , University of Wisconsin <i>Targeted Proteolysis: New Method for Removing Selected Intracellular Proteins</i>	IN PROGRESS <i>Report Enclosed</i>

1996 PROJECTS

Energy from Biomass

David Hildebrand , University of Kentucky <i>Cloning Genes Encoding Enzymes for Epoxy Fatty Acid Accumulation in Oilseeds</i>	CLOSED <i>Final Report Enclosed</i>
Yi Li , Kansas State University (Transferred to U.Conn.) <i>Genetic Improvement of Aspen for Wood Production</i>	IN PROGRESS
Kenneth Nickerson , University of Nebraska <i>Bacillus thuringiensis: Biotin Mediated Insect Toxicity Suggests Alternate Strategies for Pest Control in Energy Crops</i>	IN PROGRESS <i>Report Enclosed</i>

Electric Power from Biomass

Zong-Ming Cheng, North Dakota State University
*Purification of a Broad-Spectrum Antimicrobial Protein
and its Gene Isolation*

CLOSED
Final Report Enclosed

National Ethanol Research Institute

Ravindra Datta, University of Iowa
Development of an On-Board Ethanol Reformer

CLOSED
Final Report Enclosed

Donald L. Van Dyne, University of Missouri
*The Technical and Economic Feasibility of Converting
Ligno-Cellulosic Feedstocks to Ethanol*

CLOSED
Final Report Enclosed

Historically Black Colleges and Universities (HBCU) Initiative

Shang-Tian Yang, The Ohio State University
Keith A. Schimmel, North Carolina State A&T University
*Novel Metabolic and Process Engineering Approached for
Enhanced Propionic Acid Production*

CLOSED
Final Report Enclosed

1995 PROJECTS

Historically Black Colleges and Universities (HBCU) Initiative

Frans deBruijn, Michigan State University
Edison R. Fowlks, Hampton University
Frank Louws, North Carolina State University
Automated Fluorescent Genomic Fingerprinting of Bacteria

IN PROGRESS
Report Enclosed

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Richard Amasino	
University:	University of Wisconsin	
Agreement Number:	OR22072-75	
Project Title:	Regulation of plant senescence	
Reporting Period and Report Type:	From: 9/1/98 To:	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

1. Determine the senescence-delaying efficiency of the SAG 12 promoter compared to the SAG 13 promoter when these promoters are used to drive expression of a gene encoding a cytokinin biosynthetic enzyme IPT in transgenic plants.

We have analyzed not only the senescence-delaying phenotype of SAG13 driving IPT but have also evaluated the senescence-delaying phenotype of a senescence-specific sugar transporter promoter in this regard. We continue to confirm that the SAG12 promoter provides the greatest senescence specificity; i.e., the SAG12 promoter IPT construct only affects senescence whereas the other constructs affect other aspects of plant development.

2. Identify the regulatory elements of SAG 12 and 13 that confer senescence-specific expression and the regulatory factors that bind to these elements.

We have identified a region of the SAG12 promoter that confers senescence specific expression. This was done by a combination of deletion analysis and cloning manipulations as well as by comparison of Arabidopsis and Brassica SAG12 promoters. The latter approach provides a new and generally applicable model for the identification of regulatory elements. Regulatory

elements tend to be conserved so by establishing that the Brassica gene can function in Arabidopsis and therefore must contain regulatory elements recognized by the Arabidopsis transcription machinery, we have compared the sequences from the two species and identified a regulatory element. This work has resulted in two publications this year:

Noh, Y.-S. and R. M. Amasino. 1999. Identification of a promoter region responsible for the senescence-specific expression of SAG12. *Plant Molecular Biology* 41 (2):181-194

Noh, Y.-S. and R. M. Amasino. 1999. Regulation of developmental senescence is conserved between Arabidopsis and Brassica napus. *Plant Molecular Biology* 41 (2):195-206

3. Identify regulatory genes in the senescence program.

We have identified five mutants in which senescence is accelerated. These mutants arose in our activation tagging efforts listed under objective 5. The mutants behave dominantly which indicates that they are in fact due to gene activation. Efforts are underway to identify the genes that have been revealed by this approach.

In a related effort we have identified mutations in genes that are involved in sugar metabolism to test the hypothesis that these genes are involved in senescence. In the near future we will have homozygous mutant lines for evaluation.

4. Investigate the functions of SAG gene products.

We have studied the expression and function of a senescence-specific sugar transporter. Functional studies include a determination of sugar levels in plants that either overexpress the gene or have a loss-of-function mutation in the gene. In this next period we will publish the results of these studies.

We have collaborated with other groups to determine the effect of altered senescence on nitrogen metabolism and the expression pattern of SAG12 during pathogen responses. We have also examined several other genes for the connection between pathogen responses and senescence.

Finally we have studied the difference in protein and mRNA accumulation patterns for a SAG gene that we had discovered, ERD1.

This work has resulted in four publications this year:

Pontier, D., S. Gan, R. M. Amasino, D. Roby and E. Lam. 1999 Markers for hypersensitive response and senescence show distinct patterns of expression. *Plant Mol. Biol.* 39: 1243-1255

- W. Jordi, A. H. C. M. Schapendonk, E. Davelaar, G. M. Stoop, C. S. Pot, R. de Visser, J. A. van Rhijn, S. Gan and R. M. Amasino. Increased cytokinin levels in transgenic P_{SAG12}-IPT tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning. *Plant Cell & Environment*, in press.
- Quirino, B., J. Normanly and R. M. Amasino. 1999. Diverse range of gene activity during *Arabidopsis thaliana* leaf senescence includes pathogen-independent induction of defense-related genes. *Plant Mol. Biol.* **40**: 267-278
- Weaver, L. M., J. E. Froelich and R. M. Amasino. 1999. The chloroplast-targeted ERD1 protein declines during senescence while its mRNA increases. *Plant Physiol.* **119**: 1209-1216.

5. Saturate the genome of *Arabidopsis* with an insertion mutagen that activates nearby genes to identify genes that can promote or delay senescence and genes that affect biomass production.

See 3 above for the identification of senescence genes by this approach. We have also identified a collection of gene that affect plant stature. These are potentially useful in agriculture because much of the recent increase in crop yields come from alterations of stature such as dwarfing genes and gene that permit high density growth.

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 the regulatory elements of a gene that confers senescence specific expression. This will enable other genes to be expressed in a senescence-specific manner with a greater degree of control than was previously available. We have also identified genes that can regulate the leaf senescence program. These genes have been identified by genetic screens and the cloning of these genes is underway.

Scientific Accomplishments

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

One of our most significant accomplishments has been to examine the details of the regulation of SAG12, an *Arabidopsis* gene encoding a cysteine protease, which is expressed only in senescent tissues. Studies of the expression patterns of a variety of genes showing senescence-specific or senescence-preferential expression indicate that plant senescence involves multiple regulatory pathways. We have shown that the expression of SAG12 is specifically activated by developmentally controlled senescence pathways but not by stress- or hormone-controlled pathways. Using SAG12 as a molecular marker for the study of developmental

senescence, we demonstrated that cytokinin, auxin, and sugars can repress developmental senescence at the molecular level. Studies using promoter deletions and recombination of promoter fragments indicate that a highly conserved region of the SAG12 promoter is responsible for senescence-specific regulation, while at least two other regions of the SAG12 promoter are important for full promoter activity. Extracts from young and senescent *Arabidopsis* leaves contain factors that exhibit differential binding to the senescence-responsive promoter element.

As a first step to determine if the program of senescence-specific gene expression is conserved in plants, we isolated two SAG12 homologs (BnSAG12-1 and BnSAG12-2) from *Brassica napus*. Structural comparisons and expression studies indicate that these two genes are orthologs of SAG12. The expression patterns of BnSAG12-1 and BnSAG12-2 in *Arabidopsis* demonstrate that the senescence-specific regulation of this class of cysteine proteases is conserved across these species. Gel-shift assays using the essential promoter regions of SAG12, BnSAG12-1, and BnSAG12-2 show that the extent of binding of a senescence-specific, DNA-binding protein from *Arabidopsis* is proportional to the expression levels of these genes in *Arabidopsis*. Therefore, the expression levels of these genes may reflect the affinities of the senescence-specific DNA-binding protein for the promoter element. The comparison of promoter sequence elements across species provides a new and generally applicable model for the identification of regulatory elements.

We have further explored the range gene activity associated with leaf senescence and have identified genes that show preferential transcript accumulation during this developmental stage. The mRNA levels of a diverse array of gene products increases during leaf senescence, including a protease, a ribosomal protein, 2 cinnamyl alcohol dehydrogenases, a nitrilase and glyoxalase II. Two of the genes identified are known to be pathogen-induced. The senescence specificity of each gene was determined by characterization of transcript accumulation during leaf development and in different tissues. The increased expression of nitrilase in senescent leaves is paralleled by an increase in free indole-acetic acid (IAA) levels. Additionally, we have demonstrated that the induction of defense-related genes during leaf senescence is pathogen-independent and that salicylic acid accumulation is not essential for this induction. Our data suggests that the induction of certain genes involved in plant defense responses is an integral component of the leaf senescence program.

We have studied the expression and function of a senescence-specific sugar transporter. Functional studies include a

determination of sugar levels in plants that either overexpress the gene or have a loss-of-function mutation in the gene. In this next period we will publish the results of these studies.

An important issue in leaf senescence research is the nature of the proteases that are responsible for the turnover of leaf proteins. *Arabidopsis thaliana* ERD1 is a ClpC-like protein that sequence analysis suggests may interact with the chloroplast-localized ClpP protease to facilitate proteolysis. The mRNA encoded by the ERD1 gene has previously been shown to accumulate in response to senescence, as well as to a variety of stresses and hormones. Here we show that the ERD1 protein, in contrast to the ERD1 mRNA, strongly declines in abundance with age, becoming undetectable in fully expanded leaves. Sequence analysis also suggests that ERD1 is chloroplast targeted, and we show in an in vitro system that the native protein is properly imported, processed, and present within the soluble fraction of the chloroplast, presumably the stroma. We show that ClpP protein, which is also present in the stroma, declines with age in parallel with ERD1. These results are consistent with ERD1 and ClpP interacting, but suggest that it is unlikely that either plays a major role during senescence. Certain other chloroplast proteins decline with age coordinately with ERD1 and ClpP, suggesting that these declines are markers of an early age-mediated change that occurs within the chloroplast.

A major question in senescence about which little is known is how the process is regulated. To lay the foundation for future progress in this area we have performed a large scale mutant screen for genes that regulate senescence. We identified five mutants in which senescence is accelerated. These mutants arose in our activation tagging efforts listed under objective 5. The mutants behave dominantly which indicates that they are in fact due to gene activation. Efforts are underway to identify the genes that have been revealed by this approach. In a related effort we have identified mutations in genes that are involved in sugar metabolism to test the hypothesis that these genes are involved in senescence. In the near future we will have homozygous mutant lines for evaluation. We expect that these genetic approaches will provide the major focus for future efforts from our lab.

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.

In addition to the following publications, my lab has been asked to give two talks at the upcoming (July 2000) Gordon Conference on Plant Senescence, Abscission, and Programmed Cell Death.

Noh, Y.-S. and R. M. Amasino. 1999. Identification of a promoter region responsible for the senescence-specific expression of SAG12. *Plant Molecular Biology* 41 (2):181-194

Noh, Y.-S. and R. M. Amasino. 1999. Regulation of developmental senescence is conserved between *Arabidopsis* and *Brassica napus*. *Plant Molecular Biology* 41 (2):195-206

Pontier, D., S. Gan, R. M. Amasino, D. Roby and E. Lam. 1999. Markers for hypersensitive response and senescence show distinct patterns of expression. *Plant Mol. Biol.* 39: 1243-1255

W. Jordi, A. H. C. M. Schapendonk, E. Davelaar, G. M. Stoop, C. S. Pot, R. de Visser, J. A. van Rhijn, S. Gan and R. M. Amasino. Increased cytokinin levels in transgenic PSAG12-IPT tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning. *Plant Cell & Environment*, submitted.

Quirino, B., J. Normanly and R. M. Amasino. 1999. Diverse range of gene activity during *Arabidopsis thaliana* leaf senescence includes pathogen-independent induction of defense-related genes. *Plant Mol. Biol.* 40: 267-278

Weaver, L. M., J. E. Froelich and R. M. Amasino. 1999. The chloroplast-targeted ERD1 protein declines during senescence while its mRNA increases. *Plant Physiol.* 119: 1209-1216.

Technology Transfer

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

Our technology for regulating leaf senescence continues to be licensed and developed by several agricultural biotech companies. More details could be provided after a discussion with the Wisconsin Alumni Research Foundation (<http://www.wisc.edu/warf>).

Commercial Accomplishments

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

This past summer there was a successful field trial of our technology and a larger scale trial is planned for this coming year.

Educational Accomplishments

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

The major educational accomplishments were the training of two graduate students. I have also given several presentations on the use of biotechnology in agriculture. The most recent one may be viewed over the internet at www.biotech.wisc.edu/lectures

Additional Funding

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

None beyond our matching fund sources.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

No changes.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	H.D. Bradshaw, Jr.	
University:	University of Washington	
Agreement Number:	OR22072-63	
Project Title:	Microsatellite (SSR) marker map for <i>Populus</i> Alternate title: Increasing woody biomass for energy by molecular breeding	
Reporting Period and Report Type:	From: 1 July 1997 To: 30 June 1999	Check one: ☐ Interim Report ☒ Final Report

Project Objectives

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

The specific objectives of this proposal over a two-year period are to:

- **Construct and characterize a genomic library of *Populus* DNA enriched for SSR sequences. This library could be used to produce a very large number of SSR markers, beyond the number proposed below.**

This was completed. More than 1200 microsatellite-containing genomic clones were isolated and sequenced.

- **Develop primer pairs for at least 100 highly-polymorphic SSR markers from *Populus*.**

A total of 132 SSR primer pairs were developed.

- **Test these 100 markers for their utility by placing them on a current genome map consisting of more than 500 markers of other types.**

Most of the developed SSR markers have been mapped, and this information is included on the website.

- **Determine the informativeness of each marker by estimating levels of polymorphism in *Populus* species from the major sections within the genus and in *Salix*.**

Tests for polymorphisms have been with each marker in the three sections (4 species) of the genus *Populus* with the greatest economic importance, and in willows (*Salix*). This information is included on the website.

- **Assess synteny among mapped markers in at least 3 full-sib families from pure species and hybrid pedigrees.**

A subset of markers have been mapped in two full-sib families. Since we found no examples of markers linked in one pedigree being unlinked in another, we discontinued this line of investigation. For all practical purposes the SSR markers may be considered fixed on the *Populus* map.

- **Make SSR primer pair sequences and their map positions available to the scientific community via the World Wide Web.**

All 132 *Populus* SSR primer pairs are currently available to the public on our website, acknowledging the matching contributions of the CPBR and the PMGC. Scientists from around the world have begun using the markers to align genetic maps within the genus *Populus*, and for other purposes such as genetic fingerprinting. The URL is:

<http://poplar2.cfr.washington.edu/pmgc/ssr/pmgcssr.htm>

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.

This project produced more than 100 new genetic markers for fast-growing hybrid poplar trees. These new genetic markers have two important characteristics which were not available before. First, the markers are 'universal'; that is, they are the only set of genetic markers which have the same position on the chromosomes of all poplar species. This allows genetic maps constructed in one hybrid poplar to be compared directly with that of another, making it possible to identify genes important for rapid growth, disease resistance, and biomass feedstock quality. Second, these new SSR markers reveal more of the genetic diversity present in hybrid poplar, making them useful for such practical applications as genetic fingerprinting to assure that the correct poplar strains are planted in a particular geographic area.

Scientific Accomplishments

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

Genetic markers are widely used in plant breeding and molecular biology, primarily for constructing genetic linkage maps of the chromosomes. Perhaps the most important application of markers and maps has been the detection and characterization of quantitative trait loci (QTLs) controlling important traits such as yield, quality, and resistance to stress, diseases, and pests.

Many of the first QTL mapping studies were performed with 'anonymous' dominant genetic markers, such as RAPDs or AFLPs. While useful, these markers are limited in utility because of their dominant mode of inheritance and lack of transferability among pedigrees (due to the anonymous nature of the markers). Now that the value of QTL mapping has been demonstrated

with primitive genetic marker systems, the development of highly-informative, locus-specific, portable markers is needed.

Microsatellite (SSR) markers have become the 'gold standard' in linkage mapping studies. They are PCR-based (requiring only small amounts of crude DNA), codominant, and highly polymorphic. In addition to their value for QTL mapping, SSRs are useful for day-to-day practical purposes such as genetic fingerprinting, assessment of genetic diversity, and paternity analysis.

The goal of this project was to develop at least 100 SSR markers for trees in the genus *Populus*, so that a 'universal' genetic map for these fast-growing trees could be produced and shared among researchers around the world. This goal has been met and exceeded, with 132 SSR primer pairs now published on the World Wide Web

(<http://poplar2.cfr.washington.edu/pmgc/ssr/pmgcssr.htm>).

I have heard from at least a dozen researchers worldwide that the SSR markers are in use in their laboratories. In my own lab, we have used the SSRs for QTL mapping (Frewen *et al.* 1999), as well as for genetic fingerprinting of hybrid poplar clones for the benefit of members of the Poplar Molecular Genetics Cooperative.

The markers have proven to be versatile, with the majority of them working in all economically important sections of the genus *Populus* (Table 1). Some of the primers also work in the related genus of willows (*Salix*), another woody biomass crop (Table 1).

Thanks to joint funding between the PMGC and CPBR, a common set of 'universal' genetic markers will assure that hybrid poplar researchers around the world can compare the results of genetic experiments, speeding the genetic improvement of poplar for biomass production.

Table 1. SSR markers for *Populus*

	Locus name	Linkage group*	Primer1 seq	Primer2 seq	Known to be polymorphic in these <i>Populus</i> species*
1	PMGC14	?	FTCAGAATGTGCATGATGG	GTGATGATCTCACCGTTTG	TDT _o
2	PMGC61	C	GATCCCTCTGCACCGTTTAC	ACCCTAAATTGCTGACAAC	TD
3	PMGC93	A	ATCATGCGTTCGGCTACAGC	CTCAAACCTCAAAGTGTATAAC	TD
4	PMGC108	?	TGCAGGTGATGTCATCACCG	AACCGAATCCATGCGTCACC	TDT _o
5	PMGC204	?	GAAGATAAATTCTCCAGCTC	TAACCTTCCCCGCATGT	TD
6	PMGC223	?	CGATGAGGTTGAAGAAGTCG	ATATATGTACCGGCACGCCAC	TT _o
7	PMGC244		CTTAGTTGAAAGTCTCTTAAC	GAATAACAGTTGTTTTTCAG	DT _o
8	PMGC325	?	CGATTTATGACAGACAGCTTG	GTACCGTTGAGGTGGCTAG	TDT _o
9	PMGC333	E	CTTAGTGGTGAAGTATTC	GAGTGGGTGCTGATTCATCC	D
10	PMGC409	C	ACGTATATGAAGTTCTTGATTGC	GACAGATCATTATGATTACTACAG	TD
11	PMGC420	X	ATGGATGAGAAATGCTTGTG	ACTGGCACACTCTTTAACTGG	TD

12	PMGC422		AACCTCGAATTAAGAATAACCC	GTCTCGGTAAAGGTATTGTGCGC	TDN
13	PMGC433	D?	GCAGCATTGTAGAATAATAAAAG	AAGGGGTCTATTATCCACG	TD
14	PMGC451	?	AATTACAACCACTTTAGCATATTC	TGCCGACACATCACACATACC	TD
15	PMGC456	?	TGTAGGAGATATCCACGTGG	AACAATATGCTTCATAGCACAG	TD
16	PMGC486	?	AGAAGTTGTTGAACCCGATGGG	GCTACAAACTTTGTTGTACCC	TDT ₀
17	PMGC510	J	AGTCCTGGTCCTGGATTGG	CTACATTAATTTCCCTGTATC	TDT ₀
18	PMGC520	?	TAACCTACTAGAAAAACCTTTG	TTGCTAGCTAGCTTGTTAG	TDT ₀
19	PMGC562	?	TTTTGGGAGGGGAGTCGAG	ACAACCTCTCAACTTCCTAATC	TDT ₀
20	PMGC571	?	CTGGTACCGATGGAGAAGAC	CAAACCAACAACTCACCGTAC	TDT ₀
21	PMGC573	?	GTCATAATCGCCTATACACAG	GATTGTGAACTCGATCTAAAGG	TD
22	PMGC575	?	TAAATTCATGTAGATTGACG	CTTACTATTTTCATGGTTGTC	TT ₀
23	PMGC576	B	GCTGTCTAACATGCCATTGC	AATTTACATTTCTTTATCATCACC	TDT ₀
24	PMGC607	?	TATTTCTACAACATACCAAAACG	CATTACTCAAGCACATGCACGC	TDT ₀
25	PMGC639	?	AACAAATTTGGCCTGCAGGG	TCAAAATATTATCACTAAACGCG	TDT ₀
26	PMGC648	?	GAAGAATAGGATTACATG	ATAAACTCTCTCCTGTTGATTG	TDT ₀
27	PMGC649	?	CATCCATGATATCAAACCAAATTAG	TGTAATCCAAACATAAAATCCCAAG	TDT ₀
28	PMGC667	M	CATTCGTTCAAGTAGTTAAGGC	GGTTAAGCTACCTCTGCTAC	TD
29	PMGC683	?	CCAGCAATGATTGATTGCTCC	GAGCTTTAACTGTCCAGTAGC	TD
30	PMGC684	M	GAAATTGAATATCTCTCACTTACC	TAATACGTGAAAAGTCAGGTTTTG	TD
31	PMGC690	?	AAAGGAACTTGTTGATGTCAAG	CTTATTTTTCAATCTCGTTTGAGC	TD
32	PMGC2011	E	TCTACGAGGAAAGGGAAGGG	CTTTATAATGCATCATAAAGTTCC	TDT ₀
33	PMGC2015	?	TTTTGGCATTCAAAGACTTGCC	AGTTGATTCCATGTCGTGTCC	T
34	PMGC2020	D	TAAGGCTCTGTTGTTAGTCAG	GAGATCTAATAAAGAAGGTCTTC	TDT ₀
35	PMGC2021	I	TAAGGCTCTGTTTGTAGTCAG	GAGATCTAATAAAGAAGGTCTTC	TDT ₀
36	PMGC2030	?	TCCACAACCTCTGGCTAACC	GGACTACAATGTGCGTGACC	TDT ₀
37	PMGC2055	X	TCAATTATTTAAGCTACTCGCTC	GCAATGTGCCATAAAATGCGTC	TD
38	PMGC2060	L	CTCTCAAATGCTGATTTACCG	TCTTCAGTTGCAGTATTCAAAG	TDT ₀
39	PMGC2084	?	CCCCACCACTAGATTCAGC	GAGTGGTGATGATGGTTGCC	T
40	PMGC2088	?	TCACAAAAGGTTAACGACTTCG	CAGTACTCAGCTGCAGGTCC	TD
41	PMGC2098	A	CACAGTGCCAAAAACAGAGTGG	TCTACTTCATTGTTATTTCATGTTAC	TD
42	PMGC2105	?	ATTTCTCTAGGAAACAACAACC	CCTTAAGATGTTGCTGAACTC	TD
43	PMGC2140	H	GCTGTCAGAATCAAACACTTC	AAGCAGATAACTAAGACATGCC	TDT ₀
44	PMGC2143	?	TCATCATCCATTACTCAACTTG	GCGTAAGAAGCTATTATCGTC	TDT ₀
45	PMGC2156	B	GATCTCTCTTACATCACTCATC	GAATGTCTTTACTCCATTGTTGG	TD
46	PMGC2163	?	CAATCGAAGGTAAGGTTAGTG	CGTTGGACATAGATCACACG	TDT ₀
47	PMGC2217	Y?	ATTAGCTTCTTCTAAAGCAGC	TGACTGACTGTCTGTCTTCG	TDT ₀
48	PMGC2235	I	GCCAAAATAGTAAGTGTGATGG	CACACATTCTCTCATTCAAAGC	TD
49	PMGC2270	I	CAAAAAACATGCAGAAATCTTCAG	TTACAGCTTATATAGCACTACG	TDT ₀
50	PMGC2274	R	GGGGCTAAAATACTTGATGGG	ATCTTCTTCATCATATTTATGTTG	TDT ₀
51	PMGC2289	O	GTCTATCTGTCTGATGTCACC	AAATCTCACATTATAAAAGATTTAG	TDT ₀
52	PMGC2315		CTGTGGTATTTGTGCAATGTG	CAACAGAGCAAACCTTGAGTCG	TD
53	PMGC2316		TACAGGTCAACGCAGTTGAC	TTAGAAGTCAGTGGGATTAGG	TDT ₀
54	PMGC2321		ATTCACATGGCTACTACCATG	AAAACCTTGACATCTTGCGTG	TD
55	PMGC2328		CAAAGGTGAAGTTACAGTCAC	CCATTAGGCCATTATAGACAC	TDT ₀ N
56	PMGC2385		ATTCTTCACCTGGGCAATATG	CTTGGCTGTAAATGACGAGTC	TDN
57	PMGC2392		AAGAGAGATAGCATCACCAAG	TATGTCGAGGAAATCCTTAGC	TD
58	PMGC2408		TAGGTCACTAGAGTGCGTG	CGAAAATGGTAGCTCTAATGCC	TD
59	PMGC2418		AATTTTCTCTCTTACCGCCAG	TGATCCCTCAATGTCTTTACAG	TN
60	PMGC2419		TTTCCCTGTCATCGGCACTG	CATTGGAGACAGCTAATCAGC	TDT ₀ N
61	PMGC2420		GACACCACTTCTAAAGATGGC	ACATGCCTTAGGCTAGTTGC	TDT ₀
62	PMGC2423		AAACGGACAATGATAGTCTTTC	GGATTAGCATGTCCTGAGTC	TDN
63	PMGC2481		CAAAAGAAGGGTAGAGTCTAC	TTCTTCGGTGTGTGTTATTGC	DN

64	PMGC2499	AGAGGGTTTTCAATAACATACC	TATTGGAACCTCTCGTCGACC	TDTto
65	PMGC2500	AATGTCGACCACTCCACGC	AGAGGGTTTTCAATAACATACC	TDTtoN
66	PMGC2501	CACAGGACGTTTTGGAGCAG	AATTCGGACAGTCAGTCACC	TDTtoW
67	PMGC2515	GAAAAGGGATTGTTAATAAACCC	CCAAAATCATAAAAGACAGGGC	TDTtoNW
68	PMGC2516	TAGTTCATTATCCTTGGGCTG	GTCAGTCGCCTTGCATTGC	TDNW
69	PMGC2522	TCTGTTAATTTCTCAGCTGTTG	TGCTTTACTAAACTTTTTACTGC	TD
70	PMGC2523	TTTTTCTAATTAAAGTCTACAAAC	CAAATCATGCCGGATTATAGC	TDTtoN
71	PMGC2525	CGAGTCACAAGCTCCCAATAG	GCAGGCTGTCCTATCTGCG	TDN
72	PMGC2531	TAAGAGAATTGGGAGAGCAAC	TTTTATCTTTTCCAGTTGTCTAC	TDTtoN
73	PMGC2536	GCTGTCTAACATGCCATTGC	CATTTCTTTATCATCACCTTAAC	TDTtoN
74	PMGC2541	CATTATAGTCCTGATTGATCTTC	CACTCAAGATCGAGTCTATGG	T
75	PMGC2550	AGGTTACAACTTTGTTGTAGC	GAACAACTCTCACTGTGGTC	TDTtoNW
76	PMGC2556	ACAAAATGGTCCCCATCTTTC	GCCTACCAATACTAAGAGCC	TDN
77	PMGC2557	ACAAAATGGTCCCCATCTTTC	GCCTACCAATACTAAGAGCC	TDW
78	PMGC2558	CCAGAGAAAGAGAGTGCTTC	AATGCAGATGTCGTTGTTTGC	TDW
79	PMGC2571	TCTCGCAGATTCATGTAACCC	GACTGTATGTTGACCATGCC	TDTtoW
80	PMGC2573	TGCCTTTCTGATCAATATCGC	CTCGTTAATTAGAGTCGAATTAG	TDTtoN
81	PMGC2574	TGTCTGTTCTTATTTTCTCTG	AGTTGTATCTCAACTAGAATTAG	TDTto
82	PMGC2578	GAGAACTCGGTGACTGACTG	CAGCAACATCCACATATTAGC	TDTtoNW
83	PMGC2585	ACTGCTGTGTATTGCCCTAG	TAGTTGAAGTTGGAGCACAAC	TD
84	PMGC2598	TGAACTGGTCATCATTTGACG	CATATATACAAGTCGTTACCATC	TDNW
85	PMGC2599	ACAGTACGCAGAAAGCTTGG	TTCTGTTTCGGAGATGTTTAC	TDN
86	PMGC2603	CATACTTTCAATTTTCTTACTGC	AAGACTCACAAATGCATCTTGC	TD
87	PMGC2606	AATTTACATTTCTTTATCATCACC	GCTGTCTAACATGCCATTGC	TDTtoW
88	PMGC2607	TTAAAGGGTGGTCTGCAAGC	CTTCTTGACCTCGTTTTGAG	TDN
89	PMGC2610	AACACGCAAGAACATACATAAG	GATTAACATGTTTCGCTACGC	nd
90	PMGC2611	TGACGATTACAGTTTTTGATCG	CTCCTAATTCCTGACAACCAC	TDTtoW
91	PMGC2614	TATACAAAATGTCACCTAAAGAG	CGACATATGGTAGGCATATTTT	TDTto
92	PMGC2658	GCCCTTGAATACCATGAGCG	ACCTTCAGTAGATCAGGTTAGTG	TDTtoNW
93	PMGC2660	GTTCTATGTGTAGGAGATATCC	TAACAATATGCTTCATAGCACAG	TD
94	PMGC2675	CACACCGACAAATTATGAGTG	TTTTAGAGTGAATTTTCTGCG	TDTtoN
95	PMGC2679	GGAATCCGTTTAGGGATCTG	CGTCTGGAGAACGTGATTAG	TDTtoN
96	PMGC2691	ATTTTGAATTTGAATTATGTTGTT	TTTCAGAGTATTTTAGGGTGTC	TDN
97	PMGC2696	G	TTCTTCATGCAGGAAGGAGC	TDTtoN
98	PMGC2699	ACACACACACCAGGCTTCTC	AACCCTAATTTCAAGAATTGGG	TDTtoN
99	PMGC2702	TTTCCTCCATGTATATCAAACC	GCATCAGCACATCAGAGTTC	TDN
100	PMGC2709	GCTTATCTCTCCTTCCACCG	GTGCAGTTCAGAGTATTGTTG	TDTtoNW
101	PMGC2718	ATTGTAATTATTGAACACATGCC	ACAACTATAAATATAGGCTGCC	TDN
102	PMGC2730	ATCTACCAAACCTACATTATCTTG	GAAAACCAAAGAGTCTTCACAG	TD
103	PMGC2731	GGCTTAATATGGGTGAGGTTT	CTGGTCAACAGCTACTGCAC	TDTtoN
104	PMGC2737	CGTATAGTACTTGAAGAATCCC	AGAAGTTAAGGTAACGCTAGG	TDN
105	PMGC2737	AGATTAACCTTAGTTTGCTTGG	TTGTTATCCACTGCCAGTGC	TDN
106	PMGC2647	CTCGTTAATTAGAGTCGAATTAG	GATATGTCAAGGAAATCCTTAG	TD
107	PMGC2765	GAGATAGCATCACCTAGAGG	CATTTCAATCTTTTGTGCCGC	TNW
108	PMGC2766	AATAGGATGAGGCTTCAATAC	TGGCGCTGGTTTCTTAGCTG	TW
109	PMGC2775	ATACCGCCGCGTCCACTC	TAGCAAAGGAGAGGGTTTCC	TDTto
110	PMGC2786	CTTGAATTTCAATAGAATCGCAAG	CTAATTTAACTTGCTTGGTCTG	TD
111	PMGC2794	GTCACGCTAAGCCAGATCTC	GCTTTGCTAAGCAGGCTTTC	TDN
112	PMGC2803	AGGCCTCACTATTTTCAGCC	TAATCGCCTATACACAGGCG	TDTto
113	PMGC2804	AAAGTTTTTCAATTTCAATCCTTG	GGTCCTATCTTCAAATTCTG	TDW
114	PMGC2806	CTTTGATTGCTGATGAATGGC	GATCACAAATGCATCTTGCAAG	TD
114	PMGC2812	TGCATTATAGTCCTGATTGATC		

115	PMGC2818	AAGCTTCATCGTCCTGCTTG	CGTATCAATTCACGACTCTCG	TDToNW
116	PMGC2826	GCTTCTTTAGCGACATGCATC	GTCAGAACTGTGACAGTAACC	TDToW
117	PMGC2832	CAAGCTTGGCTTTGCTAAGC	AGGCCTCACTATTTTCAGCC	TDN
118	PMGC2838	TGGTCGGCGAGAGTGACCAG	ATTGATCTCTCTTTACATCACTC	TDNW
119	PMGC2839	AACCCATAGCAAGAAGCTAG	CAATTACCGAAGAGGATTACTG	TDToW
120	PMGC2840	TATATTGAGATCTCATCTAACAG	ACAAAATTCAATTGTGTTGTAATC	TDTo
121	PMGC2847	AATAGGTAACCAGTAATTGTCAG	TAGGCTAGTTGCCAGGACC	TDTo
122	PMGC2852	ATAATCTCCCTAGCTTAATTCC	GAATAACATGGATAATGTGTTTG	TDToN
123	PMGC2855	GGTATCTTGTTATCCACTGCC	TTTTCTCGTTAATTAGAGTCG	TDToN
124	PMGC2858	CTTACCATCTTTATCCTAATGC	TTTCAAAAAATAAAAAGCAGCGC	TDToN
125	PMGC2861	GTTTTTCATGCGACATTGAGG	TTTGATACACAAGTTTCATTGTG	TDToW
126	PMGC2862	TTTGTAATAATGAAGATTTGTAC	ATTTTTGTTCTTTTAACCAAAATTC	TDToN
127	PMGC2866	ATTGTTCAAATCCTCAGGTTT	TAGCATAGTAGCTAGCTAGTG	TDN
128	PMGC2873	TGGTTGGAATGTCTTTACTCC	ATACATTGATCTCTCTTTACATC	TDToW
129	PMGC2879	TTGATTGAGCCTCACGAGC	AAACTCCAACATTTTAAGGACC	TDNW
130	PMGC2881	CCTCACTTTCAAATTGAAGCC	AACACATAAATCTTGAAAGGAAC	TDNW
131	PMGC2885	CATGATCAAATTGGATTTGAATG	AAAGATGAACATGGCTAGCTC	TDToW
132	PMGC2889	CCCAAGATCCGATTTTGGG	CACAATGTACAAATCGCTGTC	TDToNW

Table 1 legend. The microsatellite (SSR) primer sequences in this database are derived from cloned genomic fragments of a female *P. trichocarpa* (this female has the clone number 383-2499). **Linkage group** refers to the published *Populus* genetic map whose citation follows:

Bradshaw, H.D., Jr., Villar, M., Watson, B.D., Otto, K.G., Stewart, S., & Stettler, R.F. (1994) Molecular genetics of growth and development in *Populus*. III. A genetic linkage map of a hybrid poplar composed of RFLP, STS, and RAPD markers. *Theoretical and Applied Genetics* 89(5): 167-178.

Known to be polymorphic in these species is indicated by: T = *Populus trichocarpa*; D = *P. deltoides*; N = *P. nigra*; To = *P. tremuloides*; Ta = *P. tremula*; W = *Salix* (willow).

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.

Frewen, B.E., Chen, T.H.H., Howe, G., Davis, J., Rohde, A., Boerjan, W., & Bradshaw, H.D., Jr. (1999) QTL and candidate gene mapping of bud set and bud flush in *Populus*. *Genetics* (in press).

Howe, G.T., Davis, J., Frewen, B., Sarul, P., Jekni, Z., Bradshaw, H.D., Jr., & Chen, T.H.H. (1999) Molecular genetic approaches for studying endodormancy in trees: Model systems using species and hybrids of *Populus*. *HortScience* (in press).

Bradshaw, H.D., Jr. (1999) *Populus* as a model system for forest tree genetics and genomics. Mississippi State University, Starkville, MS, 15 June.

Bradshaw, H.D., Jr. (1999) 21st Century genomics and the Poplar Molecular Genetics Cooperative. Weyerhaeuser Technology Center, Tacoma, WA, 31 March.

Bradshaw, H.D., Jr. (1999) The application of genome science to increase the production of energy from biomass. NAS/ NRC Committee to Review the R&D Strategy for Biomass-Derived Ethanol and Biodiesel Transportation Fuels, Irvine, CA, 11 February.

- Bradshaw, H.D., Jr. and Strauss, S.H.** (1999) Strategies for identifying genes that are QTLs. Plant and Animal Genome VII, Forest Tree Genome Mapping Workshop, San Diego, CA, 17 January.
- Bradshaw, H.D., Jr.** (1998) The Poplar Molecular Genetics Cooperative. Universidad de Talca, Talca, Chile, 11 November.
- Bradshaw, H.D., Jr.** (1998) Molecular breeding in *Populus*. Silvotechna Meeting, Concepcion, Chile, 13 November.
- Bradshaw, H.D., Jr.** (1998) Future applications of genetic mapping in tree breeding and selection. Shell International Renewables Limited Forestry Research, 2020 Vision Workshop for Forest Biotechnology, East Malling, Kent, U.K., 15-16 June.
- Bradshaw, H.D., Jr.** (1998) Poplar Molecular Genetics Cooperative research. Forestry Centre, U.K., 11 June.
- Bradshaw, H.D., Jr.** (1997) Genetic architecture of quantitative traits in *Populus*. IUFRO Conference on Silviculture and Improvement of Eucalypts, Salvador, Brazil, 24-29 August.
- Bradshaw, H.D., Jr.** (1997) The Poplar Molecular Genetics Cooperative. Electric Power Research Institute, Palo Alto CA, 21 August.
- Bradshaw, H.D., Jr.** (1997) Mapping quantitative trait loci in forest trees. Western Forest Genetics Association, Berkeley CA, 4 August.
- Bradshaw, H.D., Jr.** (1997) Mapping quantitative trait loci in forest trees. Institute of Forest Genetics, Placerville CA, 31 July.

Technology Transfer

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

The decision was made at the outset not to patent the markers, but to make them publicly available to encourage their use.

Commercial Accomplishments

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

Genetic fingerprinting was performed by our lab for members of the Poplar Molecular Genetics Cooperative, including Alberta-Pacific Forest Industries, Boise Cascade Corporation, Fort James Corporation, Pacifica Papers Ltd., International Paper, and Westvaco.

Educational Accomplishments

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

A graduate student, Xuesong Yu, performed the mapping experiments.

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.

None.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	H.D. Bradshaw, Jr.	
University:	University of Washington	
Agreement Number:	OR22072-89	
Project Title:	Map-based cloning of genes to increase poplar biomass	
Reporting Period and Report Type:	From: 1 October 1998 To: 30 September 1999	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

1. Determine the rust resistance or susceptibility phenotype of ~2000 hybrid poplars in the full sibship PMGC Family 545.
2. Use bulked segregant analysis to identify at least 100 additional AFLP markers linked to the *Mmd1* resistance gene.
3. Produce a fine structure (0.1cM) genetic map around the *Mmd1* rust resistance locus.
4. Isolate overlapping bacterial artificial chromosome (BAC) clones of *P. trichocarpa* DNA containing *Mmd1* and its flanking AFLP markers.
5. Construct a physical map of the *Mmd1* locus by making BAC contigs.

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 very large greenhouse experiment was used to determine the rust resistance or susceptibility of 2037 hybrid poplar (*P. trichocarpa* × *P. deltoides*) clones from a single full-sib family. Each of the clones was represented by 3 ramets (copies), and all 6000 cuttings were inoculated with the hybrid rust known as *Melampsora x columbiana* 3 (*Mxc3*). Brigid Stirling (grad student, University of Washington) and George Newcombe (pathologist, Washington State University-Puyallup) scored the resistance/susceptibility phenotype of all ramets. Of the 2037 hybrid poplar clones, 1083 were found to be resistant, and 954 were susceptible, confirming that the resistance

gene *Mmd1* is segregating 1:1 as expected from the fact that the *P. trichocarpa* female parent is heterozygous (Rr) for resistance and the male *P. deltoides* parent (ILL-101) is homozygous susceptible (rr).

Leaf tissue from all 2037 clones was harvested for DNA extraction. Two microsatellite markers, developed with prior CPBR funding, were used to identify triploid hybrids and exclude them from further study. A total of 1916 diploid hybrids remained for linkage analysis, with 966 resistant and 950 susceptible to *Mxc3*. Two DNA pools (bulks) were made from nine resistant and nine susceptible offspring. A total of 762 AFLP primers were used to screen approximately 4000 polymorphic markers by bulked segregant analysis for linkage to the *Mmd1* resistance gene. More than 120 candidate polymorphisms between the bulks were tested for very tight linkage.

Six of the AFLP markers were mapped within a 6cM window around *Mmd1*. The most tightly linked AFLP marker, called BVS1, has been cloned and sequenced to produce a sequence tagged site (STS) marker. PCR primers designed from the BVS1 sequence amplify a fragment of the expected size, and a presence-absence polymorphism in BVS1 shows only one recombination event with *Mmd1*, placing the marker an estimated 0.05cM from the resistance gene.

Thanks to Carol Loopstra, Jim Giovannoni, and Julia Vrbelov at Texas A&M University we were able to obtain a BAC genomic library of our *P. trichocarpa* female which carries the *Mmd1* resistance allele. The library contains 50,000 BAC clones of approximately 120kb each. This provides a 10-fold coverage of the poplar genome, adequate for physical mapping and, hopefully, positional cloning. The BAC library was screened with BVS1, which recognizes only the resistance allele, and 5 BAC clones were retrieved. The stage is now set for further fine-structure genetic and physical mapping around the *Mmd1* locus.

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.

Bradshaw, H.D., Jr. (1999) Quantitative genetics in the era of genomics. Mississippi State University, Starkville, MS, 16 June.

Bradshaw, H.D., Jr. (1999) *Populus* as a model system for forest tree genetics and genomics. Mississippi State University, Starkville, MS, 15 June.

Bradshaw, H.D., Jr. (1999) 21st Century genomics and the Poplar Molecular Genetics Cooperative. Weyerhaeuser Technology Center, Tacoma, WA, 31 March.

Bradshaw, H.D., Jr. (1999) The application of genome science to increase the production of energy from biomass. NAS/ NRC Committee to Review the R&D Strategy for Biomass-Derived Ethanol and Biodiesel Transportation Fuels, Irvine, CA, 11 February.

Bradshaw, H.D., Jr. and Strauss, S.H. (1999) Strategies for identifying genes that are QTLs. Plant and Animal Genome VII, Forest Tree Genome Mapping Workshop, San Diego, CA, 17 January.

- Stirling, B.V., Newcombe, G., and Bradshaw, H.D., Jr. (1999) Map-based cloning of Mmd1, a poplar leaf rust resistance gene. Plant and Animal Genome VII, Forest Tree Genome Mapping Workshop, San Diego, CA, 17 January.

Bradshaw, H.D., Jr. (1998) The Poplar Molecular Genetics Cooperative. Universidad de Talca, Talca, Chile, 11 November.

Bradshaw, H.D., Jr. (1998) Molecular breeding in *Populus*. Silvotecnica Meeting, Concepcion, Chile, 13 November.

Bradshaw, H.D., Jr. (1998) Future applications of genetic mapping in tree breeding and selection. Shell International Renewables Limited Forestry Research, 2020 Vision Workshop for Forest Biotechnology, East Malling, Kent, U.K., 15-16 June.

Bradshaw, H.D., Jr. (1998) Poplar Molecular Genetics Cooperative research. Forestry Centre, U.K., 11 June.

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.

Genetic markers have been developed which will predict the disease resistance genotype of a hybrid poplar in Family 545 with 99.95% accuracy.

Educational Accomplishments

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

A graduate student, Brigid Stirling, is receiving training in the most advanced methods in forest tree molecular genetics.

Additional Funding

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

This project is supported by the Poplar Molecular Genetics Cooperative, a consortium of industry, agency, and academic members with an annual budget of approximately \$200K.

The new science of genomics will be used to locate and isolate a gene for resistance to the poplar leaf rust disease. Poplar leaf rust is the single most important disease in limiting the biomass productivity of hybrid poplar plantations worldwide. The resistance gene, called *Mmd1*, will be located on a genetic map of the poplar chromosomes, and this positional information ultimately will be used to isolate (clone) the resistance gene. The cloned gene can be used to genetically engineer hybrid poplars for resistance to leaf rust. If this map-based (positional) cloning approach is successful for the *Mmd1* gene, the same methods may then be used to clone additional genes important for biomass yield in hybrid poplar.

Scientific Progress Report

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

Principal Investigator: Zong-Ming Cheng
University: North Dakota State University
Agreement Number: OR22072-76
Project Title: *Evaluation and Characterization of Rooting Capability of Hybrid Aspens Transformed with Rooting Genes*
Reporting Period and Report Type:
From: January 1, 1999 **To:** December 31, 1999
Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

Summary of scientific progress:

We have confirmed majority of the plants by PCR and some by Southern blot. We anticipate to finish the confirmation in 2-3 months before the spring. We have also tested microcuttings in vitro and hardwood cuttings in greenhouse. The rooting experiments conducted in the greenhouse with hardwood cutting are key experiments. We did three two experiments, one in February and one in May, together with 3500 cuttings. The most interesting data is with transformants with GH3 promoter and *iaaM* gene. There are several clones rooted more than 50%, one particular one with 91%, while the control cuttings rooted in 10-25%. If the conditions for rooting cuttings in the greenhouse were optimized, we feel optimistic that some of the transformed plants will root consistently at high frequencies, particularly with GH3-*iaaM* transformants. We have placed many plants in coolers to produce hardwood cuttings. We plan to carry out several large experiments on rooting. We will pay particular attentions to some of those which had shown high rooting frequencies. Dr. Cheng will contact with the sponsoring company to discuss results in more detail.

Four objectives were listed in the proposals, they are:

- a. To confirm the hybrid aspen plants transformed with *iaaM* and *rolB* under three promoters by PCR amplification and Southern blotting to identify single-copy transformed plants.
- b. To examine the expression of these genes by GUS assay, determine the auxin contents in

- iaaM*-transformed plants and sensitivity to the applied auxin in *rolB*-transformed plants.
- c. To evaluate the rooting capability of *in vitro* cuttings and hardwood cuttings.
- d. To analyze data of gene expression, auxin contents/sensitivity and results of rooting capability to determine the possible rooting mechanisms.

Progress made in each of these objectives are explained below.

- a. To confirm the hybrid aspen plants transformed with *iaaM* and *rolB* under three promoters by PCR amplification and Southern blotting to identify single-copy transformed plants.

Together, approximately two hundreds plants have been transformed with two genes (*iaaM* and *rolB*) under control of 35S, Win, Heat shock, GH3 promoters. All of these plants have been shown resistance to kanamycin as a selective marker. All of these plants have been tested for GUS expression by histochemical assays.

We have developed and optimized a protocol to extract DNA from aspen leaves and run the polymerase chain reactions (PCR). About 75% of *iaaM* transformed plants have been confirmed by PCR assay, and the DNA from the remaining plants have been extracted. For *rolB* gene transformants, about 50% of the plants have been confirmed by PCR.

We have also optimized the protocol Southern blot and confirmed about 20% of the transformed plants. We anticipate to complete confirmation in 2-3 months, so we will accomplish this objective. We should have completed this objective now, but we were little bit behind was that one Ph.D student (Mike Bosela) left for a postdoc job at USDA Forest Research Lab on Purdue campus, and another Ph.D student (David Dai) had his both comprehensive written and oral exams in October and December.

- b. To examine the expression of these genes by GUS assay, determine the auxin contents in *iaaM*-transformed plants and sensitivity to the applied auxin in *rolB*-transformed plants.

In the first we intended to examine the expression of these genes by GUS assays and sensitivity to the applied auxin in *rolB*-transformed plants, and in the second year to determine the auxin contents of the *iaaM*-transformed plants.

Expression of the genes by GUS was based on the fact that the target gene and GUS marker gene are linked. Transformed and non-transformed aspen plants are assayed by viewing blue colors in plant tissues after making cross sections.

Gus expression pattern in transgenic stem was evaluated with 35S-GUS and heat shock-gus. With the 35S-GUS construct, the GUS activity was found in all tissues except pith. The strongest activity was found in the phloem tissue. The heat shock promoter gave rise to a very strong expression only in epidermis and phloem. In the current-growing stem, Gus activity was

determined in epidermis, parenchyma, vascular cambium, and primary xylem for the 35S promoter. The heat shock promoter was mainly expressed in parenchyma in current-growing stems. We can see that the heat shock promoter expressed more tissue-specific, especially in mature stems. In root tips, 35S and heat shock promoters were expressed in columella, vascular, and root apical meristem with a very strong expression in root apical meristem. GUS activity was also determined after induction with heat shock (heat shock at 42 C) and wounding (prying or pinching). In most cases, the treatments induced expression of the GUS gene, strongly suggest that these promoters are inducible.

The auxin sensitivity assay was done with rolB-transformed plants. Three different kinds of plants were used: 35S-rolB, HS-rolB and non-transformed control. Five auxin (NAA) concentrations (0, 0.01, 0.1, 1.0, 10.0 μ M), were tested. Each treatment had 8-18 microshoots. Data were taken 14 days after culture. The overall experiments didn't seem to suggest the rolB-transformed microshoots become more sensitive to auxin. However, there is one treatment (HS-rolB) rooted 80%, while others ranging from 0 to 61%. We will repeat the experiment and we will test each transformant separately to determine whether there is any variation among individual plants.

c. To evaluate the rooting capability of *in vitro* cuttings and hardwood cuttings.

In vitro cutting rooting (see above)

Whether the transformed plants can be rooted by hardwood cuttings is the core question and experiment in this project. We did three trials in the greenhouse with iaaM-transformed plants. Three types of plants were tested (GH3-iaaM, HS-iaaM, and win-iaaM) along with non-transformed control plants. For heat shock and win promoters, induction of promoters was also done. The summary data are shown below.

I) GH3-IAAM

Cutting Type (# of genotypes)	Rooted cuttings/total cuttings (%)
Stock control (n=1)	25.5/96 (23.5%)
GH3-GUS control (n=1)	12.5/48 (26%)
GH3-iaaM (5/15 Experiment) (n=5)	81/632 (12.8%)
GH3-iaaM (2/7 Experiment) (n=8)	101/367 (27%)
<i>[If R2G Dist and LAA-treatment excluded</i>	<i>52/295 (18%)]</i>
GH3-iaaM (All)	182/999 (18.2%)
All cuttings	220/1143 (19%)

By analyzing the data in more detail with treatment and particularly based individual clones (transformants), several treatments rooted over 50%. One particular clone (R2G dist.) rooted at 91% (29 rooted out of 32 cuttings).

R5A Base 97A: 18.5/36 (51%) cuttings rooted
 R5A Base 97B: 15.5/48 (32%) cuttings rooted
R2G Dist: 29/32 (91%) cuttings rooted
 R3C 9/32 (28%) cuttings rooted

There were also a few GH3iaaM genotypes that rooted poorly when subjected to the primary treatment, but at high frequencies when untreated:

R4G Prox (Control) 10/22 (45%) cuttings rooted
 R1A Dist (Control) 10.5/24 (44%) cuttings rooted

II) HS-IAAM

Cutting Type (# of genotypes)	Rooted cuttings/total cuttings (%)
Stock control (n=1)	3/64 (4.6%)
HS-GUS controls (n=2)	12/96 (12.5%)
HS-iaaM transformants (n=13)	116/878 (13%)
All cuttings	131/1038 (12.6%)

Overall, there were no significant differences between the rooting frequency of treated vs. non-treated cuttings for all cutting types. Also there was less variation among all treatments, individual clones (detailed data not included).

III) WIN-IAAM

Cutting Type (# of genotypes)	Rooted cuttings/total cuttings (%)
Stock control (n=1)	2/96 (2%)
Win-GUS controls (n=1)	27/44 (61%)
Win-iaaM transformants (n=22)	204.5/1364 (15%)
All cuttings	233.5/1504 (15.5%)

By analyzing the data in detail, it didn't seem to have any trends in treatment effects or clonal effects.

- d. To analyze data of gene expression, auxin contents/sensitivity and results of rooting capability to determine the possible rooting mechanisms.

This objective is scheduled to do in the second year.

Industrial Contacts

Two telephone conversations and several email communications were made with Dr. Nick Wheeler of Weyerhaeuser. A brief introduction/summary of the project was sent to the Weyerhaeuser. Dr. Cheng has been consulted by Weyerhaeuser in propagating alder species by tissue culture.

Publications and Presentations

A Ph.D dissertation is under preparation. One "Introduction of the Project" of the project were given at the annual CPBR meeting. Several papers are in preparations.

Due to potential of the technology and concerns of disclosure in the early stage, no papers have been published. Two refereed journal papers are in final preparation and are expected to be submitted in the spring, 2000.

Technology Transfer and Commercial Accomplishments

We are in the process to test rooting of the hardwood cuttings of transgenic plants, which is the core and final test for the possibility for commercialization. We anticipate to complete the rooting test in year 2000. If the results are as we hoped and expected, we will contact the sponsoring company for licensing the technology and possibly expanding this technology to other species (see the letter of support).

Educational Accomplishments and Key Personnel Hiring or Turnover

A Ph.D student (Mr. Mike Bosela) completed his research in July, 1999 and has been employed by a postdoctoral fellow at USDA Forest Research Lab at Purdue University campus. He is currently finishing writing his dissertation. A new student will be hired in the early 2000.

Scientific Progress Report

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

Principal Investigator: Zong-Ming Cheng
University: North Dakota State University
Agreement Number: OR22072-50
Project Title: *Purification of a broad-spectrum antimicrobial protein and its gene isolation*

Reporting Period and Report Type:

From: July 1, 1996 **To:** April 30, 1999
Check one: ☐ Interim Report ☒ Final Report

Project Objectives

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

1. Final Isolation of the Antimicrobial Protein
2. Characterization of Antimicrobial Protein
3. Sequencing of the Protein
4. Cloning of the Gene Encoding the Antimicrobial Protein
5. Determination of Mechanism of Antimicrobial Protein

Scientific Accomplishments

This proposal has five objectives as listed above. We have accomplished objective 1, 2, 3 and 5. The main reason that we have not accomplished the objective 4 was that the active compounds were not proteins as indicated in the original publications in China. Since the compounds are cyclic peptides with modified amino acids, which are generally synthesized through a non-ribosomal fashion, therefore, there are no genes directly coding the cyclic peptides. Current literature suggests that such compounds are generally synthesized by a large enzyme complex by a series of biochemical reactions, and the genes for the enzyme complex are extremely difficult to identify and clone. Even if the genes encoding the enzyme complex are cloned, it would be very difficult to transfer the genes into crops and have them expressed properly. The results regarding each of these five objectives will be described below.

Objective 1. Final Isolation of the Antimicrobial Protein

Previous research in China showed that *Bacillus subtilis* TG26 produce two proteins each about 14 kD which possess strong inhibitory activities against *Fusarium graminearum*, causing wheat scab, and against other plant pathogens. After we obtained the strain TG26, we confirmed its broad spectrum anti-microbial activities (Table 1).

When the bacterium was confirmed to produce the active compounds against plant pathogens, we used ammonium sulphate to precipitate the active compounds, assumed they were proteins, and then went through a series of biochemical purification methods, including gel filtration, HPLC with various columns, two active compounds, named CF8 and CF9, were obtained. Since both peptides could not be sequenced (see below), we have used a new method to purify another three active peptides.

In this new method, *Bacillus subtilis* TG26 was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH7.0) at room temperature for 3 days. After centrifugation, the supernatant of the fermentation broth was acidified with concentrated HCl to pH 2.5, causing precipitation of crude precipitate. The crude precipitate then was extracted by methanol. The methanol extract was evaporated and the remaining dried material was suspended in 50 mM sodium phosphate buffer (pH 6.8). Because we used concentrated HCl to cause precipitation during this process, the final dried sample was neutralized by dissolving in 50 mM sodium phosphate buffer (pH 6.8), and the remaining fermentation broth was neutralized to pH 7.0 by adding sodium hydroxide. Only the crude precipitate showed activity against *Fusarium graminearum*, while the remaining neutralized fermentation broth showed no activity. This suggested that almost all the active components were precipitated by HCl.

After the precipitation showed strong antimicrobial activities, the precipitation was further separated by gel filtration which is based on size and shape of different components. The crude preparation was subjected to a Sephadex G-150 column pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.8). The flow rate was 30 ml/hour, and fractions were detected at the 280 nm absorbance using DU-640 spectrophotometer. There were two major fractions eluted. Only fractions in the first peak showed inhibitory activity against *Fusarium graminearum*. This fraction was kept and subject to further separation.

The active fractions from gel filtration were further separated by reverse-phase HPLC which is based on hydrophobicity of the proteinaceous compounds. The active fraction was loaded onto a reverse-phase C18 column. We used two mobile phases, A:100% H₂O, 0.1% trifluoroacetic acid, and B: 100% methycyanide, 0.1% trifluoroacetic acid. There was one major peak which was eluted around 38% B, while there were several peaks eluted from 50% B to 56% B. All the fractions were concentrated by speed vacuum. Methycyanide and trifluoroacetic acid were also got rid of during this process. Among the fractions eluted from 50% to 56% B, we got S1 and S4 fractions which were shown to be pure (Other two fractions, S2 and S3, however were not pure). The fraction eluted at 38% was also pure and named as S36. All S1, S4 and S36 show inhibitory activity against *Fusarium graminearum*.

Objective 2. Characterization of Antimicrobial Protein.

In all experiments, we have obtained five purified active fractions, these are CF8 and CF9 and S1, S4 and S36. Earlier research showed that the CF and CF9 could not be sequenced with many attempts by the Biotechnology Center at Cornell University and the Medical Facility Center at the University of Alabama. we will report the characterization of S1, S4 and S36 peptides. The results of CF8 and CF9 were reported earlier.

S1 and S4 fractions are related to each other. They are all water soluble, and their properties are similar. From the mass-spectrometry experiments, the molecular weight of S1 is 1438.2 daltons, while S4 is 1467.4 daltons. Therefore, S1 and S4 are peptides of about 10 to 15 amino acids. Interestingly, from the amino acid composition analysis, both S1 and S4 show the identical composition: one molecule of Ile, one molecule of Ala, one molecule of Pro, two molecules of Tyr, three molecules of Glx, and there are two additional unknown amino acids (X and Y). Based on the mass and amino acid composition, we derived that S1 and S4 have 12 amino acids and may have the following formula: (Ile-Ala-Pro-Tyr₂-Glx₃-X-Y₃). The difference between S1 and S4 may be due to different modifications.

We further performed Edman Degradation to determine the primary structure. However, it was unsuccessful. This could be due to the complicated modifications which are common in *Bacillus subtilis* products. The N-terminal may be blocked or the peptide is cyclic. We also performed NMR analysis. The NMR data also showed that there are many methyl modifications occurred in the peptide. Due to these complicated modifications, NMR study also could not determine the primary structure of S1/S4 peptides.

Objective 3. Sequencing of the Protein

As all of the experiments showed that the proteinaceous compounds are not proteins, but small peptides. So, we have sequenced the peptides, not proteins. Since we could not have the CF8, CF9, S1 and S4 sequenced, we have focused on the S36. The S36 peptide is quite different from S1 or S4 peptides. S36 peptide eluted earlier in reverse phase-HPLC. This shows that it has less hydrophobicity, and probably it may have less modifications. This is confirmed by NMR analysis, as much less methyl groups were found in the NMR data. Also, from the NMR data, only 8 amino acids were found, while S1/S4 has 12 amino acids. This was also confirmed by mass spectrometry experiment. The mass of S36 is 1035 daltons. In fact, in the NMR data, the sequence of the 8 amino acids has been lined up. The general sequence looks like this:

1	2	3	4	5	6	7	8
Asx	Serine	Glx	Serine	Threonine	Amx	Asx	Tyrosine.

The peptide is likely circularized at Asx and Tyr.

However, with the normal NMR analysis, we could not resolve the final sequence of the amino acid 1 and 7 (either being asparagine or aspartic acid), and amino acid 3 (either being glutamine or glutamic acid). So we collaborated with Dr. Kevin Mayo at the University of Minnesota's Medical School and employed the N-15 labeling technique before NMR. In this process, strain TG26 was first grown in medium with N-15 nitrogen as the sole source of nitrogen. Then, gel filtration and the reverse phase HPLC was used to purify the active fraction S36. The fraction of S36 was then subject to the NMR. By using this N-15 labeling NMR, we have revealed that both Asx are asparagine, and Glx is glutamine. The sixth amino acid is a modified amino acid. Based on the molecular weight and mass spectrometry, we deduced that this amino acid has a fatty acid chain like this: $\text{CH}_3-(\text{CH}_2)_n-\text{CH}-\text{COOH}$

Objective 4. Cloning of the Gene Encoding the Antimicrobial Protein

Since the antimicrobial compounds are not proteins, but small peptides, and the peptide S36 has a cyclic structure with a modified amino acid, they are most likely synthesized by a complex enzyme system, therefore there are no genes directly encoding these compounds. These compounds are generally synthesized through non-ribosomal, a series of biochemical reactions, which are controlled by a large enzyme complex. To clone genes encoding a large enzyme complex would be extremely difficult. It would also be very unlikely that the large enzyme complex would be functional in plant system. Therefore, we did not undertake the gene cloning.

Objective 5. Determination of Mechanism of Antimicrobial Protein

Again, since these compounds are not proteins, we have determined the mechanisms of inhibition of the peptide, especially S36. The peptide 36 was added to the culture of testing bacteria and fungi. A time course experiment was conducted to monitor the growth of bacteria and fungi. Ultra structure of *Fusarium* fungal growth was observed under a scanning electron microscope. Spore germination was significantly inhibited. Growth inhibition seems to be caused by membrane leakage, since hyphae shows "beads in a string" phenomena.

Industrial Contacts:

Two telephone conversations were made with both Dr. Jihong Liang in Monsanto company and Dr. Jeffrey Rosichan at Novartis in April and May, 1998. Dr. Cheng also discussed results with Dr. Bernard Vernooij when he visited Novartis company in Research Triangle Park in July 15, 1998. Two more email communications were made with Dr. Louis-Pierre Molleyres, the Natural Product Chemist at Novartis for possible future corporation.

During the process of submitting proposal for the 1999 competition, Dr. Cheng contacted company representatives in Mycogen Plant Sciences in St Paul and San Diego, AgrEvo, Rhone Poulenc, AgraTech Seeds, Novartis Seeds.

New Collaborations:

New collaboration was established with Dr. Kevin Mayo at the University of Minnesota's Medical School in further characterizing the antimicrobial compounds. The new techniques used include regular and N^{15} NMR. Some research was also conducted in the Dr. Jan Novak's laboratory at University of Alabama's Medical School. The later one was discontinued due to Dr. Novak's transfer to another facility.

Publications and Presentations

A Master's degree thesis was submitted to North Dakota State University. A Ph.D dissertation is under preparation. One "Introduction of the Project" and one "Final Report" of the project were

given at the annual CPBR meeting.

Due to potential of the technology and concerns of disclosure in the early stage, no papers have been published. Two refereed journal papers are in final preparation and are expected to be submitted in the spring, 2000.

Technology Transfer

Two testing agreements have been signed with Monsanto and Novartis at the earlier stage of this research. Now, research in purification and characterization has been completed. Since the results reveal that antimicrobial compounds are small cyclic peptides, not proteins as original research results indicated in China, cloning the genes encoding the enzyme complex would be extremely difficult, therefore, it would be unlikely that any genes will be cloned and be available for technical transfer.

Commercial Accomplishments

Since all of the research data so far suggest that the active compounds are cyclic peptides, and there are no genes directly encoding the cyclic peptides, we don't anticipate any commercialization of the genes encoding the enzyme complex which synthesize the compounds. Therefore, we will complete and terminate this project. We had contact with Novartis regarding utilizing the peptides as biological control agents, but Novartis has not proceeded further.

Educational Accomplishments

One master's graduate student completed in 1998. One Ph.D student is scheduled to compete in spring, 2000.

Additional Funding

\$7000 cash was obtained from the ND Agri. Exp. Station in 1996.

Key Personnel Hiring or Turnover

A MS student completed his study in 1998 and has been employed by a private breeding company. A Ph.D student was hired in 1997 and is scheduled to complete in spring, 2000..

TECHNICAL PROGRESS REPORT
to The Consortium for Plant Biotechnology Research, Inc.,

AGREEMENT NO. OR22072-59

DEVELOPMENT OF AN ON-BORDER ETHANOL REFORMER FOR AUTOMOBILES

Overall Project Period:

May, 1997 - April 30, 1999

Period for this Report:

October 31, 1998 - October 31, 1999

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SUMMARY OF PROGRESS

This research is concerned with the development of an on-board catalytic steam reformer to produce hydrogen from hydrous ethanol for use in a fuel-cell stack for propulsion of future automobiles. There is currently a considerable interest in this concept, although the majority of the research is focused on gasoline or methanol as the fuel. However, biomass-based hydrous ethanol fuel has many advantages over these fuels as discussed later. Specifically, this project seeks to identify optimum catalyst candidates and processing conditions for the hydrous ethanol reformer that can provide good activity, selectivity and stability at moderate temperatures. Furthermore, it is planned to study the thermodynamics and kinetics of the process in sufficient detail to allow design and scale up of the hydrous ethanol reformer. The desirable features of an on-board reformer are rapid start up, and light weight. The project progress, as discussed in the attached detailed report, is quite well on schedule. Dr. Ilie Fishtik, postdoctoral associate, and Mr. Anthony Alexander, graduate student, have been working on the project.

Two alternate reforming processes are being investigated, namely, steam reforming (SR) and partial oxidation reforming (POR) of ethanol. The work done so far has focussed on steam reforming. The steam reforming of ethanol is a complex catalytic process in which a variety of reactions takes place. In order to find the operational conditions under which the desired reaction is dominant, a detailed thermodynamic analysis of the ethanol steam reforming was performed. The approach used is based on the concept of "response reactions" developed by us, which have the property of being unique, i.e., being independent of the usually arbitrary initial choice of a set of independent reactions, and follows in a natural way from the fundamental equations of chemical thermodynamics. Further, sensitivity analysis provides rigorous criteria for selection of the dominant reactions which can be used subsequently in both thermodynamic and kinetic analyses. It was determined that at low water concentrations, ethanol decomposes according to

to produce methane, which is an undesirable product. The desired steam reforming reaction,

namely,

can be made predominant, however, in the temperature range 700-800K and above, as well as at higher water ethanol ratios. An increase in the amount of water also has the effect of increasing the extents of the water-gas-shift and methane steam reforming reactions

and, consequently, a reduction in the amounts of the undesired products, CO and CH₄.

For a deeper understanding of the complex chemistry of the catalytic steam reforming and partial oxidation of the ethanol a comprehensive energetic analysis has been done. Namely, based on literature and our data a plausible mechanism of the process was generated. The mechanism includes 26 surface intermediates and 90 elementary reactions. Using the unity bond index - quadratic exponential (UBI-QEP) method which has been developed by Shustorovich, the energetic characteristics of the elementary reactions, i.e., the enthalpy changes and activation barriers of the forward and reverse reactions, were evaluated. Based on the approach recently developed by us we were able to qualitatively deduce the energetically favorable reaction pathways and the nature of the most probable rate determining steps.

Further, a series of 9 catalysts was screened in order to narrow down the list of possible catalyst candidates for ethanol reforming via partial oxidation reforming or the steam reforming route. Based on the results of this screening the most appropriate catalysts have been shown to be Ni oxide based catalysts. Namely, the catalyst Ni-0920 S, Engelhard and G-90B (NiO/CaAl₂O₄/Al₂O₃), United Catalyst. These catalysts readily convert hydrous ethanol at moderate temperatures (around 500°C) to a mixture of H₂, CO₂, CO and CH₄. The last two products are undesirable products: CO is a poison for the fuel cell, while the production of CH₄ is a lost in hydrogen. Our main goal, therefore, was to minimize the amounts of CO and CH₄. Extensive experiments have shown that the temperature, water to ethanol ratio and the oxygen to ethanol ratio are the main factors that determine the product composition. Thus, at 550°C and a

water to ethanol ratio equal to 2:3 (by volume) the production of methane on G-90B catalyst can be reduced practically to zero. Under these conditions, however, there is still substantial amount of CO in the product. In order to reduce the amount of CO and concomitantly to increase the amount of hydrogen a second, WGSR reactor, was used in tandem at the temperature of around 200°C. This decreased the amount of CO substantially while increasing the amount of H₂ owing to the WGSR.

By this it was proved that the steam reforming of ethanol can be performed practically quantitatively at around 550°C and ethanol/water ratio 2/3 (by volume) according to the desirable reaction, thus, resulting in a pure mixture of hydrogen and carbon dioxide.

For the remainder of this project period, it is planned to continue the studies of the optimum conditions of the process with an emphasis on the partial oxidation reforming route and to perform a comprehensive investigation of the kinetics of the partial oxidation reforming and steam reforming routes.

DETAILED PROGRESS REPORT

Objectives

The objective of this research is to develop an on-board catalytic reforming process for the production of hydrogen from hydrous ethanol for use in a fuel-cell stack for propulsion of future automobiles. When hydrogen is used in fuel cells, there are virtually no emissions, apart from those produced by the catalytic reformer itself. Fuel cells are of great interest for future automobiles since they can provide twice the energy efficiency of conventional IC engines. Fuel cells produce the electricity needed on-board to recharge the battery-pack of an electric vehicle or to directly provide power to the drive motor.

Thus, hydrogen fuel has the potential to effectively address both the energy efficiency

and pollution concerns of today's vehicles. However, on-board storage and refueling of hydrogen presents daunting problems. Consequently, liquid fuels that can be reformed into hydrogen on-board are of interest. Although the reforming of hydrocarbons is routinely performed in the chemical industry based on either a) steam reforming or b) partial oxidation in the presence of steam, on board reforming requires some special desirable characteristics enumerated below:

- High concentration of hydrogen in the reformat gas and extremely low CO levels. The anode Pt electrocatalyst of fuel cells is poisoned by CO as low as 10 ppm.
- Reformer catalyst should be active and selective towards hydrogen generation and should suppress the undesirable side reactions such as methane formation. Further, they should be stable and resistant to deactivation by coking and should be inexpensive.
- Mobile applications demand frequent start-up and shutdowns, hence the fuel processor is required to be a light-weight system, operating at low temperature with fewer components. The time required to reach the reformer operating temperature should be small.
- System should not be extraordinarily complex, and should be amenable to straightforward control schemes.

Currently, the main focus is on the use of methanol as a fuel for on-board reforming, due primarily to its low reforming temperatures, and gasoline owing to its ease of availability. In principle, any fuel can be reformed into a mixture of hydrogen and carbon dioxide. However, hydrocarbon fuels, such as gasoline or methane, require very high temperatures for steam reforming and also cause substantial coking of catalyst. On the other hand, oxygenated fuels, particularly the lower alcohols such as methanol and ethanol, reform at lower temperatures. The advantages of ethanol are:

- It is a renewable fuel that is free available, stored, and distributed using the existing gasoline distribution infrastructure.
- It is less toxic and hazardous in case of a spill than either gasoline or methanol.
- It has a higher hydrogen to carbon ratio than gasoline and is, therefore, reformable at moderate temperatures. A high carbon content increases the reforming temperature,

coking propensity, and the amount of CO₂ in the reformat gas.

- It is a single molecule as compared with hundreds in gasoline and, consequently, is a better fuel, reformable at lower temperatures at conditions that can be specified precisely. However, its reforming temperature is higher than that of methanol due to the presence of a C-C bond.
- Its energy content is 30% higher than that of methanol.
- *Hydrous* ethanol, which is considerably cheaper than anhydrous ethanol, should make an excellent fuel for either POR or SR.
- Ethanol contains no catalyst poisons such as S, N, or heavy metals present in fossil fuels requiring an additional treatment step in reformers of these fuels.

The specific objective of this work, thus, is to develop a catalytic reformer that would use hydrous ethanol (~ 50-60 wt%), thus avoiding the energy intensive ethanol dehydration step in the production of fuel ethanol. Steam reforming (SR) and partial-oxidation reforming (POR) will be the processes investigated. SR is highly endothermic, requiring heat input. The overall reaction for SR is given by:

The second possibility is partial-oxidation reforming (POR), the overall reaction for which depending upon the amount of oxygen, is

For $x = 1$, thus

Thus, for $x = 1$, POR represents a loss of 17% of the hydrogen, and introduces 2 moles of N₂ in the product, but is overall an exothermic process, while SR is highly endothermic, requiring input of heat. These are the key issues to be considered in the selection and design of the reformer. The planned work, thus, includes research to select the appropriate reforming process, i.e., SR, POR,

or a combination thereof, and identify appropriate catalysts for good activity, selectivity, and stability at lower temperatures. This will form the basis of the design of a bench-scale ethanol reformer to be tested later. A schematic of the ethanol reformer-fuel cell system is shown in Figure 1. A schematic of the gasoline reformer being developed by Arthur D. Little, recently made public, is shown in Figure 2. It may be noticed that an additional; sulfur removal catalytic step is required in the Arthur D. Little reformer due to the presence of sulfur in gasoline.

The reformed gases for a fuel cell must be rigorously free of CO, which causes rapid poisoning of the electro-catalyst in fuel cell, with a concomitant decline in its power output. In related work, we are also developing a CO tolerant fuel cell that can also operate at higher temperatures, e.g., 150 °C, instead of the usual temperature of 80 °C.

Literature Review

Production of hydrogen by steam reforming of methane, naphtha, heavy oils, coal and alcohols (mainly methanol and ethanol) has become increasingly important in recent years. The main focus in the literature, however, is on the traditional feeds, namely hydrocarbons with a particular emphasis on natural gas and coal (Rostrop-Nielsen, 1984; Austin, 1986; Satterfield, 1991; Pena *et al.* 1996). Also a large body of information has accumulated on the catalytic steam reforming of methanol which has been the primary focus of research for transportation applications. A variety of catalysts are capable of carrying out the methanol reforming including reduced NiO-based preparations (Mizuno *et al.*, 1986), reduced CuO/ZnO shift catalysts (extensive literature is cited by Amphlet *et al.* (1985) and Su and Rei (1991)), Cu/ZrO₂ and Pd/SiO₂ (Takahashi *et al.*, 1982), Cu/ZrO₂ (Takezava *et al.*, 1987), and Pd/ZnO (Iwasa *et al.*, 1993). Kurpit (1975) developed a low-temperature reformer using copper-zinc oxide catalyst. Konig *et al.* (1988) patented a process for methanol steam reforming using a mixture comprising of a noble metal component along with TiO₂/CeO₂ and a binder. Santacesaria and Carra (1988) reported the activity of three commercial catalysts for steam reforming of methanol. The product

Figure 1. Schematic of Ethanol Reformer-Fuel Cell System for Powering Vehicles

Figure 2. A schematic of the gasoline reformer being developed by Arthur D. Little

distribution is significantly affected by the type of catalyst and the experimental conditions used. Two main mechanisms have been proposed to explain the steam reforming of methanol (Jiang *et al.*, 1993a, b): (1) the decomposition-shift mechanism, where the chemisorbed methanol is totally dehydrogenated to CO on the metal surface and then the shift reaction proceeds with the adsorbed water-derived species; (2) the formate mechanism, initially proposed by Takahashi *et al.* (1982), and based on the formation of methyl formate ester on the catalyst surface between two adsorbed species, H_3CO and HCO . The ester is hydrolyzed to methanol and formic acid. The latter decomposes directly to CO_2 and H_2 with 100% selectivity, i.e., no CO is formed. More recently a comprehensive study of the methanol steam reforming, including the process kinetics, has been performed by Idem and Bakhshi (1994a, b; 1996) on Mn-promoted coprecipitated Cu-Al catalyst.

