

**National Institute of Food and Agriculture
Biotechnology Risk Assessment Grants
Program
Annual Project Director's Meeting**



USDA APHIS-BRS
Riverdale, Maryland
June 14, 2013



United States Department of Agriculture
National Institute of Food and Agriculture



United States
Department of
Agriculture

National Institute
of Food and
Agriculture

USDA Biotechnology Risk Assessment Grants Program Annual Project Director's Meeting

Welcome to the Annual Project Director's (PD) Meeting for the USDA Biotechnology Risk Assessment Grants (BRAG) Program. This year's meeting includes awardees of proposals submitted in fiscal years 2010, 2011 and 2012.

Authority for the BRAG program is contained in section 1668 of the Food, Agriculture, Conservation, and Trade Act of 1990 (i.e., 1990 Farm Bill) and amended in section 7210 of the Farm Security and Rural Investment Act of 2002 (i.e., 2002 Farm Bill). In the Food, Conservation, and Energy Act of 2008 (i.e., 2008 Farm Bill), the authority was not repealed, so the BRAG program continued its role in supporting risk assessment research related to biotechnology. In accordance with the legislative authority in the 2002 Farm Bill, the BRAG program supports research designed to identify and develop appropriate management practices to minimize physical and biological risks associated with genetically engineered (GE) animals, plants, and microorganisms. The USDA's National Institute of Food and Agriculture (NIFA) and Agricultural Research Service (ARS) jointly administer the BRAG program.

The main purpose of the BRAG program is to support the generation of new information that will assist Federal regulatory agencies in making science-based decisions about the effects of introducing into the environment GE organisms, including plants, microorganisms (including fungi, bacteria, and viruses), arthropods, fish, birds, mammals and other animals excluding humans. Investigations of effects on both managed and natural environments are relevant. The BRAG program accomplishes its purpose by providing Federal regulatory agencies with scientific information relevant to regulatory issues.

The overall goal of the PD Meetings is to improve post-award management of competitive grants administered by NIFA and ARS. It is the intent that these meetings will enhance communication and interaction between USDA Program Staff and BRAG awardees. In turn, this will assist Program Staff in identifying success stories resulting from USDA-sponsored research in the BRAG program and facilitate the reporting of important impacts resulting from the most successful research through communications with Congress, the Secretary and Undersecretary of

Agriculture, USDA administrators, federal regulators, the scientific community, commodity groups and other stakeholders, and the general public. It is critical to identify and highlight these impacts in order to maintain funding in USDA's biotechnology risk assessment program areas, as well as to continue the recent trend of increased Congressional budget appropriations to USDA competitive grant programs that have occurred since 2008. Conducting annual meetings for awardees is just one of several approaches being implemented by NIFA to improve post-award management.

A second purpose of this meeting is to foster communication among awardees in this program and federal regulators, such as USDA Animal and Plant Health Inspection Service, U.S. Environmental Protection Agency, and the U.S. Food and Drug Administration, which have scientific interests in risk assessment research. It is anticipated that the sharing of information and the ensuing dialogue that will occur in this informal setting will allow all awardees to benefit from the experiences of their colleagues and yield greater opportunity for successful completion of their BRAG awards. In addition, it is expected that improved communication among BRAG awardees will result in better sharing of limited resources and the development of new fruitful collaborations.

We look forward to a highly successful and productive meeting, and we eagerly anticipate continued progress on your BRAG awards.

Respectfully,

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USDA
Biotechnology Risk Assessment Grants (BRAG) Program
Project Director's Meeting

June 14, 2013

USDA-APHIS-BRS Headquarters
4700 River Road
Riverdale, MD 20737

Friday, June 14, 2013

8:30 - 9:00 AM	Arrival and Poster Setup
9:00 - 9:15 AM	Welcome Shing Kwok - USDA-NIFA
9:15 - 9:35 AM	APHIS and Plant Pest Risk Assessment John Turner - USDA-APHIS
9:35 - 9:55 AM	Regulation of Microbial Pest Control Agents and Plant-incorporated Protectants by the US EPA Chris Wozniak - EPA
9:55 - 10:15 AM	Environmental Assessment of Genetically Engineered Animals at FDA Evgenij Evdokimov - FDA
10:15 - 10:35 AM	Fostering Coexistence: Landscape Approaches with Alfalfa Stephanie Greene - USDA-ARS, WA
10:35 - 10:50 AM	Break
10:50 - 11:10 AM	Maternally Induced Sterility in Fish Xavier Lauth - Center for Aquaculture Technologies
11:10 - 11:30 AM	Pollen-mediated Gene Flow in Switchgrass Neal Stewart - University of Tennessee
11:30 - 11:50 AM	Transmission Genetics of Sorghum-to-Johnsongrass Gene Transfer Andrew Paterson - University of Georgia
11:50 - 1:00 PM	Lunch - On Your Own

- 1:00 - 1:20 PM** Recombinant Newcastle Disease Vaccines: Risk for Recombination, Reversion to Virulence and Spread in Non-Target Species
Patti Miller - USDA-ARS, GA
- 1:20 - 1:40 PM** Reducing Root Suckering of Transgenic Poplar
Yi Li - University of Connecticut
- 1:40 - 2:00 PM** Assessing the Risk of European Corn Borer Resistance to Cry1F-expressing Corn
Blair Siegfried - University of Nebraska
- 2:00 - 2:20 PM** Potential Shortfall of Transgenic Pyramided Cotton for Insect Resistance Management
Yves Carriere - University of Arizona
- 2:20 - 2:40 PM** **Break**
- 2:40 - 3:30 PM** Discussion
- 3:30 - 5:00 PM** Poster Session

Posters

#	Name	Award Year	Institution	Poster Title
1	Arinder Arora	2010, 2012	Univ. of NM School of Medicine	Development Of Different Molecules For Paratransgenic Control Of Pierce's Disease
2	Catherine Bonin	2010, 2012	IA State Univ.	Fitness Comparisons Between Cultivated and Native Switchgrass (<i>Panicum virgatum</i>) in Ohio and Iowa: Implications for Future Biofuel Crops
3	Catherine Bonin	2010, 2012	IA State Univ.	Gene Flow Networks and Potential Invasiveness of Seed- producing Miscanthus Species for Biofuel Crops
4	Catherine Bonin	2010, 2012	IA State Univ.	Pollen-mediated Gene Flow up to 130 m in Small, Experimental Arrays of Cultivated and Wild Switchgrass (<i>Panicum virgatum</i>): Implications for Transgenic Field Trials
5	Carolina Concha	2012	NC State Univ.	Development and Evaluation of Male-only Transgenic Strains of the New World Screwworm Fly
6	John Burke	2010	Univ. of GA	Assessing the Fate and Impact of Crop Gene Introgression Into Wild Sunflower
7	Geoffrey Ecker	2011	Univ. of CT	Native and Cultivated Switchgrass: Distribution Patterns and Pollen Dispersal
8	Scott Fahrenkrug	2012	Recombinetics, Inc.	Towards TALEN-Mediated Genetic Containment of Genetically Engineered Livestock
9	Adam Forshaw	2010, 2012	Univ. of NM School of Medicine	Simulated Field Trial of Microencapsulation in Paratransgenic Delivery Systems
10	Aaron Gassmann	2012	IA State Univ.	Risks from Field-evolved Resistance to Bt Corn by Western Corn Rootworm
11	Fred Gould	2012	NC State Univ.	A Genomic Approach for Bt Resistance Risk Assessment
12	Alfred Handler	2011	USDA-ARS, FL	Recombinase-based Transformation Vectors for Improved Transgenic Strain Development and Ecological Safety in Tephritid Pest Species

13	Brian Lovett	2011	Univ. of MD	Functional Gene Microarray Analysis of the Effect of <i>Metarhizium</i> on Winter Wheat and Turf Rhizospheres
14	Jonathan Lundgren	2012	USDA-ARS, SD	Do RNAi based GM Crops Pose Novel Ecological Risks?
15	Hong Luo	2010	Clemson Univ.	Environmental Risk Assessment of Turfgrass Genetically Engineered for Abiotic Stress Tolerance
16	Elizabeth Maga	2010	UC Davis	Assessing Unintended Effects of a Mammary-specific Transgene Product at the Whole Animal Level
17	William Powell	2012	SUNY-ESF	American Chestnut Restoration Introduces a New Paradigm of Transgene Introgression to Save a Keystone Species
18	Jared Schmidt	2010	Univ. of NE	Synchrony of Flowering of 6 Shattercane Populations and 3 Sorghum Hybrids Under Various Management Practices
19	Anthony Shelton	2010	Cornell Univ.	A Critical Assessment of the Effect of Cry Proteins on Beneficial Arthropods Through the Use of Cry-Resistant Hosts
20	Blair Siegfried	2010	Univ. of NE	A First Glimpse of Non-target Impacts on Honey Bee and Ladybug Using In Vivo RNAi Bioassay
21	Vibha Srivastava	2010	Univ. of AR	Recombinase Mediated Targeted Gene Insertion and Marker Gene Deletion for Precise Genetic Transformation
22	Steven Strauss	2010	OR St. Univ.	Zinc Finger Nuclease Based Mutagenesis for Genetic Containment in Poplar
23	James Thomson	2010	USDA-ARS, CA	Improved recombinase technology for targeted marker free integration and founder line production for risk assessment
24	Ping Wang	2012	Cornell Univ.	Understanding the Molecular Genetic Basis of Insect Resistance to Bt-Crops

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A MULTIGENERATIONAL ASSESSMENT OF THE FATE AND IMPACT OF CROP GENE INTROGRESSION INTO WILD SUNFLOWER

Burke, J. M.; Dechaine-Berkas, J. M.; Baack, E. J.
University of Georgia
Athens, Georgia

Award Number: 2010-33522-21668

NON-TECHNICAL SUMMARY: With the commercial introduction of genetically modified crops, it is feared that engineered genes (i.e., transgenes) might escape into related wild taxa via cross-pollination (i.e., hybridization), possibly resulting in the production of more invasive weeds or causing the decline of natural populations of herbivores, pathogens, and/or competing plant populations. Previous research indicates that hybridization between crop plants and their wild relatives is widespread, and that at least some transgenes are likely to be favored in the wild. To date, however, the majority of studies have examined only short-term effects of individual crop alleles and/or transgenes in wild populations. We thus know relatively little about the likely long-term impact of crop-wild gene flow across the genome as a whole. By examining the outcome of crop-wild hybridization at both the phenotypic (i.e., visible trait) and genetic levels in multiple locations and across multiple generations, this research will provide a much more complete understanding of the types of crop-related traits that are likely to be favored in the wild, as well as the rate at which beneficial crop alleles will be incorporated into wild populations. By developing a more complete understanding of the evolution of crop-wild hybrid populations from one year to the next, we will be able to determine if and when short-term studies can of phenotypic and genetic change can be used to predict the longer-term consequences of crop-wild gene flow. Our findings will thus help to inform cost-benefit analyses relating the likely accuracy of risk assessment strategies to their economic viability.

OBJECTIVES: The primary goals of this research project are to identify the types of traits that are likely to provide a selective advantage following their transmission from cultivated sunflower into wild sunflower populations, investigate the factors favoring the establishment of and spread of beneficial crop alleles in wild populations, and to investigate the efficacy of short-term selection studies for predicting the long-term outcome of crop-wild gene flow. To this end, we seek to: (1) quantify the long-term response of crop-related traits, particularly those that are likely targets of genetic modification (e.g., enhanced growth, competitive ability, herbivore or pathogen resistance) to natural selection; (2) investigate the persistence/spread of cultivar alleles at a genome-wide scale and in the context of phenotypic traits that these genomic regions are known to influence; (3) determine whether or not selection has similar effects on phenotypic and/or allelic persistence across environments; and (4) test whether or not short-term selection experiments are valid predictors of longer-term, population-level responses to selection in crop-wild hybrid systems. This work will greatly improve our understanding of the evolution of crop-wild hybrid populations under natural conditions, and will thus have broad impacts on risk assessment for novel transgenic crops.

APPROACH: This research project will involve: (1) an analysis of phenotypic responses to selection over time in experimental crop-wild hybrid populations that have been subjected to multiple generations of natural selection, and (2) an analysis of allele frequency changes over time in these same populations using a genome-wide collection of single nucleotide polymorphisms (SNPs). For the phenotypic analyses, we will investigate the strength and direction of natural selection in the field across three generations. These analyses will be performed using the ASTER program in R to estimate lifetime fitness using a maximum-likelihood approach. Fitness in each generation will be modeled using survival to reproduction and estimated seed production. This work will be accompanied by common garden experiments in two locations to further evaluate the evolution of relevant plant phenotypes over time. In terms of tracking allele frequency changes, we will genotypically characterize the original founding population as well as a subset of individual from each location and generation of selection using a genome-wide collection of 384 SNPs using the Illumina BeadXpress platform. These data will allow us to track allele frequency changes over time on a genome-wide basis. Major frequency changes will be compared to the null expectation under neutral drift (derived from simulations). Depending on the direction of the deviation, significant outliers will be attributable to positive or negative selection. The results of this work will be further compared to QTL mapping results from this same population, thereby allowing us to connect allele frequency changes to phenotypes, and thus to our phenotypic selection analyses. The magnitude of allele frequency changes will also be used to derive estimates of the strength of selection.

PROGRESS: 2010/09 TO 2011/08

OUTPUTS: The primary goal of this project is to develop a more complete understanding of the evolution of crop-wild hybrid populations over time and to determine whether or not short-term studies of phenotypic and genetic change can be used to predict longer-term consequences of crop-wild gene flow. Our objectives are thus to: (1) quantify the long-term response of crop-related traits, particularly those that are likely targets of genetic modification (e.g., enhanced growth, competitive ability, herbivore or pathogen resistance, etc.) to natural selection; (2) investigate the persistence/spread of cultivar alleles at a genome-wide scale and in the context of phenotypic traits that these genomic regions are known to influence; (3) determine whether or not selection has similar effects on phenotypic and/or allelic persistence across environments; and (4) test whether or not short-term selection experiments are valid predictors of longer-term, population-level responses to selection in crop-wild hybrid systems. During the first year, we selected 384 easily scorable, polymorphic SNP loci from our larger 10,640 Illumina Infinium SNP array. Because the genetic map positions of all of these SNPs are known, we were able to choose a set that provides complete and relatively even genomic coverage. These SNPs were then submitted to Illumina for the production of a GoldenGate assay that will be used to genotype our experimental populations. We also completed the seed processing from our experimental hybrid populations and then germinated, transplanted, and collected tissue from all individuals that will be genotyped from the base population (G0) as well as all subsequent generations (G1-G3) that will be genotyped. DNA was extracted, quantified, and quality-checked for all of these individuals in preparation for genotyping. In addition, we established the proposed common garden studies in both North Dakota and Iowa during the spring/summer of 2011 and collected phenotypic data from ca. 600 individuals as well as a representative sample

of wild and cultivar parental individuals at each location. In addition to the above, we have collected seeds from several naturally-occurring crop-wild hybrid populations and will be exploring their utility for extending our findings to native hybrid populations. **PARTICIPANTS:** John Burke served as the Project Director, and has been responsible for oversight of the SNP array design, DNA extraction, and genotyping. Jennifer Decahine served as a co-PD and has been responsible for oversight and analysis of the common garden experiment in North Dakota as well as collection of natural populations. Eric Baack served as a co-PD and has been responsible for oversight and analysis of the common garden experiment in Iowa as well as collection of natural populations. Jonathan Corbi served as a postdoctoral research associate at UGA and took the lead on the SNP array design, seed germination, and DNA extraction. Birkin Owart served as a graduate research assistant at CWU and took the lead on data collection in field plots for the common garden experiment, data organization, and analysis. Alan McNolty, Melissa Ames, Maxwell Davis, and Allison Butler served as undergraduate research assistants at CWU and helped with the processing of field materials. Molly Tulkki and Megan Gress served as undergraduate research assistants at Luther College and helped with the establishment and maintenance of the Iowa field plots as well as data collection. **TARGET AUDIENCES:** Not relevant to this project. **PROJECT MODIFICATIONS:** Not relevant to this project.

IMPACT: 2010/09 TO 2011/08

None to date.

PUBLICATIONS (not previously reported): 2010/09 TO 2011/08

No publications reported this period

SECOND GENERATION PARATRANSGENESIS FOR CONTROL OF PIERCE'S DISEASE OF GRAPES

Durvasula, R. V.; Miller, T.
University of New Mexico
Albuquerque, New Mexico

Award Number: 2010-33120-21852

NON-TECHNICAL SUMMARY: Despite advances in public health, insect-transmitted diseases remain a leading cause of morbidity and mortality. Additionally, the global impact of diseases to agriculture exceeds \$100 billion. Currently, the best methods for control of many insect-borne diseases involve the use of pesticides which are toxic, expensive and allow evolution of insect resistance. Evolving methods for control of vector-borne diseases rely on modification rather than elimination of insects. These strategies involve either direct transformation of an insect genome or expression of gene products in the insect via transformed symbiotic microbes (paratransgenesis). Paratransgenesis is a "Trojan Horse" approach to control of disease transmission. It employs the interactions between disease-transmitting vectors, bacterial symbionts of the vectors and transmitted pathogens. Symbiotic bacteria are isolated and genetically transformed to export molecules that interfere with pathogens. The genetically altered symbionts are then introduced into the host vector where expression of engineered molecules affects the host's ability to transmit the pathogen. Pierce's Disease is a deadly disease of grapevines causing tremendous economic loss to the wine industry of California each year. It is caused by the bacterium *Xylella fastidiosa*, which is spread by xylem-feeding sharpshooters. The predominant vector of this disease in the US is the Glassy Winged Sharpshooter (GWSS), *Homalodisca vitripennis*. Pierce's Disease is prevalent within the USA from Florida to California, and outside the USA in Central and South America. In the paratransgenic approach, a commensal bacterium of *H. vitripennis*, *Alcaligenes xylosoxidans* var. *dentrificans* (AXD), is modified to export molecules that disrupt the transmission of *X. fastidiosa*, the causative agent of Pierce's disease of grapevines. Both AXD and *Xylella* colonize the anterior mouthparts (cibarium) of *H. vitripennis*, thus assuring that exported molecules from AXD contact *Xylella* and interrupt transmission to plants. Broadcast of engineered *Alcaligenes* to field sites such as vineyards with subsequent uptake by sharpshooters would result in disruption of regional *Xylella* transmission. Release of genetically engineered *Alcaligenes* could pose environmental risks: (1) *Alcaligenes* species have been associated with human diseases such as pneumonia in immunocompromised persons. (2) Potential horizontal gene transfer to other microbes of the environmental consortium could pose risks above and beyond scenarios involving release of unmodified organisms. Field application of the paratransgenic strategy for control of Pierce's disease would therefore require additional measures to contain human contact with *Alcaligenes* and minimize gene spread in the environment. This proposal introduces the concept of second

generation paratransgenics in which advanced material engineering at the nano- and micro-scale is used to target release of engineered microbes and restrict gene transcription to highly specific sites of pathogen residence within the arthropod itself, with the aim of greatly reducing the risk of foreign gene release into the environment.

OBJECTIVES: The overall aim of this three-year project is to develop a second generation paratransgenic approach to delivery of engineered *Alcaligenes xylosoxidans denitrificans* (AXD) to *Homalodisca vitripennis*, the arthropod vector of *Xylella fastidiosa*, causative agent of Pierce's disease of grapevines. This proposal will focus on strategies to contain unwanted environmental spread of recombinant bacteria and potential horizontal gene transfer (HGT) of foreign DNA. Specific Aim 1: To develop a synthetic alginate-chitosan microsphere for encapsulation and field delivery of engineered AXD to *H. vitripennis* Specific Aim 2: To establish efficacy of a synthetic alginate-chitosan microsphere in preventing environmental escape of recombinant AXD and horizontal gene transfer of foreign DNA to environmental bacteria Specific Aim 3: To establish in closed-cage settings the efficacy of a Resin-Microsphere System (RMS) in delivery of engineered AXD to the cibarial region of *H. vitripennis* **TIMELINE, DELIVERABLES** Month 6 Formulation of Alginate-Chitosan Microsphere; Bacterial stability; pH-gated release of bacteria/re-design with new polymers as needed ALC Microsphere with desired characteristics Month 18 Containment of R-AXD by ALC Microsphere Prevention of HGT and transfer within earthworm; ALC Microsphere with containment properties Month 24 Development of Resin Microsphere System Microsphere stability, water impermeability and coating of plants/ re-design as needed RMS that permits microsphere viability and water impermeability Month 36 Simulated field trial with *H. vitripennis* and grape plants Delivery of R-AXD via RMS; colonization of cibarium; no release into rhizosphere; Proof-of-concept in simulated conditions of targeted release of R-AXD **Expected Outcomes and Alternative Approaches:** We expect the alginate microspheres to provide partial protection of the R-AXD. Whereas, freezing temperatures will cause death of most control AXD, we expect a statistically significant increase in survival of the microsphere-encased population. Similar results are expected for the trials involving extremes of high UV light and aridity. We expect the alginate matrix to prevent HGT in either direction. Comparison of recombinant events between the experimental (alginate microspheres) and control (liquid co-incubation) groups should reveal a statistically significant difference that validates the hypothesis. Again, we expect that ALC microspheres will prevent release of R-AXD into the gut of the earthworm and secondary sequelae such as HGT within gut microbial consortia. There should be a very significant difference in free R-AXD in the gut lumen of experimental versus control worms. In the absence of high fluid flux, ingested ALC microspheres are expected to remain closed with no leakage of R-AXD into the gut of the worm. If the RMS performs in a similar fashion to CRUZIGARD, we expect stability of the ALC microspheres and water impermeability of the matrix itself. Since the R-AXD bacteria are stabilized within the microspheres, it is possible that they will remain viable for the entire duration of 6 months.

APPROACH: We will develop an alginate-chitosan microsphere for encapsulation of genetically modified AXD stabilized at an acidic pH of 3.7 and gated to open under high fluid flux and pH change (greater than 7.5). The associated flow of fluid at a neutral pH through the

mouthparts and anterior gut of the insect will serve as the gating mechanism that causes swelling of the alginate microspheres and release of recombinant AXD (R-AXD). Experiment 1: Synthesis and characterization of Alginate-Chitosan (ALC) microspheres. Alginate microcapsules will be synthesized and loaded. Experiment 2: Containment of AXD within ALC microspheres R-AXD will be encapsulated. Containment of R-AXD will be verified by 3 methods: (1) Fluorescence microscopy (2) Serial washes of ALC microspheres (3) Washed spheres will be immersed in fluid that approximates xylem (pH =7.5) Experiment 3: Microsphere function under extreme environmental conditions Alginate microspheres containing R-AXD will be subjected to simulated conditions of extreme environmental stress: heat, light, aridity. Experiment 4: Containment of bacteria and genetic material in the setting of microbial consortia. Populations of R-AXD contained within alginate microspheres will be exposed to microbes commonly found in soil consortia to determine the extent of horizontal gene transfer. (2) We will evaluate possible HGT from donor bacteria of the rhizosphere to recipient AXD contained in alginate microspheres. Experiment 5: Containment of recombinant AXD upon ingestion of microspheres by the earthworm, *L. terrestris*. We will evaluate the barrier functions of the alginate-chitosan microsphere in the gut of *L. terrestris*. Experiment 6: Development of a water impermeable Resin Microsphere System (RMS) for field application. In this set of experiments, we will design a RMS for containment of ALC microspheres that will (1) provide a barrier against rain, (2) permit stability of ALC microspheres, (3) permit coating of shoots of grape vines where *H. vitripennis* is likely to feed, and (4) permit probing and release of ALC microspheres during the initial phase of penetration of the shoot by *H. vitripennis*. Experiment 7: Delivery of R-AXD to the cibarium of *H. vitripennis*. For this experiment we will use adult *H. vitripennis* collected from citrus orchards at the Agricultural Operations at UC Riverside. This 3-year program will develop new tools for the delivery of engineered symbiotic bacteria in a paratransgenic system. Robust methods for containment of genetically engineered microorganisms are possible given recent developments in nano-scale material engineering and controlled release. We propose to adapt these technologies to the prevention of arthropod-borne infectious diseases while minimizing risk of transgene delivery. Several other potential applications are already under development in the Durvasula lab related to delivery of engineered symbiotic bacteria to triatomine bugs and larval stages of phlebotomine sand flies. We expect to develop these strategies toward control of other arthropod-borne agricultural diseases and continue to expand the armamentarium against these devastating global scourges.

PROGRESS: 2010/09 TO 2011/08

OUTPUTS: Current outputs for USDA 2010-33120-21852 include: MICROENCAPSULATION OF TRANSGENIC BACILLUS SUBTILIS WITHIN CHITOSAN-COATED ALGINATE MICROSPHERES, American Society of Tropical Medicine and Hygiene, Philadelphia PA, 2011, (Poster presentation). Initial results regarding encapsulation of engineered bacteria were presented via oral poster session to interested parties at the ASTMH annual meeting in Philadelphia. PARATRANSGENIC APPROACHES TO ARTHROPOD-BORNE DISEASE - ACT 1, 1st BIODESERT CONSORTIUM ON BACTERIAL SYMBIOSIS, Tunis Tunisia (Invited Seminar). In this plenary seminar, Dr. Durvasula (PI) provided a comprehensive overview of paratransgenic strategies directed at control of vector-borne disease. Molecular tools and environmental release strategies under development in the Durvasula Lab were presented to an international audience

of scientists and students from US, Europe and North Africa (Morocco, Algeria, Tunisia and Libya). PARATRANGENIC APPROACHES TO ARTHROPOD-BORNE DISEASE - ACT 2 (RISK ASSESSMENT AND MITIGATION), 1st Biodesert Consortium on Bacterial Symbiosis, Tunis Tunisia, 2011 (Invited Seminar. In this plenary seminar, Adam Forshaw (Research Fellow) introduced the idea of microbial encapsulation for risk mitigation of paratransgenic vector control strategies. Preliminary results of early encapsulation experiments were presented to an international consortium of scientists expert in the field of insect-microbial symbiosis. MICROENCAPSULATION AS A STRATEGY FOR IMPLEMENTATION AND ENVIRONMENTAL SAFE-GUARDING OF A PARATRANGENIC APPROACH TO CONTROL OF VECTOR-BORNE DISEASES (Provisional Patent, # 2011-057-02) This provisional patent outlines microencapsulation strategies that can be used to deliver genetically engineered bacteria to disease-transmitting arthropods under field conditions. Composition of microspheres and methods of encapsulation and monitoring of microorganisms are described in detail. PARTICIPANTS: Dr. Ravi Durvasula, MD (Principal Investigator) Chief of Medicine and Acting ACOS for Research New Mexico VA Health Care System Vice Chairman for VA Affairs Professor of Medicine Director, Center for Global Health University of New Mexico School of Medicine Albuquerque, NM-87131. Dr. Thomas A. Miller, PhD (Collaborator) Professor of Entomology Entomology Department University of California Riverside, CA-92521. Adam Forshaw Howard Hughes Medical Inst. Research Fellow University of New Mexico School of Medicine Albuquerque, NM-87131. Arinder K. Arora Graduate Student, Biology Department University of New Mexico, Albuquerque NM-87131. Sudeep Kumar Post Doctoral Fellow Department of Internal Medicine University of New Mexico, Albuquerque NM-87131. John A. Shelnett (Consultant) Distinguished Member of Technical Staff Sandia National Laboratories Albuquerque, NM 87106. TARGET AUDIENCES: Whereas the proposal defines a narrow audience of agriculturalists working on Pierce's Disease and regulatory authorities chartered with oversight of transgenic technologies, the impact of this project, when fully realized, extends the target audience to: 1) Entomologists with interest in control of vector-borne disease 2) Agriculturalists in several international communities (Europe, Asia, Africa and North America) who are developing field-based strategies to enhance output 3) Public health officials tasked with development of novel methods for control of vector-borne human diseases 4) Biotechnologists with a focus on environmental applications 5) Medical personnel, such as infectious disease specialists, with particular focus on vector-borne disease. 6) Global health officials with interest in global food security and agricultural output (i.e. the Gates Foundation). PROJECT MODIFICATIONS: Whereas the initial proposal describes a paratransgenic method involving *Alcaligenes* species, concerns remained about the potential adverse impact on human health, especially in immunocompromised individuals. Recent finding from the Miller Lab suggest that *P. agglomerans* plays a symbiotic role in *H. vitripennis*. Since the *Pantoea* strain E-325 has been approved for environmental dissemination and poses no threat to human and animal populations, we elected to move forward with this organism instead of the *Alcaligenes* species described in the original proposal. We do not believe that this constitutes a major change or scientific departure from our original proposal or intended project. Furthermore, we have developed robust molecular tools for the transformation of *Pantoea* strains, and have been able to make significant progress with the stated aims of this proposal.