The partial oxidation route (POR) for methanol has also been intensively studied. Base metal catalysts comprising Cu and Cr and promoted with Zn have been used extensively for the partial oxidation and decomposition of methanol due to the low reaction temperatures and the high conversion obtained. However, the thermal instability of these catalysts and their ineffectiveness under oxidizing conditions has prompted the use of noble metal catalysts (Konig *et al.*, 1988). They reported a process in which the catalytic decomposition or steam reforming of the fuel takes place under partly oxidizing conditions using a noble metal element of group VIII mixed with TiO_2 or CeO_2 . In their patent, Konig *et al.* (1988) reported high conversions of the fuel to H_2 under conditions where the formation of coke and dimethyl ether was prevented. Kumar *et al.* (1993) patented a process for the partial oxidation of methanol using a variety of catalysts, namely, NiO/ZrO_2 , $\text{NiO-Al}_2\text{O}_3\text{-CaO}$ and Cu-Pd-SiO_2 . They even tested some reforming catalysts such as Cu-SiO_2 , $\text{NiO-Al}_2\text{O}_3\text{-MgO}$ for the partial oxidation of methanol. The partial oxidation reformer also holds a oxidation/methanation catalyst for converting any carbon monoxide formed to carbon dioxide or methane. Schmitz *et al.* (1994) have developed highly active catalysts for methanol decomposition to H_2 and CO that are based on the novel technique of supported molten-salt catalysts developed in our laboratory. Specifically, they prepared catalysts by supporting molten CuCl-KCl or $\text{CuCl-ZnCl}_2\text{-KCl}$ on SiO_2 , Al_2O_3 , or ZnSiO_3 supports. The POX reaction has been also studied over Cu-ZnO and $\text{Cu-ZnO}/\gamma\text{-Al}_2\text{O}_3$ catalysts (Huang and Cheng, 1988). These authors proposed a mechanism for the POX of methanol, which assumed the dissociative adsorption of methanol as the rate determining step, while methoxide was assumed as the most abundant surface intermediate. The POX of methanol was shown to be a viable process for practical applications. A catalytic POX reformer for methanol (Kumar and Ahmed, 1995) was developed recently at Argonne National Laboratory. The reformer is robust and easily controlled by varying the methanol, water and air feed rates as the fuel-cell system responds to the rapidly changing power demands of the vehicle's drive cycle. However, few details are available regarding the catalyst employed in the reformer.

Although, as discussed above, ethanol has several important advantages as a fuel

candidate for the production of hydrogen, relatively few papers in the literature report any investigations of the steam reforming of ethanol. Garcia and Laborde (1991) and Vasudeva *et al* (1996) conducted a thermodynamic study of the reforming of ethanol. It was shown, from a thermodynamic point of view, that the steam reforming of ethanol is entirely feasible. Ethanol reforming has been studied to understand the formation of acetic acid and hydrogen. Iwasa and Takezawa (1991) have shown that, in the formation of acetic acid, acetaldehyde is the key intermediate when using Cu-based catalysts. They found that the role of support is important. Cu alone shows very high selectivities toward acetaldehyde (88% at 250°C), whereas the presence of a support (Al_2O_3 , SiO_2 , ZrO_2 , or ZnO) substantially affects this selectivity and produces several other intermediates at significant concentrations. Several catalysts based on supported oxides, noble metals and carbides were tested for ethanol steam reforming by Cavallaro and Freni (1996). The results showed acceptable performance at temperatures above 630 K. Furthermore, $\text{CuO}/\text{ZnO}/\text{Al}_2\text{O}_3$ and $\text{NiO}/\text{CuO}/\text{SiO}_2$ catalysts did not produce appreciable quantities of coke and/or unexpected oxygenates by-products even with ratios of $\text{H}_2\text{O}/\text{C}_2\text{H}_5\text{OH}$ lower than 3 mol/mol⁻¹, whereas ratios of $\text{H}_2\text{O}/\text{C}_2\text{H}_5\text{OH}$ higher than 4-5 mol/mol⁻¹ were required by most of the other catalysts tested. Noble metals (Pt, Rh) and W-based catalysts showed almost the same activity as the inexpensive $\text{CuO}/\text{ZnO}/\text{Al}_2\text{O}_3$ catalyst. Catalytic activity and reactions selectivity of platinum, rhodium-, and nickel-doped zinc oxides (M/ZnO , M: metal) prepared by a wet method have been investigated for reforming of ethanol in connection with fuel-cell systems by Ishikawa *et al.* (1994). These authors used a fixed bed-flow tubular type reactor for the catalytic reaction in the temperature range of 250-500°C. The reforming on Pt/ZnO yielded CO, CH_4 , and CO_2 as well as main products, H_2 and CH_3CHO . The reaction on Rh/ZnO somewhat depressed CH_3CHO formation, while the catalytic activity of Rh/ZnO was lower than that of Pt/ZnO. The activity of Ni-based catalysts toward hydrogen production by steam reforming of ethanol was tested by Kumar and Ahmed (Kumar and Ahmed, 1995). The product was shown to contain large amounts of CO and CH_4 . Arthur D. Little, in conjunction with the Department of Energy and the Illinois Department of Commerce and Community affairs

are developing a multi-fuel processor (Figure 2), that can also process ethanol (Mitchel *et al.*, 1995), for fuel cell vehicles. Initial studies were carried out on a 25 kW catalytic partial oxidation reformer to determine the effect of equivalence ratio, steam to carbon ratio, and residence time on ethanol conversion. Results of the POX experiments with a nickel catalyst showed: (1) 75-85% conversion of the chemical energy in the fuel to chemical energy in hydrogen and carbon monoxide (counted as hydrogen); (2) operation without significant carbon/soot formation was possible; and (3) operation was stable and reliable.

It is to be mentioned that the catalytic SR and POR are extremely complicated processes and our knowledge of their mechanisms is far from being complete. Even in the case of the steam reforming of methane, the most simple reformed molecule, there still does not exist a unanimously accepted mechanism (Wagner and Froment, 1992). This is not surprising as in the kinetic modeling of catalytic reactions, one typically takes into account the presence of many different surface species and many reaction steps, based frequently simply on guess work. Their relative importance will depend on reaction conditions (conversion, temperature, pressure, etc) and as a result, it is generally desirable to introduce “complete” kinetic fundamental descriptions using, for example, the microkinetic treatment (Dumesic *et al.*, 1993). In many cases such models are based on detailed molecular information about the elementary steps obtained from surface science or in situ studies. An example of this approach is the methanol decomposition reaction (Topsoe *et al.*, 1997).

Apparatus

A diagram of the continuous flow apparatus built to conduct ethanol steam reforming experiments is shown in Figure 3. Helium serves as the carrier gas for hydrous ethanol. A Tylan General (Model FC 280S) helium mass flow controller is used to regulate helium flow through the system. A feed consisting of various liquid ethanol to water ratios is sent to the system by a Gilson syringe pump (Model 305). The feed mixture flows through a shut-off valve to a union-T where it mixes with the helium carrier gas. The feed mixture is vaporized as it travels through 2

feet of 0.25 inch stainless steel tubing that is wrapped with heating tape and fiber glass insulation. A high pressure gauge (Omega, 0-300 psig) is placed before the three-way valve. The three-way valve allows flow to be directed either to the reactor or to the bypass line.

The reactor has a diameter of 0.5 inches and is made from SS tubing. A high temperature heating furnace (Omega CRFC-26/120 rated at 1100°C) surrounds and heats the reactor. The reactor temperature is controlled by connecting the electric furnace to a temperature controller (Omega, CN9111A). One-fourth inch stainless steel tubing is also used to connect the reactor exit to a three-way valve. This three-way valve regulates whether gas flow from the bypass line or from the reactor goes into the GC. Flexible steel tubing (1/8") is used to make the connection from the three-way valve to the GC. Both the bypass line and the lines from the reactor exit to the GC are wrapped in heating tape and fiber glass insulation. The heating tape on the vaporization line, the bypass line, and the exit line to the GC are connected to separate power regulators (variacs) to control their temperatures. Type K thermocouples are placed on the vaporization line to the reactor, the exit line from the reactor to the GC, and on the bypass line. The thermocouples are connected to an Omega™ temperature displayer (DP 465) so that the temperature of each line can be monitored. A fourth thermocouple is located within the reactor. The temperature controller connected to the reactor is used to monitor its temperature.

A SRI gas chromatograph (GC) is used to analyze the product gas stream from the reactor and the gas stream from bypass line. A Carboxen™ 1000 (60/80 mesh) column is used inside the GC. It was selected for its ability to analyze mixture of permanent gases (i.e. CO, CO₂, and CH₄) and C1-C2 hydrocarbons using helium as a carrier gas. To measure the hydrogen product, the helium carrier gas is changed for nitrogen. This procedure, however, is time consuming. In the future we are planning to purchase a hydrogen analyzer or, alternatively, to utilize carrier gas (e.g. Ar) which will enable simultaneous analysis of all of the important products.

Figure 3. Ethanol Steam Reformer Experimental Apparatus

Thermodynamics of the Steam Reforming of Ethanol

In order to have a better understanding of the effect of process variables on hydrogen production by steam reforming of ethanol, a thermodynamic study of the process was performed and a paper has been submitted for publication (Fishtik *et al.* 1997). The concept of response reactions (RERs) developed by us (Fishtik *et al.* 1994,1995,1996) was used. Within the RERs approach, it is possible to derive a unique set of reactions which can completely describe the

system's behavior. Further, from the sensitivity analysis, the set of RERs may be substantially reduced by neglecting those of RERs whose contributions the system's response is negligibly small.

We have considered 7 chemical species which were found in significant amounts in experiments on the steam reforming of ethanol (Garcia and Laborde, 1991; Kumar and Ahmed, 1995; Vasudeva *et al.* 1996; Cavallaro and Freni, 1996), namely, C_2H_5OH and H_2O as reactants, and H_2 , CH_3CHO , CO , CO_2 and CH_4 , as reaction products. The equilibrium composition of the system was computed using the ECHIMAD-P/V computer program (Geana *et al.* 1985), under the assumption that species are ideal gases. The obtained results were analyzed in terms of the RERs approach. For the C-H-O system, a particular RER is defined by 4 species. Thus, a complete set of possible RERs for the steam reforming of ethanol is equal to the number of way 4 species may be selected from a total of 7, i.e., 35 (Table 1). Based on the sensitivity analysis of the system, the RERs whose contribution is dominant to the system's response were selected. For ethanol and hydrogen, the most important RERs are given in Tables 2 and 3. The contributions of these RERs as a function of temperature and initial feed ratio are plotted in Figures. 4-8.

Based on the above thermodynamic analysis, the following conclusions were drawn. From a thermodynamic point of view, both ethanol and acetaldehyde are unstable with respect to a mixture of H_2O , H_2 , CO , CO_2 and CH_4 . At low water concentrations, ethanol decomposes according to

to produce methane and carbon monoxide, which are undesirable products. The first of these reactions is dominant at lower temperatures, while the second is favored at the higher temperatures. The desired steam reforming reaction

however, can be made predominant in the temperature range of 700-800K and above as well as at

Figure 4. Species distribution as a function of temperature at $P=1\text{atm}$, $n^0(\text{C}_2\text{H}_5\text{OH}) = n^0(\text{H}_2\text{O}) = 1\text{mol}$ obtained from Gibbs free energy minimization

Fig. 5 Relative fractions of RERs in which ethanol is involved as a function of temperature at $P = 1 \text{ atm}$, $n^0(\text{C}_2\text{H}_5\text{OH}) = n^0(\text{H}_2\text{O}) = 1 \text{ mol}$.

Fig. 6 Relative fractions of RERs in which ethanol is involved as a function of the initial amount of water at $T = 800K$, $P = 1 \text{ atm}$, $n^0(C_2H_5OH) = 1 \text{ mol}$.

Fig. 7 Relative fractions of RERs in which hydrogen is involved as a function of temperature at $P = 1 \text{ atm}$, $n^0(C_2H_5OH) = n^0(H_2O) = 1 \text{ mol}$.

Fig. 8 Relative fractions of RERs in which hydrogen is involved as a function of the initial amount of water at $T = 800\text{K}$, $P = 1 \text{ atm}$, $n^0(\text{C}_2\text{H}_5\text{OH}) = 1 \text{ mol}$.

higher water/ethanol ratios. An increase in the amount of water also has the effect of increasing the extents of the water-gas-shift and methane steam reforming reactions

and, consequently, a reduction in the amounts of the undesired products, CO and CH_4 .

Energetic Analysis of the Plausible Mechanism

Heterogeneous catalytic reactions generally proceed via a complex network of elementary steps through which the reactants are transformed into intermediates and finally into products, which is referred to as the reaction mechanism. The latter can not be deduced from first principles but is normally postulated on the basis of a general physicochemical knowledge of the system coupled with guess work. Due to the large number of surface intermediates typically

involved and, consequently, a large number of possible competing reaction pathways, the determination of a realistic mechanism is an extremely difficult task. In principle, a discrimination among the different competing mechanisms is possible if the rate constants for every elementary step were known. In this respect, of relevance are the theoretical approaches that can estimate and predict the energetic characteristics of surface reactions and hence the rate constants. A simple and reliable method of modeling the energetic characteristics of surface reactions is the unity bond index - quadratic exponential potential (UBI-QEP) method (known also as the bond order conservation - Morse potential (BOC-MP) method) which has recently been developed by Shustorovich (Shustorovich and Seller, 1998). This model is based on a limited number of assumptions and, hence, requires only a few parameters which can be obtained from experimental data. Further, the enthalpy changes and activation energies predicted by UBI-QEP approach are in satisfactory agreement with experimental data.

Normally, the procedure used is as follows. Starting from a list of species including the reactants, intermediates and products, a plausible set of elementary surface reactions is postulated. Then, using the UBI-QEP formalism, the energetic characteristics of the elementary reactions, i.e., the enthalpy change and activation barriers of the forward and reverse reactions, are evaluated. With this information in hand, one can qualitatively deduce energetically favorable reaction pathways, i.e., lowest activation energies of the elementary steps by which the reactants are transformed through intermediates into products.

Although entirely reasonable, the above described procedure, in our opinion, has an important limitation. The point is that in multiple chemical reaction systems, there exist a huge number (strictly speaking, an infinite number) of possible ways by which the reactants may be transformed through intermediates into products. The qualitative argument by which an elementary reaction may be neglected because it has a high activation barrier might not always be acceptable because the activation energy of the reverse reaction could be small and the reaction could, thus, proceed in the opposite direction. Therefore, an important aspect of the problem is to define and completely enumerate a finite and unique number of ways by which the elementary reactions. The problem of enumeration of all of the possible ways (mechanisms) by which the

elementary steps can be added up so that the intermediates are canceled, resulting in net chemical process involving only reactants and products (overall reactions), is well known in chemical kinetics, especially, in heterogeneous catalysis (Horiuti and Nakamura, 1967; Horiuti, 1973; Temkin, 1973; 1979; Milner, 1964; Happel and Sellers, 1982; 1983; 1989a; 1989b; Sellers, 1984; 1989; Mavrovouniotis and Stephanopoulos, 1992; Mavrovouniotis, 1992). In chemical processes with only one overall reaction and a given set of linearly independent elementary steps, the derivation of the mechanism is straightforward. In multiple chemical reaction systems, however, a meaningful, i.e., a unique, solution to the problem can be obtained only by defining direct mechanisms (Milner, 1964) and direct overall reactions (Happel and Sellers, 1982; 1983; 1989a; 1989b; Sellers, 1984; 1989). Recently, we have shown (Fishtik *et al.*, 1998b) that the concept of uniqueness of both direct mechanisms and direct overall reactions may be deduced from chemical thermodynamics. It appears that every reaction route that is normally postulated from purely stoichiometric considerations has a definite and unique contribution to the Gibbs free energy change of the system. This new finding has an important bearing on the status of the theory of reaction routes. Namely, this theory appears to be rigorously grounded in thermodynamics.

In a special recent study (Fishtik and Datta, 1998c) we showed how the concept of unique direct mechanisms and overall reactions may be used within the UBI-QEP formalism for a systematic discrimination among reaction routes for the selection of energetically most favorable ones. These ideas are illustrated here by applying them to the analysis of the possible reaction routes in catalytic steam reforming and partial oxidation of ethanol on nickel catalyst.

The first step in this analysis is to generate a plausible set of elementary surface reactions. Normally these elementary reactions are selected so that no more than two bonds need to be broken and two new bonds are to be formed in every elementary surface reaction. Because the structure of the reactants (ethanol, water and oxygen) is well known the generation of a set of elementary surface reactions can be easily generated “breaking” the molecules step by step into different surface intermediates followed by their recombination into products. A plausible (and limited) set of elementary reactions for ethanol steam reforming and partial oxidation is given in Table 6. The next step is to evaluate the heat of adsorption of the surface intermediates. The

necessary formulas are provided by UBI-QEP theory. The results of the calculation of the heat (enthalpy change) of the surface intermediates are summarized in Table 7. Based on these data and using the necessary thermochemical data for bond dissociation energy (enthalpy change of bond dissociation, Table 8) one can readily estimate the enthalpy changes and the activation energies of the elementary steps. The necessary formulas are again provided by the UBI-QEP.

Catalyst Screening Tests

It is well known from the literature, that metal-oxide supported metal catalysts are quite active for steam reforming. In this respect, a series of 8 catalysts of different composition and different supports were obtained from various manufacturers and screened at this initial stage for a qualitative investigation in order to determine the best candidates for the steam reforming of ethanol. Approximately 3 g of each of these catalysts was loaded into the reactor during the series of runs. The runs were carried out at atmospheric pressure in the temperature range 250-500°C with a 4/1 (by volume), or 1/1 on a mole scale ethanol/water feed at WHSV = 71.0 kg C₂H₅OH/h/kg catalyst. The main products resulting from catalytic steam reforming and/or catalytic decomposition are easily identified by GC: H₂, CO₂, CH₄, CO, CH₃CHO, and C₂H₄. In the case of experiments with incomplete conversions, these main products are accompanied by unconverted starting material, (water and ethanol), which were also quantified.

The list of catalysts which were studied, along with their characteristics and the products obtained, is summarized in Table 5. Qualitatively, the main conclusion which can be drawn from these results is that the investigated catalysts could be divided roughly into three groups based on their catalytic action: The first group (CuO/ZnO catalysts on Al₂O₃ and C support) mainly catalytically decomposed ethanol to acetaldehyde and hydrogen

The second group of catalysts (NiO/Al₂O₃, CuO/ZnO (BASF), Pt/Al₂O₃) were very active toward ethanol dehydration process

The catalytic activity of the third group of catalysts ($\text{Fe}_2\text{O}_3/\text{Cr}_2\text{O}_3/\text{C}$, $\text{NiO}/\text{CaAl}_2\text{O}_4/\text{Al}_2\text{O}_3$, NiO (BASF)) is high toward a product mixture of H_2 , CO_2 , CO and CH_4 . It is this group of catalysts which is the most appropriate so far of the catalysts investigated for the steam reforming of ethanol. The product distribution depends in a complex manner on the type of catalyst and operating conditions, especially on the temperature. Qualitatively, however, the species distribution among the products (H_2 , CO_2 , CO and CH_4) corresponds roughly to that predicted by thermodynamics (Figure 4).

Fig. 9. Yield of hydrogen as a function of temperature

In order to quantitatively estimate the possible yields of hydrogen resulting from the steam reforming of ethanol, the catalyst G-90B ($\text{NiO}/\text{CaAl}_2\text{O}_4/\text{Al}_2\text{O}_3$) was chosen for further more detailed experiments. The GC was calibrated for hydrogen at several concentrations to cover the predicted range of hydrogen production. The results are given in Fig. 9, where the yield of hydrogen, calculated with respect to the desirable reaction

is plotted as a function of temperature. It can be seen that yields up to 90% and more are possible at temperatures around 600°C .

It is to be mentioned that for those catalysts that mainly produce acetaldehyde and ethylene, an increase in temperature above 450°C decreased the amounts of both acetaldehyde and ethylene while increasing the amount of H_2 , CO_2 , CO and CH_4 . This is due to the increasing role of the steam and carbon dioxide reforming, hydrogenation and/or catalytic decomposition of acetaldehyde and ethylene as listed below:

a) Further reactions of CH_3CHO :

1. $\text{CH}_3\text{CHO} + 3\text{H}_2\text{O} = 2\text{CO}_2 + 5\text{H}_2$
2. $\text{CH}_3\text{CHO} + 2\text{H}_2\text{O} = 5\text{CH}_4 + 3\text{CO}_2$
3. $\text{CH}_3\text{CHO} + \text{H}_2\text{O} = 2\text{CO} + 3\text{H}_2$
4. $\text{CH}_3\text{CHO} + \text{CO}_2 = 3\text{CO} + 2\text{H}_2$
5. $\text{CH}_3\text{CHO} + 3\text{CO}_2 = 5\text{CO} + 2\text{H}_2\text{O}$
6. $\text{CH}_3\text{CHO} + 3\text{H}_2 = 2\text{CH}_4 + \text{H}_2\text{O}$
7. $2\text{CH}_3\text{CHO} + 2\text{H}_2 = 3\text{CH}_4 + \text{CO}_2$
8. $\text{CH}_3\text{CHO} = \text{CH}_4 + \text{CO}$

b) Further reactions of C_2H_4 :

1. $\text{C}_2\text{H}_4 + 4\text{H}_2\text{O} = 2\text{CO}_2 + 6\text{H}_2$
2. $2\text{C}_2\text{H}_4 + 2\text{H}_2\text{O} = 3\text{CH}_4 + \text{CO}_2$
3. $\text{C}_2\text{H}_4 + 2\text{H}_2\text{O} = 2\text{CO} + 4\text{H}_2$

Thus, CH_3CHO and C_2H_4 act like intermediates at higher temperatures. This also confirms the thermodynamic results that any oxygenate or hydrocarbon can, in principle, be steam reformed at higher temperatures.

Detailed experiments with Ni oxide based catalysts

As already mentioned above, based on a comprehensive catalyst screening (9 commercial catalysts) we were able to identify those catalysts that are the most appropriate for our purposes. Namely, the catalyst Ni-0920 S, Engelhard and G-90B ($\text{NiO}/\text{CaAl}_2\text{O}_4/\text{Al}_2\text{O}_3$). These catalysts can easily decompose hydrous ethanol (100% conversion) at moderate temperatures (around 500°C) to a mixture of H_2 , CO_2 , CO and CH_4 . The last two products are undesirable products: CO is a poison for the fuel cell while the production of CH_4 is a lost in hydrogen. Our main goal, therefore, was to minimize the amounts of CO and CH_4 . The extensive experiments has shown that the temperature, water to ethanol ratio and the oxygen to ethanol ratio are the main factors that determine the product composition. Based on these experiments it has been shown that at 550°C and a water to ethanol ratio equal to 2:3 (by volume) the production of methane on G-90B catalyst can be reduced practically to zero. Several representative experimental results that illustrate the effect of the operational variables on the product distribution in the system are shown in Figures 10-12. Under these conditions, however, there are still produced significant amounts of CO . In order to reduce the amount of CO and concomitantly to increase the amount of hydrogen a second, water-gas-shift-reaction (WGSR) reactor, was built

Fig. 10 The Effect of temperature on the product distribution

Fig. 11. The effect of oxygen on the product distribution

Fig. 12 The effect of feed composition on the product distribution

Fig. 13 The effect of WGSR catalyst on the yield of CO. 1 - in the absence of WGSR catalyst; 2 - in the presence of WGSR catalyst

up into our experimental system. The WGSR reactor was loaded with 5g of the CuO/ZnO catalyst C18-7-01 supported on Al_2O_3 /C (United Catalyst) and, so far, was operated at a constant temperature equal to 200°C. The effect of the WGSR catalyst was shown to be extremely beneficial having the immediate effect to substantially decrease the amount of CO and increase the amount of hydrogen due to WGSR

A typical result is shown in Figure 13.

Thus, we are in a position to claim that the main goal of the present project was achieved in principle; that is, the steam reforming of ethanol can be performed at temperatures around 550°C and a water to ethanol ratio equal to 2:3 (by volume) with a Ni oxide based catalyst practically quantitatively according to the desirable reaction resulting in a pure mixture of hydrogen and carbon dioxide.

Kinetic Studies of the Steam Reforming of Ethanol

a) Data

As already mentioned above, a detailed study of the steam reforming of ethanol was performed using the commercial catalyst G-90B ($\text{NiO}/\text{CaAl}_2\text{O}_4/\text{Al}_2\text{O}_3$). Data were collected at 12 different values of WHSV at four temperatures (475°C, 500°C, 525°C and 500°C), and at 5 different ethanol:water ratios (1:2.0, 1:2.5, 1:3.0, 1:3.5 and 1:4.0, on a mole basis). An example of a set of data is presented in Figure 14 for ethanol versus WHSV at the different temperatures for a fixed ethanol:water ratio of 1:3.0. For kinetic modeling only conversions below 15% were selected to maintain differential conditions when use can be made of

$$R_j = \frac{F_{A0} \Delta X_j}{W}$$

where R_j is the rate of formation of species j , F_{A0} is the molar flow rate of ethanol, ΔX_j is the conversion of ethanol to species j and W is the mass of catalyst in the reactor.

b) Kinetic Modeling

With the thermodynamic and kinetic information presented in Table 6, one, in principle can proceed to determine the energetically most favorable reaction pathways. Thus a complete solution of the problem can be obtained by generating a complete set of direct mechanisms and direct overall reactions. Notice that the latter are overall RERs derived above. From the complete set of direct mechanisms and overall RERs, those that involve elementary reactions with the lowest activation energy barriers are further selected. However, although straightforward and systematic in principle, this approach is almost impossible to follow due to the complexity of our system. Since the analysis by hand is essentially impossible it is necessary to use a special computer software. Work along developing such a program is in progress. Here, however, we shall use a simplified qualitative approach that is practical at this time. First, we select a minimum set of energetically most probable surface elementary reactions involving only hydrogen and oxygen, thus showing the hydrogen production pathway resulting from water. From Table 6 it is readily seen that these are:

	ΔH	E_f	E_r
$\text{H}_2\text{O(g)} + \text{S} = \text{H}_2\text{OS}$	-17	0	17
$\text{H}_2\text{OS} + \text{S} = \text{HS} + \text{OHS}$	11	21	10
$\text{OHS} + \text{S} = \text{HS} + \text{OS}$	-15	13	28
$\text{OHS} + \text{HS} = \text{OS} + \text{H}_2\text{S}$	0	15	15
$\text{HS} + \text{HS} = \text{H}_2\text{S} + \text{S}$	15	23	8
$\text{H}_2\text{S} = \text{H}_2\text{(g)} + \text{S}$	7	7	0

Notice that the dissociation of water and surface recombination of hydrogen atoms have quite large activation energies in the forward direction. These two elementary reactions, however, are stoichiometrically mandatory, i.e., necessary to account for the hydrogen and oxygen resulting from water. In a similar manner, we select energetically the most favorable ways that lead to the

C-C bond break. A detailed analysis of the data from Table 6 shows that there are several such reaction pathways. We have chosen only those in which all of the activation energies are below 15 kcal/mol. For instance, the following reaction pathway

	ΔH	E_f	E_r
$\text{CH}_3\text{CH}_2\text{OS} + \text{S} = \text{CH}_3\text{CH}_2\text{S} + \text{OS}$	-8	13	21
$\text{CH}_3\text{CH}_2\text{S} + \text{S} = \text{CH}_3\text{S} + \text{CH}_2\text{S}$	18	24	6

was rejected due to the high activation energies. Based on these considerations we concluded that the most important pathways in the C-C bond breaking are

	ΔH	E_f	E_r
a)			
$\text{CH}_3\text{CH}_2\text{OS} + \text{S} = \text{CH}_3\text{CHOS} + \text{HS}$	-1	7	8
$\text{CH}_3\text{CH}_2\text{OS} + \text{S} = \text{CH}_3\text{S} + \text{CH}_2\text{OS}$	11	12	1
b)			
$\text{CH}_3\text{CH}_2\text{OS} + \text{OS} = \text{CH}_3\text{CHOS} + \text{OHS}$	14	14	0
$\text{CH}_3\text{CHOS} + \text{S} = \text{CH}_3\text{COS} + \text{HS}$	-8	10	18
$\text{CH}_3\text{CHOS} + \text{S} = \text{CH}_3\text{S} + \text{CHOS}$	5	15	10
c)			
$\text{CH}_3\text{CH}_2\text{OS} + \text{OS} = \text{CH}_3\text{CHOS} + \text{OHS}$	14	14	0
$\text{CH}_3\text{CHOS} + \text{OS} = \text{CH}_3\text{COS} + \text{OHS}$	7	14	7
$\text{CH}_3\text{CHOS} + \text{HS} = \text{CH}_3\text{COS} + \text{H}_2\text{S}$	7	7	0
$\text{CH}_3\text{COS} + \text{S} = \text{CH}_3\text{S} + \text{COS}$	-10	4	14
d)			
$\text{CH}_3\text{CH}_2\text{OS} + \text{OS} = \text{CH}_3\text{CHOS} + \text{OHS}$	14	14	0
$\text{CH}_3\text{CHOS} + \text{S} = \text{CH}_3\text{COS} + \text{HS}$	-8	10	18
$\text{CH}_3\text{COS} + \text{OS} = \text{CH}_3\text{OS} + \text{COS}$	0	10	10

Notice, in all mechanisms these elementary reactions will be unidirectional, i.e. they will proceed only from left to right. Now, it remains to select a set of plausible elementary reactions involving C1 surface species that can explain the formation of CO(g), CO₂(g) and CH₄(g). In doing this we have to take into account that the C1 elementary reactions can proceed in both direction. Based on these consideration we have selected those elementary reactions whose activation energies in either directions are below 25 kcal/mol. The resulting reduced overall kinetic mechanism is shown in Table 9.

c) Rate Equations

Because of the lack of information on the preexponential factors and, hence, rate constants, we have modeled the kinetics of the steam reforming of ethanol using the LHHW formalism (Hougen and Watson, 1947). Even starting with the reduced kinetic mechanism given in Table 9 a complete enumeration by hand of the different possible mechanisms that produce the 13 overall RERs and the corresponding LHHW rate equations is extremely tedious. In view of this, a different approach was adopted. Namely, following Boudart (Boudart and Djega-Mariadassou, 1982), the elementary reactions that are considered to be at quasi-equilibrium before and after RDS may be combined into overall steps. Consider, for example, the following mechanism

	ΔH	E_f	σ
1. $\text{CH}_3\text{CH}_2\text{OH(g)} + 2\text{S} = \text{CH}_3\text{CH}_2\text{OS} + \text{HS}$	-24	4	1
2. $\text{H}_2\text{O(g)} + \text{S} = \text{H}_2\text{OS}$	-17	0	3
3. $\text{H}_2\text{OS} + \text{S} = \text{HS} + \text{OHS}$	11	21	3
4. $\text{OHS} + \text{S} = \text{HS} + \text{OS}$	-15	13	3
5. $\text{CH}_3\text{CH}_2\text{OS} + \text{S} = \text{CH}_3\text{CHOS} + \text{HS}$	-1	7	1
6. $\text{CH}_3\text{CHOS} + \text{S} = \text{CH}_3\text{COS} + \text{HS}$	-8	10	1
7. $\text{CH}_3\text{COS} + \text{OS} = \text{CH}_3\text{OS} + \text{COS}$	0	10	1

8. $\text{CH}_3\text{OS} + \text{S} = \text{CH}_2\text{OS} + \text{HS}$	5	10	1
9. $\text{CH}_2\text{OS} + \text{S} = \text{CHOS} + \text{HS}$	-7	10	1
10. $\text{CHOS} + \text{S} = \text{COS} + \text{HS}$	-23	0	1
11. $\text{COS} + \text{OS} = \text{CO}_2\text{S} + \text{S}$	9	15	2
12. $\text{CO}_2\text{S} = \text{CO}_2(\text{g}) + \text{S}$	6	6	2
13. $\text{HS} + \text{HS} = \text{H}_2\text{S} + \text{S}$	15	23	6
14. $\text{H}_2\text{S} = \text{H}_2(\text{g}) + \text{S}$	7	7	6

It is seen that the surface recombination of hydrogen atoms has the highest activation energy and, consequently, in the absence of other information it is natural to assume that this elementary reaction is the RDS while the remaining elementary reactions may be assumed to be at quasi-equilibrium. In this case the 12 elementary reactions that precede the RDS can be combined into one step

	ΔH	E_f	σ
1. $\text{CH}_3\text{CH}_2\text{OH}(\text{g}) + 2\text{S} = \text{CH}_2\text{CH}_2\text{OS} + \text{HS}$	-24	4	1
2. $\text{H}_2\text{O}(\text{g}) + \text{S} = \text{H}_2\text{OS}$	-17	0	3
3. $\text{H}_2\text{OS} + \text{S} = \text{HS} + \text{OHS}$	11	21	3
4. $\text{OHS} + \text{S} = \text{HS} + \text{OS}$	-15	13	3
5. $\text{CH}_3\text{CH}_2\text{OS} + \text{S} = \text{CH}_3\text{CHOS} + \text{HS}$	-1	7	1
6. $\text{CH}_3\text{CHOS} + \text{S} = \text{CH}_3\text{COS} + \text{HS}$	-8	10	1
7. $\text{CH}_3\text{COS} + \text{OS} = \text{CH}_3\text{OS} + \text{COS}$	0	10	1
8. $\text{CH}_3\text{OS} + \text{S} = \text{CH}_2\text{OS} + \text{HS}$	5	10	1
9. $\text{CH}_2\text{OS} + \text{S} = \text{CHOS} + \text{HS}$	-7	10	1
10. $\text{CHOS} + \text{S} = \text{COS} + \text{HS}$	-23	0	1

so that the mechanism may now be written as

which would provide the same LHHW rate expression as the complete mechanism. Thus, from the view point of kinetics the two mechanisms are equivalent the former being more satisfactory while the latter is more convenient. From this example it may be concluded that once the RDS has been specified the quasi-equilibrium elementary steps in the mechanism preceding and succeeding the RDS may be always substituted with a set of overall steps involving one surface species, an active site S on the catalyst, and terminal species. In other words, these overall steps are nothing but formation reactions of the surface species involved in the RDS from terminal species. It may be shown that the formation reactions are stoichiometrically unique, i.e., independent on the elementary reactions from which they are derived and therefore independent of the mechanism for a given RDS. In particular, they can be derived even from the atomic matrix. This observation suggests that within the LHHW formalism the rate equations may be derived as follows. First, we derive a complete set of formation reactions for a given set of terminal ($\text{C}_2\text{H}_5\text{OH}$, H_2O , CO , CO_2 , H_2 and CH_4) and surface species ($\text{CH}_3\text{CH}_2\text{OS}$, CH_3CHOS , CH_3COS , CH_3S , CH_2OS ,

CHOS, H₂OS, HS, OHS, OS, H₂S, COS, CO₂S, CH₄S). For our system the set of formation reactions for all of the surface species is given in Table 10. Next, based on energetic considerations we postulate a plausible candidate for RDS from Table 9. Then, from Table 10 we select different formation reactions of the surface species involved in this particular RDS and linearly combine them so as to eliminate the surface species and to obtain an overall reaction. As an example illustrating the approach assume the elementary surface reaction (reaction 10 from Table 9)

to be a candidate for RDS. It is seen that in this reaction there are involved 3 surface species CH₃CHOS, CH₃S and CHOS. From Table 10 we arbitrarily select a set of formation reaction, e.g.,

Combining these formation reactions with the RDS we have the following mechanism

Evidently, selecting different formation reactions will result in different mechanisms and different overall reactions, e.g.,

d) Fitting

Using the approach described above, a large number (about 200) of different mechanisms were derived and checked against fitting experimental data. Numerical simulations were performed with the commercial program Scientist (MicroMath Computer Software) using both the least squares method and Levenberg-Marquardt minimizer. The best fitting (R exceeding 0.98) was achieved with the set of mechanisms and rate equations given in Table 10. An example of the results of fitting is presented in Figure 15. It is to be noted that any attempts to include the adsorption terms in the LHHW rate equations failed to improve the fitting which may mean that surface coverage of the various species under reaction conditions is small. Further, inclusion of the thermodynamic term in the rate expressions didn't improve fitting, presumably because under differential conditions the rate of reverse reactions is small. The rate constants as a function of temperature are given in Table 12. All of the three rate constants satisfactorily follow the Arrhenius equation. An example of the fitting of the rate constants against Arrhenius equation is presented in Figure 16. The activation energies for the overall reactions given in Table 9 are 50.0 kJ/mol (steam reforming of ethanol), 29.9 kJ/mol (thermal decomposition of ethanol) and 69.3 kJ/mol (WGS) respectively. While activation energies for other steps on Ni are not reported in

the literature the activation energy for the WGSR may be compared with 67.1 kJ/mol obtained by Xu and Froment (Xu and Froment, 1989) on a $\text{Ni/MgAl}_2\text{O}_4$ catalyst for steam reforming of methane.

Conclusions

Our detailed theoretical and experimental research of the steam reforming of ethanol has proven the efficacy of this process for production of hydrogen rich gas for fuel sells. More specifically, we have shown

- thermodynamically, steam reforming of ethanol is feasible although a variety of side products can be obtained. Under certain conditions (temperature and ethanol to water ratios), however, the desirable reaction may be made predominant.

- the best catalyst for steam reforming of ethanol appears to be the Ni based catalysts. Along with H_2 and CO_2 , however, these catalysts produce CO and CH_4 . At around 5500C and a water to ethanol ratio equal to 2:3 (by volume) the amount of methane can be reduced practically to zero. However, there is still 8 to 14 percent CO in product.

- a WGSR reactor loaded with the CuO/ZnO catalyst C18-7-01 supported on Al_2O_3 /C (United Catalyst) and operated at a constant temperature equal to 200oC is extremely beneficial to substantially decrease the amount of CO, and increase the amount of hydrogen due to WGSR.

- mechanism of the steam reforming of ethanol may be successfully derived based on UPI-QEP energetic estimations.

- the kinetics of the process may be adequately modeled using the LHHW formalism.

Thus, it may be concluded that the main goals of the present project were successfully achieved; that is, the steam reforming of ethanol can be performed at temperatures around 5500C and a water to ethanol ratio equal to 2:3 (by volume) with a Ni oxide based catalyst practically quantitatively according to the desirable reaction resulting in a pure mixture of hydrogen and carbon dioxide. We were also able to generate adequate thermodynamic and kinetic information on selected catalyst to allow the design and development of prototypes for testing.

Figure 14. Ethanol Conversion as a function of WHSV

Figure 15. Fitting Results for the Rate of Hydrogen Production

Figure 16. Temperature Dependence of Rate Constants

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Stanton B. Gelvin	
University:	Purdue University	
Agreement Number:	OR22072-90	
Project Title:	Plant Genes Involved in T-DNA Integration and Radiation Sensitivity	
Reporting Period and Report Type:	From: 12/1/98 To: 2/29/00	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

OBJECTIVE 1: Screen the Feldmann and DuPont collections of T-DNA mutagenized *Arabidopsis* plants for mutants that are resistant to *Agrobacterium* transformation.

We have screened more than 3250 individual plants. Of these, 238 were putative *rat* mutants. Approximately 110 of these putative mutants have been or are now in the process of re-screening. We have confirmed at least 10 *rat* mutants from these.

OBJECTIVE 2: Screen the Feldmann and DuPont collections of T-DNA mutagenized *Arabidopsis* plants for mutants that are radiation hypersensitive.

Several reviewers pointed out that our initial *rat* mutant screening was a huge amount of work, and that we should not pursue this screen. We have therefore abandoned this line of experimentation.

OBJECTIVE 3: Perform genetic analyses on the *rat* mutants.

This is an ongoing line of experimentation. Of the confirmed new *rat* mutants, 8 are homozygous. Of three heterozygous lines, two are segregating (for the kanamycin-resistance marker on the T-DNA) 3:1, and one is segregating 7:1.

OBJECTIVE 4: Perform molecular analyses on the mutants.

We are in the process of isolating T-DNA/plant DNA junctions for several of the known new *rat* mutants.

OBJECTIVE 5: Perform genetic complementation of the *rat* mutants with wild-type DNA.

Because we do not have the wild-type gene yet, we have not done this analysis yet.

OBJECTIVE 6: Assay mutants for increased levels of homologous recombination.

We have not begun these analyses yet.

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 below.

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.

Congress on Molecular Plant-Microbe Interactions, Amsterdam, The Netherlands July 24-30, 1999. Invited lecture: "Plant genes involved in *Agrobacterium*-mediated transformation".

20th Annual Crown Gall meeting, Houston, TX, November 5-7, 1999. Lectures and posters: "Identification of plant genes involved in *Agrobacterium*-mediated transformation", "An *Arabidopsis* histone H2A gene is involved in T-DNA integration".

Genetic Engineering/Physiological and Molecular Plant Biology seminar, University of Illinois, Feb. 16, 2000: "Plant genes involved in *Agrobacterium*-mediated plant transformation".

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.

None to date.

Educational Accomplishments

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

Contributed to the training of three postdoctoral research fellows.

Additional Funding

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

NSF: Identification of plant genes involved in *Agrobacterium*-mediated transformation. (To Stanton B. Gelvin, Vitaly Citovsky, and Barbara Hohn). 9/1/99-8/31/04. \$4,440,751

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

Postdoctoral research fellows involved in the project:

1. Jaime Humara (Spanish government fellowship supplemented from this grant)
2. C.T. Ranjith Kumar
3. Jyothi Rajagopal
4. Ho Chul Yi (Korean KOSEF fellowship supplemented from this grant)

SCIENTIFIC ACCOMPLISHMENTS

1. SCREENING FOR *RAT* MUTANTS (Jaime Humara and Jyothi Rajagopal)

As an ongoing effort in our laboratory, we screened more than 3250 T-DNA mutagenized *Arabidopsis* plants from the Feldmann library (ecotype Ws) for resistance to *Agrobacterium* transformation (*rat* mutants). During this past year we screened an additional approximately 3000 mutagenized plants using personnel hired on other grants, and previously screened approximately 3000 plants. Our overall screening efforts have now come close to saturating the Feldmann collection (6400 total mutagenized lines; we have screened in all approximately 9000 individual plants). We had originally proposed to screen the DuPont collection of T-DNA mutagenized *Arabidopsis* plants. However, we have found that this collection has very poor germination (we have twice received this collection), and we have therefore abandoned efforts to use the DuPont collection. We have now acquired the INRA/Versailles collection of T-DNA mutagenized plants (also in the ecotype Ws) and have begun screening this collection for *rat* mutants. In addition, with the help of Dr. Ray Bressan of Purdue University, we shall soon initiate construction of a new 50,000-60,000 member collection of T-DNA mutagenized *Arabidopsis* plants (ecotype Ws). This collection should be available for screening in six to eight months. (We had started to acquire the Amasino/Sussman collection of T-DNA mutagenized plants, but abandoned efforts to use this collection when we learned that the T-DNA used to mutagenize these plants contains an active AP3 homeotic gene).

Primary screening of these lines involves germination and sterile growth of the plants in agar medium in baby food jars, sectioning of the roots into 3-5 mm segments (the shoots are placed back into medium to re-grow roots), infection of the roots for two days by an oncogenic *Agrobacterium* strain (A208), transfer of the roots to agar medium containing timentin (to kill the *Agrobacterium*) but lacking phytohormones (for selection of tumors), and incubation for 4-6 weeks. Of the approximately 3250 plants screened, we identified 238 putative *rat* mutants. Seeds of these putative mutant plants were collected and the lines are currently being re-tested for the *rat* phenotype. This secondary screening involves infection of the roots with *A. tumefaciens* A208 (to screen for tumorigenesis) and also infection with a non-oncogenic *Agrobacterium* strain (At872) containing a binary vector with a *bar* gene. The infected roots are subsequently screened for sensitivity or resistance to the herbicide phosphinothricin on callus inducing medium (CIM). We have completed secondary screening of approximately 110 putative *rat* mutants and have confirmed more than 10 *rat* mutants. Work during the second year of this grant will finish the secondary screening of these lines (as well as initiate screening of other mutagenized lines).

We have initiated genetic analysis of some of the confirmed *rat* mutants. Eight of the lines are homozygous (they do not segregate kanamycin-sensitive plants), and of three heterozygous lines tested, two segregate 3:1 (kanamycin^r:kanamycin^s), indicating a single segregating T-DNA locus, and one segregates 7:1 (an unusual segregation ratio). We shall soon initiate a co-segregation analysis of the heterozygous plants (looking for co-segregation of kanamycin-resistance with the *rat* phenotype). In addition, we shall cross the homozygous plants with a wild-type Ws plant and perform a similar co-segregation analysis of the resulting F2 progeny. Finally, we shall test the F1 progeny resulting from these crosses for dominance/recessivity of the *rat* phenotype.

We are also initiating an analysis of the confirmed *rat* mutants to determine whether transformation is blocked early or late in the process. To do this, we shall infect cut root segments with an *Agrobacterium* strain containing a binary vector (pBISN1) with

a *gusA*-intron gene. We shall assay transient GUS activity 4-6 days after infection. Low GUS activity will indicate an early transformation block-point, whereas high GUS activity will indicate a late stage (probably integration) block-point.

2. INTERACTION OF HISTONE H2A PROTEINS WITH VIRD2 PROTEIN (C.T. Ranjith Kumar)

We had previously identified a mutant, *rat5*, that contains a T-DNA insertion into the 3' untranslated region of a histone H2A gene. We recently published that this mutant is defective in T-DNA integration (Mysore, K.S., Nam, J., and Gelvin, S.B. 2000. An *Arabidopsis* histone H2A mutant is deficient in *Agrobacterium* T-DNA integration PNAS 2000 97: 948-953). Because VirD2 protein is covalently linked to the 5' end of the T-strand that enters the plant nucleus, and because we had previously published that VirD2 is involved in T-DNA integration (Mysore, K.S., Bassuner, B., Deng, X-b., Darbinian, N.S., Motchoulski, A., Ream, W., and Gelvin, S.B. 1998. Role of the *Agrobacterium tumefaciens* VirD2 protein in T-DNA transfer and integration. Mol. Plant-Microbe Interact. 11:668-683), we reasoned that VirD2 may interact with any "integration complex" of plant proteins, possibly including histone H2A.

We therefore tested VirD2, both as a bait and a prey protein, in a yeast two-hybrid system, using histone H2A (encoded by *RAT5*) as the corresponding prey or bait protein. In all situations tested, these two proteins interacted. We have determined that histone H2A is encoded by a 6-member multigene family in *Arabidopsis*. We have cloned the cDNAs and gene for most of these family members. We next determined whether the other members of the histone H2A family could interact with VirD2 in a yeast two-hybrid system. Our analysis indicated that all members of the family that were tested could interact. As controls, we showed that neither VirE2 protein (also brought into a plant on the T-strand) and a GTPase could not interact with histone H2A in a yeast two-hybrid system.

We are continuing to analyze which portions of VirD2 interact with histone H2A in this system.

3. FUNCTIONAL EQUIVALENCE OF HISTONE H2A PROTEINS IN T-DNA INTEGRATION (Ho Chul Yi)

As described above, we have previously shown that histone H2A genes make up a six-member multigene family in *Arabidopsis*, and that mutation of one of these genes (G1) in the *rat5* mutant results in the *rat* phenotype. In the *rat5* mutant, the other histone H2A genes are wild-type, yet the *rat* mutant phenotype is expressed. It could thus be argued that the different histone H2A genes are not functionally redundant. However, these genes are controlled by different promoters, and the possibility exists that the proteins may be functionally equivalent if expressed in the appropriate manner.

We therefore placed 5 of the 6 histone H2A cDNAs behind a strong CaMV 35S promoter and individually introduced each histone H2A gene into the *rat5* mutant by a flower vacuum infiltration method. Although many *Arabidopsis* ecotypes and *rat* mutants cannot be transformed by root inoculation, they are efficiently transformed by flower vacuum infiltration (Mysore, K.S., Kumar, C.T.R., and Gelvin, S.B. 2000. *Arabidopsis* ecotypes and mutants that are recalcitrant to *Agrobacterium* root transformation are susceptible to germ-line transformation. Plant J. 21:9-16). As shown

in Table I below, transformation of root segments of the wild-type plant to the phenotype tumorigenesis is very efficient, whereas transformation of the *rat5* mutant to the same phenotype is negligible. When the wild-type *RAT5* histone H2A cDNA is expressed in *rat5* mutant plants, the transformation phenotype is restored. Complementation of the *rat5* plant can also be achieved by expression of cDNAs (under the control of the CaMV 35S promoter) from the other histone H2A genes in the *rat5* mutant plant. Thus, when overexpressed from a CaMV 35S promoter, all histone H2A cDNAs tested appear to be functionally equivalent with regard to transformation and T-DNA integration. Thus, the lack of histone H2A functional redundancy in the *rat5* mutant most likely results from lack of adequate expression of the other histone H2A proteins in the root tissue.

We are continuing these experiments by introducing each of the histone H2A genes (under the control of their native promoters) into the *rat5* mutant plant to determine whether when in higher copy these genes can complement the *rat* phenotype. We are also introducing each of the histone H2A genes into wild-type plants to determine whether overexpression of each of these genes results in increased transformation of infected transgenic root segments.

Table 1.
Complementation of *rat5* Mutant Plants with
Various Histone H2A Genes

Line	Phenotype recovery (% of Ws)
Ws	100
<i>rat5</i>	0
H2A1-1-1	89
H2A1-1-2	44
H2A1-5-3	69
H2A1-8-1	37
H2A2-1-6	31
H2A2-1-10	26
H2A3-2-4	94
H2A3-2-5	78
H2A3-2-8	67
H2A3-3-1	50
H2A4-1-1	45
H2A4-5-3	91
H2A4-5-5	73
H2A4-6-9	82
H2A4-6-10	82
H2A4-7-10	55
H2A4-8-3	64
H2A4-8-7	64
H2A5-2-2	18

LAY SUMMARY OF YEAR 1 RESEARCH PROGRESS

Agrobacterium tumefaciens has the natural ability to transfer DNA to plant cells and, as such, has been exploited by scientists as a genetic engineering tool to generate transgenic plants. Although some plant species can be transformed using *Agrobacterium*, many agronomically important crops such as maize, soybean, and wheat remain recalcitrant to efficient stable transformation. Many of these "recalcitrant" crops can be transiently transformed; however, the DNA transferred from the bacterium fails to integrate and subsequently stabilize in the plants. Scientists lack an understanding of the molecular events associated with T-DNA integration. We have identified numerous plant (*Arabidopsis*) mutants that are resistant to *Agrobacterium* transformation (*rat* mutants). Among these mutants are several that can transfer but fail to integrate T-DNA. One of these integration-deficient *rat* mutants is *rat5*, a histone H2A mutant. We have determined that histone H2A interacts with a protein associated with the incoming T-DNA, and that histone H2A makes up a small multi-gene family. We are continuing to characterize the role that this gene family plays in T-DNA integration, as well as identifying other plant genes involved in the T-DNA integration process.

Scientific Progress Report

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

Principal Investigator:	Robert Haselkorn, Ph.D.	
University:	The University of Chicago	
Agreement Number:	OR 22072-92	
Project Title:	Increasing the Energy content of Plants: Molecular Genetics of Acetyl-CoA Carboxylase	
Reporting Period and Report Type:	From: October 1, 1998 (effective starting date: February 1, 1999) To: September 30, 1999	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

Switchgrass (*Panicum virgatum* L.) is a native grass in the US and Canada with a potential to become a source of renewable biomass for fuel production. Several studies, funded by the DoE's Office of Fuels Development, aiming at the development of elite cultivars by classical breeding programs and development of proper agrotech practices, are currently underway. Attempts have also been made to establish a plant regeneration system and to assess genetic variation within and among different switchgrass populations. However, no detailed knowledge of any aspect of molecular biology or genetics of the species is yet available. This knowledge will be necessary to enhance breeding programs, explore genetic diversity of germplasm and to provide the means for metabolic engineering to improve the energy content of switchgrass.

We proposed to study the molecular biology and genetics of ACCase in switchgrass with the following goals in mind:

1. Analysis of ACCases and their genes in switchgrass.
2. Assessment of genetic variation at a molecular level to understand switchgrass phylogeny, to aid breeding programs and to further genetic studies.
3. Development of a switchgrass transformation system.
4. Development of a new selectable marker for transformation of Graminae based on herbicide resistant ACCase and engineering herbicide resistance in switchgrass to reduce weed competition.

We expect the proposed research to lead to the development of new tools for breeders and the future genetic engineering of switchgrass.

Due to the reduced budget, which amounts to 40% of the funds originally requested, we were obligated to reduce the work program. The major reduction concerned the development of a switchgrass transformation system. However, some attempts at transformation are now included in the program for year two.

Progress made towards the four major objectives during the effective period of the project (February to August 1999):

1. Fragments of genes encoding plastid ACCase from 8 switchgrass cultivars have been cloned and sequenced.
2. Preliminary multiple alignments of the genomic sequences were created and phylogenetic trees were constructed.
3. A plant regeneration system was established for switchgrass based on callus derived from mature embryos.
4. A synthetic gene consisting of an optimized 35S promoter and coding sequences of wheat plastid ACCase is being constructed.

Scientific Accomplishments

Objectives 1 and 2: Analysis of ACCases and their genes in switchgrass, and assessment of genetic variation at a molecular level to understand switchgrass phylogeny.

In many respects, the well established hexaploid crop, bread wheat, is an ideal reference system for a wild polyploid switchgrass. We have a great deal of information about wheat ACCases and their genes, as well as ACCase genes from other grasses (rye, barley, lolium, maize). This information includes multiple sequence alignments and phylogenetic trees, substitution rates and approximate divergence times. Based on this experience, we concluded that the most straightforward approach is to analyze the DNA sequences of genes encoding plastid ACCase from selected cultivars of switchgrass. We know from similar analysis of other grasses, mostly wheat, that this strategy has the potential to reveal new information on the origin of genomes and the genetic relatedness between different genomes, different ecotypes and cultivars.

This information allows reconstruction of switchgrass phylogeny and an understanding of the flow of genetic information within the polyploid species of switchgrass.

We started this analysis with the tetraploid and octaploid cultivars listed in Table 1. We plan to add more cultivars in the second year. The preliminary results of our phylogenetic analysis of switchgrass are presented in Fig. 1. At this stage, we included sequences of all clones obtained to date, not all cultivars have been fully surveyed. The existence of two major types of genomes in one or multiple copies, depending on switchgrass ploidy, is one of the possible conclusions from this phylogeny. The two different genomes diverged very recently, if the molecular clock calibrated for a different grass tribe applies here.

Table 1. Switchgrass (*Panicum virgatum*) material used in this study.

Information compiled from (2) and C.M. Taliaferro, personal communication. Seeds were provided by C.M. Taliaferro, Oklahoma State University.

	Origin		Status	Abbreviated name
<u>Octaploids (2n=8x=72)</u>				
Blackwell	northern OK	upland	commercial	Bla
Caddo	central OK	upland	breeding	Cad
Cave-in-Rock	southern IL	upland	commercial	Cav
<u>Tetraploids (2n=4x=36)</u>				
Alamo	southern TX	lowland	commercial	Ala
SL 94-1 syn-1*	southern TX	lowland	breeding	SL
Kanlow	central OK	lowland	commercial	Kan
NL 94-1 syn-1*	northern OK	lowland	breeding	NL
Tennessee 104**	southwestern TN	upland	breeding	Ten

*SL 94-1 and NL 94-1 are synthetic derivatives of Alamo and Kanlow, respectively.

** Flow cytometry results (2.88pg DNA/nucleus) indicate that Tennessee 104 is a tetraploid. It's morphology is that of an upland ecotype and the site of collection was marginally "upland".

Fig. 1. Preliminary phylogenetic tree of switchgrass based on partial sequence of the *Acc-1* gene.

Multiple alignment and phylogenetic tree (neighbor joining method) were created using Clustal X (3). The alignment is 2kb long and includes 8 exons and 7 introns. Cav3 sequence (the most divergent of all switchgrass genes sequenced so far) was used as an outgroup. The names include cultivar name (Table 1) followed by sequence ID number and in most case the number of clones analyzed (in brackets).

Objective 3. Development of a switchgrass transformation system: establishing a plant regeneration protocol based on callus derived from mature embryos.

Thus far, most transgenic cereal plants have been generated by bombarding isolated pre-mature embryos with DNA-coated gold particles. During the last several months we have made significant progress in establishing wheat transformation in our laboratory based on bombardment of pre-mature embryos of cv. "Bob White" using a protocol described before (1). Although not part of this project, experience in wheat transformation will be very helpful in our work with switchgrass. We will use microprojectile bombardment to deliver DNA to callus derived from mature embryos of c. "Alamo". Plant regeneration from such callus has recently been achieved in our laboratory (Fig. 2).

We will make some attempts at switchgrass transformation by following the strategy eloped for wheat: using the *bar* gene as the selectable marker and co-transformation to create transgenic switchgrass plants with new traits. Parameters that influence transformation efficiency such as osmotic treatment and bombardment factors will be investigated. Selection and regeneration conditions will be calibrated. Even if successful, these experiments can not be completed within the duration of this project.

Fig. 2. Regeneration of switchgrass plants from callus derived from mature embryos.

Mature seeds of switchgrass cultivar 'Alamo' were surface-sterilized and placed on callus induction medium (MS salt and vitamin supplemented with 2.5 mg/L 2,4-D and 10 mg/L BA). After 3-6 weeks, calli derived from explants were transferred to hormone free MS medium for plant regeneration. Plantlets 6-10 cm high shown on this picture, were transferred to soil and are currently being grown in a greenhouse to evaluate their development and fertility. Hundreds of regenerated plants were obtained. Transformation experiments based on this plant regeneration system are underway.

Objective 4. Development of a new selectable marker for transformation of Graminae based on herbicide-resistant ACCase.

We have recently determined that the aryloxyphenoxypropionate and cyclohexanedione herbicide sensitivity determinant of wheat plastid acetyl-CoA carboxylase is located in a 400-amino acid fragment of the carboxyltransferase domain (T. Nikolskaya, O. Zagnitko, G. Tevzadze, R. Haselkorn and P. Gornicki, manuscript in preparation). A series of artificial genes consisting of the yeast *GAL10* promoter, yeast *ACCI* leader, wheat acetyl coenzyme A carboxylase (ACCase) cDNA and yeast *ACCI* 3'-tail was used to complement a yeast *ACCI* mutation. These chimeric genes encoded full length plastid enzyme, with and without the putative chloroplast transit peptide, as well as five chimeric cytosolic/plastid proteins. Four of the chimeric genes, all containing at least half of the wheat cytosolic ACCase coding region at the 5'-end, complement the yeast mutation. Aryloxyphenoxypropionate and cyclohexanedione herbicides inhibit growth of haploid yeast strains expressing two of the chimeric ACCases at concentrations below 10 μ M. This inhibition resembles the inhibition of wheat plastid ACCase observed *in vitro* and *in vivo*. Differential response to herbicides localizes the sensitivity determinant to the third quarter of the multi-domain plastid ACCase. Sequence comparison of different multi-domain and multi-subunit ACCases suggests that this region includes part of the carboxyltransferase domain and therefore that the carboxyltransferase activity of ACCase (second half-reaction) is the target of the inhibitors.

With this information in hand, we can assemble new genes for the expression of herbicide-resistant wheat plastid ACCase in transgenic plants. One approach is to use one of the already created resistant chimeras. An alternative is to introduce single amino acid changes that convert the sensitive wild-type enzyme to a resistant mutant. A screen for such mutations is underway. Construction of a proper gene to express plastid ACCase in transgenic switchgrass, wheat and other grasses is well advanced (a few cloning steps remain). The final product will include the wheat wild-type coding sequence fused to an optimized 35S promoter. It will then be used as a source of fragments needed to create additional genes (herbicide-resistant variants, different promoters). These genes will be introduced into plants using the transformation protocols discussed in the previous section. ACCase is considered to be one of the key enzymes regulating metabolite flux through the fatty acid/lipid pathway and as such is an important regulator of carbon allocation between carbohydrate and lipid. It will be important to test whether over-expression of this enzyme alters this allocation.

References.

Becker, D., R. Brettschneider, and H. Lorz. 1994. Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. *Plant J.* 5:299-307.

2. Hopkins, A. A., C. M. Taliaferro, C. D. Murphy, and D. Christian. 1996. Chromosome number and nuclear DNA content of several switchgrass populations. *Crop. Sci.* 36:1192-1195.
3. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673-4680

Publications and Presentations

None

Technology Transfer

None

Commercial Accomplishments

No

Educational Accomplishments

This project provided a significant educational opportunity for teaching students and postdocs in two important areas of plant science: evolution and plant transformation.

Additional Funding

No

Key Personnel Hiring or Turnover

Dr. Shaoxing Huang was hired as a postdoctoral research associate. Dr. Huang has significant expertise in grass transformation gained during his previous appointment at Hamburg University as a Humboldt Fellow. Dr. Huang was hired in February 1999 replacing the person for whom the funds were originally requested. The total level of effort by research associates will be the same as last year.

Scientific Progress Report

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

Principal Investigator:	David Hildebrand	
University:	University of Kentucky	
Agreement Number:	OR22072-56	
Project Title:	Cloning Genes Encoding Enzymes for Epoxy Fatty Acid Accumulation in Oilseeds	
Reporting Period and Report Type:	From: 7/1/96 To: 9/30/99	Check one: ☐ Interim Report ☒ Final Report

Project Objectives

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

1. Partially purify the enzyme which converts linoleic acid to vernolic acid (epoxygenase) to the point whereby amino acid sequence information can be gathered and used for cDNA cloning.

The epoxygenase enzyme responsible for synthesis of the epoxy fatty acid, vernolic acid, was partially purified from developing *Vernonia* seeds and thoroughly characterized biochemically. This led to the discovery that the *Vernonia* enzyme is a $\Delta 12$ desaturase-like enzyme and provided the basis of cloning its cDNA.

2. Use the information gained from Objective 1 and additional molecular approaches for cloning cDNA(s) encoding enzyme(s) responsible for epoxy fatty acid production, e.g. epoxygenase.

Previous studies with epoxy fatty acid biosynthesis in plants indicated that the enzyme responsible in *Euphorbia* is a P450 monooxygenase. Although our biochemical studies confirmed that this is the case in *Euphorbia*, we established that this is not the case in *Vernonia* as mentioned under Obj. 1. Using conserved $\Delta 12$ desaturase sequences we were able to clone epoxygenase cDNAs from developing *Vernonia* and *Stokesia* seeds.

3. Test cDNA(s) for epoxy fatty acid production in transgenic plant tissues (i.e. tobacco calli and *Arabidopsis* seed).

Testing in transgenic tobacco calli gave no clear results and it appears that tobacco calli cannot accumulate epoxy fatty acids. Testing in transgenic Arabidopsis seeds did give small but significant accumulation of the epoxy fatty acid, vernolic acid, in transgenic Arabidopsis seed. Considerable effort was expended on using yeast as a rapid test system for epoxy fatty acid biosynthetic genes. Methods have been established for using yeast for this purpose that appear promising.

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.

Good progress has been made for providing a basis of using oilseed plants as bioreactors as a renewable source of epoxides. Genetic alteration of biochemical pathways operative in seeds to effect the accumulation of high value products is a direct way to increase the profitability of crop production and provide useful materials for industry. This also provides a means to use biochemical pathways in plants to produce compounds that are costly to produce from petroleum. A considerable market currently exists for epoxy fatty acids, particularly for epoxy coatings and plasticizers. The current US plasticizer market is about 2 billion pounds per year. Presently most of this is derived from petroleum. Current value of epoxy fatty acids could be as much as \$600 million/year in the US and if a renewable and stable supply were established and materials engineers find new uses, the market would be expected to expand. In addition there is industrial interest in use of epoxy fatty acids in oil-based paints, lubricants and lubricant additives, adhesives, insecticides and insect repellants, crop oil concentrates and the formulation of carriers for slow-release pesticides and herbicides. Soybeans are currently utilized to some extent to produce epoxidized oil at a cost equivalent of \$0.3/pound of seed oil. Appropriately genetically engineered soybean oil would be more valuable than currently produced epoxidized oil plus the \$0.3/pound savings. This would enable soybeans to make considerable inroads into the petroleum-derived epoxy fatty acid market. Currently epoxidized plant oils such as soybean oil are produced by introducing an epoxy group across the double bonds of polyunsaturated fatty acids. This is a costly process and the processing costs are avoided if the biosynthetic reactions in oilseeds are altered such that the seeds themselves convert the polyunsaturated fatty acids into epoxy fatty acids.

There is no known way to produce a commercial oilseed that accumulates epoxy fatty acids by conventional breeding and

genetics. However, certain genotypes of several plant species accumulate high levels of epoxy fatty acids in the seed oil. We have studied and characterized this process biochemically. Using this information we have cloned genes responsible for epoxy fatty acid accumulation from these exotic plant species and tested them in model plant transformation systems. This will provide the basis for the transfer of gene(s) encoding epoxy fatty acid biosynthetic enzymes to major oilseeds to effect epoxy fatty acid accumulation in plant oils which can be readily produced on a commercial scale.

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 our research is to genetically engineer oilseeds for high epoxy fatty acid accumulation in triglycerides (oils). Epoxy fatty acids have unique chemical properties which makes them very valuable in the production of a wide range of industrial products. Few plant species accumulate epoxy fatty acids as the major constituents of their seed oils. *Vernonia galamensis* is one of the highest known accumulators of the epoxy fatty acid vernoleate (around 80% of the seed oil), mostly in the form of trivernolin. Some other plants such as *Euphorbia lagascae* also accumulate significant amount of vernoleate. However, most of the vernoleate in *E. lagascae* is in the form of divernolin.

The biosynthesis of vernoleate in *E. lagascae* has been partially characterized by Stymne's group (Bafor et al. 1993). They reported that the enzyme responsible for vernolic acid biosynthesis, epoxygenase, is a microsomal enzyme which utilizes linoleoyl-phosphatidyl choline as the substrate. This enzyme is considered to be a cytochrome P450 monooxygenase based on its inhibition by carbon monoxide and anti-P450 reductase antibodies, NADPH as the preferred reductant and lack of inhibition by cyanide.

In our studies with *V. galamensis* we have established that the *Vernonia* epoxygenase is also a microsomal enzyme which utilizes linoleoyl-phosphatidyl choline as the substrate. However, the *Vernonia* epoxygenase is distinctly different from the *Euphorbia* epoxygenase and typical P450 monooxygenase enzymes and it is a desaturase like enzyme. This is based on its inhibition by cyanide and anti-cytochrome b5 antibodies but lack of inhibition by anti-P450 reductase antibodies and NADH as the preferred reductant. Typically, desaturases are inhibited by CN and not CO (Jaworski and Stumpf, 1974; Nagai and Bloch, 1968). Similar results were obtained by Lee et al. (1998) with the epoxygenase of *Crepis palestina*.

Yeast is the most rapid model system to test the eukaryotic transgenic expression and analyses of genes derived from higher plants. Furthermore, it is easily manipulated by using well-established classical genetics and powerful molecular genetics. Functional expression of the epoxygenase requires the presence of a suitable membrane environment (ER) and electron donor that makes the yeast an ideal candidate for this work. Although *Saccharomyces cerevisiae* does

not form polyunsaturated fatty acids, especially linoleic acid (the precursor for the epoxygenase), under normal conditions, we have established in our lab that linoleic acid is readily incorporated into yeast lipids (e.g. phosphatidylcholine and triglycerides). Thus, yeast can be converted into an organism with the right substrate for the epoxygenase. Having the right substrate for the epoxygenase, however, is not a guarantee for successful transgenic expression of this enzyme. The physical properties of vernolic acid differ fundamentally from the common fatty acids. It is possible that vernolic acid, due to the presence of the epoxy group, would severely disrupt the membrane functions and could be very toxic to the yeast. In fact, vernolic acid, naturally present in the seeds of *Vernonia galamensis* and *Euphorbia lagascae*, is incorporated exclusively in triacylglycerols of the seed oil presumably protecting the cells from the adverse effects of vernolic acid on membranes.

To address the above question we fed a 30 mL yeast culture with 1 mM vernolic acid in the presence of a detergent. No vernolic acid could be detected when we analyzed the total lipid pool by GC. However, after separating the total lipids into their respective lipid classes by TLC, using a system that can separate monovernolin, divernolin and trivernolin, we were able to detect vernolic acid in a band (detected by spraying primulin) that comigrated with a monovernolin standard present exclusively in vernolic acid fed yeast. The presence of vernolic acid in the above band was indicated by GC and confirmed by GC-MS. No vernolic acid was detected in the polar lipids, including phosphatidylcholine. It is interesting to note that vernolic acid was detected in mono-vernolin only, the yeast seems to be able to acylate vernolic acid to the glycerol backbone at only one position, perhaps by a specific acyltransferase. Although very minor in total lipids, vernolic acid was around 20% of mono-vernolin fatty acids. We now can readily and clearly screen for yeast lines expressing epoxygenase cDNA clones by the presence of the mono-vernolin containing triacylglycerol band by TLC. This is the first successful report on testing vernolic acid in a yeast system.

Based on our biochemical characterization that *Vernonia* is a desaturase-like enzyme, we used RT-PCR strategy to clone a $\Delta 12$ desaturase-like cDNA from developing *Vernonia* embryos. A full-length cDNA was tested in yeast and was found to have $\Delta 12$ desaturase activity causing significant linoleic acid accumulation in the transgenic yeast. Because *Vernonia* epoxygenase is a desaturase-like enzyme, we expected that the *Vernonia* epoxygenase will be more homologous to *Vernonia* $\Delta 12$ desaturase than any other heterologous $\Delta 12$ desaturase. Based on this reasoning, the isolated *Vernonia* cDNA $\Delta 12$ desaturase was used to screen the *Vernonia* cDNA library at high stringency. Another full-length $\Delta 12$ desaturase-like cDNA was isolated. In collaboration with Rhéne-Poulenc, this cDNA was fully sequenced and found to have nine nucleotide and four amino acid differences, as compared with the *Vernonia* $\Delta 12$ desaturase used to screen the library. This cDNA was also expressed in yeast and like the first $\Delta 12$ desaturase-like cDNA had significant $\Delta 12$ desaturase activity in yeast. This indicated that we isolated a second *Vernonia* $\Delta 12$ desaturase isozyme. Rescreening of the *Vernonia* cDNA library with *Vernonia* $\Delta 12$ desaturase using less stringent conditions resulted in the identification of more than 10 putative epoxygenase clones. A similar approach was used to isolate *Crepis palestina* epoxygenase and *Crepis alpina* acetylenase. Both enzymes, the epoxygenase and acetylenase, are desaturase like enzymes with 58% and 56% identity respectively when compared pairwise with the *Arabidopsis* ER $\Delta 12$ -desaturase amino acid sequence. As an epoxygenase activity was not observed in genes obtained by screening the

Vernonia early seed cDNA library, an RT-PCR strategy was applied. Degenerate primers were designed from the conserved sequences of some $\Delta 12$ desaturase of several species of plants, oleate 12-hydroxylase of *Ricinus communis*, acetylenase of *Crepis alpina* and epoxygenases of *Crepis palestina* and *Vernonia galamensis*. This was based on our prior work indicating that Asteraceae epoxygenases responsible for epoxy fatty acid accumulation in the seed oil are desaturase-like enzymes. This led to epoxygenase gene cloning from *Vernonia galamensis* and *Crepis palestina*, two Asteraceae species by two independent groups and *Stokesia laevis*, another Asteraceae specie, by us.

We obtained an epoxygenase like gene from *Stokesia laevis*. This gene was 1377 bp and encoded 378 amino acids. The closest match to this *Stokesia* gene is the *Vernonia* epoxygenase, with 84% similarity. Expression vectors for yeast and soybean are being constructed in order to assess enzyme function. The ORF fragments were cut out and ligated with a yeast expression vector, pYeDP 60 or a plant expression vector wherein the *Stokesia* gene is driven by a phaseolin promoter. This construct is introduced to plants with a particle delivery system. For *Agrobacterium*-mediated and enhanced particle delivery transformation, the gene with the phaseolin promoter was inserted into a binary vector containing T-DNA left and right borders.

Work is in progress on greatly increasing oil content of soybean seeds without reductions of seed protein by increasing expression of certain lysphosphosphatidate acyltransferases and/or diacylglycerol acyltransferases (DGAT). We designed degenerate primers from the sequence information of DGAT of *Arabidopsis thaliana*, *Brassica napus* and *Mus musculus*, and tried to obtain DGAT gene from *Vernonia* and *Stokesia*. Correct sized fragments were obtained by PCR using cDNA libraries as templates. The results of BLAST searching showed that they are novel genes with high similarity to DGAT.

Studies have been conducted to assess metabolism of vernolic acid in developing soybean seeds and accumulation in seed oil triglyceride. Studies with feeding developing soybean seeds with vernolic acid indicate that vernoleate can be incorporated into soybean seed oil and can accumulate in mono- and di-vernolin. No tri-vernolin accumulation has yet been observed. This information is providing a basis for metabolic engineering soybeans for high accumulation of epoxy fatty acids in seed oil and increasing oil content.

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.

Seither, C., S. Avdiushko and D.F. Hildebrand. 1997. Isolation of cytochrome P-450 genes from *Vernonia galamensis*. pp 389-391 In: J.P. Williams, M.U. Khan and N.W. Lem, eds. Physiology, Biochemistry and Molecular Biology of Plant lipids, Kluwer Academic Publishers, Dordrecht.

Hildebrand, D.F., H. Fukushige, M. Afitlhile and C. Wang. 1998. Lipoxygenases in plant development and senescence. Chapt. 8 In Eicosanoids and Related Compounds in Plants

and Animals. A.F. Rowley, H. Kuhn and T. Schewe, eds., Portland Press Ltd. pp 151-181.

Tanos G. Hage, Craig Seither and David Hildebrand. 1999. Isolation and Functional Expression of two cDNAs from *Vernonia galamensis* (Cass.) Less. Encoding a Microsomal Oleate Desaturase (FAD2) in *Saccharomyces cerevisiae* (Accession No AF188263 and AF188264). Plant Gene Register.

Technology Transfer

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

Sequences of cDNAs and the cDNAs were transferred to Rhone Poulenc, the corporate partner.

Commercial Accomplishments

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

As described above, epoxy fatty acids are very useful in coatings and plastics as well as other industrial products. It would be very useful to genetically engineer oilseeds to provide a renewable source of these materials. We have elucidated the biochemical basis for epoxy fatty biosynthesis in plants and cloned cDNAs for one of the key genes. This will provide a necessary basis for using plants as a commercial source of this useful industrial material in the future.

Educational Accomplishments

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

This grant provided partial support for the thesis project for a MS candidate, Craig Seither. Mr. Seither successfully completed his MS thesis and has gone onto Pharmacy school. Thesis title: "Characterization of Epoxy Fatty Acid Synthesis in *Vernonia galamensis* and the Isolation of Candidate cDNA Clones". It also provided partial support for a postdoctoral scholar, David McClure, who is now working in the biotechnology industry with Abbott Laboratories.

Additional Funding

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

The Kentucky Soybean Promotion Board provided additional matching funding of \$10,000 during the second year of the project.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

Craig Seither completed a MS thesis on the project funded by the CPBR. David McClure gained training from the project that he left to go to work with the biotechnology industry. Tanos Hage joined the project in his stead.

Send completed report to :

The Consortium for Plant Biotechnology Research, Inc.

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Or sent as an email attachment to: cpbr@gate.net

Scientific Progress Report

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

Principal Investigator:	David Hildebrand	
University:	University of Kentucky	
Agreement Number:	OR22072-93	
Project Title:	Engineering oilseeds for epoxy fatty acid accumulation	
Reporting Period and Report Type:	From: 5/1/99 To: 2/28/00	Check one: ☒ [X] Interim Report ☐ [] Final Report

Project Objectives

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

1. Continue cloning and sequencing of epoxygenase cDNA candidates.

Several full-length apparent epoxygenase cDNAs have been cloned (see below) and sequenced including one from *Stokesia laevis*. The *Stokesia* gene is 1134 bp encoding 378 amino acids. It has the conserved amino acids of related di-iron oxygenases. This was based on our prior biochemical studies indicating that epoxygenases in *Vernonia* and *Stokesia* are $\Delta 12$ desaturase-like enzymes.

2. Testing of epoxygenase cDNA candidates in yeast and soybean somatic embryos.

A number of epoxygenase cDNA candidates have been tested in yeast. Those that turned out to be $\Delta 12$ desaturases functioned well in yeast in our hands, but known or probable epoxygenase cDNAs gave no detectable expression. This has been evaluated a number of ways in yeast without detectable function and other groups have found similar lack of activity of epoxide biosynthetic enzymes in yeast. We are evaluating several rapid systems for functional testing in plants as well as the more long term testing in transgenic soybean somatic embryos. Soybean transformation and testing are in progress. We are also conducting functional testing in transgenic Arabidopsis seeds as this is a reliable system for testing of seed lipid metabolic genes and more rapid than most plant seed testing systems.

3, Metabolic fate of vernolic acid in developing soybean oil accumulating tissues.

We have established culture conditions for soybean zygotic and somatic embryos in which they accumulate triglyceride as do developing seed tissues in planta. Feeding studies with such embryos using vernolic acid indicates developing soybean seed cotyledons can accumulate some vernolic acid and incorporate it into triacylglycerol mainly as mono-vernolin. This indicates that diacylglycerol acyltransferase (DGAT) from soybeans does not readily incorporate vernoleate containing diacylglycerol and/or vernoleoyl-CoA into triacylglycerol. We therefore have cloned and sequenced a full-length DGAT cDNA from developing *Vernonia* seeds. This gene encodes a protein of 522 amino acids. We have also developed a procedure for synthesis of ^{14}C vernolic acid and ^{14}C vernoleoyl-CoA (not commercially available) for detailed studies of the metabolic fate of vernolic acid in developing soybean oil accumulating tissues.

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 succeeded in cloning genes encoding enzymes involved in epoxy fatty acid biosynthesis. Vector constructs have also been made with these genes for functional testing in yeast and plant systems including soybean seed tissue. We also cloned functional relatives of epoxide biosynthetic genes that encode enzymes that synthesize polyunsaturated fatty acids. These latter genes have functioned well in the yeast test system resulting in the production of the transgenic yeast of polyunsaturated fatty acids not made by yeast not expressing these plant genes. We have established that yeast is however not suitable for efficient testing of epoxy fatty acid biosynthetic genes. We have therefore shifted some effort toward developing rapid plant-based test systems. Work is in progress for the testing of epoxide biosynthetic genes in soybean seed cotyledon tissues and we have begun work with a model plant seed test system that has a high probability of success. We have investigated the metabolism of epoxy fatty acids in oil accumulating soybean seed tissues and this has improved our understanding of what is needed for high, commercially viable accumulation of epoxy fatty acids in oilseed oils. This has lead to the cloning of an additional gene of importance in the high and selective accumulation of epoxy fatty acids in oilseed oil. Overall this research has made very significant progress toward providing the basis for genetic engineering oilseeds such as soybeans for economically viable accumulation of a renewable industrial chemical feedstock.

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 principal goal of the CPBR supported research is to genetically engineer oilseeds such as soybeans for high epoxy fatty acid accumulation in triglycerides (oils). Epoxy fatty acids have unique chemical properties, which makes them very valuable in the production of a wide range of industrial products. Few plant species accumulate epoxy fatty acids as the major constituents of their seed oils. *Vernonia galamensis* is one of the highest known accumulators of the epoxy fatty acid vernoleate (around 80% of the seed oil), mostly in the form of trivernolin. Some other plants such as *Euphorbia lagascae* also accumulate significant amount of vernoleate. However, most of the vernoleate in *E. lagascae* is in the form of divernolin.

The biosynthesis of vernoleate in *E. lagascae* had been partially characterized by Stymne's group (Bafor et al. 1993). They reported that the enzyme responsible for vernolic acid biosynthesis, epoxygenase, is a microsomal enzyme which utilizes linoleoyl-phosphatidyl choline as the substrate. This enzyme is considered to be a cytochrome P450 monooxygenase based on its inhibition by carbon monoxide and anti-P450 reductase antibodies, NADPH as the preferred reductant and lack of inhibition by cyanide.

In our studies with *V. galamensis* we have established that the *Vernonia* epoxygenase is also a microsomal enzyme which utilizes linoleoyl-phosphatidyl choline as the substrate. However, the *Vernonia* epoxygenase is distinctly different from the *Euphorbia* epoxygenase and typical P450 monooxygenase enzymes and it is a desaturase like enzyme. This is based on its inhibition by cyanide and anti-cytochrome b5 antibodies but lack of inhibition by anti-P450 reductase antibodies and NADH as the preferred reductant. Typically, desaturases are inhibited by CN and not CO (Jaworski and Stumpf, 1974; Nagai and Bloch, 1968). Lee et al. (1998) obtained similar results with the epoxygenase of *Crepis palestina*.

Yeast is the most rapid model system to test the eukaryotic transgenic expression and analyses of genes derived from higher plants. Furthermore, it is easily manipulated by using well-established classical genetics and powerful molecular genetics. Functional expression of the epoxygenase requires the presence of a suitable membrane environment (ER) and electron donor that makes the yeast an ideal candidate for this work. Although *Saccharomyces cerevisiae* does not form polyunsaturated fatty acids, especially linoleic acid (the precursor for the epoxygenase), under normal conditions, we have established in our lab that linoleic acid is readily incorporated into yeast lipids (e.g. phosphatidylcholine and triglycerides). Thus, yeast can be converted into an organism with the right substrate for the epoxygenase. Having the right substrate for the epoxygenase, however, is not a guarantee for successful transgenic expression of this enzyme. The physical properties of vernolic acid differ fundamentally from the common fatty acids. It is possible that vernolic acid, due to the presence of the epoxy group, would severely disrupt the membrane functions and could be very toxic to the yeast. In fact, vernolic acid, naturally present in the seeds of *Vernonia galamensis* and *Euphorbia lagascae*, is incorporated exclusively in

triacylglycerols of the seed oil presumably protecting the cells from the adverse effects of vernolic acid on membranes.

To address the above question we fed a 30 mL yeast culture with 1 mM vernolic acid in the presence of a detergent. No vernolic acid could be detected when we analyzed the total lipid pool by GC. However, after separating the total lipids into their respective lipid classes by TLC, using a system that can separate monovernolin, divernolin and trivernolin, we were able to detect vernolic acid in a band (detected by spraying primulin) that comigrated with a monovernolin standard present exclusively in vernolic acid fed yeast. The presence of vernolic acid in the above band was indicated by GC and confirmed by GC-MS. No vernolic acid was detected in the polar lipids, including phosphatidylcholine. It is interesting to note that vernolic acid was detected in mono-vernolin only, the yeast seems to be able to acylate vernolic acid to the glycerol backbone at only one position, perhaps by a specific acyltransferase. Although very minor in total lipids, vernolic acid was around 20% of mono-vernolin fatty acids. .

Based on our biochemical characterization that *Vernonia* is a desaturase-like enzyme, we used RT-PCR strategy to clone a $\Delta 12$ desaturase-like cDNA from developing *Vernonia* embryos. A full-length cDNA was tested in yeast and was found to have $\Delta 12$ -desaturase activity causing significant linoleic acid accumulation in the transgenic yeast. Because *Vernonia* epoxygenase is a desaturase-like enzyme, we expected that the *Vernonia* epoxygenase would be more homologous to *Vernonia* $\Delta 12$ desaturase than any other heterologous $\Delta 12$ desaturase. Based on this reasoning, the isolated *Vernonia* cDNA $\Delta 12$ desaturase was used to screen the *Vernonia* cDNA library at high stringency. Another full-length $\Delta 12$ desaturase-like cDNA was isolated. In collaboration with Rhone-Poulenc, this cDNA was fully sequenced and found to have nine nucleotide and four amino acid differences, as compared with the *Vernonia* $\Delta 12$ desaturase used to screen the library. This cDNA was also expressed in yeast and like the first $\Delta 12$ desaturase-like cDNA had significant $\Delta 12$ -desaturase activity in yeast. This indicated that we isolated a second *Vernonia* $\Delta 12$ desaturase isozyme. Rescreening of the *Vernonia* cDNA library with *Vernonia* $\Delta 12$ desaturase using less stringent conditions resulted in the identification of more than 10 putative epoxygenase clones. A similar approach was used to isolate *Crepis palestina* epoxygenase and *Crepis alpina* acetylenase. Both enzymes, the epoxygenase and acetylenase, are desaturase like enzymes with 58% and 56% identity respectively when compared pairwise with the *Arabidopsis* ER $\Delta 12$ -desaturase amino acid sequence. As an epoxygenase activity was not observed in genes obtained by screening the *Vernonia* early seed cDNA library, a RT-PCR strategy was applied. Degenerate primers were designed from the conserved sequences of some $\Delta 12$ desaturase of several species of plants, oleate 12-hydroxylase of *Ricinus communis*, acetylenase of *Crepis alpina* and epoxygenases of *Crepis palestina* and *Vernonia galamensis*. This was based on our prior work indicating that Asteraceae epoxygenases responsible for epoxy fatty acid accumulation in the seed oil are desaturase-like enzymes. This led to epoxygenase gene cloning from *Vernonia galamensis* and *Crepis palestina*, two Asteraceae species by two independent groups and *Stokesia laevis*, another Asteraceae specie, by us.

The apparent epoxygenase cDNA cloned from *Stokesia laevis* was 1.4 kb, the ORF was 1134 bp and encoded 378 amino acids. The closest match to this *Stokesia* gene is the *Vernonia* epoxygenase, with 84% similarity. Expression vectors for yeast and soybean were constructed in order to assess enzyme function. The coding region was ligated into a yeast expression vector, and several plant expression vectors. Expression experiments in yeast failed to result in any accumulation of vernoleate even in triglyceride. This was repeated with a *Vernonia* epoxygenase cDNA known to have epoxygenase activity in soybean somatic embryos and *Arabidopsis* and

again no vernoleate accumulation in yeast could be achieved. We therefore conducted studies in yeast with both the known *Vernonia* epoxygenase and the probable *Stokesia* epoxygenase and conducted epoxygenase activity measurements using our optimized and sensitive epoxygenase assay procedure. Again no epoxygenase activity was observed with either cDNA. Other groups have likewise been unable to achieve functional expression of epoxy fatty acid biosynthetic genes in yeast. It is not yet clear if yeast capable of expression epoxy fatty biosynthesizing genes are selected against (the inducible promoter being used may be slightly leaky) or epoxides are degraded immediately upon synthesis. The vernoleate feeding studies described above indicate yeast can accumulate detectable vernolic acid in triacylglyceride. However feeding exogenous fatty acid is far different metabolically than in vivo synthesis in phosphatidyl choline of the ER membranes.

As it may not be possible to achieve functional expression of plant epoxygenase in yeast, our efforts are now focused on functional testing using plant-based systems. For one of the plant expression vectors, the *Stokesia* gene is driven by a phaseolin promoter. The phaseolin promoter construct was introduced to soybean somatic embryo cultures using a particle delivery system. Multiple sets of soybean transformations have been carried out so far. Bombarded embryos are kept on solid FN media for 3 days. They are then transferred into proliferation media (liquid FN media). This media contains a high concentration of sucrose (60 mg/L), 10 mg/L 2,4-D as a plant hormone and 30 mg/L hygromycin as a selection agent. After 7 days of culture, all dead portions are removed and green parts are crushed to induce secondary somatic embryogenesis. They are transferred into fresh media and subcultured twice every 10 days. Only a few somatic embryos survived the initial experiments and they were transferred into maturation media (liquid FL-Lite). This media is hormone-free and contains 30 mg/L hygromycin. Somatic embryos that do not grow well for 3 weeks are transferred to proliferation media again. Somatic embryos that grow well under selection will be tested for epoxy fatty acid accumulation and epoxygenase activity if necessary.

A number of approaches are being taken to finally and unequivocally ascertain function of the putative *Stokesia* epoxygenase gene. Although it has not yet shown evidence of epoxygenase or desaturase activity in the yeast system, the yeast system works fine in our hands for functional expression of $\Delta 12$ desaturases. This in addition to the sequence homology provides strong evidence that the putative *Stokesia* epoxygenase gene indeed encodes for an epoxygenase. Moreover the same approach in our hands has resulted in cloning a functional *Vernonia* epoxygenase. We therefore tried a rapid plant expression system using cucumber protoplasts. For this a plant expression vector containing T-DNA left and right borders with the putative epoxygenase driven by the 35S promoter was electroporated into the protoplasts. In this system, all reactions (*Stokesia* gene, *Vernonia* epoxygenase as a positive control, and untransformed protoplasts as a negative control) showed epoxygenase activities. Cucumber protoplasts apparently have an epoxygenase- or peroxygenase-like activity that obscures new gene testing. We are therefore trying other rapid assessment systems including a viral vectoring system that can give very high transient expression in leaves especially of certain model plant systems. The putative epoxygenase is being put into a viral vector for this purpose. These systems are very rapid and can give high activity in a short time (as little as 12 h), but these cells do not normally accumulate triacylglyceride. Another approach that was considered utilizes

cyanobacteria. This latter approach has worked well for a $\Delta 6$ desaturase involved in γ -linolenic acid synthesis by Thomas' group in collaboration with Rhone-Poulenc. As we have had no problem with functional desaturase expression in yeast this may not be advantageous. The lack of triglyceride accumulation and the fact that such tissues apparently have not evolved functions for directing the epoxy fatty acids to accumulate in accumulating triacylglyceride, indicate that the much slower and labor intensive approach involving testing in oilseeds somatic or zygotic embryos may be the only recourse with a high probability of success. We are therefore putting more effort into the soybean somatic embryo testing system mentioned above as well as testing the genes in *Arabidopsis*.

We have evaluated a number of plant species that make epoxides other than vernolic acid for epoxy fatty acid biosynthesis and established growing and seed production conditions for several of these species. These unique epoxy fatty acids have other potential industrial and medicinal uses. Preliminary evaluation of the biochemical properties of these additional epoxxygenases has been performed and cDNA libraries made. In addition to the enzymes responsible for epoxy fatty acid synthesis, other enzymes are needed for high accumulation of epoxy fatty acids in triacylglycerol of seed oil. The most important of these is diacylglycerol acyltransferases or DGAT. DGAT not only can preferentially incorporate vernolic acid into triglyceride but it can selectively utilize diacylglycerols containing vernolic acid. The high accumulation of vernolic acid in *Vernonia* seed oil is dependent on high levels of di- and tri-vernolin indicating high utilization of mono- and di-vernolin containing diacylglycerol by DGAT in *Vernonia*. In order to clone such useful DGATs from *Vernonia*, we designed degenerate primers from the sequence information of DGAT of *Arabidopsis thaliana*, *Brassica napus* and *Mus musculus*, in order to clone a DGAT gene from *Vernonia*. Correct sized fragments were obtained by PCR using an RT-PCR strategy. The results of BLAST searching showed that they are novel genes with high similarity to DGAT. This gene was fully sequenced using a rapid amplification of cDNA ends (RACE) strategy. The gene was 1.8 kb, the ORF was 1566 bp and encoded 522 amino acids. This gene is closest to DGATs from *Arabidopsis thaliana* and *Brassica napus* with 70% similarity. We also obtained an apparent DGAT gene from *Ricinus communis* that accumulates a high amount of similar fatty acid, ricinoleic acid, in the seed oil. The full sequence of this gene would be determined by using the same strategy as *Vernonia*.

Other genes may be necessary for high vernolic acid accumulation in oil seeds such as soybeans in addition to epoxxygenase and DGAT. Therefore studies have been conducted to assess metabolism of vernolic acid in developing soybean seeds and accumulation in seed oil triglyceride. Studies with feeding developing soybean seeds with vernolic acid indicate that vernoleate can be incorporated into soybean seed oil and can accumulate in mono- and di-vernolin. No tri-vernolin accumulation has yet been observed. Soybean seed oil is very high in linoleic acid and *Vernonia* seed oil very high in vernolic acid. In order to better understand the relative accumulation of vernolic acid in soybean triglyceride compared with *Vernonia* triglyceride, feeding studies with developing embryos of both species with both ^{14}C linoleic and ^{14}C vernolic acids are being conducted. In order to do this we needed to establish a procedure for synthesis of ^{14}C -vernolic acid. We additionally needed to establish procedures for culturing *Vernonia* and soybean embryos at a period of maximal triglyceride synthesis and uptake of the labeled fatty acids. Both of these goals have been accomplished. This information is providing a basis for metabolic engineering soybeans for high accumulation of epoxy fatty acids in seed oil and increasing oil content while at the same time holding protein content steady. Finally work is in progress on greatly increasing oil content of

soybean seeds without reductions of seed protein by increasing expression of certain lysophosphatidate acyltransferases together with DGATs.

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.

Hage, Tanos G., Craig Seither and David Hildebrand. 2000. Isolation of two cDNAs from *Vernonia galamensis* (Cass.) Less. Encoding a Microsomal Oleate Desaturase (*FAD2*) (Accession No [AF188263](#) and [AF188264](#)) and Functional Expression in *Saccharomyces cerevisiae*. Plant Physiol. (in press).

Wang, Cunxi, Kevan Croft, Ulla Jarlfors and David Hildebrand. 1999. Subcellular localization studies indicate that lipoxygenases 1 to 6 are not involved in lipid mobilization during soybean germination. Plant Physiol. 120:227-235.

Technology Transfer

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

The complete cDNA sequences of epoxigenases and related cDNAs involved in epoxy fatty acid biosynthesis have been provided to the industrial partner, Rhone Poulenc and are being considered for patenting. Our metabolic studies being conducted under Objective 3 are providing a basis for strategies for accumulation of commercially valuable epoxy fatty acids to economically viable levels.

Commercial Accomplishments

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

Genes have been cloned that are responsible for epoxy fatty acid biosynthesis. We have also conducted studies that will facilitate the cloning of additional epoxide biosynthetic genes that encode enzymes that make additional epoxides with different industrial chemical properties. Genes have also been cloned that are important to the high and selective accumulation of epoxy fatty acids in oilseed oils. These are being used to genetically engineer oilseeds such as soybeans for high, commercially viable accumulation of epoxy fatty acids in oilseed oils. We have also made good progress in elucidating the metabolic basis of high accumulation of epoxy fatty acids in plant seed oils that will provide the basis for effective strategies for commercialization of genetically engineered plants as bioreactors for the commercial synthesis of these industrially important chemical feedstocks.

Educational Accomplishments

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

The CPBR grant provides funding for a postdoctoral scholar and an undergraduate student worker. The first postdoctoral who worked on the project, Dr. Tony Hage, accepted an academic position involving teaching and research at Notre Dame Univ. A postdoctoral scholar was recruited to replace Dr. Hage, Dr. Tomoko Hatanaka. Dr. Hatanaka has an excellent research and publication background as well as academic credentials. She will assist the PI in providing extensive training and mentoring of the students recruited to work with the CPBR funded project. An undergraduate student, Thoryn Stephens is funded by CPBR to work on the project and he is being involved in the science as much as he is comfortable with. The matching funds are providing support for two graduate students, Julia Shi and Mohamed Taha. They are just beginning their Ph.D. research programs on renewable energy related plant biotechnology research.

Additional Funding

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

The ongoing research supported by the CPBR has greatly facilitated our being able to obtain a new research grant from the United Soybean Board (USB) for energy related plant biotechnology research. The USB will provide \$80,000 for this research in the coming year and allows this as an exclusive match for the CPBR funding (see matching funding budget).

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

Dr. Tony Hage worked on the project as a postdoctoral fellow at the beginning of the project and left to accept a permanent position. Dr. Tomoko Hatanaka has joined the project in his place as postdoctoral fellow. She brings extensive expertise in plant molecular biology and biotechnology to the project. Two Ph.D. students have been hired to work with the project research objectives. They are Julia Shi and Mohamed Taha.

Isolation of two cDNAs from *Vernonia galamensis* (Cass.) Less. Encoding a Microsomal Oleate Desaturase (FAD2) (Accession No AF188263 and AF188264) and Functional Expression in *Saccharomyces cerevisiae*

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➤ **REPORT**

Plants produce a wide variety of unsaturated fatty acids and derivatives such as hydroxy and epoxy fatty acids that can accumulate in seed storage lipids. A number of the enzymes responsible for catalyzing these reactions appear to have evolved from oleate or delta-12 desaturases (Broun et al., 1998; Shanklin and Cahoon, 1998) *Vernonia galamensis* (Cass.) Less. is one of the highest known natural accumulator of epoxy fatty acid (Perdue et al., 1986; Thompson et al. 1994). It has been shown that the *V. galamensis* epoxygenase is a desaturase-like enzyme (Seither et al., 1997; Hitz, 1998; Lee et al., 1998). A PCR strategy outlined in Table 1 resulted in the isolation and functional expression of a cDNA (Accession No AF188263) encoding an oleate desaturase (vgFAD2-1) in the yeast *Saccharomyces cerevisiae*. Screening a *V. galamensis* cDNA library with the above vgFAD2-1 cDNA resulted in the isolation and functional expression in *Saccharomyces cerevisiae*, of a new member of the microsomal oleate desaturase gene family (vgFAD2-2, Accession No AF188264). A third member of the *V. galamensis* oleate desaturase was isolated by Hitz (1998). This is the first report of a microsomal oleate desaturase family with three members. Partial sequence analysis of another *V. galamensis* cDNA suggests there may be a fourth microsomal oleate desaturase.

VgFAD2-1 and vgFAD2-2 cDNA clones share more than 99% nucleotide identity in the coding region (nine nucleotides differences corresponding to two amino acids). In contrast to the ORF, the 3' untranslated region of FAD2-2 has a unique 32 bp fragment (TCAATTTCTCGTTTTTATGGTATTCGACTTTT) as compared with that of FAD2-1. This unique untranslated region can be used as gene specific probes in DNA and RNA gel blots to study the expression of *V. galamensis* FAD2s in different tissues. It has been shown that FAD2-1 of soybeans (Accession No. L43920) and FAD2-1 of cotton (Accession No. Y10112) are specifically induced during seed development. While the FAD2-2 of soybean (Accession No. L43921) and the FAD2-2 of cotton (Liu et al., 1999) are constitutively expressed in various tissues. VgFAD2-1 and vgFAD2-2 share 70 % amino acid identity with the Hitz (1998) vgFAD, less than its identity with with FAD2 sequences from *Crepis palestina* (Accession No Y16284, 88%) and *Petroselinum crispum* (Accession No. U86072, 79%). This suggests that vgFAD2-1 and vgFAD2-2

may have evolved independently from vgFAD2. VgFAD2-1 and vgFAD2-2 share about 60% amino acid identity as compared with *Vernonia galamensis* epoxxygenase (Hitz, 1998) whereas the Dupont's vgFAD2 shares 56% amino acid identity.

➤ TABLE I

Characteristics of vgFAD2-1 and vgFAD2-2 encoding a microsomal oleate desaturase

Organism:

Vernonia galamensis (Cass.) Less.

Location of gene:

Nuclear encoded (apparently)

Function:

Introduction of a cis double bond at the delta 12 position of oleic acid (18:1), esterified at the sn2 position of phosphatidylcholine (PC), to produce linoleic acid (18:2)

Source:

A cDNA lambda Zap express library constructed from poly(A)+ of developing embryos from *Vernonia galamensis*.

Isolation:

vgFAD2-1: The 5' end of the cDNA fragment was amplified by PCR, using a purified phage DNA obtained from the above cDNA Library. The forward primer for the PCR was the T3 of pBluescript and the reverse primer was the degenerate primer 5'-RTGRTGIGCIACRTG-3'. This degenerate primer was designed for a conserved histidine motif among desaturases. This cDNA fragment shared high homology to several desaturases. The complete cDNA was amplified by PCR using the same template as above. The forward gene specific primer was: 5'-AGGCGGATCCATGGGTGCAGGTGGGCGA-3' and the reverse primer was the T7 of pBluescript II KS.

vgFAD2-2: A full-length cDNA, FAD2-2, was isolated by screening the same library as above by using vgFAD2-1 as a probe.

Functional expression:

vgFAD2-1 and vgFAD2-2 cDNAs were cloned into pYeDP60 yeast expression vector behind a galactose inducible promoter according to the method described by Pompon et al. (1996). With both genes 18:2 increases from < 0.2% to ~ 10% in induced transgenic yeast similar to the expression of soybean and Arabidopsis Fad2 genes in yeast.

Features of the cDNAs:

vgFAD2-1: this clone is 1475 bp in length, including a complete ORF of 1152 bp, 95 bp 5' untranslated region and 228 bp 3' untranslated region.

vgFAD2-2: this clone is 1507 bp in length, including a complete ORF of 1152 bp, 95 bp 5' untranslated region and 260 bp untranslated region.

Features of deduced amino acid sequence:

The vgFAD2-1 and vgFAD2-2 have a nucleotide sequence of 383 bp long, and they displayed three histidine motifs (HXXXHH: residues 106 to 111; HXXHH: residues 142 to 146; HXXHH: residues 316-320) which are conserved in microsomal oleate desaturase and other membrane bound desaturases (Okuley et al., 1994).

➤ ACKNOWLEDGMENTS

This work was supported by grants from the United Soybean Board, Consortium for Plant Biotechnology Research (CPBR) and the Kentucky Soybean Promotion Board.

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Scientific Progress Report

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

Principal Investigator: L. O. Ingram, J. F. Preston, K. T. Shanmugam

University: University of Florida

Agreement Number: DE-FO 05 920R22072

Project Title: Ethanol Production from Uronic Acid - Substituted Xylose Residues in Hemicellulose Hydrolysates

Reporting Period and Report Type:

From: 2/22/99
To: 9/15/99

Check one:

☒ Interim Report
☐ Final Report

Project Objectives

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

~~Please complete.~~ SEE ATTACHED

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.

Please complete. SEE ATTACHED

Scientific Accomplishments

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

Please complete. 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.

Please complete. SEE ATTACHED

Scientific Progress Report

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

Principal Investigators: L.O. Ingram, J.F. Preston, and K.T. Shamugam

University: The University of Florida

Agreement Number: DE-FC05-920R22072

Project Title: Ethanol Production from Uronic Acid-Substituted Xylose Residues in Hemicellulose Hydrolysates

Reporting Period: From 2/22/99 To: 9/25/99

Reporting Type: Interim Report

Project Objectives

The progress reported below is for work done since the award date of 2/22/99.

The objective of this research is to develop improved biocatalysts which can rapidly and efficiently metabolize glucuronoxyllose and the complete mixture of sugars in hemicellulose hydrolysates to ethanol. The goal of this work is to increase yields, decrease fermentation times, and decrease the BOD loading from stillage and waste treatment associated with the conversion of biomass to ethanol.

Specific aims:

1. To determine the amounts of acid resistant glucuronic acid-substituted sugars in acid hydrolysates from candidate biomass species.

Progress:

- a) Hemicellulose fractions have been prepared from sweetgum (*Liquidambar styraciflua*), cottonwood (*Populus deltoides*) clones that were selected for rapid biomass formation, sugar cane bagasse, and rice hulls. Based upon ¹³C-NMR analysis, the hemicellulose fraction from sweetgum is comprised of glucuronoxylan in which the ratio of xylose to 4-O-methylglucuronic acid residues is 6.4:1.0. Similar ratios were found for sweetgum and cottonwood samples following dilute acid hydrolysis. Higher ratios of xylose to glucuronic acid were estimated in the hemicellulose fractions from sugarcane bagasse and rice hulls. The presence of undetermined carbons in the sugarcane and rice hull samples overlapped the shift values for C-4 and the O-methyl carbons of the glucuronic acid residues made their quantitation by NMR difficult.

- b) Attempts to resolve and quantify oligosaccharides containing O-methyl-glucuronic acid residues linked to xylose (GAX) using several HPLC systems (ion-pair reverse-phase with borate, ion-modified partitioning, ion-exchange) have been attempted, but have not been successful. Other chromatographic systems are being explored to allow analytical quantification of GAX in acid hydrolysates. Preparative quantities of GAX have been obtained by a combination of ion exchange and gel filtration chromatography. These will serve as substrates for the development of recombinant bacteria to convert GAX to ethanol.
2. To investigate the limitations for concurrent sugar utilization in strains LY01 of *E. coli* and P2 of *Klebsiella oxytoca* and develop new and improved strains which more rapidly complete the conversion of hemicellulose-derived sugars to ethanol.
- a) A recombinant endoxylanase derived from *Erwinia chrysanthemi* D1 has been over-expressed in *E. coli*, and has been shown to be an effective catalyst for the generation of free sugars from sweetgum hemicellulose. The parent strain will be evaluated for an α -glucuronidase to complement the xylanase and improve the conversion of hemicellulose to ethanol.
 - b) In *Escherichia coli*, expression of *xyl* operons is subject to catabolite repression and is also controlled by a regulatory protein coded by *xylR* gene. Our initial strategy is to eliminate these controls on *xyl* operon expression by replacing the native promoter sequences with a promoter sequence which is independent of any control in the cell - ~~constitutive expression. Upon expression of *xyl* operon in a constitutive manner (both~~ *xylAB* coding for isomerase and kinase, respectively, and *xylFGH* coding for xylose transport proteins), we will investigate the physiological control, if any, on simultaneous utilization of xylose and glucose. Towards this objective we have constructed a promoter probe vector in which expression of *lacZ* gene would depend on introduced promoter DNA. Using this plasmid, we have cloned random fragments from *E. coli* genomic DNA and isolated several recombinant plasmids which are *lacZ*⁺ under all the growth conditions tested. We also cloned *Zymomonas mobilis* promoter DNA which allowed expression of *lacZ* constitutively (same level of β -galactosidase activity when grown in rich medium and minimal medium; aerobic and anaerobic). Few of the selected promoter DNA have been sequenced. In a separate experiment, we identified, using *E. coli* gene arrays, a set of *E. coli* genes which are expressed constitutively. Selected DNA sequences from these various promoters are currently under investigation as sources of promoter DNA for *xyl* operon.

Layperson's Summary

The lignocellulose of woody biomass represents a significant underutilized resource for fermentative production of alternative fuels. The hemicellulose components, which constitute as much as 40% of the lignocellulosic biomass, must be converted to fermentable substrates for efficient and cost-effective processes for generating ethanol from the biomass. Pretreatment with dilute acid (0.5% sulfuric acid) at elevated temperatures (greater than 120°C) is used to hydrolyze the hemicellulose fraction in commercial processes involved in the production of ethanol from biomass. The free

sugars, principally xylose, generated by this process may be fermented to ethanol by recombinant strains of *Escherichia coli* and *Klebsiella oxytoca* that have been developed by our research group. A significant amount of the carbohydrate components of the hemicellulose are resistant to hydrolysis in dilute acid. These contain glucuronic acid residues linked to xylose, and require enzymatic digestion for their conversion to fermentable substrate. We have established that the levels glucuronic acid in the hemicellulose fraction of sweetgum and cottonwood, both of which are a significant biomass resource for production of ethanol, are at levels that render 25% of the sugars resistant to release and fermentation. Sugarcane bagasse and rice hulls, other important resources for bioconversion to ethanol, also have acid-resistant components in their hemicellulose fractions. Recombinant strains of *E. coli* have been developed which produce enzymes to assist in the complete digestion of the hemicellulose polymers, and which are better able to ferment multiple sugars derived from the digestion of the cellulose and hemicellulose fractions of lignocellulose of biomass resources. It is anticipated that this work will contribute to the development of cost-effective processes for the commercial production of ethanol from underutilized lignocellulosic biomass.

Scientific Accomplishments

1. Structural composition of hemicellulose fractions of hardwood biomass.

The composition of the hemicellulose fractions of sweetgum (*Liquidambar styraciflua*) was determined by ^{13}C -NMR analysis before and after hydrolysis in 0.5% H_2SO_4 . Growing stems of 2 to 3 cm were debarked, dried at 130 C, and pulverized in a Wiley Mill. The glucuronoxylan fraction was prepared as described by Jones et al. (1961) by extraction with benzene:ethanol in a Soxhlet apparatus for 8 hours, followed by extraction of the residue with 25% KOH for 12 hr at room temperature under N_2 . After filtration to remove the insoluble lignocellulose fraction, the soluble hemicellulose fraction, containing the glucuronoxylan, was neutralized with glacial acetic acid, and the polymers were precipitated from cold 70% ethanol. The precipitate was collected by filtration, washed with increasing concentrations of ethanol, dried, and dissolved in water for further analysis. A sample was subjected to hydrolysis in 0.5% H_2SO_4 at 122 C for 60 min. Samples (20 to 40 mg based upon total carbohydrate analysis) were dissolved in 4.0 ml 25% D_2O containing 0.62% acetone as a shift (32.07ppm) standard. A static decoupled ^{13}C spectrum was obtained in a Nicolet 300 MHz NMR spectrometer at 22 C. A transformed and phased spectrum is presented in Fig.1 below for 20,000 scans of 34.7 mg of sweetgum hemicellulose in 4.0 ml 25% D_2O .

FIG.1 ^{13}C -NMR spectrum of sweetgum hemicellulose,

The shift assignments were made from the previous studies on the ^{13}C -NMR spectra of the glucuronoxylan from *Rudbeckia fulgida* (Kardosova et al., 1998). Using the shift for the methyl carbon of acetone of 31.07 to standardize the axis, the peaks were integrated and assigned relative shift values by the FELIX software program (Table 1). The spectrum indicates that the hemicellulose fraction of sweetgum predominantly if not completely comprised of 4-O-methyl-D-glucuronoxylan.

TABLE 1 Assignments and amounts of carbon atoms in sweetgum hemicellulose.

<u>Carbon</u>	<u>ppm</u>	<u>Intensity, %</u>
Xylose nr β C1, int β C1	100.7	23.8
Glucuronate C1	98.6	3.9
Glucuronate C4	83.5	3.5
Xylose intC4	77.4	24.4
Xylose int β C5	63.9	17.5
Glucuronate OCH ₃	60.9	3.7
Acetone CH ₃	31.2	38.7

The unique shift values for the C1, C4, and O-methyl carbons of the 4-O-methyl-D-glucuronic acid residues are clearly resolved and quantifiable, and essentially equivalent in amounts, with an average contribution of 3.7% of the total. The internally linked C4 of xylose can be used to estimate the relative amounts of the xylose residues. The ratio of xylose to glucuronic acid is 24:3.7, indicating that 12.5% of the sugar residues are glucuronic acids. Since the α -(1-2) linkage of the glucuronic acid residue to the β -(1-4) xylan is resistant to the dilute acid conditions that are responsible for the cleavage of the xylan and the release of free xylose, acid hydrolysis would leave 25% of the sugars as the dimer GAX, and unavailable for fermentation to ethanol by *E. coli*.

The ^{13}C -NMR spectrum of the dilute acid hydrolysate of the glucuronoxylan from sweetgum is shown in Fig. 2.

FIG 2. ^{13}C -NMR spectrum of sweetgum hemicellulose subjected to hydrolysis in 0.5% H_2SO_4 at 122 C for 30 min.

The carbons with shift positions of 100 and 95.5 likely represent C1 β and C1 α of free xylose; the shoulders on upfield of these may represent the C1 β and C1 α of xylose that is substituted on C2 by its linkage to O-methyl-D-glucuronic acid. The C4 of the

glucuronic acid is resolved and quantifiable at 84.5 ppm. This data supports the conclusion that hydrolysis in dilute sulfuric acid (0.5% at 125 C for 30 min) releases all of the xylose with the exception of that which is substituted via its linkage to glucuronic acid.

Cottonwood (*Populus deltoides*) is a biomass candidate for conversion to ethanol that has been selected for development of rapidly growing clones. Three of these which showed rapid biomass production (Rockwood et al., 1996) were evaluated with respect to hemicellulose composition. Figure 3 shows the ^{13}C -NMR spectrum of the dilute acid hydrolysate of one of these.

FIG. 3. ^{13}C -NMR spectrum of cottonwood (clone Ken8) hemicellulose after hydrolysis in 0.5% H_2SO_4 at 122 C for 30 min.

This spectrum is very similar to that seen for the acid hydrolysate of sweetgum hemicellulose, with a prominent C4 for the O-methyl-D glucuronic acid residue at 85 ppm, and C1 β and C1 α carbons for free and substituted xylose residues. The corresponding spectra obtained for the hemicellulose hydrolysates of cottonwood clones SC71 and S13C30 were very similar to that shown for Ken8 in Fig. 3.

The compositions of the hemicellulose fractions were also determined by HPLC analysis of sugars released by dilute acid hydrolysates.

TABLE 2. Quantification of sugars released by dilute acid hydrolysis of hemicellulose fractions of sweetgum and cottonwood clones^a

<u>Source</u>	<u>Glucose^b</u>	<u>Xylose^b</u>	<u>GAX^c</u>
SG	<0.2	7.39	2.21
CW-Ken8	0.20	6.09	2.87
CW-SC71	0.44	4.40	2.29
CW-S13	0.38	6.70	2.65

a-Components were detected by differential RI after resolution on a BioRad HPX 87P column eluted with water at 70 C.

b-Glucose and xylose were assigned on the basis of retention times of standards.

c-GAX assignment is based upon the higher retention time (RT 29 min) expected for an acidic molecule.

The sweetgum source contained the least amount of glucose in the hydrolysate of the hemicellulose fraction, and would appear to provide the most homogeneous preparation with respect to glucuronoxylan. The composition based upon acid hydrolysis and HPLC analysis fully support the interpretation of the ¹³C-NMR analysis (Fig. 1). The cottonwood samples are more variable with small amounts of detectable glucose in the hydrolysates. The RT 29min component, tentatively designated GAX, is present in slightly greater quantities than in the sweetgum hemicellulose.

2. Structural composition of hemicellulose from sugarcane bagasse and rice hulls.

Sugar cane bagasse and rice hulls were obtained from BCI International, Jennings LA, from lots that have been used for the commercial production of ethanol. These were processed by the same procedures used to prepare the glucuronoxylans from hardwood lignocellulose.

The ¹³C-NMR spectrum for the hemicellulose fraction from sugarcane bagasse is shown in Fig. 4. Notable is the very low amount of signal corresponding to the C4 carbon of glucuronic acid at 85 ppm. This suggests a lower amount of 4-O-methyl-D-gluronic acid than was observed in the hemicellulose fraction of either sweetgum or cottonwood. The signal at 55 ppm corresponds to a carbon configuration not seen in the spectra of the sweetgum and cottonwood preparations, and remains to be defined.

FIG. 4. ^{13}C -NMR analysis of hemicellulose from sugarcane bagasse.

The ^{13}C -NMR spectrum for the rice hull hemicellulose fraction is shown in Fig. 5. As in the case of the sugarcane bagasse, carbons unique to O-methyl-D-glucuronic acid are present in amounts less than found in the hardwood hemicellulose fractions. Also present is an unassignable carbon shift at 57 ppm, similar to that found for sugarcane hemicellulose preparation.

FIG. 5. ^{13}C -NMR analysis of hemicellulose from rice hulls.

It is apparent that the hemicellulose preparations from sugarcane bagasse and rice hulls contain components that are different from the 4-O-methyl-D-glucuronoxylan that has been well defined as the predominant structural component of the hemicellulose fraction of hardwood lignocellulose. Further studies on the properties of the components obtained from the stillage of cultures of *E. coli* LY01 will be made to identify components that are resistant to metabolic conversion to ethanol.

3. Preparation of GAX from sweetgum hemicellulose.

4-O-Methyl-D-glucurono- α -(1-4)-D-xylose (GAX) has been isolated in preparative quantities from the dilute acid hydrolysate of sweetgum glucuronoxylan. Sweetgum sawdust was digested in 0.5% H₂SO₄ at 122 C for 30 min. Following neutralization with Ba(OH)₂ and removal of insoluble BaSO₄, oligosaccharides containing glucuronic acid residues were adsorbed onto a Dow-1-acetate column. After elution with acetic acid, these were further subjected to gel filtration on a BioRad P2 column equilibrated and eluted with 0.1 M acetic acid. Peak fractions containing carbohydrate were combined and concentrated. A further gel filtration step was performed on a P2 column eluted with water to remove acetic acid. The ¹³C-NMR analysis is shown in Fig. 6.

FIG. 6. ¹³C-NMR analysis of GAX prepared from sweetgum glucuronoxylan.

The carbons at 60.9, 83.5, and 98.6 ppm represent the O-methyl-C, C4, and C1 of the glucuronic acid residue. The carbon at 78 ppm represents the C4 of the xylose residue, and its presence in an amount nearly the same as the resolved signals for the assignable uronic acid carbons indicates that the fraction contains GAX as the predominant if not exclusive component. This procedure has provided large quantities of the GAX with which to select for ethanologenic bacteria that are able to utilize the GAX derived from the hemicellulose fractions of the biomass resources.

4. Depolymerization of hemicellulose fractions by recombinant endoxylanase from *Erwinia chrysanthemi* D1.

A plasmid bearing an endoxylanase gene derived from *Erwinia chrysanthemi* D1 has been provided by Noel T. Keen of the University of California, Riverside (Keen, 1996). This has been overexpressed in *E. coli* HB101 and purified to homogeneity. Its activity on sweetgum glucuronoxylan is shown in Fig. 7 below.

FIG. 7

Reducing Sugars Generated Over Time by *E. chrysanthemi* D1 xynA as a Function of pH for 1.0% Sweetgum Xylan in 50 mM $\text{NaC}_2\text{H}_3\text{O}_2$

The endoxylanase is clearly most active at pH 6. The linear progress curve through 40 min, followed by a decrease in the rate of product formation, points to a process in which preferred substrate becomes limiting.

The *Erwinia* endoxylanase shows greatest activity on xylan preparations that contain the greatest amounts of glucuronic acid (Table 3). Thus the hemicellulose fraction from sweetgum, which is nearly pure glucuronoxylan, is a very good substrate, especially compared to the birch and beechwood xyans, and the sugarcane hemicellulose, all of

which are relatively low in glucuronic acid. This suggests that the *Erwinia* xylanase may prefer to attack regions containing the glucuronic residues, and may assist in the digestion of hemicellulose rich in O-methyl-D-glucuronic acid.

TABLE 3. Comparative depolymerization activity of *E. chrysanthemi* D1 recombinant endoxylanase^a toward hemicellulose preparations^b

Source	Endoxylanase Activity (Units)	
	pH 6	pH 7
Sweetgum hemicellulose	1.75	0.61
Beechwood xylan	0.88	0.37
Birchwood xylan	0.46	0.41
Sugarcane bagasse hemicellulose	0.66	0.27
4-O-methyl-D-glucurono-D-xylan	1.65	0.6

^a Recombinant endoxylanase A from *E. chrysanthemi* D1 was expressed in *E. coli* BL21(DE3) as described by Keen et al. (1996). The recombinant enzyme preparation was purified to homogeneity with CM-Sephadex as verified by SDS-PAGE. For each assay, 2.8 µg of the purified recombinant enzyme in 100 µl of dH₂O was added to 500 µl of reaction mixture consisting of 1.0% (w/v) of each hemicellulose preparation given in 50 mM sodium acetate at the appropriate pH. Reducing sugars generated in the reaction mixtures were determined in 20 minute intervals by the Nelson method (1944). All reactions were run in triplicate, with the endoxylanase activities shown calculated from the mean amounts of reducing sugars determined at each time point for each reaction condition. One unit of endoxylanase activity is the amount of enzyme necessary to produce 1 µmole of xylose equivalent per hour of incubation at room temperature.

^b The preparation used were obtained as follows: Sweetgum hemicellulose was generated from 10' tall smooth bark trees grown on Oldfield Farms, Micanopy, FL. Stems were cut into 2-5 mm thick disks, each ca. 43-45 mm in diameter. The sawdust generated from the cutting of the disks was collected and subjected to the hemicellulose extraction method of Jones et al. (1961). Beechwood xylan, Birchwood xylan and 4-O-methyl-D-glucurono-D-xylan were purchased from Sigma Chemical, St. Louis, MO. Sugarcane bagasse was obtained from BCI international, Jennings, LA.

5. Simultaneous utilization of glucose and xylose

In *Escherichia coli*, the proteins needed for xylose utilization are coded by two diverging operons: *xylAB* coding for isomerase and kinase, respectively, and *xylFGH* coding for xylose transport proteins. Expression of *xyl* operons requires the presence of xylose and absence of glucose. XylR protein is a positive activator of *xyl* operon. Catabolite repression of *xyl* operons is mediated through the level of CRP-cAMP complex and the intracellular cAMP pool is controlled by the availability of glucose in the medium. This catabolite repression of *xyl* operon needs to be eliminated before

simultaneous utilization of glucose and xylose can be evaluated. Our initial strategy is to replace the native promoter sequences of *xyl* operons with promoter sequences which are independent of any control in the cell - constitutive expression. Upon expression of *xyl* operon in a constitutive manner, we will investigate the physiological control, if any, on simultaneous utilization of xylose and glucose.

Towards this objective we have constructed a promoter probe vector in which expression of *lacZ* gene (β -galactosidase) would depend on introduced promoter DNA. Using this plasmid, we have cloned random fragments from *Zymomonas mobilis* genomic DNA which allowed expression of *lacZ*. Nine clones which expressed *lacZ* and thus carry a *Z. mobilis* promoter DNA were tested for the level of β -galactosidase activity produced by the culture when the cells were grown in rich medium under anaerobic conditions. Results of these experiments are presented in the following Table. Five of the clones were further tested for the ability to produce β -galactosidase activity when grown in glucose-minimal medium, also under anaerobic growth conditions. Clones 11, 16, and 22 produced higher level of β -galactosidase activity when grown in minimal medium suggesting that the promoter is from an operon coding for small molecule biosynthesis. Clones 13 and 18 produced about same level of β -galactosidase activity irrespective of the growth medium. The *Z. mobilis* insert DNA in these plasmids was sequenced and these sequences revealed that clones 11, 15, 18 and 21 contain the same promoter DNA used for synthesis of riboflavin synthase α -chain. Clones 17 and 22 contained the same DNA coding for ubiquinone synthesis proteins. Clones 13, 14 and 16 contained unique DNA of unknown nature. The promoter DNA from clones 13 and 18 are currently being evaluated as surrogate promoters for *E. coli xyl* operons.

In order to identify constitutive promoters which are expressed at varying levels we are analyzing the expression of all the genes in *E. coli* using a gene array in which all 4,290 open-reading-frames are impregnated in a nylon membrane. The cDNAs derived from total mRNA produced by *E. coli* grown under different conditions are hybridized to the DNA in the membrane. The intensity of the spot is directly proportional to the concentration of that specific mRNA in that growth condition. Based on this analysis, we have identified a set of operons whose expression was constitutive (similar level of expression in cells grown in rich medium, minimal medium; aerobic and anaerobic). The promoter DNA from these operons are currently being evaluated as a source of promoter DNA for *xyl* operons.

Table 4 Level of expression of *Z. mobilis* promoter-driven *lacZ* in *E. coli*

Clone No.	β -galactosidase Activity	
	Rich medium	Glucose-minimal
11	1,300	3,700
13	3,500	3,500
14	500	ND
15	1,000	ND
16	1,700	3,600
17	550	ND
18	1,600	2,000
21	1,100	ND
22	220	3,300

Cells grown in rich medium (L-broth + 0.3% of glucose) or minimal medium at late-exponential phase of growth was assayed for β -galactosidase activity. Enzyme activity is expressed as nmoles per min per mg cell protein. ND-not determined.

References.

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- Kardosova, A., M. Matulova, and A. Malovikova. 1998. (4-O-Methyl- α -D-glucurono)-D-xylan from *Rudbeckia fulgida*, var. *sullivantii* (Boynton et Beadle). Carbohydrate Res. 308:99-105.
- Keen, N.T., C. Boyd, and B. Henrissat. 1996. Cloning and characterization of a xylanase gene from corn strains of *Erwinia chrysanthemi*. MPMI 9:651-657.
- Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.
- Rockwood, D.L., S.M. Pisano and W.V. McConnell. 1996. Superior cottonwood and eucalyptus clones for biomass production in wastewater bioremediation systems. Bioenergy '96-The seventh national bioenergy conference. pp 254-261.

Publications and Presentations

L. Ingram, J.F. Preston, and K.T. Shanmugam. Ethanol Production from Uronic Acid-Substituted Xylose Residues in Hemicellulose Hydrolysates. 1999 Symposium for the Consortium for Plant Biotechnology Research, Inc.

Technology Transfer

To be considered.

Commercial Accomplishments

To be considered.

Educational Accomplishments

The ^{13}C -NMR analysis of glucuronoxylan have provided a useful example for the structural identification of carbohydrate polymers. These have been used in our advanced techniques course, PCB 5136L, in which the students design and execute NMR experiments using the Virtual NMR facility operated by M. Buszko in the Department of Microbiology and Cell Science at the University of Florida.

Additional Funding

To be considered

Key Personnel Hiring

Dr. Jason C. Hurlbert was hired as a Post-Doctoral Research Associate to carry out research on the expression of genes encoding β -xylanases and α -glucuronidases with which to convert the glucuronoxylans to fermentable substrates. Due to limited funding, a second Post-Doctoral Research Associate working on the coutilization of xylose and glucose is only partly supported by this grant.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Jiming Jiang, Ph.D.	
University:	University of Wisconsin-Madison	
Agreement Number:	OR22072-95	
Project Title:	Toward cloning a functional rice centromere	
Reporting Period and Report Type:	From: 12/1/98 To: 5/31/00	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

Fully characterize the DNA composition of rice centromeres (Year One).

1. Sequenced a 68-kb rice centromeric BAC clone.
2. Analyzed the cytological locations of more than 30 rice bacterial artificial chromosome (BAC) clones containing centromeric repeats.
3. Developed a special fluorescence in situ hybridization (FISH) technique to visualize the chromosomal origin of centromeric BAC clones.
4. Several BAC clones derived from centromere 11 were identified. DNA sequences specific to this centromere were isolated.
5. Discovered a rice neocentromere.

Develop physical maps which span the centromeric regions and characterize the DNA composition of individual centromeres (Year Two).

We analyzed the fingerprinting data of ~400 rice BAC clones containing centromeric repeats.

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 main goal of the current CPBR project is to determine the DNA sequences located in the rice centromeres. The centromeres of eukaryotic species contain mainly repetitive DNA sequences. It is very difficult to localize a large DNA fragment to a specific centromere because of the presence of the repetitive DNA sequences. Using specially designed techniques and research strategies we were able to assign several large DNA fragments to specific rice centromeres. This is the first such achievement in any plant species. This progress will provide the foundation for us to discover the DNA sequences spanning an entire rice centromere. We also discovered a "neocentromere" in rice, the first *de novo* neocentromere in any plant species. Neocentromeres are extremely important tools in humans and other model organisms for centromere studies. The rice neocentromere will provide us an unprecedented tool to study the structure and function of plant centromeres in the future.

Scientific Accomplishments

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

We sequenced a centromeric bacterial artificial chromosome (BAC) clone containing a 68-kb DNA fragment derived from a rice centromere. The most dominant component of this BAC clone, 17p22, is a 155-bp satellite repeat. The high sequence similarity between the monomers of this repeat made sequence assembly difficult. We developed a cytology-based digital mapping technique. Using this technique we were able to determine the distribution of the satellite repeat and other low copy DNA elements within the BAC insert (Jackson et al. 1999). The locations of the low copy DNA elements provided anchor markers for sequence assembly. A single contig was successfully constructed from the sequencing data. DNA sequences homologous to retrotransposons were discovered in 17p22, indicating that these sequences may be derived from a degenerated retrotransposon. Part of the 155-bp rice centromeric satellite repeat is partially homologous to the maize centromeric satellite Cent-C. This dramatic discovery suggests that the major DNA components of the rice and maize centromeres are at least partially conserved after over than 100 million years of divergence of these two species.

We screened a rice BAC library using several repetitive DNA elements isolated from BAC 17p22. More than 30 BAC clones were analyzed by fluorescence in situ hybridization (FISH). These BAC clones can be divided into two groups. Clones in the first group hybridized specifically to the centromeric regions (Fig. 1a). Clones in the second group hybridized all over the chromosomes (Fig. 1b), indicating that these clones contain repetitive DNA elements which are not specific to the centromeres. We have selected several clones in the first group to isolate DNA elements which are specific to rice centromeres but are different from those in 17p22. The research strategy is to digest the DNA from these clones with several restriction enzymes and blot the DNA to nylon membranes. DNA fragments, which do not hybridized to 17p22, will be subcloned into plasmid clones and characterized using molecular and cytogenetic approaches.

Fig. 1. Two different types of rice centromeric DNA BAC clones. (a) A BAC clone hybridized only to the centromeric regions. (b) A BAC clones hybridized to all over the chromosomes.

Although the clones in the first group hybridized only to the centromeric regions, the chromosomal origins of the BACs cannot be determined because these clones hybridized to all the 12 rice centromeres. We developed a FISH technique which includes a special preanneal procedure with rice C_0t1 DNA. Using this technique we found that the signals derived from several BAC clones in the first group are much stronger in one pair of centromeres than those in other centromeres (Fig. 2). Such signals from three centromeric BAC clones were localized to specific chromosomes by co-hybridization with rice chromosome-specific DNA markers developed recently in our laboratory. This is the first time that large insert DNA clones containing centromeric repeats are mapped to specific chromosomes in plants. The chromosome-specific centromeric DNA sequences isolated from such BACs will provide anchor markers to construct contigs spanning complete rice centromeres. We have developed sublibraries from two BAC clones which were mapped to centromeres of chromosomes 3 and 11, respectively. Plasmid clones containing sequences different from these of 17p22 will be isolated and sequenced.

Fig. 2. A rice centromeric BAC showed stronger signals in the centromeres of chromosome 11 (arrows) than those in other centromeres

The 5S ribosomal RNA genes were previously mapped on the short arm very close to the centromere of rice chromosome 11. We did a high resolution FISH mapping on rice pachytene chromosomes using a 5S rDNA probe and a satellite DNA probe, pRCS2, which is specific to the centromeres of rice. Both the 5S rRNA genes and the RCS2 satellite DNA are organized into two separate domains on the early pachytene chromosome (Fig. 3b, c). One of the two 5S rRNA gene domain is clearly located between the two satellite DNA domains (Fig. 3d). This results revealed that the 5S rRNA genes are part of the rice centromere 11 because the RCS2 satellite DNA is only located in the primary constriction of rice chromosomes. We isolated four 11L (the long arm of chromosome 11) telocentric chromosomes that were derived from independent centromere misdivision events of chromosome 11. All four 11L telocentric chromosomes carry 5S rRNA genes in the centromere based on FISH analysis (Fig. 3e). The 5S rDNA signals are clearly located outside of the RCS2 signals on prometaphase 11L (Fig. 3e), confirming that the 5S rDNA is part of the centromeres of 11L telocentric chromosomes.

Fig. 3. High resolution FISH mapping of the 5S rRNA genes on early pachytene chromosomes of rice. (a) Part of a pollen mother cell at early pachytene. (b) The FISH signal derived from a 5S rDNA probe are separated into two domains. (c) The FISH signals derived a rice centromere-specific satellite DNA probe pRCS2 are also separated into two domains on the same chromosome. (d) One of the two 5S DNA domain (green signal, arrow) is located between the two RCS2 domains, indicating that this domain is located within the primary constriction. (e) Location of the 5S rRNA genes on telocentric chromosomes derived from rice chromosome 11. 1, An isochromosome 11S.11S shows both 5S rDNA (green) and pRCS2 (red) signals on both arms; 2-5, All four telocentric 11L chromosomes, which were derived from different centromere misdivision events, contain 5S rRNA genes. The 5S rDNA signals (green) always located outside of the pRCS2 signals (red) on prometaphase chromosomes.

We screened a rice BAC library using a 5S rDNA probe and probe pRCS1 that is dispersed in the rice centromeric regions. Several BAC clone hybridizing to both pRCS1 and the 5S rDNA probe was identified. Sequencing of the ends of one of these BAC clones, 22B9, indicated that only one end of the BAC insert is derived from 5S rRNA genes. About 30% of the insert of 22B9 contains non-rDNA sequences. Characterization of the non-rDNA sequences is underway. The discovery of the association of 5S ribosomal rRNA genes with the centromere of chromosome 11 provides us another approach to isolate DNA sequences specific to centromere 11. Thus, rice chromosome 11 will be the best target for us to construct a DNA contig spanning its complete centromere.

We have isolated numerous cytogenetic stocks containing rearranged rice chromosomes from a triploid rice. The rearrangements in some of the rice chromosomes presumably involved in the centromeres based on morphology of pachytene chromosomes. We recently made an extensive survey on these rearranged chromosome by FISH analysis using rice centromeric DNA probes. An abnormal metacentric chromosome in a trisomic stock, CZ37, is completely devoid of any rice centromeric DNA elements isolated previously in our lab. All the root tip cells analyzed from a CZ37 plant contained one copy of this chromosome, indicating that this chromosome has a normal transmission in somatic cells. However, this chromosome lags in majority of the anaphase I cells in meiosis, suggesting that the centromere of this chromosome is probably only partially functional. It is highly likely that this chromosome contains a neocentromere, possibly the first *de novo* neocentromere discovered in plant species. This neocentromere will provide us an unprecedented tool to study the structure and function of plant centromeres.

With a collaboration with Dr. Rod Wing's lab at the Clemson University we have started to study the fingerprinting data of BAC clones containing centromeric DNA repeats. Several BAC contigs including multiple clones were identified by grouping approximately 400 hundreds of BAC clones with at least one end homologous to previously identified rice centrmERIC repeats.

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.

Publications:

Jackson, S. A., F. Dong, and J. Jiang (1999) Digital mapping of bacterial artificial chromosomes by fluorescence in situ hybridization. *Plant J.* 17: 581-587.

Invited Seminars:

Department of Botany and Plant Pathology, Purdue University, February 10, 1999.
Molecular structure of grass centromeres.

Department of Agronomy, Yangzhou University, P. R. China, August 11, 1999.
Fluorescence-based DNA *in situ* hybridization techniques and their applications in plant genome mapping.

Toward cloning a functional rice centromere. 1999 Symposium of the Consortium for Plant Biotechnology Research. Washington, D.C.

Institute of Nuclear Agricultural Science, Zhejiang University, P. R. China, August 18, 1999.
Molecular structure of plant centromeres.

Department of Plant Pathology, Kansas State University, October 21, 1999. Centromeres of plant chromosomes.

Technology Transfer

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

Patent Submitted: Jiang, J. and F. Dong (1999) DNA sequences specific to rice centromeres.

Commercial Accomplishments

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

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Educational Accomplishments

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

One graduate student, Fenggao Dong, completed his Ph.D. degree in March, 2000. Fenggao was partially supported by the current CPBR grant during the past year and played the major role in characterizing a large DNA fragment derived from a rice centromere. Fenggao will join Cereon Genomics (Monsanto) in April of 2000.

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.

Dr. Zhukuan Cheng, hired in July of 1999 as a new postdoctoral associate by the current CPBR grant.

Dr. Alexander S. Parokonny, hired in March, 2000 as a new postdoctoral associate by the current CPBR grant.

SCIENTIFIC PROGRESS REPORT submitted to CPBR

Principal Investigator:

Gayle Lamppa

Department of Molecular Genetics and Cell Biology
University of Chicago

PROJECT #OR22072-96

TITLE:

Accumulation of products within the plastid for biomass conversion: test system with cellulase.

Reporting period: 1/1/99-12/31/99

Interim report

GENERAL SUMMARY OF PROJECT OBJECTIVES AND PROGRESS

Our immediate goal is to determine if a cellulase, endoglucanase E1 from Acidothermus cellulocystis which is a thermophilic bacterium, can be accumulated in plastids in an active form. It seems reasonable that the cytosol would not tolerate high levels of a cellulase during plant growth and development, and hence, an effective means of sequestering it may be crucial for its overexpression in vivo. We propose that the numerous chloroplasts per cell will serve as an excellent site for this purpose. Our specific aims should contribute to the production of usable and relatively inexpensive amounts of a cellulase in transgenic plants, which subsequently can be used for cellulose degradation and glucose fermentation to ethanol. **Our specific research objectives are described below, and a brief summary of the progress we have made is provided.** Our results relating to these goals are presented in greater detail in the section "Scientific Accomplishments".

Objective 1. Design gene constructs that code for a precursor fusion protein with endoglucanase E1 fused to a transit peptide with a site cleavable by the stromal processing peptidase (SPP) at the junction.

The goal of these experiments was to test the general hypothesis that transit peptides and any protein linked to them must be compatible for efficient processing and import into chloroplasts, and specifically, this would be the case for endoglucanase E1. Hence, we have separately linked E1 and its catalytic domain to two different transit peptides, and have attempted to optimize the transit peptide-mature protein junction for cleavage by SPP and import.

An array of fusion proteins (see Figure 1) was made using the transit peptide of ferredoxin (FD). We found that the efficiency of proteolytic processing and import is significantly affected by the number of amino acids immediately following the cleavage site, before the start of E1. Hence, it appears that the spacing at the transit peptide-E1 junction is critical. This is supported by the observation that an insertion after the cleavage site of 10 amino acids from the signal peptide of E1, which is normally synthesized as a precursor that is exported from Acidothermus, also promotes processing and import.

Another important observation is that a fusion protein containing only the catalytic domain (CD) of E1 is more readily cleaved and imported than a comparable precursor with full length E1.

We have extended our results with the transit peptide of FD by using the transit peptide of Rubisco activase (RBCA) fused to E1 and CD. In comparable constructs, each transit peptide confers different properties, which are discussed at greater length in "Scientific Accomplishments". Nevertheless, precursors with the transit peptide of RBCA are also cleaved by SPP and imported.

Objective 2. Expression of gene constructs and production of endoglucanase E1 in vivo.

The results presented above identified several important gene constructs encoding precursor fusion proteins that are efficiently cleaved and imported in vitro. Other constructs that are not cleaved and imported serve as useful controls for in vivo analyses. We selected five of these for transformation of tobacco. The genes were placed downstream of the nearly constitutive CaMV 35S promoter with 5' and 3' nontranslated regions as part of a cassette. This promoter was selected to assure fairly high transcription levels. The transformants are described below in the section on "Scientific Accomplishments". Leaf disks were inoculated with Agrobacterium and regenerated plants---the primary transformants---are ready for analysis. The level of E1 and

endoglucanase activity will be examined in different tissues of the transgenic plants, starting with leaves. We plan to assay total cellular protein and to also isolate plastids to determine the success of targeting E1 to the chloroplast. We already know that very little E1 activity can be detected in the transgenic plants if E1 is synthesized without a transit peptide.

Objective 3. Determine if an active endoglucanase can be recovered by in vitro translation, and if the transit peptide affects this activity.

Based on the recommendations of the reviewers of our original proposal, our revised research objectives de-emphasized this goal. However, as we began to make more complicated constructs, we realized that the addition or deletion of amino acids at the N-terminus of E1 might influence endoglucanase activity. Therefore, there was good reason to attempt in vitro expression. Furthermore, we foresaw the possibility of manipulating cellulase activity genetically if we had a relatively rapid assay for analyzing mutant constructs. These experiments have been successful. We have synthesized active E1 as well as its CD by in vitro transcription of their respective genes followed by translation of their transcripts. Unexpectedly, the precursor fusion proteins are active. Activity is inhibited by a specific mutation at the N-terminus of E1 in the precursor, and optimal activity depends on high temperature. Our studies on the import efficiency of E1 and its CD into isolated chloroplasts, using the array of constructs already created (see Objective 1 and "Scientific Accomplishments"), can now be extended to include assays for a functional enzyme after transport into the organelle in vitro.

IN SUMMARY, based on the Time Table we proposed for the first 12 months of our experiments, our studies are right on schedule. An illuminating series of fusion proteins have been synthesized and analyzed. Importantly, they demonstrate the specificity of SPP's recognition of different substrates. Further, the translocation apparatus of the chloroplast imports these substrates with different efficiency. We are beginning the analysis of E1 expression in for the primary tobacco transformants. Within 2 months we should have seed from these transgenic plants for further detailed biochemical studies. In addition, we have developed a new in vitro system for E1---and CD---expression that should help us explore in the future some of the structural features of E1 needed for activity. The in vitro expression system should facilitate testing of some of the predictions that have been made based on structural information and evolutionary comparisons of different cellulases.

LAYPERSON'S SUMMARY

Our goal is to try to accumulate large amounts of active cellulase in the chloroplasts of transgenic plants, where it will be sequestered and thus unlikely to have deleterious effects on plant growth and development. Furthermore, it might be possible to rapidly enrich for the enzyme by chloroplast isolation. We chose for our study endoglucanase E1 from Acidothermus cellulocystis, a thermophilic bacterium (courtesy of Steven Thomas, NREL). We proposed to synthesize precursor fusion proteins with signals that would specifically target E1 to the chloroplast, where the signal---called the transit peptide---would then be cleaved, releasing mature E1 within the organelle. We proposed to initially test our fusion proteins in vitro for proteolytic processing and import into isolated chloroplasts in order to select the best candidates for more costly in vivo studies using transgenic plants. Thus far we have made important steps forward in designing constructs that promote efficient cleavage of a subset of precursor fusion proteins and we also demonstrate that these precursors are imported. The junction between the transit peptide and E1 is a critical region within the structure of the precursor. In addition, different transit peptides affect processing and import to different degrees. These studies have guided our choice of constructs for transformation of tobacco. We have primary transformants

growing, and are beginning a comprehensive analysis of E1 levels and activity within chloroplasts of transgenic plants.

In parallel studies, we attempted to express active E1 in an in vitro system starting with the E1 gene. This includes in vitro transcription followed by in vitro protein synthesis. We are excited that we have obtained an active enzyme. In the future, this allows us to extend our in vitro studies to explore the import and release of active E1 in the chloroplast. We can test the many constructs that we have already synthesized in the import assay and these findings can be compared with our in vivo results. Further, we foresee that this should allow us to genetically manipulate E1 structure and rapidly analyze what changes influence its activity. Many predictions have been made based on structural analyses and evolutionary comparisons of cellulases that can be tested using the in vitro expression system, which should facilitate a comprehensive study on structure-function relationships.

SCIENTIFIC ACCOMPLISHMENTS

In this section, I describe the nature of the constructs we have designed, and discuss in greater detail our experimental results. Representative original data are presented.

Objective 1. Design gene constructs that code for a precursor fusion protein with endoglucanase E1 fused to a transit peptide with a site cleavable by the stromal processing peptidase (SPP) at the junction.

A. Precursor fusion proteins using the ferredoxin transit peptide.

1. Constructs using full-length endoglucanase E1 with insertions or a specific deletion.

We initially designed a series of constructs that encoded precursor fusion proteins with E1 linked to the transit peptide of ferredoxin (FD). The FD transit peptide is considered to possess considerable structural flexibility, and has been used previously to import some foreign proteins into chloroplasts (de Boer et al., 1991, EMBO J. 10: 2765-2772 and Pilon et al., 1992, J. Biol. Chem. 267: 19907-19913), and hence it was chosen for our first studies. Figure 1 lists each construct (#1-#11) used in our experiments to-date, and shows the structure at the transit peptide-mature protein junction of each precursor fusion protein. The results from our assays are also tabulated in Figure 1.

In the first three precursors, the transit peptide and cleavage site were left intact and an increasing number of amino acids---one, five and fifteen residues---from mature ferredoxin were included as a spacer before the start of mature E1. Construct #2 (FD+1::E1, transit peptide plus one amino acid fused to E1) was not proteolytically cleaved by recombinant SPP from *E. coli* or by a soluble chloroplast extract. Construct #3 (FD+5::E1) was cleaved very weakly by SPP, and not at all by the chloroplast extract (Figure 2). For construct #2 import into pea chloroplasts was "poor", whereas we characterized the import of construct #3 as "good". On the other hand, an increase in the spacer region to 15 residues in construct #4 (FD+15::E1) yielded a precursor that was efficiently cleaved by SPP and the chloroplast extract (Figure 2). FD+15::E1 import was characterized as "very good"; it imported into the chloroplast, processed, and most of mature E1 was found in the stromal fraction (Figure 3). These are relative levels of import that we are now quantifying by phosphorimager analyses. Lack of degradation by thermolysin treatment of the chloroplasts demonstrated that E1 was indeed sequestered within the organelle.

When we observed that construct #2 was not processed, and #3 was processed inefficiently, and further that import was relatively low, we became concerned that the unusual residues at the start of E1---Ala-Gly-Gly-Gly-Tyr (AGGGY)---might prevent or compromise recognition of the transit peptide by SPP and the chloroplast import machinery. Glycines are

well-known to alter protein conformation. Therefore, in construct #5 (FD+5::Δ5E1) these residues were deleted. Although FD+5::E1 itself was not efficiently processed or imported, the AGGGY deletion resulted in a precursor that was cleaved by SPP. However, the chloroplast extract did not remove the transit peptide. (One explanation for the discrepancy is that recombinant SPP is significantly more "robust" than SPP in the chloroplast extract, and there is thus a difference between the amount of active SPP relative to the substrate. Another possibility to consider is that features of some substrates are not recognized by SPP in the context of other factors in the chloroplast extract). FD+5::Δ5E1 was also imported into chloroplasts, and cleaved. We conclude that the very N-terminal AGGGY of E1 can have a negative effect on different steps in the import pathway if located in close proximity to the transit peptide.

To test the hypothesis that a spacer is needed between the transit peptide and E1 for efficient cleavage, as suggested from our results with construct #4 (FD+15::E1), we generated construct #6 (FD+5::sp10E1) which contains, besides the 5 residues from mature FD, an additional 10 amino acids from E1's own signal peptide. The introduction of this spacer sequence yielded a precursor that was efficiently cleaved by SPP and the chloroplast extract. It was also imported into the chloroplast, processed and 40% was found in the stroma and 60% in the membrane fraction after thermolysin treatment. That sp10E1 is found in the membrane fraction is probably due to the presence of the region from the signal peptide, which contains a number of hydrophobic residues (Ala, Val, and Pro). It will be interesting to determine if suborganellar location influences E1 activity.

2. Constructs using the catalytic domain of E1.

E1 is comprised of three domains: the N-terminal catalytic domain (CD), the serine-proline rich linker, and the C-terminal cellulose binding domain (CB), as illustrated in Figure 1A. We entertained the idea that other structural features of E1, in addition to the AGGGY sequence, were in some way affecting processing by SPP and transport into the chloroplast. To investigate this question, we decided to determine if CD alone---separated from the linker and CB---fused to a transit peptide would be a better substrate in these reactions. Hence, three new precursor fusion proteins were synthesized:

- Construct #7 (FD+1::E1CD, i.e. FD transit peptide plus 1 amino acid fused to CD),
- Construct #8 (FD+5::E1CD) and
- Construct #9 (FD+5::Δ5E1CD).

Once again, when only one amino acid followed the transit peptide in construct #7 (FD+1::E1CD), the precursor was not processed by SPP, and import was "poor". In contrast, both construct #8 (FD+5::E1CD, Figure 2) and construct #9 (FD+5::Δ5E1CD) were processed, albeit not as well as several other constructs listed in Figure 1B, or presented in Figure 2. Import was quite efficient for both precursors. The results for FD+5::E1CD are shown in Figure 3.

B. Precursor fusion proteins using the transit peptide of Rubisco activase.

Transit peptides do not share a common primary sequence. Therefore, it seemed important to extend our study and determine if an alternative to the FD transit peptide would yield a precursor containing E1 that was more efficiently recognized in the processing and import assays. We selected ribulose-bisphosphate carboxylase/oxygenase activase (RBCA) because we had found previously that at least in vitro, the RBCA precursor itself is very efficiently cleaved by SPP. Two constructs were made with the RBCA transit peptide plus five amino acids from mature RBCA:

- Construct #10 (RBCA+5::E1) and
- Construct #11 (RBCA+5::E1CD).

Construct #10 (RBCA+5::E1) was efficiently cleaved by recombinant SPP from *E. coli* and by the chloroplast extract. However, import for RBCA+5::E1 was poor. Construct #11 (RBCA+5::E1CD) contained the CD fused to the transit peptide, and it was processed very efficiently (Figure 2) and imported (Figure 3). From a comparison of the results using the RBCA+5 constructs (#10 and #11) versus the FD+5 constructs (#3 and #8), we conclude that RBCA+5 promotes more efficient processing of the E1 precursors by SPP.

Objective 2. Expression of gene constructs and production of endoglucanase E1 in vivo.

In the General Summary, I described the basic expression cassette used for transformation. Figure 1 (far right column) lists the different constructs that we have introduced into tobacco thus far using this cassette. Tobacco disks were transformed 4 months ago and analysis of tissue extracts from these primary transformants is underway. We selected the FD transit peptide::E1 constructs (#4, #6 and #8) that were positive in our in vitro processing and import assays, as well as construct #3 that was not well processed. In addition, we included construct #1; that is, the gene for E1 without a linked transit peptide. This should help us assess the importance of targeting to the chloroplast for detection of E1 activity.

Based on our in vitro results with the RBCA transit peptide constructs, we have introduced construct #11 containing the catalytic domain of E1 into tobacco as well. We will restrict ourselves to a careful analysis of lines of these transgenic plants (before developing new constructs) because of the large number of individuals that must be examined for a thorough analysis and accurate interpretation of what happens to E1 expression in vivo.

Objective 3. Determine if an active endoglucanase can be recovered by in vitro translation, and if the transit peptide affects this activity.

We have investigated whether E1 can be expressed in an active form following in vitro transcription and translation. Nine of the constructs shown in Figure 1 have been tested, and our results are presented in Table 1. Each one of these genes was inserted downstream of the T7 bacteriophage promoter, allowing for in vitro transcription and coupled translation in a TNT system (Promega). This system includes a reticulocyte lysate for synthesis of radiolabeled protein. The activity of the translation products was monitored using a MUC assay that is very similar to a β -glucuronidase (GUS) assay. Experiments were first carried out at 65° C and fluorescence measured. A complete description of the assay is given in the legend of Table 1. Table 2 demonstrates that the values obtained are within the linear range of detection.

Significantly, we were able to synthesize active E1 in the in vitro expression system. To our surprise, however, six of the precursor fusion proteins exhibited nearly as much activity as endoglucanase E1 without a transit peptide. Construct #3 (FD+5::E1) showed 85% of the activity found for E1 alone, and construct #10 (RBCA+5::E1) showed 93% of E1 activity. Precursors with only the catalytic domain of E1 were also tested. Construct #8 (FD+5::E1CD) contained 67% of E1 activity. On the other hand, construct #11 (RBCA+5::E1CD) was significantly more active, with 126% of E1 activity.

The two precursors---constructs #5 and #9---with the AGGGY deletion near the N-terminus of E1 showed very low levels of activity (9% and 11% of E1 activity, respectively), yet both were higher than the control reactions, where a background of 2% activity was found using a vector containing the gene for the native precursor of RBCA (without the E1 coding region), or no vector at all in the MUC assay.

We performed the MUC reactions at 65° C again, only we first treated the precursor fusion proteins with recombinant SPP (see Table 1, legend). There was an average of ~21% increase in endoglucanase activity after processing by SPP (Table 1). We tentatively conclude from these experiments that the transit peptide can function as a separate domain from the mature protein, and does not strongly interfere with the enzymatic activity of E1 under these in vitro conditions. Nevertheless, these are still preliminary experiments and conditions may be identified where the transit peptide has an inhibitory effect on the ability of E1 to carry out its role in cellulose degradation. Further, it might be crucial that transit peptide removal is accomplished within the organelle after import, since proteins with transit peptides may be a target for degradation, or interfere with organelle biogenesis if accumulated.

Next, MUC assays were carried out at 37° C for a subset of the constructs. Since E1 is from a thermophilic bacterium, the native enzyme is most active at elevated temperatures. Indeed, we found about a 50% drop in endoglucanase activity in the MUC assay at the lower temperature compared to 65° C.

Taken together, these results demonstrate the specificity of the reaction carried out by E1 expressed in the in vitro eukaryotic (reticulocyte lysate) system. Based on these findings, we are in an excellent position to further optimize the reaction and explore the importance of different domains and features of E1 that might be necessary for its function. Most importantly, we are currently investigating whether E1 activity can be detected in chloroplasts after in vitro import, exploiting the different constructs we have already designed. Preliminary results indicate that indeed active E1 can be detected. These results may help to direct our in vivo studies geared to accumulating high levels of E1 in the chloroplast.

IN SUMMARY, in the last 11-12 months we have developed the tools to investigate if endoglucanase E1 can be sequestered in an active form within the chloroplast. Thus far, we have utilized an in vitro approach that has served as a guide for transgenic studies, which have been initiated. The transgenic plants are ready for a comprehensive analysis. Furthermore, we have developed a new in vitro expression system that can be used to study E1 activity after in vitro import. Hence we can explore parameters that are important for activity, including determinants within the structure of E1 itself.