IMPACT: 2010/09 TO 2011/08

Our current work in microencapsulation of genetically modified bacteria for use in paratransgenic insect control has yielded several very promising results: 1) We have successfully developed an alginate-based calcium/barium hybrid microparticle with a Ca²⁺-ALG core and Ba²⁺-ALG shell that successfully contains "payload" microbes in liquid solution while maintaining microbe viability until the particles are dissolved. 2) We have successfully demonstrated "tuning" the particle release profiles, wherein microbes can diffuse from the particle at variable rates depending on cross-linker composition (Ba²⁺:Ca²⁺). 3) We have successfully encapsulated *P. agglomerans* and *B. subtilis* and have demonstrated their rescue from the microcapsule as well as enhanced viability within the capsule. 4) We have validated the premise that alginate microspheres would provide resistance to environmental insult such as ultraviolet radiation by demonstrating increased survival of encapsulated microbes when exposed to high energy UVC radiation. 5) We are currently developing a novel encapsulation formula incorporating a high-carbon dye which should further increase this UVC resistance. 6) The initial proposal aims to develop microencapsulation technology for paratransgenic control of Pierce's disease. The progress to date with encapsulated *P. agglomerans* advances the goal and we anticipate contained field trials in 2012 to evaluate efficacy, both in terms of payload delivery and risk mitigation, i.e., decreased non-target spread of payload. 7) Other applications of this approach greatly increase the global impact of this project. Microencapsulation is being developed for use in desert communities through the collaboration with the BioDesert Program (European Union collaboration). Furthermore, microencapsulation is being developed to drive paratransgenic strategies directed at arthropod vectors of human disease, such as sand flies (visceral leishmaniasis) and kissing bugs (Chagas disease). Current work on the prevention of horizontal gene transfer (HGT) within the rhizosphere from our engineered microbes has yielded similarly promising results: 1) All attempts at forcing HGT (a very low probability event in nature) of our engineered DNA plasmid to *Pseudomonas fluorescens* and native *P. agglomerans* in liquid media, soil and other HGT-promoting environments have failed. This is encouraging since it suggests that the engineered DNA is unsuitable for bacterial uptake except under very, very specific laboratory conditions and thus lowers the likelihood for environmental contamination. We are currently investigating several other microbes to confirm this hypothesis. 2) Preliminary results demonstrate that *P. agglomerans* is viable in the earthworm gut as well as the soil. We are currently investigating whether any possible HGT occurs within these systems. 3) We are preparing studies on HGT prevention utilizing alginate microspheres, which act as physical barriers containing engineered DNA from entering the rhizosphere, thereby even further decreasing the likelihood of environmental contamination.

PUBLICATIONS (not previously reported): 2010/09 TO 2011/08

1. Arora, A.K., Durvasula, R., and Miller T.A. 2012. Distinguishing between two closely related strains of *Xylella fastidiosa*. (in prep)
2. Forshaw, A.P., Miller, T.A., Arora A.K. and Durvasula, R., 2010. Microencapsulation of engineered microbes in paratransgenic control strategies. (in prep)

CHARACTERIZATION FOR FIELD-EVOLVED RESISTANCE FOR RISK ASSESSMENT OF CURRENT Bt CROP REGULATIONS

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Award Number: 2010-33522-21700

NON-TECHNICAL SUMMARY: Transgenic crops that produce Cry (from Crystal) toxins from the common soil bacterium *Bacillus thuringiensis* (Bt) are widely used for insect pest control due to their effectivity, increased yields, and low environmental impact. Increasing adoption of Bt crop technology represents an increased risk for development of insect resistance to Bt crops. Current regulatory mandates that attempt to reduce the risk of resistance evolution were developed based on models and data obtained from laboratory-selected insects. However, in order to develop a more efficient regulatory framework to minimize the risks of resistance to Bt crops, we must understand how insects may develop resistance in the field. The main goal of our project is to characterize resistance in a field-selected strain of the Fall armyworm (*Spodoptera frugiperda*), the first pest to develop resistance to transgenic Bt corn resulting in withdrawal of a Bt corn variety from the market in the location where resistance was detected. In our project, we will determine the levels of resistance against Bt corn and individual Bt toxins in these insects and determine how this resistance is transmitted. We will also determine if resistant insects display any reduced fecundity or affected development when compared to susceptible insects. The molecular mechanism involved in resistance to Bt toxins in our *S. frugiperda* strain will also be identified, which will advance the identification of resistance genes to target during development of resistance monitoring methods. Significant outcomes from our research include the determination of resistance and cross-resistance patterns, stability of resistance, fitness costs linked to resistance, and the identification of mechanisms resulting in field-evolved resistance to Bt corn in *S. frugiperda*. We anticipate that this information will allow regulators to evaluate and identify risks associated with current Bt-resistance management mandates. This process is crucial to enhance current regulatory framework aimed at delaying development of field resistance to transgenic Bt crops and to design efficient Bt resistance monitoring strategies.

OBJECTIVES: Evolution of insect resistance to transgenic crops expressing *Bacillus thuringiensis* (Bt) toxins is one of the main risks associated with the increased use of this technology. The current regulatory framework is based on models and scientific data obtained from laboratory-developed resistant insect strains. Data from field-developed resistant insects would be crucial to assess risks of current regulatory protocols. In this regard, the goal of our project is to characterize field-evolved resistance to Bt corn in the Fall armyworm (*Spodoptera frugiperda*). The rationale for using *S. frugiperda* as model for our research is that it is the first documented insect species to develop field-evolved resistance resulting in withdrawal of a Bt crop from the market (Bt corn expressing Cry1Fa in Puerto Rico). We have isolated a Bt-resistant strain of *S. frugiperda* from field individuals captured in Puerto Rico. Using this strain

as biological model, we analyze three aspects of Bt resistance that directly affect current regulations to delay evolution of resistance to Bt crops. First, we will determine levels of resistance and cross-resistance to Bt corn or purified Bt toxins currently expressed in Bt crops and alternative toxins that are active against *S. frugiperda*. Since recessive genetic transmission of resistance is key to current Bt crop regulations, we will also use backcrossings and bioassays to characterize transmission of the resistance trait in our Bt-resistant strain of *S. frugiperda*. Second, since fitness costs directly affect viability of homozygous resistant, and potentially heterozygous larvae, we will test the existence of fitness costs associated with resistance to Bt corn in our *S. frugiperda* strain by comparing development and fecundity in resistant and backcrossed populations with susceptible *S. frugiperda*. Knowledge on the molecular mechanisms resulting in resistance to Bt toxins is crucial to the identification of genes involved in resistance, which allows development of alternative control tactics as well as resistance monitoring methods. Consequently, as third objective we will characterize the resistance mechanism/s in our *S. frugiperda* strain. Due to the high levels of resistance observed for this strain, we will first test for alterations in Bt toxin binding, followed by examination of alternative potential resistance mechanisms, including alterations in Bt toxin processing and enhanced defensive state in resistant larvae. We expect to provide regulators with information on resistance and cross-resistance patterns, stability of resistance, fitness costs linked to resistance, and resistance mechanisms resulting in field-evolved resistance to Bt corn in *S. frugiperda*. This information will allow for evaluations to identify risks associated with current Bt-resistance management mandates. This process is crucial to enhance current regulatory framework aimed at delaying the risk of development of field resistance to transgenic Bt crops and to design efficient Bt resistance monitoring strategies.

APPROACH: In our first objective we will use bioassays with Bt corn and purified Bt toxins that are currently expressed in transgenic Bt crops (Cry1Fa, Cry1Ab, Cry1Ac, Cry2Ab, and Vip3A), as well as alternative Cry toxins that are active against *S. frugiperda* (Cry1Ca and Cry1Da), to determine levels of resistance and cross-resistance in our Bt-resistant strain of *S. frugiperda*. To examine the genetic transmission of the resistant trait we will use bioassays with susceptible, resistant, and backcrosses. For our second objective, we will monitor and compare development and fecundity parameters of resistant and backcrossed populations with susceptible *S. frugiperda* growing on non-transgenic corn or artificial diet. These studies will allow determination of any potential fitness costs associated with resistance to Bt corn in our *S. frugiperda* strain. In our third objective, we will characterize the resistance mechanism in our *S. frugiperda* strain. We will focus initially on potential alterations in Bt toxin binding to midgut brush border membrane vesicles (BBMV) from larvae of the resistant larvae compared to vesicles from susceptible and the F1 generation from backcrosses. In these assays, we will use Cry1Fa and toxins that share receptors with this toxin (Cry1Ac and Cry1Ba) to test for alteration of binding to specific binding sites in the BBMV. Ligand blots will also be used to further test alterations in toxin binding in resistant insects. If no binding differences are identified, we will compare Cry1Fa toxin processing and midgut regeneration in the larval gut of susceptible and resistant *S. frugiperda* larvae to identify potential alternative resistance mechanisms.

PROGRESS: 2010/09 TO 2013/02

Target Audience: Our research was designed to cause a change in knowledge on field-evolved insect resistance to transgenic crops to lead a change in actions by regulatory agencies in the US and abroad that are involved in evaluating risk assessment for current and novel transgenic crops expressing Cry toxins from *Bacillus thuringiensis* (Bt). Information from our project is of interest to researchers from national and international research groups from government, academic, and industry backgrounds. Summaries of our project goals and outputs have also been disseminated through popular press, including Farm World, Southeastern Farm Press, and Tennessee Land Life and Science. Audiences for these publications include general public and farmers.

Changes/Problems: One of the objectives (Aim 1) in the project included bioassays with transgenic turf (*Paspalum*) expressing the cry1Fa gene from *Bacillus thuringiensis*. However, we were unable to perform these bioassays due to issues with intellectual property protection affecting this transgenic variety. As a substitute, we included purified CryBb toxin in our bioassays. In Aim 3 we substituted Cry1Ab for Cry1Da in the binding assays since Cry1Ab is more informative (shares binding sites with Cry1Fa, the toxin produced by transgenic maize) and it is produced by some transgenic maize varieties. Binding assays with Cry1Ea (Aim 3) were not performed since we found in bioassays (Aim 1) that this toxin was inactive against our insects.

What opportunities for training and professional development has the project provided? The project has allowed the PI to interact with national and international collaborators, resulting in collaborative projects formalized in presentations and publications. The project has provided the backbone and the support for the PhD degree of a student. This student (Siva Jakka) was responsible for performing the proposed research under direct supervision by the PI. Through the completion of the project objectives the student was trained in research methods used worldwide to study resistance in insects. The student also was responsible for developing insect bioassay and culturing protocols to attain the project goals. This student has successfully completed his Thesis and attained his PhD degree. An undergraduate student was supported part-time by the project to maintain the insect colonies and perform research supervised by the graduate student and PI. Over time, the student was trained in analysis of fitness costs and bioassay methods. Due to her direct involvement in the research, this student will be included as co-author in two publications in preparation.

How have the results been disseminated to communities of interest? During the project performance period we have disseminated data from our research through presentations at US universities, biotech industry, and annual meetings of national (Entomological Society of America) and international (Society for Invertebrate Pathology) scientific societies. Through these presentations we contacted a research group in Japan with some shared research interests, which resulted in a collaborative project and a peer-reviewed publication. In addition, we were invited to share our data at symposia on “strategies to monitor and reduce resistance to *Bacillus thuringiensis* among targeted insects” (Biotechnology Summit celebrated in Merida, Mexico), and the 2nd International Symposium on Insect Midgut Biology (Guangzhou, China). Audiences at these meetings included students (undergraduate and graduate), research staff and laboratory directors from national and international research groups, as well as researchers from biopesticide and biotechnology companies. Summaries of our project goals and outputs were also disseminated through popular press, including Farm World, Southeastern Farm Press, and Tennessee Land Life and Science. Audiences for these

publications include general public and farmers. What do you plan to do during the next reporting period to accomplish the goals? Nothing Reported

IMPACT: 2010/09 TO 2013/02

What was accomplished under these goals? From eggs originally collected in Puerto Rico we developed a strain of *Spodoptera frugiperda* (fall armyworm) displaying high levels of field-evolved resistance to transgenic corn expressing Cry1Fa toxin from *Bacillus thuringiensis*. After continued selection of with transgenic Bt corn we characterized the genetics of resistance, tested the existence of fitness costs associated with resistance, determined patterns of cross-resistance to Bt pesticides, and identified the mechanism of resistance in this *S. frugiperda* strain. Completion of this work has resulted in relevant changes in knowledge that we expect will result in changes in actions related to risks assessment for Bt crops to increase their durability. We found resistance to Cry1Fa corn in this strain to be transmitted as an autosomal recessive trait. In bioassays comparing susceptible and resistant larvae we detected cross-resistance to transgenic corn producing Cry1Ab toxin and to purified Cry1Ac toxin. In contrast, we did not detect differences in susceptibility between the strains when using commercial Bt products or Bt toxins that do not share binding sites with Cry1A toxins in *S. frugiperda*. Based on this cross-resistance phenotype we determined toxin binding to midgut brush border membrane vesicles from susceptible and resistant larvae and found that Cry1Fa and Cry1A binding was dramatically reduced in resistant insects. We tested expression levels of multiple proposed Cry1A toxin receptors and found dramatically reduced levels of an alkaline phosphatase and an aminopeptidase in the midgut of resistant larvae. During these tests, we also collaborated with a Japanese group to identify an ABCC2 transporter protein as a functional receptor for Cry1A and Cry1Fa toxins, yet we did not find associations between this receptor and resistance to Cry1Fa corn in our *S. frugiperda* strain. An important component of resistance evolution that is not directly considered in current Insect Resistance Management (IRM) plans to delay evolution of resistance to Bt crops is the potential existence of fitness costs in resistant insects. These fitness costs can help select against homozygous resistant and even heterozygous insects in the field. Among the diverse studied biological parameters, we only detected delayed larval development in resistant compared to susceptible insects when reared on artificial diet, corn, cotton, or soybean plant material. Moreover, resistance was stable even after more than 10 generations without selection, which may help explain why field-evolved resistance in Puerto Rico remains stable years after Bt corn was withdrawn from the regional market. Our data represent the first molecular characterization of a mechanism responsible for field-evolved resistance to a transgenic Bt crop and support assumptions of current IRM models. Specific geographic and environmental conditions may help explain evolution of field resistance to Bt corn in Puerto Rico. Our identification of the resistance mechanism opens new avenues of research to develop sensitive and economical high-throughput resistance monitoring approaches.

PUBLICATIONS (not previously reported): 2010/09 TO 2013/02

1. Type: Journal Articles Status: Published Year Published: 2013 Citation: Tanaka, S., Miyamoto, K., Noda, H., Jurat-Fuentes, J. L., Endo, H., and R. Sato (2013) "The ATP-binding cassette transporter subfamily C member 2 in *Bombyx mori* larvae is a functional receptor for Cry toxins from *Bacillus thuringiensis*?" *FEBS J.* 280(8): 1782-1794.

2. Type: Conference Papers and Presentations Status: Published Year Published: 2011 Citation: Jurat-Fuentes, J.L., Perera, O.P., Castagnola, A., Oppert, C., and Jakka, S. "Proteomics and genomics of resistance to Bt toxins and transgenic Bt crops? Invited speaker, "State-of-the-art Molecular Research of Global Interest" symposium, 59th Annual Meeting of the Entomological Society of America, Reno (NV), November 2011
3. Type: Conference Papers and Presentations Status: Published Year Published: 2012 Citation: Jurat-Fuentes, J.L. "Characterization of resistance mechanisms to Dipel and transgenic Bt corn to improve resistance monitoring? Invites speaker, "Strategies to Monitor and Reduce Resistance to Bacillus thuringiensis among Targeted Insects" symposium, organized by International Center for Genetic Engineering and Biotechnology and Universidad de Nuevo Leon, Merida (Mexico), March 2012.
4. Type: Theses/Dissertations Status: Accepted Year Published: 2013 Citation: Jakka, S. R. K. 2013. Characterization of field evolved resistance to transgenic Cry1Fa maize in Spodoptera frugiperda. Ph.D. Thesis.
5. Type: Conference Papers and Presentations Status: Published Year Published: 2011 Citation: Poster presentation presented by S. Jakka; co-authors: C. Oppert, C. Blanco, M. Portilla, and J. L. Jurat-Fuentes. Title: 'Characterization of field-evolved resistance to transgenic Bt corn in Spodoptera frugiperda'; Date: August 2011 at the 44th Annual Meeting of the Society for Invertebrate Pathology
6. Type: Conference Papers and Presentations Status: Published Year Published: 2012 Citation: Poster presentation by Jakka, S.R.K. Co-authors: Knight, V.R., and J.L. Jurat-Fuentes, 'Testing for cross-resistance in Spodoptera frugiperda (J. E. Smith) with field-evolved level resistance to Bt maize', November 2012, Annual Meeting Entomological Society of America, Knoxville, TN.
7. Type: Conference Papers and Presentations Status: Published Year Published: 2012 Citation: Poster presentation by Gong, L. Co-authors: Jakka, S.V.R., Hu, M.Y., and Jurat-Fuentes, J.L. 'Characterization of the mechanism responsible for field-evolved resistance to Bt corn expressing Cry1Fa toxin in Spodoptera frugiperda from Puerto Rico', September 2012, 2nd International Symposium on Insect Midgut Biology, Guangzhou, China.
8. Type: Conference Papers and Presentations Status: Published Year Published: 2012 Citation: Oral presentation by Jurat-Fuentes, J.L. Co-authors: Jakka, S.V.R., and Gong, L. 'Mechanism of field-evolved resistance to transgenic Bt corn in Spodoptera frugiperda', August 2012, Annual Meeting Society for Invertebrate Pathology, Buenos Aires, Argentina.
9. Type: Conference Papers and Presentations Status: Published Year Published: 2012 Citation: Oral presentation by Jakka, S.V.R. Co-authors: Oppert, C., Blanco, C., Portilla, M., and Jurat-Fuentes, J.L. 'Characterization of field-evolved resistance to transgenic Bt corn in Spodoptera frugiperda', August 2012, Annual Meeting Society for Invertebrate Pathology, Buenos Aires, Argentina
10. Type: Conference Papers and Presentations Status: Other Year Published: 2012 Citation: Jurat-Fuentes, J.L., 'Resistance mechanisms to Bt corn? Invited speaker at Nanjing Agricultural University; Nanjing, China. September 2012
11. Type: Conference Papers and Presentations Status: Other Year Published: 2012 Citation: Jurat-Fuentes, J.L., Research on Bt mode of action and resistance?, invited speaker at the Institute for Vegetables and Flowers, Chinese Academy of Agricultural Sciences; Beijing, China, September 2012

12. Type: Conference Papers and Presentations Status: Other Year Published: 2012 Citation: Jurat-Fuentes, J.L., ?Plant protection and biofuels: a view from the bug side?, Invited speaker at the monthly Tennessee Plant Research Center Colloquium, Knoxville (TN), February 2012.
13. Type: Journal Articles Status: Other Year Published: 2013 Citation: Jakka, S. R. K., Gong, L., Sheets, J., Hasler, J., Blanco, C. and Juan L. Jurat-Fuentes ?Field-evolved resistance to Bt corn in *Spodoptera frugiperda* is associated with reduced toxin binding? In preparation for submission
14. Type: Journal Articles Status: Other Year Published: 2013 Citation: Jakka, S. R. K., Knight, V., and J. L. Jurat-Fuentes ?Lack of fitness costs in *Spodoptera frugiperda* with field-evolved resistance to Bt corn?, In preparation for submission
15. Type: Journal Articles Status: Other Year Published: 2013 Citation: Jakka, S. R. K., Knight, V., and J. L. Jurat-Fuentes ?Testing for cross-resistance in *Spodoptera frugiperda* (J. E. Smith) with field-evolved resistance to Bt corn? In preparation for submission

REDUCING ROOT SUCKERING OF TRANSGENIC POPLAR

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Award Number: 2010-33522-21697

NON-TECHNICAL SUMMARY: Poplar is one of the most important forest, bioenergy and pulping crops in the US and the world. Transgenic technology offers a powerful tool to improve productivity and quality of poplar trees. However, root suckering is very common in poplar and extremely difficult to manage. Poplar suckers help spread transgenes and transgenic plants, and also contribute to the invasiveness of some poplar species, which creates additional environmental concerns for transgenic poplars. No molecular containment technologies that can reduce root sucker-mediated spread of transgenes and transgenic progenies have been developed. We propose to develop a molecular tool to repress root sucker development and to evaluate its effectiveness in poplar. The proposed technology may help reduce environmental concerns and problems associated with root sucker-mediated spread of transgenes or transgenic progenies from poplar plants.

OBJECTIVES: Objective 1) Construction of root sucker repressing genes. Objective 2) Production of transgenic poplar plants using the sucker repressing genes. Objective 3) Evaluation of effectiveness of the root sucker repressing genes in poplar. Objective 4) Characterization of growth and development patterns of the transgenic poplar that express the root sucker repressing genes.

APPROACH: We will use a root specific gene promoter to control expression of a set of genes that may repress root suckering of poplar plants. We will use tobacco and *Populus tremula* X *P. alba* as a model plant to test the effectiveness of the proposed root sucker repressing genes. We will characterize root suckering potential, biomass production and other changes in growth and developmental patterns, if any, observed in the transgenic plants.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: We have constructed all proposed genes and used these genes to transform tobacco plants. We have produced more than 60 independent transgenic tobacco lines for each construct. We have also started transformation of poplar plants. **PARTICIPANTS:** Nothing significant to report during this reporting period. **TARGET AUDIENCES:** Nothing significant to report during this reporting period. **PROJECT MODIFICATIONS:** Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

Data regarding the effectiveness of the proposed technology has not been obtained at this time but we have made significant progress. Successful development of a root sucker repressing gene

technology could reduce gene flow problems due to undesirable spread of root suckers from crop plants such as poplar.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

1. Ye, X. V. Busov, N. Zhao, R. Meilan, L. M. McDonnell, H. D. Coleman, S. D. Mansfield, F. Chen, Y. Li, and Z-M Cheng (2011): Transgenic Populus trees for forest products, bioenergy, and functional genomics. *Critical Reviews in Plant Sciences*. 30: 415-434.
2. Li, Y and Duan, H (2011): Molecular approaches for transgene containment and their potential applications in horticultural crops. In: *Transgenic horticultural crops: challenges and opportunities*. Mou. B. and Scorza R. (ed.) CRC Press. 289-299.
3. Kausch A, Hague J, Oliver M, Li Y, Daniell H, Mascia P, Watrud L, and C. Neal Stewart, Jr. (2010): Transgenic biofuel feedstocks and strategies for biocontainment. *Biofuels*. 1(1), 163-176.

CROP-WILD GENE FLOW IN SORGHUM AND RELATIVE FITNESS OF THE SHATTERCANE X SORGHUM F2 POPULATION

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Award Number: 2010-33522-21658

NON-TECHNICAL SUMMARY: Grain sorghum is an important food and feed crop throughout the world. The reduced digestibility of sorghum seed relative to other grains makes it a less efficient resource, even though it is highly adapted to growth in semiarid environments common to Africa, India, and the Southern and Western Great Plains of the USA. There has been considerable interest in modifying the quality traits of grain sorghum using transgenic technology to enhance its nutritional value to both humans and animals raised for human consumption. Development of sorghums with improved digestibility traits is highly desirable, but using transgenic technology to accomplish this is implicitly risky because there are several related species capable of interbreeding with sorghum, and it is not clear whether those traits will make weedy relatives even weedier. There has been no research conducted to determine how likely these traits will be transferred (gene flow) to the weedy relative called shattercane, or if they were transferred, how they would affect subsequent generations of the weed (potential weediness). Lack of such knowledge is an important problem because it limits our fundamental understanding of gene transfer and potential hybridization between grain sorghum and shattercane. This limits our ability to assess the potential risks of introducing genetically modified grain sorghum into US agroecosystems. The proposed research will provide regulators the information needed to make science based decisions about several issues that may arise as a result of deploying genetically modified sorghum. Specifically, the proposed research will provide the baseline research to predict the probability of pollen-mediated gene flow from grain sorghum to shattercane and the potential for sorghum genes (traits) to become stable in the wild population. Our pollen-mediated gene flow results also will provide valuable information for identifying the appropriate isolation distance for managing the genetic purity of hybrid and elite inbred lines of grain sorghum. Finally, our results will be useful for developing good stewardship practices to minimize the escape of herbicide tolerant sorghums currently being developed using non-transgenic technologies.

OBJECTIVES: The long-range goal of this research is to develop best management practices to maintain the utility and value of genetically modified grain sorghum and minimize the risk of transgene escape to wild shattercane populations. The objectives of the proposed research, which is the next step toward attaining that goal, is to predict the potential for pollen-mediated gene flow from genetically modified grain sorghum to shattercane and to assess the fitness of the shattercane x grain sorghum F2 population relative to shattercane. The central hypothesis is that several domestication traits of grain sorghum (e. g. lack of dormancy) may reduce the fitness of shattercane x sorghum F2 populations and increase the mean escape time of a transgene. Coupled with management practices that minimize pollen transfer, it may be possible to

minimize transgene escape to shattercane. This hypothesis was formulated on the basis of our preliminary research conducted to quantify 1) several fitness components of shattercane x grain sorghum F1 hybrids and their parents, and 2) the rate of outcrossing between grain sorghum and shattercane in situ, and on published literature describing the key factors controlling pollen-mediated gene flow and transgene escape. The rationale for the proposed research is that an understanding of pollen-mediated gene flow from non-transgenic grain sorghum to wild shattercane populations in situ will provide the background data needed to assess the risk of pollen-mediated gene flow from genetically modified grain sorghum to wild shattercane. Results of this research also will provide valuable science-based information useful for identifying management practices that could minimize the biological risk of deploying transgenic sorghum. The proposed research will further existing knowledge of characteristics, rates, and methods of gene transfer from genetically modified sorghum to wild relatives and addresses questions such as, "how likely will a transgene from grain sorghum escape to the wild shattercane population" and "what is the probability distribution of escape time of a transgene" We plan to accomplish the objective of this proposal by pursuing the following specific objectives: 1. Quantify the synchrony of flowering of multiple genotypes of grain sorghum and shattercane in situ, 2. Quantify duration of pollen viability and its settling velocity in several grain sorghum hybrids, 3. Quantify the fitness of the shattercane x grain sorghum F2 population relative to the wild type shattercane, 4. Utilize results from the above and our prior research in models to predict pollen-mediated gene flow and the probability distribution of escape time. It is our expectation that results will be useful to Federal regulatory agencies in making science-based decisions about introducing genetically modified grain sorghum in the USA and for agronomists developing management strategies to maximize the value and longevity of genetically modified grain sorghums.