PUBLICATIONS AND PRESENTATIONS

No publications have resulted from this study, but we anticipate---based on the results presented in our Progress Report---that a manuscript will be submitted by late 1999, describing our precursor fusion proteins for E1 and in vitro analyses. I presented the experimental plan and goals of this project at the CPBR Symposium, March 1999.

TECHNOLOGY TRANSFER

Research Agreement is being finalized with Novartis Inc. and the University of Chicago (ARCH Development Inc).

COMMERCIAL ACCOMPLISHMENTS

None to-date.

EDUCATIONAL ACCOMPLISHMENTS

Training of two postdoctoral research associates in plant molecular biology and biochemistry.

ADDITIONAL FUNDING

None for this project.

KEY PERSONNEL HIRING or TURNOVER

- 1) Dr. Rong Guan Jin joined my laboratory in January, 1999, to work on this project, 100% effort.
- 2) Dr. Stefan Richter, in my laboratory for 3 years, is training and working with Dr. Jin, 50% effort.
- 3) Dr. Rong Zong joined my laboratory in July, 1999, to work on this project, 60% effort.

All of these individuals are highly skilled scientists with backgrounds and expertise that complement one another. Dr. Richter is a microbiologist, molecular biologist and has received training in my laboratory in protein biochemistry. Dr. Jin is an expert in working with transgenic plants and tissue culture. Similarly, this is Dr. Zong's major area of expertise, although both of these individuals have research broad training. I'd like to emphasize that I now have the key personnel in place to make excellent progress over the next year using the tools we have assembled.

A nsit Spacer β-1,4-endoglucanase E1 of peptide *Acidothormus cellulolyticus*

B

Construct	Transit Peptide-E1 Junction	Processing SPP ¹⁾ Extr. ²⁾	Import	Activity	Transgene started
1 E1	M AGGGYW-	N.T.	N.T.	Yes	Yes
2 FD+1::E1	-RVTAM A.....	-	Poor	Yes	No
3 FD+5::E1	-RVTAM ATYKV.....	+	Good	Yes	Yes
4 FD+15::E1	-RVTAM ATYKVTLITKESGTV	++	Very Good	Yes	Yes
5 FD+5::ΔSE1	-RVTAM ATYKV.....	++	Good	No	No
6 FD+5:: ^{SP10} E1 ³⁾	-RVTAM ATYKV.....	++	V. Good	Yes	Yes
7 FD+1::E1CD	-RVTAM A.....	-	Poor	Yes	No
8 FD+5::E1CD	-RVTAM ATYKV.....	++	V. Good	Yes	Yes
9 FD+5::Δ5 E1CD	-RVTAM ATYKV.....	++	V. Good	No	No
10 RBCA+5::E1	-SMTVK AAENE.....	++	Poor	Yes	No
11 RBCA+5::E1CD	-SMTVK AAENE.....	+++	Good	Yes	Yes
Transit Spacer ⁴⁾ E1 Peptide					

¹⁾ Recombinant SPP; ²⁾ Chloroplast extract; ³⁾ SP10: 10 aa of C-terminus of E1 signal peptide; ⁴⁾ N-terminus of mature FD or RBCA

Figure 1. Expression constructs for accumulation of E1 in chloroplasts. (A) Schematic representation of the expression construct. (B) Characterization of E1 expression constructs. Relative amount of precursor processed: >75%, +++; 75-25%, ++; 25-5%, +; <5%, -. N.T., not tested; FD, ferredoxin; RBCA, Rubisco activase.

Figure 2. Processing of each E1 construct by recombinant SPP (Richter and Lamppa, 1998, *PNAS* 95, pp. 7463-7468) and a chloroplast extract from pea (Abad et al., 1989, *J. Cell Biol.* 90, pp. 117-124). The name of each construct is given at the top of each panel. The results are tabulated in Figure 1B. Lanes 1, [³⁵S]methionine-labeled precursor fusion proteins. Lanes 2, processing with recombinant SPP. Lanes 3, processing with chloroplast extract. p, precursor; m, mature E1 or E1CD.

Figure 3. In vitro chloroplast import of E1 precursor fusion proteins. [^{35}S] methionine-labeled precursors were incubated with pea chloroplasts for 20 min, and then stromal and membrane fractions of chloroplasts with or without thermolysin treatment (indicated as -/+) were separated and analyzed by SDS-PAGE (Lamppa, 1995, *Meth. Plant Mol. Biol.*, pp. 141-171). The name of each construct is given at the top of each panel. The results are included in Figure 1B. T, 1 μl translation product; S and M, stromal and membrane fraction, respectively; p, E1 fusion precursor; m, mature E1 or E1CD.

Table 1. Relative activities of E1 expression constructs in vitro¹⁾.

Constructs	Relative amount of released MU					
	at 65 °C		at 65 °C		at 37 °C	
	Units ³⁾	% ⁴⁾	upon SPP processing ²⁾		Units ³⁾	% ⁵⁾
			Units ³⁾	% ⁴⁾		
1 E1	54.98±0.34	100			25.14±0.59	46
3 FD+5::E1	46.76±0.73	85	57.53±0.61	105	22.36±0.41	48
4 FD+15::E1	41.60±0.62	76	54.66±1.36	99		
5 FD+5::Δ5E1	6.30±0.82	11	7.34±0.08	13		
6 FD+5::_{SPI0}E1	50.11±0.72	91	58.87±0.77	107		
8 FD+5:: E1CD	37.00±0.16	67	41.92±0.69	76	15.37±0.20	41
9 FD+5::Δ5 E1CD	5.22±0.14	9	11.38±0.30	21		
10 RBCA+5::E1	51.13±0.41	93	65.48±0.34	119	23.50±0.39	36
11 RBCA+5:: E1CD	69.06±0.45	126	83.64±0.28	152	24.75±0.28	30
PreRBCA	1.00±0.09	2				
Control⁶⁾	1.07±0.10	2				

Table 2. MU standard.

[MU] in nM	Units ³⁾
40.0	156.74±1.70
16.0	62.31±0.53
8.0	30.37±0.30
4.0	15.15±0.16
2.0	7.73±0.18
1.0	3.91±0.12
0.5	2.08±0.13
0.25	1.02±0.07
0.125	0.56±0.05

¹⁾ Relative activity was examined by hydrolysis of the substrate 4-methylumbelliferyl β-D-cellobioside (MUC) which liberates the fluorescent product 4-methylumbelliferone (MU)(MUC assay, protocol kindly provided by S.R. Thomas, National Research Energy Laboratories). All constructs are under T7 promoter control and were expressed in coupled transcription/translation reactions (50 μl standard reactions, TNT System, Promega). Translation product (5 μl) was added to 200 μl 0.5 mM MUC in reaction buffer (100 mM sodium chloride, 50 mM sodium acetate, pH 5) and incubated at 65 °C or 37 °C for 30 min. To stop a reaction, 200 μl of 150 mM glycine•NaOH, pH 10, were added. The relative amount of released MU was measured as fluorescence using 365 nm excitation and 456 nm emission filters. All translation products were also radiolabeled using [³⁵S]methionine in TNT standard reactions and quantified upon SDS-PAGE. A specific factor was calculated for each E1 construct and used to normalize fluorescence values.

²⁾ Translation products were processed by recombinant SPP before MUC assay (Richter and Lamppa, 1998, *PNAS* 95, pp. 7463-7468).

³⁾ Fluorescence units, means of five measurements.

⁴⁾ Relative amount of MU released by E1 was taken as 100%.

⁵⁾ Relative amount of MU released at 65 °C was taken as 100 %.

⁶⁾ Master mix of TNT System.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Yi Li	
University:	University of Connecticut	
Agreement Number:	OR22072-97	
Project Title:	<i>Genetic Improvement of Seed Productivity for Biomass Crops</i>	
Reporting Period and Report Type:	From: 12/98 To: 12/99	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

1. Construction of the seed specific promoter/iaaM, ipt or GUS fusion genes.

We have constructed the IFR436 promoter/iaaM, IFR436 promoter/ipt and IFR436 promoter/GUS, oleosin promoter/iaaM, oleosin promoter/ipt fusion genes as proposed.

2. Production of IFR436 promoter/iaaM, IFR436 promoter/ipt and IFR436 promoter/GUS, oleosin promoter/iaaM, oleosin promoter/ipt transgenic canola plants.

We have produced a large number of transgenic plants with the IFR436 promoter/iaaM, IFR436 promoter/ipt and IFR436 promoter/GUS genes (see Table 1)

3. Analyses of yield and chemical compositions (i. e., contents of total starches, proteins, and lipids) of the resulting transgenic seeds.

To be done in Year 2000.

4. Analyses of patterns of transgenic seed development, patterns of fusion gene expression, and contents of auxin and cytokinin.

We have established analytical methods to measure auxin and cytokinin contents in small amounts of plant tissues such as in developing seeds. We will analyze the levels of auxin and cytokinin in developing seeds of transgenic plants in Year 2000.

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.

To improve seed productivity of bioenergy crops, we have constructed several useful genes and produced a large number of transgenic canola plants. Some interesting phenotypes have been observed in the resulting transgenic plants although they need to be confirmed at the second and third generations. For example, we have observed that the ipt transgenic plants produce more seed pods and the size of the seedpods is larger than the wild-type ones.

Scientific Accomplishments

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

Although setting up a new lab at UConn hampered our productivity of this project significantly, we made satisfactory progresses in 1999. Our progresses are summarized in the following paragraphs:

a) Production of A Large Number of Transgenic Canola plants

We have used the Moloney protocol (Moloney et al., 1989) for canola transformation. Briefly, we have been using cotyledonary petioles of *B. Napus* as explants. The cells of cut surfaces of petioles, which undergo organogenesis, are susceptible to topical infection by *Agrobacterium* (Moloney et al., 1989). After 72 hr infection with *Agrobacterium* the explants were transferred to a selective regeneration medium. Within 3-4 weeks, transgenic shoots emerged and then the shoots were rooted within 7-10 days after transferred into a rooting medium. For the initial several months in 1999, our canola transformation efficiencies were very low. More recently, we have made significant improvement on the transformation efficiencies. We have so far harvested seeds from 15 transgenic plants. Many of the transgenic plants are currently growing in greenhouses at various developmental stages. Confirmation of transgenic plants were done using Southern blot analysis for putative IFR436 promoter-ipt and IFR436 promoter-iaaM plants and using histochemical staining of GUS enzyme activity for the GUS plants. The number of independent transgenic plants for each gene construct produced is summarized in Table 1.

Table 1 Summary of Transgenic Plants Produced

Construct	Number of Independent Transgenics
IFR436 promoter/iaaM	46
IFR436 promoter/ipt	57
IFR436 promoter/GUS	25
*SAUR promoter/GUS	13

*SAUR promoter/GUS fusion gene has been used to establish the canola transformation protocol in our laboratory because high expression levels of SAUR promoter/ GUS fusion in callus, leaf and stem tissues makes the gene a convenient marker to monitor transformation efficiencies.

Because artifacts are often produced from tissue culture and subsequent regeneration processes, we have decided to examine the changes in growth and developmental patterns of the transgenic plants at the second and third generations. However, I would like to mention two

interesting but yet to be confirmed phenotypes observed in T0 transgenic plants. One is that 2 independent IFR436 promoter-iaaM plants produce seedless pods. The seedless phenotype is not very surprising because relatively high auxin levels in developing ovaries or seedpods can lead to formation of seedless fruits. On the other hand, we expect that some of the IFR436 promoter/iaaM plants with appropriate IAA levels in developing seeds will exhibit increased seed productivity (see the rationale described in the original proposal). The second one is that 3 independent IFR436 promoter-ipt plants produce more seedpods per plant and the seedpods are larger in size than the wildtype ones. The size of the IFR436 promoter/ipt seeds is indistinguishable from that of the wildtype seeds the stem diameters of the transgenic plants are also significantly larger than those of wildtype plants.

b) Construction of Seed Specific Promoter/iaaM or ipt Fusion Genes

The construction of the oleosin promoter/iaaM and oleosin promoter/ipt will be finished soon. The anticipated effects of these fusion genes are similar to the IFR436 promoter iaaM and IFR436 promoter-ipt genes because the oleosin gene promoter is also active at early stages of seed development (for more detailed descriptions, see the original proposal). We will soon transform canola plants with the newly constructed fusion genes.

c) Establishment of Analytic Methods for Auxin and Cytokinin in Small Amount of Tissues

In order to determine relationship of increased levels of plant hormones and number and size/weight of seeds, we have recently obtained USDA competitive grant to purchase a HPLC and a GC/MS (about \$150,000 for these two instruments). We have established analytical methods to analyze auxin and cytokinin contents in small amounts of tissues such as developing seeds using heavy isotope-labeled plant hormones as internal standards. The HPLC has been used for purification of plant hormones from plant materials and the GC/MS has been used for identification and quantification. We will analyze auxin and cytokinin levels in transgenic seeds at different developmental stages.

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.

Publications

No publication has been produced because the project started less than a year ago.

Presentations

Invited seminar: Molecular and genetic approaches to the effects of auxin and gravity on higher plants, University of Rhode Island, February 1999;

Invited seminar: Temporal and spatial control of plant hormone concentration in transgenic plants, Department of Animal Science, University of Connecticut, April 1999;

Invited Lecture: "Molecular action of auxin: from earth to space". In the section of "New Frontier in Plant and Animal Genetic Research" University of Connecticut College of Agriculture Excellence Committee Workshop, UConn, May 1999;

Invited Lecture: "Plant Biotechnology and Agriculture" 1999 Advisory Broad Meeting of The Department of Connecticut State Department of Agriculture, Hartford, June 1999;

Invited Symposium Lecture: Manipulation of Endogenous Plant Hormones and Its Applications in Agriculture and Horticulture, US-Sino Symposium on Biotechnology, Xian, China, July 1999;

Invited Lecture: Plant Gene Transfer and Crop Improvement. Northwest University of Agriculture, Chongqing, China, July 1999;

Invited Lecture: Manipulation of Plant Hormone Contents and Its Applications in Crop Improvement, Beijing Forestry University, September 1999;
Invited Lecture: Temporal and Spatial Control of Hormone Gene Expression and Its Applications in Agriculture, Horticulture and Forestry, Guanxi University, Naning, China, September 1999;
Invited Lecture: Plant Biotechnology and Agriculture, Guanxi Providential Academy of Agricultural Science, Naning, China, September 1999;
Invited Lecture: Plant Gene Transfer and Crop Improvement, Institute of Botany, Chinese National Academy of Sciences, Kunming, China, September 1999.

Technology Transfer

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

No disclosure or patent application has been filed because the project started less than a year ago

Inventions: None

Disclosures: None

Patents Applied for: None

Patents Granted: None

Licensing Agreements: None

Commercial Accomplishments

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

No commercial accomplishments can be reported at this time because the project started less than a year ago.

Educational Accomplishments

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

Two graduate students, Ms. Anurina Mitra and Mr. Jason Li, have been working on the project and been trained in the field of plant biotechnology (e.g., molecular cloning, plant transformation and plant tissue culture). A postdoctoral research associate, Dr. Yan Wu, has also spent portions of her efforts on the project and received training in the field of plant biotechnology.

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.

Ms. Anurina Mitra (a MS student), Mr. Jason Li (a Ph. D.student), and Dr. Yan Wu (a research associate) have been working on the project.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigators:	Lars G. Ljungdahl and Xin-Liang Li	
University:	University of Georgia	
Agreement Number:	OR22072-64	
Project Title:	Novel Hydrolytic Enzymes from Anaerobic Fungi Degrading Biomass	
Reporting Period and Report Type:	From: July 1, 1997 To: June 30, 1999	Check one: ☐ Interim Report ☒ Final Report

Project Objectives

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

Objective 1. Obtain a complete collection of genes (cDNAs) encoding cellulases, hemicellulases, and other enzymes that are required for efficient breakdown of cellulose and hemicelluloses by anaerobic fungi.

The most important aspect of the project has been to isolate as completely as possible the genes coding for the full range of enzymes participating in plant cell wall breakdown by the anaerobic fungus *Orpinomyces* PC-2. At the beginning, there were eight such cDNAs (six cellulases, one xylanase, and one beta-glucanase) cloned and sequenced. We have over the two years expanded the list substantially. As many as ten cellulases, one beta-glucanase, one beta-glucosidase, one xylanase, one mannanase, one acetyl xylan esterase, and three phenolic acid esterases have been isolated and sequenced (see Table 1 of Scientific Accomplishments).

To further isolate genes responsible for the important hydrolases produced by *Orpinomyces*, we have obtained more than two hundred different clones that produce such enzymes. Although we believe that these represent a complete spectrum of hydrolytic enzymes produced by the fungus, we have recently taken advantage of a novel procedure of isolating more sequences of *Orpinomyces* hydrolases. The procedure uses the two-repeated peptide regions in the protein-binding domain of enzymes of the cellulase/hemicellulase complex. They were used to design degenerate nucleotide primers. A large number of unknown sequences have been amplified by PCR using these primers and cDNAs of *Orpinomyces* as templates. Sequencing of the amplified products revealed that at least 10 additional enzymes not previously isolated are present in the cellulase/hemicellulase complex of *Orpinomyces*. Using the partial sequences as

screening probes, we have now obtained four more cDNAs coding for three more cellulases and one mannanase. Sequencing of the cDNAs are in progress, as of other cDNAs for which we have only partial sequences.

Objective 2. Develop gene expression systems for producing large quantities of cellulases, xylanases, beta-glucanases (lichenase), and phenolic esterase of anaerobic fungi.

We have focused on developing protein production systems using *E. coli* and *Aureobasidium pullulans* as hosts. Several *Orpinomyces* cellulases, xylanase A, and *C. thermocellum* FaeZ have been successfully overexpressed in *E. coli*. Development of large-scale production processes for supplying these products is in progress.

The construction of a expression plasmid and development of transformation procedures for the yeast-like fungus *A. pullulans* have been accomplished. Several heterologous proteins have been expressed using this system. Preparation of an international patent is under way. Please refer to Scientific Accomplishments for details.

More recently, we have succeeded the transformation of *Trichoderma reesei*, the commonly used cellulase production fungus. The fungus produces high levels of cellulase and addition of highly active cellulase and hemicellulase genes should make the fungus more potent for the conversion of lignocellulose to fermentable sugars, a critical step for the production of energy from plant biomass.

Objective 3. Determine physiochemical properties of the recombinant hydrolases derived from anaerobic fungi.

A number of enzymes derived from both anaerobic fungi and the thermophilic anaerobic bacterium *C. thermocellum* have been purified after they are cloned in and expressed in heterologous hosts. These enzymes include cellulase E and F, beta-glucosidase A (BglA), acetylxyylan esterase A, two feruloyl esterases, and a mannanase. The work has resulted in several articles already published (see publication list) and in various stages of preparation.

Objective 4. Test products containing single enzymes and/or enzymes in combination in industrial applications.

We have engaged in collaboration of testing of the enzyme products in feed, food, pulp, textile, and energy industries. For a list of tests and the performances of our enzymes, please refer to the Scientific Accomplishments.

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 obstacle of economical conversion of lignocellulosic biomass to energy has been identified as the efficient conversion of the biomass to fermentable sugars. To overcome this obstacle, we have launched this project aiming to search for the efficient biocatalysts (enzymes) and to produce them in a cost-effective way for the conversion process. Many different ecosystems have been examined, which resulted in the identification of group of microbes, the anaerobic fungi in the rumen and caecum of herbivores. The fungi produce highly active

enzymes that efficiently mineralize plant cell wall biomass. This project has achieved the isolation of the genes responsible for the major enzymes for the degradation. In addition, the project has opened the possibilities for the production of such enzymes and for the implementation of these enzymes into bioprocesses for the economical conversion of biomass into fuels and other chemicals.

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 unique environment with excellent sources of plant hydrolytic microbes is the rumen of bovines and the intestine of other herbivorous animals. In this environment the breakdown of plant material occurs within hours after ingestion. It has long been known that bacteria and flagellates are agents for the degradation of plant tissue. However, rather recently, in 1975, Orpin described the first anaerobic fungus *Neocallimastix frontalis*. Seventeen species of anaerobic fungi have been isolated and described (Orpin, 1975, 1994). In our laboratory we have isolated 5 different anaerobic fungi from the rumen of a cow (Borneman et al. 1989). Investigations of the microbiology and biochemistry of plant tissue degradation in rumen have revealed that the anaerobic fungi are responsible for the initial attacks and that their population increases rapidly and proportionally to the amount of recalcitrant fibers in the diet. Two of our isolates, *Neocallimastix* MC-2, and *Orpinomyces* PC-2, have been subjects of intensive investigations by us. Lately, we have concentrated our work on the *Orpinomyces* species and Table 1 shows the hydrolytic enzymes we have until now cloned, sequenced, and characterized from this fungus. In addition to these enzymes we have evidence that *Orpinomyces* PC-2 produces at least six additional plant-degrading enzymes. Part of our proposed work involves the isolation and characterization of these enzymes. We are now confident that anaerobic fungi produce a full spectrum of cellulases, and hemicellulases. They, in addition, seem to break bonds between lignin and hemicellulase; a process which opens up plant tissue for attack by the cellulolytic/hemicellulolytic enzymes.

The enzymes from *Orpinomyces* PC-2 have in several cases, been found superior to equivalent enzymes from other sources e.g., having much higher specific activities. For example, XynA of *Orpinomyces* PC-2 has a specific activity of 3,500 U/mg of protein against birchwood xylan, a commonly used xylanase substrate, whereas specific activities of xylanases from other sources range from 20 to 600 U/mg (Table 2). The same is true for the β -(1,3),(1,4)-glucanase of *Orpinomyces* (Chen, et al. 1997, Table 3) and a cellulase of *Neocallimastix patriciarum* (Denman et al., 1997).

Table 1. Hydrolytic enzymes cloned and sequenced from *Orpinomyces*

Enzyme ^a	Size AA	Binding ^b sequence	Optimum pH range	Most active temp. range (°C)	Reference
CelA	459	NCRPD	4.3-6.8	30-50	Li et al., 1997b
CelB	471	NCRPD	4.8-7.6	30-50	Li et al., 1997a
CelC	449	NCRPD	4.6-7.0	30-45	Li et al., 1997b
CelD	455	NCRPD	4.5-7.0	30-50	Unpublished
CelE	477	NCRPD	4.5-7.5	30-50	Chen et al., 1998
CelF	432	CBD	4.5-6.5	30-50	Unpublished
CelG	Incomplete	None	4.5-7.0	30-50	Unpublished
CelH	491	NCRPD	ND	ND	Unpublished
CelI	Incomplete	NCRPD	ND	ND	Unpublished
CelJ	Incomplete	NCRPD	ND	ND	Unpublished
BglA	663	None	5.5-8.0	45-55	Unpublished
XynA	362	NCRPD	5.5-7.5	45.50	Li et al., 1997a
LicA	245	None	4.5-8.0	35.55	Chen et al., 1997
ManA	574	CBD/NCRPD	ND	ND	Unpublished
AxeA	313	None	7.0-8.5	30-45	Blum et al., 1999
FaeA	330	None	5.5-8.0	30-50	Unpublished
FaeY ^c	285	NCRPD	5.5-7.5	55-65	Unpublished
FaeZ ^c	287	NCRPD	ND	ND	Unpublished

a) Cel stands for cellulase; Bgl, β -glucosidase; Xyn, xylanase; Lic, β -glucanase (lichenase); Man, mannase; Axe, acetyl xylan esterase, and Fae, feruloyl esterase.

b) NCRPD stands for noncatalytic repeated peptide domains which has been suggested to function equivalent to the dockerin domains of the cellulosomes of *C. thermocellum*; CBD means cellulose binding domain.

c) FaeY and FaeZ are feruloyl esterases from *Clostridium thermocellum*.

Table 2. Specific activities of xylanases from various sources

Organism	Specific activity $\mu\text{mol} / \text{min} / \text{mg}$	Reference
<i>Orpinomyces</i> sp. strain PC-2	3,500	This proposal
<i>Clostridium acetobutylicum</i>	22.4	Lee et al., 1987
<i>Trichoderma longibrachiatum</i>	130	Roger and Nakas, 1989
<i>Trichoderma viride</i>	152	Ujiie and Yaguchi, 1991
<i>Aspergillus sydowii</i>	204	Ghosh and Nanda, 1994
<i>Aspergillus fischeri</i>	588	Raj and Chandra, 1996

Table 3. Specific activities of beta-(1,3)(1,4)-glucanases from various sources

Organism	Specific activity $\mu\text{mol} / \text{min} / \text{mg}$	Reference
<i>Orpinomyces</i> sp. PC-2	6,170	Chen et al., 1997
<i>Fibrobacter succinogenes</i>	1,372	Erfle et al., 1988
<i>Bacillus subtilis</i>	2,600	Wolf et al., 1995
<i>Streptococcus bovis</i>	338	Ekinici et al., 1997

It was mentioned above that *T. reesei* produces separate enzymes, whereas anaerobic bacteria, such as *C. thermocellum*, produce large complexes called cellulosomes. We have recently found that anaerobic fungi produce free separate enzymes as well as cellulosomal type complexes. These complexes have been isolated and found to be of 2 sizes (Fig. 1). The smaller has a molecular mass of between 2 to 3 million Da. The bigger has a mass of about 100 million Da and is a polymeric form of the smaller complex. This is similar to previous findings regarding the cellulosome from *C. thermocellum*. This was ascertained using SDS-PAGE analysis, which showed that the two differently sized complexes had almost identical banding pattern (Fig. 2). The number of protein bands on the gel suggests that at least twenty protein components (polypeptides) are present in the complexes of *Orpinomyces* (Fig. 2). We can only account for 10 of the components therefore more than half of the components of the *Orpinomyces* complex has yet to be cloned and characterized. Referring to Table 1, it is to be noted that many of the enzymes contain NCRPDs (dockerins), others have CBDs, or do not have any of these (Fig. 3). The NCRPDs, or dockerins, are found in enzymatically active subunits of the cellulosomal complex and they function to bind the enzymes to scaffolding subunits of the complex. We have preliminary evidence for perhaps four scaffolding polypeptides in the *Orpinomyces*' complex. The enzymes of Table 1 lacking an NCRPD, are free enzymes.

The genes encoding the enzymes listed in Table 1 have been isolated from an *Orpinomyces* PC-2 cDNA library (Chen et al., 1995). The library was constructed using mRNA isolated from the fungus grown on Avicel and xylan and therefore should contain all the potential cell wall hydrolytic genes. To achieve the goal of isolating genes coding for a full spectrum of hydrolytic enzymes of the fungus, several cDNA library screening strategies were employed. Initially, screening for enzyme-producing plaques using various natural and synthetic substrates allowed us to isolate cellulase A (CelA), CelB, CelC, CelE, CelF, xylanase A (XynA), acetyl xylan esterase A (AxeA), and lichenase A (LicA). There is no assurance of isolating all functional genes by activity screening because some genes are either not expressed or active enzymes are not formed, due to differences between fungi and *E. coli* in terms of translation, protein folding, glycosylation, and/or proteolytic degradation. Also, as was the case for feruloyl esterase, there was no substrate available for activity screening. As a second approach, we isolated the enzyme of interest to obtain its amino terminal and partial internal amino acid sequences. Using the protein sequences, we were able to design probes to isolate cDNAs by reverse genetic procedures. Genes of FaeA and BglA were isolated by these procedures. A third approach was to take advantage of the NCRPD sequences, which contain two highly conserved sequences of about 40 amino acids each. Two degenerate oligonucleotides were designed, synthesized, and used to amplify all possible flanking regions of NCRPD specific cDNAs. Using the cDNA library as template and the oligonucleotides as primers, a pool of PCR products were amplified and cloned into a PCR cloning vector. Sequencing of 50 insert-containing

plasmids revealed 19 distinct clones (Fig. 4), some of which matched the NCRPD regions of the cellulase and xylanase genes already isolated by activity screening whereas eleven were new sequences. These eleven sequences represented partial sequences of genes encoding eleven components of the *Orpinomyces* cellulosomal complexes and therefore they are excellent DNA hybridization probes for isolation of the corresponding full-length genes by library screening. By using this procedure, we have isolated three new cellulases, CelH, CelI, and CelJ and mannase gene, *manA* (Table 1). A goal of the work proposed is to find the 7 additional putative enzymes with NCRPDs.

A major goal for the currently funded project was to isolate feruloyl esterase genes from anaerobic fungi. We have previously found feruloyl esterases in these fungi and demonstrated that they enhance the hydrolysis of hemicellulase by xylanase (Borneman et al., 1991, 1992, and 1993). As shown in Table 1 we have successfully isolated the gene *faeA* coding for the feruloyl esterase FaeA from *Orpinomyces* cDNA library. Significantly, the sequence data when analyzed were found to have homology with unknown domains in XynY (Fontes et al., 1995) and XynZ (Grepinet et al., 1988) of the *C. thermocellum* cellulosome. We have now confirmed that these domains have feruloyl esterases activity. This discovery allowed us to expand our work to include two highly thermostable feruloyl esterases from *C. thermocellum* (see below).

1. Develop heterologous gene expression systems for producing large quantities of recombinant enzymes.

Enzymes produced by the anaerobic fungi have high specific activities and unique properties. However, the fungi do not produce them in amounts high enough for industrial applications. In addition, the technology to grow anaerobic fungi in fermenters is lacking. Thus a major goal of this proposal was to produce anaerobic fungal enzymes economically. This can be done by developing heterologous production systems using recombinant biotechnology.

E. coli is commonly used in both laboratory and industrial scale processes for recombinant protein production. The technology to use this bacterium is well developed. Several genes of anaerobic fungi are expressed in *E. coli* and active enzymes have been obtained (Gilbert et al., 1992; Fanutti et al., 1996; Chen et al., 1997; Li et al., 1997a,b). One example of very high level of expression is that of *Orpinomyces* XynA (Li et al., 1997a) with the NCRPD deleted using plasmid pET 21b and the *E. coli* host strain BL21. The recombinant protein intracellularly located is fully soluble and active. We have achieved a production of the xylanase in 5-L fermenters using defined media as high as 17,000 U/ml (Fig. 5). This level is probably the highest xylanase production ever achieved. The result shows that genes from the fungi can be expressed in *E. coli* as active enzymes and in exceptionally high yields. Down-stream processes for xylanase recovery from *E. coli* cells, partial purification, and concentration have been recently established in the laboratory. The xylanase is intensively studied for use in pulp bleaching, feed, and bakery applications (see below).

The FaeZ located as a functionally unknown domain of *C. thermocellum* XynZ has a cellulose binding domain (CBD) next to its carboxy side (Fig. 3). Two forms of the enzyme with and without the CBD have been produced as intracellular proteins in *E. coli*. Both forms are fully soluble and catalytically active on FAXX and FAX3, two native xylan fragment esters with ferulic acid isolated from wheat bran (Borneman et al., 1991). The enzymes also cleave ferulic acid from intact wheat bran, sugar beet pulp, and mechanic soft wood pulp. This activity is greatly enhanced in the presence of a xylanase (Fig. 6), suggesting a strong synergistic effect between xylanase and feruloyl esterase on hemicellulose hydrolysis.

The expression of LicA of *Orpinomyces* in *E. coli* has not been as straightforward as that of *Orpinomyces* XynA or the *C. thermocellum* FaeZ. This is due to a high (95%) inclusion body formation. Even though LicA has extremely high specific activity (5,000 U/mg, Chen et al., 1997), only 200 U/ml in shake flask cultures and 800 U/ml in fermenters were achieved. These levels are too low for commercial production. Alternative expression systems using *Aureobasidium pullulans*, *Trichoderma reesei*, and *Bacillus* species are therefore presently explored. *A. pullulans*, a yeast-like fungus, grows rapidly on cheap defined media. Most importantly, one extracellular protein, XynA, constitutes over 90% of proteins secreted when the fungus grows on xylose, xylan, or wheat bran as carbon source (Li and Ljungdahl, 1994, 1996, 1997). More recently, we have cloned and sequenced 1.5 and 0.8 kb genomic sequences upstream and downstream, respectively, of the *xynA* gene. Using these functional elements, we have constructed an *A. pullulans* expression vector (Fig. 7) containing regions facilitating single and double crossovers, a *xynA* promoter and terminator for inducible expression, a XynA signal peptide region facilitating excretion of proteins of interest into cultural medium, and a hygromycin B gene as a selection marker. Several genes including those coding for the *Orpinomyces* LicA, an *E. coli* β -glucuronidase, and a pig leptin have been tested in this system. Heterologous proteins were detected in all cases. The levels of the heterologously expressed proteins, however were not high.

T. reesei has been extensively used for production of many industrial enzymes, particularly cellulases for various industrial applications. Advantages of using this fungus as a protein production host include existence of highly inducible promoters such as that of cellobiohydrolase I, well established large-scale fermentation conditions, and safe protein products for food and feed consumption. We have recently engineered a vector that may allow the expression of the genes of our interest and secretion of the heterologous proteins (Fig. 8). We have recently succeeded the transformation of *T. reesei* using integration expression. We are currently testing the production levels of two enzymes, LicA of *Orpinomyces* and FaeZ of *C. thermocellum* using the system.

2. Characterization of plant cell wall-degrading enzymes.

Knowledge of physiochemical properties of enzymes is critical in determining their suitability for industrial applications. These properties include amino acid sequence, size, specific activity, domain structure and organization, temperature and pH profile and stability, glycosylation, sensitivity to metal ions, substrate and product specificity, substrate binding, and product inhibition. For example, pulp bleaching processes prefer high temperature and high pH conditions. Other applications have other temperature and pH requirements. Thus, enzymes with different properties are required for different applications.

a. **Cellulases.** Ten cellulases and one β -glucosidase have been isolated from *Orpinomyces*. Some of these contain CBDs while others contain NCRPDs, but surprisingly the catalytic domains of the ten cellulases belong only to hydrolases of family 5 and 6 (Chen et al., 1998, Xue and Denman, 1999). CelA, CelC, CelG, CelH, and CelI are family 6 enzymes whereas CelB, CelE, CelJ are family 5 hydrolases. The family 6 enzymes share between 50-85% homologies, suggesting that they evolved through gene duplications and shufflings (Ljungdahl et al., 1998). They behave as both endoglucanases and cellobiohydrolases (Li et al., 1997) but yield different profiles of products, reflecting their highly specialized specificity in attacking cellulose. The family 5 enzymes are typical endoglucanases, only previously found in anaerobic bacteria.

The intact form of CelF expressed in *E. coli* has been purified and characterized (Chen et al., 1999). The mature CelF consists of a CBD and a catalytic domain joined by a linker (Table 1, Fig. 3). The purified enzyme has both cellobiohydrolase and endoglucanase activities and cellobiose and cellotriose are major products of the hydrolysis of CMC. The enzyme has highest activity between pH 5.8-6.2 and at 50°C. CelF is a typical fungal enzyme.

The β -glucosidase (BglA, Table 1) consists of 657 amino acid residues with a calculated mass of 75 kDA (Ximenes et al., 1999). No significant homology was found between BglA and β -glucosidases of either aerobic fungi or bacteria. Neither CBD nor NCRPD is present in BglA, suggesting that the enzyme is free. It converts cellooligosaccharides and cellobiose to glucose. The enzyme is expressed in *S. cerevisiae* and substantial percentage of the active enzyme is secreted into the culture medium. Three forms of the enzyme were formed having differing glycosylation. K_m and V_{max} against cellobiose as substrate are 0.31 mM and 6.45 μ mol/min/mg at 37°C and pH 6.0. K_i of glucose inhibition is 3.6 mM. These kinetic parameters are more favorable for converting cello-oligosaccharides to glucose, than those of enzymes from other fungi, such as *Trichoderma* and *Aspergillus* species. This has been demonstrated by comparing levels of glucose production by *Trichoderma* crude cellulases in the presence of either *Orpinomyces* or *Aspergillus* β -glucosidases (Chen et al., 1999). These data strongly indicate that *Orpinomyces* BglA might be used efficiently for biomass conversion.

b. **Hemicellulases.** As shown in Table 1, we have cloned and sequenced enzymes that hydrolyze various types of hemicelluloses. Besides a β -glucanase (Chen et al., 1997) and a xylanase (Li et al., 1997a) previously reported we have achieved the cloning, sequencing and characterization of one acetyl xylan esterase, three feruloyl esterases, and a novel mannanase (Table 1). In contrast to the mannanase of *N. patricairum* (Fanutti et al. 1996) which is homologous to bacterial enzymes the catalytic domain of the *Orpinomyces* ManA is homologous to aerobic fungal mannanase. It contains both a CBD and a NCRPD (Fig. 3).

AxeA cDNA was isolated by screening the library using β -naphthyl acetate as an indicative substrate (Blum et al., 1999). The cDNA consists of 1067 bp with an open reading frame of 939 nucleotides coding for a polypeptide of 313 amino acid residues. The recombinant enzyme was able to remove acetate from native xylan alone but the ability was greatly enhanced in the presence of the XynA cloned from the same fungus. At pH 6.0 and 40°C, K_m and V_{max} are 0.90 mM and 785 μ mol/min/mg, respectively, using *p*-nitrophenyl acetate as substrate. *AxeA* has a 56% amino acid identity with the catalytic domain of BnaA, an acetyl xylan esterase of *N. patriciarum* (Dalrymple et al., 1997), but the *Orpinomyces* *AxeA* is devoid of a NCRPD found at the carboxy terminus of the *Neocallimastix* BnaA. This suggests that BnaA is a component of a complex while the *AxeA* is a free enzyme.

We are especially proud with the success of the cloning of a feruloyl esterase cDNA from *Orpinomyces* and the discovery that the two unknown domains of XynY and XynZ, components of the *C. thermocellum* cellulosome, are in fact feruloyl esterases. FaeZ/CBD, the polypeptide expressed in *E. coli* containing the feruloyl esterase and the CBD domains of XynZ (Fig. 3) has a K_m of 5 mM and a V_{max} of 12.5 μ mol/min/mg against FAXX at pH 6.0 and 60°C.

3. Tests of enzymes in industrial applications.

The enzymes under study have potential applications in a number of industries, including feed and food, pulp and paper, textile and energy. Strategies are being developed to test these enzymes in as many industries as possible.

Xylanases are currently used in industrial scales for pulp bleaching to cut down the consumption of chlorinated chemicals and subsequent toxic effluent from paper mills. Current products may be contaminated with cellulases causing yield loss and they have low specific activity and acidic pH profile. *Orpinomyces* XynA recombinantly produced in *E. coli* has a specific activity of 3,500 U/mg and no cellulase activity. It is active at pH 8.0 (Saha et al. 1999). Both laboratory and pilot-scale tests revealed that a dosage of 1 gram of XynA per ton of pulp give satisfactory results when pH and temperature were controlled at 8.0 and 50°C, respectively.

XynA has also been tested in fish and chicken diets in China and in the Departments of Poultry and Animal Sciences at the University of Georgia. In both cases, it significantly improved feed conversion rates when wheat or wheat by-products were used as cereal ingredients. The improvement was equal or more than what the competitive enzyme products developed by other enzyme companies such as Novo Nordic of Denmark and BASF of Germany.

Ferulic acid is a potent antioxidant and preservative for food products and at 0.05% it can protect lipids in food products from oxidation. Agricultural by-products such as wheat bran, rice bran, and sugar beet pulp, are rich in ferulic acid esterified to hemicelluloses. We have developed an enzymatic method using both xylanase and feruloyl esterase to make free ferulic acid from these materials. Up to 5-gram ferulic acid per kg of wheat bran is released. The technology may be used for the manufacturing of natural ferulic acid from plant materials, and one of our goals is to make the process economical.

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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.

Presentations:

- 1997 Jan Japan Science and Technology Corporation. The 5th JST International Symposium, New Frontiers in Microbiology. Presentations: The tungsten-selenium-iron formate dehydrogenase and other enzymes of the acetyl-CoA pathway in acetogenic clostridia.
- 1997 Feb Tokyo University of Pharmacy and Life Sciences. Invited seminar: Cellulolytic bacteria and fungi.
- 1997 Feb Tokyo Electric Power Company. Invited seminar: Cellulolytic and other hydrolases of *Clostridium thermocellum* and anaerobic fungi.
- 1997 Sep Fourth International Conference on Carbon Dioxide Utilization, Kyoto, Japan. Scientific Board Member, Chairman of Biological CO₂ Fixation Session. Delivered two presentations.
- 1997 Sep The Consortium for Plant Biotechnology Research, Inc. Conference of Electric Power from Biomass Industrial Research Needs Meeting.
- 1997 Nov The Consortium for Plant Biotechnology Research, Inc. 1997 symposium, Washington, D.C. Invited Presentation: Novel hydrolytic enzymes from anaerobic fungi degrading biomass.
- 1997 Dec Atlanta/Athens Chapter of the von Humboldt Association of America: Invited Dinner Talk: GRA and Georgia Biotechnology Center.
- 1998 May Annual Meeting Am. Soc. Microbiology, Atlanta, GA. Presented symposium lecture: The Cellulase/Hemicellulase Complex of Anaerobic Fungi.
- 1998 Jun Gordon Conference: The Molecular Basis of Microbial One-Carbon metabolism: Discussion Leader A Molecular Physiology and Biochemistry: Pathway and Enzymes.
- 1998 Aug Natl. Renewable Energy Laboratory. Golden, CO Presented invited lecture. The cellulolytic system of the anaerobic fungus *Orpinomyces* PC-2.
- 1998 Sept MIE Bioforum Genetics, Biochemistry and Ecology of Cellulose Degradation. Suzuka City, Japan. Invited Plenary Lecture. "The cellulase/hemicellulase enzyme system of the anaerobic fungus *Orpinomyces* and aspects of further cellulase research."

- 1998 Sept National Taiwan Ocean University Inst. Marine biology. Invited Lecture. Acetogenesis and anaerobic CO₂ fixation.
- 1998 Sept Academic Sinica, Botany Department. Invited Lecture. The cellulosome of *Clostridium thermocellum* and the cellulase/hemicellulase complex of the anaerobic fungus *Orpinomyces* PC-2.
- 1999 Mar Marshall University, Department of Biochemistry and Molecular Biology. Invited Lecture. "The Acetyl-CoA Pathway in Anaerobic Acetogenic Bacteria."
- 1999 Jun Invited seminar at RTG conference in Prokaryotic Diversity: The cellulosomal systems of *Clostridium thermocellum* and anaerobic fungi.
- 1999 Jul Gordon Conference: Molybdenum and Tungsten Enzymes. Discussion Leader of New Mo and W Enzymes.
- 1999 Jul Gordon Conference: Cellulases and cellulosomes. Discussion Leader of "Microbial Cellulase systems - Free cellulases and/on Cellulosomes": and invited presentation. The cellulosome of the polycentric, anaerobic fungus *Orpinomyces*.

Publications:

- X.-L. Li, H. Chen, and L.G. Ljungdahl (1997).¹ Monocentric and polycentric anaerobic fungi produce structurally related cellulases and xylanases. *Appl. Env. Microbiol.* 63:628-635
- Chen, H., X.-L. Li and L. G. Ljungdahl (1997). Sequencing of a 1,3-1,4- β -D-glucanase (lichenase) from the anaerobic fungus *Orpinomyces* strain PC-2: properties of the enzyme expressed in *Escherichia coli* and evidence that the gene has a bacterial origin. *J. Bacteriol.* 179:6028-6034.
- Li, X.-L., C. Huizhong, and L. G. Ljungdahl (1997). Two cellulases, CelA and CelC, from the polycentric anaerobic fungus *Orpinomyces* strain PC-2 contain N-terminal docking domains for a cellulase-hemicellulase complex. *Appl. Environ. Microbiol.* 63:4721-4728.
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- Kataeva, I., Li, X.-L., Chen, H., Choi, S.-K., and Ljungdahl, L. G. (1999) Cloning and sequence analysis of a new cellulase gene encoding CelK, a major cellulosome component of *Clostridium thermocellum*: Evidence for gene duplication and recombination. *J. Bacteriol.* 181:5288-5295.

- Blum, D. L., Li, X.-L., Chen, H., and Ljungdahl, L. G. (1999). Characterization of an acetyl xylan esterase from the anaerobic fungus *Orpinomyces* sp. strain PC-2. *Applied Environ. Microbiol.* 65. No. 9 *Appl. Environ. Microbiol.* 65 (9): 3990-3995.
- Blum, D.L., I.A. Kataeva, X.-L. Li, and L.G. Ljungdahl. (2000) Feruloyl esterase activity of the *Clostridium thermocellum* cellulosome can be attributed to previously unknown domains of XynY and XynZ. *J. Bacteriol.* 182:XXX-XXX.

Technology Transfer

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

A total of eight patent applications were filed over last 5 years and three of them have been issued. The others are in at various stages of reviewing process by PCT Office and US Patent and Trade Office.

A licensing agreement was signed in 1999 between the University of Georgia Research Foundation, Inc.(UGAF) and Aureozyme, Inc. UGAF will receive up front and annual licensing fees and royalty payments from Aureozyme.

Patent applications filed during the funding period:

- Li, X.-L., H. Chen, E. A. Ximenes, and L.G. Ljungdahl, 1998. Beta-glucosidase and coding sequences and protein from *Orpinomyces*. Patent applied for May 5, 1998.
- Li, X.-L., H. Chen, and L.G. Ljungdahl, 1998. *Orpinomyces* cellulase CelF protein and coding sequences. Patent applied for May 10, 1998.
- Li, X.-L., Ljungdahl, L.G. and Chen, H. 1998. *Orpinomyces* CelE and coding sequences. Patent applied for May 30, 1998.
- Blum, D., Kataeva, I., Li, X.-L., and Ljungdahl, L.G., 1998. Phenolic acid esterases coding sequences and methods. Patent applied for Sept. 7, 1

Commercial Accomplishments

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

Partially due to the funding and funding from Georgia Research Alliance, Aureozyme was established in 1997. Since then, the company has attracted two rounds of angel investment for the total of \$1.20 million from two individual investors. Several enzyme products are in pipe-lines of development. These include two xylanases, five cellulases, one beta-glucanase, and two feruloyl esterases. The first enzyme sold at large scales was a feruloyl esterase. We have also developed a novel protein expression system using a fast-growing aerobic fungus. A PCT patent application is in preparation.

Educational Accomplishments

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

We have been actively involved in the education at University of Georgia both at the graduate and undergraduate levels. Two Ph.D. candidates, David Blum and Eduarado Ximines and three undergraduate students were supported by the joint funding from CPBR, Aureozyme, and Georgia Research Alliance. Postdoctoral Associates were also trained by working on the funded project.

Additional Funding

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

Because of progress made during the funding period, Aureozyme has secured another round of finance from the same investors totaling \$600,000. Also because of the progress, we, in collaboration with Dr. Mike Azain in the Department of Animal Science and Dr. Nick Dale have been awarded another Georgia Research Alliance grant to test our enzymes on feed application in chicken industry, one of the largest industries in Georgia. We have also submitted a new CPBR/Aureozyme proposal in 1999. The proposal has been ranked in the top five projects recommended by both scientific and corporate panels for funding.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

There was no change or hiring of key personnel in the laboratory during the period but Aureozyme had lured Mr. Kenneth Janoski to fill the President and Chief Executive Officer position. This has been the most critical addition to the company. Mr. Janoski has many years of experience in managing chemical and technology consulting businesses.

Fig. 1. Gel filtration chromatography of purified cellulase/hemicellulase complexes from *Orpinomyces* PC-2

Fig.. 2. SDS-PAGE analysis of the peaks containing cellulase/hemicellulase complexes after gel filtration (see Fig. 4). Proteins of peak 1 (lane 1, 30 μ g and lane 2, 150 μ g) and peak 2 (lane 3, 150 μ g) were dissociated and separated by SDS-PAGE. Protein bands in the gel were visualized by Coomassie blue staining.

Fig. 3. Domain organizations of various hydrolases of *Orpinomyces* and *C. thermocellum*.

Cloned and sequenced by activity screening

CelA	GKWGVLNNDWCMIDNRRCSSNNNNCSSSITSQGYPCCSNNNCKVEYTDNDGKWGVEN
CelB	GQWGVENGWCGIKSSCGNNQRQCWSERLGYPCCYTTNAEYTDNDGRWGVEN
CelC	GNWGVENFDWCFIDESRCNPGYCKFEALGYSCCKGCEVVYSDGDNWGVEN
CelD	GQWGIENNDWCLIQPSKCNNSQCKFNALGYSCCSNCNSVYSDNDGQWGIEN
CelE	GKWGVENGWCGIKSSCDNNQRYCWSERLGYPCCYTTNVEYTDNDGRWGVEN
XynA	GTWGVENNQWCGCGVEACSGKITAQGYKCCSDPKCVVYYTDDDGNWGVEN

Cloned and sequenced by NCRPD-specific amplifications

RP2	GKWGVENGWCGIGGTIVDNDSCFAKSLGYSCSSCDVVYTDNDGNWGVEN
RP3/RP4	GKWGVENGWCGIPDPEPTCWAERLGYPCCTTTTTVEYTDSDGNWGVEN
RP7/RP14	GKWGVGNWCGIPVPEACWSEKLGYPCCSSSSAKVEYTDADGNWGVEN
RP12	GKWGVENGWCLLKSKCAEEETWTGMNDGYAKCSGCDVSYSDDSGNWGVEN
RP15/27	GKWGVENGWCGCGKAPSPKPKKCSARIVAQGYKCCSSCLPVMYEDSDGNWGVEN
RP19	GKWGVENGWCGIEDCWSTKLGYPCKVTTDIVYTDGDGNWGVEN
RP18/23/24	GKWGVGNWCGIPDACWAEKLGYPCTTTTKVEYTDADGNWGVEN
RP29	GKWGVENNEWCGCGNGGKVCVGAQGYPCQSTKQVEYVDDGDNWGVEN
RP31	GKWGVENNQWCGCGVEACPAKITAQGYKCCSDPKCVVYYTDDDGNWGVEN
RP37	GKWGVENGWCGIEDSSSSATCWSEALGYPCSSSSAKVEYTDADGNWGVEN

Fig. 4. List of sequences isolated from an *Orpinomyces* cDNA library using activity screening and NCRPD-specific PCR amplification.

Fed batch culture of *E.coli* BL21(DE3) with PET/xynA

Fig. 5. Production of the *Orpinomyces* xylanase A using *E. coli* as a host in 5-L fermentors.

Fig. 6. Release of ferulic acid from unprocessed wheat bran by 0.2 U feruloyl esterase Z (FaeZ) of *C. thermocellum* in the presence of various amounts of the *Orpinomyces* XynA

Fig. 7. Map of the Vector (pXL21) used for transformation of *A. pullulans*. Symbols: Hyg, hygromycin B resistance gene; Am, ampicillin resistance gene; XynA, xylanase A gene; Tcyc, terminator sequence; XynA SP, XynA signal peptide; XynA P, XynA gene promoter.

Fig. 8. Illustration of the gene expression cassette for production of heterologous proteins by *Trichoderma reesei*

Scientific Progress Report

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

Principal Investigator:	Frank J. Louws Ph.D.	
University:	North Carolina State University	
Agreement Number:	OR22072-41	
Project Title:	<i>Automated Fluorescent Genomic Fingerprinting (auto-rep-PCR) of Bacteria</i>	
Reporting Period and Report Type:	From: 1/15/96 To: 7/14/99	Check one: ☐ Interim Report ☒ Final Report

Project Objectives

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

To analyze the genetic diversity of xanthomonad pathogens of economic importance in North Carolina and develop a region-specific database for species, subspecies and/or strain identification and classification.

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.

Bacterial pathogens can be regarded as criminals that attack economically important crop plants to steal farmer profits and reduce food, feed and fiber supplies. We employed protocols known as rep-PCR genomic fingerprinting to generate DNA fingerprints of these pathogens. The DNA fingerprints generated are analogous to UPC codes used in (e.g.) grocery stores and are therefore highly amendable to computer-assisted data storage and analysis. We characterized the DNA fingerprint profiles of economically important pathogens that attack stone fruit crops (including peaches), strawberries, cabbages and other crucifers, and tomatoes. The genetic fingerprints were found to be diagnostic for each pathogen population. Development of a database now allows for rapid identification of these pathogenic bacteria in future cases of disease problems.

Understanding the genetic diversity of the "enemy" and the population dynamics of these

pathogens will provide enhanced capabilities to implement integrated disease management strategies to limit crop losses.

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 NCSU program collected numerous bacterial pathogens associated with disease problems of strawberries (*Xanthomonas fragariae*), stone fruit crops (*X. arboricola* pv. *pruni*), cabbage (and other cole crops; *X. c.* pv. *campestris*) and tomatoes (*Pseudomonas syringae* pv. *tomato*). The strains were characterized using a genomic fingerprinting protocol known as rep-PCR.

CHARACTERIZATION OF STRAWBERRY ANGULAR LEAF SPOT PATHOGENS

Xanthomonas fragariae strains that cause angular leaf spot of strawberry do not persist in North Carolina from one year to the next but are introduced on plants imported from nurseries in Canada or other areas. The bacterial pathogen systemically infects plants and may not cause symptoms until NC temperature conditions in early spring are conducive to disease onset. Growers often implement a spray program to limit disease loss but NC research has demonstrated intensive applications of pesticides (specifically copper sprays) are not effective. Therefore, emphasis must focus on limiting plant infection during the plant production phases. Diagnostic and detection assays need to be developed and implemented. Knowledge of the genetic diversity of this population would provide a basis for developing diagnostic methods, designing detection protocols, and initiating plant breeding programs to develop host resistance.

Thirty-four isolates of *X. fragariae* were analyzed by rep-PCR. The rep-PCR genomic fingerprints highlight that this population, in North Carolina, appears to be highly clonal. The North Carolina population could be partitioned into two rep-PCR genomic groups based on polymorphisms of a few amplified DNA fragments. The collection also included strains from several global regions where strawberries are grown and a total of four rep-PCR groups were found. The limited genetic diversity that was found in this population has practical implications for diagnostic and plant breeding programs. Limited diversity suggests a diagnostic probe or assay and host genetic resistance should be effective for all strains within the population.

CHARACTERIZATION OF STONE FRUIT LEAF SPOT PATHOGENS

Xanthomonas arboricola pv. *pruni* (Xap) affects a range of stone fruit crops including peaches. The pathogen incites a leaf spot and causes a fruit spot – rendering fruit unmarketable for most retail outlets. Growers spray bactericidal pesticides to manage this disease but additional work is needed to better understand the biology and epidemiology of this pathogen. Also, diagnostic protocols need to be developed and implemented to limit the introduction of the pathogen into new orchards.

Forty-six putative Xap strains isolated from stone fruit hosts (32 from 9 peach varieties, 5 from nectarine, 2 from apricot, 1 from prune, 5 from plum and 1 from almond) in NC (34), GA (2), VA (1), SC (1), Brazil (5) and Uruguay (2) had nearly identical rep-PCR genomic fingerprint profiles. The “pruni” population was similar to the strawberry population in that there was very little genetic diversity observed, based on a low number of polymorphic bands. Again, this information is of practical value in that diagnostic probes should be effective for all pathogenic strains. Such probes could be useful to limit the introduction of the pathogen in new orchards and to ask fundamental questions about the biology and epidemiology of the pathogen.

CHARACTERIZATION OF CRUCIFER BLACK ROT PATHOGENS

Black rot caused by *Xanthomonas campestris* pv. *campestris* (Xcc) is the most important disease of crucifer crops worldwide and is a recurrent problem in the Carolinas and surrounding states. Black rot can affect most crucifer crops including cabbage, cauliflower, kale, radish, broccoli, Brussels sprouts, Chinese cabbage, turnip, collards, mustards, and several common weed species. In our region, black rot tends to be most common on farms that have limited options for adequate rotation. However, the disease appears in fields and farms with no history of crucifer crop production, implicating contaminated seed or transplants as a source of inoculum.

Fifty-two, 39, 25 and 35 isolates of Xcc were obtained (fall, 1996) from 60 symptomatic leaves sampled from a collard (FLD A) and cabbage (FLD C) field in SC, and a collard (FLD G) and red cabbage (FLD J) field in NC, respectively. FLD C but not FLD A, G and J had a recent history of cole-crop production. Rep-PCR genomic fingerprint analysis differentiated 9 distinct lineages among the 151 isolates and 8 ATCC Xcc strains. Most FLD A (31) and FLD C (32) isolates and ATCC 33440 were in lineage 1. Other FLD A (20) and C (6) isolates were in lineage 5, lineage 7 (FLD A,1) and 8 (FLD C,1). ATCC 29497, 33442, 33436 belonged to lineage 7. All FLD G isolates clustered in lineage 4. Most FLD J (28) isolates and ATCC 31600 and 31922 belonged to lineage 9. Other FLD J isolates belonged to lineage 3 (6) and 1 (6). ATCC 33913 (type strain) and 31313 clustered together in lineage 2. Characterization of genetic diversity within and among fields detailed the lineage composition and structure of Xcc populations in natural epidemics. The ability to differentiate lineages provides a foundation to pursue basic questions related to the ecology of different lineages and the interaction of different lineages with brassica plants having known resistance/susceptibility to the pathogen.

CHARACTERIZATION OF 123 ATCC *XANTHOMONAS* STRAINS

A collaborative project was conducted with Dr. George Lacy at Virginia Polytechnical Institute and State University. We characterized 123 xanthomonad strains that currently comprise the American Type Culture Collection (ATCC). Genomic fingerprints were generated from the 123 strains and the dendrograms generated were compared to "species" groups delineated by DNA-DNA hybridization experiments. The rep-PCR data has proven to be an effective tool to rapidly group xanthomonad strains into groups consistent with those obtained by DNA hybridization work (which is used to define a bacterial species).

The rep-PCR genomic fingerprint profiles were used toward compiling a global xanthomonad database in cooperation with F.J. de Bruijn and J.L.W. Rademaker. The global database demonstrated a high congruency between rep-PCR genomic fingerprint profiles and DNA homology similarity values. This observation discovered through the characterization of hundreds of xanthomonad strains will have a substantial impact on basic taxonomical concepts in bacteriology and detailed in publications authored by J.L.W. Rademaker, F.J. Louws, and F.J. de Bruijn among others.

CHARACTERIZATION OF TOMATO BACTERIAL SPECK PATHOGENS

Bacterial spot caused by *X. axonopodis* pv. *vesicatoria* was an occasional problem in NC fields during the time of this study. However, a more prominent problem in NC tomato fields was bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (Pst). Therefore, we focussed our rep-PCR work on this population. We believe this pathogen is introduced into production fields primarily on contaminated seeds. The pathogen can incite substantial defoliation, but more importantly, it can infect fruit causing superficial black specks rendering the fruit unmarketable. Growers routinely apply copper-based pesticides but we have documented most field strains are resistant to copper. We initiated a study to assess the genetic diversity in order to acquire baseline data on this important pathogen.

Over 160 strains were collected from Pst epidemics in western NC and were found to be highly similar to one another. Selected strains within each population were assayed e.g. for pathogenicity and copper and antibiotic sensitivity. The population was found to be highly homogenous based on genetic and phenotypic assays. The rep-PCR genomic fingerprint database and other data provided an understanding of this population and aided the implementation of recommendations for our growers and raised important questions for future research.

COLLABORATION WITH MICHIGAN STATE UNIVERSITY AND HAMPTON UNIVERSITY

Numerous genomic fingerprints generated at NCSU were electronically transferred to MSU. The fingerprints were correctly identified in a prototype database at MSU. This work highlighted the real potential of designing an internet-based protocol for bacterial identification. Unique fingerprints generated at NCSU are included in the full database generated at MSU. Dr. Louws also spent two full days working closely with Dr. E. Fowlks, sharing information and conducting rep-PCR genomic fingerprinting at the Hampton laboratory of Dr. Fowlks.

Dr. Louws also coordinated work done in collaboration with the University of Florida. Tomato and pepper bacterial spot strains (*Xanthomonas species*) representing populations throughout the Caribbean and Central America were characterized by rep-PCR genomic fingerprinting and numerous phenotypic/biochemical assays. The majority off the population throughout the Caribbean/Central America region comprised a group of strains known as "group A" (*X. axonopodis* pv. *vesicatoria*). Furthermore, this group of strains could be partitioned into numerous rep-PCR genomic lineages. In several cases, island or country-specific lineages were observed as well as field-specific lineages. This data provided a basis to speculate about the relative importance of evolutionary forces that structure a population including the rate of migration and genetic drift. A unique group of strains were found in the study and through the use of the *Xanthomonas* rep-PCR database, these strains were identified as *X. gardneri*, a genotype that has not been recorded since 1957.

Several publications, including an honored invitation to write a review chapter for Annual Reviews of Plant Pathology, were coordinated among NCSU and MSU collaborators highlighting work done as outlined below.

COLLABORATION WITH INDUSTRIAL PARTNERS

Dr. Louws has kept in close contact with the industrial partners. He coordinated a site visit to the Heinz Research Station in Bowling Green Ohio to conduct a 2-day hands on training for Heinz personnel. Heinz used the rep-PCR to identify and assess diversity of tomato bacterial pathogens. Dr. Louws also spent time in California with Rogers Seed Inc (now Novartis Seed). He had considerable follow-up correspondence with personnel at Rogers concerning the practical implementation of rep-PCR at their research facility. Finally, Dr. Louws and Jan Rademaker have conducted 2 workshops at the National American Phytopathology Meetings (1996 and 1997) that was attended by people of diverse industrial backgrounds.

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.

Publications in Refereed Journals:

- Tuang, F. N., Rademaker, J. L. W., **Louws, F. J.**, Alcocilja, E. C., de Bruijn, F. J. 1999. Classification of bacterial rep-PCR genomic fingerprints using a backpropagation neural network. *FEMS Microbiology* 177:249-256.
- Bouzar, H., J.B. Jones, R.E. Stall, **F.J. Louws**, M. Schneider, J.L.W. Rademaker, F.J. de Bruijn and L.E. Jackson. 1999. Multiphasic taxonomy of bacteria causing spot disease on tomato and pepper plants in the Caribbean and Central America reveals distinct predominance of *Xanthomonas axonopodis vesicatoria* strains. *Phytopathology* 89:328-335.
- Rademaker, J.L.W., B., Hoste, **F.J. Louws**, K., Kersters, J. Swings, L. Vauterin, P. Vauterin, F.J. de Bruijn 1999. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *Int. J. Systematic Bacteriol.* accepted.
- Rademaker, J.L.W., M.H. Schultz, **F.J. Louws**, U. Rossbach, L. Vauterin, J. Swings, F.J. de Bruijn. Molecular systematics of xanthomonads by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. *Int. J. Systematic Bacteriol.* submitted.

Book Chapters (peer reviewed):

- Louws, F.J.**, J.L.W. Rademaker, and F.J. de Bruijn. 1999. The three Ds of PCR-based genomic analysis of phyto bacteria: Diversity, detection and disease diagnosis. *Annual Review of Phytopathology* 37:81-125.
- Rademaker, J.L.W., **F.J. Louws**, and F.J. de Bruijn. 1999. Computer assisted pattern analysis of electrophoretic fingerprints and database construction. In: Akkermans, A.D.L., J.D. van Elsas and F.J. de Bruijn. (eds). *Molecular Microbial Ecology Manual*. Kluwer Academic Publishers, Dordrecht, Suppl. 4 Chapter 7.1.3. pp..
- Rademaker, J.L.W., **F.J. Louws**, and F.J. de Bruijn. 1998a. Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. In: Akkermans, A.D.L., J.D. van Elsas and F.J. de Bruijn. (eds). *Molecular Microbial Ecology Manual*. Kluwer Academic Publishers, Dordrecht. Suppl. 3 Chapter 3.4.3. pp. 1-26.
- Louws, F.J.**, M. Schneider and F.J. de Bruijn. 1997. Assessing genetic diversity of microbes using repetitive sequence-based PCR (rep-PCR). In: *Nucleic acid amplification methods for the analysis of environmental microbes*. G. Toronzos., ed. Technomic Publishing Co., Inc., Lancaster, PA. pg 63-94.
- de Bruijn, F.J., J. Rademaker, M. Schneider, U. Rossbach and **F.J. Louws**. 1996. Rep-PCR Genomic Fingerprinting of Plant-Associated Bacteria and Computer-Assisted Phylogenetic Analyses. In: *Biology of Plant-Microbe Interactions*. G. Stacey, B. Mullin and P. Gresshoff, eds. *Int. Soc. Plant-Microbe Interactions*. pg. 497-502.

Research Abstracts:

- Louws, F.J.**, J.P. Smith and A.P. Keinath. 1998. *Xanthomonas campestris* pv. *campestris* lineages in commercial cole-crop fields. *Phytopathology* 88:S56.
- Louws, F.J.**, J.L.W. Rademaker, V.K. Stromberg, R.L. Gherna, F.J. de Bruijn and G.H. Lacy. 1997. Rep-PCR genomic fingerprinting of 123 ATCC xanthomonad strains. *Phytopathology* 87:S60.

- Rademaker, J.L.W., **F.J. Louws**, M.H. Schultz, U. Rossbach, L. Vauterin, J. Swings, and F.J. de Bruijn. 1997. Molecular systematics of xanthomonads by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. *Phytopathology* 87:S81.
- Rademaker, J.L.W., **F.J. Louws**, F.N. Tuang, M.H. Schultz, U. Rossbach, L. Vauterin, J. Swings, E. Alocilja and F.J. de Bruijn. 1997. Computer-assisted "on-line" identification of xanthomonads by a rep-PCR database or artificial neural network. *Phytopathology* 87:S81.
- Jones, J.B., H. Bouzar, **F.J. Louws**, J. Rademaker, M. Schneider, F.J. de Bruijn, and R.E. Stall. 1997. Genetic variation of xanthomonads isolated from tomato in the Caribbean and Central America as determined by rep-PCR. *Phytopathology* 87:S49.
- Rademaker, J.L.W., **F.J. Louws**, U. Rossbach, L. Vauterin, J. Swings, and F.J. de Bruijn. 1997. Classification and identification of *Xanthomonas* by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. ASM General Meeting, Miami, FL.
- Louws, F.J.**, J.L.W. Rademaker, U. Rossbach, L. Vauterin, J. Swings, and F.J. de Bruijn. 1996. Genomic fingerprinting and computer-assisted pattern analysis of inter- and intra-subspecies diversity of *Xanthomonas*. *Phytopathology* 86: S78.

Invited Speaker; Workshops And Presentations (Research):

a) Invitational Workshops:

- Louws, F.J.** PCR-based genome analysis to assess genetic diversity and classify phyto bacteria. *IV: Discussion: The Fine Line of Species Determination*. American Phytopathology Society Annual Meeting. Montreal, Quebec, August 9, 1999.
- Louws, F.J.** and G. Holmes. Remote Diagnosis *IV: Workshop – Disease Management Products – Supporting Decision Making in IPM*. American Phytopathology Society Annual Meeting. Montreal, Quebec, August 7, 1999.
- A workshop for Plant Pathology scientists and other professionals; 2 concurrent workshops with ~ 100 people in attendance; handouts provided.
- Louws, F.J.**, and J.L.W. Rademaker. Rep-PCR: a rapid technique for bacterial identification and assessment of genetic diversity. American Phytopathology Meetings workshop on "Rapid diagnostic assays for plant pathogens". Rochester, NY, August 12, 1997.
- A workshop for Plant Pathology scientists and other professionals; Edited an Associated Workshop Manual (80 copies).
- Louws, F.J.** and J.L.W. Rademaker. Rep-PCR for rapid identification of tomato pathogens. H.J. Heinz Co. Bowling Green, OH. Dec 3-4, 1996.
- Louws, F.J.**, J.L.W. Rademaker, and F.J. de Bruijn. Rep-PCR: a rapid technique for bacterial identification and assessment of genetic diversity. American Phytopathology Meetings workshop on "Rapid diagnostic assays for plant pathogens". Indianapolis, IN, 1996.

b) Research Presentations (not highlighted in abstracts above):

- Louws, F.J.** Shifty Enemies- Population Structure of Phyto bacteria and Deployment of

Host Resistance. NCSU-Plant Genetics and Breeding Seminar Series. Dec 2, 1998.

Louws, F.J. Population structure and genetic diversity of phyto bacteria: Implications for disease management. NCSU Dept. Plant Pathology Seminar series. Nov 3, 1997.

Louws, F.J., J.L.W. Rademaker, and F.J. de Bruijn. Computer-assisted analysis of xanthomonad genomic fingerprints: Classification and Identification. NCR-178 National Bacteriology Working Group, Athens, GA, Mar 21-22, 1997.

Louws, F.J., J.L.W. Rademaker, and F.J. de Bruijn. Genomic fingerprinting xanthomonads to assess genetic diversity and for diagnosis. 12th Annual International Tomato Disease Workshop. Columbus Ohio, Dec 5-6, 1996.

Louws, F.J., J.L.W. Rademaker, E.R. Fowlks and F.J. de Bruijn. Genomic fingerprinting and computer-assisted analysis of genetic diversity of bacteria. Consortium for Plant Biotechnology, Inc. Annual Symposium. Washington, D.C. November 19, 1996.

Louws, F.J., DNA fingerprinting phytopathogenic bacteria. Rogers Seed Co. Gilroy, CA. Sept. 19, 1996.

Louws, F.J., Population structure and genetic diversity of *Xanthomonads*. NCR-178 National Bacteriology Working Group, Athens, GA, Mar 29-30, 1996.

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.

Teaching Activities:

a) Advisor or major contributor to student training for use of rep-PCR in their work/thesis:

i) Post Doctoral Students and Technicians:

Gloria Abad. Trained to use rep-PCR to characterize a population (~300 strains) of plant pathogenic soil-borne bacteria of complex diversity.

Tammy Abernathy: Trained to use rep-PCR to characterize xanthomonad populations from various hosts.

ii) Graduate Students:

Karen Stamen (MS candidate; Dept. Botany). Trained to use rep-PCR to characterize rhizosphere bacteria as part of her thesis work.

Thomas Mitchell (Ph.D. candidate; Dept. Plant Pathology) . Employed rep-PCR to verify integrity of a bacterial strain that was mutagenized.

Anna Romero (Ph.D. candidate; Dept. Plant Pathology). Trained to use rep-PCR to characterize a group of unique xanthomonad strains pathogenic for pepper.

iii) Undergraduate Students:

Diane Grigsby. Trained to use rep-PCR to characterize xanthomonads as part of this study. Diane's experience, in part, provided the basis for her to acquire a technician position at the Baylor College of Medicine, TX.

Christy Taylor. Trained to use rep-PCR to characterize xanthomonads as part of this study. Christy mastered the protocols well and, in part, her lab experience provided the basis for her to acquire an advanced technician position in microbiology on campus.

Suzanna Waddy. Trained to use rep-PCR to characterize xanthomonads as part of this study. Suzanna mastered the protocols well and, in part, her lab experience provided the basis for her to acquire an advanced technician position in genetics on campus.

b) Guest Lecturer:

- "Molecular approaches to bacterial identification, classification and diagnostics", for a graduate level course: Bacteria and their interactions with plants (PP 503); P. Lindgren and D. Ritchie). North Carolina State University. Nov 24, 1999.
- "Rep-PCR characterization of bacteria and computer-assisted genome analysis", lab section graduate level course: Bacteria and their interactions with plants (PP 503L); P. Lindgren and D. Ritchie). North Carolina State University. Nov 24, 1999.
- "Molecular approaches to bacterial identification, classification and diagnostics", for a graduate level course: Bacteria and their interactions with plants (PP 519; P. Lindgren and D. Ritchie). North Carolina State University. Nov 24, 1997.
- "Principles of Bacteriology". PP 318 ; L. Grand. North Carolina State University. April 21, 1997.
- "Molecular approaches to bacterial identification, classification and diagnostics", for a graduate level course: Bacteria and their interactions with plants (PP 519; P. Lindgren and D. Ritchie). North Carolina State University. Nov 26, 1996.

c) Community College Training Activity (featured rep-PCR as part of each 3-hr presentation):

- "Managing Plant Diseases". Central Carolina Community College. Pittsboro, NC. Nov 23, 1998.
- "Biology and Management of Vegetable Diseases". Central Carolina Community College. Pittsboro, NC. March 17, 1998.
- "Management of Plant Diseases: Emphasis on Biological-based management strategies". Central Carolina Community College. Pittsboro, NC. Nov 10, 1997.
- "Biology, Identification and Integrated Management of Plant Diseases". Central Carolina Community College. Pittsboro, NC. Mar 18, 1997.
- "Disease management in sustainable farming systems". Central Carolina Community

d) Extension And Public Service Contributions

(presentations made at extension and grower meetings highlighting rep-PCR genomic fingerprinting research and providing disease management recommendations).

- "Bacterial Disease Control". Tomato Extension Meeting. Rowan Co. Feb 3, 1999.
- "Black rot: Is it coming back?". Southeast Vegetable & Fruit Expo. Greensboro, NC, Dec. 17, 1997.
- "Identifying and managing diseases of strawberry". Insect and Disease Clinic: Southeastern Strawberry Expo, Raleigh, NC. Nov 14, 1997.
- "Disease Management Strategies". Area Cabbage Field Day. Tabor City, NC. May 22, 1997.
- "Black rot IPM". Elizabeth City, NC. February 24, 1997.
- "Managing diseases of vegetables". Sanford, Lee Co., NC February 6, 1997.
- "Managing diseases of vegetables". Derby, Richmond Co., NC February 4, 1997.
- "Managing diseases of tomatoes". Salisbury, Rowan Co., NC February 3, 1997.
- "Black rot of cabbage and other crucifers". Tabor City, NC. January 30, 1997.
- "DNA forensics: Identifying criminal bacteria". Annual Southeast Vegetable and Fruit Expo. Greensboro, NC, Dec 11, 1996.
- "Identifying and managing diseases of strawberry". Insect and Disease Clinic: Southeastern Strawberry Expo, Raleigh, NC. Nov 15, 1996.
- "Control of angular leafspot of strawberry". Southeastern Strawberry Expo, Raleigh, NC. Nov 14, 1996.

Additional Funding

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

- **F. J. Louws.** Population structure and genetic diversity of phyto bacteria: Theoretical and practical investigations. NCSU Faculty Research and Professional Development Funds. Oct 10, 1997. \$5,000.00.
- **F.J. Louws.** Hardware And Software To Design Worldwide Databases Of Bacterial Genomic Fingerprints. Feb 1998. \$7,114.00. College of Agriculture and Life Sciences, NCSU.
- Wilson, M., J.B. Jones and **F.J. Louws.** 1998-1999. Integrated biological and chemical control of bacterial spot and speck of tomato. Southern Regional Extension IPM Committee. (\$121,282; NCSU=\$19,311 over 2 years).

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

This project depended primarily on undergraduate students and technicians to accomplish the work reported.

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Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Lee R. Lynd Michael J. Antal, Jr.	
University:	Dartmouth College University of Hawaii	
Agreement Number:	OR22072-65	
Project Title:	Pretreatment Process for Cellulosic Biomass	
Reporting Period and Report Type:	From: October 1, 1998 To: August 31, 1999	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

The overall goal of the project is to characterize the performance and identify the key mechanisms involved in LHW pretreatment of lignocellulosic biomass. "Performance" is defined in terms of the key characteristics of successful biomass pretreatment:

- *Fiber reactivity*—can the pretreated fiber (cellulose) be readily converted (> 90%) to ethanol in a reasonable amount of time?
- *Pentosan recovery*—can the original biomass pentosan content (hemicellulose) be preserved in a form that can be readily converted to ethanol (i.e. monomeric or oligomeric pentoses)?
- *Hydrolyzate inhibition*—can the formation of sugar degradation products (e.g. furfural, hydroxymethyl furfural) and other biological growth inhibitors (e.g. acetic acid) be minimized such that conversion of pretreated biomass can occur without preliminary hydrolyzate detoxification?
- *Solids concentration*—can the above criteria be met at pretreatment solids concentrations—defined as the dry weight of biomass as a percentage of the total weight of reactants (biomass + water)—necessary for commercial-scale operation ($\geq 15\%$ solids)?

Consistent with these criteria, the PIs focussed their efforts toward characterizing liquid hot water pretreatment (LHWP) at conditions of varying temperature, time, and solids concentration. Steam pretreatment was also investigated. Sugar cane bagasse was the predominant feedstock. During the reporting period, the PIs completed 17 LHWP experiments with temperatures ranging from 173°C to 230°C, times ranging from 1 to 46 minutes, severity parameters (log Ro) ranging from 3.1 to 4.5, and solids concentrations ranging from 1% to 7.7%. Three steam pretreatment runs were completed. The results for the reporting period will be presented and discussed in the context of the overall project (15 steam pretreatment runs and 22 LHW pretreatment runs).