APPROACH: We propose to conduct four experiments and develop a model of the pollen-mediated gene flow from sorghum to shattercane. Abridged details of these activities follow: Exp 1. Field experiments will be conducted to quantify the proportion of shattercane seeds that will pollinate in sync with grain sorghum. Six shattercane populations and three sorghum hybrids differing in maturity will be sown (the sorghum at three planting dates) and exposed to tillage or no till treatments. Ten plants from each treatment combination will be marked and followed at regular intervals to determine the start and end time of pollination. Results will be used to quantify the overlap in pollination, the duration of pollination, and synchrony of first day, peak day, and pollination period. Exp 2. Laboratory experiments will be conducted to quantify duration of pollen viability of both shattercane and sorghum. Pollen from plants in exp 1 will be collected and exposed to either shade or sunlight for periods ranging from 1 min to 6 hours, after which pollen viability and germination will be quantified. These results will then be compared to calculated pollen germination based on the literature. Exp 3. The length of time a wind dispersed pollen grain remains aloft is determined in part by the gravitational settling velocity of the grains in still air. Settling velocity (V_s) will be estimated using Stokes law and compared to an empirical measure of settling velocity obtained using a settling tower. Pollen will be collected from plants used in experiment 1. Exp 4. Field experiments will be conducted to quantify the fitness of the shattercane x grain sorghum F2 population relative to the shattercane parent populations. Crosses between the six shattercane populations and three sorghum hybrids will be

made in the greenhouse and the F1 seeds and shattercane populations will be grown to maturity in the field, selfed, and seed collected. Seeds from each F1 hybrid (F2 seeds) will then be sown and seedling emergence, growth and development, and seed production will be measured the following year. The F2 fitness will then be compared to that of its shattercane parent and the proportion of offspring produced will be used to calculate the relative fitness of the F2 population (s' , the ratio of expected offspring of the F2 to the expected offspring of the shattercane parent). Modeling Gene Flow. Results obtained from Experiments 1-3 will be used to parameterize a published model and predict pollen-mediated gene flow from a source grain sorghum population to shattercane plants. The model will then be evaluated by comparing predicted gene flow to observed outcrossing rates observed in our preliminary research. Mean escape time and probability distribution of escape based on possible transgene selection coefficients and leakage parameters will be modeled using a range of realistic values for the number of wild type individuals present within and at various distances from a source population. These results could then be used to identify the optimal isolation distance based on a predetermined level of risk.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: Grain sorghum is an important food and feed crop throughout the world. The reduced digestibility of sorghum seed relative to other grains makes it a less efficient resource, even though it is highly adapted to growth in semiarid environments common to Africa, India, and the Southern and Western Great Plains of the USA. There has been considerable interest in modifying the quality traits of grain sorghum using transgenic technology to enhance its nutritional value to both humans and animals raised for human consumption. Using transgenic technology in sorghums is implicitly risky because there are several related species capable of interbreeding with sorghum, and it is not clear whether introduced traits will make weedy relatives even weedier. There has been no research conducted to determine how likely these traits will be transferred (gene flow) to the weedy relative, shattercane, or if they were transferred, how they would affect subsequent generations of the weed (potential weediness). Lack of such knowledge is an important problem because it limits our ability to assess the potential risks of introducing genetically modified grain sorghum into US agroecosystems. This research will provide regulators the information needed to make science based decisions about several issues that may arise as a result of deploying genetically modified sorghum. Specifically, the research will provide the baseline research to predict the probability of pollen-mediated gene flow from grain sorghum to shattercane and the potential for sorghum genes (traits) to become stable in the wild population. Our pollen-mediated gene flow results also will provide valuable information for identifying the appropriate isolation distance for managing the genetic purity of hybrid and elite inbred lines of grain sorghum. Finally, our results will be useful for developing good stewardship practices to minimize the escape of herbicide tolerant sorghums currently being developed using non-transgenic technologies. Our specific objectives were to: 1. Quantify the synchrony of flowering of multiple genotypes of grain sorghum and shattercane in situ, 2. Quantify duration of pollen viability and its settling velocity in several grain sorghum hybrids, 3. Quantify the fitness of the shattercane x grain sorghum F2 population relative to the wild type shattercane, 4. Utilize results from the above and our prior research in models to predict pollen-mediated gene flow and the probability distribution of escape time. To date, we are concluding

the second year of field experiments at two locations to quantify the synchrony of flowering of six populations of shattercane growing with three sorghum hybrids planted at three different sowing dates. Results of this experiment will be published in the refereed literature. 2012 was the first year of a field experiment to evaluate the fitness of a shattercane x sorghum F2 population relative to the parent lines. Results of these experiments have been and will be presented at annual professional meetings in 2012. **PARTICIPANTS:** Professor Lindquist is the Project Director for this work. Dr. Bernards has left the University of Nebraska and will only participate peripherally. Mr. Jared Schmidt is conducting this research as part of his PhD program in Agronomy at UNL. Dr. Pedersen is providing support in crossing shattercane x sorghum in the greenhouse. Dr. Lorenz will provide quantitative genetics support. Mr. Darren Binder is a Research Technologist providing field research support for this project. **TARGET AUDIENCES:** Our target audience will be policy makers making decisions about regulated sorghums and farmers in the Great Plains and world-wide that could benefit from improved sorghum production practices. Several policy makers from USDA, USEPA, USFDA and other organizations were in attendance at the BRAG project directors meeting where our initial results were presented in June, 2012. **PROJECT MODIFICATIONS:** Due to the difficulty in making sufficient crosses using hand emasculation, only one shattercane population and one sorghum hybrid were used to create the shattercane x sorghum F2 population used in the 2012 fitness research.

IMPACT: 2011/09 TO 2012/08

Previous research showed that synchronously flowering shattercane can receive pollen from sorghum at a distance of at least 200 m from a relatively small sized source population. Proportion of florets within a shattercane panicle receiving sorghum pollen was as high as 2.46% at that distance. Within the source, rate of hybridization was up to 16%. Both wind speed and direction were important predictors of the probability of hybridization, especially at distances greater than 60 m from the source. Results of our flowering synchrony research suggest that shattercane and sorghum flowering are nearly completely synchronous regardless of shattercane population, sorghum maturity group, or planting date.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

1. Lindquist, J. L. 2012. Pollen-mediated gene flow in sorghums: Implications for herbicide resistant sorghum. Proceedings of the 6th International Weed Science Congress, Hangzhou, Peoples Republic of China.
2. Schmidt, J. J., J. F. Pedersen, M. L. Bernards, A. J. Lorenz. J. L. Lindquist. 2012. Flowering synchrony of grain sorghum and shattercane. Proceedings of the Weed Science Society of America. 52:249.
3. Schmidt, J. J., J. F. Pedersen, M. L. Bernards, J. Lindquist and A. J. Lorenz. 2011. Synchrony of flowering in grain sorghum and shattercane. Proceedings of the North Central Weed Science Society. 66:98.

ENVIRONMENTAL RISK ASSESSMENT OF PERENNIAL GRASSES GENETICALLY ENGINEERED FOR ABIOTIC STRESS TOLERANCE

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Award Number: 2010-33522-21656

NON-TECHNICAL SUMMARY: Perennial grasses, such as turfgrass, forages and biofuel plants are essential components of agriculture and environment. Genetic improvement of perennials using biotechnology approaches is important to the turfgrass industry, biofuel production and the environment. However, considering the invasiveness of perennial grasses, the use of genetically modified cultivars, despite of the implementation of gene containment scheme, raises additional concerns about the potential greater ecological impact relative to the more domesticated food crops. This project focuses on genetic engineering of creeping bentgrass for enhanced abiotic stress resistance. Field trial study will be conducted to evaluate the direct or indirect effects of the transgenes on host biochemistry, physiology, and consequently the potential impacts on non-target organisms and environmental and ecological systems. Data obtained will help better evaluate environmental safety and appropriate use of transgenes to facilitate perennial species plantation in stressful environments, and provide guidelines to genetically engineer perennials for other target traits of interest.

OBJECTIVES: The major objective for this project is to evaluate environmental risk associated with engineered abiotic stress tolerance in transgenic perennial turfgrass using metabolic, vacuolar, cytoplasmic and chloroplast protein genes. As tolerance to one stress often has effects on multiple stress responses and other physiological phenotypes, we expect that the overall secondary and non-target effects of the introduced foreign genes in perennial plants could be better evaluated. In this project, we will: 1) Study how the manipulation of various molecular mechanisms would impact plant response to adverse environmental conditions. 2) Evaluate how transgene expression would impact weediness and invasiveness of genetically engineered perennials. 3) Assess how transgenic perennials with enhanced abiotic stress tolerance would interact with weeds and forages. 4) Study how stress tolerant transgenic perennials would impact non-target soil chemistry.

APPROACH: We will first produce transgenic creeping bentgrass plants with modified metabolism, vacuolar proton pump, sumoylation and electron transfer pathway. Transgenic plants expressing different transgenes will be evaluated for their performance in response to various environmental stresses. Field study will then be conducted to evaluate, under stressed and non-stressed conditions, whether alteration occurs in transgenic perennials for fitness traits. The field trial study will also investigate how transgenic plants develop under stressed and non-stressed conditions when grown together with weeds as well as legume forages. This will address potential hazards from abiotic stress tolerant transgenic grasses such as the ability to increase density locally, invade new habitats, and/or become a weed management problem or positively

impact weed management. Finally, the effects of transgenic perennials with enhanced abiotic stress tolerance on soil chemistry will be assessed to study potential impact of transgenic perennials on sustainable agriculture and environmental protection.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: The objective of this research project is to evaluate environmental risk associated with engineered abiotic stress tolerance in transgenic perennial turfgrass by manipulating various molecular pathways. We have completed all the chimeric gene constructs and introduced them into creeping bentgrass. Transgenic plants expressing different transgenes have been generated and analyzed in greenhouse to evaluate their performance under environmental adversities. We have also propagated enough plant materials and initiated field trial study under an USDA-APHIS permit. **PARTICIPANTS:** Hong Luo (PI), Haibo Liu (Co-PI) have been responsible for organizing and supervising all the research activities for this project. Zhigang Li (a postdoctoral Research Associate) and Qian Hu (Research Associate) were in charge of conducting chimeric gene construction, plant tissue culture, plant transformation and transgenic analysis. Graduate students, Man Zhou, Shane Reighard, Shuangrong Yuan and Ning Yuan were involved in the experiments of this research project, and received training in molecular and cell biology, plant tissue culture and plant genetic transformation. **TARGET AUDIENCES:** Plant biotechnologists in academia, industry and regulatory agencies, turfgrass breeders, turfgrass industry. **Project modifications:** No modification to this project. **PROJECT MODIFICATIONS:** Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

Since we just initiated our field trial study, we do not have data for environmental risk assessment on the transgenic turfgrass generated at this time. Improved environmental stress resistance in crops is one of the major goals in agricultural biotechnology. Environmental safety evaluation of transgenic turfgrass engineered for enhanced abiotic stress tolerance will facilitate plantation of transgenic perennial species in stressful environments, and provide guidelines to genetically engineer perennials for other target traits of interest.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

1. Li, Z., Li, D., Hu, Q., Zhou, M., Luo, H. (2012) Manipulation of miR156 genes leads to modified plant morphology and enhanced abiotic stress tolerance in transgenic creeping bentgrass (*Agrostis stolonifera* L.). American Society of Plant Biologists Southern Section 2012 Meeting, March 3-5, Myrtle Beach, SC.
2. Zhou M., Hu Q., Li Z., Chen C.-F., and Luo H. (2011) Expression of a novel antimicrobial peptide penaeidin4-1 in creeping bentgrass (*Agrostis stolonifera* L.) enhances plant fungal disease resistance. PLoS ONE 6(9):e24677.
3. Li, Z., Hu, Q., Zhou, M., Vandenbrink, J., Li, D., Menchyk, N., Reighard, S.R., Norris, A., Liu, H., Sun, D., Luo, H. (2012) Heterologous expression of OsSIZ1, a rice SUMO E3 ligase enhances broad abiotic stress tolerance in transgenic creeping bentgrass. Plant Biotechnology Biotechnology. In press.
4. Li, Z., Zhou, M., Hu, Q., Reighard, S., Yuan, S., Yuan, N., San, B., Li, D., Jia, H. and Luo, H. (2012) Manipulating expression of tonoplast transporters. In: Plant Salt Tolerance: Methods and

Protocols, *Methods in Molecular Biology*, vol. 913, DOI 10. 1007/978-1-61779-986-0 24, Shabala, S. Cuin, T.A. (eds), Springer Science + Business Media, LLC, pp359-369.

5. Luo, H. (2012) An integrated dual recombination system for use in producing clean transgenic plants. 2012 World Congress on In Vitro Biology. June 3-7, Bellevue, WA. Oral presentation.

WHOLE ANIMAL ASSESSMENT OF UNINTENDED EFFECTS OF FOREIGN GENE PRODUCTS ON HOST AND NON-TARGET ORGANISMS

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Award Number: 2010-33522-21776

NON-TECHNICAL SUMMARY: Genetic engineering involves the introduction of a new gene into an animal to cause a direct and specific effect; however, any undesired and indirect effects of the gene and its product must be identified in order to assess the safety of the animals and food products resulting from them, something that has not been well studied at the whole animal level. We hypothesize that the use of animal-wide scale analyses to monitor the types of bacteria and metabolites present will give insight into the impact on the animal when a foreign gene product is present or consumed. We will use milk from genetically engineered goats that produce lysozyme, a protein normally found in human milk that slows or stops the growth of bacteria. This milk is intended for human use to promote the growth of beneficial bacteria in the gut upon consumption and thereby improve health. The specific objectives of the work are 1) to determine if the presence of lysozyme can alter the bacteria present in the udder of the animals and thereby affect the biochemistry of the animal by altering metabolite levels; and 2) to determine if the nature of the changes in the bacterial populations in the gut after consumption of the milk by non-target organisms (young goats being raised on the milk) alters the animals' biochemistry. Based on the desired function of lysozyme, we expect to be able to relate changes in bacteria to any indirect effects on the whole animal. This type of information will be useful to government regulators in assessing the safety of genetically engineered food animals and their products.

OBJECTIVES: We generated transgenic dairy goats expressing the antimicrobial human lysozyme (HLZ) in their milk, one of the main antimicrobial components of human milk that promotes the health of the newborn by protecting against infection and establishing a healthy gut microbiota, with the goal of improving milk safety and intestinal health upon consumption of the milk. Characterization of these animals and their milk has demonstrated that our transgene is functioning as intended resulting in milk with bacteriostatic properties that can modulate intestinal bacterial populations. In this, or most other transgenic livestock applications, systemic unintended effects of a transgene product have not been well documented. This particular transgene product, lysozyme-rich milk, is ultimately intended for human consumption, however, presence of the transgene product may alter normal bacterial populations and thus metabolites in both the host (lactating dams) and in non-target organisms (kid goats being raised on the milk). We hypothesize that global-scale analysis methods such as microbial and metabolite profiling methods will be useful in assessing direct and indirect effects of transgene products at the whole animal level. Two specific aims will be addressed using our HLZ transgenic goats to determine 1) if the metabolic profile of the host and non-target animals consuming the milk is altered and 2) the nature of the microbial populations in the udder of the host organism and along the length

of the intestinal tract of non-target organisms. This type of information will be useful in evaluating the scope of pleiotropic effects of transgenes and their products on an entire organism.

APPROACH: We will determine if the presence of our transgene product (human lysozyme (HLZ) milk) alters host (lactating dams) and non-target organism (kid goats being raised on HLZ milk) biochemistry by conducting metabolite and microbial profiling experiments. Metabolite profiling will be carried out on serum of lactating transgenic and control does at peak lactation and that collected from kid goats being raised on HLZ or control goat milk at three time points; 2 months of age, at weaning and one month post-weaning to determine if any changes vary with age and after the consumption of milk has stopped. Metabolomic profiling will be carried out using gas and liquid chromatography followed by mass spectroscopy for identification and quantification of metabolites. Based on the function of our transgene, impact at the level of the intestine of our non-target organisms and in the udder of our host organisms will be investigated. Intestinal segments will be taken from kid goats consuming HLZ or control milk at two months of age to assess the physical state of the intestine by histology and to collect intestinal contents for microbial profiling. DNA will be extracted from the intestinal sample and feces from kid goats collected at weaning and one month post-weaning as well as from the milk from lactating transgenic and control does for microbial profiling analysis and for PCR analysis to detect any possible transgene transfer to gut or milk bacteria. Microbial profiling will be carried out using 16s rRNA gene sequencing to determine the types and relative frequency of bacterial species present in all samples. Specific changes in bacterial populations and any potential transgene transfer will be correlated with the metabolite data. This approach will produce information on the global nature of indirect effects in the whole animal and will be useful for regulators in assessing the scope of transgene impact.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: This work is designed to gather data regarding unintended consequences of transgene expression at the whole animal level by conducting global types of analyses to determine if a transgene product can impact aspects of an animals' physiology other than those intended by the function of the particular transgene. A line of transgenic goats expressing lysozyme (a key antimicrobial component of human milk but lacking in the milk of dairy animals) in their milk has been generated with the intent of using the milk to improve human health. These transgenic goats will be used to investigate the physiology of the host (serum metabolite profile and milk microbial profile of lactating does) and non-target organisms (serum metabolite profile, fecal microbial composition and state of the intestine of kid goats consuming the milk at 2 months of age and at one month post-weaning). Work on both Aims 1 (metabolomics) and 2 (microbial profiling and gut histology) has been conducted over the past year. Serum samples from lactating does at peak lactation and kid goats at both time points has been collected and submitted for metabolic profiling analysis (Aim 1). A feeding trial was carried out with goat kids and intestinal samples collected for the one month post-weaning time point (Aim 2). Histological sections from the two month time point have been analyzed and fecal and gut contents from both time points have been prepared for microbial analysis. Milk samples from transgenic and control does at early, peak and late lactation have been collected, prepared and analyzed via 16S rRNA gene sequencing (Aim 2). A summary of work completed to date

was disseminated at the 2012 BRAG Project Director Meeting. Two graduate students were mentored and two undergraduate interns were trained as they all contributed work to this project. PARTICIPANTS: Elizabeth Maga- PI: Directed experiments, processed and analyzed gut content and fecal samples from two month time point using 16S rRNA gene sequencing. Aided in analysis of 16S rRNA gene sequencing data from lactating does and validating Illumina-based sequencing for microbial profile analysis. Jim Murray- CoPI: Trained graduate student to conduct histological analysis of goat intestinal segments. Elizabeth McInnis- Graduate Student: Collected, processed and analyzed microbial profiling data for goats in early, mid and late lactation. In final stages of thesis preparation reporting the results of the microbial profile of transgenic and control goat milk at different stages of lactation. Merritt Clark- Graduate Student: Ran feeding trial for kid goats consuming milk from birth to post-weaning. Collected and is processing samples for histological analysis and microbial profiling of the intestine for the post-weaning time point and analyzed histological sections from the two month time point. Brianna Benedetto and Priscilla Mendoza- Undergraduate interns: Provided care for and fed transgenic and control milk to goat kids on the feeding trial and assisted with sample collection. TARGET AUDIENCES: Government Regulators Scientists in the field of transgenic research PROJECT MODIFICATIONS: Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

Lysozyme acts as an antimicrobial by cleaving a specific link in the cell wall of bacteria resulting in the slowing of bacterial growth or in bacterial death due to cell lysis. Therefore, the expression of lysozyme in the mammary gland is expected to have an impact on the number and type of bacteria present in the milk. This was indeed found to be true using 16S rRNA gene sequencing to profile the bacterial population of raw milk at early, mid and late lactation. The microbial community of milk from transgenic animals changed over time in a similar fashion to the community found in control milk with some subtle differences at each time point. There was only one significant difference among phyla, with control milk having significantly more Proteobacteria at early lactation, and several differences at the class, order and family levels at all stages of lactation. All differences identified were in bacteria normally found in milk and the environment and thus pose little risk. This data will be related to metabolic profile of the lactating host to determine if these differences at the level of the udder are having an impact on the whole animal. In terms of effects on non-target organisms, when culture-dependent tests were used, kid goats fed milk from lysozyme transgenic goats until two months of age had higher levels of coliforms and *E. coli* in the duodenum and ileum than did kids fed milk from non-transgenic control goats, however, the differences were not statistically different. When the culture-independent method of 16S rRNA gene sequencing of clone libraries was used, this difference was significant. Kid goats fed milk from transgenic goats had significantly more Proteobacteria (the phylum housing coliforms and *E. coli*) and Verrucomicrobia than control-fed goats. The number of sequences generated with clone libraries was limiting due to cost, hence the use of next generation sequencing technologies (Illumina platform) was investigated and was found to give a more comprehensive microbial profile by allowing for a greater number of reads to be generated for a comparable cost to the sequencing of clone libraries. The milk from transgenic goats was also able to improve the state of the intestine by increasing gut surface area as kid goats fed milk from transgenic animals for two months had significantly taller villi and

deeper crypts in the ileum. Overall, the expression of the transgene is impacting the host (microbial populations of milk) and non-target organism (kid goat intestine) in a beneficial or neutral manner. Work to date demonstrates that global scale analysis methods can detect differences between transgenic and control samples at the whole animal level and may be useful in determining if any unintended consequences of transgene expression exist.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

McInnis, E. A., Murray, J. D. and Maga, E. A. 2012. Profiling of bacteria from the milk of human lysozyme transgenic goats and control goats at early lactation. *Transgenic Res.* 21:921-922.

A MODE OF PLASTID INHERITANCE SURVEY IN MEDICAGO TRUNCATULA ASSESSING THE FEASIBILITY OF TRANSGENE CONTAINMENT IN PLASTIDS

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Award Number: 2010-33522-21672

NON-TECHNICAL SUMMARY: *Medicago sativa* (alfalfa) is the fourth largest U.S. crop by land area, with a history of gene-flow problems. Plastid localization is an effective tool for transgene containment in crops with strict maternal plastid inheritance, but its use depends on the availability of tools for plastid transformation. *M. sativa* transmits plastids maternally, paternally or biparentally. Currently no information exists detailing the genes responsible for plastid inheritance nor has plastid transformation been reported in alfalfa. As the first step towards engineering plastid inheritance in alfalfa, we propose a survey of natural variability of plastid inheritance in *Medicago truncatula*, a related diploid model species, since the tetraploid *M. sativa* is not suitable for genetic analyses. We have already demonstrated maternal and biparental plastid inheritance in a small subset of *M. truncatula* accessions. We now propose to extend the screen to 30 lines to encompass a broader spectrum of inheritance modes. In addition, we shall determine the dominant-recessive relationships between the maternal and biparental modes of inheritance as well as the number of genes in a segregating population. Scoring of plastid types in the progeny will be based on polymorphic DNA markers and on visual markers introduced by plastid transformation. Information on plastid inheritance phenotypes will be useful for association mapping of the relevant genes and for selecting or engineering alfalfa for strict maternal plastid inheritance. Plastid transformation in alfalfa developed through this project will enable better containment through plastid localization of transgenes, therefore meeting the objectives of the BRAG risk management research.

OBJECTIVES: Specific objectives of the research for the two-year period are the following: (1) Screening the 30 deeply sequenced *Medicago* lines for mode of plastid inheritance; (2) Testing mode of plastid inheritance in the Borung x Paraggio F1 hybrids and in the F2 generation; (3) Marking the plastids of the Jemalong 2HA line with a transgenic aurea spectinomycin resistance gene; (4) Testing the mode of plastid inheritance in reciprocal crosses within the Jemalong 2HA line using the aurea spectinomycin resistance gene as marker.

APPROACH: (1) Screening the 30 deeply sequenced *Medicago* lines for mode of plastid inheritance. The 30 deeply sequenced accessions of the HapMap project will be obtained and used as pollen parents in crosses with the A17 mother. The mode of plastid inheritance (maternal, biparental, paternal) will be evaluated in the seed progeny by ptDNA markers. (2) Testing the mode of plastid inheritance in the Borung x Paraggio F1 hybrids and in the F2 generation. When crossed with ecotype A17 as a pollen parent, ecotype Borung exhibited a predominantly biparental mode of plastid inheritance and Paraggio a predominantly maternal mode of plastid inheritance. The objective will be to determine the dominant-recessive

relationships between the maternal and biparental modes of inheritance as well as the number of genes involved in crosses with the A17 mother. (3) Marking the plastids of the Jemalong 2HA line with a transgenic aurea spectinomycin resistance gene. Medicago-specific plastid transformation vectors will be constructed with the aurea aadA gene as selective marker. The vector DNA will be introduced into leaves by the biolistic process, and the spectinomycin resistance marker will be used to recover transplastomic clones. The transplastomic leaves will have a golden-yellow color due to post-transcriptional interference with chlorophyll accumulations. (4) Testing the mode of plastid inheritance in reciprocal crosses within the Jemalong 2HA line using the aurea spectinomycin resistance gene as marker. Transplastomic aurea 2HA lines will be crossed with the wild type, and the mode of plastid transmission determined in reciprocal crosses using the leaf color as marker.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: ACTIVITIES:

(1) The target for the genetic analyses of plastid inheritance is Paraggio, a cultivar, which showed low levels of plastid transmission as pollen parent. We are now constructing suitable F2 populations and tester lines to test segregation for the maternal and biparental modes of plastid inheritance.

(2) The objective of plastid transformation in *Medicago truncatula* is to provide genetic and phenotypic markers in crosses. The plastid transformation vectors carry the aurea-aadA gene and highly-expressed GFP genes. The transforming DNA is introduced into alfalfa leaves by the biolistic protocol and putative transplastomic clones are selected by spectinomycin resistance. Plastid transformation is in progress.

EVENTS: PI in 2012 gave lectures and disseminated information at the following events and locations:

(1) Plant and Animal Genome Conference XX, San Diego, CA, January 14-18, 2012, Plant Organellar Genetics Workshop organizer and speaker

(2) 7th Annual Tripartite Meeting of the Americas between the University of Sao Paulo, Rutgers University, and The Ohio State University, Columbus, Ohio, May 31 - June 2, 2012; speaker

(3) Annual Project Director's Meeting for Biotechnology Risk Assessment Grants (BRAG) Program, Riverdale, MD, June 5-6, 2012; speaker PARTICIPANTS: Mr. Csanad Gurdon, a Ph.D. student in the Plant Biology Graduate Program developed PCR-based plastid DNA markers and assembled and annotated plastid genomes from Illumina and SOLiD reads of total genomic DNA. He now conducts the plastid inheritance study in crosses with cultivar Paraggio, and will screen additional *Medicago truncatula* lines for plastid inheritance.

Dr. Tarinee Tungsuchat-Huang, a Research Associate, took on the supervision of the *Medicago truncatula* plastid transformation project.

Two undergraduate genetics major students contribute to plastid transformation in *Medicago truncatula*. Ms. Megan Radler successfully tested novel GFP-expressing spectinomycin resistance markers in tobacco. These genes are already used for plastid transformation in *Medicago*. Ms. Kanak Verma, another undergraduate student, is testing variant kanamycin resistance genes in tobacco, to be used in future plastid transformation vectors.

Collaborations

PI is collaborating with Dr. Brigitta Dudas at the Agricultural Biotechnology Center, Godollo, Hungary, on a parallel study of plastid inheritance in *Medicago sativa*. The Maliga laboratory shared information that facilitated the development of plastid markers in *Medicago sativa* and provided plastid transformation vectors to obtain genetically marked lines. In Godollo, plastid-encoded spectinomycin resistance mutations were isolated in *Medicago sativa*, which modify restriction endonuclease cleavage sites. These AFLP markers are now utilized as universal DNA markers in a survey on the mode of plastid inheritance. The collaborative effort yielded one joint publication. **TARGET AUDIENCES:** (1) The target audience is plant biotechnologists and plant breeders, particularly those interested in forage crops and transgene containment.

(2) Regulatory agencies, who should be aware of the genetic diversity available in *Medicago* for the mode of plastid inheritance. **PROJECT MODIFICATIONS:** Originally, we were planning to screen the 30 deeply sequenced *Medicago truncatula* lines for their mode of plastid inheritance. We now decided to screen *Medicago truncatula* ssp. *tricycla* lines instead, in the hope of capturing more diversity. The seed was obtained from Dr. Stephanie L. Greene, USDA, ARS National Temperate Forage Legume Germplasm Resources Unit, Prosser, WA.

IMPACT: 2011/09 TO 2012/08

The complete plastid genome sequence of *Medicago truncatula* cultivars Borung, Jemalong 2HA and Paraggio has been deposited in GenBank.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

Maliga, P. (2012) Plastid transformation in flowering plants. In *Genomics of Chloroplasts and Mitochondria* (Bock, R. and Knoop, V. eds). Springer, pp. 393-414.

RECOMBINANT NEWCASTLE DISEASE VACCINES: RISK FOR RECOMBINATION, REVERSION TO VIRULENCE AND SPREAD IN NON-TARGET SPECIES

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Award Number: 2010-33522-21696

NON-TECHNICAL SUMMARY: Billions of poultry vaccines made with live genetically modified viruses are currently being used in China and Mexico. Concerns over their ability to recombine or swap genes with viruses in the environment, increasing their ability to cause disease, exist. We propose to study this topic in a controlled experimental environment. We hypothesize that by co-infecting hosts with the modified vaccine virus along with a wild type virus, we will show whether these vaccines are stable and safe or that recombination is possible. The objectives are to evaluate if 1) a host infected with a Newcastle disease virus (NDV) containing one avian influenza (AI) gene and a wild type AI virus can recombine 2) a modified NDV can revert from a vaccine virus to a virulent virus and 3) these modified vaccine viruses can infect and transmit in wild bird species they were not intended for. We expect 1) to be able to produce recombination between a modified NDV containing an AI gene and a wild type AI virus, but do not expect that the occurrence of recombination will be common 2) that modified NDV without the AI gene will not revert to being a virulent NDV and 3) the non-target species will be infected with and be able to transmit these vaccines. These studies, which will optimize the possibility of producing dangerous viruses from genetically modified viruses and wild viruses, may determine regulatory rules concerning the licensing of these vaccines in the U.S.A and potentially other areas of the world.

OBJECTIVES: Evaluate three risks associated with recombinant Newcastle disease viruses being used as live vaccines in poultry and to provide data to regulatory agencies (Center for Veterinary Biologics) and researchers to allow them to consider whether this class of vaccine is safe and effective for use in the U.S. market.

APPROACH: We will use commercially available live vaccines from China or Mexico formulated with Newcastle disease viruses (NDV) containing the H5 hemagglutinin (HA) protein for avian influenza and/or NDV recombinants with H5 inserts made in our laboratory. We will utilize an NDV that has been modified by reverse genetics to include an attenuated hemagglutinin-neuraminidase (HN) or fusion (F) and HN genes from a virulent NDV. An established cell culture protocol that uses products from egg based studies will be performed to determine if 1) the avian influenza HA gene inserted in the NDV genome can recombine, by homologous or non-homologous recombination, with low pathogenic H5 and non H5 influenza viruses and 2) if the recombinant NDV (rNDV) containing an attenuated HN and/or F and HN genes from a virulent strain can revert back to a virulent virus. A wild type NDV, documented to have increased in virulence in nature in 1998, will be tested along side the rNDV. The protocol

uses 14-day-old specific pathogen free (SPF) embryonated chicken eggs (ECE) and favors the growth of virulent viruses in cell culture, avoiding having to make multiple passages of egg fluids. Any viruses that form plaques in cell culture without the addition of an extraneous protease potentially have an increase in virulence and will have the HN and F genes sequenced to compare with parent virus. Selected viruses will be evaluated in embryos and birds to define the change in virulence. To assess non-target species infection for specific aim three, the three most common wild avian species associated with poultry houses; pigeons, starlings, and house sparrows, will be tested experimentally with rNDV and rNDV-H5 used in the first two specific aims to determine susceptibility to infection and for the potential of the virus to transmit and change within these species. Selected viruses recovered after infection will be viewed in the same egg based study to evaluate virulence.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: Specific aim 1: Co-infection of 14 day-old (n=900) embryonating eggs with a recombinant LaSota vaccine virus containing the HA subtype H5 (rLaSota-AI/H5) gene of avian influenza virus and wild type avian influenza viruses of subtypes H5, H6 or H9 has been completed. The rLaSota-AI/H5 was co-infected with AIV subtype H5, H6 and H9 (300 eggs/co-infection). Allantoic fluid from 800 embryonating eggs that died between 24 and 72 h post-infection was collected and is currently being tested for cytopathic effects and plaque formation in cell culture. Specific aim 2: Assessing the potential of recombinant Newcastle disease viruses (attenuated fusion cleavage sites) to revert back to virulence in a host organism has also been completed. Nine hundred 14 day-old embryonating eggs were inoculated with five different Newcastle disease viruses (Wild type Lasota, rLaSota, rM, rZJ1-Lento, and Wild-type Australia). 106 allantoic fluid samples positive for virus from the inoculated eggs have been tested for cytopathic effects in cell culture. 70 of these 106 samples produced cytopathic effects. 20 of these 70 have been sequenced. The remaining viruses are currently being sequenced and the fusion cleavage site will be analyzed. Specific aim 3: Recombinant Newcastle disease virus vaccines are able to infect pigeons and spread to contact birds has been partially completed. Infection and transmission experiments in pigeons have been completed. Two groups of sparrows have been infected with two recombinant NDV vaccines. Oropharyngeal and cloacal swabs were collected from all birds on days 2, 4, 6, 8, and 10 pi and samples are stored at -80 degrees for further processing (virus isolation and titration). **PARTICIPANTS:** Nothing significant to report during this reporting period. **TARGET AUDIENCES:** Nothing significant to report during this reporting period. **PROJECT MODIFICATIONS:** Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

Samples for Specific Aim 1 are undergoing analysis. However, the high mortality rates observed in eggs co-infected with rNDV-LaSota and AIV of subtypes H5, H6 and H9 are likely resultant of AIV replication, as all three strains are known to cause embryo death. Future screening of allantoic fluids from these eggs in cultures of MDCK cells that do not support NDV infection without the addition of trypsin will discern if viruses with increased virulence is present. Of the 20 out of 70 samples that have sequenced for Specific Aim 2, no reversion to virulence or changes in the fusion cleavage site have been observed. The mortality rates observed in these 70

embryonating chicken eggs inoculated with rNDV vaccines are somewhat expected for low virulence NDV isolates. Screening of these allantoic fluids in cell cultures without addition of trypsin suggested potential increase in virulence; however, the definitive answer is obtained by sequencing of the F gene cleavage site. The remaining 50 samples will be evaluated. For Specific Aim 3, none of the pigeons showed clinical signs after being infected with the Newcastle disease strains, however, the birds shed virus and uninfected pigeons placed in contact with infected pigeons were infected and shed virus for two or more days. The sparrow samples are yet to be analyzed. These preliminary findings suggest that our system is suitable to assess the risk associated with recombinant NDV vaccines.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

No publications reported this period

A CRITICAL ASSESSMENT OF THE EFFECT OF CRY PROTEINS ON BENEFICIAL ARTHROPODS THROUGH THE USE OF CRY-RESISTANT HOSTS

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Award Number: 2010-33522-21772

NON-TECHNICAL SUMMARY: A long-term goal of our work is to provide science-based information to regulatory agencies on the environmental impacts of insect resistant genetically-modified (IRGM) plants on nontarget organisms, especially beneficial arthropods of important crop pests species. Without such information, regulatory agencies cannot ensure the environmental safety of the products they regulate and will be subjected to criticisms. Recent examples of studies showing negative environmental effects of IRGM plants have had tremendous negative effects on regulatory systems. For example, there are two recent publications purporting to show negative effects on nontarget natural enemies by insecticidal crystal (Cry) proteins derived from *Bacillus thuringiensis* (Bt) and expressed in IRGM plants. In both publications, we believe there were serious problems in the methodology that led to erroneous conclusions. These and other studies showing a negative effect failed to take host quality into account and separate direct and indirect effects of Cry proteins. Nevertheless such studies cause serious challenges to the regulatory authorization of Bt plants that could unnecessarily prevent or delay the environmental and economic benefits such plants can offer. We propose to conduct tests that will overcome common methodological problems in testing Cry proteins against nontarget natural enemies by using a unique resource, strains of pest insects that have evolved resistance to Cry proteins expressed in commercial Bt plants. In addition, we will also examine whether there are differential effects on nontarget organisms by the same Cry protein when it is expressed in different plants. Results from these tests will provide unambiguous information on the potential effects of these proteins on natural enemies and will expand the knowledge base for biosafety studies on nontarget organisms. The studies fit in well to the goals of the BRAG program by providing regulators with sound information that can be used to conduct risk analyses.

OBJECTIVES: A long-term goal of our work is to provide science-based information to regulatory agencies on the environmental impacts of insect resistant genetically-modified (IRGM) plants on nontarget organisms, especially beneficial arthropods of important crop pests species. Without such information, regulatory agencies cannot ensure the environmental safety of the products they regulate and will be subjected to criticisms. Recent examples of studies showing negative environmental effects of IRGM plants, although criticized for their design and methods, have had tremendous negative effects on regulatory systems. We propose to conduct tests that will overcome common methodological problems in testing Cry proteins against nontarget natural enemies by using a unique resource, strains of pest insects that have evolved resistance to Cry proteins expressed in commercial Bt plants. Results from these tests will provide unambiguous information on the potential effects of these proteins on natural enemies

and will greatly expand the knowledge base for biosafety studies on nontarget organisms. The objectives of our proposal are to determine effects on predators and parasitoids by Cry proteins that are expressed in commercialized Bt cotton and Bt corn varieties, and to determine whether the plant host can modify the effect. These objectives can be stated by the following null hypotheses. 1. Predators and parasitoids will be not be affected by Cry toxins their hosts have ingested when fed on commercialized Bt corn and Bt cotton. 2. Predators and parasitoids that feed on hosts that fed on a Bt expressing plant will not be differentially affected based on the plant species.

APPROACH: Our project is unique for the following reasons: 1) the use of four resistant strains of Lepidoptera and a multitude of diverse parasitoids and predators will allow us to draw more general conclusions about the risk of commercial Cry proteins to natural enemies than has been previously possible; 2) the Cry proteins used in our proposed studies represent the only lepidopteran-active Cry proteins that are on the market today that are expressed in commercial Bt plants. In addition to these two, our proposal is unique and appropriate to the goals of BRAG for another reason, i.e. we will test whether there are differences in the natural enemies reaction to a Cry protein when the same Cry protein is expressed in two very different species of plants (i.e. cotton and broccoli). This last item will provide a unique insight into the potential role of a plant's chemistry to modify a Cry protein's effect on the host and its natural enemy. This is especially relevant because it is expected that the currently expressed proteins in corn and cotton will be also utilized in other crops in the future. In our case, we will be working with the following systems: 1.Herculex I corn expressing Cry1F; Cry1F-resistant FAW and ECB. 2.Bollgard I cotton expressing Cry1Ac; Cry1Ac-resistant CL (CL-1) 3.Bollgard II cotton expressing Cry1Ac+Cry2Ab; Cry1Ac+Cry2Ab resistant CL (CL-2). 4.Cornell Cry1Ac broccoli; Cry1Ac-resistant CL (CL-1). The natural enemies will be selected for their importance on controlling lepidoptera on these crops.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: We have obtained and currently rear the following species of insects that are resistant to the respective Bt proteins: fall armyworm to Cry1F; European corn borer to Cry 1F; cabbage looper to Cry 1Ac and Cry2Ab. We also are rearing lacewings, ladybird beetles and a parasitoid. With these insects in place, we have completed one set of test showing that consumption of resistant fall armyworm does not harm ladybird beetle, thus showing the safety of Cry1F. A manuscript is being submitted by the end of the year. **PARTICIPANTS:** The following individuals in my program worked on this project: H. Collins, Tian Jun-Ce, Aki Seto. Other people who were involved in the project include Rick Hellmich and Steve Naranjo from USDA ARS, and J. Romeis from Agroscope in Switzerland. **TARGET AUDIENCES:** The main target audience are regulators who assess the safety of transgenic crops, and the scientific community who researchers these crops. **PROJECT MODIFICATIONS:** Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

Our tests demonstrate conclusively the safety of Cry1F to a predator and help to explain that some other studies had spurious results due to poor experimental design.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

A publication on fall armyworm and Cry1F proteins will be submitted by the end of 2011.

ASSESSING THE RISK OF EUROPEAN CORN BORER ADAPTATION TO TRANSGENIC MAIZE

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Award Number: 2010-33522-21673

NON-TECHNICAL SUMMARY: Estimation of resistance risk for transgenic plants expressing Bt proteins has been limited by the lack of well-characterized resistant colonies of target pest species. In this application, we describe two strains of European corn borer (ECB) with high levels of resistance to the toxins that are expressed by Bt corn hybrids. Specific research described in this proposal is designed to: 1) refine our understanding of the genetic basis of resistance; 2) identify diagnostic genetic markers that detect mutations linked to larval Cry1Ab and Cry1F resistance traits described above and which can be used for high-throughput screening of field populations; 3) more accurately assess fitness costs associated with resistance under more relevant ecological conditions. The rationale for the proposed research is that an understanding of resistance risk will promote the sustainability of transgenic corn expressing Bt proteins for European corn borer control. With combined expertise in the biology, ecology, genetics and management of European corn borer, the principal investigators are uniquely qualified to undertake the proposed study.

OBJECTIVES: Specific research described in this proposal is designed to: 1) refine our understanding of the genomic architecture of resistance and identify structural or gene expression changes that are linked to larval Cry1Ab and Cry1F resistance traits; 2) identify diagnostic molecular genetic markers that detect mutations linked to larval Cry1Ab and Cry1F resistance traits described above and which can be used for high-throughput screening of field populations; 3) more accurately assess fitness costs associated with resistance under more relevant ecological conditions.

APPROACH: Our existing AFLP-based map for pedigree FQ4 will be saturated with SNP markers we have already designed. Resulting fine mapping of the QTL for Cry1F on LG12 will allow assembly of a BAC-based physical map consisting of clones from our existing library. A combined SNP linkage and physical-map will similarly be applied to the identification and refining of QTL associated with Cry1Ab resistance traits. Refined maps will be used as a scaffold onto which to assign differentially-expressed genes identified in Objective 2 for predictions of co-segregation with the QTL. Pyrosequencing and assembly of a representative ECB midgut transcriptome will serve as a scaffold for identifying transcripts from short reads. Collect quantitative and qualitative gene expression information by Illumina G2 sequencing from individual full-sib progeny showing segregating Bt resistance phenotypes. Assemble Illumina G2 reads against long read EST scaffolds, predict quantitative gene expression differences that co-segregate with resistance traits, and predict putative SNPs with differentially expressed genes for

further fine mapping in Objective 1. Fitness costs arise in the absence of Bt toxin when individuals with resistance alleles have lower fitness than homozygous susceptible individuals. The evolution of resistance to Bt crops can be delayed, and in some cases prevented, by the presence of costs. Importantly, fitness costs often vary with ecological conditions, and accurate parameterization of costs requires making measurements under ecologically relevant conditions. Preliminary assessments of fitness costs have been completed. However, the identification of strains with field-derived resistance alleles presents a unique opportunity to measure fitness costs for strains likely to resemble Bt-resistant populations that may arise in the field, and to do so under ecologically relevant conditions by rearing insects on corn plants.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: OUTPUTS: Objective 1. Two pedigrees were established by crossing individual Cry1F resistant females with a susceptible male. Resulting F1s were subsequently used to generate 4 replicates of reciprocal backcrosses to the Cry1F resistant line, and resulting backcross progeny were phenotyped by exposure to a diagnostic Cry1F concentration. Backcross progeny were reared to early 5th instar on non-Bt diet, and midgut tissue dissected from at least 24 larvae from each the "resistant" and "susceptible" phenotypes from each family. Total RNA was extracted from midgut tissue for 4 backcross families and stored at -80 degrees C, and DNA was isolated from the remaining larval tissues and stored at -20 degrees C. DNA from all families have been genotyped using ~300 single nucleotide markers, and approximately 300 additional SNP markers are yet to be screened. Total RNA from two families was submitted for sequencing on the Illumina HiSeq at Iowa State University, and RNAseq analysis is pending. Objective 2. Transcriptome sequencing of midgut tissue from Cry1Ab and Cry1F resistant larvae has been completed using 454 and Illumina sequencing platforms. A hybrid assembly of sequencing data resulted in approximately 124,713 contigs with an average length of 984 bp. In addition, we have completed a replicated RNASeq experiment to compare transcript abundance from midgut tissue of Cry1F resistant and susceptible larvae that have been exposed to either Cry1F-treated or untreated diet. Results of the comparisons are still in progress but almost 4,000 genes have been identified with significant differences in expression. The transcriptomes developed from these efforts will be utilized as a template for expression QTL mapping of resistance traits described in objective 1. Objective 3. Estimating fitness costs. For fitness comparisons of Cry1F resistant and susceptible strains we have completed 5 generations of crossing, backcrossing and selection to obtain resistant and susceptible strains of similar genetic backgrounds. On plant survival of larvae resistant and susceptible to the Cry1F toxin were examined, using three different non-Bt maize lines at the V9-V10 stage (two of these lines are susceptible to European corn borer, and one has moderate resistance). Survival did not differ between resistant and susceptible larvae, indicating a lack of fitness costs for resistant larvae. This experiment was repeated using reproductive plants, and again, survival of resistant and susceptible larvae did not differ for any of the maize lines. Currently, resistant and susceptible larvae are developing on the three maize lines, and upon emergence of adults, survival and life history data will be collected to determine if fitness costs exist for resistant insects developing to adulthood on non-Bt maize plants. **PARTICIPANTS:** This proposal represents a collaborative effort among scientists from the University of Nebraska (PI Siegfried), Iowa State University (coPI Gassman), and USDA-ARS (coPI's Coates and Hellmich). Funding from this proposal

currently supports a post-doctoral associate at Iowa State and a senior research technician at the University of Nebraska. **TARGET AUDIENCES:** Nothing significant to report during this reporting period. **PROJECT MODIFICATIONS:** Because of the difficulty in maintaining the Cry1Ab resistant colony of *O. nubilalis* and in obtaining isogenic resistant and susceptible strains for genomic and fitness comparisons, we have focused our efforts on the Cry1F resistant strain.

IMPACT: 2011/09 TO 2012/08

We have now completed year two of a three year project are on track to complete nearly all the stated objectives which will provide answers to questions directly pertaining to the risk of resistance development to Bt proteins in the European corn borer (i.e., genetic architecture of resistance, resistance allele frequency, and fitness costs). The transcriptome sequencing efforts that have already been completed will allow us to characterize genetic markers for direct assay of Bt resistance in ECB populations which will assist in development of future diagnostic assays for resistance. The research will directly assist in the formulation of resistance management strategies for transgenic crops by providing relevant biological data that has previously been unavailable in formulating resistance management models.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

1. Khajuria, C., L.L. Buschman, M. Chen, B.D. Siegfried, and K.Y. Zhu. 2011. Identification of a novel aminopeptidase P-like gene (OnAPP) possibly involved in Bt toxicity and resistance in a major corn pest (*Ostrinia nubilalis*). PLoSONE 6(8):e23983.
2. Siegfried, B.D. and R.L. Hellmich. 2012. Understanding successful resistance management: The European corn borer and Bt corn in the United States. GM Crops and Foods doi.org/10.4161/gmcr.20715.

POLLEN-MEDIATED GENE FLOW IN SWITCHGRASS

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Award Number: 2010-39211-21699

NON-TECHNICAL SUMMARY: The incorporation of transgenic switchgrass into the U.S. portfolio of bioenergy feedstocks is arguably necessary to reach guidelines set forth in U.S. energy policies. Because there currently exist nontransgenic agronomic plantings and native populations of switchgrass in North America, and due to the possibly invasive character traits of switchgrass feedstock varieties, there are underlying risks involved with pollen-mediated spread of switchgrass transgenes. However, basic knowledge on pollen dispersal distributions and agronomic x wild relative hybrid fitness in switchgrass is lacking. Here, we propose to (1) estimate field-to-field pollen-mediated gene flow of switchgrass, (2) experimentally characterize the extent of pollen-mediated gene flow, and (3) experimentally assess fitness of hybrids of agronomic switchgrass and their wild relatives. Knowledge gained will help develop best management practices for future plantings of transgenic switchgrass, and will be useful to both regulators and conservation biologists. Our approach will also serve as a model for the incorporation of transgenic varieties of other bioenergy feedstocks into the landscape.

OBJECTIVES: Objective 1: Describe field-to-field pollen-mediated gene flow of switchgrass in non-transgenic commercial fields at the agronomic level. Objective 2: Experimentally describe the extent of pollen-mediated gene flow from non-transgenic or transgenic to non-transgenic plants. Objective 3: Experimentally describe the crossability and fitness of hybrids of agronomic switchgrass and their wild relatives.

APPROACH: We will approach these objectives by (1) using diagnostic molecular markers of a new improved nontransgenic variety of switchgrass to be planted in agronomic settings, (2) monitoring the dispersal of fluorescent marker genes from transgenic switchgrass in areas lacking native switchgrass, and (3) performing hybrid crosses of agronomic switchgrass with wild relatives and measuring fitness traits of offspring. The project we outline here focuses on furthering existing knowledge with respect to characteristics, rates, and methods of gene transfer that may occur between genetically-engineered plants and related wild and agricultural organisms.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: Sites for switchgrass pollen flow experiments have been identified, and work has been initiated. Pollen-distance study has been established in Oliver Springs, TN. Source plants (clones) and receptor plants (different clones) have been planted, and plants are firmly established. Pollen flow data are currently being collected (summer/fall 2012), and preliminary findings indicate that the red fluorescent protein (RFP) is expressed in shed pollen and is viewable with filtered microscopy techniques. The field-to-field pollen flow/hybridization

portion of the study has been established at two farm sites: Alcoa, TN and Vonore, TN. Leaf tissue has been collected from cv. Alamo and cv. EG1102 (improved Kanlow) individuals in representative fields. RNA and DNA has been extracted, and screening of potential diagnostic markers has begun. RAPDs, which are useful in distinguishing native switchgrass populations, are not capable of distinguishing between Alamo and Kanlow bulk samples. PARTICIPANTS: Not relevant to this project. TARGET AUDIENCES: Not relevant to this project. PROJECT MODIFICATIONS: Not relevant to this project.

IMPACT: 2011/09 TO 2012/08

Other markers, in particular SNPs, hold more promise, are currently being pursued via underway Illumina sequencing efforts, for which downstream bioinformatics work will commence in October 2012. Breeding studies have just been initiated; seeds from native switchgrass populations have been collected, as have seeds from agronomic cultivars, and seeds of a wild relative (*Panicum dichotomiflorum*) have been obtained from USDA ARS-GRIN. Targeted crosses between agronomic switchgrass cultivars and native congeners and conspecifics are currently underway, and resulting seeds will be collected in Fall 2012. Character traits of germinants will be compared in Spring 2013.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

Nageswara-Rao, M., C.N. Stewart, and C. Kwit. Accepted. 2012. Genetic diversity and structure of natural and agronomic switchgrass (*Panicum virgatum* L.) populations. *Genetic Resources and Crop Evolution*.

GENETIC CONTAINMENT OF PLANTS USING ZINC-FINGER NUCLEASES

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Award Number: 2010-33522-21736

NON-TECHNICAL SUMMARY: Gene flow from transgenic plants presents an important barrier to field research and commercial use. A major strategy for avoidance of this problem in forestry crops is genetic engineering of sexual sterility. However, current methods are too inefficient or likely to lead to reversion to fertility. We propose to overcome this problem with zinc-finger nucleases (ZFNs) that will produce null mutations in genes essential for sexual reproduction. We will impart sterility with ZFNs by targeting two genes, LEAFY (LFY) and AGAMOUS (AG) orthologs from poplar, known to be critical to fertility in most plant species. We will use the heat-inducible recombinase ParA and codA genes to remove the ZFNs and minimize chimerism, respectively. This work should provide the first test of ZFN approaches to genetic containment in any plant. The Strauss, Tzfira, and Thomson laboratories have conducted a number of preliminary studies and have the needed tools in place for completion of the project goals.

OBJECTIVES: 1. Collaborate with DOW Agrosiences to create and validate effective ZFNs. The PtLFY and PtAG genomic sequences were derived from the conserved DNA sequences of *P. trichocarpa* and the 717-1B4 and 353-53 poplar clones that we will use for transformation. 2. Develop a binary vector system for recombinase-mediated removal of the ZFNs. The basic design is to flank the independent ZFN expression cassettes with a heat-inducible ParA recombinase system and positive/negative selection marker fusion. 3. Create a binary construct that contain four ZFNs directed against both the AG and LFY genes. All four ZFNs will be cloned into the RCS-MCS site in the recombinase-based vector. 4. Regenerate ZFN-mutagenized transgenic poplars and screen them for mutations using high resolution melting (HRM). We will transform the two target poplar genotypes as has been done routinely in the Strauss laboratory for nearly two decades. However, instead of the normal callogenesis/selection period in the dark for the first three weeks, we will also heat shock the developing calli each day during the second half of this period. 5. Clone and sequence a sample of mutated loci. Prior to retransformation with the FT gene to induce early flowering, we will confirm putative biallelic mutants at one or more loci identified by HRM by cloning and sequencing. We will only screen enough regenerants to find biallelic mutants; the focus of this project is on assessing sterility from the gene mutants, not on determining mutation efficiency or optimizing mutagenesis. 6. Re-transform a sample of transgenic poplars that have biallelic mutations with an FT gene to induce precocious flowering. The Strauss laboratory has been employing the FT gene to accelerate flowering for several years. 7. Analyze the floral morphology of ZFN-mutated poplars. The Strauss laboratory will use standard dissecting microscopy and thin-sections and normal light microscopy to describe the nature of floral mutations observed.