Scientific Accomplishments

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

Tables and figures referred to in the following text are provided at the end of this section.

Results Summary

- Both steam pretreatment and LHW pretreatment were capable of producing highly reactive fiber. With temperatures $\geq 215^{\circ}\text{C}$ and reaction times ≥ 5 minutes, steam pretreatment yielded fiber achieving $\geq 90\%$ SSF conversion. LHW pretreatment achieved similar results with comparable temperatures and even lower reaction times (≥ 2 min).
- LHW pretreatment recovered more xylan than did steam pretreatment for comparable reaction temperatures and times. At 220°C and 2 minutes, 48% of feed xylan was recovered after steam pretreatment; as much as 90% was recovered following LHW pretreatment.
- The top performing LHW pretreatment runs with respect to the cumulative effectiveness metrics resulted in average SSF conversions of 80% and xylan recoveries of 80%. Hydrolyzate inhibition was significant in most runs.
- The kinetics of steam pretreatment inherent in the severity parameter may not adequately describe LHW pretreatment phenomena— 217°C and 2.3 minutes could not be traded off with 173°C and 46 minutes, though both have log Ro values of 3.8.
- In general, SSF conversion increased with increasing LHW pretreatment temperature for a given reaction time.
- Reaction times above two minutes for pretreatments (steam or LHW) at 220°C did not significantly affect SSF conversion.
- SSF conversions were higher for steam pretreatment at 220°C than at 200°C . At 200°C , SSF conversion increased significantly with increasing reaction time ($\sim 30\%$ at 2 minutes; 78% at 10 minutes). At 220°C , SSF conversion increased only marginally with increasing reaction time (85% at 2 minutes; 90% at 10 minutes).
- SSF conversion increased as a function of pretreatment time for LHW pretreatment at 220°C and 1% solids. At solids concentrations $\geq 3\%$, SSF conversion appeared to decrease with increasing reaction time, but reaction times were limited to ≤ 3 minutes for these runs.
- For a reaction time of 2 minutes, LHW pretreatment at 1% solids resulted in higher xylan recovery than at higher solids concentrations.
- Xylan recovery decreased as a function of time for LHW pretreatment at 1% solids and for steam pretreatment. Extended time runs (> 5 min) are necessary to determine the behavior of LHW pretreatment at higher solids concentrations ($\geq 3\%$) as a function of reaction time.
- Fiber reactivity increased with increasing xylan dissolution. Achieving $\geq 90\%$ SSF conversion, required that $\geq 90\%$ of the xylan must be removed from the fiber.
- Xylan recovery decreased with increasing xylan dissolution. For LHW pretreatment, when $\geq 90\%$ of xylan was dissolved, recovery was $\leq 80\%$. When all of the xylan was dissolved, recovery was $< 30\%$.

- Hydrolyzate monomeric xylan fraction and furfural production increased as a function of increasing xylan dissolution, indicating that hydrolysis of dissolved xylan is chiefly responsible for xylan degradation.
- For both LHW and steam pretreatment, the extent of xylan dissolution increased with increasing time.
- For a reaction time of 2 minutes, LHW pretreatment at 1% solids resulted in higher xylan recovery than at higher solids concentrations. Xylan recovery decreased as a function of time for LHW pretreatment at 1% solids and for steam pretreatment. Extended time runs (>5 min) are necessary to determine the behavior of LHW pretreatment at higher solids concentrations ($\geq 3\%$) as a function of reaction time.
- The degree to which LHW pretreatment hydrolyzates inhibited glucose fermentation rate and yield increased with increasing pretreatment temperature.
- The degree of fermentation rate and yield inhibition increased with increasing solids concentration. For a reaction time of 2 minutes, pretreatment at 1% solids resulted in non-inhibitory hydrolyzate, while higher solids concentrations resulted in hydrolyzates that were completely inhibitory.
- LHW pretreatment reaction time affected the degree of hydrolyzate inhibition: at 1% solids and 220°C, rate inhibition increased as a function of time—no inhibition at 2 minutes, 75% at 5 minutes, and 90% at 10 minutes.

Fiber Reactivity

A conventional batch simultaneous saccharification and fermentation (SSF) system was used to determine the fiber reactivity of the pretreated sugar cane bagasse. *Trichoderma reesei* cellulase (Iogen) supplemented with β -glucosidase (Novozyme 188) in a ratio of 4 IU β -glucosidase/FPU cellulase served as the hydrolyzing enzyme. *Saccharomyces cerevisiae* strain D5A, provided by NREL (Golden, CO), was the fermenting organism. SSF was carried out in 250 mL serum vials filled to a working volume of 100 mL. The vials were incubated at 37°C and agitated at 140 RPM on an orbital shaker. The growth medium consisted of 10 g/L yeast extract and 20 g/L peptone in distilled water. Growth continued to completion before the experiments were terminated.

SSF conversion data for steam pretreatment and liquid hot water (LHW) pretreatment are listed in Tables 1a and 1b, respectively. It should be noted that final conversion based on ethanol production is often higher than that based on residual cellulose. This may be attributed to low measurements for initial cellulose content, low values for the glucose content in the enzyme mixture, or a high estimate for carbohydrate allocated to cell production, or some combination of these. Unless otherwise noted, residual cellulose values will be used in subsequent reporting and discussion.

Both steam pretreatment and LHW pretreatment were capable of producing highly reactive fiber. With temperatures $\geq 215^\circ\text{C}$ and reaction times ≥ 5 minutes, steam pretreatment yielded fiber achieving $\geq 90\%$ SSF conversion. LHW pretreatment achieved similar results with comparable temperatures and even lower reaction times (≥ 2 min).

The severity parameter ($R_o = \text{time}[\text{min}] \cdot \exp((T[^\circ\text{C}]-100)/14.75)$) is often used as a pretreatment ordinate to combine the effects of temperature and time. The commonly accepted notion is that, within a defined range of temperatures (160°C-240°C), temperature and time can be traded off. The parameter was developed to describe steam pretreatment behavior, but the parameter has been used for hydrothermal pretreatment in general. Figure 1 presents SSF conversion as a function of log R_o for both steam and LHW pretreatment runs. From the figure, one can see that, fiber reactivity generally increases with increasing log R_o , leveling off above a log R_o value of 4. There is, however, considerable scatter in the data, especially for the LHW runs—at a log R_o value of 3.8, for example, the low conversion value is 32% (173°C, 46 min) while the high is 86% (217°C, 2.3 min). This suggests that the kinetics of steam pretreatment may not adequately describe LHW pretreatment phenomena. The data just cited, for example, indicate that 217°C and 2.3 minutes cannot be traded off with 173°C and 46 minutes. It is possible that

experimental variance contributed to scatter in the data. For example, replicate steam pretreatment runs at 200°C and 2 minutes differed by as much as 25% when error bars are taken into account (Figure 1).

Figure 2 presents SSF conversion as a function of reaction time for LHW pretreatment at various temperatures. The data indicate that, in general, conversion increased with increasing temperature for a given reaction time. At 220°C, reaction times above two minutes did not significantly affect SSF conversion.

Steam pretreatment data are presented in Figure 3 as a function of time at 200°C and 220°C. At each reaction time—2, 5, and 10 minutes—conversions were higher for the higher pretreatment temperature. At 200°C, SSF conversion increased significantly with increasing reaction time (~30% at 2 minutes; 78% at 10 minutes). At 220°C, SSF conversion increased only marginally with increasing reaction time (85% at 2 minutes; 90% at 10 minutes).

When LHW pretreatment data are plotted as function of time at 220°C for various solids concentrations (Figure 4), SSF conversion increased with reaction time at 1% solids. At solids concentrations $\geq 3\%$, SSF conversion appeared to decrease with increasing reaction time, but reaction times were limited to ≤ 3 minutes for these runs. Additional experiments should be performed at longer reaction times to determine whether SSF conversion continues to decrease with time for solids concentrations $\geq 3\%$. As will be presented in the next section, the overall data indicate that SSF conversion increases with the amount of xylan removed from the fiber (Figure 6).

Pentosan Recovery

Xylan recovery data for steam and LHW pretreatment are listed in Tables 2a and 2b, respectively. From these data, one can see that, for comparable reaction temperatures and times, LHW pretreatment recovered more xylan than did steam pretreatment. For example, at 220°C and 2 minutes, 48% of feed xylan was recovered after steam pretreatment (AP980610); as much as 90% was recovered following LHW pretreatment (AP981118A).

It should be noted that xylan recovery was determined by balancing both the dissolved xylan and the xylan remaining in the pretreated fiber with the feed xylan. Thus, 100% xylan recovery could be obtained without any xylan being dissolved. If no xylan were removed, however, the pretreatment would likely not yield reactive fiber—Figure 6 indicates that fiber reactivity increased with increasing xylan dissolution. It is clear that to achieve greater than 90% conversion, greater than 90% of the xylan must be removed from the fiber. Figure 7a indicates that xylan dissolution has an associated cost—xylan recovery decreased with increasing xylan dissolution. For LHW pretreatment, when $\geq 90\%$ of xylan was dissolved, recovery was $\leq 80\%$. When all of the xylan was dissolved, recovery was $< 30\%$. Hydrolytic degradation of dissolved xylan likely accounts for the decrease in xylan recovery as a function of dissolution. Figures 7b and 7c indicate that for LHW pretreatment, the amount of xylan hydrolyzed to monomeric xylose and degraded to furfural increased as xylan dissolution increased.

Figure 8 indicates that for LHW pretreatment, the extent of xylan dissolution is a function of temperature—for a given time, xylan dissolution increased with increasing temperature. The same is true of steam pretreatment (Figure 9). Figure 10 depicts xylan recovery as a function of reaction time at 220°C for steam pretreatment and for LHW pretreatment at various solids concentrations. For a reaction time of 2 minutes, LHW pretreatment at 1% solids resulted in higher xylan recovery than at higher solids concentrations. Xylan recovery decreased as a function of time for LHW pretreatment at 1% solids and for steam pretreatment. Extended time runs (> 5 min) are necessary to determine the behavior of LHW pretreatment at higher solids concentrations ($\geq 3\%$) as a function of reaction time. The xylan recovery data correspond with Figure 5 which indicates that the extent of xylan dissolution increased with increasing time for 1% solids. For steam pretreatment, xylan dissolution decreased from 2 to 5 minutes, but increased from 5 to 10 minutes.

Hydrolyzate Inhibition

To evaluate the level of hydrolyzate inhibition, side-by-side glucose fermentations were carried out using *S. cerevisiae* D5A in nutrient medium prepared with a liquid phase consisting of either distilled water or the pretreatment hydrolyzate. Fermentation was carried out in 150 mL serum vials filled to a working volume of 50 mL. The vials were incubated at 37°C. Inhibition was measured by comparing glucose fermentation rate and final conversion for flasks with hydrolyzate

in varying dilutions and flasks without. The growth medium consisted of 0.15% (volume) corn steep liquor (Corn Products), 1mM MgSO_4 , and 20 g/L glucose.

Figures 11 and 12 presents rate and final yield inhibition levels, respectively, as a function of time for LHW pretreatment at various temperatures. Both rate and yield inhibition and were dependent on temperature—increasing temperatures resulted in increased levels of inhibition. Only two of the sixteen runs presented in the figures produced hydrolyzates that did not inhibit fermentation rate. Most had rate inhibition levels $>80\%$. Five runs exhibited no yield inhibition. Only three hydrolyzate inhibition points using corn steep liquor media were obtained for steam pretreatment. These data are not presented as the resulting hydrolyzates were likely diluted by condensate accumulation in the bottom of the reactor during steam pretreatment. (To obtain meaningful data for steam pretreatment runs, one must extract liquid from the resulting pretreated fiber. This was not performed during the reporting period, but will be performed in the coming months.)

Solids concentration had a strong effect on both rate and yield inhibition levels. Figures 13 and 14 indicate that the degree of rate and yield inhibition increased with increasing solids concentration. For a reaction time of 2 minutes, pretreatment at 1% solids resulted in non-inhibitory hydrolyzate, while higher solids concentrations resulted in complete inhibition. At 1% solids and 220°C , rate inhibition increased as a function of time—no inhibition at 2 minutes, 75% at 5 minutes, and 90% at 10 minutes.

Cumulative Effectiveness

SSF conversion for steam and LHW pretreatment is plotted as a function of xylan recovery in Figure 15. Effective pretreatments lie in the upper right corner of the graph. This plot illustrates that LHW pretreatment was much more effective than steam pretreatment—four LHW pretreatment points lie in the 80%-90% region for both SSF conversion and xylan recovery. The top combined effectiveness for a steam pretreatment run resulted in a SSF conversion of 85% and a xylan recovery of 48%. The best combined values for a LHW pretreatment run were 80% and 90% for SSF conversion and xylan recovery, respectively.

Values for the effectiveness metrics—SSF conversion, xylan recovery, and hydrolyzate rate and yield inhibition—are totaled for the LHW pretreatment runs in Figure 16. In this plot, 4.0 represents a perfect score (note: no inhibition received a value of 1; complete inhibition received a value of 0). Two of the 18 runs scored higher than 3.5. The top performing run resulted in an SSF conversion of 80%, a xylan recovery of 90% and no inhibition with respect to either rate or yield.

Discussion

To assess the overall performance and potential of LHW pretreatment, it is useful to compare the results obtained in this study to the current state-of-the-art process, dilute acid pretreatment. Table 3 lists effectiveness metric values for dilute acid, steam, and LHW pretreatment. Both dilute acid and LHW pretreatment are divided into two sub-categories—conventional and modified—that refer to reactor configuration. “Conventional” refers to a reactor configuration where the solid and liquid phases experience the same temperature and residence time. Examples are a dilute acid slurry reactor and a LHW pretreatment batch reactor. “Modified” refers to a configuration where the solid and liquid phases experience different temperatures or residence times, or both. The “modified” dilute acid configuration refers to NREL’s 2-stage, differentiated-temperature, liquid flow-through dilute acid system. The “modified” LHW reactor refers to Mok and Antal’s liquid flow-through reactor.

The CPBR project has clearly differentiated LHW pretreatment from steam pretreatment and has demonstrated that LHW pretreatment has great potential. LHW pretreatment has the clear advantage of recovering more xylan than steam pretreatment. The same is true of dilute acid pretreatment. Both dilute acid and LHW pretreatment are capable of achieving SSF conversions 80% and pentosan recoveries of 80% in reactor configuration where the liquid and solid phases experience the same temperature and residence time. Steam pretreatment produces reactive fiber ($\geq 80\%$) but does not recover high amounts of pentosan ($\leq 65\%$).

It seems likely, however, that to achieve fiber reactivity values greater than 90% and pentosan recoveries greater than 90%, a modified reactor configuration that differentiates the solid and liquid phases will have to be employed. Results from this study show that to achieve high fiber

reactivity, one must dissolve most of the xylan during pretreatment. But, the results also indicate that xylan recovery decreases with increasing xylan. Thus, it seems that in a reactor where the liquid and solid phases experience the same temperature and residence time, extremely high fiber reactivity cannot be obtained without compromising xylan recovery.

Degradation of soluble xylose likely occurs before the solid phase xylan is completely dissolved. This might be due to xylan solubility limits being reached, after which hydrolysis of soluble xylan and subsequent degradation of xylose becomes rate-limiting. Alternatively, xylan may experience mass transfer limitations that hinder dissolution. The "modified" reactors in Table 3 recover large amounts of pentosan. This is probably due to the fact that dissolved pentosans are continuously removed from the reactor by the flow-through liquid phase. Additional research should be conducted to determine the solution behavior of xylan—mass transfer limitations, dissolution rates, solubility limits, and soluble xylan hydrolysis kinetics. Such research will serve to inform the optimum reactor configuration capable of producing highly reactive fiber and high pentosan recoveries.

Future Work

The following experimental conditions will be completed in the next two months:

Item No.	Mode	T (°C)	t (min)	Solids (%)	Rationale
1	steam	220	2		To provide hydrolyzate with which to evaluate inhibition with BCI's recombinant strain of <i>Klebsiella oxytoca</i> . Hydrolyzate will be pressed from steam pretreated biomass prior to cold water flush.
2	LHW	220	2	10	To complete data set to evaluate effect of solids concentration on LHW pretreatment. Hydrolyzate inhibition will be evaluated with BCI's recombinant strain of <i>Klebsiella oxytoca</i> .
3	LHW	220	5	10	Same as Item 2.
4	LHW	220	10	10	Same as Item 2.
5	LHW	200	2	10	To complete data set to evaluate effect of temperature and time on LHW pretreatment.
6	LHW	200	5	10	Same as Item 5.
7	LHW	200	10	10	Same as Item 5.

Tables

Table 1a. SSF conversion for steam pretreatment runs.

UH ID	T °C	Time min	SSF Conversion		Final	Residual Cellulose
			Theoretical	Ethanol		
			72 hr	200 hr		
AP980115	200	2.0	26.5 ± 1.	35.1 ± 1.5	38.5 ± 2.	41.0 ± 3.9
AP980213	200	2.0	22.7 ± 0.4	27.2 ± 0.5	29.3 ± 0.4	15.7 ± 12.6
AP990309A	200	2.0	16.8 ± 0.4	22.4 ± 1.4	30.5 ± 1.6	19.8 ± 3.0
AP971219	200	2.0	23.5 ± 0.4	28.5 ± 0.2	31.6 ± 0.1	25.6 ± 9.6
AP971230	200	5.0	49.7 ± 0.1	63.8 ± 2.0	74.3 ± 1.3	58.8 ± 7.9
AP971222	200	5.0	49.5 ± 0.5	62.5 ± 4.0	69.6 ± 8.7	56.7 ± 8.7
AP990309B	200	10.0	46.3 ± 3.8	69.9 ± 5.3	81.4 ± 0.2	78.7 ± 0.5
AP980320	210	5.0	55.6 ± 1.2	75.9 ± 2.3	85.7 ± 1.9	82.6 ± 3.0
AP980723	213	10.0	37.5 ± 1.0	63.2 ± 0.1	78.8 ± 1.3	56.1 ± 1.7
AP980218	215	5.0	73.9 ± 1.1	101.6 ± 5.0	105.7 ± 2.6	95.2 ± 6.7
AP980610	220	2.0	64.7 ± 4.7	92.6 ± 5.3	101.6 ± 5.6	84.9 ± 1.7
AP971211	220	2.0	68.3 ± 1.8	86.1 ± 1.6	93.0 ± 2.2	84.8 ± 1.8
AP970929	220	2.0	69.3 ± 1.3	86.9 ± 4.1	87.2 ± 4.5	84.7 ± 4.6
AP980618	220	5.0	68.9 ± 4.6	96.4 ± 1.1	104.8 ± 3.3	90.3 ± 1.2
AP980729A	220	10.0	44.3 ± 0.0	84.9 ± 1.2	98.4 ± 1.4	90.5 ± 1.0

Table 1b. SSF Conversion for LHW pretreatment runs.

UH ID	Solids %	T °C	Time min	SSF Conversion			Residual Cellulose
				Theoretical	Ethanol	Final	
				72 hr	200 hr		
AP981015B	1.0	173	46.0	22.1 ± 0.	23.1 ± 12.6	38.4 ± 0.7	32.3 ± 1.0
AP980529	1.0	180	2.0	8.7 ± 1.0	11.6 ± 2.9	14.4 ± 2.9	19.7 ± 9.2
AP980325	4.8	210	2.0	36.7 ± 9	49.9	55.4 ± 2.0	44.4 ± 7.5
AP981002B	2.8	210	2.1	50.2 ± 0.	70.9 ± 7.5	81.7 ± 7.0	67.8 ± 1.4
AP981002C	3.9	212	2.1	49.4 ± 2.2	69.0 ± 4.4	79.2 ± 4.3	84.2 ± 3.1
AP980709	2.5	214	1.5	35.2 ± 8.1	49.1 ± 9.5	57.0 ± 10.1	41.1 ± 6.0
AP981223D	7.0	215	2.0	77.6 ± 8.0	97.5 ± 9.3	106.0 ± 10.3	78.6 ± 0.6
AP981007	4.8	217	2.0	59.2 ± 2.3	83.9 ± 0.3	96.3 ± 0.6	87.0 ± 0.2
AP981015A	5.9	217	2.3	54.0 ± 2.1	75.6 ± 3.1	86.5 ± 3.0	86.5 ± 1.6
AP981125B	1.0	218	2.0	57.1 ± 0.0	80.7 ± 0.3	89.7 ± 0.6	67.4 ± 3.3
AP981125A	1.1	218	10.0	70.1 ± 1.3	104.2 ± 2.4	106.5 ± 1.8	94.7 ± 0.7
AP990309D	3.1	218	10.0	44.0 ± 6.6	72.0 ± 4.5	81.2 ± 4.2	60.8 ± 11.0
AP990311A	7.5	220	1.5	68.9 ± 4.4	106.0 ± 2.6	107.0 ± 1.8	97.5 ± 3.5
AP981118A	1.1	220	2.0	64.7 ± 0.1	89.3 ± 2.0	95.4 ± 2.8	79.7 ± 2.4
AP980910B	2.4	220	2.0	59.0 ± 8.6	74.9 ± 10.6	78.5 ± 10.9	84.3 ± 2.8
AP981021C	3.0	220	2.0	72.0 ± 1.3	95.9 ± 2.6	104.5 ± 0.1	94.4 ± 0.3
AP981021A	3.3	220	2.1	73.7 ± 3.9	103.9 ± 0.9	115.3 ± 1.4	93.4 ± 1.8
AP10002A	2.7	220	3.0	69.1 ± 0.6	84.3 ± 10.3	93.1 ± 14.1	91.0 ± 1.4
AP980903B	1.4	220	5.0	60.2 ± 8.1	75.9 ± 11.2	79.4 ± 11.7	90.2 ± 2.6
AP981217A	5.7	224	1.5	52.9 ± 4.1	72.8 ± 4.1	82.9 ± 4.8	59.1 ± 5.7
AP980723B	1.7	225	2.0	82.5 ± 1.0	105.8 ± 2.7	114.5 ± 1.4	87.1 ± 0.3
AP981112A	2.4	230	1.0	64.9 ± 1.2	86.9 ± 2.4	92.8 ± 2.6	80.7 ± 1.4

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Table 2a. Xylan dissolution, degradation, recovery, and profile for steam pretreatment runs.

UH ID	T °C	Time min	Dissolved Xylan %	Furfural/ Feed Xylose g/g	Xylose Recovery	Extract Monomer Fraction	Flush Monomer Fraction
AP990309A	200	2.0	15%	0.00	89%	0.0	0.0
AP990309B	200	10.0	89%	0.06	12%	0.0	0.6
AP980723	213	10.0	91%	0.10	12%	1.0	1.0
AP980610	220	2.0	88%	0.04	48%	0.0	0.5
AP980618	220	5.0	79%	0.17	26%	0.0	0.7
AP980729A	220	10.0	100%	0.13	2%	1.1	0.8

Table 2b. Xylan dissolution, degradation, recovery, and profile for LHW pretreatment runs.

UH ID	Solids Conc. ^a %	T °C	Time min	Dissolved Xylan	Furfural/ Feed Xylose g/g	Xylan Recovery	Extract Monomer Fraction ^b	Flush Monomer Fraction ^b
AP981015B	1.0	173	46.0	66%	0.00	91%	0.4	0.0
AP980529	1.0	180	2.0	18%	0.00	139%	0.0	0.0
AP981128C	0.9	195	2.0	43%	0.00	101%	0.0	0.0
AP981002B	3.5	210	2.1	89%	0.01	77%	0.2	0.2
AP981002C	5.3	212	2.1	88%	0.01	75%	0.2	0.0
AP980709	2.6	214	1.5	50%	0.01	77%	0.1	0.0
AP981223D	7.0	215	2.0	66%	0.02	98%	0.2	0.3
AP981007	5.5	217	2.0	79%	0.02	81%	0.3	0.0
AP981015A	7.7	217	2.3	94%	0.04	54%	0.4	0.3
AP981125B	1.0	218	2.0	78%	0.01	87%	0.1	0.0
AP981125A	1.1	218	10.0	100%	0.14	15%	0.7	0.0
AP990309D	3.1	218	10.0	84%	0.02	78%	0.2	0.0
AP990311A	7.5	220	1.5 ^c	100%	0.04	28%	0.4	0.5
AP981118A	1.1	220	2.0	82%	0.02	90%	0.2	0.2
AP980910B	2.6	220	2.0	85%	0.03	3%	0.3	0.3
AP981021C	3.2	220	2.0	93%	0.06	67%	0.5	0.0
AP981021A	3.7	220	2.1	94%	0.05	61%	0.5	0.4
AP10002A	3.0	220	3.0	94%	0.04	65%	0.3	0.0
AP980903B	1.4	220	5.0	92%	0.04	68%	0.3	0.0
AP981217A	5.7	224	1.5	70%	0.01	91%	0.1	0.1
AP980723B	1.7	225	2.0	95%	0.02	65%	0.3	0.0
AP981112A	2.6	230	1.0	84%	0.02	83%	0.2	0.2

^aSolids concentration defined as dry mass feed/extract. ^bMonomer fraction defined as mass xylose/(mass oligomeric xylan + xylose). ^cReaction time for AP990311A is likely understated—depressurization required 20 minutes.

Table 3. Comparison of combined effectiveness metrics for dilute acid, steam, and liquid hot water pretreatment.

Metric	Dilute Acid		Steam	LHW	
	Conventional	Modified ^b		Conventional	Modified ^c
Fiber Reactivity	80% (90%)	≥90%	≥80%	80-90%	?
Pentosan Recovery	80% (90%)	≥95%	≤65%	80-90%	≥95%
Hydrolyzate Inhibition	Strong	?	Yes	Moderate ^d	?
Solids Concentration	35% (50%)	5-10%	>50%	≤10%	<1%

^aConventional refers to pretreatment where liquid and solid phase have the same residence time and temperature.

^bModified dilute acid configuration refers to NREL's 2-stage, differentiated-temperature, flow through system.

^cModified LHW configuration refers to liquid flow through reactor. ^dNote: this based on previous Antal LHW reactor.

Figure 1. SSF conversion as a function of log Ro.

Figure 2. SSF conversion as a function of reaction time for LHW pretreatment at various temperatures

Figure 3. SSF conversion as a function of reaction time for steam pretreatment at 200°C and 220°C

Figure 4. SSF conversion as a function of reaction time for LHW pretreatment at various solids concentrations.

Figure 5. Xylan dissolution as a function of reaction time at 220°C for steam pretreatment and LHW pretreatment at various solids concentrations.

Figure 6. SSF conversion as a function of xylan dissolution.

Figure 7a. Xylan recovery as a function of xylan dissolution.

Figure 7b. Hydrolyzate xylan monomer fraction as a function of xylan dissolution.

Figure 7c. Furfural formation as a function of xylan dissolution.

Figure 8. Xylan dissolution as a function of reaction time for various LHW pretreatment temperatures.

Figure 9. Xylan dissolution as a function of reaction time for steam pretreatment at 200°C and 220°C.

Figure 10. Xylan recovery as a function of reaction time at 220°C for steam pretreatment and LHW pretreatment at various temperatures.

Figure 11. Hydrolyzate rate inhibition as a function of reaction time for LHW pretreatment at various temperatures.

Figure 12. Hydrolyzate yield inhibition as a function of reaction time for LHW pretreatment at various temperatures.

Figure 13. Hydrolyzate rate inhibition as a function of reaction time at 220°C for LHW pretreatment at various solids concentrations.

Figure 14. Hydrolyzate yield inhibition as a function of reaction time at 220°C for LHW pretreatment at various solids concentrations.

Figure 15. SSF conversion as a function of xylan recovery.

Figure 16. Cumulative LHW pretreatment effectiveness.

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.

A manuscript for the research presented in this report is in progress. Anticipated completion date is December 31, 1999.

Technology Transfer

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

There is nothing new to report.

Commercial Accomplishments

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

There is nothing new to report.

Educational Accomplishments

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

There is nothing new to report.

Additional Funding

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

There is nothing new to report.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

There is nothing new to report.

Send completed report to :

*The Consortium for Plant Biotechnology Research, Inc.
P.O. Box 20634
(Express Delivery address: 10 Sylvan Drive, Suite 21)
St. Simons Island, GA 31522
Phone: 912.638.4900 Fax: 912.638.7788*

Or sent as an email attachment to: cpbr@gate.net

Scientific Progress Report

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

Principal Investigator:	Kenneth W. Nickerson	
University:	University of Nebraska at Lincoln	
Agreement Number:	OR 22072-47	
Project Title:	Bacillus thuringiensis: biotin-mediated insect toxicity suggests alternate strategies for pest control	
Reporting Period and Report Type:	From: To: November, 1999	Check one: ☐ Interim Report ☒ Final Report

Project Objectives

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

Please complete.

ATTACHED

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.

Please complete.

ATTACHED

Scientific Accomplishments

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

Please complete.

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.

ATTACHED

Please complete.

Technology Transfer

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

Please complete. None Yet

Commercial Accomplishments

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

Please complete. None Yet

Educational Accomplishments

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

Three students received extensive training in microbial physiology.
Please complete. They were: 1/ Mr. Jeffrey Swanson, an M.S. candidate in entomology;
2/ Ms. Dana Kadavy, A PhD candidate in Bio Sci; and 3/ Dr. Narasimhan Sudarsan.

Additional Funding

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

Based on the availability of CPBR starter funds, we recently were
Please complete. successful in getting \$50,000 from the Nebraska Corn Board.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

Unfortunately, Dr. Mary Ann Pfannenstiel had to resign to pay closer
Please complete attention to a family biotech start up company. Dr. N. Sudarsan was hired
as of 5/98. He worked on the project until we ran out of money (Spring '99).

Send completed report to :

*The Consortium for Plant Biotechnology Research, Inc.
P.O. Box 20634*

*(Express Delivery address: 10 Sylvan Drive, Suite 21)
St. Simons Island, GA 31522*

Phone: 912.638.4900 Fax: 912.638.7788

Or sent as an email attachment to: cpbr@gate.net

Directly Related Subject Matter

- 1/ Du, C., and K.W. Nickerson. 1996. The *Bacillus thuringiensis* insecticidal toxin binds biotin-containing proteins. *Appl. Environ. Microbiol.* 62:2932-2939.
- 2/ Du, C., and K.W. Nickerson. 1996. *Bacillus thuringiensis* HD-73 spores contain surface CryIAc toxin: physiological and pathogenic consequences. *Appl. Environ. Microbiol.* 62: 3722-3726.

Other Papers which acknowledged CPBR support

- 3/ Jensen, E.C., H.S. Schrader, B. Rieland, T.L. Thompson, K.W. Lee, K.W. Nickerson, and T.A. Kokjohn. 1998. Prevalence of broad host range lytic bacteriophages of *Sphaerotilus natans*, *Escherichia coli*, and *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 64: 575-580.
- 4/ Cho, S.C., P.H. Dussault, A.D. Lisec, E.C. Jensen, and K.W. Nickerson. 1999. Jasmonate and homojasmonate lactones as possible extracellular quorum sensing molecules for *Candida albicans*. *J. Chem. Soc. Perkin Trans 1*, 193-196.
- 5/ Kadavy, D.R., B. Plantz, C.A. Shaw, J. Myatt, T.A. Kokjohn, and K.W. Nickerson. 1999. The microbiology of oil flies. *Appl. Environ. Microbiol.* 65: 1477-1482.
- 6/ Kaiser, A., M. Vollmert, D. Tholl, M.V. Graves, J. Gurnon, W. Xing, A.D. Lisec, K.W. Nickerson, and J.L. Van Etten. 1999. *Chlorella* virus PBCV-1 encodes a functional homospermidine synthase. *Virology* 263: 254-262.

LAYPERSON'S SUMMARY

As part of his PhD thesis (University of Nebraska, 1996), Cheng Du made the provocative observation that the insecticidal protein toxins produced by *Bacillus thuringiensis* had a strong affinity for all proteins which had the vitamin/coenzyme biotin covalently attached to them. Furthermore, he noted that the model insect system *Manduca sexta* (tobacco hornworm) had two biotin-containing proteins as part of the gut system to which Bt toxins initially attach. While this evidence was suggestive, it did not prove that the biotin recognition by Bt toxin was even involved in the pathogenicity mechanism by which Bt kills insect cells. It could be just a coincidence. Part of the problem is that all known biotin-proteins in insects are sequestered in the mitochondria whereas the first stages of toxin binding occur on the outer surface of the cytoplasmic membrane, facing the gut contents. Could the Bt toxin even come in contact with a biotin-protein until after the insect had been dead a long time?

Our approach was to characterize all the biotin-containing proteins from the susceptible *Manduca sexta* larvae and then find out where they were located in the cells lining the larval gut. We were going to do this by preparing a cDNA library containing all the genes from *M. sexta* as expressed in a bacterium, screening thousands of them to see which one had the gene of interest, and then characterizing both the gene and the protein. This approach has only been partly successful. It took us much longer to make the insect cDNA library than we had anticipated and, consequently, we have not yet finished screening it. This part of the project is on hold. We have, however, laid a much better foundation for the next stage of the project: a/ We have generalized the observation of biotin-containing proteins in the insect gut to other Lepidopteran insects. Something which is general in nature is more likely to be important. b/ We have prepared the first cDNA library from the European corn borer. and c/ We have clarified that a very prominent 1 kb band in insect mRNA (and thus in the cDNA library) is obtained from mitochondrial DNA, not insect chromosomal DNA.

PROJECT OBJECTIVES

- 1/ From a cDNA library from Manduca sexta brush border cells to identify cDNA corresponding to putative biotin-containing target(s) which bind Bt toxins.

We have made substantial progress but unfortunately we have not yet cloned either of the putative receptors. Until recently there were few good cDNA libraries from insects, primarily because of the exceptionally long untranslated 3' portions of their mRNAs. Clontech's Smart III cDNA kit is designed to overcome this difficulty and we made rapid progress after it was introduced (December, 1998). We now have a cDNA library for the European corn borer Ostrinia nubilalis. The unamplified library contained 5×10^5 PFU with ca. 96% white plaques showing that they had inserts while the amplified library has 15 ml of a lysate containing 5×10^9 PFU/ml.

This library is now available for the right person to do the screening. All the protocols were worked out by my collaborator, Prof. V. Sekar, Madurai University, during the summer of 1997. Our plan was that he would return for the summers of 1998 and 1999 to complete the screening. Unfortunately, on both occasions he was denied a visa to enter the US. We are hoping that this situation will soon be straightened out and that he can join us for a sabbatical year starting in August of 2000.

We have shown that the biotin-containing putative receptor story worked out in Manduca sexta by Cheng Du (Du and Nickerson, 1996) also applies to BBMV from the European corn borer, O. nubilalis. This was the work of a M.S. student named Jeffrey Swanson. It led directly to our decision to make a cDNA library from ECBs. However, Jeff Swanson chose to stop at the M.S. level rather than pursue a PhD and we have not been able to attract another student to replace him yet.

- 2/ To show that the ability of *Bacillus thuringiensis* toxins to bind biotin proteins is causally related to the presence of biotin proteins in Brush Border Membrane Vesicles (BBMVs) of susceptible insects.

This part of the project also made some progress but eventually bogged down and is currently on hold. Two approaches were to be taken. The first was to design a Bt mutant by site directed mutagenesis in which the amino acid residues thought to be critical for biotin binding were replaced by alanine. The modifications in the CryIAc toxic were to be at positions: V-531, Y-533, S-535, T-558, and V-598. Unfortunately, we had a break down in communications with our corporate sponsor, Ciba-Geigy at Research Triangle Park, NC, and the indicated mutagenesis was conducted not in the Lepidopteran active CryIAc but in the Coleopteran active Cry IIIA. We are still trying to get the multiple alanine mutant constructed for CryIAc.

The second part was to be a microscopy approach using streptavidin to detect where in insect BBMVs the biotin-containing proteins were located. Before she left, Dr. Mary Ann Pfannenstiel pursued this project far enough to show that standard fixation procedures destroyed exposed biotins and she then tried to develop an alternative, milder fixation protocol. We have not worked in this area since she left.

In sum, neither objective was finished. Both are on hold awaiting the arrival of a new graduate student or technician. Alternatively, when Dr. Nickerson returns from sabbatical (August 2000) he will tackle them himself.

SCIENTIFIC ACCOMPLISHMENTS

1/ Cloning two genes from a *Manduca sexta* cDNA library encoding biotin-containing proteins.

Our first attempts did not work. We obtained a cDNA library prepared from *M. sexta* gut tissue as a gift from Prof. Michael Wells. It was a lambda zap library and had been prepared in 1994. The lambda lysates from *E. coli* XL1Blue MRF were screened by plaque western lifts with streptavidin-alkaline phosphatase. There was a very low background color because *E. coli* contains some biotin proteins of its own. Two of ca. 15,000 plaques were positive. These two plaques were used to infect *E. coli* SOLR Strain (which does not contain a nonsense suppressor). In the presence of a helper phage the cloned gene is excised and inserted into the plasmid pBluescript suitable for sequencing. However, when sequenced, both plaques appeared to contain truncated genes corresponding to proteins of <20 kDa.

At this point we learned that cDNA libraries from Lepidopteran insects were notorious for their high frequency of truncated genes. This defect likely occurs because their mRNAs have long untranslated regions and the greater length of the mRNAs makes it less likely that the entire mRNA was copied. We then decided that we needed to make our own cDNA library using a protocol designed especially for long mRNAs. On the positive side, we knew that our streptavidin based screen worked. The *E. coli* background was very low and, at least in two cases, biotin was attached to the insect protein. This latter point had been inferred from the universal biotin attachment sequence (AMKM) but it was nice to confirm.

2/ Equating *Manduca sexta* and *Ostrinia nubilalis* larvae

All of our previous work on biotinylated proteins as potential toxin receptors was done with the tobacco hornworm *Manduca sexta*. We wanted to see if the same story was true for larvae of the European corn borer *Ostrinia nubilalis*. We did this for two reasons. First, we wanted to make sure the presence of biotinylated proteins in the BBMV's was generally true for Lepidopteran insects and not characteristic only of *M. sexta*. Second, we wanted to make our research more relevant to transgenic corn.

Western blot analysis of BBMV's prepared from ECB larvae showed that both streptavidin and neutravidin reacted with proteins of ca. 85 and 120 kDa. Furthermore, when the BBMV proteins were separated on SDS-7.5% PAGE instead of the SDS-10% PAGE which we had used previously, the 120 kDa biotin-containing protein was clearly separated from the 120 kDa Cry 1Ac binding protein. As has been shown in several other insects, the 120 kDa Cry 1Ac binding protein appears to be aminopeptidase N because it also reacted with anti-aminopeptidase polyclonal antibodies (courtesy of Don Dean, Ohio State University). Thus, with regard to streptavidin reactive proteins, *M. sexta* and ECB larvae appear to be functionally identical.

3/ Preparation of a cDNA library from European Corn Borers

Initially we tried to use Clontech's Smart II kit because this system uses a modified oligo dT primer that anneals to the junction of the poly A tail and the rest of the mRNA, thus avoiding problems with very long poly A tails. In addition, it employs a novel 5' cap oligonucleotide so that only full-length cDNAs are amplified. However, we were still having problems with the linkers needed to insert the ds cDNA and with the in vitro packaging step. Both these problems were corrected in the Clontech Smart III kit and we made rapid progress once that kit was available.

Our cDNA library was prepared from roughly 200 neonate larvae of ECB. It is a lambda gt11 expression library. The unamplified library contained 5×10^5 PFU of which 96% were white plaques indicating DNA insertion. The amplified library has 15 ml of lysate containing 5×10^9 PFU/ml.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Basil Nikolau	
University:	Iowa State University	
Agreement Number:	OR22072-73	
Project Title:	<i>How Do Plants Generate Acetyl-CoA?</i>	
Reporting Period and Report Type:	From: 1/1/99 To: 6/30/99	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

Genetically reduce the accumulation of specific enzymes required for acetyl-CoA generation

Determine the effect of each genetic alteration on:
 the accumulation of acetyl-CoA-derived phytochemicals
 the expression of acetyl-CoA-generation genes
in vivo measurements of the generation of different acetyl-CoA pools

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.

Our specific goals are aimed at identifying the mechanisms by which two physically distinct pools of acetyl-CoA are synthesized in plants. These two pools, one in the plastids and the other in the cytosol, are the precursors for the synthesis of a large number of plant chemicals that have utility in nutritional and industrial applications. Specifically, the plastidic acetyl-CoA-pool is used to produce fatty acids, and hence seed oils; and the cytosolic acetyl-CoA-pool is used to produce pigments, waxes and many compounds that plants use for defense from biotic and abiotic stresses.

We are determining the role of five enzymes in generating these two pools of acetyl-CoA. These enzymes are: pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALDH), acetyl-CoA synthetase (ACS), plastidic pyruvate dehydrogenase complex (pPDHC), and ATP-citrate lyase (ACL).

Our previous studies have enabled us to formulate the following three hypotheses:

1. pPDHC, rather than ACS, is the key enzyme that generates the plastidic acetyl-CoA-pool during seed development.
2. ACL is the enzyme that generates the cytosolic pool of acetyl-CoA.
3. The coordinate action of PDC, ALDH and ACS generates a specialized plastidic pool acetyl-CoA.

To test these hypotheses we are isolating and characterizing the genes of *Arabidopsis* that code for PDC, ALDH, ACS, pPDHC and ACL. These cloned genes will be used to generate transgenic plants that show reduced PDC, ALDH, ACS, pPDHC and ACL expression. These transgenic plants will be used to determine the effect of each of these genetic manipulations on the plant's ability to generate acetyl-CoA. Hence, these genetic manipulations will test the above hypotheses.

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 specific scientific achievements are described below in reference to each of the acetyl-CoA-generating enzyme:

Acetaldehyde dehydrogenase (ALDH):

Full-length cDNA clones of AtALDH1, AtALDH2, AtALDH3, and AtALDH4 were completely sequenced. The AtALDH1 and AtALDH3 proteins contain predicted mitochondrial targeting sequences at their N-termini and their sequences cluster with other plant mitochondrial ALDHs following phylogenetic analysis; AtALDH2 does not contain a mitochondrial targeting sequence and clusters with cytosolic ALDHs. Blast analysis revealed that AtALDH4 has a high similarity with mammalian fatty aldehyde dehydrogenase. In phylogenetic analyses AtALDH4 clusters with mammalian microsomal ALDHs. It is therefore hypothesized that AtALDH4 is involved in the alpha oxidation of fatty acids. The AtALDH1, AtALDH2, AtALDH3 and AtALDH4 genes were physically mapped and gene structures determined via comparisons to the *Arabidopsis* genome sequence. To link the AtALDH1, AtALDH2 and AtALDH3 proteins with functions antisense binary plasmids were constructed in which each of these three genes was driven by the napin or d35S promoter and transformed into *Arabidopsis* Columbia ecotype. Transgenic T1 plants are under analysis.

Pyruvate decarboxylase (PDC):

Full-length cDNA clones of AtPDC1 and AtPDC2 are completely sequenced. A phylogeny tree was constructed by comparing plant and fungal PDC sequences. The AtPDC1 and AtPDC2

genes were physically mapped and gene structures determined via comparisons to the *Arabidopsis* genome sequence (Figure 2). To link the AtPDC1 and AtPDC2 proteins with functions, antisense binary plasmids are being constructed in which both of these genes are driven by the napin or d35S promoter. Transformation experiments are underway.

Acetyl-CoA synthetase (ACS):

Much of our effort on ACS has been directed towards completing the analysis of the ACS protein and on determining the effects of down regulation of the ACS protein in *Arabidopsis* plants.

Protein chemistry of ACS. ACS is a 70 kDa, monomeric protein. Enzyme activity is dependent on acetate, the Mg^{+2} salt of ATP, and CoASH as substrates. Potassium strongly increases enzyme activity as does preincubation with dithiothreitol (DTT). The enzyme is inhibited by diamide and this is reversed by DTT suggesting that there is a key disulfide linkage that is important in enzyme activity.

Regulation of ACS activity in *Arabidopsis*. We used antisense expression of the ACS cDNA to create a group of *Arabidopsis* mutants with reduced ACS levels. By means of our sensitive enzyme assay and the antibody we have prepared against the ACS protein we have demonstrated that these plants express ACS levels ranging between wild type levels down to about 10% of wild type levels. These plants show no morphological differences when compared to wild type plants. When the ACS level is lowered below about 20% of the wild type level, the plants are delayed in flowering and some are extremely stunted and never reach a reproductive phase. As these plants are taken through 4 generations of crossing, the phenotypes (including ACS enzyme levels) return toward wild type. Lipid analysis in a group of plants with altered ACS levels are underway.

Plastidic pyruvate dehydrogenase complex (pPDHC):

Cloning the genes for pyruvate dehydrogenase (PDH). This enzyme is composed of four subunits, E1 α , E1 β , E2 and E3. We have isolated and sequencing cDNA clones coding for all four of these subunits. These analyses have shown that in *Arabidopsis* whereas the E1 α and E2 subunits appear to be each coded by a single gene, the E1 β and E3 subunits are each coded by at least two genes.

Biochemical analysis of pPDHC mutants. We have produced *Arabidopsis* plants, which should under accumulate pPDHC. This has been done by expressing the antisense E1 RNA. We have had difficulty characterizing these plants because we can not differentiate between the plastid and mitochondrial form of the E1 subunit. Antibody produced against the plastid E1 subunits cross-reacts with the mitochondrial E1 subunit. In order to overcome this problem we have decided to repeat these experiments using the E2 subunit. We have identified a short region the plastid E2 subunit that is not found in the mitochondrial subunit. A oligopeptide has been produced based on this sequence and polyclonal antiserum has been prepared in rabbits against this oligopeptide. This antibody is specific for the plastid E2 subunit. We are presently producing *Arabidopsis* plants that are expressing the antisense E2 RNA.

ATP-citrate lyase (ACL):

ACL accumulation was altered in transgenic *Arabidopsis* plants by the expression of ACL antisense RNAs. We made gene constructs in which *ACL-A1* or *ACL-B2* cDNAs are fused to the CaMV 35S promoter in opposite orientation from normal. The resulting transgenes were introduced into the *Arabidopsis* genome by *Agrobacterium*-mediated transformation, and transformants were selected on the basis of resistance to kanamycin, a trait physically linked to each transgene. We obtained over 100 independent transgenic lines, as indicated by kanamycin resistance in the T1 generation and substantiated by further testing in the T2 generation. Over 50 independent lines were selfed, and selected T2 lines were propagated to the T3 generation.

The focus of the past six months was the generation and characterization of these transgenic lines. In particular, the phenotype of the plant lines at multiple developmental stages has been characterized. The most severe phenotype among the plant lines that survive and bare progeny,

consists of a much reduced plant body, shorter and thinner inflorescence stalks, smaller (or non-opening) flowers, reduced and occasionally early senescing petals, and anthers appear to have a problem with timing or mechanisms of dehiscence. If siliques are present, they are much reduced, partially filled with seed or empty. The seed within siliques is often smaller, and appear desiccated. Dehiscence within filled siliques is often premature (ie siliques will dehisce when half of the seed is mature and the other half is green). Anthers appear to have a problem with timing or mechanisms of dehiscence. Leaves are very reduced and often have anthocyanin production.

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Two genes coding for each of the two subunits of ACL have been isolated. These are termed ACL-A1, ACL-A2, ACL-B1 and ACL-B2. To investigate the expression patterns of each of these genes, promoter-GUS transgenes are being prepared.

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Technology Transfer

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None.

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Research undertaken in conjunction with this grant is providing opportunities to educate and train undergraduate and graduate students in research. In addition, this grant is providing continued research training of post-doctoral research associates. This grant is providing multidisciplinary training in the areas of plant molecular biology, biochemistry, genetics with the aim of solving a complex problem in plant metabolism.

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NONE

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Scientific Progress Report

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

Principal Investigator: Basil Nikolau

University: Iowa State University

Agreement Number: OR22072-73

Project Title: *How Do Plants Generate Acetyl-CoA?*

**Reporting Period and
Report Type:**

From: 8/1/98
To: 11/30/99

Check one:

☒ Interim Report
☐ Final Report

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Commercial Accomplishments

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Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	John B. Ohlrogge and Christoph Benning	
University:	Michigan State University	
Agreement Number:	OR22072-98	
Project Title:	DNA Discovery of Genes and Networks Which Control Plant Seed Storage Products	
Reporting Period and Report Type:	From: 10/1/98 To: 9/30/99	Check one: ☒ Interim Report ☐ Final Report

Project Objectives:

Progress made in first year is summarized below in italics and underlined and also is presented with figures in the following section.

I. Partial Sequencing of cDNAs from an *A. thaliana* Seed Library: Approximately 12,000 of the estimated 20,000 genes of *A. thaliana* are already partially sequenced (Rounsley et al., 1996). We plan to expand this database by sequencing at least 6,000-8,000 clones from an *A. thaliana* developing seed library prepared for the purpose of this project. *This objective has been accomplished.*

II. Identification of Arabidopsis Genes Specifically Expressed in Developing Seeds: The sequence data generated will be analyzed with the objective to assemble "contigs" covering the respective cDNAs and to identify cDNAs encoding proteins with similarity to those of known function. PCR products from sequenced clones will be applied to glass slides in high density DNA microarrays. Equipment that can array and analyze over 10,000 DNA samples in an area of 2 x 6 cm on a glass slide has recently been installed at MSU. Probing these arrays with fluorescent labeled first strand bulk cDNA from seeds of different developmental stages and vegetative tissues will allow us to identify *A. thaliana* genes specifically expressed in seeds. *This objective has been accomplished except that seeds of different developmental stages have not yet been analyzed.*

III. Global Expression Analysis in Selected Mutants and Transgenic Plants: The two stages mentioned above are a prerequisite to initiate the actual analysis of metabolic regulation by differential gene expression in developing seeds. This will be done by comparing global gene expression in selected mutants of *A. thaliana*, and transgenic plants of *A. thaliana* and in some cases of *Brassica napus*. For this purpose, microarrayed cDNAs of *A. thaliana* as described above will be probed with fluorescent

labeled first strand bulk cDNAs from wild type and mutants or different transgenic plants. In particular, two biochemically well characterized seed mutants, wri1 (Focks and Benning, 1998) and tag1 (Katavic et al., 1995), with low oil content or altered fatty acid composition will be tested. Both mutants show a complex phenotype at the level of seed metabolism that is not yet understood and may be the consequence of altered regulation of seed metabolism. The experiment should provide clues to answer the question, whether WRI1 or TAG1 encode regulators of seed metabolism. Preliminary experiments for this objective have been started.

Scientific Accomplishments:

This report is based on a presentation covering work done during year one of the funding period given at the National Plant Lipid Cooperative meeting at South lake Tahoe, June, 1999.

Abstract: Transcriptional Profiling of Seed Development using cDNA Microarrays.

T. Girke, J. Todd, J. White* C. Benning*, and J. Ohlrogge

Department of Botany and Plant Pathology, Michigan State University

*Department of Biochemistry, Michigan State University

Little is known about the regulation of fatty acid and lipid synthesis in seeds. To analyze the controlling machinery of these pathways, we monitored the seed-wide gene expressions from Arabidopsis using the cDNA microarray technique. A non-redundant set of about 3,000 expressed sequence tags (ESTs) from a seed-specific library of Arabidopsis and a collection of ESTs involved in glycerolipid synthesis was placed in high-density arrays on coated microscope slides. These arrays were probed with first strand cDNA fluorescent probes reverse transcribed from mRNAs of different sources. The reliability of each microarray experiment was tested with a complex set of controls, including mRNA quality controls, sensitivity controls and specificity controls.

For monitoring the large-scale gene expressions we first analyzed the seed specific expressed cDNAs by comparisons of probes from seed with those from leaves and roots. Second, we plan to investigate the alterations of gene expression during seed development with probes from different time stages of developing seeds. Third, we will use selected mutants with alterations in seed oil composition to identify new genes involved in the regulation of fatty acid and lipid deposition in seeds. We expect that the microarray data of these experiments will guide us to uncover new approaches to engineer plant lipid metabolism.

Content of storage products

- The above figure shows the developmental profile of developing Arabidopsis seeds.

cDNA Library from Developing Arabidopsis Seeds

ESTs Analyzed by Hybridization:	27,648
ESTs Sequenced (250-350 bp):	11,068
ESTs Processed:	10,395 (100%)
Contigs	1,301 (64%)
Singlets	3,729 (36%)
Unique RNAs <	5,030
BLASTX Analysis: Score < 100	2,456 (24.8 %)

The Seed Microarray Project

- The initial arrays were produced from 2700 cDNA clones selected from 5000 clones sequenced at MSU and selected to be unique. The second generation arrays include approximately 5000 cDNA clones sequenced both at MSU and by InCyte.

10,000 cDNAs from a developing seed library of Arabidopsis have been partially sequenced from the 5'-end. A data base has been established as the basis for further Bioinformatic Analysis.

The Arabidopsis Seed EST Database

Summary of Seed EST Database Contents

Code	Code Description	Number	Percent
A A	amino acid metabolism	141	3.4
C	carbohydrate metabolism	268	6.3
C D C	cell division cycle	8	0.2
C H P	chaperonin, heat shock, folding	29	0.7
C S K	cytoskeleton	58	1.4
C W	cell wall	82	2.0
D	defense, disease	17	0.4
D E V	development	127	3.0
D N A	DNA modifying enzymes	31	0.7
H O R	hormone biosynthetic enzymes	21	0.5
L	lipid metabolism	170	4.0
M T	membrane, transporters, receptors	92	2.2
N M	N metabolism	15	0.4
N S H	non-significant homology	1043	24.8
N U C	nucleus	17	0.4
N U M	nucleotide metabolism	24	0.6
O X	oxygen detoxifying enzymes, peroxidases	66	1.6
P A	proteinases, ubiquitin	133	3.2
P S	photosynthesis	99	2.4
R B	ribosome, protein translation	142	3.4
R N A	acting on RNA	60	1.4
R S	respiration	36	0.9
S E M	secondary metabolism	79	1.9
S M	S-metabolism	7	0.2
S P	storage protein	610	14.5
S T D	signal transduction (kinases, calmodulin)	153	3.6
T	transcription factors	91	2.2
T O N	tonoplast	17	0.4
T R F	trafficking	12	0.3
U F	unidentified function	559	13.3
Total		4205	100.0

Arabidopsis Seed Array (7680 spots)

Arabidopsis mRNA: ●Leaves ●Seeds

Clones were spotted in duplicate. This figure shows the reproducibility of the duplicate patterns.

An *Arabidopsis* Microarray

Approx. 20% of elements show >2 fold difference

525 EST's, each spotted four times. Probes prepared from mRNA isolated from *Brassica napus* seed (Cy3) and leaf (Cy5).

PCR amplified cDNA clones are spotted in arrays onto pretreated glass slides at a density of up to 10,000 per cm². Two polyA RNA samples are prepared from different tissues, developmental seed stages, or mutant and wild-type embryos. These are reverse transcribed into cDNA. The abundance of RNAs corresponding to the cDNAs on the array can be compared in two different samples following the labeling with two dyes and hybridization of the two complex probes to the array. Data are acquired using a two channel laser scanner.

Differentially Expressed Genes

- This figure indicates the ratio of expression in seeds vs. leaves for several well known tissue specific genes.

Arabidopsis Seed Array: Controls

Arabidopsis mRNA: ● Leaves ● Seeds

Synthetic control clones with 100%, 90% and 80% sequence identity were produced and used to indicate the level of cross-hybridization.

Human cDNA clones were spotted and their mRNA spiked into the plant mRNA population to estimate the sensitivity of the technique.

Expression Profile Arabidopsis seeds vs. leaves

This figure is a plot of the expression patterns in leaves versus seeds. The plot gives the ratio of expression from probes synthesized from leaf and seed mRNA. Red spots indicate clones involved in lipid metabolism.

Expression Profile B. napus seeds vs. leaves

Brassica napus

B. napus mRNA: ○Leaves ●Seeds

We have also demonstrated that *Brassica napus* RNA cross-hybridizes well with our *Arabidopsis* arrays.

Mutants of *Arabidopsis* are available which are deficient in the accumulation of seed oil. E.g., the *wrinkled 1* mutant produces shrunken seeds with a low oil content (right panel). The affected gene is thought to be involved in the developmental regulation of seed carbohydrate metabolism.

WT

wri1-1

Expression Profile *wrinkled1-1*

wri1-1 seeds

Note that the difference between mutant and wild type is much less than between leaf and seed comparisons shown in the previous expression profiles.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Brenda Oppert	
University:	Kansas State University	
Agreement Number:	OR22072-99	
Project Title:	<i>Evaluation of Insect Serpins as Biopesticides</i>	
Reporting Period and Report Type:	From: 6/30/99 To: 09/30/1999*	Check one: ☒ Interim Report ☐ Final Report

*Please note that an extension was approved for Year One until March 31, 2000. Matching funding from Dow AgroChemical is expected October, 1999.

Project Objectives

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

Objective #1: Assess the potential of insect serpins to control the Indianmeal moth, *Plodia interpunctella*.

Progress to date: Jayme Morris, Research Associate in the Department of Biochemistry, Kansas State University, has been expressing different serpin constructs in *E. coli* for use in biological and *in vitro* assays.

Objective #2: Evaluate the potential of insect serpins to delay resistance development to the microbial pesticides from *Bacillus thuringiensis* (Bt).

Progress to date: This objective was scheduled during year 2 of the funding.

Objective #3: Provide *M. sexta* serpins for bioassay of the corn rootworm, *Diabrotica* spp., and corn earworm, *Helicoverpa zea*.

Progress to date: We have continued to dissect corn rootworm larvae for the evaluation of corn rootworm digestive proteinases in larvae fed corn or an artificial laboratory diet. Analysis of corn rootworm digestive proteinases will begin in October, 1999.

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.

Previously, research funded by this proposal showed that an insect-derived protein that inhibits digestive enzymes was effective in reducing the activity of digestive enzymes from the Indianmeal moth, a problem pest of stored grains and products. When the protein was added to the Indianmeal moth diet, larvae either developed more slowly or died. The gene encoding this protein could be transferred into cereal grains to provide protection from Indianmeal moth infestation.

Work during the second half of the reporting period was stalled due to a delay in obtaining matching funding. However, progress was made on the expression of insect proteins in preparation for future testing and bioassays with the corn rootworm. In addition, personnel in Manhattan worked with Dow AgroChemical personnel in San Diego to obtain extracts of corn rootworm gut fluid to evaluate prior to bioassay.

Scientific Accomplishments

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

Three constructs of *Manduca sexta* serpins were expressed in a recombinant *E. coli* system. Proteins were purified and collected for future testing in the Indianmeal moth and corn rootworm.

Rootworms were reared on corn or on an artificial diet were shipped to Manhattan, where they were dissected and guts were extracted for future analysis. Gut extracts were placed in buffer in aliquots and frozen at -20°C .

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.

Oppert, B. "Serpins as Potential Biopesticides." Presented to Dow AgroChemicals, San Diego, CA, August, 1999.

Technology Transfer

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

No technology transfer was initiated during the reporting period.

Commercial Accomplishments

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

The discovery of the potential for serpins with chymotrypsin inhibitory activity to affect the growth and development of *P. interpunctella* larvae may lead to the development of a new biopesticide for this pest of stored grains and products.

Educational Accomplishments

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

This project has served as instruction for teaching basic biochemical, microbiological, and insect bioassay concepts to undergraduate students. The project has also provided training in these areas for research associates and personnel working in the laboratory.

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.

Jayne Morris, Research Associate in the Department of Biochemistry, is assisted in this project by Ms. Michele Zuercher, laboratory technician for Dr. Oppert, and Kris Hartzler, KSU student in the Department of Entomology.

Send completed report to :

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Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Brenda Oppert	
University:	Kansas State University	
Agreement Number:	OR22072-99	
Project Title:	<i>Evaluation of Insect Serpins as Biopesticides</i>	
Reporting Period and Report Type:	From: 10/01/1998 To: 03/31/2000*	Check one: ☒ Interim Report ☐ Final Report (Year 1)

*Please note that an extension was granted for Year 1 funding to 03/31/2000. This report is a compilation of the Interim Report on 06/30/99 and data obtained since that date. A list of abbreviations follow the References.

Project Objectives

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

Objective #1: Assess the potential of insect serpins to control the Indianmeal moth, *Plodia interpunctella*.

Progress to date: Previous research with *P. interpunctella* digestive proteinases indicated that major activities were attributed to serine proteinases, predominately trypsin- and chymotrypsin-like proteinases, with minor elastase-like proteinases. Accordingly, three serpin constructs inhibiting trypsin, chymotrypsin, and elastase proteinases were chosen to test with *P. interpunctella* larvae. Recombinant serpins were produced and purified. In *in vitro* tests, the serpin with chymotrypsin-inhibitory activity was the most effective in inhibiting digestive proteinase activity of *P. interpunctella*. In bioassays, the chymotrypsin inhibitor was effective in slowing larval development and increasing mortality of *P. interpunctella* larvae.

Objective #2: Evaluate the potential of insect serpins to delay resistance development to the microbial pesticides from *Bacillus thuringiensis* (Bt).

Progress to date: This objective will be completed during year 2 of the funding.

Objective #3: Provide *M. sexta* serpins for bioassay of the corn rootworm, *Diabrotica* spp. (Note: this objective initially included the corn earworm, *Helicoverpa zea* in serpin bioassays. However, Dow scientists have indicated a more immediate target is the corn rootworm, and collectively we have chosen to concentrate on this insect pest).

Progress to date: Working with Dow's San Diego laboratory, we have evaluated digestive proteinases of western corn rootworm fed corn or an artificial diet. No significant differences were observed in gut pH or proteinase activities in larvae fed either diet, thus validating the use of artificial diet in the bioassay of *M. sexta* serpins. Preliminary analysis of serpin variants identified several potential serpins for testing with western corn rootworm larvae.

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.

During the reporting period, research funded by this proposal showed that an insect-derived protein that inhibits digestive enzymes was effective in reducing the activity of digestive enzymes from the Indianmeal moth, a problem pest of stored grains and products. When the protein was added to the Indianmeal moth diet, larvae either developed more slowly or died. The gene encoding this protein could be transferred into cereal grains to provide protection from Indianmeal moth infestation.

Corn rootworm reared in the laboratory on corn roots or an artificial diet displayed no major differences in gut physiology. These results indicated that the artificial diet, a more convenient and accurate method of testing potential control proteins, can be used to test inhibitors of digestive enzymes. Several inhibitors were identified that will be tested with western corn rootworm larvae to assess their potential as insect control proteins.

Scientific Accomplishments

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

Literature Review

Review of *Plodia interpunctella* digestive proteinases. At the initiation of this project, data on *P. interpunctella* digestive proteinases was summarized. As much of this data was performed at GMPRC and is unpublished, and because the data have relevance to the present study, details are included in this report.

In work by Dr. Karl Kramer, Research Chemist at GMPRC, proteinase inhibitors were bioassayed with *P. interpunctella* larvae, using a wheat germ artificial diet (89.7% wheat germ, 0.15% sorbic acid, 0.15% methyl-p-hydroxy-benzoate, 10% glycerol). Each treatment consisted of 1.65 g of diet, divided into 10 equal portions in a microcentrifuge tube. One egg was added per tube. All inhibitors were tested at 1% (w/w). Results are the average larval weight at 7 days, $\pm$ S.E. (Table 1).

Antipain, CTI, E-64, E-64 + SBBI, and LP were the most effective inhibitors. Antipain and leupeptin target serine and cysteine proteinases, and E-64 can inhibit both serine and cysteine proteinases in lepidopteran insects. Trypsin inhibitors SKTI and SBTI had a small but insignificant effect. Similarly, SBBTI and AP, which target both trypsin and chymotrypsin, had

no significant effect. Interestingly, individually SBBI and E-64 had no effect, yet SBBI combined with E-64 reduced the growth of IMM larvae. LP was the most effective inhibitor, resulting in 100% mortality.

Table 1. Effect of proteinase inhibitors on the growth of *P. interpunctella* larvae.

Treatment	Mean Larval Weight (n)
Control (no inhibitor)	9.59 ± 0.87 (7)
Soybean bean Bowman Birk trypsin inhibitor (SBBTI)	10.06 ± 0.88 (9)
Soybean Kunitz trypsin inhibitor (SKTI)	9.15 ± 1.19 (5)
Lima bean trypsin inhibitor (LBTI)	9.04 ± 0.55 (8)
Aprotinin (AP)	9.00 ± 0.68 (8)
Antipain	7.30 ± 0.96 (7)
Corn trypsin inhibitor (CTI)	6.68 ± 0.83 (8)
E-64	6.64 ± 0.73 (8)
1% E-64 + 1% SBBTI	4.75 ± 0.90 (8)
Leupeptin (LP)	no larva survived

Another experiment in Dr. Kramer's laboratory measured the mean development time and mortality of *P. interpunctella* larvae on untreated or inhibitor-treated diets, with the diet and procedure the same the previous bioassay (Table 2).

Table 2. Effect of proteinase inhibitors on the development and survival of *P. interpunctella*.

Treatment	Days from Egg Hatch to Adult Eclosion (n) ¹	Mortality (%)
Control (no inhibitor)	19.5 ± 0.7 (10) a	0
1% CTI	21.1 ± 0.4 (8) a	11
1% CTI + 0.1% E-64	19.9 ± 0.3 (10) a	0
1% LBTI + 0.1% E-64	20.6 ± 0.7 (8) a	11
1% SBBTI + 0.1% E-64	20.4 ± 0.6 (9) a	10
1% SKTI + 0.1% E-64	20.3 ± 0.4 (10) a	0
1% AP + 0.1% E-64	20.6 ± 0.6 (8) a	11
1% AP + 1% SKTI + 0.1% E-64	21.0 ± 0.4 (10) a	0
0.1% LP	25.0 ± 0.8 (5) b	38
1% LP	0	100
0.1% E-64	19.7 ± 0.7 (7) a	22

¹Treatments with the same letter are not significantly different, as analyzed by Tukey, $\alpha < 0.05$.

In this study, 0.1 and 1% leupeptin significantly slowed the growth and increased mortality of *P. interpunctella* larvae. Combinations of various inhibitors with E-64 (0.1%) had no effect. Although 0.1% E-64 caused a slight increase in mortality, development time was similar to the control group.

The effect of leupeptin on the *in vitro* activity of gut extract from *P. interpunctella* larvae with the synthetic trypsin substrate N- α -benzoyl-L-arg p-nitroanilide (BAPNA) was substantial (Fig. 1). The lowest amount tested (50 ng) inhibited the gut extract by 52%.

In another study, the effect of reducing reagents dithiothreitol and β -mercaptoethanol on the BAPNA-activity of gut extract from Bt-susceptible and resistant *P.* was measured (unpublished data). A 10-20 % reduction in activity was observed with proteinases from Bt-susceptible insects (unpublished data). However, less than 5% reduction was observed with gut extracts from Bt-resistant insects. Sulphydryl reducing agents will commonly stimulate the activity of cysteine proteinases, but some enzymes, such as cathepsin B, are inhibited. This data is only useful to illustrate differences in digestive-proteinases from Bt-susceptible and -resistant *P. interpunctella*.

Another *in vitro* study of Bt-susceptible *P. interpunctella* proteinases indicated that the best inhibitors of BAPNA activity were LP (IC_{50} 2.7 pM), SBBTI (0.1 μ M), antipain (0.2 μ M), and some inhibition with chymostatin, TLCK, aprotinin, SKTI, potato inhibitors (PI) I and II, and E-64 (Oppert *et al.*, 1996). Interestingly, hen's egg white cystatin, a specific cysteine proteinase inhibitor, was similar to E-64 in the inhibition curve. No inhibition was observed with EDTA, bestatin, iodoacetamide, TPCK, elastatinal, calpain inhibitor I, and leech elastase inhibitor. The best inhibitors of chymotrypsin activity, as measured with the synthetic substrate N-succinyl-alala-pro-phe p-nitroanilide (SAAPFpNA) were PI-I (0.5 μ M), PI-II (0.9 μ M), and some inhibition with SBBTI, SKTI, chymostatin (μ M levels) and leech elastase inhibitor and PMSF (mM levels). No inhibition was observed with antipain, AP, TPCK, TLCK, E-64, leupeptin, pepstatin, cystatin, EDTA, iodoacetamide, bestatin, elastatinal, benzamidine, and gut extracts from Bt-resistant insects.

Similar results with inhibitors were reported in a comparison of gut digestive enzymes from Bt-susceptible and -resistant larvae (Johnson *et al.*, 1990). These results suggest that the major digestive proteinases in *P. interpunctella* are trypsin-and chymotrypsin-like, but cysteine proteinases may also play a minor role in digestion.

Review of *Diabrotica sp.* digestive proteinases. Several enzymes have been characterized from rootworm, including acetylcholinesterase from WCR beetles (Gao *et al.*, 1998) and lipase from SCR eggs (Krysan and Guss, 1973). Fifteen digestive proteinase activities were isolated for WCR larvae using ion exchange chromatography and gelatin zymograms (Gillikin *et al.*, 1992). Hydrolysis of radiolabeled substrates had pH optima from 4 to 7. Most of the activities were reduced by cysteine proteinase inhibitors, including egg white cystatin. At least eight proteinases were described from midguts of SCR larvae (Edmonds *et al.*, 1996). Approximately 2/3 of the activity was inhibited by E-64 or cystatins, and 1/3 was inhibited by pepstatin, an aspartic proteinase inhibitor.

Various peptides and proteins have insecticidal activity against rootworm larvae. *Bacillus thuringiensis* toxin Cry3B2 was active in SCR larvae (Donovan *et al.*, 1992). Cysteine proteinase inhibitors from soybean were more inhibitory to gut enzymes from third instar WCR larvae than E-64 (Zhao *et al.*, 1996). Cystatin from rice was inhibitory *in vitro* to gut extracts and *in vivo* to growth of larvae of SCR (Edmonds *et al.*, 1996). Peptides from the venom of the

spider *Tegenaria agrestis* were paralytic to rootworm beetles (Johnson *et al.*, 1998). Toxins from *Photorhabdus luminescens* have potency in SCR similar to Bt toxins in Lepidoptera (Guo *et al.*, 1999).

Year 1 Research Results

Production of recombinant serpins. Serpins were expressed in an *E. coli* expression system. Plasmids containing each mutant construct or variant were transformed into *E. coli* JM-109 cells, using standard molecular biology procedures as previously described (Jiang *et al.*, 1995). Each construct or variant contained a metal affinity binding amino-terminus (Met-His₆) as described in Jiang and Kanost (1997). Recombinant serpins were purified using Ni-NTA resin (Qiagen) and imidazole elution. Purified serpins were evaluated by sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis (PAGE) and Coomassie staining (Fig. 2). Three additional preparations have been performed to obtain serpins for bioassay. The concentration of mutant serpins varied with each preparation, from approximately 0.3 to 1.2 µg/ml, with volumes ranging from 0.5 to 1.5 ml per preparation. Serpin variants were kindly supplied by Dr. Haobo Jiang for testing with *Diabrotica virgifera* gut extracts for inhibition of caseinolytic activity (Table 3).

Table 3. Serpin variants, predicted or known reactive sites, and inhibitor activity.

VARIANT	REACTIVE SITE*	INHIBITORY ACTIVITY
A	FIT <u>R</u> QARL	Bovine pancreatic trypsin, Human plasmin, <i>Metarhizium anisopliae</i> PR2
B	IVP <u>A</u> SLIL	Human cathepsin G, Porcine pancreatic elastase, Human neutrophil elastase, <i>Tritirchium album</i> proteinase K, <i>Bacillus licheniformis</i> subtilisin, <i>Metarhizium anisopliae</i> PR1
C	FIIE SYSS	<i>Tritirchium album</i> proteinase K, <i>Metarhizium anisopliae</i> PR1
D	RGIR PRPS	none
E	RVVK KKRF	<i>Metarhizium anisopliae</i> PR2
F	IAV <u>V</u> DSID	Porcine pancreatic elastase, Human neutrophil elastase, <i>Bacillus licheniformis</i> subtilisin
G	IVGI TSIQ	<i>Bacillus licheniformis</i> subtilisin, <i>Diabrotica virgifera</i> crude gut extract
H	FIT <u>Y</u> VESI	Bovine pancreatic α-chymotrypsin, Human cathepsin G, <i>Tritirchium album</i> proteinase K
I	IV <u>A</u> L SLEF	Bovine pancreatic α-chymotrypsin, Human cathepsin G, Porcine pancreatic elastase, Human neutrophil elastase
J	LTDR CCSD	<i>Metarhizium anisopliae</i> PR2, <i>Manduca sexta</i> prophenoloxidase activating enzyme
K	IT <u>T</u> Y SKHF	Bovine pancreatic α-chymotrypsin, Human cathepsin G, <i>Tritirchium album</i> proteinase K
Z	GI <u>A</u> Y LSAV	Bovine pancreatic α-chymotrypsin, Human cathepsin G, <i>Diabrotica virgifera</i> crude gut extract

*Predicted P1, P1' sites are bolded; Known P1 sites are underlined.

Serpin-1B mutants were selected based on previous studies with *P. interpunctella* digestive enzymes that indicated trypsin-, chymotrypsin-, and elastase-like activities were important components of digestion (Johnson *et al.*, 1990; Christeller *et al.*, 1992; Oppert *et al.*, 1996). The mutant variant cDNA's selected were:

- Alaserpin (A), which has demonstrated activity against elastase enzymes.
- Pheserpin (F), which has demonstrated activity against chymotrypsin enzymes.
- Lysserpin (K), which has demonstrated activity against trypsin enzymes.

In vitro assay of serpin activity with *P. interpunctella*. Purified mutant serpins were tested in a microplate assay for the inhibition of purified mammalian enzymes. Enzymes and substrates were obtained from Sigma Chemical Co. Construct F was tested with 0.1 μ g α -chymotrypsin (bovine pancreas) and the substrate SAAPFpNA (0.1 mM), and construct K was tested with 1.0 μ g trypsin (porcine pancreas) and BApNA (3.7 mM). Construct A was not tested with a purified enzyme. Buffer (0.1 M Tris, pH 7.5, 5 mM CaCl₂, 0.1M NaCl) was added to adjust the sample volume to 200 μ l. Aliquots (20 μ l) of all eluant fractions of serpin were tested for inhibitory activity, and active fractions inhibited from 77-100% of the total activity (data not shown).

Midgut extracts were obtained from 4th instar *P. interpunctella* larvae. Larvae were ice-anaesthetized, posterior and anterior ends were removed, and the entire gut was placed in dissection buffer (200 mM Tris, pH 8.0, 20 mM CaCl₂) and frozen at -20 °C until used. Samples were spun at 12,000 x g, and the supernatant was used in the assay for serpin activity.

A general protease substrate, BODIPY-TR-X (fluorescently-labeled casein, Molecular Probes) was used to examine the inhibition of the caseinolytic activity of *P. interpunctella* gut enzymes by recombinant serpins. The optimal pH for hydrolysis was approximately 9 (Fig. 3). Therefore, assays with recombinant serpins were performed at pH 9, using a universal buffer system (Frugoni, 1957), unless otherwise noted.

Construct F, with chymotrypsin inhibitory activity, reduced the caseinolytic activity of *P. interpunctella* gut extract by 16% at the highest level tested (Fig. 4). Although this is not a strong reduction in activity, the casein substrate is hydrolyzed by proteinases from different mechanistic classes. The inhibition is an indication that some *P. interpunctella* digestive proteinases are sensitive to the inhibitor. No inhibitory activity was observed with the K construct. This may be due to inhibitor insensitive enzymes, or alternatively a result of insufficient inhibitor concentration in the preparations. We did not test the A construct in this set of experiments, as our previous data indicated that elastase proteinases are a minor component of digestion.

The inhibition of serpins on *P. interpunctella* gut extract and their respective substrate were studied *in vitro*. As with the general casein substrate, inhibition was observed in a dose dependent manner with construct F and SAAPFpNA, but no inhibition was observed with construct K and BApNA (Fig.5). To examine if the trypsin inhibitor lost activity due to hydrolysis by chymotrypsin enzymes in the extract, constructs F and K were combined and assayed for inhibitory activity to BApNA hydrolysis. A slight increase in inhibition was observed at the highest concentrations, but the increase in inhibitory activity was not significant.