APPROACH: 1. Collaborate with DOW Agrosiences to create and validate effective ZFNs. The sequencing is already done and a number of conserved ZFN-suitable target regions identified. These sequences will be the targets for design of two pairs of highly effective ZFNs using the Dow Agrosiences pipeline. 2. Develop a binary vector system for recombinase-mediated removal of the ZFNs. Located inside the recombinase recognition sites is the inducible recombinase gene, the positive/negative selectable marker and a unique RCS-MCS derived from pRCS2 for cloning of up to seven independent plant expression cassettes (discussed below). 3. Create a binary construct that contain four ZFNs directed against both the AG and LFY genes. The RC-MCS is derived from the pRCS2 series of vectors developed in coPI Tzfira's laboratory. The four ZFNs, each driven from a double 35S promoter and the 35S terminator, will be inserted into the RCS-MCS version of this vector by the Tzfira laboratory essentially as described. 4. Regenerate ZFN-mutagenized transgenic poplars and screen them for mutations using high resolution melting (HRM). The final protocol will be adjusted based on preliminary studies to begin prior to the grant period that will employ a GUS reporter version of the base recombinase vector. These studies will seek to identify the longest possible duration of pre-heat shock callus development that is feasible (to maximize ZFN action), but that can yield a majority of non-chimeric and GUS-negative shoots able to grow in the presence of 5-FOA. 5. Clone and sequence a sample of mutated loci. To facilitate cloning of PCR products that contain the ZFN target sites, the Tzfira laboratory has agreed to reproduce and distribute to all collaborators a TA-cloning vector suitable for blue/white colony screening. This method will reduce costs considerably compared to purchase of commercial TA-cloning kits, and increase speed of analysis. For each of the biallelic mutants, at least 7 independent colonies will be sequenced to provide a probability of 1% that a double mutation would be missed. 6. Re-transform a sample of transgenic poplars that have biallelic mutations with an FT gene to induce precocious flowering. Although clone 353-53 produces a higher frequency of normal appearing catkins and flowers than does 717-1B4, both of the target clones have given rise to single flowers and/or inflorescences. The laboratory is also working to produce a system for graft-induction of flowers produced on non-transgenic scions, though this has not been successful to date. Thus, we currently plan to retransform the ZFN-mutated transgenic poplars with our heat-inducible FT genes, which will produce flowering poplars within one year from co-cultivation. 7. Analyze the floral morphology of ZFN-mutated poplars. The effects of complete knock out should be strong and dramatic, and thus visible with a dissecting microscope.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: This project was delayed in starting due to extended negotiations between officials at Dow AgroSciences and Oregon State University over a material transfer agreement to obtain the ZFNs for the study, several months of design, testing, and then delivery of the ZFNs to OSU, and delays in final vector construction due to departure of coPI Tzfira from the project. As a result of the loss of Tzfira, we were forced to produce the constructs at Oregon State University, slowing progress. After many months of work, efforts to produce a recombinase construct that would remove ZFNs with heat induction have been postponed due to problems in cloning of a functional selectable marker by coPI Thompson at USDA. He is still working to produce this construct, but it will be used to only a limited extent in this project, if at all. However, as detailed below, we have now produced a vector with heat-inducible ZFN genes together with GFP to

enable monitoring of chimerism, and they are being transformed into plants currently on a large scale. Thus the core project goal of determining the rate of ZFN mutagenesis will be accomplished. The main results this year were: 1. We transferred ZFNs into a vector with a heat-shock inducible promoter that will drive ZFN expression to allow induced mutation to occur during early callus formation. It also has a constitutive eGFP reporter to help us monitor transformation and chimerism. All of the four test ZFNs have been produced and have been transformed into plant tissues. 2. After upgrading our qPCR machine to the StepOnePlus system, we calibrated the HRM system and found that the HRM system is sensitive enough to detect one SNP (i.e. a single base pair change) within a ~1,10bp amplicon. Since ZFN nucleases tend to induce larger base pair changes (i.e. indel mutations up to several tens of base pairs), our HRM targets within PtLFY and HRM analysis system are very promising for detection of nuclease-induced indels. 3. We tested the effect of a variety of concentrations of 5-FC on growth and survival of wild-type and codA-transgenic poplar tissues in culture (kill curve). We found that low concentrations of 5-FC (ranging from 50 to 500mg/L) promoted wild-type shoot regeneration. However, high concentrations (above 750mg/L) severely inhibited wild-type shoot regeneration. 4. We transformed plants with a construct containing the GFP gene to study the chimerism of transgenic-calli over time, and to guide our timing of heat shock. 5. Although we were able to monitor the GFP express over the first three weeks (calli-induction stage), the study was suspended due to the selectable marker problems discussed above. It will be resumed with the new heat-induced GFP construct just created. 6. We transformed our four new heat-shock inducible eGFP/ZFN vector into poplar clones 717 and 353. Currently, we have co-cultivated 2,000 explants for each clone and construct, which should enable us to get about 500 potentially transgenic calli. We were able to identify eGFP expression as early as two days after heat-shock. There were three conference presentations related to containment technologies.

PARTICIPANTS: The participants are S. Strauss as PI, J. Thomson at USDA as coPI, C. Ma as transformation technician, Amy Klocko as molecular biologist (constructs), K. Ault as accounts/purchasing manager, and H. Lu as graduate assistant (mutagenesis efficiency).

TARGET AUDIENCES: The target audience is other plant scientists and transgenic biotechnologists, especially those interested in ZFN effectiveness, genetic containment technologies, and tree/forest biotechnology.

PROJECT MODIFICATIONS: The objectives/approach were modified given the changes in the project that are described under the Outputs section of this report.

IMPACT: 2011/09 TO 2012/08

The project has no outcomes as of yet. The system elements have been studied and the constructs should be produced shortly, after which their effectiveness for mutagenesis will be determined.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

Vining, K.J., R. Contreras, M. Ranik and S.H. Strauss. 2012. Genetic Methods for Mitigating Invasiveness of Woody Ornamental Plants: Research Needs and Opportunities. HortScience 47:1210-1216.

GENE FLOW AND FITNESS STUDIES OF SWITCHGRASS: IMPLICATIONS FOR NEW BIOFUEL CROPS

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Award Number: 2010-33522-21703

NON-TECHNICAL SUMMARY: Switchgrass (*Panicum virgatum*) is a native perennial grass that has been cultivated for forage, soil conservation, and prairie restoration, with planned rapid expansion to millions of acres to meet the demand for cellulosic biomass. Transgenic traits that have been examined in field trials include increased biomass, drought tolerance, increased nitrogen use efficiency, herbicide tolerance, and reduced lignin content. Current cultivars may not be invasive, but effects of further breeding and massive increases in propagule pressure may lead to weed problems. To address existing knowledge gaps, we will document patterns of pollen dispersal, population dynamics, and the relative competitive ability of cultivars vs. wild biotypes and crop-wild hybrids in a variety of locations and environmental conditions. Fitness traits of switchgrass biotypes will be measured in both cropping and non-cropping (marginal lands) ecosystems in Ohio and Iowa. We will use seed addition experiments and mathematical models to investigate how further cultivar improvement is likely to affect growth rates of feral populations relative to wild ones. This research addresses one of the two highest priorities in the Request for Applications by this program. Ecological information about the potential for transgenic switchgrass to become weedy is urgently needed by USDA's Biotechnology Regulatory Services. Also, a basic understanding of pollen- and seed-mediated gene flow will help with designing requirements for field trials in which strict confinement is mandated.

OBJECTIVES: One portion of this research focuses on the potential for genes from cultivated switchgrass to spread to wild or feral switchgrass populations via pollen and seeds. We will investigate ploidy levels of wild and cultivar populations; flowering times of wild and cultivar populations; pollen-mediated gene flow from cultivar populations to wild plants; and recruitment of volunteers adjacent to cultivar populations. To examine the potential weediness of new switchgrass cultivars, we will compare wild and cultivated genotypes with respect to seed traits (percent germination, longevity, and multi-year dormancy); first-year performance (survival and fecundity in seed addition experiments); and longer-term competitive ability (clonal growth and annual fecundity).

APPROACH: First, we will characterize ploidy levels of wild populations in Ohio and Iowa to determine the potential for gene flow. If molecular markers can be identified, we will also examine the extent of pollen-mediated gene flow from known source populations. We plan to use common garden experiments to investigate the seed longevity, survival, and reproductive success of wild vs. cultivated switchgrass in different locations, competition treatments, and habitats. Data from these studies will be used to estimate population growth rates of these genotypes as well as future cultivars that have hypothetical changes in seed longevity, survival,

and fecundity. Anticipating high-yielding transgenic cultivars that will be available within the next few years, we plan to use seed addition experiments and demographic matrix models to investigate how further cultivar improvement is likely to affect population growth rates of feral vs. wild populations.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: Presentations at national meetings: 1) Stottlemeyer A, Snow AA, Sweeney P, Miriti MN, Heaton, EA. Flowering phenology, ploidy, and fitness differences between cultivated and native switchgrass (*Panicum virgatum* L.): implications for future biofuel crops. Annual Meeting of the Botanical Society of America, Columbus, OH, July 10, 2012. 2) Palik DJ, Snow AA, Sweeney P, Stottlemeyer A, Miriti MN, Heaton EA. Relative competitive abilities of cultivated vs. wild switchgrass (*Panicum virgatum*): implications for new biofuel cultivars. Annual Meeting of the Botanical Society of America, Columbus, OH, July 9, 2012. 3) Mutegi E, Stottlemeyer A, Snow AA, Sweeney P. Genetic diversity and population structure in remnant prairie populations of switchgrass (*Panicum virgatum*) and comparisons with cultivar genotypes. Annual Meeting of the Botanical Society of America, Columbus, OH, July 9, 2012. PARTICIPANTS: Allison Snow, lead PD Emily Heaton, PD Maria Miriti, PD Amy Stottlemeyer, graduate research assistant Evans Mutegi, postdoctoral researcher Patricia Sweeney, postdoctoral researcher George Patrick, senior research associate Destiny Palik, graduate research assistant Hsiao-chi Chang, graduate research assistant Emily Lewis, research assistant TARGET AUDIENCES: Not relevant to this project. PROJECT MODIFICATIONS: Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

Switchgrass (*Panicum virgatum*) is a native perennial grass that has been cultivated for forage, soil conservation, and prairie restoration, with planned rapid expansion to millions of acres to meet the demand for cellulosic biomass. Transgenic traits that have been examined in field trials include increased biomass, drought tolerance, increased nitrogen use efficiency, herbicide tolerance, and reduced lignin content. Current cultivars may not be invasive, but effects of further breeding and massive increases in propagule pressure may lead to weed problems. To address existing knowledge gaps, we are documenting patterns of pollen dispersal, population dynamics, and the relative competitive ability of cultivars vs. wild biotypes in a variety of locations and environmental conditions. Our experiments will be completed in 2013, but preliminary results suggest that current cultivars are not likely to be more invasive plants than those from native populations in Ohio and Iowa. The cultivars are able to cross-pollinate with native populations, which are genetically distinct from the cultivars based on DNA markers. Thus, it may be desirable to avoid massive plantings of switchgrass near small, remnant prairie populations. We also found that cultivars can establish volunteer populations, which implies that they could establish free-living populations near cultivated fields. A basic understanding of pollen- and seed-mediated gene flow will help with designing requirements for field trials of transgenic cultivars in which strict confinement is mandated.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

Stottleyer, A. L. 2012. Investigating hybridization potential, components of fitness, and volunteerism in wild and cultivated *Panicum virgatum* L. (switchgrass). PhD Dissertation, The Ohio State University.

RECOMBINASE-MEDIATED TARGETED GENE INTEGRATION AND EXCISION FOR MARKER-FREE TRANSGENIC CROPS

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Award Number: 2010-33522-21715

NON-TECHNICAL SUMMARY: Transgenic crops hold great promise in meeting the future needs of food, fiber and energy. Some of the potential risks associated with transgenic plants are (1) the unintended introduction of genetic aberrations during the process of transformation, and (2) presence of the selection marker genes. Quite often, insertion of transgene (intended modification) is accompanied with DNA repair process leading to the incorporation of unintended and unnecessary alterations within the locus or in flanking sequences, which cannot be removed. Similarly, marker genes cannot be removed, unless a specific strategy is incorporated at the time of transformation. Thus, strategies to introduce precision in the gene-integration process and the subsequent marker-removal process would not only contribute to the quality of the transgenic plants, but also towards preventing the potential risks to environment. This project is focused on developing the approach for precise site-specific integration of transgene constructs consisting of gene-of-interest and selection marker gene, followed by precise excision of the undesirable marker genes. The resulting transgene locus is predicted to contain only the gene-of-interest flanked by a pair of recombination sites (34 bp each). The proposed technology will allow development of higher-quality transgenic clones that will circumvent biological and environmental risks associated with the highly-expressed selection marker genes, the inherent component of the transformation process.

OBJECTIVES: The overall goal of this project is to develop a method for generating transgenic crops free of selection marker genes. The proposed molecular strategy expands beyond marker removal and covers targeted gene integration. Thus the proposed technology will allow removal of marker genes from precise integration structures, and develop technology for generating precise site-specific, marker-free transgene locus. The strategy involves the use of site-specific recombination systems, FLP-FRT and Cre-lox, to perform site-specific transgene integration followed by marker excision. In case further optimization is necessary, the strategy will employ a negative selection system, dh1A, to streamline isolation of marker-free locus. To accomplish the overall goal, we will address following objectives: (1) Develop transgenic lines using FLP/FRT-mediated site-specific integration (2) Heat induce cre gene in the selected lines to initiate excision of marker genes. Determine the efficiency of excision. (3) Test the utility of dh1A gene as a negative selection marker for improving the efficiency of the clean locus recovery.

APPROACH: The project involves standard rice transformation and molecular biology methods. The transgenic rice lines will be developed using particle-bombardment mediated rice transformation (biolistic). The transgenic lines will be evaluated for the presence and expression of genes using standard protocols such as PCR, RT-PCR and Southern hybridization. The

success of the project will be determined by the success in obtaining marker-free site-specific integration lines. The objective is to generate these lines in the first generation without the need to analyze the progeny.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: Using two target lines (A and B), multiple site-specific integration (SSI) lines were developed (>10). FLP-FRT recombination was utilized for SSI of a GUS gene. Each of these SSI lines was analyzed by PCR and Southern hybridization to isolate precise integration of a single copy of the transgene (GUS). The SSI locus was equipped with heat-inducible Cre activity and strategically located loxP sites to remove all DNA (including marker genes) flanking GUS gene. SSI lines (young plants) were heat-treated at 42 C for 3 hours to induce cre activity, and then allowed to grow normally. DNA from adult plant was isolated to analyze marker excision. This analysis indicated that Cre-mediated DNA excision was extremely efficient with none or only trace amount of marker genes detectable. T1 seeds borne on these lines were germinated and analyzed on Southern blot for locus structure. This analysis revealed that all lines transmitted marker-free SSI (MFSSI) locus (fully excised) to the next generation at a very high efficiency. This data indicated that FLP-FRT mediated SSI and heat-induced Cre mediated marker excision is a robust approach for generating marker-free SSI locus, in which gene-of-interest flanked with two oppositely oriented loxP sites is present. Finally, we analyzed transgene expression before and after marker-excision, and found that transgene activity is not altered through the marker-excision process. Thus stable transgenic lines are produced by this novel transformation platform. **PARTICIPANTS:** 1) Soumen Nandy, Postdoc 2) M. Aydin Akbudak, postdoc 3) Vibha Srivastava, P.I. **TARGET AUDIENCES:** Not relevant to this project. **PROJECT MODIFICATIONS:** Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

This project developed and validated the molecular approach for directing precise transgene integration into a specific genomic site, and then removing marker genes to generate a 'clean' transgenic line. The developed method/ approach is robust, and the transgenic lines produced in this method display transgene stability.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

1. Nandy S and Srivastava V (2012) Marker-free site-specific gene integration in rice based on the use of two recombination systems. *Plant Biotech. J.*10: 904 - 912.
2. Nandy S and Srivastava V (2011) Site-specific gene integration in rice genome mediated by the FLP-FRT recombination system. *Plant Biotech J.* 9: 713-21.

IMPROVED RECOMBINASE TECHNOLOGY FOR TARGETED MARKER FREE INTEGRATION AND FOUNDER LINE PRODUCTION FOR RISK ASSESSMENT

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USDA, ARS, Pacific West Area
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Award Number: 2010-33522-21773

NON-TECHNICAL SUMMARY: The proposed project will demonstrate the use of an innovative Recombinase Mediated Cassette Exchange (RMCE) technique using two novel unidirectional recombinase enzymes. The RCME research will develop transgenic founder lines in the species *Camelina sativa* and provide molecular characterization as a baseline for future genetic and assessment studies for the Brassicaceae family. This strategy removes genomic positional effects and allows direct comparison of transgene expression lines. Effective *Camelina sativa* founder lines and recombinase pairs will be published and made publicly available.

OBJECTIVES: The research objectives are to reduce potentially negative effects of transgene insertion and genomic presence. The aim of this proposed research is to investigate the use of novel unidirectional recombinases Bxb1, CinH, ParA and phiC31 to implement a Recombinase-Mediated Cassette Exchange (RMCE) technique for precise integration with simultaneous marker removal. The specific goals are: 1) To identify the most efficient pair of recombinase enzymes for dual unidirectional RMCE. 2) To demonstrate proof of concept with dual unidirectional RMCE in *Camelina sativa*. 3) To generate transgenic founder *Camelina sativa* lines containing the RMCE genetic platform for precise biotech risk assessment.

APPROACH: Single copy transgenic *Camelina sativa* founder lines will be generated containing the selection gene cassette flanked by fused recognition sites. An exchange vector will be biolistically transformed into the various *Camelina sativa* founder lines. Recombinase mediated cassette exchange will be examined by negative selection and used to score the most efficient pairs. The most effective *Camelina sativa* founder lines and recombinase pairs will be published and made publicly available.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: This project is working towards demonstrating the use of an innovative Recombinase Mediated Cassette Exchange, RMCE, technique where two novel unidirectional recombinase enzymes are used simultaneously. Three different selective agents have been used to successfully produce transgenic *Camelina* plants. The most effective selection strategies appear to be hygromycin using the hptII gene and glufosinate ammonium, BASTA, with the bar gene. Sulfadiazine with the sulI gene has had limited success in producing transgenic plants, with only one confirmed transgenic line being produced. To date, we have successfully produced a number of transgenic *Camelina* plants containing the TAG foundation construct. Our RMCE strategy includes a negative selection scheme using the codA negative selectable marker. We have performed experiments showing that the codA gene can effectively be used in *Camelina*.

Transgenic TAG GBC and GHC plants grow on glufosinate and hygromycin media, respectively, but die on media containing 5FC. The biolistic version of the pEXCH vector needed for the next step of the RMCE strategy is complete. Using information from our previous experiments in yeast and plants, we have selected Bxb1 as the integrase and CinH as the excisionase for the EXCH vector. We are expressing Bxb1 using a strong constitutive potato ubiquitin promoter, and CinH using a germline-specific promoter. This strategy will allow selection of site-specific insertion in the first generation and confirmation of excision in the subsequent generation. We are currently designing a second version of the pEXCH intended for Agro-bacterial transformation to allow wider utilization of this technology, complimenting the biolistic version constructed. Outreach for this project included presenting results at the June 2012 World Congress on In Vitro Biology in Bellevue, WA as well as the 2012 BRAG conference in Washington DC. PARTICIPANTS: Meridith Cook was hired as post-doctoral researcher for this project. TARGET AUDIENCES: Outreach for this project included presenting results at the January 2012 Plant and Animal Genome conference in San Diego, CA; June 2012 World Congress on In Vitro Biology in Bellevue, WA; June 2012 Citrus Research Board New Technologies conference in Emeryville, CA; July 2012 Soybean precision genome modification workshop Minneapolis, MN; as well as the 2012 BRAG conference in Washington DC. PROJECT MODIFICATIONS: Not relevant to this project.

IMPACT: 2011/09 TO 2012/08

To date, the necessary TAG transformation constructs for production of founder lines have been completed with the addition of an Agrobacterium version of the pEXCH. As the TAG constructs contain the various selection gene cassettes for positive BAR, nptII, hptII, sulI, codA and DHLA markers and success of the project relies on their functionality, testing these various marker systems in Camelina has been a priority. Results have confirmed that the BAR and Hyg are viable positive selection markers while codA is a useful option for conditional negative selection. We have optimized selection of transformed Camelina using these selective agents, and have found that 15 mg/L of glufosinate ammonium and 20 mg/L of hygromycin in MS medium are the most effective selection levels. To date, we have successfully produced a number of transgenic Camelina plants containing the TAG foundation construct. Transformation efficiency with the floral dip method varies, but we generally observe an efficiency of approximately 1 to 2 percent. For the construct pCTAGGBC, glufosinate-resistant, we have produced 13 PCR positive lines, and for pCTAGGHC, hygromycin-resistant, we have produced 14 PCR positive lines, Fig 1. We have confirmed transformation in the T2 generation for many of these lines, and have determined copy number using Southern blot analysis, Fig 2. We are continuing to screen transformants in order to identify single-copy lines. Studies on the markers have confirmed that camelina tolerates the Bar:codA and hptII:codA combination. The 5-fluorocytosine, the selection agent for the codA gene, appears to be harmless to wild-type Camelina at concentrations up to 1000 mg/L and toxic to plants containing the gene at 300 to 500 mg/L as previously predicted in literature, Fig 3. Analyses of GusPlus gene has shown success and this screenable marker may be use as a backup if needed. The DsRed and DHLA markers are in progress. Currently we are in the process of retransformation with the pEXCH vector to study the RMCE efficiency for gene targeting and marker removal.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08
No publications reported this period

PERENNIAL GRASSES FOR BIOENERGY: POLLEN AEROBIOLOGY, BIOCONTAINMENT, AND PLANT GENETICS

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Storrs, Connecticut

Award Number: 2011-33522-30817

NON-TECHNICAL SUMMARY: The current energy crisis has generated public and private investment in new sources of energy derived from plants. Switchgrass (*Panicum virgatum*) is one species that has been genetically engineered to optimize biofuel production. However, the long-term ecological risks from these engineered grasses must be studied carefully because this native species grows as wild, feral, and cultivated populations over much of the U.S. Thus, wind-blown pollen from a genetically engineered switchgrass could spread to other switchgrass populations, causing gene flow and negative impacts in natural and cultural landscapes. This project has three research initiatives that will describe the switchgrass gene flow process, especially with regard to pollen movement and gene flow. The research will determine if forests can contain wind-blown pollen (and transgenes) by acting as a pollen trap. The project will help regulators and stakeholders predict and control ecological risks and gene flow from engineered switchgrasses.

OBJECTIVES: The U.S. needs more renewable energy, and perennial C4 grasses are an attractive resource for conversion to biofuels and other forms of bioenergy. Genetic engineering (GE) can create novel traits to optimize C4 grasses for bioenergy purposes. However, future environmental release of these GE grasses raises questions that must be addressed by federal regulators. For example, we need to understand the potential for pollen-mediated gene flow and plant dispersal that might produce hazards such as new weeds, invasives, or deleterious changes in native grass populations. Our proposed research will support predictive risk assessments and biocontainment strategies for the perennial, bioenergy grass *Panicum virgatum* (switchgrass). The three main research objectives are: 1) characterizing switchgrass flowering and pollen biology, 2) measuring pollen source strength, transport, and the ability of forests to act as pollen traps (biocontainment), and 3) characterizing switchgrass ecotypes (e.g. upland vs. lowland, ploidy level), interspecific hybridization, and dispersal patterns in five Northeastern states. If our study shows that forests can mitigate transgene flow, it could be an important tool for stakeholders in the Northeast where large isolation distances are nearly impossible, but forests are abundant. These projects build upon our previous research and preliminary data. The expected outputs include peer-reviewed publications and practical recommendations for pollen containment in experimental field trials.

APPROACH: Methods for Objective 1: In the first year, we will plant switchgrass in a 70 m x 70 m field surrounded by forest at the Univ. of Connecticut Agronomy Research Farm. The experimental field will have crop rows parallel to the windbreak. Switchgrass will be a non-transgenic, northern-adapted cultivar such as Blackwell. An identical control field will be planted

at a university farm nearby where there are no forests or wind barriers. We will measure time of panicle development, first anthesis, last anthesis, growing degree days, and number of flowers per panicle. Pollen viability relative to humidity and time after anthesis will be measured using established methods. Grasses will be cut at the end of each growing season, dried and weighed for biomass. Research Objective 2: Using the two fields described, we will deploy micrometeorological instruments and pollen collection devices. Each instrumentation station includes a 10 Hz sonic anemometer, a fast-response thermocouple, a radiant thermometer for remote ground surface temperatures, and a continuously recording pollen sampler. The station in the planted field will observe the meteorological conditions in the source field. Inside the tree barrier will be two instrumentation stations on a tower. The instruments have to be deployed for extended periods of time to capture favorable wind and pollen conditions. The second control field will have the same instrumentation to collect data during the flowering season. Pollen flux will be computed at the instrumentation stations using the microclimatological measurements with programs written in Mathematica. Pollen source strength will be computed from field data. Research Objective 3: Switchgrass plants will be collected along the coast of MA, NH, RI, and ME. Isolated populations from inland roadsides (at least 10 miles from the coast) will be collected separately. In brief, the procedure will be: 1) extraction of total genomic DNA, 2) conducting PCR using 55 primer pairs, 3) labeling PCR products, and 4) obtaining DNA sequences. Alleles will be scored using GeneMarker v1.95 software. These results will be compared against an existing dataset. Switchgrass ecotype (upland vs. lowland) and ploidy level will be determined.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: Ecological risk assessment is a framework in which science-based knowledge about crop biology, novel traits (transgenes) and the environment is used to predict environmental impacts. Our research program provides information relevant to ecological risk assessment for *Panicum virgatum* (switchgrass), a native grass that has high potential for lignocellulosic biofuel production. Activities in the first year of this project have included: 1) installing switchgrass fields and instrumentation at research plots, 2) field surveys to understand switchgrass distribution, 3) collecting individuals to assess population genetics, and 4) measuring aspects of pollen biology. Events have included presentations at conferences and workshops. The project has also provided training and mentoring to one postdoctoral research associate, one PhD graduate student, and two undergraduates. **PARTICIPANTS:** Co-Principal Investigator: Dr. Thomas Meyer, Associate Professor, Natural Resources and Environment, University of Connecticut; Graduate student: Geoffrey Ecker; Postdoctoral Fellowship: Patrick Lienin. **TARGET AUDIENCES:** Scientists, risk assessors, land managers, biotechnology companies, crop breeders, federal agencies. **PROJECT MODIFICATIONS:** Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

Switchgrass (*Panicum virgatum*) is a native grass that is cultivated for livestock forage, ornamental gardens, erosion control, and wildlife habitat restoration. Recently, switchgrass has been engineered to improve its utility as a lignocellulosic biofuel crop. Unfortunately, little is known about its ecology and distribution in the Northeastern US. This gap in knowledge is a

barrier to ecological risk assessments and the development of new management practices. The main objectives of this project are to: 1) characterize switchgrass flowering and pollen biology, 2) measure pollen movement, 3) assess methods to reduce pollen movement, and 4) characterize switchgrass ecotypes and population genetics in the Northeast. Our previous work has shown that switchgrass is native to the New England coastal ecoregion, but it is also found in specific inland habitats (Ahrens, Ecker and Auer, 2011). This knowledge has led to questions about the potential for pollen mediated gene flow between populations. We collected information about switchgrass pollen biology and applied a Lagrangian modeling approach to predict pollen movement in different wind conditions. The results from two case studies suggested that viable pollen grains can be carried up to 3-6 km by wind (Ecker, Meyer and Auer, manuscript in review). These results suggest that pollen mediated gene flow could occur between the native, feral, and cultivated switchgrass populations observed in the Northeast.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

1. Ecker, GI, Meyer, T and CA Auer. Pollen longevity and dispersion models for switchgrass (*Panicum virgatum* L.) Manuscript in review (2012)
2. Auer, C. 2012. Understanding the receiving environment: Switchgrass and creeping bentgrass in New England. USDA-APHIS (oral presentation)
3. Auer, C. 2012. Ecological risk assessment for switchgrass: What do we know about the receiving environment Switchgrass I: State of the Science, Samuel Roberts Noble Foundation, Oklahoma (oral presentation)
4. Ahrens, C., Meyer, T., and C. Auer. 2012. Modeling current switchgrass (*Panicum virgatum*) distribution in New England for ecological risk assessment. Botanical Society of America (oral presentation)
5. Ahrens, C., Meyer, T., and C. Auer. 2012. Modeling current switchgrass (*Panicum virgatum*) distribution in New England. Connecticut Conference on Natural Resources (oral presentation)

RISK ASSESSMENT FOR INSECT RESISTANCE TO PYRAMIDED BT COTTON

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Award Number: 2011-33522-30729

NON-TECHNICAL SUMMARY: Transgenic crops that produce insecticidal *Bacillus thuringiensis* (Bt) toxins for pest control can increase agricultural profitability while reducing reliance on insecticide sprays. However, widespread use of these crops increases the risk that evolution of resistance by pests will cut short the success of this approach. Negative consequences of resistance include increased pesticide use and loss of profits for farmers and industry. The "pyramid strategy" based on two-toxin Bt cotton is currently extensively used to reduce the risk of resistance. However, monitoring data shows that some populations of cotton bollworm (CBW), *Helicoverpa zea*, which is an important pest of multiple crops in the U.S., have evolved resistance to the toxins Cry1Ac and Cry2Ab used in pyramided Bt cotton. Furthermore, we recently discovered that lab selection of CBW with Cry1Ac increased survival on pyramided Bt cotton that produces Cry1Ac and Cry2Ab. This unexpected finding highlights the need for better knowledge of pest biology to assess the risk of resistance to pyramided Bt cotton. To allow regulators to better assess risks associated with current Bt-resistance management mandates and enhance the regulatory framework for pyramided Bt crops, we will determine the genetic and molecular basis of CBW resistance to the Bt toxin Cry1Ac, and how such resistance increases survival to other Bt toxins and pyramided Bt cotton, or decreases fitness in absence of Bt toxins. Data from this research will be used in models to improve resistance management strategies for CBW and other pests.