The effect of pH on inhibition patterns was examined. At lower concentrations of the F construct, the inhibition of SAAPFpNA activity by *P. interpunctella* gut extracts was greater at pH 9 than at pH 8 (Fig. 6). The opposite was true with construct K and BApNA, although the inhibition at pH 8 was less linear (Fig. 7). Interestingly, the inhibition BApNA hydrolysis by gut extract with constructs K and F was greatly enhanced at pH 8 (Fig. 8), which may be an indication of substrate or inhibitor stability or increased inhibitor affinity at the lower pH.

In vivo assay of serpin activity with *P. interpunctella*. The bioassay procedure was a modification of the single larva bioassay (Johnson *et al.*, 1991), using semi-hydrated cereal/wheat germ (Grape Nuts®, Post) rolled into a flat, thin “piecrust” and sectioned into approximately 0.5 cm cubes. We use this assay because, neonate larvae readily consume the cereal-based diet. Diet cubes were treated with different serpins by applying solutions onto the diet using a micropipetor, allowing solutions to completely “soak” into the cube. Treated diet cubes were placed in 16 well assay trays, and eggs were added to each well. Mortality was calculated from the number of survivors after 10 d from treated samples compared with controls treated with Ni-NTA column elution buffer (no serpin). *P. interpunctella* strains used in this study were 688-s, collected from farm storage bins in Riley County, Kansas, in June of 1988 and susceptible to Bt toxins, and 198-r, selected for resistance from 688-s using Bt subsp. *entomocidus* (HD-198) (McGaughey and Johnson, 1992). We chose 198-r insects because they lack a major gut serine proteinase, and resistance to Bt in this strain was described as proteinase-mediated (Oppert *et al.*, 1997).

Each serpin construct was tested in a preliminary bioassay for the effect on 688s and 198s larvae. The concentration of constructs K and A were adjusted to those that produced a 50% inhibition *in vitro* of BApNA or SAAPLpNA, respectively, by *P. interpunctella* gut extract. Due to the limited quantity of the F construct at the time of the bioassay, we used a concentration that produced a 25% reduction in SAAPFpNA activity by gut extract. The results from the bioassay indicated an increased mortality for both Bt-susceptible and -resistant larvae when they were fed the F construct, even though the concentration applied was comparable to 50% less inhibition of substrate than the K and A constructs (Table 4). In addition, larvae developed slower on the K-treated diet (data not shown). Unfortunately, the data are not conclusive because the controls treated with elution buffer from the affinity column were toxic to larvae. We are currently trying to solve the problem with the control.

Table 4. Survival of *P. interpunctella* larvae on diets treated with elution buffer (control) or serpin constructs K, A, or F.

Colony	Percent Mortality			
	Control	K	A	F
688s	100	0	6	50
198r	94	13	19	50

Guts were obtained from surviving larvae using the dissection procedure previously described. The proteolytic activity from these samples will be analyzed using the Proteinase Activity Blot Assay described in the proposal. Results from these comparisons will identify proteinases that are down-regulated or over-expressed in response to the ingestion of serpins, providing information about the compensatory responses of *P. interpunctella* to ingested proteinase inhibitors. This data may explain why doses mutant serpins A and K that produced a 50% inhibition of gut proteolytic activity *in vitro* exhibited low toxicity in *P. interpunctella* larvae.

Analysis of *Diabrotica* sp. digestive proteinases.

WCR larvae were shipped on corn roots from Dow AgroSciences, San Diego. Third instar WCR larvae have a mean weight of 11 mg (Jackson, 1985). Gut extracts were prepared from corn root-reared WCR larvae weighing 9.5-10 mg using the same previously described

techniques for *P. interpunctella* (see “*In vitro* assay of serpin activity with *P. interpunctella*”). Larvae were also shipped on artificial diet (Marrone *et al.*, 1985). However, larvae died at first or second instar due to fungal contamination. Therefore, larvae were reared on corn roots and transferred to artificial diet 48 hr prior to dissection. These larvae weighed approximately 9 mg, and extracts contained more food than those reared solely on corn roots (unpublished observation).

A comparison of the pH of gut contents from WCR larvae reared on corn roots or artificial diet indicated no significant differences. Measurements were made using three different techniques: 1) exuded guts on pH indicator paper, 2) guts homogenized into water and probed with a microelectrode, and 3) force-feeding a pH indicator dye to larvae. The pH paper yielded measurements of 6.0-6.2 for corn-reared larvae and 5.8-6.0 for diet-fed larvae. The microelectrode measured 6.06 for larvae fed either corn roots or artificial diet. Both corn- and diet-reared larvae fed pH indicator dye had a gut pH measurement of 6.0. Therefore, there was no measurable difference in gut pH from larvae fed corn roots or artificial diet.

The hydrolysis of fluorescently-labeled casein was compared with extracts from corn-reared or diet-reared WCR larvae (Fig. 9). Two microliters of gut extract were added to a microplate well in Universal buffer at various pH values (Frugoni, 1957). Fluorescently-labeled casein (BODIPY-TR-X, Molecular Probes) was added, and the relative fluorescence was measured at timed intervals. Data represent the mean of duplicate measurements. Regardless of the incubation time, diet-reared insects had generally greater caseinolytic activity than corn-reared insects. This correlated to our observation that diet-reared insects had ingested more food. However, overall pH profiles were similar in corn- or diet-fed larvae.

A number of para-nitroanilide substrates were screened for activity with WCR larval gut extract. Minor activities were observed with BApNA and SAAPFpNA (Fig. 10). BApNA activities increased in alkaline pH, while SAAPFpNA activities were variable over the entire pH range. Hydrolysis of SAAPFpNA appeared to have 3 major peaks of activities in both corn- and diet-reared larvae, at acidic, neutral, and alkaline pH values. This would suggest multiple chymotrypsin-like enzymes in the WCR.

WCR gut proteinases were analyzed with 4-12% ZBC zymogram gels (Invitrogen, San Diego) containing casein substrate (Fig 11). Please note that molecular mass markers are included for reference only and cannot be used to estimate the relative molecular mass of proteinase activities due to the presence of casein in the gels (casein can interact with proteins and affect their mobility). Gut proteinases appeared to be sensitive to SDS in the electrophoresis buffers, as prewashing prior to development was necessary for optimal activity. Six distinct proteinase activities were observed in diet-fed larvae (P1-P6), while corn fed larvae lacked the P4 proteinase, a relatively minor activity. With both extracts, activities of all proteinases were higher in the presence of reducing buffer, indicating that these are all thiol-stimulated proteinases, characteristic of cysteine proteinases. Activities were enhanced in acidic buffer (pH 4.2), but all were observed to a lesser degree in neutral (pH 7.3) and alkaline (pH 10.5) buffers.

Various inhibitors were selected for testing with WCR extract from corn-fed larvae to further characterize the proteinase activities. All inhibitors were obtained commercially from Sigma Chemical Co. and Boehringer Mannheim, with the exception of equestatin. Equestatin was provided by Dr. Vito Turk (Department of Biochemistry and Molecular Biology, J. Stefan Institute, Ljubljana, Slovenia) in an ongoing collaboration with our laboratory to test the range of activity of equestatin in insects. Dr. Turk has permitted the testing of equestatin with *Diabrotica virgifera* gut extracts.

The most potent inhibitors of WCR activities were E-64, leupeptin, equistatin, and chymostatin (Fig. 12, Table 5). E-64 has previously been demonstrated to inhibit WCR digestive proteinases (see Literature Review) and inhibits cysteine proteinases (Table 5). Leupeptin inhibits both cysteine and serine proteinases. Chymostatin is a specific inhibitor of chymotrypsin-like serine proteinases and some cysteine proteinases. Equistatin, isolated from the sea anemone *Actinia equina*, is classified as a thyropeptide, a group of proteins that inhibit cysteine and cation-dependent proteinases (Lenarcic and Turk, 1999). Thyropeptides contain thyroglobulin type-1 domains similar to cysteine-rich domains found in thyroglobulin. Other inhibitors effective against WCR digestive proteinases were TLCK, TPCK, and serpin 1G (to be discussed in detail at the end of the report).

Table 5. Fifty percent inhibition constants for inhibitors of casein hydrolysis by WCR gut extract.

Inhibitor	Target Proteinase	IC ₅₀ (μM)
E-64	¹ papain and other cysteine proteinases like cathepsin B and L	0.004
equistatin	papain, cathepsin D	0.026
leupeptin	¹ serine and cysteine proteases, such as trypsin, papain, plasmin, and cathepsin B	0.029
chymostatin	¹ α-, β-, γ-, δ-chymotrypsin	0.060
TLCK	¹ trypsin and many other serine and cysteine proteases such as bromelain, ficin, and papain	1.99
serpin 1G	<i>Bacillus licheniformis</i> subtilisin	3.58
TPCK	¹ chymotrypsin and many other serine and cysteine proteases such as bromelain, ficin, and papain	24.0
PMSF	¹ serine proteases (chymotrypsin, trypsin, and thrombin) and cysteine proteases such as papain (reversible by DTT treatment)	1.44 x 10 ³
pepstatin	¹ aspartic (acid) proteases such as pepsin, renin, cathepsin D, chymosin, and many microbial acid proteases	2.19 x 10 ³

¹As per Boehringer Mannheim Proteinase Inhibitors Technical Guide (<http://biochem.roche.com>).

²Gordon and Seglen, 1993

Although chymostatin had previously been referenced as an inhibitor of WCR proteinases (Gillikin *et al.*, 1992), we examined the inhibition with regard to a specific effect on chymotrypsin-like activities in the WCR. We observed a chymotrypsin-like activity of approximately 100 kDa molecular mass in activity blot analyses with SAAPFpNA (data not shown-the activity was very low and difficult to detect in reproduced images). Inclusion of chymostatin in zymogram buffers reduced all proteinase activities (data not shown). When chymostatin was included in a microplate analysis of WCR gut extract hydrolysis of SAAPFpNA, there was little inhibition observed, except at the highest concentration tested (Fig. 13). The chymotrypsin-specific inhibitor TPCK, however, specifically inhibited the

SAAPFpNA-hydrolysis by WCR extract in a dose-dependent manner similar to that observed with casein. Therefore, we suggest that the inhibition by chymostatin is not mediated via a chymotrypsin-dependent mechanism.

Gilliken *et al.* (1992) reported a minor inhibition with 10 mM EDTA. We observed no inhibition with 1 mM EDTA but saw an increase in caseinolytic activity up to 170 percent with dilutions to 0.1 mM (data not shown). Pepstatin was also reported to inhibit 60% of total proteolytic activity (Gilliken *et al.*, 1992). At the highest concentration tested, 0.05 mM pepstatin caused an approximately 20% inhibition (Fig. 12). The previous study used radiolabeled-BSA as the substrate, but our results generally are consistent.

We are in the initial stages of analyzing the effects of serpins on WCR gut proteinases. Micromolar concentrations of serpins 1K and 1G prevented the hydrolysis of fluorescent casein by WCR gut extract from corn-reared larvae (Fig. 14). The assays have been corrected with a control containing serpin elution buffer as the inhibitor. The elution buffer control resulted in strong inhibition of WCR gut proteinases (Fig. 15). Some inhibition was seen in controls containing BSA, but this was only observed after longer incubation times (data not shown). Inhibition by the elution buffer is mostly due to the presence of imidazole in the buffer (data not shown). Addition of the elution buffer to the assay buffer resulted in a shift from pH 6.3 to 7.5, and we believe that the inhibition is due to this pH shift. Serpin samples are being dialyzed to remove the imidazole and will be retested with WCR extract. A comparison of serpin 1G to the other inhibitors tested indicated that the IC_{50} was in the micromolar range, similar to TLCK and TPCK (Table 5).

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Guo, L., Fatig III, R. O., Orr, G. L., Schafer, B. W., Strickland, J. A., Sukhapinda, K., Woodsworth, A. T. and Petell, J. K. 1999. *Photorhabdus luminescens* W-14 insecticidal activity consists of at least two similar but distinct proteins. Purification and characterization of toxin A and toxin B. *J. Biol. Chem.* 274: 9836-9842.

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Abbreviations:

AP - aprotinin
BapNA - N-benzoyl-L-arginine-p-nitroanilide
BSA - bovine serum albumin
CTI - corn trypsin inhibitor
E-64 - L-trans-epoxysuccinylleucylamide [4-guanidino] butane
EDTA - ethylenediamine tetraacetic acid
LP - Leupeptin
PMSF - phenylmethylsulfonyl fluoride
PAGE - polyacrylamide electrophoresis
PI - potato inhibitor
SAAPFPNA - N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide

SDS - sodium dodecyl sulfate
SCR - southern corn rootworm
SKTI - trypsin inhibitor (Kunitz) from soybeans
SSBBI - soybean Bowman-Birk trypsin inhibitor
TLCK - tosyl-L-lysine chloromethyl ketone
TPCK - N-tosyl-L-phenylalanine chloromethyl ketone
WCR - western corn rootworm

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.

Kanost, M., Oppert, B. and James, K. "Evaluation of insect serpins as biopesticides." Presented at the Consortium for Plant Biotechnology Research, Inc., Washington, DC, March, 1999.

Oppert, B. and Kanost, M. "Serpins as potential biopesticides." Presented to Dow AgroChemicals, San Diego, CA, August, 1999.

Technology Transfer

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

No technology transfer was initiated during the reporting period.

Commercial Accomplishments

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

The discovery of the potential for serpins with chymotrypsin inhibitory activity to affect the growth and development of *P. interpunctella* larvae may lead to the development of a new

biopesticide for this pest of stored grains and products. Investigations of gut proteinases in *Diabrotica virgifera* reared on different diets provides information needed in the successful bioassay of proteins. Knowledge of specific digestive proteinases of *D. virgifera* can provide new targets for control.

Educational Accomplishments

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

This project has served as instruction for teaching basic biochemical, microbiological, and insect bioassay concepts to undergraduate students. The project has also provided training in these areas for research associates and personnel working in the laboratory.

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.

Karen James, Research Associate in the Department of Biochemistry, Kansas State University, initiated and completed the work described in this report. She was assisted by KSU students Kris Hartzler and Fernando Estrada. Ms. James resigned due to relocation, and recently Jamie Morris-Hardeman was hired to replace her. Ms. Morris-Hardeman will continue to be assisted by Kris Hartzler and laboratory technician Michele Zuercher.

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Fig. 1. Effect of leupeptin on the gut activity of *P. interpunctella* larvae with the substrate BApNA.

Fig. 2. Coomassie-stained SDS-PAGE of the fractions obtained from Ni-NTA-agarose affinity chromatography of recombinant serpins. a) Construct F; b) Construct K; c) Construct A.

Fig. 3. Hydrolysis of BODIPY-TR-X by *P. interpunctella* gut extract at different pH values.

Fig. 4. Inhibition by recombinant serpins (construct F) of the hydrolysis of BODIPY-TR-X by *P. interpunctella* gut extract.

Fig. 5. Inhibition of Indianmeal moth gut extract hydrolysis of the substrates SAAPFpNA (serpin F construct) or BApNA (serpins K or K+F).

Fig. 6. Inhibition of Indianmeal moth gut extract hydrolysis of SAAPFpNA by serpin construct F at pH 8 or 9.

Fig. 7. Inhibition of Indianmeal moth gut extract hydrolysis of BApNA at pH 8 or 9.

Fig. 8. Inhibition of Indianmeal moth gut extract hydrolysis of BApNA by construct K+F at pH 8 or 9.

Fig. 9. Hydrolysis of fluorescent casein by corn rootworm extract.

Fig. 10. Hydrolysis of para-nitroanilide substrates by WCR gut extracts.

Fig. 11. Caseinolytic activity of WCR gut proteinases at acidic (4.2), neutral (7.3), and alkaline (10.5) pH, with (+) or without (-) L-cysteine in the buffer. M=molecular mass markers; c=corn root-reared larvae; d= artificial diet-reared larvae.

Fig. 12. Inhibition of the hydrolysis of fluorescent casein by WCR extract from corn-fed larvae.

Fig. 13. Inhibition of the hydrolysis of SAAPFpNA by selected proteinase inhibitors.

Fig. 14. Serpin inhibition of the hydrolysis of fluorescent casein by WCR extract.

Fig. 15. Effect of elution buffer (EB), bovine serum albumin (BSA), and serpins 1G and 1K on the hydrolysis of fluorescent casein by WCR gut proteinases. The amount of EB was the same as that added in the corresponding serpin assay.

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Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Dr. Peter J. Reilly	
University:	Iowa State University	
Agreement Number:	OR22072-71	
Project Title:	<i>Glucoamylase Mutagenesis to Increase Glucose Yield and Reduce Energy Use</i>	
Reporting Period and Report Type:	From: 7/1/98 To: 12/31/99	Check one: ☐ Interim Report ☒ Final Report

Project Objectives

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

a. Carry out multisequence alignments of all new glucoamylase (GA) sequences (to be done in the first year of the grant).

In the two years since the proposal for this grant was written, only one new GA sequence, that from *Thermoanaerobacterium thermosaccharolyticum*, has been published. In the proposal, we mentioned that since five or more GA sequences were being published each year, we would have several to enter into a new multisequence alignment within the first year of the grant. It has not been worth the effort to carry out a new alignment for just one new sequence.

b. Sequence the *Lactobacillus amylovorus* GA (to be done in the first eighteen months of the grant).

When the grant proposal was submitted, we had already received the gene for the *L. amylovorus* GA from a group in the Department of Food Science at McGill University in Montreal. After we obtained the grant, we started work on this gene, contained in a plasmid in *Escherichia coli*, and found that it had no open reading frame. Furthermore, partial sequencing of the DNA contained in restriction fragments of the same sizes as those supposedly holding the gene gave sequences that were not homologous with GA. When we asked for the same gene from a different source, we were told that the freezer holding all the group's cloned material thawed during the disastrous 1997 Quebec ice storm and that everything was discarded.

Although the McGill group was planning to reclone the GA gene and then give the clone to us for sequencing, we decided not to wait but instead to do a more difficult but hopefully more rewarding project, partly to take the place of work on the fourth area (below). This was the production of the *Clostridium* (now *Thermoanaerobacterium*) *thermosaccharolyticum* GA, its crystallization, and determination of its three-dimensional structure. We have successfully grown the organism anaerobically, a new skill in our laboratory, and have produced pure glucoamylase, but in quite low concentrations. We are varying culture conditions in an attempt to increase glucoamylase concentrations so that we have enough to crystallize it. We expect to bring this project to completion.

c. Produce and characterize the *Methanococcus jannaschii* GA (to be done over the two years of the grant).

This is the area where we made the most progress. We worked in two areas:

One, which has yet to bear results, is the culturing of *M. jannaschii* itself, the determination of the best conditions for it to produce GA, and the production, purification, and then the characterization of the enzyme's properties. We bought the lyophilized culture from the DSM culture collection in Braunschweig, Germany and transferred it to Professor Lacy Daniels of the Department of Microbiology at the University of Iowa, who has extensive experience in growing methanogens, but he has been unable to make it grow.

The second area is to produce the *M. jannaschii* GA from a standard production strain such as *E. coli*. To do this we bought the gene, but then realized that the codon usage in *M. jannaschii* was very different from that in *E. coli*, and that the latter was very unlikely to express the enzyme with the coding used by *M. jannaschii*. We therefore completely synthesized the gene using the codons favored by *E. coli*, transformed *E. coli* with it, and have successfully expressed GA in very substantial amounts, the first time the *M. jannaschii* GA (or apparently any archeal GA) has ever been obtained in the laboratory. To this point, however, we do not have the full enzyme, but only a truncated form. We have attempted to find out the reason for this, but have so far been unsuccessful. We expect to continue work in this area, as we feel that success would be a major achievement.

d. Carry out further site-directed mutagenesis on *Aspergillus awamori* GA to improve its selectivity (to be done over the two years of the grant).

We realized very soon after receiving the grant that further work in this area would bring us sharply diminished returns for the effort expended, since during the previous grant we had increased the glucose yield from about 96% to about 97.5%, close to the theoretical maximum, given that much of the remaining 2.5% reduction of yield was caused by the presence of byproducts and impurities not produced in the GA-catalyzed hydrolysis of starch dextrin. The reviewers of the proposal that led to this grant to some extent realized this. Therefore we have not pursued this line of attack, but instead put our effort into the revised second area and the nonrevised third area.

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 been attempting to produce glucoamylase, an enzyme that makes glucose from starch, from two microorganisms that grow at very high temperatures in the absence of air. In fact one of them is found in deep-sea thermal vents under very high pressures and grows from carbon dioxide and hydrogen. We would like to find out whether glucoamylase from these sources acts differently and has a somewhat different three-dimensional shape than the glucoamylase from fungi that is used industrially in the process to make high-fructose syrup. There are two reasons to do this: 1) It may be possible to replace the currently used glucoamylase with one of these enzymes, which should be much more stable and therefore perhaps cheaper to use; 2) We would learn where the glucoamylase family of enzymes came from, since the high-temperature microbes making the glucoamylases we are investigating are very primitive.

We have successfully made both glucoamylases, but so far we can obtain one of them only in very low concentrations, while the other one is not fully formed. We expect to continue working with them until we are successful.

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 has already been covered in the Project Objectives section above.

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.

Publications:

Reilly, P. J. Protein Engineering of Glucoamylase to Improve Industrial Performance—A Review. *Starch/Stärke*, 51, 269 (1999).

Reilly, P. J., in *Handbook of Food Enzymology*, J. R. Whitaker, A. G. J. Voragen, G. Beldman, and D. Wong, eds. Marcel Dekker, New York. Glucoamylase. Accepted for publication.

Presentations at Technical Meetings:

P. J. Reilly. Glucoamylase Mutagenesis to Reduce Energy Use. Consortium for Plant Biotechnology Research Annual Meeting, Washington, DC, March 1999.

H.-L. Liu, Y. Doleyres, P. M. Coutinho, C. Ford, and P. J. Reilly*. Mutations of Glucoamylase to Improve Its Selectivity and Thermostability. 217th ACS National Meeting, Anaheim, CA, April 1999.

P. J. Reilly. The Impact of Protein Engineering on Enzymatic Conversion of Starch. 50th Starch Convention, Detmold, Germany, April 1999.

P. J. Reilly. The Effects of Mutations on Starch-Degrading Enzymes. Frontiers in Carbohydrate Research 6, Purdue University, West Lafayette, IN, May 1999.

Invited Lectures and Seminars:

Iowa BioDevelopment, Indian Hills Community College, Ottumwa, IA, November 1998.

Genencor International, Inc., Palo Alto, CA, January 1999.

Lehrstuhl für Technologie der Kohlenhydrate, Technische Universität Braunschweig, Braunschweig, Germany, April 1999.

Departments of Molecular Genetics & Cell Biology, University of Chicago, Chicago, IL, May 1999.

Technology Transfer

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

We have submitted one patent application to the U.S. Patent Office and to many international patent bodies that claims improvements in glucoamylase selectivity and thermostability through site-directed mutagenesis. Most of the claims, covering nearly all of our significant mutations, have been preliminarily accepted by the USPO, acting for all the patent bodies. The patent has been licensed to Genencor International, Inc.

Commercial Accomplishments

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

We have collaborated throughout this project and through several previous CPBR-financed projects with Genencor, to the benefit of both sides. They have given us excellent advice on the routes to take that would be of industrial benefit, and we have been able to increase their knowledge of glucoamylase and related enzymes. We have given Genencor samples of our most promising mutated glucoamylases for testing.

Educational Accomplishments

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

One postdoctoral fellow, Saber Khan, and three M.S. students, Zhiliang Fan, Pinghua Feng, and Chandrika Mulakala, were supported on this project, Dr. Khan from July

1999 to December 1999, Ms. Fan from July 1998 to May 1999, and Mr. Feng and Ms. Mulakala from January 1999 to December 1999. Ms. Fan received an M.S. degree in December 1999.

Additional Funding

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

We have arranged to receive \$15,000 each from Genencor International, Inc., BC International, Inc., and the Center for Advanced Technology Development of Iowa State University from March 2000 to March 2001. This will extend our knowledge and techniques used to model and mutate glucoamylase to cellobiohydrolase I and II, which are closely related exo-hydrolases. We have submitted a preproposal to extend this new project with CPBR funding. Although it is on a somewhat different topic, this new project clearly has evolved directly from the current project.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

There was no change in the identities of the Principal Investigator or co-Principal Investigator.

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Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Keith A. Schimmel	
University:	North Carolina A&T State University	
Agreement Number:	OR22072-52	
Project Title:	Novel Metabolic and Process Engineering Approaches for Enhanced Propionic Acid Production	
Reporting Period and Report Type:	From: September 1, 1996 To: August 31, 1997	Check one: ☐ Interim Report ☒ Final Report

Project Objectives

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

1. Develop an extraction system that has a large partition coefficient for propionic acid, a high selectivity for propionic acid over acetic acid, and can operate at a pH as close to 6 as possible.

Extractive experiments were conducted in eight systems: Alamine 304-1 (a triauryllamine, Henkel Corporation) or TOPO (tri-n-octyl-phosphine oxide) in each of hexane, oleyl alcohol, kerosene, and 2-octanol. Different concentrations of extractants were used: 5, 10, 20, 30% (v/v) of Alamine 304-1 in all diluents; 10, 20, 30% (w/w) of TOPO in hexane, oleyl alcohol, and 2-octanol; but 5, 7.5, and 10% (w/w) of TOPO in kerosene because of the limited solubility of TOPO in kerosene. At pH = 4, 30% of TOPO in hexane had the highest partition coefficient (5.198), whereas 30% Alamine in oleyl alcohol had a partition coefficient of 4.168. As the pH increased, the partition coefficients significantly decreased. At pH = 5, 30% of TOPO in hexane showed the highest partition coefficient (1.260), whereas 30% Alamine in oleyl alcohol showed a partition coefficient of 0.786. At pH = 6, the same trend was followed with lower partition coefficients.

2. For the most promising systems identified in "1", determine the toxicity for *Propionibacterium acidipropionici*.

Four systems were selected for toxicity tests based on the partition coefficients of those systems: 30% (w/w) TOPO in hexane, 30% (w/w) TOPO in 2-octanol, 30% (v/v) Alamine in oleyl

alcohol, and 30% (v/v) Alamine in 2-octanol. Among the four systems tested, 30% (v/v) Alamine in oleyl alcohol showed the least toxicity to the bacteria, whereas 30% (w/w) TOPO in hexane showed the largest toxicity to the bacteria.

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.

Propionic acid is used in the manufacture of herbicides, chemical intermediates, artificial fruit flavors, pharmaceuticals, cellulose acetate propionate, and preservatives for food, animal feed, and grain. Its present U.S. market is over 300 million lbs per year and is growing at 4% annually. Currently, almost all propionic acid is produced by petrochemical processes. The overall project goal was to develop a process to economically produce propionic acid from low-value agricultural commodities and food processing byproducts, such as corn fiber and corn steep liquor. Toward this end, a new solvent extraction process was developed for separation and recovery of propionic acid from the fermentation broth. This extraction process requires low energy and is highly efficient in recovering and concentrating carboxylic acids from the fermentation broth. The new system provides lower solvent toxicity and a higher separation factor than previously used extraction systems.

Scientific Accomplishments

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

Introduction

Propionic acid is used in the manufacture of herbicides, chemical intermediates, artificial fruit flavors, pharmaceuticals, cellulose acetate propionate, and preservatives for food, animal feed, and grain. Currently, almost all propionic acid is produced by petrochemical processes. Its present U.S. market is over 300 million lbs per year and is growing at 4% annually. The project goal was to develop a process to economically produce propionic acid from low-value agricultural commodities and food processing byproducts, such as corn fiber and corn steep liquor.

The corn wet milling industry has experienced enormous growth recently because of the increasing demands for high-fructose corn syrup, ethanol, and other bio-products derived from corn dextrose. About 25 to 30 percent of the 35.7 million tons of corn annually processed by the corn wet-milling industry goes into corn fiber and corn steep liquor, which have low values and limited use as animal feed. There is an urgent need to develop new and better uses of corn fiber and corn steep liquor. It is thus desirable to use the surplus corn byproducts to produce valuable biochemicals, such as propionic acid.

Extractive fermentation processes requires low energy and are highly efficient in recovering and concentrating carboxylic acids from fermentation broth. However, extractive fermentation, is a relatively new technology that has not yet been used in industry. Extractive fermentation has been extensively studied for alcohol fermentation. More recently, extractive fermentation and other types of integrated fermentation-separation processes, such as electrodialysis fermentation, have also been studied for several organic acid fermentations, including homolactic and acetic acid fermentations. However, very little has been done with heterofermentation, such as propionic acid fermentation, that has more than one major acid product. Extractive fermentation using conventional extractants suffers from severe solvent toxicity and a low separation factor and may

require formidably expensive solvent regeneration procedures. Development of an extraction system that overcomes these problems was the goal of this project.

Extraction Experiments

Extraction experiments were conducted on eight systems: Alamine 304-1 (a tri-laurylamine, Henkel Corporation) or TOPO (tri-n-octyl-phosphine oxide) in each of hexane, oleyl alcohol, kerosene, and 2-octanol. Different concentrations of extractants were used: 5, 10, 20, 30%(v/v) of Alamine 304-1 in all diluents and 10, 20, 30% (w/w) of TOPO in hexane, oleyl alcohol, and 2-octanol, but 5, 7.5, and 10% (w/w) of TOPO in kerosene because of the limited solubility of TOPO in kerosene.

Extraction Experimental Procedures

Vials with Teflon caps were used for the experiments. Each vial contained 2 ml of acid solution and 2 ml of extraction solution. Acid solution contained propionic acid in the concentration of 20 g/L and acetic acid in the concentration of 10 g/L. Acid solutions were adjusted to appropriate pH value using NaOH (5 N). Extraction solutions contained either Alamine or TOPO in each of the four diluents in different concentrations. Vials were shaken (100 cycle/min) for 24 hours. Vials were settled for 24 hours to separate the organic phase from the aqueous phase.

After the phase separation, 100 μ l of the aqueous phase were taken to mix with 900 μ l of mobile phase solution for HPLC analysis. A HP 1100 HPLC and an Alltech C18 column were used for the analysis with a temperature of 40 $^{\circ}$ C, a mobile phase of 60% Acetonitrile and 40% phosphate buffer (0.05 M), a flow rate of 1 ml/min, and a sample amount of 10 μ l.

Extraction Experiments Results and Conclusions

The partition coefficient pH isotherms are presented for both propionic acid and acetic acid in Figures 1-8. From Figure 6 can be seen that at a pH of 4, 30% of TOPO in hexane showed the highest partition coefficient (5.198). Figure 4 shows that 30% Alamine in oleyl alcohol was the best Alamine system at a pH of 4 with a partition coefficient of 4.168. As the pH increased, the partition coefficients significantly decreased. At pH = 5, 30% of TOPO in hexane showed the highest partition coefficient (1.260), where 30% Alamine in oleyl alcohol showed the partition coefficient of 0.786. At pH = 6, the same trend was showed except with lower partition coefficients.

Figure 1: Partition coefficients versus pH for Alamine 304-1 in 2-octanol.

Figure 2: Partition coefficients versus pH for Alamine 304-1 in hexane.

Figure 3: Partition coefficients versus pH for Alamine 304-1 in kerosene.

Figure 4: Partition coefficients versus pH for Alamine 304-1 in oleyl alcohol.

Figure 5: Partition coefficients versus pH for TOPO in 2-octanol.

Figure 6: Partition coefficients versus pH for TOPO in hexane.

Figure 7: Partition coefficients versus pH for TOPO in kerosene.

Figure 8: Partition coefficients versus pH for TOPO in oleyl alcohol.

Toxicity Test

Four systems were selected for toxicity tests based on the partition coefficients of those systems: 30% (w/w) TOPO in hexane, 30% (w/w) TOPO in 2-octanol, 30% (v/v) Alamine in oleyl alcohol, and 30% (v/v) Alamine in 2-octanol.

Toxicity Test Experimental Procedure

A synthetic lactose medium was used. This medium contained the following (per liter): 10g of yeast extract (Difco), 5g of Trypticase (BBL), 0.25 g of K₂HPO₄, 0.05g of MnSO₄, and ~ 16g of lactose. The medium was sterilized at 121 °C and 15 psig for 20 min. Extractive solutions were sterilized by filtration (Nalge Disposable Filterware, Nalge Co. Rochester, NY 14602). *Propionibacterium acidipropionici* purchased from ATCC was used as seed culture.

Toxicity tests were conducted in 60 ml vials with Teflon-lined caps. All test vials were heat sterilization at 121 °C and 15 psig for 20 min. Each test vial contained 50 ml medium with seed culture and 10 ml of specific extractive solution. Control vials contained medium and the bacteria only. All tests were conducted in triplicate. The vials were placed on a shaker at 25 °C for 48 hours. Cell densities were measured by OD₅₄₀.

Toxicity Test Results and Conclusions

Table I shows the experimental results. Among the four systems tested, 30% (v/v) Alamine in oleyl alcohol showed the least toxicity to the bacteria, whereas 30% (w/w) TOPO in hexane showed the largest toxicity to the bacteria.

Table I. Toxicity tests

(The spectrophotometer was adjusted to 0 using control vials at time = 0)

Vial #	Extractive solution (10 ml)	Medium (ml)	OD ₅₄₀ (48 hours)
1	30% TOPO/hexane	50	0.009
2	30% TOPO/hexane	50	0.005
3	30% TOPO/hexane	50	0.003
4	30% TOPO/2-octanol	50	0.040
5	30% TOPO/2-octanol	50	0.045
6	30% TOPO/2-octanol	50	0.047
7	30% Alamine/oleyl alcohol	50	0.170
8	30% Alamine/oleyl alcohol	50	0.144
9	30% Alamine/oleyl alcohol	50	0.205
10	30% Alamine/2-octanol	50	0.078
11	30% Alamine/2-octanol	50	0.084
12	30% Alamine/2-octanol	50	0.075
13	control	50	0.864
14	control	50	0.738
15	control	50	0.513

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.

Conference Proceedings

Schimmel, K.A., Ilias, S., Wei, J., "Synthetic Membrane Technology: Research on a Critical Technology for Industrial Ecology," Fourth Annual HBCU/Private Sector - Energy Research and Development Technology Transfer Symposium, Greensboro, NC, April 2-4, 1996.

Conference Presentations

Yang, S.-T., Schimmel, K.A., "An Energy-Efficient Extractive Fermentation Process for Enhanced Propionic Acid Production from Plant Biomass," The Consortium for Plant Biotechnology Research 1996 Symposium, Washington, DC, November 19-21, 1996.

Journal Articles

Schimmel, K.A., Gao, C., "Extraction Systems for Propionic/Acetic Acid Mixtures," (Submitted to *Biotechnology and Bioengineering*, 1999).

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.

One post-doctoral research associate, one chemical engineering graduate student, and one undergraduate chemical engineering student have been impacted by the project. The black female undergraduate student has gone on to pursue graduate work in chemical engineering at the University of Delaware, which has one of the top chemical engineering graduate programs in the world. The project results have enhanced the content of four courses: Bioseparations (CHEN 608), Pollution Prevention (CHEN 622, formerly Industrial Ecology, GEEN 655), Chemical Engineering Independent Study (CHEN 510), and Introduction to Biochemical Engineering (CHEN 605).

Additional Funding

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

Schimmel, K.A., S. Ilias, "Synthetic Membrane Technology: Education and Research on a Critical Technology for Industrial Ecology - Renewal," Lucent Technologies Industrial Ecology Faculty Fellowship Program, \$50,000, 11/96-12/97.

L.M. Niedziela, K.A. Schimmel, et al., "Enhancing Biotechnology Instructional Capabilities at North Carolina A&T State University," North Carolina Biotechnology Center, \$170,000, 8/97-7/98.

Schimmel, K.A., "Biotechnology Quality Improvement Partnerships," NSF/SUCCEED, \$10,000, 9/97-8/98.

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.

Principal Investigator:	David A. Somers, Ph.D.	
University:	University of Minnesota	
Agreement Number:	OR22072-66	
Project Title:	<i>Incorporation of Value-Added Traits Into Alfalfa for Biomass Energy</i>	
Reporting Period and Report Type:	From: 1/1/1998 To: 12/31/99	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

See submitted report.

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 goal of this project is to produce a biodegradable plastic polymer known as polyhydroxybutyrate (PHB) in leaves of alfalfa plants. This plastic is made naturally by many different bacteria. Because of it can be completely degraded by microorganisms, PHB is an attractive source of nonpolluting plastic that could be used in a variety of consumer products. Production of PHB in plants would be less expensive than production by bacteria in fermentors. Three enzymes are needed to produce PHB in plants. The genes for these enzymes were previously isolated from the bacterium *Alcaligenes eutrophus* and manipulated by our industry partners for use in plant cells. The genes were introduced into alfalfa and the amount of PHB produced in over 200 different plants was measured. The amount of PHB in leaves varied widely from 0.024-0.2% of the leaf dry weight. All plants had normal growth and vigor suggesting that there was no deleterious effects from PHB production. Further tests were done on 20 plants with the highest accumulation of PHB. Two methods of microscopy were used to visualize granules of PHB in leaf cells. Only plants receiving the three bacterial genes had PHB granules. Ten plants were used to pollinate plants from the alfalfa variety UMN2966, which is adapted to the midwestern U. S. Seeds resulting from these

crosses were planted and the plants will be analyzed for production of PHB. The results from the first year show that significant amounts of PHB can be produced in alfalfa plants and that production has no detectable detrimental affect on the plants.

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 submitted report.

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 the reporting period.

Technology Transfer

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

None during the reporting period.

Commercial Accomplishments

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

None during the reporting period.

Educational Accomplishments

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

A post-doctoral scientist has been carrying out research for the project. This scientist has become familiar with a number of new techniques and procedures including: plant tissue culture, plant molecular biology, gas chromatography, mass spectroscopy, epi-fluorescence microscopy, transmission electron microscopy, and plant breeding techniques. Two undergraduate students have worked with this scientist and have been exposed to most of the techniques listed and have been actively involved in plant propagation, nucleic acid extraction, chemical extraction of PHB, and gas chromatography data analysis. The research was presented to five small groups of non-scientists (high school students, high school teachers and USDA-ARS secretaries) during tours of the laboratory.

Additional Funding

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

None during the reporting period.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

A post-doctoral scientist, Dr. Saruul Purev, was hired in October 1998 to carry out the project research and is continuing with the project during the second year.

Incorporation of Value-added Traits Into Alfalfa for Biomass Energy.

Year-One Progress Report.

During the first year of our project we (1) demonstrated the transfer of polyhydroxybutyrate (PHB) genes from *Alcaligenes eutrophus* into alfalfa genome, (2) quantified PHB-contents in leaves of transgenic alfalfa, (3) initiated analysis of polymer composition, (4) analyzed the cellular localization of PHB in alfalfa leaves, (5) obtained seeds for PHB-progeny analysis, and (6) initiated crossing of PHB-producing transgenic plants with alfalfa being developed for biomass production.

Engineering expression of genes for PHB synthesis in alfalfa. Two vectors (pMON8 carrying all three PHB genes and pMON9 with two PHB genes modified to form PHB-V copolymer), were introduced separately into *Agrobacterium* plant transformation binary vector by triparental mating. *Agrobacterium* strains containing each plasmid were used to inoculate alfalfa leaf explants. Regeneration of alfalfa plants on media with antibiotic was carried out in about 10 weeks. Transgenic nature of regenerated plants was confirmed by PCR assay using NPT marker gene-specific primers. Ninety percent of total 114 pMON8 plants and 98 of pMON9 plants analyzed were transgenic. Selected alfalfa plants were transferred into soil and grown in the green house for further analysis. Transgenic plants had normal wild-type growth.

Analysis of PHB content in transgenic alfalfa. Quantitation of PHB in leaves of alfalfa was carried out by using gas chromatography. Analysis of transesterified chloroform extracts of leaves from two-months-old transgenic plants showed presence of a novel compound that eluted with the same retention time as methylhydroxybutyrate. The compound was not detected in untransformed alfalfa plants. Based on the PHB amount in the leaves, transgenic plants varied widely and were generally lower than the amounts previously reported in *Arabidopsis* (14% of dry weight). PHB amount ranged from 8 to 350 µg/g of fresh weight (0.024 - 0.2% of the leaf dry weight). Comparison of two groups of transgenic alfalfa showed that PHB amount was higher in the leaves of pMON-8 plants transformed with *Agrobacterium* containing all three PHB genes than the pMON-9 plants carrying two PHB genes modified to form PHB-V copolymer. (Table 1). Average accumulation of PHB in the leaves of pMON-8 and pMON-9 were 89.4 µg/g FWT and 41.2 µg/g FWT, respectively.

Gas chromatography of pMON-9 plants showed that only one had a novel compound that eluted with the same retention time as methylhydroxyvalerate. We selected about 20 plants with highest PHB content for detailed characterization.

Appearance of PHB granules. Transmission electron microscopy of leaf samples of PHB-producing plants revealed that PHB accumulated as agglomerations of electron-lucent granules of 0.2 - 0.4 µm. PHB-granules of small round form were easily distinguished from starch that appears as oval singular granules. (Fig. 1). PHB granules were correctly located in the chloroplasts as the PHB-genes were engineered to be plastid-targeted. Normal growth and vigor of transgenic plants suggested no deleterious effect of PHB production in these plants. Bacterial PHB granules stained with Nile Blue A emitted red fluorescence at excitation wavelengths of 546 nm. Similar granules of about 0.3 µm in diameter were detected in PHB-

producing plants with epifluorescence microscopy. (Fig 1). No fluorescing granules were found in untransformed alfalfa.

Progeny analysis of PHB-producing plants. To study the inheritance of transgenes we crossed PHB-producing transgenic plants with the alfalfa line 2966 which is adapted to the Midwest. Seeds obtained from both crosses currently are growing for F1 progeny analysis. We also initiated crossing plants accumulated the highest concentrations of PHB with plants being developed for biomass production (two alfalfa lines UMN-3202 and UMN-3145).

Table 1. PHB accumulation in leaves of transgenic alfalfa plants containing all three PHB genes (pMON-8) and two PHB genes modified to form PHB-V copolymer. Measurements are made on mature 50-60-days-old leaves.

Fig. 1. Transmission electron micrographs of thin sections from PHB-producing transgenic plants (2) and wild type alfalfa (1). Leaf mesophyll cells with agglomerations of electron-lucent granules (→) in the chloroplasts. (C-cytoplasm, V-vacuole, S-starch). Visualization of PHB granules by epifluorescence microscopy of leaf tissues stained with Nile Blue A: PHB-producing transgenic alfalfa (4) and wild-type alfalfa (3).

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Dr. Martin H. Spalding	
University:	Iowa State University	
Agreement Number:	OR22072-62	
Project Title:	Coordinated Expression of Multiple Anti-Pest Proteins	
Reporting Period and Report Type:	From: 7/1/97 To: 6/30/99	Check one: ☐ Interim Report ☒ Final Report

Project Objectives

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

Goal: The overall goal of this research is development of a single-gene system for the coordinated expression of multiple secreted proteins by using a "spacer" peptide from the periplasmic carbonic anhydrase of *Chlamydomonas* to link the proteins into a single translation unit.

Specific Objectives:

1. Linking of two reported proteins with the pCA1 spacer peptide and expression of the combined polypeptide in tobacco. **Progress.** Using green fluorescent protein (GFP) and chloramphenicol acetyltransferase (CAT) as linked reporter proteins, we have been able to recover processed and active GFP in leaves of transgenic tobacco plants and secreted from tobacco suspension culture cells. We also have been able to detect CAT activity in transgenic plants and suspension cultured cells, but we have been unable to detect any CAT protein. We suspect the CAT protein is being degraded in the endomembrane system.
2. Characterization of components necessary for pCA1 spacer processing in tobacco. **Progress.** We have been unable to begin work on this objective, because all of our effort has been directed at the first objective.

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.

Our research goal is development of a system to combine multiple genes for anti-pest proteins into a single gene in biomass crops, where disease and insects cause substantial yield reductions. This will be valuable where two proteins act together in disease protection or where multiple insect toxins increase the spectrum of insects affected and decrease the incidence of toxin resistance. We so far have been able to achieve partial success in that we have recovered from a hybrid, two-protein gene one of the two proteins in an active state.

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 grant began officially on 7/1/97. However, funds were received from the CPBR on 10/1/97 and from the corporate partner, Mycogen, on 12/1/97. These funds were used to hire a postdoctoral associate (see below) on 12/7/97. In the subsequent nineteen months, substantial progress was made toward our first objective. Two plasmid vectors were constructed linking reporter genes together with the pCA1 spacer. The reporter genes used in these vectors were a modified jellyfish green fluorescent protein (GFP) gene and a bacterial chloramphenicol acetyl transferase (CAT) gene. The coding sequences for these proteins were fused with an *Arabidopsis* basic chitinase signal sequence, the pCA1 spacer and a nopaline synthase (*nos*) terminator in the *Agrobacterium* binary vector pBIN m-gfp5-ER (obtained from Dr. Jim Haseloff, Cambridge) under control of the CaMV-35S promoter. The order of the fusion in both vectors is: CaMV-35S::ER-signal::GFP::pCA1-spacer::CAT:*nos*-terminator. The difference between the two vectors is that one contains an ER retention signal (HDEL) at the expected carboxy-terminus of GFP and the other does not. These vectors were transfected into *Agrobacterium*, which was then used to transform tobacco.

Putative tobacco transformants were identified by growth on kanamycin-containing medium. Apparent GFP fluorescence was observed in some of the regenerated plantlets, and GFP fluorescence was confirmed in the putative transformants by spectrofluorometric analysis of soluble extracts. Furthermore, when SDS-PAGE immunoblot analyses of protein extracts from putative transformants were performed using commercial anti-GFP antiserum, immunoreactive bands similar in size to control GFP were observed. Immunoreactive bands of lower molecular mass also were observed in some putative transformants, but no immunoreactive bands were observed at the mass expected for the primary translation product. CAT activity (assayed as acetylation of radiolabeled chloramphenicol) was detected in extracts from transgenic plants, but no anti-CAT immunoreactive protein was detected.

To facilitate further investigation of the GFP/spacer/CAT fusion protein expression, transgenic tobacco callus was converted to suspension cultures. Analysis of the resulting suspension cultures indicated that anti-GFP immunoreactive protein could be detected primarily in the suspension medium, anti-CAT immunoreactive protein could not be detected at all and CAT activity could be detected only in the suspension cells. These results indicate that the GFP protein was processed from the fusion protein and secreted, but that the CAT protein, if processed, did not accumulate and probably was not secreted.

The GFP secreted from the tobacco suspension cells was approximately 24 kD, whereas the expected size of GFP is 29 kD. An identical GFP construct lacking the pCA1 spacer and CAT (i.e., GFP alone, but with an ER retention signal) yielded protein of the correct size retained in the cells, as well as smaller anti-GFP immunoreactive proteins (similar to those from the fusion construct) both in the cells and secreted to the suspension medium. This indicates some truncation at the carboxyl end of the protein (the end adjacent to the pCA1 spacer) was likely occurring but that this truncation was not specific to the processing of the GFP/spacer/CAT fusion protein. Although not previously reported, it is possible that the carboxyl end of GFP may be inherently unstable under the conditions found in the endomembrane system. We have not yet been able to obtain carboxyl end sequence from the processed and secreted GFP to determine whether a carboxyl end truncation has occurred.

Because we were unable to detect any CAT protein, we do not know if the detected CAT activity resulted from processed protein or from the unprocessed fusion protein. Our hypothesis is that the CAT protein or spacer/CAT-fusion released by processing of GFP may have been degraded, and that the unprocessed fusion protein was not abundant enough to detect immunologically but exhibited sufficient CAT activity to be detected with a sensitive assay.

Taken together, the preliminary results suggest that expression of the fusion product was occurring and that some of this product was being processed to release at least the GFP from the fusion protein. Even though we are not certain what was happening with regard to processing of the CAT portion of the fusion protein, the original goal of using the pCA1 spacer in the development of a single-gene system for the coordinated expression of multiple, secreted, anti-pest proteins in transgenic biomass crop plants still looks very promising.

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.

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.

The funding for this project has partially supported one postdoctoral associate, who has had primary responsibility for carrying out the research. In addition, an undergraduate student, Ms. Ashlesha Sharma participated in the research on this project from 1/12/98 to 5/15/98. Ms. Sharma was the recipient of a one-semester RABS (Research Assistantships in Biological Sciences) Fellowship from the Howard Hughes Medical Institute Education Initiative at Iowa State University.

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.

A postdoctoral associate, Dr. Yoshiko Nakamura, was hired on 12/7/97 to work on this project. She received her Ph.D. from University of Tsukuba in 1996 and had continued as a postdoctoral associate in the Institute of Biological Sciences at the University of Tsukuba until joining Iowa State University. The CPBR and Mycogen funding provided only partial funding for Dr. Nakamura, so she also was partly supported by and worked part-time on a USDA project on signal transduction in *Chlamydomonas*.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Dr. Friedrich Srienc	
University:	University of Minnesota	
Agreement Number:	OR22072-77	
Project Title:	<i>Biodegradable Plastics from Yeast and Plants</i>	
Reporting Period and Report Type:	From: 7/1/98 To: 2/15/00	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

Objective 1: To modify specific PHA pathway enzymes with respect to specificity and functionality. Novel polymerases will be engineered with broader substrate specificity. Polymerases will be fused to a NADH dependent reductase which is expected to favor PHB synthesis kinetics.

Progress: The focus of this objective has been slightly altered. Instead of aiming to alter the polymerase specificity in order process different precursor types, we focused on the generation of different precursor types for PHA synthesis. In addition, instead of aiming to make PHA synthesis NADH dependent we engineered strains the can convert NADH to NADPH.

Objective 2: To increase the levels of PHB pathway enzymes to divert precursors from primary metabolism towards PHB and learn how this affects cell physiology. A divergent promoter will be used to introduce dual gene combinations into yeast resulting in defined gene dosage. Additional genes will be introduced using chromosomal integration.

Progress: The divergent promoter approach proved to be very successful. Using this approach we developed yeast strains that can store approximately 10 % PHB and more which is one order of magnitude larger then in previous constructs.

Objective 3: To investigate PHB formation in *S. cerevisiae* in the context of ethanol formation. PHB formation occurs in yeast during the same metabolic state when ethanol is produced. Yeasts engineered for high PHB productivity will be used to quantitate the co-production potential with ethanol.

Progress: Several experimental, kinetic studies have been carried out. Theoretical pathway analysis demonstrated that PHB can be synthesized in several different pathway routes.

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.

PHAs are naturally occurring, biodegradable plastics. These polymers can attain specific physical properties depending on the primary monomer sequence. Therefore it is of interest to design reaction schemes that can lead to a controlled synthesis of the polymer sequence. One approach is the development of polymerases that can recognize and polymerize specific monomer precursors. With such enzymes one can synthesize specific polymers if defined precursors can be made. We have been successfully able to alter the monomer precursor pool through a developed nutrient switching strategy using our available polymerase enzymes. This strategy leads to a controlled synthesis of the micro-architecture of PHA granules at the nanometer length scale. Furthermore, we have been able to synthesize block-copolymers that consist of molecules in which the monomer sequence alternates between two different types of monomers. This work led to numerous publications and to a patent application. In addition, we have developed an experimental technique that permits expression of multiple pathway genes at the same time. Using this approach we have engineered *Saccharomyces cerevisiae* strains that can accumulate the polymer up to 10% of the cell dry weight. This level represents an improvement of approximately one order of magnitude. Moreover, the study has shown which steps need to be engineered to achieve even higher levels.

Scientific Accomplishments

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

1. During this project period several papers have been published which are based on research accomplished during the preceding project period. These includes publication (1) which describes the targeting and expression of PHA synthesis genes in plant peroxisome and the demonstration the PHA can be made to relatively high levels in this organelle. Furthermore, publication (2) describes a quantitative model for PHB synthesis. This model is very useful for guiding the experimental work since PHB synthesis conditions can be simulated on a computer.
2. Within objective 1 we have focused on developing experimental strategies that can generate different types of monomer precursors within a cell in a controlled way. This work was very successful as we have been able to demonstrate that with reaction engineering it is possible to control the microstructure of PHA both at the nanometer length scale as well as at the molecular scale. The study has shown that block-copolymers can be synthesized in this way. A key element in this study is the development of a population balance model that describes the polymerization process. With the help of the model experimental conditions could be predicted for obtaining

polymers of the desired kind. This work is documented in publications (3) (5) (6) (7) and (8), in the presentations (1), (2) and (3), and in thesis (3) (see below). Furthermore, the University of Minnesota has filed a patent application on this technology (see Patent application (3) below).

3. With the help of a developed divergent promoter it is possible to express from a single promoter two pathway genes. Thus two genes can be expressed from a single plasmid and problems of plasmid stability in a multiplasmid system are circumvented. The expression polymerase together with the reductase enabled to increase intracellular PHB levels to approximately 10%. This level appears to be strongly plasmid copy number dependent since in the microscope cells are seen that contain much higher PHB content. This variation in PHB content is likely the result of plasmid copy number distribution in the cell population (see presentations (5) (6) and thesis (2)).
4. Kinetic studies of the PHB synthesis process have revealed that PHB synthesis is restricted to certain combinations of substrates used during cell growth. Ethanol can be a byproduct during PHB synthesis, however it serves more often as a substrate. A theoretical analysis of the stoichiometry of the yeast metabolic reaction network revealed that there are many possible routes to synthesize PHB. It is clear that a more systematic approach will lead to faster advances in this work. In order to investigate this in further detail we will need to have access to a gas chromatograph which will permit determination of oxygen consumption rates and CO₂ production rates to delineate the PHB pathway used by cells (see presentation (8)).

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.

Publications

1. Hahn JJ, Eschenlauer AC, Sleytr UB, Somers DA, Srien F. Peroxisomes as sites for synthesis of polyhydroxyalkanoates in transgenic plants. *Biotechnol. Prog.* 15(6):1053-1057 (1999, Nov-Dec).

Abstract

Bacterial genes responsible for poly(3-hydroxybutyrate) (PHB) biosynthesis were targeted to plant peroxisomes by adding a carboxy-terminal targeting sequence. The enzymes evidently were transported into peroxisomes, retained their catalytic activity, and reacted with peroxisomally available precursors because PHB synthesis in transgenic plant cells was localized to peroxisomes. Up to 2 mg/g fresh weight PHB was produced in suspension cultures of Black Mexican Sweet maize cells after biolistic transformation with three peroxisomally targeted

bacterial genes. An equilibrium effect is proposed to explain the unexpected existence of (R)-3-hydroxybutyryl-CoA in plant peroxisomes.

2. Leaf TA, Srienc F. Metabolic modeling of polyhydroxybutyrate biosynthesis. *Biotechnol. Bioeng.* 57(5):557-570 (1998)

Abstract

A mathematical model describing intracellular polyhydroxybutyrate (PHB) synthesis in *Alcaligenes eutrophus* has been constructed. The model allows investigation of issues such as the existence of rate-limiting enzymatic steps, possible regulatory mechanisms in PHB synthesis, and the effects different types of rate expressions have on model behavior. Simulations with the model indicate that activities of all PHB pathway enzymes influence overall PHB flux and that no single enzymatic step can easily be identified as rate limiting. Simulations also support regulatory roles for both thiolase and reductase, mediated through AcCoA/CoASH and NADPH/NADP⁺ ratios, respectively. To make the model more realistic, complex rate expressions for enzyme-catalyzed reactions were used which reflect both the reversibility of the reactions and the reaction mechanisms. Use of the complex kinetic expressions dramatically changed the behavior of the system compared to a simple model containing only Michaelis-Menten kinetic expressions; the more complicated model displayed different responses to changes in enzyme activities as well as inhibition of flux by the reaction products CoASH and NADP⁺. These effects can be attributed to reversible rate expressions, which allow prediction of reaction rates under conditions both near and far from equilibrium. Copyright 1998 John Wiley & Sons, Inc.

3. Kelley, A. S., Jackson, D. E., Macosko, C., Srienc, F. Engineering the composition of co-polyesters synthesized by *Alcaligenes eutrophus*. *Polymer Deg. Stabil.*, 59: 187-190 (1998).

Abstract

Co-polyesters of 3-hydroxybutyrate and 3-hydroxyvalerate were produced by *Alcaligenes eutrophus* H-16 from valeric or propionic acid under both air and knallgas (80:20 H₂:O₂) atmospheres. Selectivity for poly-3-hydroxyvalerate (PHV) synthesis over poly-3-hydroxybutyrate (PHB) is favored under hydrogen, presumably because high NADH levels under knallgas inhibit breakdown of PHV monomer precursors to PHB precursors. Switching the atmosphere from knallgas to air and back again to knallgas was shown to change the bacterial metabolism back and forth, producing different monomer precursors for polymer synthesis. The experiments suggest that this metabolic switching may be used to produce polyhydroxyalkanoates with blocky regions within the polymer or polymer granules with shells of different polymer composition.

4. Jackson, J. K., Srienc, F. Effects of recombinant modulation of the phbCAB operon copy number on PHB synthesis rates in *Ralstonia eutropha*. *J. Biotechnol.* 68: 49-60 (1999).

Abstract

We have increased the gene dosage of the poly(3-hydroxybutyrate) biosynthesis operon in *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) to test whether PHB synthesis rates may be

increased by recombinant methods. The native *R. eutropha* phbCAB operon was inserted into the broad-host-range vector pKT230. This PHB operon-containing plasmid, and a control plasmid containing the identical broad-host-range replicon but not the PHB genes, were transferred to *R. eutropha* H16. Analysis of whole-cell lysates indicated that the strain harboring the operon-containing plasmid possessed beta-ketothiolase and acetoacetyl-CoA reductase specific activities that were 6.0 and 6.2 times elevated, respectively, as compared to the control strain with a single operon. After growth on fructose, PHB synthesis rates were sharply dependent on the type of carbon source offered during the PHB accumulation phase under nitrogen limitation. In the case of the strain harboring the control plasmid, and in comparison to fructose as carbon source, PHB accumulation was 2.15, 2.83, and 2.60 times faster when resuspended in nitrogen-free medium with lactate, acetate, or 3-hydroxybutyrate, respectively. The strain harboring the PHB operon-containing plasmid synthesized PHB at a lower specific rate in each case. During exponential growth on fructose, the strain harboring the control plasmid was again more efficient at forming PHB. These results suggest that increasing the intracellular concentration of PHB precursors may be a superior alternative to raising the levels of PHB enzymes for enhancing PHB productivity in *R. eutropha*.

5. Kelley, A. S., Srienc, F. Production of two phase polyhydroxyalkanoic acid granules in *Ralstonia eutropha*. *Int. J. Biol. Macromol.*, 25: 61-67 (1999).

Abstract

To synthesize layered granules consisting of selected phases of PHB homopolymer and PH(B-co-V) copolymer, *Ralstonia eutropha* was grown on fructose and limited quantities (1 g/L) of valeric acid. Exhaustion of the valerate resulted in a carbon source shift and a shift in the PHA composition within the cell. This methodology can be used to produce "layered" granules, whose composition reflects this shift in the PHA monomer pool. Valerate was exhausted from the medium after 12 hours producing a core of PH(B-co-V) (51% HV), which was contained within a shell of PHB from the remaining fructose. Differential scanning calorimetry (DSC) showed two glass transitions, confirming the presence of two distinct polymer phases within the layered granules. Transmission electron microscopic (TEM) images stained with RuO₄ revealed a heavily stained copolymer core within a lighter stained PHB shell. Production of layered granules allows for the control of domain sizes, potentially providing metabolic control over the physical properties of the resultant polymer.

6. Mantzaris, N. V., Kelley, A. S., and Srienc, F. "A Population Balance Model describing the Dynamics of Molecular Weight Distributions and the Structure of PHA Copolymer Chains. Part I: Steady-State Analysis", *Chem.Eng.Sci.* (submitted), (1999).

Abstract

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters produced by many bacterial species under growth-limited conditions when the carbon source is present in excess. The polymerization step is catalyzed by a polymerase enzyme, which elongates the growing polymer chains by successively adding monomer units. The molecular weight distribution of the polymer chains depends on three rates: a) the rate by which the polymerization process is initiated, b) the elongation rate, and c) the termination rate, that determines the rate by which the actively growing chains stop elongating, thus becoming inactive. We have developed a population balance model that can predict the dynamics of active and inactive PHA polymer chain molecular weight distributions in a non-growing *Ralstonia eutropha* cell population. We have

considered two types of elongation rate dependence on the molecular weight. In the first model we assumed that the elongation rate is constant, whereas in the second model, the elongation rate was taken to be a linearly decreasing function of the molecular weight. The steady-state version of the model in conjunction with the available experimental data was used to compute the steady-state active chain molecular weight distribution and the termination rate as a function of polymer molecular weight for each of the two elongation rate models. For both elongation models the steady-state active chain distribution was found to be a monotonically decreasing function of the polymer molecular weight, whereas the termination rate exhibited a maximum. In the case of constant elongation rate, the decrease of the termination rate after the maximum was reached, was less pronounced than in the case of the linearly decreasing elongation rate model. For the decreasing termination rate pattern an analytical solution has been developed which is in excellent agreement with experimental data. The proposed model reveals important details of the polymerization process that are difficult to obtain experimentally. Thus, the model should be useful for the optimization and control of reaction conditions to experimentally synthesize polymers.

7. Mantzaris, N. V., Kelley, A. S., Daoutidis, P. and Srienc, F. "A Population Balance Model describing the Dynamics of Molecular Weight Distributions and the Structure of PHA Copolymer Chains. Part II: Transient Behavior", *Chem.Eng.Sci.* (submitted), (1999).

Abstract

The nature of the carbon source used during bacterial synthesis of polyhydroxyalkanoates (PHA) leads to the formation of different polymer types. To investigate the possibility and the experimental conditions for block copolymer formation via biosynthesis, we have used the previously developed mathematical model to describe transient synthesis situations that result from switching the nature of the carbon source. Based on the assumption of equal time intervals for each stage of the carbon source switching process, the best conditions for maximizing the final concentrations of di- and tri-block copolymers is predicted. In addition, the structure and molecular weight distribution of the obtained block copolymers is analyzed. Due to the fact that the predicted conditions fall into the range of feasible bioprocessing manipulations, it is expected that such block copolymers can be synthesized. The developed population balance framework should be generally useful for polymerization processes with similar reaction mechanisms.

8. Mantzaris, N. V., Kelley, A. S., Daoutidis, P., and Srienc, F. "An Optimal Carbon Source Switching Strategy for the Production of PHA di-block Copolymers with *Ralstonia eutropha*" *AIChE J.* (submitted), (1999).

Abstract

During polymerization in a non-growing cell population of *Ralstonia eutropha*, alternating between two different carbon sources (fructose and fructose/valeric acid) could lead to the production of block copolymers consisting of blocks of homo-poly-3-hydroxybutyrate (PHB) and polyhydroxybutyrate-co-valerate (PHBV) copolymer. We address the problem of finding the optimal number of carbon source switches and corresponding switching times that maximize the final concentration of di-block copolymers (PHB-PHBV and PHBV-PHB). The problem under consideration is mathematically formulated in the mixed-integer nonlinear programming (MINLP) framework, which allows the decomposition of the original problem into the primal

and master problems. The primal problem corresponds to the original problem for a fixed number of carbon source switches, whereas the master problem consists of finding the number of carbon source switches that maximizes the optimum solutions of all possible primal problems. The global optimum was obtained for 39 carbon source switches. It corresponds to a mass fraction of 50.6% of final di-block copolymer concentration over the final total polymer concentration.

Presentations

1. **Srienc F. Reaction Engineering of PHA synthesis. Invited presentation, International Symposium on Biological Polyhydroxyalkanoates, September 9-11, 1998, Tokyo, Japan.**

Abstract

At the molecular level, cellular reactions depend on the structure and on the chemo-physical environment of individual enzymes participating in a pathway. Direct modification of individual enzymes or rearrangement of the spatial localization of the enzymes in the context of a cell can critically affect the metabolic flux through a reaction sequence. According to these principles there are several ways in which polyhydroxyalkanoate (PHA) synthesis can be manipulated and engineered. First, specific PHA pathway enzymes can be changed to obtain catalysts with desired specificity and functionality. Second, the levels of pathway enzymes can be adjusted to divert precursors from primary metabolism towards biopolymer formation. Third, the cellular physiological state can be adjusted through specific cultivation conditions to affect critical metabolic control points that determine the flux through specific pathways. Although these manipulations represent standard tools available in metabolic engineering, their application becomes particularly challenging if applied to eukaryotic cells where the metabolism is compartmentalized into specific organelles.

The presentation will address application of these tools to engineer the reactions leading to the formation of PHAs. Specifically, it will focus on designing the PHA pathway in the context of eukaryotic cells such as *Saccharomyces cerevisiae* and plants. While formation of PHA in engineered cells can serve as a convenient marker to probe for the availability of precursors in specific cell environments, it is shown that significant amounts of biopolymer accumulation can result if its synthesis is engineered into cellular organelles such as peroxisomes.

2. **Kelley, A. S., Srienc, F. *in vivo* Engineering of Polymer Synthesis. Poster, International Symposium on Biological Polyhydroxyalkanoates, September 9-11, 1998, Tokyo, Japan.**

Abstract

Phase separated blends of polyhydroxybutyrate-co-hydroxyvalerate (PH(B-co-V)) with homo-PHB may be used to improve the poor physical properties of PHB homopolymer. Copolymer granules could be mixed with PHB granules to form these blends; however properties are improved if there are chemical bonds joining the two phases. If granules are synthesized layer upon layer, granules could be made with a "core" of PH(B-co-V) and a "shell" of PHB

homopolymer. It is believed that during this metabolic change a transition region forms between the two phases where synthesized chains reside simultaneously in both phases. This would chemically tie the two phases together, thus gaining the stiffness of PHB and the flexibility of PH(B-co-V) in the overall material. Physical properties of PHB such as impact strength could be improved similar to high impact polystyrene.

To test this concept we have manipulated the cellular precursor pool from conditions of copolymer synthesis to that for PHB synthesis. These two polymers have been made in the same granules within *R. eutropha* by feeding a limiting amount of valeric acid concomitantly with fructose. Core PH(B-co-V) and shell PHB granules were synthesized by the cells. Differential scanning calorimetry measurements support the presence of two distinct phases. However, when valeric acid was added to the medium after PHB synthesis had commenced, only one polymer phase was synthesized. Apparently the metabolic switching from PHB precursors to copolymer precursors is not abrupt enough to generate phase separation and the polymers mix. The transitory period between the two distinct metabolic states appears to be longer for the PHB to PH(B-co-V) switch, preventing phase separation. To confirm that the two different polymer phases were present in the same granules, isolated polymer granules have been visualized with transmission electron microscopy. Distinct core-and-shell granules have been detected using ruthenium tetroxide vapor staining to contrast the different polymer phases. This contrast is believed to arise because the RuO_4 vapor diffuses more readily into the less crystalline copolymer phase than the PHB phase, resulting in a darker stain of the copolymer phase. This work demonstrates that layered granules can be synthesized with *R. eutropha*. Because of the small domain size and chemical bonding between phases, core-and-shell granules could make an ideal choice for blending with PHB to improve the physical properties of the homopolymer.

3. Kelley, A. S., Mantzaris, N. V. and Srienc, F. "Bacterial Synthesis of Block Copolymers", *AIChE Annual Meeting*, Dallas, Texas, Oct. 31-Nov. 5, 1999.

Abstract

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters produced by many bacterial species under growth-limited carbon excess conditions. The synthase enzyme responsible for polymerization in *Ralstonia eutropha* can incorporate both R-3-hydroxybutyrate and R-3-hydroxyvalerate into the growing polymer chain. Butyrate monomers are formed by the condensation of two acetylCoA molecules, which are intermediates of sugar metabolism. However, an odd chained fatty acid, such as valeric acid, must be provided to synthesize valerate monomers.

One strategy to control the microstructure of the biodegradable polymer is to alternate the supply of substrates between an excess of sugar (fructose), and limiting amounts of valerate with and excess of sugar. This would lead to the synthesis of pure polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-valerate (PHBV) copolymer, respectively. Transmission electron microscopy (TEM) images reveal that layered granules are formed using media containing an excess of fructose and limited amounts of valeric acid. The core of the granule contains PHBV copolymer while the shell layer contains PHB synthesized after valerate exhaustion. Furthermore, multi-layered granules were synthesized by intermittent feeding of valeric acid throughout polymer synthesis.

The presence of multiple layers implies either the existence of block copolymers in single polymer chains, the existence of blends of two polymers in the same granule, or a combination of the two possibilities. Increasing cell concentration and decreasing the amount of valeric acid

added decreases the time period of PHBV synthesis. Thus the switching period can be further decreased to produce block copolymers. Various analytical techniques are being applied such as Nuclear Magnetic Resonance (NMR), TEM, and polymer fractionation to study this possibility.

4. Mantzaris, N. V., Kelley, A. S., and Srienc, F. "A Population Balance Model describing the Dynamics of Molecular Weight Distributions and the Structure of PHA Copolymer Chains", *AIChE Annual Meeting*, Dallas, Texas, Oct. 31-Nov. 5, 1999.
5. Carlson, R.P. and Srienc, F. (1998) Utilizing the Bi-directional GAL1-10 Promoter to Co-express Multiple Pathway Genes in *Saccharomyces cerevisiae*, American Institute of Chemical Engineers National Meeting, Miami Beach, Florida.

Abstract

To engineer novel biochemical pathways in a foreign host, it is advantageous to be able to introduce multiple genes. Unlike prokaryotes, eukaryotes don't typically express polycistronic messages, so it is more difficult to introduce multiple genes into a eukaryotic system. We have utilized the divergent nature of the *Saccharomyces cerevisiae* GAL1-10 promoter to co-express two genes on a series of yeast plasmids. This bi-directional promoter regulates two genes by initiating transcription in opposite directions from a single, centrally located region. When one of the two promoter gene slots is used to express the marker gene, green fluorescence protein (GFP), it is possible to follow gene expression as well as estimate gene copy number by monitoring cell fluorescence. This is particularly useful for the identification and isolation of cells which contain a desired gene copy number. In addition, we have applied this system in *Saccharomyces cerevisiae* to express the bacterial genes involved in the multi-step production of the biopolymer poly-beta-hydroxybutyric acid (PHB). We have previously shown in *S. cerevisiae* that the expression of PHB synthase alone results in the low level production of PHB (Microbiology (1996), 142, 1169-1180). However, by using the divergent promoter to co-express multiple genes from the PHB pathway, we've been able to significantly increase levels of PHB.

6. Carlson, R.P., Leaf, T.A., and Srienc, F. (1998) Utilizing the Bi-directional GAL1-10 Promoter to Co-express Multiple PHB Pathway Genes in *Saccharomyces cerevisiae*, Poster Presentation, International Symposium on Biological Polyhydroxyalkanoates, Tokyo, Japan.

Abstract

The ability to introduce multiple genes into a foreign host offers a significant advantage for the metabolic engineering of novel biochemical pathways. Since eukaryotes don't typically possess a polycistronic system like that found in prokaryotes, it is more difficult to clone multiple genes into a eukaryotic system. We have utilized the bi-directional nature of the *Saccharomyces cerevisiae* GAL1-10 promoter to simultaneously express two genes from a series of yeast plasmids. This divergent promoter regulates two separate genes by initiating transcription in opposite directions from a single, centrally located region. For instance, this system permits the quantification of gene copy number and an estimation of gene expression by using one of the two gene slots to express the reporter gene green fluorescence protein (GFP). Furthermore, we have used this system in *Saccharomyces cerevisiae* to express the bacterial genes involved in the production of poly-beta-hydroxybutyric acid (PHB). We have previously shown in *S. cerevisiae* that the expression of the PHB synthase enzyme alone results in the low level production of

PHB (Microbiology (1996), 142, 1169-1180). Using a combination of plasmid borne and chromosomally integrated GAL1-10 constructs, we have performed a systematic study of how gene copy number effects PHB levels. Co-expression of the three PHB pathway genes from GAL1-10 promoter constructs significantly increases levels of PHB.

7. Carlson, R.P., Penttilä, M., and Sreenc, F., (1999) Effects of Cofactor Imbalances on Pathway Fluxes in *Saccharomyces cerevisiae*, American Institute of Chemical Engineers National Meeting, Dallas, Texas.

Abstract

The coenzymes NADH and NADPH are generated and/or consumed in many catabolic and anabolic reactions respectively. These coenzymes which carry reducing equivalents play a fundamental role in the redox balance of numerous metabolic pathways. Perturbations in the concentrations of NAD(P)H / NAD(P)⁺ such as those caused by the introduction of a catabolic or anabolic pathway can often lead to an unfavorable redox balance. We have cloned the bacterial poly-beta-hydroxybutyric acid (PHB) pathway into *Saccharomyces cerevisiae* which has resulted in a significant accumulation of PHB during physiological states when an excess of NADH is expected. Because PHB synthesis consumes NADPH, we have over expressed the glutamate dehydrogenase gene GDH2. This gene when expressed with the native GDH1 gene is capable of transferring electrons from NADH produced during catabolic processes like glycolysis to NADPH which can be used for anabolic processes like the production of PHB. The effects of this metabolic manipulation in the context of PHB synthesis kinetics will be presented and discussed.

8. Carlson, R.P., Fell, D., and Sreenc, F., (2000) Metabolic pathway analysis of *Saccharomyces cerevisiae* producing poly-β-hydroxybutyric acid . Worldcongress on Biotechnology, Berlin, Germany.

Abstract

Genetic engineering techniques have made it possible to design and alter metabolic reactions taking place in cells. This is particularly useful for biotechnology when the metabolic reaction network is altered for the purpose of producing specific compounds. However, metabolic networks are complex. It is useful therefore to have theoretical tools that can guide experimental alterations.

We have used elementary mode analysis to examine a metabolic pathway model of *Saccharomyces cerevisiae* that has been genetically engineered to produce the bacterial storage compound poly-b-hydroxybutyrate (PHB). The model includes the reaction network of the intermediary metabolism. It takes account of both reversible and irreversible reactions as well as of cell compartmentalization. The stoichiometry of the reaction network connects the production and/or consumption of eight external metabolites (glucose, acetate, glycerol, ethanol, PHB, CO₂ and ATP). Elementary mode analysis reveals that in wild type cells there are 274 basic reaction sequences that can balance the external metabolites. When the PHB pathway is included in the recombinant reaction network the number of possible basic reaction sequences increases to 474. Out of these modes, 200 basic reaction sequences produce PHB with theoretical PHB yields up to 0.67. The analysis also suggests further modifications of the reaction network to improve strain efficiency. For instance, when naturally absent ATP-citrate lyase activity is added to the reaction network, the number of possible basic reaction sequences is

further increased to 1215, out of which 650 reaction pathways are able to produce PHB. The most efficient pathway for PHB formation has an improved theoretical yield of 0.84. The analysis is also very valuable for analyzing experimental data by delineating the pathways used in different growth phases since not all external metabolites are metabolized in different growth stages.

Theses:

1. Jackson J.K., (1998) Recombinant modulation of the *phbCAB* operon copy number in *Ralstonia eutropha* and modification of the precursor selectivity of the *Pseudomonas oleovorans* polymerase I, Masters Thesis, University of Minnesota, Minneapolis, Minnesota.
2. Carlson, R.P, (1999) Utilizing the Bi-directional GAL1-10 Promoter to Co-express Two Genes in *Saccharomyces cerevisiae*, Masters Thesis, University of Minnesota, Minneapolis, Minnesota.

Abstract

Metabolic engineering of novel pathways often requires the expression of multiple genes in a heterologous host. Unlike prokaryotes, eukaryotes don't typically express polycistronic messages, so it is more difficult to introduce multiple genes into a eukaryotic system. We have utilized the divergent nature of the *Saccharomyces cerevisiae* GAL1-10 promoter to co-express two genes on a series of yeast plasmids. This bi-directional promoter regulates two genes by initiating transcription in opposite directions from a single, centrally located region. The system's ability to facilitate multi-step pathway engineering was demonstrated using the bacterial genes involved in the production of the biopolymer poly-beta-hydroxybutyric acid (PHB). Previously, it has been shown that the expression of PHB synthase alone in *S. cerevisiae* results in low level production of PHB (Microbiology (1996), 142, 1169-1180). However, by using the divergent promoter system to co-express multiple genes from the PHB pathway, it has been possible to significantly increase PHB levels. The role of sugar composition and concentration on polymer accumulation was also investigated. When the cultures were grown on a combination of glucose and galactose, the specific PHB levels were inversely related to the initial glucose concentration. In addition to pathway engineering, the divergent promoter system is useful with integration plasmids targeted for non-unique sites. When one of the two promoter gene slots is used to express the reporter gene, green fluorescence protein (GFP), it is possible to follow gene expression as well as estimate gene copy number by assaying for cell fluorescence. This is particularly useful for the identification and isolation of cells containing a desired gene copy number.