OBJECTIVES: The US EPA (2001) has mandated the refuge strategy to delay evolution of pest resistance to Bt crops, but fundamental knowledge required to assess the risk of resistance to pyramided Bt cotton is not available. To address this knowledge gap, we will accomplish the following objectives: 1) Determine the mode of inheritance of cotton bollworm (CBW) resistance to Bt toxin Cry1Ac 2) Evaluate cross-resistance associated with CBW resistance to Cry1Ac 3) Determine survival and dominance of resistance on two-toxin cotton plants of different ages 4) Evaluate fitness costs and incomplete resistance on cotton plants of different ages 5) Determine if CBW resistance to Cry1Ac is linked with cadherin mutations 6) Develop and apply resistance risk assessment models using results from Objectives 1-5. We expect to provide regulators with information on resistance to commercially grown cotton that produces two Bt toxins, cross-resistance patterns, effects of seasonal declines in Bt toxin concentrations on resistance, fitness costs and incomplete resistance affecting the evolution of resistance and its stability, mechanisms of resistance to pyramided Bt cotton in CBW, and implications for resistance evolution of different scenarios of deployment of Bt corn and cotton. This information will allow regulators to identify risks associated with current Bt-resistance management mandates. This process is crucial to enhance current regulatory framework aimed at delaying the

risk of resistance to pyramided Bt crops and designing effective Bt resistance monitoring strategies.

APPROACH: We will comprehensively characterize factors affecting resistance risk using a stable, resistant CBW strain that has enhanced survival from neonate to adult on cotton plants producing either one or two Bt toxins. Understanding the mode of inheritance of resistance is critical for designing resistance management strategies. We will use three independent and complementary approaches to characterize inheritance of resistance: artificial diet bioassays, plant bioassays and molecular analyses. Cross-resistance between toxins in pyramided Bt cotton increases the risk of resistance. We will use two independent and complementary approaches to characterize cross-resistance associated with resistance to Cry1Ac: diet bioassays to evaluate cross-resistance to single Bt toxins, and plant bioassays to evaluate cross-resistance to commercial, two-toxin cultivars. We will also test for resistance to three sprayable formulations of Bt toxins recommended for CBW control. We will analyze CBW survival and dominance of resistance on commercially available two-toxin Bt cotton plants of two different ages. This will enable evaluation of the effects of seasonal declines in toxin concentrations as the plants age. We will also evaluate fitness costs and incomplete resistance on cotton plants of different ages, because experimental and theoretical analyses show that these factors can play a key role in delaying resistance to Bt crops. Knowledge of the molecular genetic basis of resistance can yield powerful tools for monitoring and managing resistance to Bt crops, thereby facilitating regulatory decisions. Although many genes might confer resistance to Cry1Ac in CBW, we will test for linkage with cadherin first, because mutations in this gene confer resistance to Cry1Ac in the closely related congener *H. armigera*, and in two other major cotton pests. Computer-based simulation models enable exploration of the risk of resistance to Bt crops under various scenarios. Most previous models of resistance to pyramided Bt crops assume high levels of redundant killing in two-toxin Bt cultivars, which occurs when one toxin produced by a cultivar kills insects resistant to the other toxin. However, our preliminary data show that this assumption may not hold in CBW. We will thus develop and use models to explore the consequences of the absence of redundant killing.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: During this first year of this grant, we are primarily working on the following Objectives: 3) Determine survival and dominance of resistance on two-toxin cotton plants of different ages, 4) Evaluate fitness costs and incomplete resistance on cotton plants of different ages, 5) Determine if *Helicoverpa zea* (CBW) resistance to Cry1Ac is linked with cadherin mutations, and 6) Develop and apply resistance risk assessment models. An experiment is currently ongoing to analyze CBW survival and dominance of resistance on commercially available two-toxin Bt cotton plants of two different ages. The toxins produced by the Bt plants are Cry1Ac and Cry2Ab. The goal is to evaluate effects of seasonal declines in toxin concentrations as the plants age on the dominance of resistance. We will also evaluate potential changes in fitness costs and incomplete resistance on cotton plants of different ages. Knowledge of the molecular genetic basis of resistance can yield powerful tools for monitoring and managing resistance to Bt crops, thereby facilitating regulatory decisions. Although many genes might confer resistance to Cry1Ac in CBW, we are assessing linkage with cadherin first, because

mutations in this gene confer resistance to Cry1Ac in the closely related congener *H. armigera* and in two other major cotton pests. To identify potential cadherin mutations involved in resistance to Cry1Ac, we have: 1) obtained the full-length cDNA sequence of cadherin from a Cry1Ac-susceptible strain, BE-S by 5' and 3' RACE; 2) obtained three cadherin alleles of full-length cDNA sequence from pooled RNA samples from the BE-S strain; 3) obtained five cadherin alleles of full-length cDNA sequence from five resistant GA-R larvae (resistant to Cry1Ac); 4) aligned the eight full-length cDNA sequences to identify single-base mutations and resulting in amino acid alterations. Following this work, we plan to 1) develop a PCR-RFLP technique to differentiate the resistant and susceptible alleles based on the five amino acid mutations; 2) set up 10 families from single-pair crosses between the BE-S and GA-R strain; 3) use a discriminating dose of Cry1Ac to treat the backcross progeny of the 10 single-pair families; 4) analyze the genotypes of survivors and dead individuals using the developed PCR-RFLP technique; and 5) determine if the resistance to Cry1Ac is linked to the cadherin mutations. We have developed codes for a simulation model to explore the risk of resistance to two-toxin Bt cotton under various scenarios. The model has been successfully validated by comparing results from our simulations to results obtained by other authors. Most previous models of resistance to pyramided Bt crops assumed high levels of redundant killing in two-toxin Bt cultivars, which occurs when one toxin produced by a cultivar kills insects resistant to the other toxin. We are currently using simulations to explore the consequences of a lower extent of redundant killing on the risk of resistance evolution to two-toxin cotton. PARTICIPANTS: One PhD student was funded by this project to perform work related to Objective 5. Two research specialists and three undergraduate students were funded by this project to perform Objective 3 and 4. TARGET AUDIENCES: Nothing significant to report during this reporting period. PROJECT MODIFICATIONS: Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

No outcomes/impacts to report in this new project.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

No publications reported this period

**FOSTERING COEXISTENCE: INDUSTRY-DRIVEN FIELD AND LANDSCAPE
RESEARCH ON POLLEN-MEDIATED GENE FLOW IN GENETICALLY
ENGINEERED ALFALFA**

Greene, S.; Martin, R.; Boydston, R.; Walsh, D.
USDA/Agricultural Research Service
Prosser, Washington

Award Number: 2011-33522-30733

NON-TECHNICAL SUMMARY: On January 27, 2011, USDA-APHIS announced the complete deregulation of glyphosate-resistant alfalfa, colloquially known as Roundup-Ready alfalfa (RRA). Grower demand for RRA seed surged immediately, and acreage of RRA hay and seed is predicted to increase rapidly. Following a previous period of RRA deregulation (2005-2007), transgenes have been detected in conventional alfalfa, suggesting that current practices are not sufficiently protective to mitigate gene flow from RRA to conventional alfalfa and alfalfa seed. Certain key markets for U.S.-produced alfalfa hay and seed, including many export markets and the organic market, have little to no tolerance for the presence of transgenes. This project seeks to broaden our understanding of pollen flow biology and how it might influence the movement of the RRA transgene into conventional fields. Through nine specific research objectives, we intend to assess the role of feral alfalfa in transgene transmission; the impact of pollinators on pollen-mitigated gene flow; and the flow of transgenes from genetically engineered RRA hay fields to conventional alfalfa seed production fields in different environments. Our trans-disciplinary (genetics, molecular biology, entomology, weed science), multi-state (Washington, Oregon, California, Idaho), multi-institutional (USDA-ARS, Washington and Oregon; Washington State University) team brings a comprehensive skill set to the task of formulating science-based strategies for co-existence of GE alfalfa, conventional alfalfa, and other crops. Data we will gather in pursuit of these nine objectives should provide substantial information to help ensure hay and seed production for both GE sensitive and non-sensitive markets can continue to prosper in the United States.

OBJECTIVES: With the deregulation of Roundup-Ready alfalfa (RRA) in February 2011, there is an urgent need to complete and implement coexistence strategies to protect the export seed market and other alfalfa markets that are sensitive to the adventitious presence (AP) of transgenic traits. Extended conversations with alfalfa producers and breeding companies have led to the following objectives of our project: (1) to examine how leaf cutter and alkali bees transmit RRA across commercial seed fields and how that will impact proposed harvest strategies that separate seed for non AP- and AP- sensitive markets and to examine the persistence of RRA pollen in honeybee hives; (2) to characterize fitness parameters such as seed production, seed dormancy and viability, longevity in the seed bank, seedling establishment and plant persistence, in feral and feral-RRA hybrid alfalfa to determine how important and to what extent control strategies are required; (3) to track RRA transgene flow from RRA hay and seed production fields planted during the previous deregulation (seed fields were removed in 2007) into feral alfalfa to understand the role feral alfalfa plays as a transgene reservoir and vector for long distance

transgene dispersal; (4) to study the transmission of the RR transgene from RRA hay fields to conventional seed fields to refine isolation distances by taking into account landscape variables. Our objectives support the following goals: (1) understand the role feral alfalfa plays in transmitting the RRA transgene in important seed and hay areas in the states of Washington, Idaho and California; (2) test the relative efficacy of current stewardship practices in limiting the movement of the CP4 EPSPS transgene into the environment; (3) develop a greater understanding of the role pollinators play in transgene flow. Anticipated outputs include conducting and analyzing experiments and surveys that will allow us to confirm, refine and build on current management recommendations for isolating hay and seed fields, controlling feral alfalfa and managing pollinators to support GE-sensitive and non-sensitive alfalfa production in the United States

APPROACH: (1) Feral alfalfa populations will be surveyed in Washington in two growing regions and across three site categories. Transects will be used to select individual survey sites. Ten individual sites with an area of 5 m x 100 m within each region and site type along the transect will be surveyed for a total of 60 sites (2) Extent of pollen-mediated transfer of RR trait from RRA hay and seed fields to feral alfalfa, mixed pastures, and CA hay and seed fields in Fresno County, CA, and Canyon County, ID will be surveyed. Test sites will be RRA hay fields and RRA seed fields that were planted during the earlier deregulation (hay fields may still be present, but seed fields will be gone), and isolated from other RRA fields at least 15 km. Sampling design will be concentric polygons around source fields, for a total of 16 zones that sample an 8 km radius. Sampled leaves and seed will be tested for RRA. (3) Experiments to compare establishment and persistence of seed and transplants of RRA cultivar to its CA cultivar counterpart; and to examine fitness and persistence of RRA-feral hybrid transplants will be set up at four selected sites within each region and three site types (total of 24 sites). (4) Experiments to determine alfalfa seed longevity among selected sites at depths of 0.5 cm and 5 cm will be set up. (5) Experiments to determine the effect of selected auxin inhibitor herbicides on alfalfa seed viability will be conducted. (6) Leafcutting (LF), alkali (AL) bees, and harvested alfalfa seed will be surveyed for RRA. We will sample LC bees at 4 locations at 3 time periods. To survey AL bees, we will sample 13 AL bee beds. Bees, pollen balls, cells will be analyzed for RRA pollen. Seed will be collected from field-margins and from the interior of the field and tested for RRA. (7) Quantify AL bee population density and study flight paths to determine pollinator's role in distribution of RRA pollen by counting the nesting holes of the bees and by collecting soil cores in fall after the bees have gone dormant. Study flight patterns, including height, distance, and bearing, through utilization of pane traps. (8) Quantify alfalfa pollen viability within honey bee (HB) hives by placing HB colonies randomly in an RR alfalfa seed field and removing after 3 weeks and placing at a site not containing alfalfa, and destructively sampling at 7 and 14 days. Bees and hive comb will be sampled for the presence of RR pollen. (9) Extent of pollen-mediated transmission from RRA hay fields to conventional seed fields will be examined at 5 locations each, in WA, ID, and CA. Target site will have RRA hay field (planted during 2005-2007) located either 0-2 km, 2-4 km, 4-6 km, 6-8 km, or 8-10 km away. For each trap field, 50-gram seed samples harvested in 2007, 2008, 2009, 2010, 2011 will be assayed. All experiments and surveys will be analyzed using appropriate methods and results reported broadly in research and trade publications and at meetings with the aim of disseminating

the information broadly among the alfalfa community. Evaluation of project outputs will be based on the number of papers, reports and presentations given during the course of the project.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: A project work plan was developed with input from the alfalfa Round Up Ready (RRA) technical committee in December, 2011. Primers were obtained from Monsanto and procedures worked out to confirm the presence of the RRA transgene using PCR. A crossing block was established in May, 2012 to develop alfalfa F1 populations of the following crosses: Round Up Ready (RRA+) x feral alfalfa, RRA- x feral, RRA+ x falcata, RRA- x falcata. To determine sampling height for leaf cutter bees, a grid network of 10 foot wood posts was placed in a candidate alfalfa seed field in July, 2012. Three sets of 110 in2 sticky glue traps were placed onto these posts for 24 hours in 1 ft intervals starting at 2 feet and going up to 10 feet. The majority of bees were captured between 2 and 4 feet. Fluorescent dyes were tested as a means for marking bees that originated within specific bee domiciles. Recovery of marked bees was approximately 30% and the furthest a marked bee was observed from its domicile was 582 feet. Sixty alkali bees laden with pollen on their way back from foraging in alfalfa seed fields were collected from each of seven bee beds between July 6 and July, 2012. Individual bees were collected and placed in an individual vial and the vials and placed in freezers. This fall the bees will be tested for the presence of the Round-Up Ready gene in the pollen they collected with an AgraStrip GMO RUR Test Strip. Alfalfa from the surrounding alfalfa forage and seed fields has also been collected and similar tests will be conducted to identify which fields in the area contain Round-Up Ready GE alfalfa. The effects of four growth regulator type herbicides (2,4-D, triclopyr, aminopyralid, and dicamba) on seed development and viability of RR-alfalfa is being determined on a grower field near Touchet, WA. RR-alfalfa plants containing green seed pods and still flowering were treated with herbicides July 25, 2012. Nontreated control plants were included for comparison. Plants will be rated for injury at 10-14 days after herbicide application and above ground plant material will be harvested and air dried. After drying, seed pods will be removed and threshed to remove seed. Seed will be separated into several developmental categories ranging from small undeveloped seed to fully ripened seed and germination of each seed category will be determined in Petri dish assays. Information about the project was shared at invited presentations at the Science of Geneflow Conference, Washington D.C. 9/7/2011; USDA ARS Administrator's Council, Beltsville, MD 12/7/2011; Idaho and Oregon Alfalfa and Clover Seed Grower Association, Caldwell, ID 1/12/2012; Western Alfalfa Seed Growers Association, Las Vegas, 1/16/2012 and International Sprout Growers Association, Vancouver BC 8/24/2012.

PARTICIPANTS: An alfalfa Industry Technical Committee was formed consisting of representatives of the following major genetic suppliers: Forage Genetics International, Pioneer Hybrid, Dairyland, and Cal West Seed.

TARGET AUDIENCES: The alfalfa industry is interested in this project, including the sprout growers as evidenced from the number of invitations to make a presentation at their association meetings.

PROJECT MODIFICATIONS: Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

Development of the work plan and interaction with the alfalfa industry has increased our understanding of how to set up our landscape-scale experiments. We are currently identifying

study locations and collaborators for our 2013 experiments. Hybrid seed produced in 2012 will be used in 2013 and 2014 to examine the relative fitness of RRA/feral hybrids. Data obtained from 2012 pollinator studies suggested that leaf cutters can best be sampled between 2 and 4 feet. We feel we now have a fairly robust method for determining bee flight and foraging distance with leafcutting bees for our 2013 studies. PCR procedures have been worked out so can now be used to routinely validate positives found by the AgraStrip GMO RUR Test Strips. Full implementation of the project was limited since we have not yet received the funds to date (8/14/2012), from NIFA. However, we are submitting a no cost extension so we can complete the project as outlined.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

1. Greene, S.L. 2011. Importance of gene flow to germplasm conservation and development. p. 20-22. Proceedings of the science of gene flow in agriculture and its role in co-existence. Septemebr 7, 2011, Washington D.C.
2. Greene, S.L. 2012. Tracking the alfalfa Round up Ready Gene: Implications for Coexistence p. 53-55. Proceedings for the Western Alfalfa Seed Growers Association, Jan. 16, 2012, Las Vegas, NV.

RECOMBINASE-BASED TRANSFORMATION VECTORS FOR IMPROVED TRANSGENIC STRAIN DEVELOPMENT AND ECOLOGICAL SAFETY IN TEPHRITID PEST SPECIES

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Award Number: 2011-39211-30769

NON-TECHNICAL SUMMARY: The goal of this research is to improve the development and ecological safety of genetically engineered (GE) insects created for improved biologically-based control programs including SIT and conditional lethality strategies. A major concern for GE insect release programs is ensuring stability and consistent expression of transgenes to maintain strain attributes and minimize ecological risks. To address potential instability a new transposon vector that allows its post-integration immobilization will be tested in three tephritid fruit fly pest species. Random genomic insertion is also problematic for GE strain development due to suppression of transgene expression at particular genomic sites, and mutations resulting from vector insertions that negatively affect host fitness and viability. To target transgene vectors to genomic insertion sites having minimal effects on gene expression and host fitness, a recombinase-mediated cassette exchange (RMCE) strategy that allows stabilization of the target site will be tested in the tephritid species, and stabilized target-site strains developed for conditional lethal control. In particular, will be the molecular and organismal evaluation of multi-transgenic lethality traits that should prevent the unwanted survival of GE insects due to resistance arising to a single lethal component. By seeking to improve transgene expressivity and stabilization of transposon-based vector systems, this proposal specifically addresses issues related to new GE insects by reducing their unintended survival and spread into the population after field release.

OBJECTIVES: The overall objective of the proposed research is to modify transgene vectors for more efficient and flexible use in tephritid fruit fly species, to test their function, and to create a series of stabilized target-site strains that can be used to create transgenic strains for biologically-based control programs. We expect this new generation of transformation vectors to increase the efficiency of transgenic strain development and strain effectiveness, while improving their ecological safety. Use of these strategies, if not the genetic components themselves, may be extended to other transgenic organisms as well. The specific objectives for this project are: 1) to create new recombinase-mediated cassette exchange (RMCE) target-site and donor vectors for efficient use in tephritid flies including promoter replacement for reporter genes, testing of new hetero-specific FRT and loxP recombinations sites for initial and repetitive gene insertion/replacements in target-sites, and creation of stabilization donor vectors that allow immobilization of the piggyBac target-site transposon vector; 2) to test the modified RMCE target vector and stabilization system in the Caribbean (*Anastrepha suspensa*), Mexican (*A. ludens*), and the Mediterranean (*Ceratitis capitata*) fruit fly species; 3) to create a series of target-site strains in these tephritid species that will be characterized initially in terms of strain fitness

and transgene marker expression, with optimal strains genetically mapped and the target-site molecularly characterized; and 4) to test the influence of target-site position effects on transgene expression relative to homologous resident genes, with efficacious target-sites tested for conditional lethal effector gene expression using the dominant temperature-sensitive *AsProsβ2-1* mutation, and the tetracycline-suppressible *As-hid-ala2* cell death gene.

APPROACH: To enhance transgene stability a new transposon vector that allows post-integration immobilization will be tested in three tephritid pest species, the Caribbean fruit fly, *Anastrepha suspensa*, the Mexican fruit fly, *Anastrepha ludens*, and the Mediterranean fruit fly, *Ceratitis capitata*. Transposon vectors include terminal inverted repeat sequences that are required for their mobilization (after binding to a transposase enzyme), and this vector allows the deletion of one of the terminal sequences after its initial genomic insertion thereby immobilizing the remaining vector sequences. The random nature of transposon-mediated genomic insertions will be addressed by developing a vector system that targets transgene vectors to defined genomic insertion sites based on a recombinase-mediated cassette exchange (RMCE) strategy. For this, transposon vectors will include FRT and loxP recombination site sequences that allow subsequent insertions into the same site by RMCE. Initial RMCE insertions will include sequences that allow stabilization of the initial transposon vector insertion. Several RMCE sites will be integrated into the three tephritid species and tested for transgene expression, host fitness and mating competitiveness. Those identified as having optimal sites will be stabilized to create target site strains for subsequent transgene insertions that can be used for the sterile insect technique (SIT) and conditional lethality. Constructs for transgenic conditional lethality systems will be inserted into target sites and tested individually and in combination to assess efficiency and stability of marker and lethal gene expression.

VALIDATION OF A MATERNALLY MEDIATED STERILIZATION PLATFORM FOR REPRODUCTIVE CONTAINMENT OF GE FISH WITH INITIAL APPLICATION TO TILAPIA

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Award Number: 2011-33522-31032

NON-TECHNICAL SUMMARY: With human population rising and wild fish stocks on a decline, increased productivity in aquaculture will be required to fill the looming gap between supply and demand for seafood. Aquaculture also needs to confront new market challenges such as maintained high quality and reduced impact on the environment. Transgenic fish with advantageous traits (i.e. improved growth rate, better food conversion ratio with reduced waste, resistance to disease, and enhanced nutritional benefit) are under development to promote a sustainable aquaculture industry capable of large-scale, low cost production with minimal impact on the environment. The risk of gene flow from transgenic fish represents a concern related to commercial deployment of these valuable organisms. A solution would be to render the fish sterile. Technologies exist to control fertility in finfish, but they are logistically demanding, negatively impact culture performance, or do not result in 100% sterility. We have developed a new approach, termed "Maternal Sterile Technology" (MST) and designed for efficient, large-scale production of sterile finfish. Our technology relies on the fish's own protein machinery to eliminate the few embryonic cells destined to become sperm and eggs. Without those cells, the embryos develop into adults lacking gametes and are sterile. The technology can be stacked with other attractive transgenes. Our technology was validated in zebrafish and in this proposal we seek to evaluate this approach in tilapia, one of the most widely cultivated fish in the world.

OBJECTIVES: We seek to test a new Maternal Sterile Technology (MST), designed to induce sterility in finfish. Our overall objective is to generate MST lines of tilapia, carrying a unique transgene engineered to selectively kill primordial germ cells in early embryonic development. The resulting sterile fish would lack gametes. This is realized by targeted expression of an apoptotic-inducing gene in primordial germ cells (PGCs) via a specific 3'UTR. This transgenic construct (a Maternal Sterility Construct; MSC) is only expressed in females, from an oocyte specific promoter, causing her progeny to be sterile. MSC-males will not express the transgene, and will be fertile and able to propagate the transgenic line. The specific objectives are to: 1) Targeted ablation of PGCs in tilapia (Sept 2011-March 2012). We will evaluate the ability to target and ablate the PGCs in tilapia embryos. We will produce and inject in one cell stage zebrafish and tilapia embryos, synthetic capped mRNA coding for the tilapia apoptotic-inducer Bax fused to 3'UTR of nanos and study ablation of green fluorescent protein labeled PGCs. 2) Identification and characterization of oocyte-specific promoters in tilapia (Sept 2011- Sept 2012). We will clone and test tilapia oocyte-specific promoters to identify those that are the most robust. We will assay the relative level of endogenous gene expression for at least six selected oocyte-specific gene candidates. We will select three genes with the most robust expression,

clone their proximal promoter regions, generate reporter constructs, and assay their ability to drive expression of the green fluorescent protein in zebrafish and tilapia oocytes exclusively. The 2 most robust promoters will be selected for generation of the MSC transgenes. 3) Establish functional lines of MSC-tilapia (August 2012-2013). We will assemble and inject MSCs to establish multiple stable transgenic lines of tilapia. The level of bax-nos 3'UTR mRNA level in oocytes from different females will be measured to identify the lines with the strongest expression level. Transgenic males will be confirmed fertile and capable of passing on the transgene to the next generation. We will use physiological (assessment of secondary sexual characteristic and reproductive capacity), cellular (histology of the gonad) and molecular (presence or absence of germ cell specific gene expression) investigations to assay the varying penetrance of the sterility phenotype in the progeny of transgenic MSC-female tilapia. 4) Confirm stable inheritance of the sterility phenotype between generations and in varied genetic backgrounds (Jan 2013 - Sept 2014). We will confirm this sterile phenotype can be propagated through the MSC-male, which should produce fertile progeny with a normal sex ratio, allowing generation of new MSC-female (which would produce sterile progeny). We will cross sibling MSC-male and MSC-female tilapia with a growth hormone transgenic tilapia line (with dramatic growth enhancement). The progeny (both paternal and maternal progeny) will be raised and sterility level scored at the cellular and molecular level.

APPROACH: This project seeks to create and evaluate transgenic lines of tilapia, with males capable of passing on the transgene to the next generation and females producing sterile and all-male progeny. This is accomplished with a transgenic construct (termed a Maternal Sterility Construct; MSC) which consists of 3 elements: a maternal, oocyte-specific promoter, an apoptosis-inducing gene, and a 3'UTR to restrict cell death activity to primordial germ cells (PGCs;) First, we will clone and characterize from tilapia (i) the 3'untranslated region of nanos (nos 3'UTR), a germ cell specific gene, (ii) the proapoptotic gene Bax, and (iii) a suite of oocyte-specific promoters. Synthetic capped mRNA encoding GFP:nos 3'UTR and Bax:nos 3'UTR will be produced in vitro and microinjected in one-cell stage embryos to test the ability of the 3'UTR to drive apoptotic gene expression and ablation, respectively, in PGCs. Positional analysis and counts of GFP-labeled cells under fluorescent microscopy will be used to evaluate functionality of each element cloned. Six candidate oocyte-specific tilapia genes have been identified; three with the strongest levels of gene expression in the oocyte, as measured by qRT-PCR, will have different lengths of proximal promoter regions cloned to test their ability to drive expression of GFP in the oocytes of zebrafish and tilapia. The two most promising promoters will be used to construct MSCs for testing in tilapia. At least two MSCs will be used to generate transgenic lines, in an effort to identify an MSC driving apoptosis in PGCs at levels sufficient to induce 100% sterility. One cell stage tilapia embryos will be microinjected to establish at least 10 stable transgenic lines for each MSC. Founder fish will be identified using PCR from germ cell DNA extracts. Founder males will be propagated, and F1 progeny genotyped and sexed. We will quantify the level of transgene expression in the eggs from F1 females and progeny embryos. We will score the sterility phenotype at the physiological, cellular and molecular level in the progeny of F1 females. Gonads will be sectioned and stained to study the overall cellular architecture and number of germ cells. The expression level of the germ cell specific gene vasa will be measured by quantitative real time PCR, and compared to nontransgenic controls. Penetrance of the

sterility phenotype will be determined by the percentage of sterile fish identified in the progeny of three sibling transgenic female in each line. In addition to confirming MSC-females produce sterile progeny, we will prove the MSC can be passed on through the male lineage. Transgenic F1 MSC-males from the best performing MSC-female line will be crossed with wild type females; the resulting progeny should be fertile, and inherit the MSC from the father, confirming that MSC-males can propagate the line. The resulting F2 MSC-females will be bred with a line of homozygous growth hormone transgenic tilapia, confirm that the MSC-females carry a stable grandchildless phenotype, and that this trait can be stacked with another transgene

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: To investigate the possibility that we can achieve maternal specific primordial germ cell expression and produce generations of sterile tilapia, we cloned 3'UTR of tilapia nanos gene (tnos 3'UTR) and tilapia bax (tbax). We generated synthetic mRNAs (tbax:tnos 3'UTR; GFP:tnos 3'UTR) from expression constructs and injected this RNA into zebrafish embryos to test its Primordial Germ Cell (PGC) ablation capacity. Furthermore, we cloned the regulatory upstream region of candidate oocyte specific tilapia genes and tested their ability to drive expression of a GFP reporter gene in transgenic lines of zebrafish. We established germ line transgenic zebrafish carrying 4 reporter constructs (SDAD-1:eGFP:tnos 3'UTR; Bucky Ball:eGFP:tnos 3'UTR; Zar1:eGFP:tnos 3'UTR and ZPC5: eGFP:tnos 3'UTR) and compared PGC-GFP expression levels. Finally, we generated 3 Maternal Sterility Constructs (MSCs: tZar1, tZPC5, tBucky Ball) and developed efficient protocols for sperm and egg collection, fertilization, and tilapia embryo rearing. We finally injected selected constructs into one cell stage tilapia embryos, fin clipped at one month of age followed by DNA extraction and tested for the presence of the transgene.

PARTICIPANTS: The PI, X Lauth designed the constructs, cloning and screening strategies, performed crosses and microinjections experiments, analyzed data and coordinated the research. The CoPI, J.Buchanan analyzed and reviewed the data. The Research Associate T. Tran performed all PCR (QPCR) for selection/identification of transgenic lines. Investigator Jason Stannard initiated the Tilapia breeding program. Research Associates T. Tran and A. Fujimoto and Technician S. Karimi maintained the zebrafish and tilapia lines. **TARGET AUDIENCES:** We have a presentation scheduled for the upcoming World Aquaculture Society (WAS) meeting in Nashville, Tennessee, February 21-25, 2013 **PROJECT MODIFICATIONS:** Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

mRNA microinjection experiments into one cell stage zebrafish embryos indicated the following: 1) the regulatory 3'UTR of the tilapia gene nanos provides primordial germ cell expression; 2) the ectopic over-expression of the tilapia pro-apoptotic gene bax fused to tilapia nanos 3'UTR ablates primordial germ cells. 3) Bax:nanos 3'UTR treated fish all developed as phenotypic males with testes reduced to an empty tube-like structure. Thus, tilapia bax and tilapia nanos 3'UTR are operationally functional in zebrafish. Based on these results we are confident that our sterility constructs will deliver a sterilizing phenotype in transgenic lines of tilapia. While GFP expression studies in transgenic lines of zebrafish continues, we confirmed GFP expression in PGCs in the progeny from transgenic females but not transgenic males as expected in a maternal oocyte-specific expression. tZPC5 proximal promoter drove a high level

of PGC GFP expression in zebrafish and thus, the corresponding MSC construct was injected into tilapia embryos. We identified 3 transgenic tilapia carrying the construct tZPC5:tbax:tnos in their fin DNA. Work will continue to confirm germ line transmission of the MSC construct and to establish additional founder tilapia lines.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

No publications reported this period

AN INVESTIGATION INTO THE POTENTIAL RISKS OF RELEASE OF TRANSGENIC NEW WORLD SCREWORM FLY COCHLIOMYIA HOMINIVORAX

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Award Number: 2011-33522-30730

NON-TECHNICAL SUMMARY: Background: The New World screwworm (NWS) fly, *Cochliomyia hominivorax*, is a very serious pest of warm-blooded animals. Starting in the 1950s, the Sterile Insect Technique (SIT) was used to progressively eradicate the NWS from U.S.A., Mexico and Central America. The SIT involves rearing of millions of male and female NWS that are sterilized by exposure to radiation prior to being released into the wild. If a wild type female fly mates with a sterilized male fly, no offspring will be produced. By repeatedly releasing sterilized flies, the wild NWS population is gradually reduced in size until the fly has been eradicated from the targeted area. The economic benefits of this highly successful program have been substantial, with an estimated annual savings to the US livestock producers of \$896 million. Currently, the USDA-APHIS and the Panamanian government jointly operate a NWS mass-rearing and sterilization facility in Pacora, Panama. Sterilized male and female NWS are released continually in a "buffer zone" in Southern Panama to prevent re-introduction from South America. APHIS has identified several advantages to incorporating transgenic NWS strains into the control program. Firstly, strains that carry stable fluorescent proteins will make it much easier to confirm that flies caught in the "buffer zone" were those released from the factory and not due re-invasion from Columbia. Secondly, strains that facilitate the release of only males would significantly increase the efficiency of SIT and thus reduce the costs of the program. Although it is only necessary to release sterilized males for SIT to work, both male and female flies are currently released as there is no easy way to separate out the female flies. We are currently developing transgenic NWS strains that carry genetic systems that cause female flies to die unless tetracycline is added to the diet. Project Goals. Obtain data for a future risk assessment of (1) Fluorescent protein-marked strains and (2) "male-only" strains. The data obtained from this project will be essential for regulatory agencies to evaluate the potential risks of incorporating engineered strains into the Screwworm Eradication Program. Methods. Various life parameters of transgenic NWS strains will be compared to the wild type strains. In particular we will evaluate longevity, potential to mate with a related fly species, stability (i.e. loss of the engineered gene), and efficacy of the female-killing gene in different genetic backgrounds and over time. Outcomes/Impacts. This project will provide: (1) Engineered strains of NWS that facilitate male-only releases. (2) Data on stability of the strains, mating behavior, fitness and other properties that will be essential for regulatory agencies to assess the risk of future field releases of these strains. Anticipated Benefits: Strains that increase the efficiency of SIT for control of NWS. This will reduce the costs of the ongoing NWS control program in Panama and facilitate any future eradication efforts in countries that are not free of NWS (e.g. some Caribbean islands). The latter pose a risk of re-invasion to screwworm-free countries.

OBJECTIVES: Goal 1: Evaluation of transgenic strains of the New World screwworm (NWS) fly, *Cochliomyia hominivorax*, carrying fluorescent marker genes Objectives a) Adult longevity at cooler temperatures: We will compare longevity at various temperatures of the transgenic strain to wild-type. b) Assessment of the potential for outcrossing: We will determine if transgenic males will mate with and transfer genetic material to females from a closely related species, the secondary screwworm fly *Cochliomyia macellaria*. c) Stability under field-cage conditions: Transgenic strains will be reared in large field cages for several generations and the presence of the transgene and green fluorescent protein (GFP) expression will be monitored. d) Development and assessment of "stabilized" lines: If the existing GFP-marked lines are unstable we will make "stabilized" GFP-marked lines where one piggyBac end has been excised. Transgene stability will then be assessed as described above in objective (c). Timeline: (a) and (b) Initiate and complete in year 1. (c) Initiate in year 1 and complete in year 2. (d) Initiate in year 2 and complete in year 3. Goal 2: Evaluation of male-only transgenic NWS strains Objectives a) Development of transgenic male-only strains. Transgenic strains will be made that carry tetracycline-repressible female-lethal genetic systems. b) Assessment of the fitness of the transgenic male-only strains. We will measure various fitness parameters such as fertility, fecundity, larval survival and pupal weight and compare to wild-type. Additionally longevity at various temperatures will be measured as described above. c) Assessment of the mating behavior of the transgenic male-only strains, potential for outcrossing and male-competitiveness. We will analyze the mating behavior of transgenic males compared to wild-type males and determine if transgenic males can compete effectively. We will also determine the potential for outcrossing with *C. macellaria* as described above. d) Assessment of the stability of the transgenic lines, including "stabilized" lines. Transgene stability in the male-only strains, including stabilized lines, will be assessed as described above for the GFP-marked strains. e) Assessment of the efficacy of "male-only" transgenic strains in different genetic backgrounds. Transgenic males will be crossed to females from wild-type strains that have been collected from various locations. f) Assessment of the potential for "male-only" lines to lose effectiveness over time. Transgenic lines reared for several generations in large cages and assessed for loss of female-specific lethality. Timeline: (a) Initiate in year 1 and complete in year 2. (b, c) Initiate in year 1 and complete in year 3. (d-f) Initiate in year 2 and complete in year 3. Expected Outputs: 1) Development of stable "male-only" transgenic New World Screwworm strains. 2) Data on the fitness, stability, outcrossing potential, efficacy in different genetic backgrounds and any loss of efficacy over time. Such data will be necessary for regulatory agencies to evaluate the potential risks of future field releases of the engineered strains.

APPROACH: Development of stable NWS strains carrying tetracycline-repressible female lethal genes. Several different DNA constructs containing a repressible female-lethal genetic system have been made. The key component of these systems is female-specific expression of the tetracycline-repressible transactivator or tTA. Female flies die when raised on diet that lacks tetracycline due to overproduction of tTA. Transgenic NWS strains carrying some of the DNA constructs have been developed and are being bred to homozygosity. The success of these experiments will be evaluated based on the % of the flies that develop as males when reared on diet that lacks tetracycline. The transgene contains the left and right ends of the piggyBac transposon. Both piggyBac ends and a source of piggyBac transposase are required for re-

mobilization of the transgene. As the transgenic strains do not make piggyBac transposase, the integrated DNA should be stable. However, to eliminate the risk of re-mobilization, one of the piggyBac ends will be excised using a recombination system. The success of this step can be readily evaluated by molecular analyses and loss of marker genes. Evaluation of the fitness, mating behavior, stability and efficacy of the transgenic GFP and "male-only" strains: a) Fitness parameters that will be measured for the male-only lines include average pupal weight, ratio of males to emerged adults, average fertility, fecundity, larval productivity and longevity. b) To provide data for a risk analysis, we will determine if transgenic males will mate with females from the closely related species *Cochliomyia macellaria*, the secondary screwworm fly. We will measure the egg production, % egg hatch rate (if any), % of larvae that develop to adults (if any) and adult fertility. c) To assess transgene stability, strains will be reared in large field cages for several generations and the presence and chromosomal location of the transgene in randomly selected flies (20 pools of 10 flies each pool) will be determined by molecular analysis of genomic DNA. d) The male mating competitiveness will be evaluated using cages that will contain 25 transgenic males, 25 wild-type males and 75 wild-type females. The offspring will be scored as either fluorescent (indicating presence of GFP transgene) or non-fluorescent. e) To evaluate if the efficacy of the female-killing system varies in different genetic backgrounds, mating cages will be set with virgin wild-type females and transgenic fertile males. Wild type NWS strains have been collected from several different geographical locations. If there is a genetic background effect, there will be a significant variation between crosses in the % of flies that develop as females on diet that lacks tetracycline. f) To assess the risk that a transgenic line could lose effectiveness over time, lines will be reared on medium supplemented with tetracycline for several generations in large cages. Several hundred embryos will be collected from each generation and raised on normal medium that lacks tetracycline. The % that develop as females will be calculated.

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: Activities: Several Transgenic New World screwworm lines were made and evaluated. The initial lines showed either partial or no decrease in female viability when raised on standard diet. Based on data obtained at NCSU from transgenic *Drosophila melanogaster* and *Lucilia cuprina*, the single component system was modified from that described in the grant application. To date, one transgenic New World screwworm line has been obtained with the modified system and this has been bred to homozygosity. On standard diet 98-100% of the females die. On diet supplemented with tetracycline equal numbers of males and females develop. Events: Dr Scott gave a presentation on this project at the XXIV International Congress of Entomology" in Daegu, South Korea, August 19-25, 2012. The title of the presentation was "Development of "male-only" strains of the New World screwworm fly, *Cochliomyia hominivorax*". Dr Scott also gave a talk at the Project Director's meeting for the Biotechnology Risk Assessment Grants (BRAG) Program (June 5-6, 2012) in Riverdale, MD. The title of the talk was "Development and evaluation of male-only transgenic strains of the New World screwworm fly" Dissemination: In May (21-25) 2012, Dr. Scott visited the New World screwworm mass rearing facility in Pacora, Panama and met with several staff from USDA-APHIS and USDA-ARS. Discussions focused on the future development of male-only strains and the potential advantages of transgenic strains for the genetic control program.

PARTICIPANTS: Dr. Maxwell J. Scott (PD). Designed gene constructs, analyzed data and supervised Dr. Li. Dr. Felix Guerrero (co-PD). Supervised Dr. Palavesam and analyzed data. Dr. Fang Li. (postdoctoral fellow) Made all of the FL series of single component gene constructs. Generated transgenic *Drosophila melanogaster* and *Lucilia cuprina* carrying the FL transgenes and evaluated female viability on standard diet and diet supplemented with tetracycline. Analyzed data. Dr. Azhahianambi Palavesam (postdoctoral fellow). Generated transgenic New World screwworm lines with the FL gene constructs. Supervised the Panamanian technicians who bred lines to homozygosity and performed female lethality tests on diet +/- tetracycline. Analyzed data. Partner Organization: USDA-ARS. The co-PD (Guerrero) works for the USDA-ARS as do the technicians in Panama (not supported by this grant) who rear the transgenic New World screwworm lines. **TARGET AUDIENCES:** Target Audiences: The development of male-only strain of the New World screwworm would provide several advantages for the ongoing sterile insect program that is run by USDA-APHIS. For example, diet costs should be lowered if female lethality occurs early in development (ie diet is only consumed by males). Further, sterile male-only releases would improve the efficiency of population suppression. In the long term, cattle ranchers and other livestock owners would benefit from a more efficient genetic control program. Efforts. Dr. Scott is a co-instructor for a new graduate level course offered at NCSU called " Principles of Genetic Pest Management". The course is being offered in the fall semester 2012, beginning on August 16, 2012. Thirteen graduate students (mix PhD and MS) from a wide range of disciplines from both sciences and humanities are enrolled in the course (eg Entomology, Economics, Communication). The course is required for the six doctoral students supported by the new NSF IGERT program on "Genetic engineering and society: the case of transgenic pests". **PROJECT MODIFICATIONS:** Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

Change in knowledge: One of the initial single component gene constructs (FL3) was more effective at inducing female lethality in *Drosophila* than in blowfly species (New World screwworm and *Lucilia cuprina*). This was at first surprising as most of the parts of the gene construct were of blowfly origin. However, one part of the gene construct was of *Drosophila* origin, providing a possible explanation for our observations. **Change in action:** The FL3 single component gene construct was modified taking into account the knowledge gained from our initial experiments. Specifically the part from *Drosophila* was replaced with a functionally equivalent part from *Lucilia*. The modified single component system is significantly more effective at reducing female viability in New World screwworm and *Lucilia cuprina*.

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

No publications reported this period

ASSESSING THE IMPACT OF GENETICALLY MODIFIED METARHIZIUM ANISOPLIAE

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Award Number: 2011-33522-30742

NON-TECHNICAL SUMMARY: Environmental change, resistance to insecticides and the rising human population have all contributed to increasing problems from insects. Genetically modified (GM) fungi, particularly *Metarhizium* spp, represent a major new arsenal for combating pests and diseases. However, for these tools to be used safely and effectively, we need a better understanding of what could happen to GM fungi in the wild. In this study, we will assess the impact of GM fungi on all functionally known geochemical, ecological and environmental processes in the soil. We will also perform field tests on genetic modifications that are designed to ensure that a gene introduced into a microbe to boost its performance could never escape and spread to other microbes. The resulting genetically modified microbe would combine fitness with a level of safety greater than that of inefficient non-genetically modified biocontrol agents.

OBJECTIVES: Genetic engineering has greatly increased the insect killing power of the model pathogen *Metarhizium robertsii*. Before deploying such technology, risk assessment requires that we can examine the transformed genotype in its interactions with its environment. In previous USDA-BRAG funded work, we modeled dispersal scenarios following introduction of the fungus to grasslands (turf) and agricultural (winter wheat) field sites. In this study, we propose to determine how an intensive deployment of wild type and transgenic *M. robertsii* impacts ecological processes in natural and agricultural environments. Using this background information we will field test individual failsafe systems based on site specific recombination and terminator strategies. We will determine whether genetic containment can be relied on in field conditions to limit off site dispersal, persistence, pathogenic effects and transfer of genes between pathogens. This study will also provide a model for other biocontrol agents potentially allowing them to be used elsewhere in the world while satisfying regulatory, advocacy, or scientific organizations.

APPROACH: In this study we will assess the impact wild type and genetically modified fungi (*Metarhizium robertsii*) have on soil environments using a global-scale microarray methodology that is able to assess all functionally known geochemical, ecological and environmental processes including N, C, S and P cycling, metal reduction and resistance, and organic xenobiotic degradation. We will also test strategies that provide genetic containment for a "first generation product". We will: 1) block production of spores on cadavers and mitigate spread of/and gene flow from transgenically hypervirulent organisms by inserting sporulation genes in the antisense configuration in tandem with virulence-enhancing genes, and 2) use recombinases to eliminate sporulation genes or transgenes when the fungus has passed through an insect. The impact of this research is expected to extend far beyond *M. robertsii* in: 1) providing insight into

the intimate relationships between genes, organisms and the environment; 2) providing a model for analyzing microbial communities, metabolic potential, and diversity in the rhizosphere, and 3) permit informed risk assessment and testing of containment methods.

DEVELOPING A FRAMEWORK FOR ASSESSING THE RISKS OF IN PLANTA RNAI ON NON-TARGET ARTHROPODS

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Award Number: 2011-33522-30749

NON-TECHNICAL SUMMARY: One of the biggest hurdles to developing RNAi as pest management tool is the lack of a formalized ecological risk assessment. We believe that an essential component of estimating risk of this technology will involve toxicity testing under a worse-case scenario of exposure that is similar to the testing currently used for transgenic plants that express Bt toxins and chemical pesticides. The studies described in this proposal are designed to answer questions directly pertaining to the risk of RNAi to non-target arthropods that are at greatest risk of exposure because of a shared environment and common molecular targets as measured by DNA sequence. The early-tier testing methods described in this proposal are intended to measure specific risk endpoints under a worst case scenario and not meant to reflect real-world exposures. Our studies will enhance the likelihood of the identification of potential hazards involved with the RNAi maize. The overall goals of this proposal are to adopt the current ecological risk assessment (ERA) framework developed for *Bacillus thuringiensis* (Bt) crops and integrate it into the risk evaluation process of insect-resistant crops that employ RNA interference (RNAi) for insect control. Specifically we propose to establish a standardized protocol to evaluate the potential hazards of RNAi crops to non-target arthropods, to test risk hypotheses at early-tier assessments. From this information we will develop credible risk evaluations for the scientific community and regulatory agencies to provide guidance for the future risk analyses at advanced tiers.

OBJECTIVES: The overall objective of this proposal is to adopt the current ecological risk assessment (ERA) framework developed for *Bacillus thuringiensis* (Bt) crops and integrate it into the risk evaluation process for insect-resistant crops that employ RNA interference (RNAi) for insect control. Specifically we propose to establish a standardized protocol to evaluate the potential hazards of RNAi crops to non-target arthropods, and to test risk hypotheses at early-tier assessments. From this information we will develop credible risk evaluations for the scientific community and regulatory agencies to provide guidance for the future risk analyses at advanced tiers. To achieve our overall goals, we will pursue the following specific objectives:(1) Clone and sequence vacuolar ATPase (V-ATPase) subunit a, the target gene for control the western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae), with transgenic RNAi maize from selected surrogate species of non-target arthropods representing different habitats and diverse ecological functions.(2) Develop a standard in vivo RNAi-toxicity test using a worst case scenario.(3) Carry out early-tier risk assessment of arthropod-resistant transgenic RNAi maize on selected non-target arthropods using the RNAi-toxicity test developed in Objective 2.

APPROACH: Objective 1: The working hypothesis for this objective is that the primary structure of housekeeping genes such as V-ATPase is highly conserved across diverse taxonomic groups. This hypothesis will be tested by using well-established / standardized cloning protocols, mainly RACE (rapid amplification of cDNA ends)-PCR, to obtain the complete open reading frames (ORFs) of V-ATPase subunit-a in selected surrogate non-target arthropods, followed by identification of a precise V-ATPase region to target for subsequent RNAi-toxicity testing using readily available bioinformatics tools. Objective 2: The working hypothesis for this objective is that homology, dose, and exposure are the key factors that define the worst case scenario for the RNAi-toxicity test. In such tests, sequence homology between target and non-target arthropods of arthropod-active genes dictates the specificity of transgenic RNAi maize. This hypothesis will be tested by performing both dose- and homology-response RNAi analyses to determine the worst case scenario which will be followed by optimization of dietary RNAi for selected surrogate species and testing stages. The RNAi response, i.e., risk assessment endpoint, will be measured at the transcriptional level using a readily available qRT-PCR and at translational level using a commercially available polyclonal antibody. Objective 3: This is the final stage of proposed study and is intended to test the early-tier risk hypothesis and make a recommendation for the future risk assessment and risk analysis of transgenic RNAi maize. The risk hypothesis is that the active ingredient of the arthropod-resistant RNAi maize has no transcriptional and/or translational impacts on non-target arthropods under the worst case scenario. This hypothesis will be examined in each selected surrogate species and testing stages using a standard RNAi-toxicity test developed in Objective (2).

PROGRESS: 2011/09 TO 2012/08

OUTPUTS: The Aims of objective-1 include 1) selection of appropriate surrogate species for non-target arthropods that represent different habitats and diverse ecological functions within the transgenic RNAi maize system, and 2) Clone and sequence V-ATPase subunit A, which is the target sequence for transgenic maize expressing dsRNA, from surrogate species selected in Aim-1. The Working Hypothesis is that the primary structure of housekeeping genes such as V-ATPase is highly conserved across diverse taxonomic groups. For the first year, we have completed both aims. Based on reviewers' suggestions, we removed European corn borer, *Ostrinia nubilalis*, a non-target herbivore, from the list of surrogate species. For Aim-2, we have successfully obtained the complete open reading frames (ORFs) of V-ATPase subunit-A in selected surrogate non-target arthropods (NTAs) using RACE (rapid amplification of cDNA ends)-PCR and standardized molecular cloning techniques. A 400bp region which has the highest sequence similarity among target and nontarget surrogate species has been selected as the target region for the subsequent in vivo RNAi toxicity assay. **PARTICIPANTS:** This proposal represents a collaborative effort among scientists from the University of Kentucky (PD Zhou) and University of Nebraska (Co-PD Siegfried). Funding from this proposal currently supports a post-doctoral research associate at the University of Nebraska and two research technicians at the University of Kentucky. **TARGET AUDIENCES:** Agricultural biotechnologists in academia, industry, and federal regulatory agencies. Ultimately, information acquired from this research should be disseminated to general public through risk analysis and risk communication processes. **PROJECT MODIFICATIONS:** Nothing significant to report during this reporting period.

IMPACT: 2011/09 TO 2012/08

Although we are only 1 year into a three year project, we are on track to complete all the stated objectives which will provide answers to questions directly pertaining to the potential risk of RNAi crops to non-target arthropods within the transgenic RNAi maize system. The conclusion of our initial cloning efforts of the V-ATPase subunits allows us to develop the individual in vivo RNAi toxicity bioassays for selected NTAs respectively which will provide molecular evidence for the evaluation of early-tier risk hypotheses. Our preliminary findings have been reported at the XXIV International Congress of Entomology (Zhou and Siegfried, Developing a framework for assessing the risks associated with RNAi crops on non-target organisms, Invited symposium talk, Deagu, Korea, August 19 - 25, 2012).

PUBLICATIONS (not previously reported): 2011/09 TO 2012/08

No publications reported this period

ANTIBODY-BASED PARATRANSGENICS FOR PIERCE'S DISEASE: ADVANCED METHODS FOR TRANSMISSION BLOCKING AND ENVIRONMENTAL MONITORING

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Award Number: 2012-33522-19935

NON-TECHNICAL SUMMARY: Paratransgenic strategies are under development for control of Pierce's disease transmission by sharpshooter vectors. Critical elements of the approach have been refined. *Pantoea agglomerans*, a symbiotic bacterium of *H. vitripennis* that maintains physical proximity to the causative agent *Xylella fastidiosa*, has been characterized and genetically transformed. Putative antibodies have been designed to disrupt *Xylella* transmission. Field dispersal approaches for genetically modified *Pantoea* are being modeled. The paratransgenic platform might have far-reaching applications in control of agricultural vector-borne diseases and similar approaches are under development for control of whitefly, thrip and locust-borne diseases. Field application of these technologies- still a future prospect- mandates rigorous risk assessment and mitigation strategies. The team of Durvasula, Kang and Miller has been pioneering paratransgenic approaches for over a decade. In this proposal, we introduce a dual risk mitigation strategy involving (1) an entirely novel molecule, the REDantibody, as a tool for both transmission blockade AND environmental monitoring of GMO spread and (2) a novel bioencapsulation approach with tunable nano-materials for containment of GMO release into the environment. The unique properties of this antibody- stability, embedded fluorescence, visual colorimetric detection and adaptability to a variety of pathogen repertoires- will be coupled with advanced microencapsulation techniques for bacterial containment and transgene stability to create a singular technology that could propel field application of paratransgenic control for Pierce's disease and, perhaps, other devastating vector-borne diseases of agriculture, worldwide.

OBJECTIVES: The overall aim of this four-year collaborative project involving University of New Mexico, Queen Mary College University of London in the UK and University of California at Riverside is to develop an advanced paratransgenic approach with recombinant antibodies directed at the transmission of *Xylella fastidiosa*, the causative agent of Pierce's disease, by the arthropod vector, *Homalodisca vitripennis*. This proposal will build upon ongoing paratransgenic studies of *H. vitripennis* (USDA/CSREES BRAG 2004-39454-15205, USDA/APHIS 08-8500-0510-GR and USDA/BRAG 2010-33120-21852) with particular focus on (1) development of recombinant antibodies that disrupt transmission of *Xylella* (2) engineering fluorescent antibodies that permit environmental monitoring of genetically modified symbiotic bacteria of *H. vitripennis* and (3) incorporation of microencapsulation technology to minimize unwanted environmental release of genetically transformed bacteria. An Environmental Monitoring System (EMS) for engineered organisms via embedded fluorescent tags will be developed as a platform technology that can be used in a variety of transgenic and paratransgenic systems for control of vector-borne agricultural diseases. This risk mitigation strategy will derive from the recently

patented affinity fluorescent protein REDantibody described by two of the investigators on this proposal (Durvasula and Kang). The coupling of the Environmental Monitoring System (EMS) with second-generation bioencapsulation techniques (derived from USDA/BRAG 2010-33120-21852) will result in the most advanced paratransgenic approach to date. This dual risk mitigation strategy, aimed at reducing environmental spread of transgenic organisms while providing robust tools to track their release and spread, is designed for field applications of paratransgenic control. Indeed, the Pierce's Disease application will be the prototype for this method of pathogen control. However, several other paratransgenic strategies aimed at agricultural and human diseases, under development in our laboratories, will be propelled toward field use as a result of this platform. Specific Aim 1: To express a recombinant REDantibody that recognizes key surface-exposed epitopes of *Xylella fastidiosa*, causative agent of Pierce's Disease, via *Pantoea agglomerans* E325, a symbiotic bacterium of the Glassy Winged Sharpshooter (GWSS), *Homalodisca vitripennis* Specific Aim 2a: To demonstrate, in closed-cage settings, the uptake of *P. agglomerans* transformed with REDantibody by *H. vitripennis* and colonization of the anterior cibarium of the arthropod Specific Aim 2b: To demonstrate, in closed-cage settings, the efficacy of selected REDantibodies in blocking transmission of *X. fastidiosa* by *H. vitripennis* Specific Aim 3: To develop a dual risk mitigation strategy that employs (1) fluorescent and colorimetric properties of REDantibodies within an Environmental Monitoring System (EMS) that tracks genetically modified bacteria in the rhizosphere and (2) second generation bioencapsulation technology using gated nano-materials to prevent escape of engineered bacteria into the environment

APPROACH: In the first set of experiments we will select single chain antibody (scFv) candidates that target *X. fastidiosa* MopB and other surface proteins. We will verify that: (1) the scFv is expressed and assembled by *E. coli* (2) selected scFv will be converted to REDantibody format for expression and secretion in *E. coli* (3) the selected REDantibody constructs will be transferred to *P. agglomerans* and tested for stability and (4) the expression and secretion of REDantibody from *P. agglomerans* will be determined. This is the first attempt to map the surface of *X. fastidiosa*. In the second series of experiments, we will deliver recombinant *P. agglomerans* (rec. Pa) to *H. vitripennis* and evaluate the efficacy of the REDantibody in blocking transmission of *X. fastidiosa*. We will use a contained facility at UC Riverside in which Chardonnay grape vines will be treated with transformed *P. agglomerans* and subjected to infestation by GWSS. In these experiments, we will evaluate (1) the uptake and localization of recombinant *P. agglomerans* in the cibarium of GWSS (2) persistence of recombinant *P. agglomerans* in the cibarium of GWSS after initial exposure to the bacteria (3) impact of REDantibody on development and transmission of *X. fastidiosa* by GWSS. In the final series of experiments, we will evaluate selected bacteria that comprise the rhizosphere of Chardonnay grape plants. Furthermore, dispersal studies will involve contained environments only. In all studies, we aim to compare direct visualization of bacterial populations with more advanced molecular and fluorescent techniques to optimize an Environmental Monitoring System (EMS). We will quantify (1) survival and dispersal of pSCR-189b-transformed *P. agglomerans* in the soil (2) extent of horizontal gene transfer between pSCR-189b-transformed *P. agglomerans* and the bacteria commonly found in rhizosphere consortia. We will then repeat these trials using the alginate-chitosan microencapsulation approach, with CRUZIGARD resin to

coat shoots of grape plants, with the expectation that release of bacteria into the rhizosphere and HGT will be greatly reduced or eliminated.