3. Nikolaos Mantzaris "Cell growth dynamics and control based on cell population balance models" PhD Thesis, University of Minnesota, Minneapolis, Minnesota. (February 2000)

Technology Transfer

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

Patents

1. Srienc F., Somers D.A., Hahn JJ., Eschenlauer A.C. (1998) "Polyhydroxyalkanoate synthesis in plants", U.S. Patent application Serial No. 09/052,607
2. Srienc F., Somers D.A., Jackson, J.K. (1998) "Modified polyhydroxyalkanoate polymerase", U.S. Patent application Serial No. 09/052,689
3. Kelley, A. S., Mantzaris, N. V., Srienc, F. (1999) "Novel Biopolymers and Biopolymer Blends, and Methods for Producing Same" filed September 9, 1999, U. S. Patent Pending, U.S. Serial Number 09/392,465.

Commercial Accomplishments

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

Our study has attracted the interest of several companies. The 3M Corporation will likely fund the patent filing costs (Patent (3)) and provide future research support which is being currently negotiated. In addition, Dow Chemicals will provide a research grant to support this work.

Educational Accomplishments

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

Dr. Timothy Leaf, graduated in 1998, accepted a position with Kosan Biosciences, CA.

Dr. J.J. Hahn, graduated in 1998, accepted a position with Cargill, MN.

Mr. K. Jackson, graduated in 1998, accepted a position with Biotechnical Resources, WI.

Mr. Aaron Kelley, graduate student, is currently working on this project as his PhD thesis project.

Mr. Ross Carson, graduate student, completed his Masters degree working on this project. He was accepted into the PhD program of the Chemical Engineering Department and is working now on this project as his PhD thesis project.

Dr. Nikolaos Mantzaris just defended his PhD thesis. He is currently searching for a faculty position.

Additional Funding

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

A proposal entitled "Biosynthesis of nanostructured polyesters" has been submitted to the National Science Foundation in August 1999. The proposal is pending.

Dow Chemical will support this project with a grant of \$40,000. The details are currently being finalized.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

The main work of this project in this reporting period has been carried out by Aaron Kelley, Ross Carlson, and Nikolaos Mantzaris with some overlap with T. Leaf, JJ. Hahn and K. Jackson who left the group in 1998 after graduation.

Send completed report to :

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Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Dr. Steven H. Strauss	
University:	Oregon State University	
Agreement Number:	OR22072-78	
Project Title:	<i>Genes Controlling the Transition Between Vegetative and Reproductive Phases in Forest Trees</i>	
Reporting Period and Report Type:	From: 1/1/99 To: 6/1/99	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

Objective 1: Isolate partial poplar homologs to known flowering time genes.

Computer searches were performed through GenBank, a poplar cambial EST database, and TIGR databases to identify flowering time-related genes that display sufficient evolutionary conservation useful for isolation of poplar homologs. Searches are ongoing as new sequence information becomes available. We have designed and synthesized degenerate primers to screen for poplar homologs to *TFL* (*TERMINAL FLOWER*), *CLF* (*CURLY LEAF*), *LD* (*LUMINIDEPENDENS*), *LHY* (*LATE ELONGATED HYPOCOTYL*), *CO* (*CONSTANS*), *CRY1* and *CRY2* (*CRYPTOCHROME1* and 2), *SPY* (*SPINDLY*), *PHYA* (*PHYTOCHROME A*), and *PHYB* (*PHYTOCHROME B*). Isolation of poplar homologs to *FCA* and *FLC* (*FLOWERING LOCUS C*) will be initiated soon. In prior and associated work, we isolated poplar homologs to the *Arabidopsis* genes: *APETALA1* (*API*), *AGAMOUS* (*AG*), *LEAFY* (*LFY*). Through this project to date, we have isolated poplar homologs to the *Arabidopsis* genes: *CO*, *CRY*, *PHYA*, and *PHYB*. A detailed summary and the number of homologs to each isolated gene is provided in the Scientific Accomplishments.

Objective 2: Study the sequences of the isolated homologs to assure they are evolutionary homologs, and analyze their genome structure via Southern blots to see if they are parts of gene families.

In prior work, Southern analysis demonstrated that two genes encode homologs to *API* (*PTAPI-1* and *PTAPI-2*), two genes encode homologs to *AG* (*PTAG1* and *PTAG2*), and one gene encodes a homolog to *LFY* (*PTLF*). Full-length cDNAs to each of these genes were also

isolated and phylogenetic analysis has confirmed the relationship of each gene to its *Arabidopsis* homolog. BLAST searches, sequence alignments, and hypothetical translation of the poplar *CO*, *PHYA*, *PHYB*, and *CRY* homologs have been conducted to verify their evolutionary relationship to corresponding gene families; Southern analysis is being initiated on these genes to determine each gene's family size in poplar.

Objective 3: Study expression in different tissues over developmental and maturation gradients.

Tissues for expression analysis are being collected from a clonally-propagated male and female poplar (age range 1-6 years) over the current growing season. Pre-dormancy vegetative buds were collected for expression analysis in October, 1998. In late March and late April, 1999, post-dormancy vegetative buds and new shoots, respectively, were collected. The remaining tissue sources will be collected during the current growing season at appropriate developmental points. These tissues include: (1) new vegetative buds, (2) new inflorescence buds, (3) summer shoots, (4) and seeds (female only). Preparation of RNA for expression studies has been initiated from collected tissues.

Objective 4: Isolate a full length cDNA from 1-2 genes if they show a promising pattern of expression.

Not Initiated Yet.

Objective 5: Begin transformation of altered cDNAs for 1-2 genes to study the effects of overexpression and inhibition in *Arabidopsis* and poplar.

Not Initiated 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.

The delay between vegetative growth and initiation of flowering remains a major hurdle to progress in breeding, marker aided selection, and genetic engineering of forest trees. Utilizing information from studies in model plant species, we have begun isolation of genes from forest trees to gain new insights into the control of flowering and juvenility. We are isolating *Populus trichocarpa* (poplar) genes which are functional equivalents of *Arabidopsis* genes shown to affect or act as indicators of floral timing, including *TFL*, *CLF*, *LD*, *LHY*, *CO*, *SPY*, and *CRY1* and 2. In prior work, we have successfully isolated homologs to *API* (*PTAPI-1* and *PTAPI-2*), *LFY* (*PTLF*), *AG* (*PTAG1* and *PTAG2*). Under this project, we have isolated poplar fragments to *CO* (*PiCOL1* and *PiCOL2*), *PHYA* (*PiPHYA*), *PHYB* (*PiPHYB1* and *PiPHYB2*), and *CRY* (*PiCRYa*, *PiCRYb* and *PiCRYd*). These genes are involved in aspects of floral timing, floral meristem identity, and floral organ identity. For expression studies, three of seven planned tissue collections have been completed and the remaining four will be collected during the current growing season as new vegetative buds, new inflorescence buds, summer shoots, and seeds (female only) develop.

Scientific Accomplishments

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

Poplar flowering time gene homolog isolation.

Candidate floral timing genes were identified based on searches of DNA sequence databases. Based on these searches, genes have been aligned for at least two plant families and found to contain conserved motifs to aid isolation of poplar homologs. Genes we are pursuing or have isolated include:

<i>TFL</i> (<i>TERMINAL FLOWER</i>)	<i>CRY1</i> and 2 (<i>CRYPTOCHROME1</i> and 2)
<i>CLF</i> (<i>CURLY LEAF</i>)	<i>LHY</i> (<i>LATE ELONGATED HYPOCOTYL</i>)
<i>LD</i> (<i>LUMINIDEPENDENS</i>)	<i>API</i> (<i>APETALA1</i>)
<i>SPY</i> (<i>SPINDLY</i>)	<i>AG</i> (<i>AGAMOUS</i>)
<i>CO</i> (<i>CONSTANS</i>)	<i>LFY</i> (<i>LEAFY</i>)
<i>PHYA</i> (<i>PHYTOCHROME A</i>)	<i>FCA</i>
<i>PHYB</i> (<i>PHYTOCHROME B</i>)	<i>FLC</i> (<i>FLOWERING LOCUS C</i>)

Degenerate primers have been designed to the above genes, for isolation of poplar homologs from genomic DNA and cDNA libraries. Progress towards isolation of each gene is as follows:

***CO*:** A Swedish poplar EST database focusing on wood-forming tissues of poplar (PopulusDB; <http://www.biochem.kth.se/PopulusDB/>) is publicly accessible. Poplar EST data from hybrid aspen (*Populus tremula* x *tremuloides*) and *Populus trichocarpa* are available. Searches of this database identified three ESTs displaying similarity to *CO* which were used to aid design of degenerate *CO* primers, along with reported *CO* sequences and other plant EST information. PCR amplified products were obtained from male and female genomic DNA, as well as an female early inflorescence cDNA library. Four separate sized products, resulting from different primer combinations, were subcloned from the cDNA library amplifications and sequenced. All four products were identified as poplar *CO* homologs. At least two poplar *CO* genes were identified and designated *PtCOL1* and *PtCOL2*. Three of the four products appear to be variants of *PtCOL1*; one appears to represent an expressed pseudogene and thus at least three *CO* genes may be present in poplar.

***TFL*:** Degenerate primers were generated based on known *TFL* family sequences. A single, repetitively-amplified band was observed during PCR from early female inflorescence cDNA libraries. The product was subcloned and sequenced. All isolated clones failed to show homology to *TFL*. Despite the availability of diverse sequences, *TFL* from poplar has proven surprisingly hard to amplify. We have PCR-amplified the *Arabidopsis TFL* gene and are initiating a screen of our small insert genomic library (described below), using the *Arabidopsis* gene as a probe, to isolate poplar *TFL*.

***CRY*:** Degenerate primers to *CRY* were designed to amplify *CRY1* and *CRY2* sequences. A band of the expected size was amplified from the small insert genomic DNA library,

subcloned, and sequenced. PCR products of three separate *CRY* genes were isolated. All three genes are homologs of the cryptochrome family, but assignment to the *CRY1* or *CRY2* subfamily is inconclusive with the sequence obtained. The three genes have been tentatively designated *PtCRYa*, *PtCRYb*, and *PtCRYd*. Based on preliminary results, *PtCRYb* and *PtCRYd* are alleles of the same gene, while *PtCRYa* represents a second poplar *CRY* gene. Isolation of additional coding sequence for the *CRY* genes is being initiated, which will allow assignment of each gene to the *CRY1* or *CRY2* subfamily.

***CLF*, *SPY*, and *LHY*:** Isolation of homologs to these three gene families is underway. Using degenerate primers, *CLF* and *SPY* bands were amplified from the female inflorescence cDNA library, subcloned, and sequenced. All isolated clones failed to show homology to either gene. We are repeating PCR amplifications of the genes using the OptiPrime PCR optimization kit. Recently, we have obtained amplified products of the expected sizes to *CLF* and *SPY* using a subset of the optimization buffers. Subcloning and analysis of these products is underway. Degenerate primers to *LHY* were designed and utilized, but amplified products were not observed. New degenerate primers were recently designed and will be tested in conjunction with the OptiPrime PCR optimization kit.

***LD*:** Degenerate primers have been synthesized and PCR amplifications performed from genomic DNA of male and female poplar trees. Three repetitively-amplified PCR bands have been observed from both DNA sources; these bands failed to show homology to *LD*. Using sequence information from a soybean EST homolog of *LD* and shared information on a maize *LD* sequence, we are designing new degenerate primers and will subsequently reinstate screening for poplar *LD* homologs.

***PHYA* and *PHYB*:** Degenerate primers to *PHYA* and *PHYB* genes were designed and synthesized. PCR amplified products were obtained from male and female genomic DNA, as well as a female early inflorescence cDNA library. Products were subcloned and sequenced. Initial sequence results identified isolation of one *PHYA* (*PtPHYA1*) and two *PHYB* (*PtPHYB1* and *PtPHYB2*) genes. Screening for additional *PHY* genes is underway.

***API*, *AG*, and *LFY*:** In a related project, we have isolated poplar homologs to these three genes through screening of cDNA libraries. These genes are involved in aspects of floral timing, floral meristem identity, and/or floral organ identity. Two poplar homologs to *API* (*PTAPI-1* and *PTAPI-2*) have been isolated. Two alleles to *PTAPI-1* have been identified. Full-length cDNAs to each *PTAPI* gene have been isolated. Two poplar homologs to *AG* (*PTAG1* and *PTAG2*) have been isolated. Full-length cDNAs and genomic clones to *PTAG1* and *PTAG2* have been isolated. One poplar homolog to *LFY* (*PTLF*) has been isolated. A genomic clone and full-length cDNA to *PTLF* have been isolated. Southern analysis has been conducted on each gene and these isolates represent the entire gene family for each class. Phylogenetic analysis has confirmed their relationship to the appropriate families. Suitable regions of sequence are present in each gene to design gene-specific probes for expression studies.

***FCA* and *FLC*:** Isolation of a poplar homolog to *FCA* is ongoing. The *FLC* gene (also known as *FLF*) has recently been isolated and demonstrated to play a central role in vernalization-based regulation of flowering time. *FLC* is a member of the well conserved MADS

box gene family. We will be obtaining the *FLC* clone from Dr. Richard Amasino (U. Wisconsin-Madison; personnel communication) and subsequently initiate screening for the poplar homolog.

Construction of small-insert genomic library.

For some gene families, only one region of significant conservation useful for primer design has been available. To overcome this liability, we generated a small insert poplar genomic DNA library. This allows the use of a floral timing gene-specific primer and a flanking vector primer for amplification of floral timing genes. Genomic DNA inserts are 3 kb in size on average. This library was successfully used to isolate poplar homologs to *CRY*. Amplification of *CLF* and *SPY* bands has recently been obtained from this library and they are currently being analyzed.

Tissue collections for expression analysis.

For expression studies, tissue sources for RNA extraction need to be collected at seasonal time points due to the perennial nature of poplar trees. Tissue is being collected from clonally-propagated trees over a 1-6 year age range. The male tissue source is hybrid poplar (*Populus trichocarpa* x *deltooides*) clone 24-305 and the female tissue source is hybrid poplar (*Populus trichocarpa* x *deltooides*) clone 15-29. Trees are growing at Fort James plantations at Clatskanie, OR, and Scapoose, OR (Lower Columbia River Basin). These tissue sources will be utilized for all tissue collections. In October, 1998, we collected pre-dormancy vegetative bud tissue from the above male and female hybrid poplar genotype. In late March, 1999, post-dormancy vegetative bud tissue was collected. In late April, 1999, new shoots were collected. Additionally, during the current growing season the following tissues will be collected: (1) new vegetative buds, (2) new inflorescence buds, (3) summer shoots, (4) and seeds (female only).

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.

A poster entitled "Isolation of Flowering Time Genes" was presented at the Tree Genetic Engineering Research Cooperative (TGERC) Annual Meeting (November 4-5, 1998, Seattle, WA). The poster was directed towards the TGERC representatives, a mixture of scientific and nonscientific backgrounds. The poster outlined the project to be done, the scientific background and rationale, and possible applications of the projects results. At the CPBR meeting in March, 1999, a seminar talk entitled "Genes Controlling the Transition Between Vegetative and Reproductive Phases in Forest Trees" and a poster entitled "Genes Controlling Flowering and Maturation in Trees" were presented. The CPBR talk was an update on the project directed towards a combination of scientific and business research personnel. The poster was displayed at the Congressional reception and was directed towards political representatives. A poster was presented at the Oregon State University Graduate Student Conference (April 7, 1999) entitled "Isolation and Expression Analysis of the *TERMINAL FLOWER 1* Homolog in *Populus*". Conference attendees were primarily graduate students and faculty from all academic science departments of Oregon State University. Finally, a poster entitled "Isolation of Poplar Flowering Time Genes" was presented at the annual meeting for the Northwest Scientific Association

(March 25-28, 1999, Tacoma, WA). The audience was composed primarily of forest conservationists and forest researchers.

Technology Transfer

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

No technology transfer arrangements have resulted from the work yet

Commercial Accomplishments

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

No commercial applications have currently resulted from the work yet.

Educational Accomplishments

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

New educational aspects since the last Progress Report occurred in the form of attendees at scientific conferences where aspects of the project's work were presented (see Publications and Presentations section).

Additional Funding

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

No additional funding has been generated as a result of the project yet.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

No personnel turnover has occurred since the last Progress Report.

Projected Work for Year two

Principal Investigator: Dr. Steven H. Strauss
University: Oregon State University
Project Title: Genes Controlling the Transition Between Vegetative and Reproductive Phases in Forest Trees

Objective 1: Isolate partial poplar homologs to known flowering time genes.

We will continue to isolate poplar homologs to floral timing genes. Currently, we have isolated poplar flowering time homologs to seven gene families; multiple homologs to a subset of these families have been identified. Our target is to isolate poplar flowering time genes representing ten to fifteen families, and obtain as many members of each gene family as feasible. Through this project and prior studies, we have isolated poplar homologs to *API*, *AG*, *LFY*, *CO*, *CRY*, *PHYA*, and *PHYB*. Additional families we are pursuing include *TFL*, *CLF*, *LHY*, *SPY*, *LD*, *FCA*, and *FLC*. For poplar genes we isolate, we will attempt to identify additional family members based on results of Southern analysis (Objective 2). Isolation of genes will continue during year one and into year two.

Objective 2: Study the sequences of the isolated homologs to assure they are evolutionary homologs, and analyze their genome structure via Southern blots to see if they are parts of gene families.

Sequence analysis has confirmed the evolutionary relationship of isolated poplar flowering time genes to *API* (*PTAP1-1a*, *PTAP1-1b*, and *PTAP1-2*), *AG* (*PTAG1* and *PTAG2*), *LFY* (*PTLF*), *CO* (*PtCOL1* and *PtCOL2*), *CRY* (*PtCRYa*, *PtCRYb*, and *PtCRYd*), *PHYA* (*PtPHYA1*), and *PHYB* (*PtPHYB1* and *PtPHYB2*) families. In prior studies, Southern analysis was completed for poplar homologs to *API*, *AG*, and *PTLF*, confirming all members have been isolated. During the remaining portion of year one and the start of year two, Southern analysis will be conducted on the remaining isolated members, as well as additional poplar genes that are isolated. If additional family members are indicated by Southern analysis, their isolation will be pursued (Objective 1). For all isolated genes, phylogenetic analysis will be utilized to verify their evolutionary relationship to flowering time gene families.

Objective 3: Study expression in different tissues over developmental and maturation gradients.

Three of seven tissue isolations have been completed. At the appropriate developmental stages, the remaining four tissue collections will be conducted. These will occur during the current growing season, covering the remaining portion of year one and the beginning of year two. RNA isolations have been initiated on collected samples and will be completed during year two as sample collection is completed. During year two, gene-specific primers will be designed to each isolated gene and primer specificity verified against isolated templates. Following isolation of all RNA samples, real-time PCR will be conducted on each isolated gene to analyze expression in various tissues (see Progress Report) of a clonally-propagated male and female poplar (age range 1-6 years). Based on observed expression patterns, one or more candidate genes may be identified for analysis in Objectives 4 and 5. As appropriate, *in situ* analysis may be conducted on

gene(s) for which detailed expression analysis is needed. Writing of publications will commence in year two following completion of expression data for isolated gene families.

Objective 4: Isolate a full length cDNA from 1-2 genes if they show a promising pattern of expression.

Genes from Objective 3 will be identified if any show expression patterns correlating with onset of reproductive competence. Up to two genes will be selected and their full-length cDNAs isolated from RNA of tissues showing maximum levels of expression. Isolation would occur during the second to third quarter of year two.

Objective 5: Begin transformation of altered cDNAs for 1-2 genes to study the effects of overexpression and inhibition in *Arabidopsis* and poplar.

Full-length cDNAs isolated in Objective 4 will be cloned into a plant binary vector and expressed under the control of a strong constitutive promoter, such as CaMV 35S, to induce early flowering. Depending on the RNA expression pattern indicated in Objective 3, each cDNA will be placed in sense (if the gene appears to positively affect flowering) or antisense (if the gene appears to negatively affect flowering) orientation. Sense constructs will be placed in *Arabidopsis* and poplar, while antisense constructs will be placed in both poplar and *Arabidopsis* (if sufficient blocks of conserved sequence are present in the latter species). Subcloning and transformation of the constructs will be initiated towards the end of year two. Results from the transformation will be obtained after the end of the second year of funding.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Eric W. Triplett	
University:	University of Wisconsin-Madison	
Agreement Number:	OK 22072-70	
Project Title:	Engineering Sinorhizobium for increased alfalfa biomass	
Reporting Period and Report Type:	From: 7/01/98 To: 2/29/00	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

Objective 1. Enhance alfalfa biomass as a result of H₂ oxidation.

In 1999, we did observe a statistically significant yield increase of alfalfa of 2.7%. This increase was significant at the 2.5% level. This increase was observed only with plant inoculated with a strain of *Sinorhizobium meliloti* that possesses pHUTFXPAR and was not observed with any of the control treatments.

Objective 2. Enhance nodulation competitiveness through TFX production.

In 1999, we observed a significant increase in nodule occupancy by the inoculum strain when is contained pHUTFXPAR. This increase was as much as 40% and was not observed with strains lacking pHUTFXPAR.

Objective 3. Transfer of pHUTFXPAR to other strains in soil and the rhizosphere.

These experiments will begin this year.

Objective 4. Effect of pHUTFXPAR in *Sinorhizobium* on non-target microorganisms in the rhizosphere by culture-independent means.

We have shown that TFX production can decrease the diversity of TFX-sensitive bacteria in the rhizosphere (Robledo et al. 1998a). This work is now in progress with pHUTFXPAR constructs. This work has been made easier by the development of an automated approach in this laboratory (Fisher and Triplett 1999).

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.

From our first year of work, we have evidence that we can improve the biomass of alfalfa with this technology. With this technology, the plants more efficiently use their energy so that they can be more productive. We are now studying whether this biomass increase can be observed in one more year of study prior to beginning the commercialization process.

Scientific Accomplishments

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

In our original proposal, we described the construction of a broad host range plasmid, pHUTFXPAR, that possessed three sets of genes that coded for three important phenotypes (Kent et al. 1998). The first set of genes codes for trifolitoxin (TFX) production and resistance (Breil et al. 1993). Trifolitoxin is a peptide antibiotic that inhibits a specific group of α -proteobacteria including most of the genera that fix nitrogen in plant root nodules (Triplett et al. 1994). We have shown that the production of this antibiotic allows an inoculum strain to be more competitive for nodulation versus indigenous strains and other inoculum strains in soil including under agricultural conditions (Robledo et al. 1997; Robledo et al. 1998b). Thus, we can solve the significant problem of making superior inoculum strains more competitive for nodulation (for reviews see Maier and Triplett 1996; Scupham et al. 2000; Triplett 1999).

The second set of genes on this plasmid that is important in this work is a partitioning locus confers complete plasmid stability in the absence of selection pressure (Kent et al. 1998). That is, selection pressure is not necessary in order to maintain this plasmid in the cell. This plasmid is then suitable for field use since field application of antibiotics is not practical.

The third set of genes is the yield enhancement cassette. This set of genes codes for the uptake hydrogenase phenotype that oxidizes the dihydrogen generated during the nitrogenase reaction (for review see Maier and Triplett 1996). In soybean, this hydrogen cycling by root nodule bacteria can enhance yield significantly (for review see Evans 1987).

Once constructed, we tested pHUTFXPAR for the three phenotypes of interest in the laboratory and found that they were conferred by this plasmid (Kent et al. 1998). We then transferred pHUTFXPAR to *Sinorhizobium meliloti* PC-2 by conjugation. Strain PC-2 is a very high yielding strain and is itself genetically engineered (Bosworth et al. 1994; Scupham et al. 1996). Thus we were hoping to improve a strain of *Sinorhizobium* that was already very high yielding on alfalfa. We inoculated alfalfa seeds with *S. meliloti* PC-2 (pHUTFXPAR) as well as other derivatives of PC-2 that contained two or fewer of the phenotypes conferred by pHUTFXPAR.

Table 1 below shows the yield enhancement of alfalfa upon addition of the inoculum strain possessing pHUTFXPAR. This yield increase of 2.7% was lower than expected but was significant at the 2.5% level. Given the ease of use of this technology, this would be a very inexpensive means for farmers to enhance the yield of alfalfa. Furthermore, this result was obtained on a soil where nitrogen is probably not most limiting to plant productivity. To obtain yield enhancement under such conditions with a *Sinorhizobium* strain is remarkable. We are hoping to obtain similar results in 2000 with this same planting.

Table 1. Alfalfa yield response at Arlington, WI to inoculum strains with pHUTFXPAR. These are the results from the 1999 harvests of the alfalfa planted in 1998. Seeding year yield (1998 yield) is not shown and is typically too variable and too low to be included in yield estimates.

Strain	Yield (Mg/Ha)	% change from WT
Uninoculated	12.29	
Wild-type	12.29	
pHUTFXPAR	12.62	2.7*
pHUPAR	12.47	1.5
pTFXPAR	12.46	1.4

*Statistically significant at the 2.5% level.

We have also shown that the nodulation competitiveness of PC-2(pHUTFXPAR) is enhanced compared to various controls (Fig. 1). Note in Table 1 that a significant yield increase was observed only with the addition of both the TFX production and uptake hydrogenase phenotypes. As expected, the addition of a nodulation competitiveness phenotype is absolutely essential if yield enhancements from inoculum strains are desired. In addition, Table 1 shows that the uptake hydrogenase phenotype is also necessary for increased yield. So the genetic construction of better root nodule bacteria for agriculture requires that attention be paid to yield enhancement and nodulation competitiveness.

Fig. 1. Increased nodulation competitiveness observed from inoculum strain possessing pHUTFXPAR compared to a control strain with pHUPAR (lacking the TFX production and resistance genes).

With the second year of funding, we will complete the 2000 harvests for the alfalfa field trials already in place. This will give us more data and much more power in our statistical analysis. In addition, we will focus on the risk assessment issues concerning the effects of pHUTFXPAR on soil microorganisms in soil. Our preliminary data suggests that TFX production alters the community diversity of that narrow group of organisms sensitive to TFX but has no discernible effect on other organisms (Robledo et al. 1998a). This will be done by a culture-independent method that we have recently developed (Fisher and Triplett 1999). We will

also determine the frequency of transfer of pHUTFXPAR to soil organisms. These risk assessment questions must be addressed prior to commercialization of this technology.

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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.

Scupham, A.J., E.A. Robleto, and E.W. Triplett. 2000. Solving the competition problem: genetic and field approaches to enhance the effectiveness of the *Rhizobium*-legume symbiosis. In: Prokaryotic Nitrogen Fixation: a Model System for the Analysis of a Biological Process, E.W. Triplett, ed., Horizon Scientific Press, Norfolk, UK, pp. 251-278.

Triplett, E.W. (Ed.). 2000. Nitrogen fixation: a model system for the analysis of a biological process. Horizon Scientific Press, Norfolk, UK, 792 pp.

Triplett, E.W. 1999. Antibiosis as a means to enhance nodulation competitiveness by *Rhizobium* inoculum strains under agricultural conditions. In: Highlights of Nitrogen Fixation Research, E. Martinez and G. Hernandez, eds., Plenum Publishers, New York, pp. 225-229.

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Kent, A.D., M.L. Wojtasiak, E.A. Robleto, and E.W. Triplett. 1998. A transposable partitioning locus used to stabilize plasmid-borne hydrogen oxidation and trifolitoxin production genes in a *Sinorhizobium* strain. Appl. Environ. Microbiol. 64:1657-1662.

Invited Speaker, 11th International *Trifolium* Conference, Madison, WI, June 11, 1998
Invited Speaker, Consortium for Plant Biotechnology Research, Washington, DC, March 3, 1999
Invited Speaker, APS/CPS Annual Meeting, Montreal, Canada, August 1999
Invited Speaker, 12th International Congress on Nitrogen Fixation, Foz do Iguacu, Brazil,
September 14, 1999
Seminar, University of Georgia, Athens, December 9, 1999
Invited Speaker, Agricultural Microbes I Conference, San Diego, CA, January 14, 2000

Technology Transfer

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

U.S. patents issued on this work:

"Plasmid for transformation of root nodule bacteria", issued January 12, 1999, no. 5,858,762.

"Enhanced inoculant for soybean cultivation", issued May 25, 1999, no. 5,906,929.

Commercial Accomplishments

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

A third year of field data to be collected in the summer of 2000 will determine whether LiphaTech commercializes this technology. If LiphaTech decides to commercialize, they will begin to get the data necessary for regulatory approval through EPA. The approval process requires about one year. If a positive decision comes from EPA, LiphaTech (no earlier than the 2002 field season) will begin test marketing this technology in several environments prior to large scale commercialization.

Educational Accomplishments

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

Dr. Eduardo Robleto obtained his Ph.D. degree on this project. Leo Iniquez is now the student working on this project. An undergraduate, Josh Morrison is also getting terrific experience working with us. A new postdoc will be hired whose education will be enhanced by working on this project.

Additional Funding

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

No other funding has been generated for this project during this period.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

Dr. Eduardo Robleto was the person in the Triplett laboratory who had the day to day responsibility of leading this work. However, Dr. Robleto left the Triplett laboratory in August 1999. He has been replaced by a special student, Leo Iniquez who is expected to enter graduate school this summer. Leo worked with Eduardo closely for two summers before he left. Josh Morrison is a senior undergraduate who has been working with us on this project since its beginning. Patrick Riggs works part-time on this project primarily in assisting Leo with the field work.

Send completed report to :

The Consortium for Plant Biotechnology Research, Inc.

P.O. Box 20634

(Express Delivery address: 10 Sylvan Drive, Suite 21)

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Or sent as an email attachment to: cpbr@gate.net

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Eric W. Triplett, Ph.D.	
University:	University of Wisconsin-Madison	
Agreement Number:	OR22072-100	
Project Title:	<i>Associative Nitrogen Fixation by Diazotrophic Endophytes in Switchgrass</i>	
Reporting Period and Report Type:	From: 12/1/98 To: 11/30/99	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

1. Inoculate a collection of switchgrass accessions from remnant prairies with 15 diazotrophic bacteria that are candidates for increased switchgrass growth in the absence of N fertilizer.

Progress: Given our recent results with our maize model system described below, we no longer believe that these experiments are worthwhile. As described below, we now intend to pursue experiments that enhance bacterial nitrogen fixation in maize and switchgrass by sucrose additions to the stem since our model system shows that this is the primary limiting factor for increased nitrogen fixation by endophytes in grasses. If the sucrose stem infusion work is successful, we will then add specific transgenes to maize to enhance sucrose availability in the apoplast of stems and roots during vegetative growth.

2. Isolate diazotrophs from the stems of the switchgrass accessions and re-inoculate into accessions to determine whether any provide a benefit to switchgrass in the presence of absence of added N.

Progress: We have isolated two *Pantoea agglomerans* strains from switchgrass during the last year. These strains have very high nitrogenase activity in culture under microaerobic conditions. We have placed a constitutively expressed GFP gene in both strains and have inoculated the GFP-containing strains onto switchgrass plants. These plants are now growing in the greenhouse.

3. If genotype x strain combinations are observed that enhance switchgrass growth in the absence of added N, prepare appropriate *nif* mutants to determine whether the benefit observed is the result of nitrogen fixation.

Progress: As sucrose limitation in the apoplast seems to be the limiting factor in this work, all of our efforts are directed toward this goal. Thus, we are less interested in genotype differences at the moment. However, we are preparing *nifH* mutants of diazotrophic endophytes from maize and switchgrass in order to have the appropriate negative control. We are also preparing *glnA* mutants of these diazotrophs which, according to recent preliminary work, should be capable of exporting high amounts of ammonia under nitrogen-fixing conditions.

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.

Most grasses cultivated for agriculture, such as maize, wheat, and switchgrass, require large amounts of nitrogen fertilizer for high yields. However, the nitrogen used for grass cultivation is one of the highest costs in the production of these crops. In addition, the nitrogen fertilizer applied is not entirely used by the plant which causes a large portion of it to pollute ground water. This results in unsafe levels of nitrate in drinking water in many rural areas of the United States.

A solution to these dual problems of the expense of nitrogen fertilizer production and its application as well as the pollution problems that arise from the use of these fertilizers is the development of nitrogen fixation in grasses. Biological nitrogen fixation is a process performed by bacteria whereby the bacteria convert dinitrogen gas (which makes up 80% of our atmosphere) to ammonia, a form of nitrogen that is readily used by plants. These bacteria sometimes form associations with plants that allow them to provide all of the nitrogen needs of the plant. This eliminates the need for nitrogen fertilizers. Unfortunately, such bacterial-plant associations exist only in sugarcane among the grasses. As grasses are very important to human and animal food consumption as well as to sustainable biomass production for the generation of electric power, there is increasing interest in expanding the beneficial bacterial nitrogen fixation process to some of the most economically important grasses.

Our most significant accomplishment during this year of effort was our ability to determine those factors that most limit nitrogen fixation by bacteria that reside naturally inside grass plants. Now that we know these factors, we can concentrate our efforts on generating new grass germplasm that maximizes the possibility for nitrogen fixation in grasses. In addition, we have shown that such bacteria reside in switchgrass.

Scientific Accomplishments

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

There were three significant accomplishments in our first year of support. The first is the discovery of nitrogen-fixing endophytes in switchgrass. Two isolates of *Pantoea agglomerans* were identified that fix nitrogen in culture. We have recently labeled these isolates with GFP and inoculated these onto switchgrass seedlings. In a series of experiments, we will determine how

these isolates enter switchgrass as well as the ability of these strains to provide any growth benefit in the presence or absence of added N.

The second major accomplishment concerns very similar work with maize that will have direct application to our switchgrass work. We have also worked with maize for several reasons. First, maize is an excellent model system for such work. Lots of genetic tools are available for maize that are unavailable switchgrass. For example, many hybrid and inbred lines are available. The genetic diversity of maize is enormous and is easily accessible to investigators. Mutants are also freely available or easily prepared by chemical or transposon mutagenesis. Tissue culture and transformation methods are available that includes the generation of sterile plants. The availability of sterile plants will soon be essential to develop an understanding of the role of diazotrophic endophytes in grasses.

In our work with maize, we have found that diazotrophic endophytes do re-enter the plant upon inoculation and will produce nitrogenase inside maize seedlings provided that a carbon source (0.1% sucrose) is added to the medium. This has enormous implications for the development of nitrogen fixation in grasses using endophytes.

We have also found that the diversity of microorganisms inside maize is much larger than originally thought. We are the first to take a culture independent look at the prokaryotes in grasses. In this examination, we have found a wide array of *Bacteria* but have also found evidence for *Archaea* living inside maize. The vast majority of organisms discovered in maize are not known to be diazotrophic.

These two observations in maize suggest two problems related to the ability of maize and switchgrass to obtain fixed nitrogen from diazotrophic endophytes. First, the enormous diversity of prokaryotes in maize suggests that diazotrophs in maize may be competing with a large number of non-diazotrophs for resources within the plant preventing them from thriving and providing fixed N to the plant. The second issue is that when seedlings are provided with a carbon source, nitrogenase protein is produced by the diazotrophs inside the tissue. This suggests that carbon supply to the bacteria is limiting expression of nitrogen fixation within the plant. Both of these problems can be addressed and both of them are of interest to us in future work.

A third fascinating result from the previous year of work is the discovery that certain switchgrass plants can survive long periods without any nitrogen fertilizer. We planted some switchgrass plants collected from remnant prairies in November 1998 that remain alive to this day (December 17, 1999) without ever having been given nitrogen fertilizer. Granted these plants are not robust but given the negligible nitrogen supply provided to them in the washed sand and vermiculite substrate, their continued growth is impressive. We will soon be examining some of these plants for their ability to fix N₂. We also intend to propagate these plants clonally in the field next year.

Educational Accomplishments

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

Dr. Marisa Chelius completed her Ph.D. degree while on this project in August 1999. She is continuing as a postdoc in the Triplett laboratory. Ms. Jennifer Henn completed an undergraduate research project in Biology 152 on this work.

Additional Funding

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

We have obtained an additional \$114,000 in support from Cargill for this project since November 1998. This is in addition to the original \$125,000 match received for this work. Patrick Riggs, the technician on this project, is now paid 50% by intramural state funds.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

The personnel on this project include Dr. Marisa Chelius and Mr. Patrick Riggs in Eric Triplett's laboratory. In addition, 10% of Mr. Andy Beal from Prof. Mike Casler's research program is supported by this program. Mr. Patrick Riggs began as an undergraduate working on this project from November 1998 until May 1999. Upon receipt of his B.S. degree last May, Patrick began working as a full-time technician on this project. An undergraduate in the Triplett lab, Jennifer Henn, is also working part-time on this project.

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.

Poster

Presented at the 12th International Congress on Nitrogen Fixation, Foz do Iguacu, Brazil, September 13-17, 1999, "Production of NifH by *Klebsiella pneumoniae* in maize".

Talks

Seminar, "Building better bacteria (and plants) for Agriculture, University of Georgia, Athens, December 9, 1999

Invited Speaker, title to be announced but will include endophyte work, Agricultural Microbes I Conference, San Diego, CA, January 14, 2000

Publications

Chelius, M.K. 2000. Diazotrophic endophytes associated with maize. In: Prokaryotic Nitrogen Fixation: a Model System for the Analysis of a Biological Process, E.W. Triplett, ed., Horizon Scientific Press, Norfolk, UK, pp. 779-792.

Triplett, E.W. (Ed.). 2000. Nitrogen fixation: a model system for the analysis of a biological process. (A compilation of 44 chapters written by experts from around the world.) Horizon Scientific Press, Norfolk, UK, 792 pp.

Chelius, M.K. and E.W. Triplett. 2000. Immunolocalization of dinitrogenase reductase produced by *Klebsiella pneumoniae* in association with *Zea mays*. Appl. Environ. Microbiol. (in press, February issue).

Chelius, M.K. and E.W. Triplett. 2000. *Dyadobacter fermentans* gen. nov., sp. nov., a novel gram-negative bacterium isolated from surface-sterilized *Zea mays* stems. Inter. J. System. Bacteriol. (in press, April issue).

Chelius, M.K., P. Riggs, J. Henn, and E.W. Triplett. 2000. Isolation and re-infection of diazotrophic *Pantoea agglomerans* strains from wild accessions of switchgrass. Manuscript in preparation.

Riggs, P., J. Henn, and E.W. Triplett. 2000. Synergistic growth effects of grasses in the presence and absence of added N by inoculation with mycorrhizae and diazotrophic bacteria. Manuscript in preparation for Plant and Soil.

Riggs, P., S.M. Kaeppler, and E.W. Triplett. 2000. Yield enhancement of maize under agricultural conditions by inoculation with diazotrophic bacteria. Manuscript in preparation for Plant and Soil.

Technology Transfer

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

A licensing agreement between the Wisconsin Alumni Research Foundation (WARF) and Cargill, Inc. has been negotiated on this project. With continued funding, Cargill will have first right of refusal on all inventions. It is my understanding that these rights are exclusive.

Commercial Accomplishments

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

We are working closely with Cargill, Inc. on this work. The scientific accomplishments described above are certainly pertinent to the commercial accomplishments. In addition to the work described above, we have also found that certain bacteria enhance the yield of maize in the field. A publication describing this work is now in preparation. The purpose of doing this work is to begin the product stream early in order to provide a comfort level for farmers applying bacterial inoculants on grasses as well as to generate income as early as possible to continue support of the work.

We have also found that mycorrhizal inoculation on maize can improve the ability of the diazotrophic bacteria to enhance maize yield in the presence or absence of nitrogen fertilizer. This work is also in preparation for publication.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Donald L. Van Dyne	
University:	University of Missouri	
Agreement Number:	OR22072-58	
Project Title:	Estimating the Economic Feasibility of Converting Ligno-Cellulosic Feedstocks to Ethanol and Higher Value Chemicals Under the Refinery Concept: A Phase II Study	
Reporting Period and Report Type:	From: November 1, 1996 To: July 31, 1999	Check one: ☐ Interim Report ☒ Final Report

Project Objectives

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

The overall objective of the proposed research is to continue evaluating the economic feasibility of converting ligno-cellulosic materials into ethanol and various types of higher value added chemicals under the Refinery Concept. Specific objectives include:

1. To estimate the economic feasibility of a ligno-cellulose-to-ethanol and higher value chemical, "refinery-type", manufacturing plant in Missouri. *This objective has been completed.*
2. To estimate the economic feasibility of a ligno-cellulose-to-ethanol and higher value chemical, "refinery-type", manufacturing plant in Georgia drawing feedstocks primarily from within the state. *This objective has been completed.*
3. To estimate the cost of producing ethanol when using the enzymatic process relative to costs when using acid hydrolysis. *This technology is not ready for commercial application and thus, was not completed.*
4. To evaluate the economic feasibility of co-producing ethanol and four to six higher value chemicals from ligno-cellulosic feedstocks, e.g., crop residues and switchgrass. *This objective has been completed.*
5. To estimate the environmental benefits of commercialization of various ligno-cellulosic derived chemicals in accord with the Kyoto Protocol. *This objective has been completed*

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.

Several important conclusions were reached as the result of this research, including:

- Acid hydrolysis technology -- both dilute and concentrated -- is closer to current commercial use than other technologies.
- The production of ethanol as a single product from ligno-cellulosic (LCF) resources -- using dilute acid hydrolysis -- is not economic when priced at \$1.25 per gallon.
- Co-production of ethanol and higher value chemicals can be highly profitable, assuming markets exist for these products.
- The optimum size biorefinery located in Missouri would process about 4,360 tons of feedstocks daily, while producing 47.5 million gallons of ethanol and 323 thousand tons of furfural annually. Most of the feedstocks would be crop residues.
- The Missouri plant would cost about \$455 million to construct, generate annual income of \$281 million and have an annual pre-tax net profit of \$108 million.
- Up to 6,000 additional jobs would be generated from the operation of the LCF-to-ethanol plant. In addition, there would be significant increases in tax base, personal income, and increased total economic activity.
- The optimum size LCF-based plant in Georgia would be considerable larger and process about 7 thousand dry tons of feedstocks daily, mostly wood and wood residues.
- The Georgia plant would produce almost 85 million gallons of ethanol and 437 tons of furfural annually.
- The Georgia plant construction costs are estimated at \$632.5 million. Annual operating costs are estimated to be \$142.9 million. The cash flow (total returns minus operating costs) is estimated to be \$108 million annually.
- Various higher value chemicals (for which CPBR has provided funding) could be co-produced with ethanol. This biorefinery complex would provide flexibility which should enhance the overall economic feasibility.
- Carbon dioxide and nitrous oxide are two of the six harmful emissions identified in the Kyoto Protocol that could be significantly reduced by producing and using ethanol and higher value chemicals from LCF resources via a biorefinery complex.

Scientific Accomplishments

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

1. The Economic Feasibility of Converting Ligno-Cellulosic Resources into Ethanol and Higher Value Chemicals, Missouri¹

The primary purpose of this study has been to estimate the economic feasibility of converting ligno-cellulosic feedstocks (LCF) into ethanol, using the technology that is nearest to commercialization today. Results of the study B based on dilute acid conversion technology B indicate that when ethanol and higher value chemicals such as furfural are co-produced, the project can be quite profitable. However, a plant producing ethanol as a sole product is not economic based on this technology and an ethanol price of \$1.25 per gallon. **This result implies that a way of making ethanol economically viable B without federal subsidy B is to co-produce it with other higher value products in a Abiomass refinery plant.**

An important objective of the study was to estimate the optimum size processing plant, given the LCF resource base in Missouri. Of particular importance was evaluating the economies-of-size of larger plants versus increasing feedstock costs, an unavoidable consequence of hauling feedstocks over greater distances. Size economies were identified as being the dominant economic factor, thus feedstocks were assumed to be hauled rather long distances in the base case scenario. The optimum size plant processed an estimated 4,360 tons of feedstocks daily, producing an estimated 47.5 million gallons of ethanol and 323 thousand tons of furfural annually.

The optimum size processing plant would have an estimated investment cost of \$455 million, generate annual income of \$281 million, and have an annual pre-tax net profit of \$108 million. After providing a 15 percent return on investment annually Aoff the top@ to the original investors, it still would provide a 22.5 percent return on investment over the 15 year project lifetime. The net present value of the estimated lifetime income stream would be \$177 million after repaying the original construction cost of \$455 million.

Crop residues (corn stover, wheat straw and milo stover) were the feedstock of choice because of higher levels of hemi-cellulose B used in producing the higher value furfural B and low levels of lignin relative to other feedstocks considered. In addition, some woody residues were used in the optimum base case solution. However, no dedicated energy crops were used, primarily because: 1) they were assumed to be more expensive than both crop residues and woody biomass, and 2) their composition, compared to other feedstocks, would include higher levels of cellulose, which is a feedstock for ethanol, and lignin, which is used as process energy. Both of these have lower value uses and less valuable products than the furfural produced from hemi-cellulose.

The optimum location, based on the use of a GAMS mathematical optimization model, was in Carroll County, MO, a major crop production area in west central Missouri.

Plant operations would provide significant employment opportunities and a large stimulus to economic activity in the west central Missouri region. Up to 6,000 additional jobs might be created as the result of operating this processing plant. Only about 2,700 of those would be in Missouri, although many of the Missouri jobs would be seasonal and involve feedstock harvest. The largest number of jobs would be in the chemicals industry and located elsewhere in the U.S.

¹ Complete research results are included in, "The Economic Feasibility of Converting Ligno-Cellulosic Feedstocks to Ethanol and Higher Value Chemicals," A Report to the Consortium for Plant Biotechnology Research, Inc., September 1999.

Operation of an ethanol/furfural plant in Missouri should not result in offsetting job losses in the state because all ethanol and liquid fuels are currently imported. Thus, any offsetting job losses should be in other states that produce ethanol and/or produce and refine petroleum, however both of these industries are capital- not labor-intensive.

Personal income would also increase as the result of plant operations. Up to \$155 million might be expected annually, again with much of the increase realized by personnel in the chemicals industry.

Plant operations also would bring increased tax revenues to the local area, to Missouri and to the U.S. treasuries. Local and state tax benefits (income, sales, excise tax on liquid fuels, and property taxes) could increase by an estimated \$10.8 million per year, excluding increased taxes that might accrue to other states. Also, in order to attract the plant, concessions might be made by local governments in the level of real and personal property taxes to be collected, thus reducing these estimated revenues.

The conversion of municipal solid waste (MSW) into ethanol was not as economically attractive as when using crop residue feedstocks because: 1) the primary product produced would be ethanol, not as profitable as furfural, and 2) tipping fees of \$30 per ton are not high enough to pay for the necessary additional equipment and labor used in sorting the feedstock prior to processing. In comparison, tipping fees approximately twice that level are quite economically attractive for an MSW-to-ethanol project under construction in Orange County, New York.

Development of an LCF-to-ethanol industry would be highly complementary to the existing grain-to-ethanol industry. It would simultaneously help to provide much larger volumes of high quality ethanol than can be provided from grain to augment the approximately 155 billion gallons of gasoline used in the U.S. annually.

Finally, an LCF-to-ethanol project in Missouri should provide significant incentives for agglomeration in attracting other plants and businesses opportunities.

2. The Economic Feasibility of Converting Ligno-Cellulosic Resources into Ethanol and Higher Value Chemicals, Georgia²

The research on objective number 2 uses the same methodology outline in the Missouri study, but includes the LCF resource base in Georgia. As with the Missouri study, the annual quantities of feedstocks assumed to be potentially available for use by the plant were estimated for each county. To reflect a variety of considerations such as competition for the feedstocks and the feasibility of collecting and transporting them, only 50% of the estimated milling residues and other removals were assumed to be available to the plant and only 25% of the logging residues. Also, only 10% of the estimated crop residues were assumed to be available to the plant. Additional assumptions include:

- Softwoods are somewhat concentrated in the Southeastern counties of Georgia, hardwoods are concentrated in the Central/Central-Eastern counties, and crop residues are located in the counties near a line running from the Southwestern corner of the state to the Central-Eastern part of the state. The lack of feedstocks in the Northern half of the state suggest the most economically feasible would not be located there due to high transportation costs.

² Complete research results are included in " The Economic Feasibility of Converting Ligno-Cellulosic Feedstocks to Ethanol and Higher Value Chemicals, Georgia," A Report to the Consortium for Plant Biotechnology Research, Inc., In Preparation.

- The model was run considering locating a plant in each of the 159 counties in Georgia. Allowing the size of the plant to vary anywhere from 500 tons to 10,000 tons per (TPD) of feedstock use. This provides an estimate of those counties in which an LCF plant would be most profitable. Of all the counties, only Jones County (located near the center of Georgia) had a positive net present value (NPV). Note that the NPV is calculated using a 15 percent discount rate, so a zero NPV implies investors earn a 15 % return on their capital.
- The optimal size plant in Jones County would use slightly more than 7 thousand dry tons per day of feedstock materials, with about 49% and 42% being softwood and hardwood, respectively. The remaining 9% is crop residue.
- The optimal plant would produce almost 85 million gallons of ethanol, and 437,000 tons of furfural annually. Additional smaller volumes of co-products also would be produced.
- The plant construction cost is estimated at \$632.5 million. The annual operating costs are expected to be \$142.9 million.
- The cash flow (total returns minus operating costs) is estimated at \$108 million annually.
- These base performance data assume the feedstock availability as indicated above. If a larger percentage of feedstocks are available, the plant profitability will increase because of decreases in transportation costs of feedstocks to the processing plant.

3. Process Integration for a Ligno-cellulosics-Based BioRefinery

The Ligno-Cellulosics-Based BioRefinery Strategy

The basic premise for the ligno-cellulosics-based biorefinery is that the three primary components in ligno-cellulosic feedstocks (LCF) can be converted to a wide variety of commercial chemical products using a number of non-mutually exclusive conversion pathways. The primary chemical components, cellulose, hemicellulose, and lignin are all present in any plant derived material. The differences among plant resources are differences of relative quantity, with only minor differences in detailed structure. In essence, a ligno-cellulosic, is a ligno-cellulosic, is a ligno-cellulosic.

The chemical processing required to recover any one of these basic components, or their derivatives is typically very sensitive to economies of scale, leading to very large processing facilities. In general, current ligno-cellulosic processing facilities, such as pulp mills, furfural plants, or grain processing operations focus on a single fraction of the ligno-cellulosic feedstock, with the remainder either treated as a lower value co-product, or a waste material.

The biorefinery approach considers all of the feedstock as a resource for intermediate chemicals. The term “intermediate chemicals” in this context means products that are further combined or converted into finished consumer products, but not the products themselves. It must be emphasized that this approach is not new. Phillips and Humphrey (1983) provided a road map for LCF utilization that is valid today. Our effort is to indicate strategies that make the utilization of LCF profitable.

The biorefinery approach is to deliberately utilize every component in the feedstock for the highest value products possible. This requires a more diverse strategy for integrating the facility's

production processes and to marketing the final products. The term "refinery" is chosen deliberately to reflect the wide variety of interconnected products produced from a single, complex feedstock as is the case in a modern petrochemical plant.

Ligno-cellulosic Components and their Intermediate Products

Cellulose: The primary component in most ligno-cellulosic materials is cellulose. Cellulose is a high molecular weight polymer of glucose that is durable and capable of forming strong fibers. Cellulose fibers are the main component in paper, cotton thread, and in chemical derivatives such as carboxymethyl cellulose and cellulose acetate. Cellulose may also be hydrolyzed to recover monomeric glucose, a highly fermentable sugar.

The primary substrates for industrial fermentations are glucose or sucrose, mostly derived from molasses or starch hydrolysis. From 25 to 70 percent of the total cost of a fermentation product is the carbohydrate source for the fermenting organisms (Crueger and Crueger, 1989). Glucose derived from abundant ligno-cellulosic wastes such as paper, wood and food processing residues, and harvest stovers has the potential to provide fermentable sugars at a cost lower than glucose derived from starch. The keys to achieving low cost fermentable sugars from ligno-cellulosics lie in the hydrolysis process used and in the utilization of the other components of the feedstock.

The previous study (Van Dyne, Kaylen, and Blase, 1999) showed that the use of the TVA dilute acid hydrolysis process to make fermentable sugars for ethanol fermentation, combined with the production of furfural, is a venture that requires a large initial investment, but produces highly profitable returns. The key to the profitability, however, is the value of the furfural as a co-product. In part, the relatively low value of ethanol affects profitability, but also the capital and operating costs for the cellulose hydrolysis result in lesser savings in fermentation substrate costs, if the pentose derived products are ignored.

One of the goals of the current study was to evaluate the potential savings in cellulose hydrolysis costs gained by using the NREL enzymatic hydrolysis process. Based on the current status of the process and recommendations presented by the Review Committee at the Ethanol Program Multi-Year Technical Plan review meeting in July, 1999, it is our opinion that the NREL process is not yet close enough to commercialization to be evaluated (Clements, personal communication, 1999). The process requires enzyme productivities and activities that have not yet been demonstrated. In addition, the process depends on high yields of ethanol from xyloses that have not yet been achieved, and are antithetical to the capturing of the value of the xylose as furfural and other derivatives. For these reasons, the evaluation of the NREL enzymatic process for cellulose hydrolysis was not done.

Hemicellulose: The hemicellulose component of LCF is also a fibrous polymer, but its primary monomers are five carbon sugars, pentoses. The hemicellulose fraction is rather easily hydrolyzed to produce free sugars and a cellulose-lignin residue. The pentose sugars are not generally regarded as fermentable sugars, although some organisms can utilize them (Szmant, 1986). A significant thrust in the USDOE ethanol program has been the development of pentose/hexose fermenting organisms for making ethanol. To date the yields for the pentoses continue to be low.

The same reaction conditions that lead to hemicellulose hydrolysis also lead to the formation of an industrial chemical, furfural. In fact, hemicellulose hydrolysis is the only commercial route to furfural used today. Furfural is presently used as an industrial solvent, as a monomer in certain phenol thermoset polymers and as a source of furfural alcohol and tetrahydrofuran (Chenier, 1992).

In the 1930's and 1940's furfural was emerging as a key chemical intermediate. It is relatively easy to produce, and it has a rich derivative chemistry hardly equaled by any other compound (Dunlop and Peters, 1953). Furfural was the industrial source for both adipic acid and

hexamethylene diamine, the raw materials for nylon 6,6. The last furfural-based nylon 6,6 plant closed in 1961. While the rise of the petrochemical industry in the 1950's has eclipsed the importance of furfural as an industrial intermediate, the rise of the LCF biorefinery must be based in large part upon its renewed use.

Lignins: The lignin fraction of LCF provides the glue that holds the cellulose and hemicellulose fibers together. Chemically, lignin is a thermoset polymer made up of substituted phenol units. The network structure of the thermoset encapsulates the fibers to provide strength to the plant. Because of a relatively low oxygen content and a low carbon:hydrogen ratio, lignin has a high heating value, similar to low rank coal. In fact, it is the remains of the lignin fraction of fossil plants that form coal. In most LCF utilization schemes lignin is viewed as a fuel source.

There are a number of industrial uses for lignin, ranging from lignosulfonates used as binders, in drilling muds and as dispersing agents to the production of vanillin, an artificial flavoring (Szmant, 1986). Also, lignin can be reused as an adhesive in the production of reconstituted wood products.

There is the opportunity, however, to utilize the lignin fraction of LCF as the source for aromatic intermediates in the overall biorefinery complex. The isolated lignin fraction can be depolymerized through thermochemical liquefaction methods into a range of phenols, cresols, and other aromatic compounds. There is one commercial facility in the U.S. using this chemistry for converting lignite into industrial aromatics, but to date there appears to be only one firm developing the technology for biomass conversion processes (Clements, personal communication, 1999).

Commodity Chemicals from Biochemical Conversion Processes

There is an extraordinary range of chemical products that can be produced through biochemical conversion (fermentation). About 40 fermentation product chemicals have sufficient production to be considered commodity (large volume) chemicals today, or offer the possibility of becoming commodity chemicals in the future (Clements, *et al.* 1983; Leeper and Andrews, 1991; Landucci, *et al.*, 1994). Currently, the most important fermentation chemicals, in terms of volume, are ethanol, citric acid, and fructose. Significant new candidates for commercial production include acetaldehyde, acetic acid, acrylic acid, adipic acid, succinic acid, and 1,3 propane diol.

Commodity chemicals are the focus of this work because the economy of scale for the hydrolysis process favors large facilities. This economy of scale can then be transferred both to the production of furfural derivatives and to the production of fermentation products. In addition, each of the commodity products mentioned is also a chemical intermediate for other products. The structure of the families of primary, intermediate and final products has been reported many times for the petrochemical industry, but one of the best descriptions for LCF utilization was presented by Phillips and Humphrey (1983) and is shown in Figure 1.

The network structure of chemicals and their derivatives shown in Figure 1 demonstrates the multi-product and multi-derivative opportunity for a biorefinery operation. The LCF feedstock can be used to address most of the products now derived from petroleum resources. With the addition of aromatic compounds from lignin liquefaction, the product slate of a petrochemical complex can be replicated. In fact, much of the existing infrastructure now found in the petrochemical industry will still be needed for the LCF biorefinery.

There is an important environmental advantage to the production of a number of the more important commodity and derivative chemicals in the biorefinery. Much of the chemical work that takes place in the petrochemical plant is the addition of chemical functionalities (alcohol, acid, etc.) through the addition of oxygen to the hydrocarbon molecules. Most of the processes are catalytic, and in a number of cases they involve toxic intermediate radicals such as cyanide or halides, or chemicals

like phosgene. These process chemistries are summarized in a number of references, including those by Heaton (1994), Myers (1986), Szmant (1989), and Weissermel and Arpe (1997).

The LCF materials begin with a high oxygen content and the biochemical work is removal of oxygen in the form of carbon dioxide and the creation of alcohol, acid, aldehyde and other functionalities. The biochemical work is enzymatically catalyzed and is highly specific and selective. Process conditions are mild (near ambient temperature and atmospheric pressure) and in an aqueous medium. A disadvantage of most biochemical processes is that the final products are generally present in relatively low concentration.

The Structure of a Phase II BioRefinery Based on Ligno-Cellulosic Feedstocks

The initial report presented by Van Dyne, *et al.* (1999) described an facility that produced ethanol and furfural from LCF. The main process elements are hemicellulose hydrolysis and furfural production, cellulose hydrolysis to fermentable sugars, and the production of ethanol from the fermentable sugars. We refer to this type of operation as a Phase I biorefinery.

A Phase II biorefinery incorporates chemical processing elements to make one or more furfural derivatives and a fermentation facility based upon conventional fermentation ethanol, but with provision for production of a number of other fermentation products. The Phase II fermentation section will require fermentors that can be operated over a range of temperatures and pHs and at conditions from anaerobic to aerobic, depending on the organisms and products.

The Phase II biochemical downstream processing scheme will incorporate additional unit operations, including extraction, absorption, crystallization, etc., depending upon the range of products to be produced in the facility. The essential element of the Phase II biochemical plant is its flexibility in operation. Although it will be a commodity chemical facility, it will have some of the flexibility of a multi-product specialty chemicals operation.

The strategy of *ab initio* design for multiple products from a single fermentation facility is not presently being used. A significant part of this study has been the development of guidelines for selecting compatible product lines and designing flexibility into the plant flowsheet. The evaluation of the economic advantages or penalties created by creating in product flexibility are part of a continuing study of the Phase II approach.

This report summarizes through examples the application of the Phase II approach to the production of nylon 6,6 from furfural, and production of acetic acid/propionic acid and lactic acid in conjunction with an ethanol facility. The production of poly-D-3-hydroxybutyrate) P(3HB) was also examined and found not to be compatible with integration into an ethanol facility. The fermentor for P(3HB) is an air-lift fermentor, instead of a batch or CSTR continuous fermentor. In addition, the P(3HB) product is an intracellular solid, requiring cell disruption and a unique product recovery and purification system.

Biomass-to-Nylon Process Overview

The biomass-to-nylon process takes advantage of the fact that the hemicellulose fraction is readily hydrolyzed to its component sugars, called xyloses. The reaction conditions most commonly used are reaction with dilute sulfuric acid at a temperature of about 160 C. The same reaction conditions that hydrolyze the hemicellulose also can further convert the xyloses to furfural, an industrial chemical used in refining of motor oils and for making certain plastics.

Furfural in fact has many uses, but important to this discussion is that furfural can be converted into both of the precursors of nylon 6,6 or into the raw material for nylon 6. The original process for making nylon 6,6 was based on furfural. The last of these plants closed in 1961 because of the artificially low price of petroleum.

The sequence proposed for this plant integrates the production of nylon from waste biomass materials via the production of furfural, with waste management and power production using carpet wastes and other organic waste materials. The combining of waste management and polymer production in essence creates profit centers at both the front end and the back end of the production facility. More importantly, the resulting plant is a wholly integrated processing station, benefitting from utility cascades and using process effluents internally. As an example, the production of the nylon precursors requires hydrogen which is a product of the gasification reactions used to destroy waste materials in the waste management/power production part of the plant. Similarly, the nylon plant produces carbon monoxide, carbon dioxide, and methane which are products in the fuel gas made in the gasifier section of the waste management plant.

In the case of the biomass conversion, there are additional opportunities. The hydrolysis of the hemicellulose takes about one-third of the total biomass fraction. The remaining two-thirds, cellulose and lignin, can be used for power production, but this discards an additional valuable resource, the cellulose.

The hydrolysis of cellulose to glucose can be carried out through chemical processing or by enzymatic processing. The rotting of wood in the forest is the natural version of enzymatic hydrolysis that can be used for the preparation of glucose. The cost of the enzymes is high, and the process is rather slow. Chemical methods, particularly the use of mineral acids and higher temperatures than those required for hemicellulose hydrolysis, lead to commercially viable production of glucose from cellulose. The original acid hydrolysis process for cellulose, called the Schöller process, was used extensively before and during World War II to produce ethanol for fuel.

An attractive addition to the biomass-to-nylon process is the hydrolysis of cellulose to glucose. Once the glucose is available as a fermentable sugar, essentially the same process equipment can be used to produce ethanol, acetic acid, acetone, butanol, succinic acid, or other fermentation products. The preliminary proposal here is to include the production of ethanol along with the biomass-to-nylon operation. This provides a means of profitably benefiting from the use of an additional one-third of the biomass feed as an industrial chemical product. The overall biomass-to-nylon portion of the facility can be illustrated by the following material balance shown in Table 1.

The process strategy is to create a highly integrated waste management, power production, and chemicals and fiber production complex. The three aspects of the complex complement each other in terms of exchange of inputs and products, making the sum much more profitable than the parts. In essence, the effluents from one segment of the operation are the inputs for another segment. The result is an extremely efficient, near-zero discharge facility. The several technologies involved are all proven- the competitive advantage lies in the integration of the parts.

Table 1: Inputs and Outputs for Converting Biomass to Nylon and Ethanol

InputsProducts

Wood - to - Furfural and Ethanol

2000 lb of woody biomass
 81 lb of sulfuric acid
 41 lb of lime
 12 lb nutrients
 3152 lb water
 1638 lb steam

168 lb of furfural
 153 lb of ethanol
 135 lb of carbon dioxide
 1142 lb of LCF (50% moist.)
 49 lb biogas
 221 lb gypsum (50% moist)
 5043 lb stillage
 13 lb metals and waste

Utilities: 3240 lb/hr steam
 5360 lb/hr cooling water
 165 kW electrical

Furfural - to - Nylon Option 1

168 lb of furfural
 14 lb of hydrogen
 128 lb hydrogen chloride
 172 lb sodium cyanide
 16 lb water

214 lb nylon
 49 lb CO
 205 lb NaCl
 30 lb ammonia

Furfural - to - Nylon Option 2

168 lb furfural
 21 lb of hydrogen
 128 lb of hydrogen chloride
 172 lb of sodium cyanide
 203 lb of adipic acid

459 lb nylon
 49 lb of CO
 205 lb NaCl
 32 lb water

Utilities (est.): 3500 lb/hr steam
 1500 lb/hr cooling water
 200 kW electrical

The preliminary estimated costs for the several batteries involved in nylon 6,6 production at a feed rate of 3900 tons per day of wood are:

Battery	3900 tpd (n=0.4)	3900 tpd (n=0.6)
Wood preparation	\$ 3.2 million	\$ 5.8 million
Primary hydrolysis	63.3 million	114.6 million
Furfural	17.1 million	30.9 million
Intermediates	50.0 million	50.0 million
Nylon	50.0 million	50.0 million

It is interesting to compare the production of nylon 6,6 from furfural with current petrochemical production methods (Weissermel and Arpe, 1997). The starting material is cyclohexane, the product of hydrogenation of benzene. The cyclohexane is then air oxidized to a 1:1 mixture of cyclohexanol and cyclohexanone, known as KA. The KA is oxidized further using nitric acid,

with the emission of a mixture of nitrogen oxides, or by air oxidation in an acetic acid solvent. The adipic acid is also used to produce the hexamethylene diamine needed for nylon 6,6 through reaction with ammonia.

The furfural-based production of nylon 6,6 involves fewer TRI listed chemicals, and has fewer difficult emissions. Nylon 6,6 from a biorefinery promises to have fewer overall environmentally damaging emissions than the petrochemical route.

Production of Acetic Acid/Propionic Acid in Association with Fermentation Ethanol

Currently, the primary method for commercial production of acetic acid, the eighth most important organic chemical in terms of tonnage produced, is by the oxidation of acetaldehyde (Weissermel and Arpe, 1997). Acetaldehyde, in turn, is made by the partial oxidation of propane/butane mixtures or by the oxidation of ethylene in the presence of aqueous hydrochloric acid. There exists an earlier process for making acetic acid, the catalytic dehydrogenation of ethanol. Acetic acid is a common fermentation product, with the bulk of fermentation derived acetic acid sold in the form of vinegar.

Propionic acid is less important commercially, but is used as a fungicide for some grains and in the production of cellulose propionate. Commercially, propionic acid is produced by the catalytic addition of carbon monoxide and water to ethylene (Szmant, 1989). There are a number of reports of the production of propionic acid through fermentation of lactose (Jin and Yang, 1998) and direct conversion of hemicellulose by bacterial fermentation (Ramsay, *et al.*, 1998).

The work of Glatz and co-workers (1996, 1998) is of specific interest to this study. They have developed an extractive fermentation system that co-produces acetic acid and propionic acid from glucose. Although the development work is still at the laboratory scale, there is sufficient information in the literature to create the preliminary flowsheet shown in Figure 2. Also shown in the figure is the basic flowsheet for the commercial production of ethanol by fermentation.

The similarities in process structure are obvious when comparing the ethanol process and the acetic/propionic acid process. Because the two acids are much less volatile than ethanol, extraction is preferred for their recovery from the fermentation broth. However, the other unit operations required for the two processes are essentially identical. There will be differences in operating conditions, but, in principle, the same equipment can be used in either process.

Production of Lactic Acid in Association with Fermentation Ethanol

L+ lactic acid produced by fermentation has a long history of use as a food additive (Crueger and Crueger, 1989). The world production of lactic acid has been about 20,000 metric tons annually. However, there has been an explosion of interest in the production of lactic acid as the monomer for making poly lactic acid (PLA). This polymer is biodegradable and has processing characteristics and performance very similar to polystyrene.

The current commercial production of lactic acid involves batch fermentation of glucose, or lactose with a calcium oxide pH buffer, followed by precipitation of calcium sulfate and recovery of the lactic acid product. The process steps leading up to final product recovery are shown in Figure 2.

Again, there is considerable commonality in the required unit operations for ethanol production. In fact, only the precipitation reactor is unique to the lactic acid process. Not shown, however, is the final product recovery and purification steps needed to provide polymer grade lactic acid.

Summary of Potential Guidelines for Designing the Fermentation Section of a Biorefinery

Preliminary guidelines for selecting potential fermentation based products for a LCF biorefinery include:

1. Glucose or pentoses should be the most desirable fermentation substrate;
2. Fermentation products should be extracellular;
3. Fermentors should be either batch, fed batch, or CSTR designs;
4. Preliminary product recovery should require steps such as filtration, distillation, and extraction;
5. Final product recovery and purification steps will be product unique;
6. Mixed biochemical and chemical processing steps will likely create the greatest competitive advantages; and
7. Biochemically based processes can be expected to be more environmentally benign than traditional petrochemical processes.

Unresolved questions for the Phase II Multi-Product Fermentation Facility include:

1. whether or not the entire fermentation facility can/should be able to change from one product to another;
2. if multiple products can be run in parallel, with shared use of common unit operations;
3. how to manage scheduling of unit operations; and
4. how to minimize in-plant inventories, while accommodating necessary change-overs between different products in the same piece of equipment.

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4. Evaluation of the NREL Enzymatic Hydrolysis Process

Objective 4 of this study was to evaluate the potential savings in cellulose hydrolysis costs that might be realized by using the NREL enzymatic hydrolysis process. Based on the current status of the process and recommendations presented by the Review Committee at the Ethanol Program Multi-Year Technical Plan review meeting in July, 1999, it is our opinion that the NREL process is not yet close enough to commercialization to be evaluated (Clements, personal communication, 1999). Since this research is directed toward technologies that are ready for commercialization, this objective will not be done at this time.

5. Expected Impacts on Kyoto Protocol³

In December of 1997 in Kyoto, Japan, the Parties to the UN Framework Convention on Climate Change agreed to reduce greenhouse emissions. Central to the Kyoto Protocol is a set of binding emissions targets for developed nations. The start of the binding period for the U.S. will be 2008. The U.S. emissions target, if in terms of an average, would be over the period 2008 to 2012. Those emissions targeted for reduction include:

³ Complete research results are included in, "The Expected Impacts on the Kyoto Protocol Resulting from the Use of A BioRefinery," A Report to the Consortium for Plant Biotechnology Research, Inc., In Preparation.

- Carbon dioxide, CO₂,
- Nitrous oxide, N₂O, or NO_x,
- Methane, CH₄,
- Sulfur hexafluoride, SF₆,
- Perfluorocarbons, PFC's - (a class of gasses) and
- Hydrofluorocarbons, HFC's (a class of gasses).

The latter three of these, synthetic substitutes for ozone-depletion chlorofluorocarbons (CFC's) are relatively minor but are highly potent and last for long periods of time in the atmosphere. They do not occur in the LCF to ethanol process that is study simulates. Likewise, methane is not a factor in this type of process. Consequently, only carbon dioxide and nitrous oxide are relevant to this study.

Parameters of importance concerning carbon dioxide:

- Combustion of fossil fuels results in 98% of U.S. CO₂ emissions,
- Corn-to-ethanol plants release approximately 33% of the weight of the corn as CO₂,
- Carbon is absorbed by plants and large amounts are used by forests which serve as "sinks",
- Ethanol produced from biomass nets 90% less CO₂ emissions to the atmosphere than gasoline,
- CO₂ will likely double relative to its pre-industrial level about midway through the next century, if emissions growth continues unabated,
- Combustions of fossil fuels accounts for about 75% of total anthropogenic emissions of carbon worldwide, and
- Scientists usually measure CO₂ in carbon units, primarily tons.

Nitrous oxide, although less well understood with regard to sources and absorption than CO₂, has the following relevant parameters:

- Natural sources include bacterial breakdown of nitrogen compounds in soils and fluxes from ocean upswellings,
- Nitrogen fertilizers and the combustion of fuels are the primary man-made sources,
- The second most important source of anthropogenic NO_x emissions is energy consumption,
- Animal waste is second only to commercial fertilizer as a source of NO_x emissions, and
- Crop residues emit NO_x directly to the atmosphere as they decay,

Impacts of Ligno-cellulose Ethanol

In this discussion of impacts relevant to the Kyoto Protocol the focus will be on CO₂ and NO_x. Comparisons will be made on these two target emissions between ligno-cellulosic (LCF) ethanol and gasoline. Further, two new fuels recently approved – P-series fuel and oxygenated diesel – primarily formulated with some of the products from the LCF-to-ethanol plant will be compared with gasoline respect to CO₂ and NO_x.

Ligno-cellulosic Derived Ethanol Compared to Gasoline

In a recent study undertaken by five national DOE laboratories, biomass fuels appeared to have the greatest potential to reduce greenhouse gas emissions of the eight common fuels analyzed, one of which was gasoline. In addition, the study states, "Whereas ethanol derived from corn may

actually produce higher levels of CO₂ equivalent emissions than conventional gasoline (depending on the fuel used to power the distillation plant, and other factors), LCF-based ethanol (from wood wastes, switchgrass, agricultural residues, municipal solid wastes), can reduce carbon emissions by about 90% for both light-duty and heavy-duty vehicles.....” Clearly, ethanol derived from LCF resources, like that produced by a plant similar to the one simulated in this analysis would be highly desirable in helping the U.S. meet its target emissions for carbon under the Kyoto Protocol.

LCF-Ethanol's Impact on Nitrous Oxide

In this analysis no assumptions have been made about land use changes. Rather a small percent of the crop residues that now: 1) deteriorates and is incorporated into the soil profile, or 2) is burned is assumed to be harvested, hauled to the plant and utilized as feedstock in the production of ethanol and furfural. Hence, little change is effected with regard to the impact of those residues on nitrous oxide in the environment. Minor changes accrue from: 1) the use of some of the residues that otherwise would have been burned, 2) the burning of fuel in the harvesting of the biomass and in the transportation of it to the processing plant and 3) the emitting of NO_x in process of producing the ethanol and furfural. The first of these would have a favorable impact while the latter two would have a negative affect. Overall, they are so minor that they do not justify analytical consideration which, if it were done, would show that the first partially offsets the other two.

If the assumptions were changed so that crops now producing the residues used as feedstocks were replaced with energy crops, the impact on NO_x would be sufficient to justify further attention. In that case, larger amounts of nitrogen fertilizers would likely be used to increase the volume of biomass produced per than are now is the case with the existing land use pattern. However, that is not necessary because an abundance of crop residue is available within trucking distance of the plant.

LCF-Ethanol in Alternative (E-85) Fuels

In response to a petition filed by the Pure Energy Corporation, the U.S. Department of Energy added three specific blends of methyltetrahydrofuron (MTHF), ethanol and hydrocarbons (known as P-series fuels) to the approved fuels for flexible fuel vehicles (FFV's). The P-series fuels are comprised of 60-to-100 percent non-petroleum components. Specifically, the volume composition of the most regular P-series fuels is: 32.5% pentanes plus, 32.5% MTHF, and 35.0% ethanol. In cold weather, the percentages are 16.0, 26.0, and 47.0, respectively, plus 11.0% normal butane.

The sources of several of the components of this Pure Energy fuel are relevant to this project. MTHF, which is currently produced from furfural, will be produced with thermochemical conversion technology from LCF resources. This will be integrated with an ethanol production system. In the process, LCF resources are converted into both five- and six-carbon sugars. In turn, these are bifurcated into fermentation and thermochemical pathways to produce ethanol and MTHF, respectively. To insure that the ethanol feedstock is produced from biological materials, DOE has limited the ethanol used in the P-series fuels to those produced from this source. The plant described in this process is similar to the one simulated in this study. Thus the similarity of the production outputs makes consideration of the impact of this fuel on carbon and nitrous oxide worthwhile. Fortunately, comparative data are available on these parameters.

Pure Energy provided additional environmental data to DOE, that was used in approving the fuel is noteworthy for additional reasons. For example, the grams per mile of CO from Pure Regular measured in the Federal Test Procedure and US06 test are 1.081 and 6.15, respectively. These compare to those from regular unleaded gasoline of 1.427 and 12.07, respectively.

The P-series fuels received a favorable rating with respect to nitrous oxide, also. The same tests revealed that the NO_x in grams per mile from Pure Regular in the Federal Test Procedure and US06 test are 0.064 and 0.057, respectively. These compare to those from unleaded gasoline of

0.095 and 0.077, respectively. Clearly, the P-series fuels have an advantage with respect to nitrous oxide emissions, also.

DOE concluded that, "Both the criteria pollutant emissions test results and the greenhouse gas analysis support Pure Energy Corporation's claim of substantial environmental benefits arising from the use of the P-series fuels." Further, the company estimates that the P-series regular fuel, on a life cycle basis, will reduce carbon dioxide emissions by at least 63 percent. Finally, "DOE's evaluation of the full fuel cycle greenhouse gas emissions of the P-series fuels confirmed that, over their entire production, distribution and end-use cycle, the P-series fuels will result in greenhouse gas emissions 45 to 50 percent below those of reformulated gasoline. "In sum, DOE concluded that the P-series fuels would yield "substantial environmental" benefits."

LCF-Ethanol in Oxygenated Diesel

In addition to the P-series fuel, the Pure Energy Corporation has filed a patent covering Oxygenated Diesel blends. The company plans to commercialize the fuel, which contains up to 15 percent ethanol, in the year 2000. Tests conducted at the Southwest Research Institute indicate that emissions from these fuels compare favorably with reference fuels.

Carbon emissions from oxygenated diesel. The average emission values for transient emissions testing, measured in g/hp-hr, were determined for blends with 15% ethanol (5.7% oxygen) and 10% ethanol (3.9% oxygen) as well as a reference fuel. In these units, the OC for a 15% ethanol blend was 1.835 and for a 10% ethanol blend was 2.027. Similar readings for the reference fuel in the 15% ethanol comparison was 2.525 and in the 10% ethanol comparison was 2.536.

Nitrous oxide emissions from oxygenated diesel. Improvements in NO_x were noted in the same tests. For the 15% ethanol blend the value was 4.545 compared with the reference fuel emissions of 4.766. In the case of the 10% ethanol blend the nitrous oxide value was 4.471 compared with 4.661 for the reference fuel.

Overall improvements in emissions. For the 15% ethanol blend, CO was reduced by 27%, NO_x by 5% and PM by 41%. In the case for the 10% blend the values were a CO reduction of 20%, NO_x of 4% and PM by 27%. Clearly, emissions were improved with this application of ethanol.

Summary of LCF-Ethanol Impacts on Kyoto Protocol

Measured in terms of CO₂ and NO_x, LCF-ethanol receives a favorable rating in many comparisons. These include comparisons 1) with unleaded gasoline, and 2) in the two new fuels developed by the Pure Energy Corporation. Both the ethanol used in both fuels and the furfural used in one of them could be produced in a process similar to that simulated in this study. Hence, their impacts on the environment are noteworthy. From an environmental point of view, ethanol is an improved fuel relative to unleaded gasoline and as an ingredient in the two new Pure Energy fuels. Most important is the fact that it fares well with the two applicable greenhouse emissions that are relevant in the Kyoto Protocol.

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.

- Kaylen, Michael, Donald L. Van Dyne, Youn-Sang Choi, Melvin Blase. "Economic Feasibility of Producing Ethanol from Lignocellulosic Feedstocks," **Bioresource Technology**, (In publication process).
- Van Dyne, D.L., M.G. Blase, & L.D. Clements. 1999. "Developing a Strategy for Returning Agriculture and Rural America To Long-Term Full Employment Using Biomass Refineries: A Conceptual Analysis," **Perspectives on New Crops and New Uses**, Proceedings of the Fourth National Symposium New Crops and New Uses: Biodiversity and Agricultural Sustainability, J. Janick, ed., ASHS Press, Alexandria, VA.
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- Van Dyne, D.L., L.D. Clements & M.S. Kaylen. "Opportunities for Economic Development and Rural Sustainability by Producing Industrial Products from Agricultural Resources," for presentation at the Corn Utilization and Technology Conference, St. Paul, MN, June 5-7, 2000, invited and forthcoming.
- Van Dyne, D.L. & M.G. Blasé. "The Economic Feasibility of Converting Ligno-Cellulosic Feedstocks into Ethanol and Higher Value Chemicals," Presented at the Consortium for Plant Biotechnology Research, Inc., 1999 Symposium, Marriott at Metro Center, Washington, DC, March 3, 1999.
- Van Dyne, D.L. & M.G. Blasé. "New Crops vs. New Uses," Invited Paper to the Biodiversity and New Crops Conference, Sponsored by the USDA/AARC and the New Uses Council, Phoenix, AZ, November 8-11, 1998.

Van Dyne, D.L., M.S. Kaylen & M.G. Blase. "Estimating the Optimum Biomass Refinery Size for Converting Ligno-Cellulosic Feedstocks into Ethanol and Furfural," Presented at Bioenergy '98, Great Lakes Regional Biomass Energy Program, Madison, WI, October 1998.

Ponte, M.X & D.L. Van Dyne. "Enhanced Biofuels Production: Development of More Efficient Production and Product Combinations," Presented at Bioenergy '98, Great Lakes Regional Biomass Energy Program, Madison, WI, October 1998.

Van Dyne, D.L., M.S. Kaylen & M.G. Blase. "The Economic Feasibility of Converting Renewable Ligno-Cellulosic Feedstocks into Ethanol and Higher Value Chemicals," Paper presented at the 1998 ASAE Annual International Meeting, Orlando, FL, July 1998.

Van Dyne, D.L., M.S. Kaylen & M.G. Blase. "The Economic Feasibility of Converting Lignocellulosic Feedstocks to Ethanol and Higher Value Chemicals," Presented at the 1998 Fuel Ethanol Workshop and Trade Show International, Marriott Hotel & Century Center, South Bend, Indiana, July 8-10, 1998.

Technology Transfer

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

No technology has been commercialized to date. However, several companies have expressed interest in the research project.

Commercial Accomplishments

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

None to date.

Educational Accomplishments

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

One student conducted his Ph.D. dissertation research on evaluating the economic feasibility of producing ethanol and higher value chemicals from LCF resources. Additionally, several students (undergraduate, M.S., & Ph.D.) worked on data/information collection and modelling for the research.

Additional Funding

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

Additional matching funding in the amount of \$50,000 was received from the Missouri Division of Energy.

Key Personnel Hiring or Turnover

List any changes in key personnel during the reporting period.

No changes in key personnel during life of project.

Send completed report to :

The Consortium for Plant Biotechnology Research, Inc.

P.O. Box 20634

(Express Delivery address: 10 Sylvan Drive, Suite 21)

St. Simons Island, GA 31522

Phone: 912.638.4900, Fax: 912.638.7788

Or sent as an email attachment to: cpbr@gate.net

Scientific Progress Report

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

Principal Investigator: Richard D. Vierstra

University: University of Wisconsin-Madison

Agreement Number: OR 22072-69

Project Title: Targeted Proteolysis: New Method for Removing Selected Intracellular Proteins

Reporting Period and Report Type:

From: 7/1/98
To: 6/30/99

Check one:

☒ Interim Report
☐ Final Report

Project Objectives

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

The selective depletion of intracellular proteins is a powerful tool in biological research and has many agronomic benefits. In contrast to the genetic methods currently available to attenuate protein levels, we are exploring a conceptually new approach that exploits proteolysis for selective protein removal. It involves engineering components within the ubiquitin/26S proteasome proteolytic pathway in a way that facilitates degradation of normally stable proteins. *In vitro* studies have demonstrated the potential of this targeted proteolytic approach (Scheffner *et al.*, 1992; Gosink and Vierstra, 1995). The immediate objective of this proposal is to test and develop this approach for use in intact plants. If successful, our research would provide a new tool for selectively eliminating proteins and thus would have far-reaching commercial applications for various biomass crops.

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 ubiquitin/26S proteasome pathway plays a prominent role in plant cell biology by degrading most abnormal proteins and many important short-lived regulators. In the pathway, ubiquitin functions as a reusable signal for proteolysis (Vierstra, 1996; Hershko and Ciechanover, 1998). *Via* an ATP-dependent cascade of reactions, multiple ubiquitins first become covalently attached to proteins targeted for degradation. The resulting ubiquitin-protein conjugates then serve as intermediates for selective proteolysis by the 26S proteasome; this complex breaks down the target protein into amino acids but releases the ubiquitin moiety

intact for reuse.

Selection of target proteins for ubiquitination, and thus commitment for degradation is achieved by two complex families of enzymes called E2s (or ubiquitin-conjugating enzymes) and E3s (or ubiquitin- protein ligases). E2s and E3s function by facilitating the transfer of activated ubiquitin from E1 (or ubiquitin-activating enzyme) to the target protein. This transfer produces a ubiquitin-E2 or -E3 intermediate, both of which can serve as the proximal donor of ubiquitin depending on the target protein. Recent studies by us and others have shown that target specificity for E2s and E3s can be manipulated by modifying their target recognition domains. We demonstrated that E2 specificity can be redirected simply by replacing the native target recognition domains at the C-terminus with domains that recognize other proteins. As examples, we showed that appending Protein A or peptide hormone sequences onto various *Arabidopsis* E2 cores allowed for the ubiquitination of antibodies or a hormone receptor with high specificity *in vitro* (Gosink and Vierstra, 1995). Addition of the 26S proteasome ultimately lead to the degradation of the ubiquitinated proteins. Likewise, Scheffner *et al.* (1992) showed that a protein-interaction site could be fused to the active-site core of a human E3. This engineered E3 then promoted the *in vitro* degradation of a target recognized by the interaction site.

These examples raise the intriguing possibility that by exploiting domains derived from natural or synthetic binding partners, it may be possible to design E2s and/or E3s capable of enhancing degradation of *any* intracellular protein. Although the initial results with this targeted proteolytic approach appears promising, the system remains to be demonstrated as effective *in vivo*. Our goal in this proposal is to engineer an easily manipulated experimental system using intact cells (yeast, *Saccharomyces cerevisiae*) and plants (*Arabidopsis thaliana*) that will help define the approach's efficacy and limits, while at the same time allow us to develop and demonstrate potential commercial applications. Ultimately we hope to engineering disease resistance by engineering plants "preimmune" to a viral pathogen by engineering E2s and/or E3s that can recognize and ubiquitinate viral proteins essential to their life cycle.

Our approach has been to develop in yeast an inducible, two-plasmid system, one plasmid will express a target protein either alone or fused to β -galactosidase, whereas the other would express an E2 and/or E3-fusion with affinity to the target. Through simple enzymatic or immunochemical assays for the target or the fused β -galactosidase, we would be able to assess whether the presence of the re-directed E2 could attenuate the level of the target *in vivo*. This approach will allow us to rapidly screen through many interacting pairs and thoroughly characterize those that work. In the past year, a number of ligand/target pairs were created with E2s that exploited several well-characterized protein-protein interactions, including: (i) the leucine zipper of *jun* that interacts with *fos* or its leucine zipper; (ii) binding domain of Mdm2 that binds to p53; and (iii) phage display peptides that bind to the *sarc* homology-3 (SH3) domain in *c-src*.

Several of the E2-ligand/target were first expressed in *Escherichia coli*. The resulting protein were tested *in vitro* for their retention of enzymatic activity (*i.e.*, formation of an E2-ubiquitin intermediate) and for their ability to bind to the target through the appended recognition domain. The most promising was E2-Mdm2 ligand which would ubiquitinate its target, recombinant p53 with high specificity *in vitro*. The E2-Mdm2 ligand and p53 target

were then introduced into yeast. p53 was expressed on a low copy CEN plasmid and the E2-Mdm2 was expressed at high levels the Cu²⁺-inducible *CUP1* promoter on a high copy 2 μ plasmid. Levels of p53 were monitored by immunoblotting with anti-p53 antibodies. Unfortunately, we found that the levels of p53 were unchanged by expression of the E2-Mdm2 ligand. In fact no ubiquitination of the target was evident even with the ligand. Subsequent analyses revealed that the E2-Mdm2 fusion was unstable and failed to be adequately expressed. These data, along with mounting evidence that E2s rarely participate in ubiquitination by themselves (Hershko and Ciechanover, 1998), led us to abandon this approach and focus our studies on E3s as the source of target specificity.

Because so little is known about E3s in plants, our first objective was to characterize the various E3 families to find those most appropriate for manipulation. By comparison with those described in yeast in animals, two types of E3s have emerged. One type exists as a complex of polypeptides with distinct subunits responsible for binding the substrate and the activated E2-ubiquitin donor. Examples of this type include the anaphase-promoting complex (or APC) and the SKP1/CDC4/F-Box (or SCF) complex that participate in the timely removal of a number of cell cycle regulators (Hershko and Ciechanover, 1998; Tyers and Willems, 1999). For the SCF complex, a family of F-Box protein isoforms appears responsible for recognizing individual substrates.

The other E3 type consists of a single polypeptide with docking sites for both the substrate and the E2-ubiquitin complex. The first of this type to be identified was E6-associated protein (or E6-AP), discovered to participate in the ubiquitination and degradation of p53 upon its association with the papillomavirus protein E6 (Scheffner et al., 1993). Subsequent searches of DNA databases identified numerous animal and yeast proteins that share significant similarity with E6-AP, especially within a 350-amino acid C-terminal region surrounding a conserved Cys (referred to as the HECT domain for Homology to E6-AP C-Terminus) (Huibregtse et al., 1995; Schwarz et al., 1998). During their reaction cycle, HECT E3s form an E3-ubiquitin thiol-ester intermediate in which the ubiquitin moiety is linked to the HECT-domain Cys (Scheffner et al., 1995).

By searching various DNA databases, we identified several HECT E3s in *Arabidopsis thaliana*. Two of these, designated UPL1 and 2 are 405-kDa, making them two of the largest proteins in this plant species (Bates et al., 1999). The corresponding genes are 13-kbp in length and are situated 26-cM apart on chromosome I. They are over 95% identical within both the introns and exons, suggesting that the two loci arose from a recent gene duplication. The C-terminal HECT domain of UPL1 is necessary and sufficient to conjugate ubiquitin *in vitro* in a reaction that requires the positionally-conserved cysteine within the HECT domain, E1, and an E2 of the UBC8 family. Work is now underway to determine the functions of UPL1 and 2 in plants using various reverse genetic strategies.

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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.

Publications:

Bates, P., J. Doelling, and R.D. Vierstra (1999) UBL1 and 2, 405-kDa ubiquitin-protein ligases from *Arabidopsis thaliana* related to the HECT-domain protein family. *Plant J.* (In press).

Seminars:

RhoBio, division of Rhone Poulenc. Evry France. "Use of ubiquitin in plant biotechnology" March, 1999.

Technology Transfer

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

Patent Issued:

"Ubiquitin conjugating enzyme (E2) fusion proteins." Vierstra, R.D., and M. Gosink. Wisconsin Alumni Research Foundation. Patent number 5,851,791 (1998).

Commercial Accomplishments

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

None yet applicable

Educational Accomplishments

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

Dr. Mark Gosink, the lead scientist on the project, became proficient in a number of molecular techniques and bioinformatic methods. Based on his new skills, Mark was offered and accepted a job at Parke Davis Pharmaceuticals as head of a new program in bioinformatics.

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.

Dr. Mark Gosink left the project to develop a bioinformatics program at Parke Davis Pharmaceuticals. Adam Durski was hired recently as a technician to assist in the analysis of E3 proteins and genes.

Scientific Progress Report		
Submitted to The Consortium for Plant Biotechnology Research, Inc., in accordance with the requirements of the Research Agreement cited.		
Principal Investigator:	Jack M. Widholm	
University:	University of Illinois at Urbana-Champaign	
Agreement Number:	OR22072-79	
Project Title:	<i>A New Selectable Marker and Promoters of Plant Origin</i>	
Reporting Period and Report Type:	From: 7/1/98 To: 12/31/99	Check one: ☒ Interim Report ☐ Final Report

Project Objectives

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

The overall objectives of this project are to improve the efficiency of producing transformed plants, to produce plants that are considered to be environmentally safe by preventing expression of undesirable selectable marker genes in the plants and to express useful genes in the plants at the desired levels using plant derived sequences that are not patented by others. Our specific plans to accomplish these objectives are:

Objective 1: Develop the tobacco ASA2 gene as a selectable marker in many species.

The results thus far show that the ASA2 gene driven by the 35S CaMV promoter can be used to select transformed soybean and *Astragalus sinicus* hairy roots since the transformed roots are quite resistant to the toxic Trp analog 5-methyltryptophan. Results with tobacco leaf disks or Arabidopsis plants have not given clear-cut results.

Objective 2: Develop the tobacco ASA2 promoter for tissue culture specific and constitutive expression in many species.

The 1.3 kb ASA2 gene promoter fragment has been found to be a strong constitutive promoter in tobacco, Arabidopsis, *Datura innoxia* and *A. sinicus* plants.

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 begun work demonstrating that indeed the naturally occurring ASA2 gene from tobacco can impart resistance to a toxic tryptophan analog so that this gene, which is quite different from the antibiotic or herbicide resistance usually used in gene transfer experiments, may be an effective selectable marker.

So far the results using the tobacco ASA2 gene promoter to drive gene expression show that high level expression can occur in most tissues of certain species indicating that this plant promoter may be useful for causing high level expression of useful genes in plants.

Scientific Accomplishments

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

Selectable marker analysis

A number of experiments have been carried out to determine if expression of the tobacco ASA2 gene (Song et al. 1998) in transgenic plant systems will lead to resistance to the toxic tryptophan (Trp) analog, 5-methytryptophan (5MT). Transformation of soybean cotyledons and *A. sinicus* seedlings with *A. rhizogenes* harboring a binary vector carrying ASA2 driven by the CaMV 35S promoter produced numerous hairy roots that would grow in the presence of 5MT concentrations that prevented hairy root growth on the controls. Molecular analysis, including Southern, showed that all the resistant hairy root lines carried the ASA2 gene and the roots of both species and the shoots formed on the *A. sinicus* roots had greatly increased free Trp levels. The anthranilate synthase activity found in the resistant roots was also more resistant to feedback inhibition by Trp.

A. tumefaciens transformation of tobacco leaf disks and Arabidopsis plants followed by selection for 5MT-resistance has not produced clear-cut results. The work is continuing and in both cases we are also selecting for kanamycin resistance with a cotransformed *nptII* gene so lines carrying ASA2 should be obtained for direct testing.

Thus these results indicate that the use of ASA2 as a selectable may be possible with certain species.

Promoter analysis

Transgenic plants of *Nicotiana tabacum* (Xanthi), Arabidopsis (Columbia), and *Datura innoxia* containing the GUS reporter gene driven by different size fragments of the ASA2 promoter, produced by the *Agrobacterium tumefaciens* mediated transformation method, have been used for GUS histochemical assays to analyze the ASA2 promoter activity. Well known strong promoters such as CaMV 35S and actin were used as controls to evaluate ASA2 promoter

activities. The 1.3 Kb ASA2 promoter was constitutive and as strong as CaMV 35S and actin in all three different species and much stronger than the 606 bp ASA2 promoter. Interestingly, this result was different from the result of the transient expression assay using tobacco where the 606 bp ASA2 promoter was stronger than the 1.3 Kb ASA2 promoter (Song et al. 1998). These transient/stable transformation results strongly suggest the existence of a positively acting transcription factor(s) with binding site(s) between 1.3 Kb and 606 bp upstream of the ASA2 gene and a negatively acting transcription factor(s) whose binding site(s) should be within 606 bp upstream of ASA2 promoter. The 2.3 Kb ASA2 promoter showed the tissue specific activity similar to that found in transient expression assays.

These ASA2 promoter deletion-GUS fusion constructs were also used to produce hairy roots from which shoots can develop with the forage legume, *Astragalus sinicus* (Chinese milk vetch) (Cho et al. 1999). As with the other species the 1.3 Kb promoter showed high level expression in both roots and shoots while the others, 606 and 2.3, were weak.

These results indicate that the 1.3 Kb ASA2 promoter can be used to drive high level gene expression in several species.

References

Song, H.-S., J.E. Brotherton, R.A. Gonzales and J.M. Widholm (1998) Tissue culture specific expression of a naturally occurring tobacco feedback-insensitive anthranilate synthase. *Plant Physiol.* 117:533-543.

Cho, H.-J., J.M. Widholm, N. Tanaka and Y. Murooka (1998) *Agrobacterium rhizogenes*-mediated transformation and regeneration of the legume *Astragalus sinicus* (Chinese milk vetch). *Plant Sci.* 138:53-65.

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.

Cho, H.-J., H.-S. Song, J.E. Brotherton and J.M. Widholm. 1998. Analysis of tobacco anthranilate synthase gene promoter using *Agrobacterium rhizogenes*-mediated *Astragalus sinicus* transformation system. *In Vitro Cell. Dev. Biol.* 34:63A.

Song, H.-S. and J.M. Widholm. 1998. Single-stranded DNA binding protein regulating expression of feedback-insensitive anthranilate synthase gene in *Nicotiana glauca*. *Plant Physiol. Suppl.* 117:156.

Cho, H.J., H.-S. Song, J.E. Brotherton and J.M. Widholm. 1998. Analysis of tobacco anthranilate synthase gene promoter using *Agrobacterium rhizogenes*-mediated *Astragalus sinicus* transformation system. 1998. Crown Gall Meeting, Lafayette, IN.

Brotherton, J.E. and J.M. Widholm. 1999. Metabolic consequences of overexpression of the tobacco feedback-insensitive anthranilate synthase ASA2 gene. Am. Soc. Plant Physiol. Abstracts 134.

Technology Transfer

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

The patent application was submitted before the project began. Submitted Invention Disclosure in Dec., 1995, entitled "Selectable marker and promoter for plant tissue culture transformation", filed Provisional Patent Application in July 1996 and Patent Application in July 1997. A portion of the claims concerning the promoter was allowed in 1998 and patent number 5,965,727 was issued Oct. 12, 1999. The selectable marker portion of the original application is still being examined by the U.S. Patent Office.

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.

None.

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.

A postdoctoral Research Associate, Junghee Kim was hired to replace another postdoctoral Research Associate, Hee-Sook Song, who left in the fall of 1998 to take a position at Dekalb Genetics.

P-1081

Analysis of tobacco anthranilate synthase gene promoter using *Agrobacterium rhizogenes*-mediated *Astragalus sinicus* transformation system. H.-J. CHO*, H.-S. Song, J.E. Brotherton and J.M. Widholm. Department of Crop Sciences, University of Illinois, Urbana, IL 61801. E-mail: <hjcho@uiuc.edu>

Anthranilate synthase [AS] is a key enzyme in the tryptophan biosynthetic pathway that catalyzes the first reaction in the pathway which synthesizes tryptophan from chorismate. AS is feedback inhibited by the end product, tryptophan. Tryptophan analogs, such as 5-methyltryptophan, have been used to select resistant plant cell cultures that usually have increased free tryptophan levels due to the presence of a feedback-insensitive AS. In previous unpublished work, we found a wild type (unselected) tobacco (*Nicotiana tabacum*) cell line (AB-15-12-1) which was resistant to 5-methyltryptophan and contained feedback-insensitive AS that was expressed in the cultured cells but not in regenerated plants. We have reported on the cloning and characterization of the ASA2 gene that encodes a feedback-insensitive AS α -subunit and on studies of the promoter region of this gene. An approximately 2.3kb ASA2 promoter has been cloned from the 5MT-resistant AB15-12-1 genomic DNA.

In this experiment, we further characterized stable expression of the *gus* marker gene controlled by the various ASA2 promoter regions using *Agrobacterium rhizogenes*-mediated transformation of a leguminous plant, *Astragalus sinicus*, a system which shows high transformation efficiency. The *A.rhizogenes* DC-AR2 strain harboring a binary vector was used to transform plant cells in wound sites and transformation was monitored by histochemical GUS assay. Histochemical GUS assays showed that the activity of the 1.356kb ASA2 promoter fused to a *gus* gene was strong and showed constitutive expression patterns in hairy roots, callus, and wounded stem and leaf tissues. GUS activity of the 2.252 kb ASA2 promoter fused to a *gus* gene was very weak compared to that of 1.356 ASA2 and CaMV 35S promoters but was strong especially in the vascular cylinder and root tip. Whole plants transformed with 1.356kb ASA2-*gus* showed very similar constitutive expression patterns compared to the CaMV 35S promoter. Whole plants are being regenerated for further ASA2 promoter expression pattern studies.

This work was supported by funds from the Illinois Agricultural Experiment Station and the Illinois Council on Food and Agricultural Research.

P-1082

Evaluation of promoters for biolistic transformation of embryogenic *Pinus strobus*. A.M. DINER¹, A. Zipf² and R.Ward², ¹U.S.D.A. Forest Service, ²Dept. of Plant and Soil Science, Alabama A&M University, Normal, AL 35762. E-mail: <adiner@asnaam.aamu.edu>

Three gene promoters individually linked to the GUS marker gene were evaluated in somatic embryogenic cultures of eastern white pine (*Pinus strobus* L.). Cultures were bombarded using the BiolisticTM PDS-1000 particle delivery system PDS-1000 (DuPont, Wilmington, DE). Promoters were monocot-enhanced ubiquitin promoter (Ubi1), *Chlorella* viral adenine methyltransferase promoter (amt), and monocot-enhanced (maize) alcohol dehydrogenase promoter (adh1). Cultures were bombarded once at 6.3 cm with 1 ug gold particles coated with 7 ug DNA, 5 days following subculture to freshly prepared maintenance medium. Assays were two (adh), one (amt) and seven (Ubi1). Replicates per assay ranged from 4 to 15. Each replicate consisted of a 2.5 cm diameter culture centrally located on plated medium. Foci of GUS-expressing transformed sites were assayed after 1 to 20 days. Sample size assayed per bombarded culture was approximately one-quarter of the total tissue per target culture sample. All bombarded tissues expressed GUS at 24 hours. Numbers of sites expressing GUS were determined for each bombarded culture. GUS activity was expressed in single cells and in small clusters of 3-6 cells, the latter suggesting mitosis following transformation of single cells. Irrespective of the promoter employed, a diffuse blue color frequently extended through cells neighboring a strongly GUS-positive cell in a tissue. Promoters differed significantly in frequency of GUS expression. The Ubi1 promoter showed greater frequency of GUS-expression than did the amt promoter (0.7 x Ubi1) which, in turn, was more effective than the dh1 promoter (0.05 x Ubi1). Expression decreased through 22 days to an undetectable level.

P-1083

Transient expression of a beta-glucuronidase (GUS) gene under the control of a peanut metallothionein-like gene promoter in green beans. D. V. BELIAEV and R. L. Smith, Plant Molecular and Cellular Biology Program, University of Florida, Gainesville, FL 32611. E-mail: <DVB@ICBR.IFAS.UFL.EDU>

To isolate a peanut promoter specifically active in pods we carried out a differential display experiment using RNA isolated from pods and seeds of peanut cv. Altica. Cloned cDNAs differentially amplified in pods but not in seeds were hybridized to RNA from various peanut tissues. From them POD3 cDNA was chosen for further analysis because it hybridized only to RNA isolated from pods. A genomic clone and the 5'-end of the cDNA corresponding to it were isolated and sequenced. A 65aa long POD3 ORF is homologous to the metallothionein-like gene family. Primer extension experiments pointed out two transcription start sites 5bp apart from each other. A translational fusion of the POD3 to the GUS gene was made in such a way that 32 N-terminal amino acids of POD3 ORF plus 8 more amino acids were fused to GUS protein. The fusion took place in the third exon of the POD3 genomic sequence, therefore, for the chimeric mRNA to be correctly translated the two introns of the POD3 have to be spliced out. The construct contains 1.2kb of the promoter region. Its efficient expression in different tissues of the green bean and peanut fruits has been demonstrated. Since the transient expression is difficult to quantify, we are currently carrying out the experiments on stable transformation of alfalfa and peanut with this construct.

P-1084

Cloning and Expression of a soybean cDNA Encoding Cystathionine- γ -Synthase. C.A. HUGHES¹, J.S. Gebhardt², A. Reuss², M. MacDonald², A. Samuels¹ and B.F. Matthews², ¹Morgan State University, Dept. of Biology, Baltimore, Maryland 21251 and ²USDA/ARS, SARC, Beltsville, Maryland 20705 E-mail: <chughes@moac.morgan.edu>

The essential amino acid content of dietary proteins is very important because they are required to ensure proper growth in humans and livestock that are unable to synthesize them. We are interested in determining the regulatory mechanism(s) that control the biosynthesis of an essential amino acid, methionine, which is not well understood in plants. Since methionine content is deficient in soybean, it is important to understand the role of CS, a branch point enzyme that leads to the synthesis of methionine. A soybean leaf cDNA library was screened using a radiolabeled Arabidopsis CS cDNA probe. A soybean cDNA clone was identified that encodes a protein of 536 amino acids with a predicted molecular weight of 58 kDa. The deduced amino acid sequence of the encoded protein corresponds well to the Arabidopsis (80% identity), but not to *E. coli* and other microorganisms. The first 152 amino acids are rich in serine and threonine representing about 20% of the amino acids and probably depicts a transit peptide for targeting the protein to the chloroplast. Northern blot analysis revealed that CS mRNA was detected in extracts of 8day old leaves and cotyledons, light grown material contained more CS mRNA, especially light grown cotyledons. [Supported by NSF Grant # IBN-9602190].

tical parenchyma, endodermis and pericycle. In contrast, chitooligosaccharide alone failed to elicit such a response in the root tissues. These findings demonstrate that rice roots perceive Nod factors and that these lipochitooligosaccharides, but not simple chitin oligomers, act as signal molecules in activating *MIENOD12* in cortical parenchyma as in legumes. Exogenous application of N-naphthaleneacetic acid mimicked the Nod factor-elicited tissue-specific expression of *MIENOD12* in roots while cytokinins inhibited it, thus evidencing that Nod factors, auxin and cytokinins probably act on similar signaling elements responsible for the regulation of *MIENOD12* activation in rice. Taken together, results of the study suggest that at least a portion of the signal transduction machinery important for legume nodulation likely exists in rice.

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Session 48, Regulation of Gene Expression

Effects of a chloroplast-encoded mutation on the expression of specific genes in tobacco. Archer, Kathleen. Dept. Biology, Trinity College

A critical event in the life of a plant is the transition from heterotrophy to phototrophy during seedling development. During this transition, proplastids initiate a dramatic change in structure and function, eventually maturing into photosynthetic chloroplasts. The maturation process requires both the production of nuclear-encoded chloroplast proteins, and the production of photorespiratory peroxisomal and mitochondrial proteins. How these events are coordinated is poorly understood, but it is clear that chloroplasts play a major role in the control process. To better understand how chloroplasts regulate the events leading to a photosynthetic cell, we have characterized a chloroplast-encoded mutation (*Vir-c*) in tobacco. The mutation is associated with decreased activities or protein levels for specific nuclear-encoded proteins involved in photosynthesis and photorespiration. We wanted to know if the decreased activities resulted from a reduction in specific mRNAs, and so we are evaluating mRNA levels during seedling development for the following proteins: LHCP, rubisco, catalase and glycolate oxidase. As a control, we included isocitrate lyase, which is present in glyoxysomes during the heterotrophic phase and for which we have measured normal activity in the *Vir-c* mutant. Our results to date show that mRNA for LHCP is much reduced in the mutant seedlings, and as expected, isocitrate lyase mRNAs are present in similar amounts for both the wildtype and *Vir-c* mutant. The mutation thus influences the transcription of at least one nuclear gene, and the affect does not extend to all nuclear genes. Experiments with the remaining genes of interest are in progress. This work is supported by grant # 96-35311-3811 from the NRI Competitive Grants Program/USDA.

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Session 48, Regulation of Gene Expression

Structure and expression analysis of a group 3 LEA protein gene in rice embryo. Wang, Lian-Du, Chen, Peng-Wen, Chen, Liang-Jwu. Institute of Molecular Biology, National Chung Hsing University, Chung, Mei-Chu. Institute of Botany, Academia Sinica

A cDNA clone *Ose730*, encoding a group 3 late-embryogenesis abundant (LEA) protein was isolated from rice embryos at the stage of 10-days after pollination (DAP). The deduced translation product consists of 200 amino acids with eight conserved 11-amino acid tandem repeated structure, a typical structural motif of the group 3 LEA protein. While high levels of *Ose730* transcript expressed in developing embryos from 9-DAP through 40-DAP, none was detected in endosperms, anthers, leaves, and embryos of the dried seed. The induction pattern for *Ose730* transcript was different in roots and embryos of germinating seeds when treated with abscisic acid. The *Ose730* transcript in roots was rapidly induced and peaked at 4 hr after imbibition then declined gradually. On the contrary, the induction of *Ose730* transcript in germinating embryos was gradually increased to the maximum level at 72 hr after imbibition. In situ hybridization with antisense RNA probe revealed most the *Ose730* transcript accumulated in the coleoptile of immature embryos. A genomic clone of 3734 bp consisting of 2332 bp 5' flanking sequences and two introns with 92 and 212 bp respectively in the coding region was isolated and sequenced. Several putative 5' regulatory sequence elements were identified and their roles in transcriptional regulation will be discussed. This study was supported by grant NSC86-2313-B-005-049-A17 from the National Science Council, Taiwan, ROC.

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Session 48, Regulation of Gene Expression

In situ and protein analysis of the *A. thaliana* catalase gene family. Frugoli, Julia A. Balaban, Dorota McClung, C. Robertson. Dartmouth College Thomas, Terry. Texas A&M University

The catalase gene family in *Arabidopsis thaliana* includes at least three genes encoding three monomers which combine in tetramers to form at least sevenzymes. The expression of the catalase genes and protein monomers is organ specific and regulated by developmental, light and circadian signals. In order to more precisely define the expression patterns of the catalase genes, we have designed gene specific RNA probes for *in situ* hybridization. Northern analysis showed that all three catalase genes are expressed at some level in every organ;

in situ hybridization analysis shows more detailed patterns of tissue specificity, especially in flowers. Results of protein expression studies using monomer specific antibodies will also be presented. These results are consistent with postulated roles for catalase (for example, that CAT2 encodes the major photorespiratory catalase), yet also suggest that there are some as yet poorly appreciated catalase functions. This work was supported by USDA NRI-CCP#94-37304-1044 and 96-02632.

762

Session 48, Regulation of Gene Expression

Gene dosage and genetic imprinting play roles in cosuppression phenomenon. Mishra, Krishna K. Handa, Avtar K. Department of Horticulture, Purdue University

Introduction of the fruit pectin methylesterase (PME) sense-transgene under the control of CaMV 35S promoter causes cosilencing of the endogenous fruit PME and its sense-transgene in fruits of many independent transgenic tomato plants. Transformant C92-250-1, that exhibited cosuppression in the fruits from the primary transformed plant, showed cosuppression only in self pollinated progenies homozygous for the transgene. To understand the genetic factor(s) regulating stability of cosuppression in developing transgenic fruits, genetic analysis of this transformant was performed. The homozygous cosuppressed C92-250-1 lines were back-crossed with the parental wild-type and an additional tomato cultivar. Characterization of patterns of the fruit PME and its sense-transgene expression in fruits from several F1 and F2 plants confirmed association between homozygous state of transgene and cosuppressed phenotype, indicating a role of the sense-transgene dosage. However, fruits from about 10% the S2 and F2 progenies, homozygous for the sense-transgene, showed wild-type fruit PME activity suggesting reversion of the cosuppressed phenotype. RNA-blotting analyses of these putatively reverted fruits revealed accumulation of transgene transcript throughout fruit development and the levels of fruit PME mRNA similar to that of the wild-type fruits. Interestingly, the plants exhibiting non-cosuppressed fruit phenotype segregated in 1:1 ratio for the cosuppressed and non-cosuppressed phenotypes. Basis of this segregation ratio is under investigation. Taken together, these results suggest a role of gene dosage and genetic imprinting in cosuppression phenomenon. This research was supported by USDA/NRI 94-37304-1110 and USDA/North Central Biotechnology Initiative 96-34340-2711 grants.

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Session 48, Regulation of Gene Expression

Single-stranded DNA binding protein regulating expression of a feedback-insensitive anthranilate synthase gene in *Nicotiana sylvestris*. Song, Hee-Sook, Widholm, Jack M. Dept. of Crop Sciences, Univ. of Illinois at Urbana-Champaign

We are proposing that the expression of a feedback-insensitive anthranilate synthase (AS) gene, *ASA2*, in *Nicotiana sylvestris* is regulated by interactions between the designated *ASA2* promoter region and a single-stranded DNA binding protein(s) in 5-methyltryptophan-resistant (5MT^r) cultured cells. We have reported that high levels of tobacco *ASA2* transcriptional expression were detected only in 5MT^r cultured cells and the designated region (-2252 to -606) of the tobacco *ASA2* promoter was responsible for the tissue-culture specific expression. *Nicotiana sylvestris* exhibits similar patterns of *ASA2* gene expression to those found in tobacco. The 1356 bp (-1356 to -1) tobacco *ASA2* promoter fragment drove high levels of GUS expression in 5MT^r *N. sylvestris* cultured cells and very low expression in 5MT^r cultured cells and leaves, while the 606 bp fragment (-606 to -1) controlled strong constitutive expression. Gel mobility shift assays show strong DNA-protein binding interactions between the designated region of the denatured tobacco *ASA2* promoter (-1123 to -934) and a DNA binding protein(s) in nuclear extracts of only 5MT^r cultured cells. These results indicate that a single-stranded DNA binding protein(s) in 5MT^r *N. sylvestris* cultured cells is involved in regulation of the tissue culture-specific *ASA2* gene expression. Further characterization of the interactions between the *cis-trans* acting elements is being carried out.

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Session 48, Regulation of Gene Expression

Molecular regulation of developmentally controlled senescence. Noh, Yoon-Sun, Amasino, Richard M. Department of Biochemistry, University of Wisconsin

SAG12 an Arabidopsis gene encoding a cysteine proteinase specifically expressed during developmentally controlled senescence was used as a molecular marker to study the regulation of senescence. Promoter deletion studies indicated that a *cis*-acting element between -747 and -570 from the transcription start site is necessary not only for the senescence-specific expression of *SAG12*, but also for *SAG12* repressions by auxin, cytokinin, and sugars. Structural and functional homologs of *SAG12* from *B. napus* showed very high homologies to *SAG12* only at this upstream promoter region implying the functional importance of this promoter region. Internal promoter deletion analysis for this region narrowed down the senescence-specific *cis*-acting element to 33 bp region of the promoter. Studies using gel-shift analysis revealed the presence of a senescence-specific transcription factor which binds sequence-specifically to the 33 bp

Analysis of tobacco anthranilate synthase gene promoter using *Agrobacterium rhizogenes*-mediated *Astragalus sinicus* transformation system. Hyeon-Je Cho*, Hee-Sook Song, Jefferey E. Brotherton and Jack M. Widholm. Department of Crop Sciences, University of Illinois, Urbana, IL 61801. E-mail:<hjecho@uiuc.edu>

Anthranilate synthase [AS] is a key enzyme in the tryptophan biosynthetic pathway that catalyzes the first reaction in the pathway which synthesizes tryptophan from chorismate (1). AS is feedback inhibited by the end product, tryptophan. Tryptophan analogs, such as 5-methyltryptophan, have been used to select resistant plant cell cultures that usually have increased free tryptophan levels due to the presence of a feedback-insensitive AS (2). In previous work, we found a wild type (unselected) tobacco (*Nicotiana tabacum*) cell line (AB-15-12-1) which was resistant to 5-methyltryptophan and contained feedback-insensitive AS that was expressed in the cultured cells but not in regenerated plants. We have reported on the cloning and characterization of the ASA2 gene that encodes a feedback-insensitive AS α -subunit and on studies of the promoter region of this gene. An approximately 2.3kb ASA2 promoter has been cloned from the 5MT-resistant AB15-12-1 genomic DNA (3).

In this experiment, we further characterized stable expression of the *gus* marker gene controlled by the 0.606, 1.356 and 2.3kb ASA2 promoter regions using *Agrobacterium rhizogenes*-mediated transformation of a leguminous plant, *Astragalus sinicus*, a system which shows high transformation efficiency (4). The *A.rhizogenes* DC-AR2 strain harboring a binary vector was used to transform plant cells in wound sites, and transformed hairy roots and transgenic plants were analyzed the expression patterns by histochemical and fluorescent GUS assay. GUS assays showed that the activity of the 1.356kb ASA2 promoter fused to a *gus* gene was strong and showed very similar constitutive expression patterns compared to the CaMV 35S promoter. GUS activity of the 2.252 kb ASA2 promoter fused to a *gus* gene was weak compared to that of 1.356kb ASA2 and CaMV 35S promoters but showed the tissue specific expression in the vascular cylinder, root tip, callus, young shoot and leaf base but not in the matured leaf. Whole plants transformed with 0.606 kb ASA2-*gus* showed constitutive expression patterns as like 1.356kb ASA2 and CaMV 35S promoters but the expression level was about 35 % compared that of 1.356kb promoter.

This work was supported by funds from the Illinois Agricultural Experiment Station and the Illinois Council on Food and Agricultural Research.

(1) Widholm, J.M. (1973) Measurement of the five enzymes which convert chorismate to tryptophan in cultured *Daucus carota* cell extracts. *Biochim. Biophys. Acta* 320:217-226

(2)Widholm, J.M. (1972) Cultured *Nicotiana tabacum* cells with an altered anthranilate synthetase which is less sensitive to feedback inhibition. *Biochim. Biophys. Acta* 261:52-58

(3)Song, H-S., Brotherton, J.E., Gonzales, R.E., Widholm, J.M. (1998) Cloning and characterization of *Nicotiana tabacum* anthranilate synthase genes.1115:125, *Plant Physiol.*

(4)Cho,H-J., Widholm, J.M., Tanaka, N., Nakanishi,Y., Murooka,Y. (1998) (accepted) *Agrobacterium rhizogenes*-mediated transformation and regeneration of the legume *Astragalus sinicus*. *Plant Sciences*.

catalyzing an extradiol ring cleavage leading to BA. Whereas in case of the fly agaric the tyrosinase was described and the DD was cloned by others, we characterized the tyrosinase from higher plants involved in BL biosynthesis. Studying the condensation reaction by AA feedings to hairy roots cultures and seedlings of *Beta vulgaris*, neither AA specificity nor stereoselectivity was observed. The spontaneous character of this decisive step was further confirmed by PAL inhibition as well as by BA application to broad beans which led to BX formation. The glucosyltransferases catalyzing the regioselective transfer of glucose to the 5-OH- and 6-OH-group of betanidin (5-GT and 6-GT) have been characterized and cloned. Both GTs accepted also flavonoids as substrates which might indicate that these GTs are phylogenetically related to flavonoid GTs. The recombinant 5-GT has been expressed and exhibited both betanidin and flavonoid GT activity, but did not accept CD. The BC often occur as acyl derivatives. The aromatic acyltransferases characterized use only 1-O-acylglucosides as donors, but not acyl-CoA thioesters. The detection of DD in higher plants as well as betanidin or CD glucosylation are the final steps to be proven in BL biosynthesis.

606 Session 44, Enzymology & Metabolism
Sucrose synthesis in dormant tubers of *Helianthus tuberosus*. Martinez-Noel, Giselle Battaglia, Marina Pontis, Horacio G. Centro de Investigaciones Biológicas FIBA PROBIOP - CONICET Presenter: Pontis, Horacio G. fiba@mdq.com.ar

Fructans are composed of linear or branched chains of fructosyl units linked to sucrose. In tubers of Jerusalem artichoke (*Helianthus tuberosus*) they accumulate in late summer and early autumn reaching a degree of polymerization of about 35 fructosyl units. During tuber dormancy there occurs an hydrolysis and depolymerization of fructans with the result that at the end of the winter there are only polymers of up to DP 6. During polymer hydrolysis fructose is produced, however, a very particular characteristic of dormant tubers of J. artichoke is that no free fructose is found, and moreover the total amount of fructose present in the tuber is constant throughout the dormancy. It has been hypothesized that these two facts may be explained if the fructose produced by hydrolysis is used for synthesis of sucrose that acts as an acceptor for the transfer of fructosyl residues catalyzed by the enzyme fructan-fructan fructosyl transferase. We have measured the activities of sucrose synthase (SS) and also sucrose-phosphate synthase (SPS) hitherto not described in J. Artichoke tubers. Even more, we have been able to measure the activity of two isoforms of SPS that have a very different reactivity towards glucose-6-P and Pi. SS is 10 times more active than SPS. These results, however, could not tell which of two sucrose enzymes is responsible for sucrose synthesis during dormancy.

607 Session 44, Enzymology & Metabolism
Two inactivating factors of phospho and dephospho nitrate reductase in oat (*Avena sativa* L.) leaves. Kenis, Juana D. González, Claudio A. Kirkwood, Catriona A. Plant Physiology Laboratory, F.C.E.F. y N. Universidad Nacional de Córdoba Presenter: González, Claudio A. jkenis@impsat1.com

The nature and properties of two inactivating proteins of phospho(p)- and dephospho(dp)- nitrate reductase (NR) were studied in oat (*Avena sativa* L.) leaves. Plants were grown on vermiculite under continuous light of 60 Wm⁻², at 24 ± 2°C for 7 days. Plants were watered with 30 mM KNO₃ 24 hs before harvesting. IFs and NR were obtained from the first leaf of each plant. After 120 min incubation of crude extract at 4°C both p- and dp-NR lost activity at different rates suggesting that the two were inactivated. BSA (2%) stabilized p- and dp-NR especially at 4 - and to a lesser extent at 30 - °C. As purified corn NR remained stable after a 30 min incubation at 30°C with boiled (but not fresh) crude extracts from oat leaves, inactivation of NR was not due to thermolability. TLCK (100 μM), a trypsin-like proteinase inhibitor, also protected both forms of NR from inactivation especially at 4°C. Chromatography of crude extract on DEAE-Sephacell, showed two peaks of inactivating activity (named IF₁ and IF₂) with differential sensitivity to TLCK. To partially characterize those IFs, crude extract was fractionated twice (0-45%, IF₂, and 45-70%, IF₁) with (NH₄)₂SO₄ and each fraction was chromatographed on DEAE-Sephacell. IF₁, which eluted as a single peak at about 250 mM NaCl, was active at 30 (but not at 4) °C even in the presence of BSA and TLCK. It was inactivated only after boiling for 10 min at 100°C. IF₂ eluted as a single peak at 650mM NaCl. It was active at both 4 and 30°C but only in the absence of BSA and TLCK, and completely inactivated after a 1 min treatment at 100°C. Results suggest that instability of NR in crude extracts from oat leaves is caused by two IFs, one of them being a trypsin-like proteinase. Their preference for p- or dp-NR is discussed.

608 Session 44, Enzymology & Metabolism
Metabolic consequences of overexpression of the tobacco feedback-insensitive anthranilate synthase ASA2 gene. Brotherton, Jeffrey E. Widholm, Jack M. University of Illinois, Department of Crop Sciences Presenter: Brotherton, Jeffrey E. brothert@uiuc.edu

The *Nicotiana tabacum* ASA2 gene encodes a naturally occurring feedback insensitive α-subunit of anthranilate synthase. This gene is overexpressed in tobacco suspension cultured cells selected for resistance to the toxic tryptophan (Trp) analog 5-methyltryptophan (5MT) resulting in higher levels of intracellular free Trp. A mutant tobacco line (AB), that occurred spontaneously without 5MT-selection, overexpresses the ASA2 gene, is 5MT-resistant and has high free Trp in suspension cultured cells but not in regenerated plants. Newly initiated suspension cultured cell lines from progeny of a regenerated AB plant overexpress the ASA2 gene and have the associated properties. This demonstrates that the ASA2 overexpression trait is heritable though the pattern of inheritance is unclear since suspension cultured cell lines derived from the progeny of crosses to wild type plants have a range of intermediate levels of 5MT-resistance and free Trp levels. Other potential pathway products have not yet been shown to increase as free Trp increases. Tobacco suspension cultured cell lines that were derived from progeny of crosses between an AB line plant and plants transformed with a 2.3 kb portion of the ASA2 gene promoter region driving GUS were not GUS positive as expected. This suggests that the 2.3 kb ASA2 promoter driven GUS expression pattern seen in whole tobacco plants may not accurately mirror ASA2 expression. Other approaches will be needed to define the normal cellular function of the feedback-insensitive ASA2 gene product and how it may participate in partitioning the metabolites of the Trp pathway branch among the many important Trp, indole and anthranilate derived secondary products.

609 Session 44, Enzymology & Metabolism
Rice sucrose-phosphate synthase is regulated by calcium dependent phosphorylation. Pagnussat, Gabriela C. Salerno, Graciela L. Centro de Investigaciones Biológicas (FIBA) PROBIOP-CONICET Presenter: Salerno, Graciela L. fiba@mdq.com.ar

Sucrose-phosphate synthase (SPS; UDP-Glucose: D-Fructose-6-P-2-Glucosyl transferase, EC 2.4.1.14) has a key role in sucrose biosynthesis. As described in maize and spinach, the enzyme is allosterically activated by Glc-6-P and inhibited by Pi. Light modulation of SPS occurs by protein phosphorylation. Illumination of leaves results in dephosphorylation (activation) of SPS, which shows low affinity for the inhibitor Pi. Darkening of leaves results in phosphorylation (inactivation) of the enzyme, showing high affinity for the inhibitor. The regulatory properties of SPS *in vitro* and *in vivo* differ among species and cultivars as reported in soybean and tobacco. Rice SPS biochemical properties were studied in our laboratory showing important differences respect to those from maize and spinach leaf SPSs. Although light regulation was detected, the mechanism of that modulation was unknown. The aim of this work was to study the occurrence of protein phosphorylation as a mechanism to explain the diurnal variation of rice SPS activity and to characterize the kinase(s) involved. A synthetic peptide corresponding to the rice SPS site containing Ser-162 was designed to characterize putative SPS kinases in rice leaves. Kinase activity showed calcium dependence in crude extracts and was higher in plants harvested during the dark period than in plants collected in the light. An SPS kinase activity was partially purified and characterized. Immunoprecipitation experiments using anti rice SPS polyclonal antibodies demonstrated that the kinase is capable of phosphorylating SPS *in vitro*. The effect of inhibitors and bivalent ions suggests that the characterized kinase may belong to the CDPK family.

610 Session 44, Enzymology & Metabolism
Purification and characterization of sucrose synthase from stem cambial tissues of loblolly pine (*Pinus taeda* L.) trees. Sung, Shi-Jean S. Kormanik, Paul P. USDA-Forest Service, Southern Research Station, Tree Root Biology, Athens, GA Xu, Dian-Peng Black, Clanton C. Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA Presenter: Sung, Shi-Jean S. sung@negia.net

In the southeastern U.S. loblolly pine stems grow actively from May through early November and taproots are most active from September through mid-December. During active growth sucrose synthase (SS) is the dominant sucrolytic enzyme in stem and root cambial tissues of loblolly pine seedlings and trees (Sung et al. 1989 Tree Phys 12:243; Sung et al. 1993 Tree Phys 16:1003). We used a four-step procedure for the purification to apparent electrophoretic homogeneity of SS from loblolly pine stem cambial tissues. Loblolly pine trees aged 10 - 30 years were felled in late summer and xylem-side cambial zone tissues were immediately scraped into liquid nitrogen. Supernatants of crude extracts were precipitated with 7% - 17% polyethylene glycol (mw 4000) followed by DEAE-Sephacel CL-6B, Sephadex G-200, and UDPglucuronic acid agarose chromatography. The purified protein is a 540 kD hexamer of 94 kD subunits; has a pI of 6.1; exhibits K_m values of 74 mM for sucrose with a curvilinear response and 41 μM for UDP in the cleavage direction and 70 μM for UDPglucose and 2.3 mM for fructose in the synthesis direction. It optimally cleaves sucrose at pH 7.0 - 7.5 and synthesizes sucrose at pH 7.5 - 8.5. Rabbit antibodies against purified loblolly pine tree stem SS cross reacted with SS from stem and root cambial tissues of November-harvested first year loblolly pine, slash pine, shortleaf pine, longleaf pine, and Virginia pine seedlings. It also cross reacted with SS from crops such as immature rice grains, onion bulb - com

RECEIVED DEC 08 1999

Metrics Report for Year One Matching Company

Matching Company: Westvaco	Principal Investigator: H.D. Bradshaw, Jr.
Company Contact (name, phone, fax, email): Les Pearson (843)851-4773 (phone) (843)875-7185 L.XPEARS@westvaco.com	Project Title: Map-based cloning of genes to increase poplar biomass

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor				OK				high/exc.		
	0	1	2	3	4	5	6	7	8	9	10
Scientific accomplishments made by the project									✓		
Degree to which the project's original objectives have been accomplished									✓		
Degree to which the company's original expectations have been met										✓	
Frequency of communication between the company and the project PI									✓		
Quality of communication between the company and the project PI										✓	

Comments:

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for	0	1
Patents issued	0	1
Licenses obtained	0	0
Products created	1	1
Products improved	0	0
Processes created	1	1
Processes improved	1	1
Instances of new thinking which created a framework/base for new products or processes	1	1
Dollar value of above instances of new thinking	\$ see comments	\$ -
Instances enabling commercialization of products in terms of regulatory adherence	1	1
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)		

Comments: Initial product created is a marker system to screen for a disease resistant trait. Full \$ value will be in the eventually identification and isolation of one gene(s) involved and increased understanding of disease resistance mechanisms in trees.

C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$ see comments	\$	\$
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)	0	5	10
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)	see below		
Number of jobs with increased value			
Number of new businesses created	0	1	1

Comments: Ultimate application of this technology may impact a significant proportion of poplar acreage in the Pacific North West. Technology is also applicable to poplar plantations throughout the U.S. Advances of this kind will be key to maintaining the economic competitiveness of the U.S. forest industry.

D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$250K	\$50K	\$150K	\$300K
Facilities – space, equipment Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.	\$4,000	\$200	\$200	\$200
Supplies Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$0	\$0	\$25	\$150
Legal Costs Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.	\$50	\$0	\$0	\$50
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments: Project is fundamental research. Cost estimates are for commercialization of products based on increased understanding of disease resistance mechanisms in forest trees.

This form was completed by:

LES PEARSON

Les Pearson

9/13/99

Name

Signature

Date

Metrics Report for Year One Matching Company

Matching Company: Blandin Paper	Principal Investigator: Zong Ming Cheng
Company Contact (name, phone, fax, email): Mr. John McCoy 218-327-6386 Mr. Jim Marshall 218-327-6387 Jim.Marshall@upm-kymmene.com	Project Title: Evaluation and characterization of rooting capability of hybrid aspens transformed with rooting genes.

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor				OK				high/exc.	
	0	1	2	3	4	5	6	7	8	9 10
Scientific accomplishments made by the project										✓
Degree to which the project's original objectives have been accomplished									✓	
Degree to which the company's original expectations have been met								✓		
Frequency of communication between the company and the project PI								✓		
Quality of communication between the company and the project PI									✓	

Comments:

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for	0	2
Patents issued	0	2
Licenses obtained	0	2
Products created	0	5
Products improved	0	5
Processes created	NA	
Processes improved	NA	
Instances of new thinking which created a framework/base for new products or processes		
Dollar value of above instances of new thinking	\$	\$
Instances enabling commercialization of products in terms of regulatory adherence		
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)		

Comments:

- C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$	\$	\$
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)			
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)			
Number of jobs with increased value			
Number of new businesses created			

Comments:

- D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.				
Facilities - space, equipment Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.	\$	\$	\$	\$
Supplies Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$	\$	\$	\$
Legal Costs Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.	\$	\$	\$	\$
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments:

BLANDIN HAS HAD TO CUT EXPENSES IN FORESTRY R&D. WE REGRET VERY MUCH THAT OUR INVOLVEMENT IN DR. CHENG'S WORK HAD TO STOP AS THIS WAS THE MOST FAVORABLE ONE WE WERE INVOLVED WITH AS PAST MEMBERS OF CPBR. WE RECOMMEND HIS WORK TO BE CONTINUED.

This form was completed by:

John McCoy

Name

FORESTRY MANAGER

BLANDIN PAPER CO.

Signature

2/3/00

Date

Metrics Report for Year One Matching Company

Matching Company: BC International Corporation	Principal Investigator: Ingram, Preston & Shanmugam
Company Contact (name, phone, fax, email): Joseph Glas, Ph.D. ph: (781) 461-5700 Fax: (781) 461-2626 jglas@bcintl.org	Project Title: Ethanol Production from Uronic Acid - Substituted Xylose

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor/					OK					high/exc.	
	0	1	2	3	4	5	6	7	8	9	10	
Scientific accomplishments made by the project												X
Degree to which the project's original objectives have been accomplished												X
Degree to which the company's original expectations have been met												X
Frequency of communication between the company and the project PI												X
Quality of communication between the company and the project PI												X

Comments:

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for		1
Patents issued		
Licenses obtained		
Products created		
Products improved		
Processes created		
Processes improved		1
Instances of new thinking which created a framework/base for new products or processes		
Dollar value of above instances of new thinking	\$	\$ more than 1 mil/plant
Instances enabling commercialization of products in terms of regulatory adherence		
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)		

Comments:

Value estimated to be 10% increase in revenues

C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$ 1 Mil	\$ 3 Mil	\$ 10 Mil
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)	5	15	50
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)			
Number of jobs with increased value	20	60	200
Number of new businesses created			

Comments:

Potential to increase profits by 10%

D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$ 20K	\$ 60K		
Facilities - space, equipment Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.	\$ 5K	\$ 20K	\$	\$
Supplies Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$ 5K	\$ 20K	\$	\$
Legal Costs Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.	\$	\$	\$	\$
Other Costs Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments:

This form was completed by:

JEFF GATTO

Name

Signature

9/30/99

Date

Metrics Report for Year One Matching Company

NOTE
E-mail
→

Matching Company: <i>William Bio-Pharm</i>	Principal Investigator: <i>GAYLE LAMPPA</i>
Company Contact (name, phone, fax, email): <i>GARY WELCH</i> <i>(309) 347-9271</i> <i>(309) 347-3800 (fax)</i> <i>gk.welch@energy.twc.com</i>	Project Title: <i>Accumulation of Products within the Plasmid for Biomass Conversion: Test System with Cellulase</i>

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor			OK					high/exc.		
	0	1	2	3	4	5	6	7	8	9	10
Scientific accomplishments made by the project									X		
Degree to which the project's original objectives have been accomplished									X		
Degree to which the company's original expectations have been met											X
Frequency of communication between the company and the project PI										X	
Quality of communication between the company and the project PI											X

Comments:

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for		
Patents issued		
Licenses obtained		
Products created		
Products improved		
Processes created		
Processes improved		
Instances of new thinking which created a framework/base for new products or processes		
Dollar value of above instances of new thinking	\$	\$
Instances enabling commercialization of products in terms of regulatory adherence		
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)		

Comments:

This project is in very early research stages. High risk - High potential long term return on investment.

C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$	\$	\$ 10,000
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)			
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)			
Number of jobs with increased value			
Number of new businesses created			

Comments: *technology is several years away from commercialization.*

D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$2000	2-3	2-3	
Facilities - space, equipment Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.	\$ —	\$	\$	\$
Supplies Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$ —	\$	\$	\$
Legal Costs Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.	\$ —	\$	\$	\$
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments:

William could not implement the technology. We would potentially be a customer of a company implementing the technology.

This form was completed by:

CARY K. WELCH

Name

John K. Welch

Signature

11/24/99

Date

Metrics Report for Year One Matching Company

Matching Company: Monsanto	Principal Investigator: Basil Nikolau
Company Contact (name, phone, fax, email): Tom Savage Phone: (530) 792-2246 Fax: (530) 792-2453 Email: thomas.j.savage@monsanto.com	Project Title: How do plants generate acetyl-CoA

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor					OK		high/exc.				
	0	1	2	3	4	5	6	7	8	9	10	
Scientific accomplishments made by the project												X
Degree to which the project's original objectives have been accomplished												X
Degree to which the company's original expectations have been met												X
Frequency of communication between the company and the project PI									X			
Quality of communication between the company and the project PI										X		

Comments:

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for	1	2
Patents issued		
Licenses obtained		
Products created		2
Products improved		
Processes created		
Processes improved		
Instances of new thinking which created a framework/base for new products or processes	2	2
Dollar value of above instances of new thinking	\$100,000	\$100,000
Instances enabling commercialization of products in terms of regulatory adherence		
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)		

Comments:

The investigators have done an excellent job of isolating genes encoding important enzymes of acetyl-CoA generation. They are currently evaluating the specific role each of these genes play in generating different acetyl-CoA pools within plants. This information is critical for commercial exploitation of the genetic information so far obtained. Thus, we very strongly encourage CPBR to continue to support this project.

C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$10,000	\$20,000	\$50,000
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)	50	100	250
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)	50	100	250
Number of jobs with increased value			
Number of new businesses created			

Comments:

D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time <i>Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.</i>	10	10	10	20
Facilities – space, equipment <i>Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.</i>	\$1000	\$1000	\$1000	\$2000
Supplies <i>Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.</i>	\$200	\$200	\$200	\$400
Legal Costs <i>Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.</i>	\$100	\$100	\$100	\$100
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments:

This form was completed by: Tom Savage

THOMAS J. SAVAGE

Name

Thomas J. Savage

Signature

NOVEMBER 21, 1999

Date

Metrics Report for Year One Matching Company

Matching Company: <i>Dow AgroSciences</i>	Principal Investigator: <i>DR. Brian Oppert</i>
Company Contact (name, phone, fax, email): <i>GREG A. DRAFFERT</i> <i>5501 OBERLIN DR</i> <i>883352 - 4527</i> <i>926224-1213 DOW AGRO SCI</i>	Project Title: <i>Evaluation of Serpins as</i> <i>Biopesticides</i>

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor				OK				high/exc.		
	0	1	2	3	4	5	6	7	8	9	10
Scientific accomplishments made by the project								✓			
Degree to which the project's original objectives have been accomplished							✓				
Degree to which the company's original expectations have been met								✓			
Frequency of communication between the company and the project PI						✓					
Quality of communication between the company and the project PI								✓			

Comments:

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for	0	1
Patents issued	0	1
Licenses obtained	0	0
Products created	0	1
Products improved	0	2
Processes created	0	3
Processes improved	0	1
Instances of new thinking which created a framework/base for new products or processes	1	1
Dollar value of above instances of new thinking	\$ —	\$ 53,000
Instances enabling commercialization of products in terms of regulatory adherence	0	1
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)	0	1

Comments: *Project should help characterize some products in development.*
Will contribute to research project at this stage

C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$ —	\$ —	\$ —
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)	1	1	1
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)	—	—	—
Number of jobs with increased value	—	—	—
Number of new businesses created	—	—	—

Comments:

Too early to determine impact

D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	37% of 1 person	57%	—	—
Facilities – space, equipment Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.	\$ —	\$ —	\$ —	\$ —
Supplies Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$ 1	\$ 5	\$ —	\$ —
Legal Costs Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.	\$ 1	\$ —	\$ —	\$ —
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments:

Dr. Oppert is doing some foundational studies which will allow us to understand the role of protein control agents in insect gut. It is difficult to answer this part but these studies will have an impact on the control of insects with transgenic plants. With conventional insecticides these studies had a major impact.

This form was completed by:

type

Name

Signature

Date

Dr. Oppert

[Signature]

3/24/00

Matching Company: <i>Weyerhaeuser</i>	Principal Investigator: Steve Strauss
Company Contact (name, phone, fax, email): <i>Nicholas Wheeler</i> <i>360-330-1738, Fax 360 330 1742</i> <i>nick.wheeler@weyerhaeuser.com</i>	Project Title: Genes Controlling the Transition Between Vegetative and Reproductive Phases in Forest Trees

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor						OK						high/exc.	
	0	1	2	3	4	5	6	7	8	9	10			
Scientific accomplishments made by the project									✓					
Degree to which the project's original objectives have been accomplished								✓						
Degree to which the company's original expectations have been met									✓					
Frequency of communication between the company and the project PI									✓					
Quality of communication between the company and the project PI										✓				

Comments:

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for	NA	
Patents issued	NA	
Licenses obtained	NA	
Products created	NA	
Products improved	NA	
Processes created	?	
Processes improved	?	
Instances of new thinking which created a framework/base for new products or processes	1	
Dollar value of above instances of new thinking	\$?	\$
Instances enabling commercialization of products in terms of regulatory adherence	NA	
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)	NA	

Comments: *my sense is that this work remains exploratory at this time and it may be too early to estimate long-term value.*

C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$ NA	\$?	\$?
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)	NA		
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)	NA		
Number of jobs with increased value	NA		
Number of new businesses created	NA		

Comments: I think the project could ultimately have utility & result in new jobs in a technical center, but it is too early to say.

D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$ 1 my			
Facilities - space, equipment Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.	\$ 0	\$?	\$	\$
Supplies Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$ 0	\$ 1,000.00	\$	\$
Legal Costs Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.	\$ 0	\$?	\$	\$
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments: we intend to assist with some propagation work

This form was completed by:

Name Nicholas Wheeler Signature Nicholas Wheeler Date 6/17/99

Matching Company: Westvaco Corporation	Principal Investigator: Steve Strauss
Company Contact (name, phone, fax, email): H. Dayton Wilde (hdwilde@westvaco.com) (843) 851-4822 Fax (843) 875-7185	Project Title: Genes Controlling the Transition Between Vegetative and Reproductive Phases in Forest Trees

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor		1	2	3	4	OK		6	7	8	high/exc.		10
Scientific accomplishments made by the project											✓			
Degree to which the project's original objectives have been accomplished											✓			
Degree to which the company's original expectations have been met														✓
Frequency of communication between the company and the project PI												✓		
Quality of communication between the company and the project PI												✓		

Comments: The researchers have made significant progress in isolating genes, although the vagaries of research have resulted in some of the most interesting flowering genes still awaiting isolation. The research that will show whether these genes will be useful, testing the genes for their effects on flowering, is pending.

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	Anticipated
Patents applied for	N/A	
Patents issued	N/A	
Licenses obtained	N/A	
Products created	N/A	
Products improved	N/A	
Processes created	N/A	
Processes improved	N/A	
Instances of new thinking which created a framework/base for new products or processes	N/A	
Dollar value of above instances of new thinking	\$ N/A	\$
Instances enabling commercialization of products in terms of regulatory adherence	N/A	
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)	N/A	

Comments:

This research is highly exploratory, and it is too early to predict commercial benefits.

- C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$ N/A	\$	\$
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)	N/A		
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)	N/A		
Number of jobs with increased value	N/A		
Number of new businesses created	N/A		

Comments:

This research is highly exploratory, and it is too early to predict commercial benefits.

- D. Estimate the corporate investment *in addition to the cash matching funds provided* that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time <i>Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.</i>	N/A			
Facilities – space, equipment <i>Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.</i>	\$ N/A	\$	\$	\$
Supplies <i>Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.</i>	\$ N/A	\$	\$	\$
Legal Costs <i>Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.</i>	\$ N/A	\$	\$	\$
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments:

Although we have begun to make estimates of these costs, this information is not for release outside the company.

This form was completed by:

Name William H. Rottmann

Signature

Date

William H. Rottmann 6/28/99

Matching Company: MONSANTO COMPANY	Principal Investigator: Steve Strauss
Company Contact (name, phone, fax, email): DR. DAVID N. DUNCAN 314/737-5212	Project Title: Genes Controlling the Transition Between Vegetative and Reproductive Phases in Forest Trees

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor						OK						high/exc.	
	0	1	2	3	4	5	6	7	8	9	10			
Scientific accomplishments made by the project									X					
Degree to which the project's original objectives have been accomplished									X					
Degree to which the company's original expectations have been met								X						
Frequency of communication between the company and the project PI										X				
Quality of communication between the company and the project PI										X				

Comments:

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for	?	?
Patents issued	?	?
Licenses obtained	0	5-10
Products created	0	5-10
Products improved	0	5-10
Processes created		
Processes improved		
Instances of new thinking which created a framework/base for new products or processes		
Dollar value of above instances of new thinking	\$	\$
Instances enabling commercialization of products in terms of regulatory adherence	0	5-10
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)		

Comments:

C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$ 0	\$ 1-3MIL	\$ 50MIL
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)			
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)			
Number of jobs with increased value			
Number of new businesses created			

Comments:

D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time <i>Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.</i>				
Facilities – space, equipment <i>Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.</i>	\$ 0	\$ 10 MIL	\$ 15MIL	\$ 40MIL
Supplies <i>Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.</i>	\$	\$	\$	\$
Legal Costs <i>Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.</i>	\$	\$	\$	\$
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments:

This form was completed by:

Name David N. Duncan

Signature

Date

7/22/99

Matching Company: International Paper	Principal Investigator: Steve Strauss
Company Contact (name, phone, fax, email): Bryan Kaphammer Phone: (912) 826-6904, fax: (912) 826-6700 e-mail: bryan.kaphammer@ipaper.com	Project Title: Genes Controlling the Transition Between Vegetative and Reproductive Phases in Forest Trees

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor							OK						high/exc.	
	0	1	2	3	4	5	6	7	8	9	10				
Scientific accomplishments made by the project										X					
Degree to which the project's original objectives have been accomplished									X						
Degree to which the company's original expectations have been met									X						
Frequency of communication between the company and the project PI									X						
Quality of communication between the company and the project PI										X					

Comments: This project is making excellent progress towards its objective of understanding flowering control in trees.

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for	NA	
Patents issued	NA	
Licenses obtained	NA	
Products created	NA	
Products improved	NA	
Processes created	NA	
Processes improved	NA	
Instances of new thinking which created a framework/base for new products or processes	NA	
Dollar value of above instances of new thinking	\$NA	\$
Instances enabling commercialization of products in terms of regulatory adherence	NA	
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)	NA	

Comments: This research is of a fundamental nature for which the downstream benefits are too early to see.

C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$NA	\$	\$
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)	NA		
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)	NA		
Number of jobs with increased value	NA		
Number of new businesses created	NA		

Comments: This research is of a fundamental nature for which the downstream benefits are too early to see.

D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	NA			
Facilities - space, equipment Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.	\$NA	\$	\$	\$
Supplies Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$NA	\$	\$	\$
Legal Costs Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.	\$NA	\$	\$	\$
Other Costs	NA			
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments: It is inappropriate to give out this kind of cost information

This form was completed by:

Bryan Kaphammer

July 20,
1999

Name

Signature

Date

Matching Company: Boise Cascade Corporation	Principal Investigator: Steve Strauss
Company Contact (name, phone, fax, email): Lawrence K. Miller Phone: 509-544-6535; FAX: 509-545-9964 Email: larry_miller@bc.com	Project Title: Genes Controlling the Transition Between Vegetative and Reproductive Phases in Forest Trees

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor							OK					high/exc.
	0	1	2	3	4	5	6	7	8	9	10		
Scientific accomplishments made by the project								X					
Degree to which the project's original objectives have been accomplished									X				
Degree to which the company's original expectations have been met									X				
Frequency of communication between the company and the project PI										X			
Quality of communication between the company and the project PI										X			

Comments:

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for		
Patents issued		
Licenses obtained	COMPLETELY	UNKNOWN
Products created		
Products improved		
Processes created		
Processes improved		
Instances of new thinking which created a framework/base for new products or processes		
Dollar value of above instances of new thinking	\$PROPRIETARY	\$PROPRIETARY
Instances enabling commercialization of products in terms of regulatory adherence		
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)		

Comments:

C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$	\$	\$
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)	NOT	KNOWN	AND
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)	TOTALLY	PRO-	PRIETARY
Number of jobs with increased value			
Number of new businesses created			

Comments:

D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.				
Facilities – space, equipment Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.	\$	\$	\$	\$
Supplies Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$	\$	\$	\$
Legal Costs Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.	\$	\$	\$	\$
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments: THE ABOVE INFORMATION IS NOT KNOWN AT THIS TIME. IF IT WERE, IT WOULD BE COMPLETELY PROPRIETARY.

This form was completed by:

Lawrence K. Miller

Signature

Date

Lawrence K. Miller 6/17/99

Metrics Report for Year One Matching Company

Matching Company: RhoBio	Principal Investigator: Dr. J. Winkler
Company Contact (name, phone, fax, email): Dr. George FREYSSINET 33 4 72 85 2549 Tel 33 4 72 85 2350 Fax	Project Title: New selectable marker and promoters of plant origin

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor				OK				high/exc.		
	0	1	2	3	4	5	6	7	8	9	10
Scientific accomplishments made by the project									X		
Degree to which the project's original objectives have been accomplished									X		
Degree to which the company's original expectations have been met									X		
Frequency of communication between the company and the project PI								X			
Quality of communication between the company and the project PI								X			

Comments:

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for		2
Patents issued		
Licenses obtained		
Products created	1	2
Products improved	2	
Processes created		1
Processes improved	1	
Instances of new thinking which created a framework/base for new products or processes		
Dollar value of above instances of new thinking	\$	\$
Instances enabling commercialization of products in terms of regulatory adherence		
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)		

Comments:

C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$	\$	\$ 200
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)	1	5	5
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)			
Number of jobs with increased value			
Number of new businesses created			

Comments:

D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	0.5	1	5	5
Facilities – space, equipment Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.	\$	\$	\$	\$
Supplies Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$	\$	\$	\$
Legal Costs Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.	\$	\$	\$	\$
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments:

This form was completed by:

Dr. George FREYSSINET November 21, 1999

Name Signature Date

Metrics Report for Year One Matching Company

Matching Company: <i>WESTVACO</i>	Principal Investigator: <i>Dr. Jack Widholm</i>
Company Contact (name, phone, fax, email): <i>DAVID WILDE</i> <i>843-851-4822</i> <i>david.wilde@westvac.com</i>	Project Title: <i>A New Selectable MARKER</i> <i>and PROMOTERS OF PLANT ORIGIN</i>

Please complete this report and add any comments you wish. When finished, seal it in an envelope and return to the PI who sent it to you. The PI will forward the report to CPBR in partial fulfillment of reporting requirements. The information you provide will be kept confidential by CPBR and used solely for federally-required statistical reporting. Your cooperation is greatly appreciated.

A. Rate the project to date on the items below using a scale of 0 (low/poor) to 10 (high/excellent).

	low/poor				OK				high/exc.			
	0	1	2	3	4	5	6	7	8	9	10	
Scientific accomplishments made by the project										X		
Degree to which the project's original objectives have been accomplished									X			
Degree to which the company's original expectations have been met									X			
Frequency of communication between the company and the project PI						X						
Quality of communication between the company and the project PI						X						

Comments: *The part of the project dealing with promoter analysis has gone well. Selectable marker development is lagging somewhat, but this is the more difficult part of the project. Communication has been OK considering the length of time it took to obtain a Research Agreement.*

B. Estimate the numbers of the following that have resulted from or can reasonably be expected to result from this research project.

	to date	anticipated
Patents applied for	<i>1</i>	
Patents issued		<i>1</i>
Licenses obtained		
Products created		<i>1</i>
Products improved		
Processes created		<i>1</i>
Processes improved		
Instances of new thinking which created a framework/base for new products or processes	<i>2</i>	
Dollar value of above instances of new thinking	\$	\$ <i>?</i>
Instances enabling commercialization of products in terms of regulatory adherence		<i>1</i>
Instances enabling the evaluation of technologies in a defensive sense (e.g., understanding the competition)		

Comments:

- C. Estimate the future economic impact of the use and/or commercialization of the results of this project. Please list estimates in annual terms at the future times indicated.

	In 3 years	In 5 years	In 10 years
Product sales (thousands of dollars)	\$	\$	\$
Number of jobs created (include R&D, manufacturing, distributing, marketing, etc.)			
Number of jobs retained (include R&D, manufacturing, distributing, marketing, etc.)			
Number of jobs with increased value			
Number of new businesses created			

Comments: *this project examines fundamental tools of plant biotechnology. Successful completion will lead to improvements and alternatives to presently used tools. Because the technology is not enabling, the value will be derived from*

- D. Estimate the corporate investment in addition to the cash matching funds provided that the company has made and is likely to make to accomplish the technology transfer and commercialization of results of the project. List amounts in thousands of dollars annually at the future times indicated.

	Current/ to date	In 3 years	In 5 years	In 10 years
Personnel Time Include R&D personnel, personnel involved in liaison with CPBR project, implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	0	5	1	
Facilities - space, equipment Include R&D facilities and facilities involved in implementing the technology transfer and producing, marketing, distributing the new or improved products and/or processes, etc.	\$ 0	\$ 5	\$ 2	\$
Supplies Include R&D supplies involved in implementing the technology transfer, and in producing, marketing, distributing the new or improved products and/or processes, etc.	\$ 0	\$ 5	\$ 1	\$
Legal Costs Include legal costs involved in implementing the technology transfer and in protecting and producing the new or improved products and/or processes, etc.	\$ 1	\$ 1	\$ 1	\$
Other Costs				
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$
Item _____	\$	\$	\$	\$

Comments:

This form was completed by:

DAVID WILDE
Name

David Wilde
Signature

12/1/99
Date