RISKS FROM FIELD-EVOLVED RESISTANCE TO BT CORN BY WESTERN CORN ROOTWORM

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Award Number: 2012-33522-20010

NON-TECHNICAL SUMMARY: Transgenic corn producing insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt) has been adopted rapidly by farmers, providing control of key insect pests and reducing use of conventional insecticides. The development of Bt resistance by pests is the single greatest threat to this technology. A key pest controlled by Bt corn is the western corn rootworm *Diabrotica virgifera virgifera*. Beginning in 2009, field populations of western corn rootworm were identified in Iowa with resistance to corn that produces Bt toxin Cry3Bb1. Because resistance may spread and develop independently in other regions of the United States, these first cases of resistance present a risk Bt corn containing Cry3Bb1 and to other types of Bt corn, if resistance to Bt toxin Cry3Bb1 also confers resistance to other commercialized Bt toxins. Additionally, these first cases of resistance highlight the potential vulnerability of other types of Bt corn targeting western corn rootworm. Currently, there is uncertainty about how to best measure resistance and the rate at which resistance will spread. A multidisciplinary research team has been assembled to address these risks and uncertainties. We are proposing research that is directly relevant to the biotechnology risk assessment grant's stated purpose of generating new information to assist federal regulatory agencies in making science-based decisions. We will determine the best method for measuring resistance and will test for cross resistance to other Bt toxins. The potential for resistance to spread and for the independent evolution of resistance will be evaluated in part by measuring whether or not, in the absence of Bt corn, Bt-resistant insects are at a disadvantage relative to susceptible insects (i.e. whether or not fitness costs of resistance are present). We also will measure the inheritance of resistance to understand how effective refuges may be at delaying resistance. We will apply multiple genomic tools to develop molecular markers and to identify genes associated with resistance, which will enable monitoring and detection of resistance before it reaches levels that threaten Bt corn in additional fields.

OBJECTIVES: A multidisciplinary team has been assembled that will study Bt resistance from the level of the gene to the population, and in doing so, will assess the risks that field-evolved resistance to Cry3Bb1 corn presents to single-trait and pyramided-trait technologies. These goals will be accomplished by completing the following objectives. 1) Measure resistance and cross resistance in the laboratory and in the field; 1a) Test which of several laboratory methods best characterizes resistance in the field; 2b) Measure resistance and cross resistance in fields with a history of cultivation of Bt corn and documented injury to Bt corn by western corn rootworm; 2) Measure inheritance of resistance and fitness costs of resistance for strains of Cry3Bb1-resistant western corn rootworm collected from the field; 3) Conduct quantitative trait locus analysis

based on single nucleotide polymorphisms to identify candidate genes and markers associated with Bt resistance in western corn rootworm; 4) Analyze the midgut transcriptome for western corn rootworm strains with field-evolved Bt resistance 4a) test for the mechanisms of resistance; 4b) develop molecular markers Outputs from this research will include field experiments, laboratory experiments, and molecular analysis. Through objective 1, we will conduct experiments in fields with Bt-resistant western corn rootworm to test the level of resistance and cross resistance, which will directly assess the risk to other single traits and pyramided traits. Furthermore, by testing several laboratory and greenhouse bioassays we will provide critical information on how to best characterize resistance. In objective 2, we will conduct laboratory experiments with field-collected, Cry3Bb1-resistant strains to test the inheritance of resistance and to measure fitness costs of resistance, both of which will quantify risks associated with the persistence of resistance in the field, and the rate at which resistance may spread or evolve independently in other populations. Molecular markers developed through objectives 3 and 4 will enable resistance to be monitored in the field before it reaches levels that cause field failures. Elucidating the molecular basis of resistance also will provide a better understanding of potential cross resistance and provide fundamental knowledge that may be applied to combat resistant populations with new technologies.

APPROACH: During years 1 and 2 of the project, a range of bioassays will be used to test resistance of western corn rootworm to Bt corn. Methods will include single plants held in a growth chamber, seedling mats held in a growth chamber, potted plants held in the greenhouse, and diet-based bioassays. Survival in bioassays will be analyzed with analysis of variance, and for diet-based assays, calculations of LC50s. During years 2 and 3 of the project, we will conduct on-farm research in six of the problem fields evaluated with laboratory bioassays. Currently, there is not a standard plant-based assay used by academic scientists or industry, and there is a lack of comparative data for bioassays, including side-by-side comparison of bioassay methods and comparisons of different Bt events in bioassays. Importantly, few data link bioassay results with injury in the field. Data will provide critical information on cross resistance among Bt events and will provide EPA and industry with a clear assessment of the merits of various bioassays for resistance monitoring. We will measure fitness costs of resistance using strains with field derived resistance to Cry3Bb1 corn. Strains will be reared on non-Bt potted plants in the greenhouse. We will measure survival to adulthood, head capsule width, longevity, fecundity and egg viability. We will conduct selection experiments to measure fitness costs by measuring the level of resistance to Cry3Bb1 corn over time, with declines in resistance demonstrating the presence of fitness costs. Data will be analyzed with standard parametric statistics such as analysis of variance. For Bt crops, the inheritance of resistance traits is a critical factor affecting the rate of resistance evolution. As the recessive nature of a resistance trait increases, the delays in resistance expected under the refuge strategy become greater, with the longest delays expected for resistance traits that are completely recessive. Understanding fitness costs of resistance is directly relevant to resistance management policy because fitness costs will delay the evolution of resistance and its subsequent spread. To understand the genetic basis of Cry3Bb1 resistance, a pedigree-based QTL mapping approach will be used. GBS in tandem with high-throughput Illumina HiSeq 2000 v. 3 sequencing is expected to generate ~1.0 million reads per individual. Two experiments will test differences in gene expression using high-throughput RNA-Seq

methods. Experiment 1 will identify constitutive differences in gene expression between resistant and susceptible western corn rootworm strains when they feed non-Bt corn. Experiment 2 will identify genes that are differentially regulated in larvae from resistant strains fed Cry3Bb1 corn. Understanding the mechanism(s) of resistance to Cry3Bb1 toxin will be crucial to future resistance management and mitigation strategies. When resistance alleles first occur in a population, they are at low frequency and are challenging to detect via traditional bioassay methods. Molecular markers for resistance genes will enable routine monitoring of populations and detection of resistance alleles prior to control failure.

TALEN-MEDIATED CHROMOSOME TARGETING FOR MONOSEXING AND GENETIC CONTAINMENT IN LIVESTOCK

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Award Number: 2012-33522-19766

NON-TECHNICAL SUMMARY: Genetic engineering could provide for dramatic improvements in the sustainability of agricultural animal production. The use of genetically engineered animal products would be facilitated by methods that 1) reduce the risk of GE animals to the environment; 2) reduce the potential for transfer of transgenes beyond breeding stock; and 3) demonstrate that GE animals will not have an effect on wild species. We hypothesize that editing of the pig genome can be used to develop lines of animals that either produce only females, or lines of pigs that fail to undergo sexual maturation unless managed in a breeding facility with pre-established protocols for puberty-induction. Monosexing and infertility can be used to effectively control the dispersion of genetics from engineered animals. Such control will facilitate the introduction of engineered animals into the U.S. Biomedical and Food Agriculture marketplace.

OBJECTIVES: Genetic engineering can be used to develop lines of pigs that either produce only females, or that are incapable of undergoing sexual maturation without intervention. We will use TAL-effector nucleases (TALENs) to direct either single-basepair changes, or to direct integration of expression cassettes to specific genetic loci in pig genome. In our first aim we will assess two loci on the swine Y-chromosome for their target-ability and amenability to express transgenes intended to disrupt male sperm function. Boars containing this modification should only be capable of producing daughters. For aim two we will implement a method for TALEN-mediated, reversible sterilization based on targeted inactivation of a gene required for maturation. Pigs treated in this way are predicted to remain pre-pubertal and infertile unless treated with a compound to induce sexual maturation. The consequence will be that the animals will only be able to be propagated in a breeding facility with pre-established protocols for puberty induction.

APPROACH: We will develop molecular tools, including gene editing technologies, that are intended to make modifications to the swine genome in cultured swine cells. Cells will be treated with these molecular tools and will be analyzed using such molecular techniques as PCR, Southern analysis, and sequencing. Cells containing the desired modifications will be cloned to produce pigs, which will be maintained and bred by standard husbandry practices. Special attention will be paid to the efficiency and precision of the genome modification technologies under investigation.

GENOMIC APPROACHES FOR BT RESISTANCE RISK ASSESSMENT AND IMPROVEMENT OF REGULATORY TRIGGERS

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Award Number: 2012-33522-19793

NON-TECHNICAL SUMMARY: The US government views insecticidal properties of the bacterium, *Bacillus thuringiensis* (Bt), as a "public good" and has taken actions to ensure that toxin genes from this organism that are moved into transgenic crops are used in a manner that decreases the risk of pests evolving resistance and eroding this public good. Organic farmers have used this special insecticide for over 50 years. The US-EPA and USDA concluded that monitoring for resistance could improve resistance management practices and decrease resistance risk. However, current monitoring methods are inadequate. We will use new genomic tools in concert with field data to better assess both the current extent of Bt resistance in *Heliothis virescens* and *Helicoverpa zea* moths and the rate at which resistance is increasing, if at all. This will be accomplished by developing tools that detect changes in the frequencies of alleles of candidate Bt resistance genes and also detect changes in genetic sequences that confer Bt resistance but are not in genomic regions associated with currently identified Bt resistance candidate genes. We have annually archived thousands of samples of *H. virescens* and *H. zea* from 1993 until 2011, and will use these valuable samples to predict future changes in resistance from past patterns of change and current planting patterns of Bt cultivars. While the proposed research focuses on Bt resistance, the tools developed could also be used to improve monitoring of resistance to future insecticidal crop traits as well as for monitoring weed resistance relevant to transgenic, herbicide tolerant crops.

OBJECTIVES: We will use genomic tools in concert with field data to better assess both the current extent of Bt resistance and the rate at which resistance is increasing, if at all. This will be accomplished by developing genomic tools that detect changes in the frequencies of alleles of candidate Bt resistance genes. Furthermore, the genomic techniques developed will be able to detect changes in genetic sequences that confer Bt resistance but are not in genomic regions associated with currently identified Bt resistance candidate genes. This work will be possible because our lab has been archiving samples of *H. virescens* and *H. zea* from Mississippi and Louisiana from 1993 to the present. Other areas have been sampled, but not as regularly. Our lab-strains of *H. virescens* with known genes for resistance to Bt toxins will serve as positive controls for the robustness of the genomic techniques. Specific Goals: 1) Address the untested hypotheses that; a) field populations of *Helicoverpa zea* are accumulating Bt resistance genes more rapidly than populations of *Heliothis virescens*, b) the rate at which Bt resistance is evolving in *H. zea* has decreased since the introduction of dual toxin cultivars, c) in both *H. virescens* and *H. zea* alleles for resistance to pyrethroids have decreased in frequency. 2) Assess the risk of future emergence of economically important Bt resistance in field populations based on time series analysis of allele frequencies from archived material and the newly implemented

resistance management requirements for multi-toxin Bt cultivars. Supporting Objectives: 1) Determine if there have been changes in the frequency of alleles of any candidate genes for Bt or pyrethroid resistance in *H. zea* and *H. virescens* between 1993 and 2012. 2) Determine if there are genomic signatures of response to Bt selection in regions of the genome that are not associated with Bt resistance candidate genes. 3) If genomic changes are found in *H. zea* based on objectives 1 and/or 2, collect surviving larvae from Bt and non-Bt corn to determine if survivors from Bt corn show enrichment in specific alleles. 4) Use all data from objectives 1, 2, and 3 to predict future levels of resistance to Bt cultivars.

APPROACH: Our objectives will be accomplished by developing tools that detect changes in the frequencies of alleles of candidate Bt resistance genes and also detect changes in genetic sequences that confer Bt resistance but are not in genomic regions associated with currently identified Bt resistance candidate genes. The RADtag methodology will be used for this part of the research. We have annually archived thousands of samples of *H. virescens* and *H. zea* from 1993 until 2011, and will use these valuable samples to predict future changes in resistance from past patterns of change and current planting patterns of Bt cultivars.

AN ADAPTIVE FRAMEWORK FOR NON-TARGET RISK ASSESSMENT OF RNAI-BASED, INSECT RESISTANT GM CROPS

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Award Number: 2012-33522-19728

NON-TECHNICAL SUMMARY: Unique aspects of RNAi-based insect resistant crops challenge the current approach to risk assessment of genetically modified (GM) crops to non-target organisms. One primary concern is that the stability of double-stranded and small interference RNA (dsRNA and siRNA; the basis of RNAi) within the environment is unclear and it is unknown what components of food webs within agroecosystems are directly exposed to the dsRNA. This is particularly important because the small nucleotide sequences targeted by specific siRNAs can be expressed by many organisms, thus dramatically increasing the number of potential targets of the insecticide relative to Bt-based GM crops and pesticides. In short, current knowledge gaps prevent predicting which species are actually at risk of toxicity. We will pair novel genetic methods for examining food webs and genome sequencing with traditional approaches used to establish exposure pathways to develop an exposure-based framework for assessing which species are at risk of ingesting insecticidal RNAi, especially as produced by GM crops. The overall goal of this proposal is to determine the likelihood of exposure to and toxicity of interference RNA to a corn-based arthropod food web. Specifically, this research will establish which species are at risk through consuming dsRNA containing corn tissue under field conditions, and whether dsRNAs are transferred to higher trophic levels via consuming herbivorous prey. The research will establish crucial infrastructure that can be used to establish risk of both existing RNAi-based GM crops and pesticides as well as future constructs. Specific Objectives: 1) Use PCR-based gut content analysis to establish trophic linkages to corn within an arthropod community, 2) Establish whether dsRNA passes to higher trophic levels (predators and parasitoids) via consuming herbivores, and 3) Sequence the genomes of key taxa from corn to determine whether sequence homologies exist that place these organisms at risk from crop-produced dsRNA. Diets of a corn arthropod community will be analyzed by searching in their stomachs for corn DNA. From this, a food web will be created that can help predict which species are exposed to GM corn plants and RNAi. Next, in the laboratory we will feed RNAi to plant-feeding insects (mites, caterpillars, and aphids), and determine whether the RNA persists in the herbivores and can thereby affect predators and parasitoids. Based on these datasets, we will select five species that are highly exposed to RNAi-expressing corn plants, and sequence their entire genomes. From this, we can predict whether these species are at risk of non-target effects of new RNAi molecules. This project will produce the necessary infrastructure to evaluate the exposure and potential toxicity of future RNAi-based GM crops and pesticides to a suite of ecologically relevant non-target species in a format that is adaptive and transparent to the public.

OBJECTIVES: The overall goal of this proposal is to determine the likelihood of exposure to and toxicity of interference RNA to a corn-based arthropod food web. Specifically, this research

will establish which species are at risk through consuming dsRNA containing corn tissue under field conditions, and whether dsRNAs are transferred to higher trophic levels via consuming herbivorous prey. The research will establish crucial infrastructure that can be used to establish risk of both existing RNAi-based GM crops as well as future constructs. Specific Objectives 1. Use PCR-based gut content analysis to establish trophic linkages to corn within an arthropod community. 2. Establish whether dsRNA passes to higher trophic levels (predators and parasitoids) via consuming herbivorous prey. 3. Sequence the genomes of key taxa from corn to determine whether sequence homologies exist that place these organisms at risk from crop-produced dsRNA.

APPROACH: Approach. 1) Diets of a corn arthropod community will be analyzed using qPCR-based Gut content analysis to generate a quantitative food web based on taxon abundance, frequency of corn consumption and the quantity of corn DNA consumed. Species most exposed to dsRNA expressed in GM corn will be identified. 2) Insecticidal dsRNA will be administered to three non-target herbivores with different feeding ecologies. These pests will be fed to a suite of relevant natural enemies, whose fitness will be measured. The presence of the dsRNA will be assessed using targeted qRT-PCR. 3) Of species most exposed to the RNAi-expressing corn, specific indicator taxa will be selected from key functional groups and their genomes will be sequenced in conjunction with the i5k project, an effort to sequence 5,000 insect genomes. Sequence homologies between current and future RNAi targets can be screened against this database for targeting non-target toxicity assays.

TRANSMISSION GENETICS OF SORGHUM TO JOHNSONGRASS GENE TRANSFER

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Award Number: 2012-33522-19790

NON-TECHNICAL SUMMARY: Building on prior BRAG-supported results and engaging new resources, integrative genetic, phenotypic, and ecological/evolutionary studies are proposed to provide baseline information about: the expected fate and stability (persistence) of sorghum (*S. bicolor*) transgenes that escape into Johnsongrass (*S. halepense*); the efficacy of genetic techniques to restrict gene transfer such as the targeting of transgenes to specific genomic islands of differentiation that are recalcitrant to interspecific gene flow; and the efficacy of mitigation measures to limit the spread of introgressed transgenes such as linkage to alleles that reduce fitness in the wild. The primary focus of this proposal is BRAG program area 3. Gene Transfer to Domesticated and Wild Relatives, while also addressing elements of program area 1, Management Practices to Minimize Environmental Risk.

OBJECTIVES: Our goal is to reveal the genome-wide transmission genetics of gene transfer between sorghum and Johnsongrass (*S. halepense*), one of the world's most noxious weeds and a paradigm for the potential dangers of crop-weed introgression. Since *S. bicolor* ($2n=2x=20$) and *S. halepense* ($2n=4x=40$) differ in ploidy, gene transfer between these species utilizes unreduced gametes formed by sorghum (reviewed in Warwick and Black, 1983; Tang and Liang, 1988). Using the fully-sequenced reference genotype, BTx623, we have produced *S. bicolor* x *S. halepense* tetraploid F1 hybrids and their F2-selfed progeny that closely mimic the early-generation progeny from natural crosses between these species that would lead to transgene escape. Objectives including genetic mapping will clarify the transmission genetics of each region of the genome in this population; QTL mapping will clarify the relationship of specific chromosomal regions to traits that are important to the fitness of *S. halepense* in the wild; and targeted resequencing will provide complementary fine-scale evidence toward precise delineation of loci or small regions responsible for genomic incompatibilities or QTL effects. The overall outcome of these integrative genetic, phenotypic, and ecological/evolutionary studies will be to provide objective and comprehensive baseline information about the expected fate and stability (persistence) of sorghum transgenes that escape into Johnsongrass; evaluation of the efficacy of genetic techniques to restrict gene transfer such as the targeting of transgenes to specific genomic islands of differentiation that are recalcitrant to interspecific gene flow; and mitigation measures to limit the spread of introgressed transgenes such as linkage to domestication genes or other genes that reduce fitness in the wild (Gressel and Al-Ahmad, 2006).

APPROACH: The overall hypothesis to be tested herein is that there exist specific regions of the *S. halepense* genome that are recalcitrant to gene flow from sorghum, and therefore may be exploited as landing pads for safe transgene release. Such genomic islands of differentiation will

be of especially great interest if they are also accompanied by concentrations of QTLs that differentiate between cultivated sorghum and *S. halepense*, potentially providing dual containment mechanisms by combining fundamental genomic incompatibilities with reduced fitness associated with introgression. Three experiments are proposed: 1. Genetic mapping of *S. bicolor* x *S. halepense* F₂-selfed progeny will elucidate genome-wide transmission genetics in natural crosses that would lead to transgene escape; 2. Phenotyping and mapping will reveal the genomic distributions of traits and underlying gene/QTL loci that may aid in transgene containment by escaped *S. bicolor* alleles conferring reduced fitness to wild *S. halepense* recipients; 3. Re-sequencing of selected genomic regions in *S. halepense* and *S. bicolor* diversity panels will provide complementary fine-resolution evidence of genomic regions that are recalcitrant to gene flow between these species, due to either fundamental genomic incompatibilities and/or reduced fitness associated with introgression. The overall outcome of these integrative genetic, phenotypic, and ecological/evolutionary studies will be objective and comprehensive baseline information about the feasibility of several possible approaches (and combinations thereof) to sorghum transgene containment including the use of chromosomal rearrangements that differentiate among taxa, the targeting of transgenes to other specific genomic locations that are recalcitrant to transfer, and the notion of linking transgenes to domestication genes.

EVALUATING ENVIRONMENTAL IMPACTS OF MATURING AMERICAN CHESTNUT TREES PRODUCED BY TRANSGENIC RELATIVE TO CONVENTIONAL BREEDING

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Award Number: 2012-33522-19863

NON-TECHNICAL SUMMARY: This project will evaluate environmental impacts of maturing transgenic American chestnut trees (*Castanea dentata*), one of the most economically and ecologically important tree species in our country's history prior to the advent of chestnut blight. American chestnut was the chosen organism for this study because of its value to the environment, to the timber industry, to agriculture, and because it is an American heritage tree species. Over three billion American chestnuts trees were lost to due to the introduction of the chestnut blight into the U.S. from Asia. Over a century of research has failed to restore this giant of the eastern forests, but modern biotechnology techniques are offering a new hope by allowing researchers to add blight resistance genes to the American chestnut. But before these trees can be returned to our forests, tests need to be developed to ensure there are no risks beyond those of traditional breeding. Developing these tests is the main theme of this research, as well as moving the American chestnut closer to restoration. Our previous funded BRAG research on immature chestnut trees established two shelterwood and two open field plots consisting of a standard panel of American, Chinese (*C. mollissima*), hybrid, and backcrosses, as well as several transgenic types of American chestnut trees. The transgenic trees included empty vector controls (transgenic tree with no resistance genes), and trees with single and stacked resistance-enhancing genes. To date, the differences between transgenic and conventionally-bred trees have not exceeded variation found between conventionally-bred cultivars for the environmental impacts evaluated in our previous study. In this follow-up study, the trees will be four years of age and are entering their reproductive age with the production of male catkins and pollen. In the context of this milestone, we will assess 1) gene stability in the transgenic parent trees and their seedling offspring, 2) compare the biochemical content of stems, leaves, pollen, and nuts between the transgenic and conventionally-bred chestnuts, and relate these results to: 3) growth and survival of select plant eating insects 4) decomposition of leaf litter, and 5) growth characteristics of the trees. Our hypothesis is that differences will be seen in the transgenic trees but their environmental impact will not be significantly different than the inherent variation among conventionally-bred trees. The results will inform regulators on the environmental impact of genetically engineered trees.

OBJECTIVES: The long term goal of this project is to develop assessment tools that answer the following question posed in the RFA as it applies to genetically engineered trees: "Is there an effect above and beyond what might occur with an organism that has similar traits, but was developed using other technologies" To make this comparison, we will compare transgenic American chestnut (*Castanea dentata*) trees to a standard panel of trees: the non-transgenic

American chestnut clone derived from tissue culture, wild-type American chestnut trees grown from seed, chestnut hybrid trees (*C. dentata* x *crenata*) and backcross trees (*[C. dentata* x *mollissima]* x *dentata*) produced by traditional breeding techniques, and blight resistant Chinese chestnut (*C. mollissima*) trees. American chestnut was the chosen organism for this study because of its value to the environment, to the timber industry, to agriculture, and because it is an American heritage tree species. The following objective will be accomplished. Objective 1: Determine the stability of transgene expression over time and between generations. Objective 2: Compare metabolite profiles of stems, leaves, pollen, and nuts between the transgenic events, empty-vector transgenic controls, isogenic non-transgenic controls, and the standard panel of conventionally-bred chestnuts. Objective 3: Compare the effects of transgenic trees, empty-vector transgenic controls, isogenic non-transgenic controls, and the standard panel of conventionally-bred chestnuts on the growth and survival of select herbivorous insects and relate any differences to phytochemical and/or nutritional (see Objective 2) variation among genotypes. Objective 4: Examine and compare decomposition of leaf litter between the transgenic events, empty-vector transgenic controls, isogenic non-transgenic controls, and the standard panel of conventionally-bred chestnuts. Objective 5: Examine the growth characteristics of the transgenic events, empty-vector transgenic controls, isogenic non-transgenic controls, and the standard panel of conventionally-bred chestnuts. The objectives in this proposal will be addressed in the context of the BRAG program area #4, "Environmental assessment research designed to provide analysis which compares the relative impacts of animals, plants, and microorganisms modified through genetic engineering to other types of production systems." Comparing the effects of the transgenic, hybrid, and wild-type trees on selected key non-target organisms, as well as analyzing each plant's overall fitness, will allow regulators to determine whether or not the risk to the environment is substantially the same among the regulated and non-regulated trees. The expected outcomes that are being tested are that there will be no significant difference between the transgenic trees and trees modified by conventional means.

APPROACH: Each objective has specific methods outlined in the proposal, such as the use of PCR, RT-qPCR, mass spectrophotometry, insect feeding bioassays, carbon and nitrogen analysis, growth trait measurements, etc. But the overall theme of the analysis is to compare the transgenic chestnut trees to traditionally-bred chestnut trees. The key to this is the establishment of the "standard panel" of non-transgenic trees, which can determine the normal variability of the traits being tested in each objective. The standard panel in this project includes clonal lines of American chestnut that are non-transgenic, but were produced from tissue culture, American chestnut seedlings, hybrid chestnuts (Chinese x American), B1 backcross chestnut ((Chinese x American) x American), and Chinese chestnut trees. The transgenic events being tested included empty vector controls (just selectable and visual marker genes), single, and stacked resistance-enhancing transgenes. Several statistical methods will be used as appropriate to each objective, such as Univariate analysis of variance will be used to assess differences in all traits among the means. Pearson's product moment correlations to assess relationships between traits. Principal component analysis will be used as a multivariate tool to gain an overall understanding of the variation in the traits among the trees in multidimensional space and to identify relationships among traits. Hierarchical cluster analysis will be used to group trees with similar traits. If the traits being tested in the transgenic American chestnut trees fall within the variability of the

standard panel, then the transgenic trees will not pose any additional risk than trees produced by these conventional means. If the trait fall outside the variability of the standard panel, then further assessment would be warranted. This type of analysis will inform regulators of the risk level of the transgenic trees and aid in decisions on deregulation.

GENE FLOW NETWORKS AND POTENTIAL INVASIVENESS OF PERENNIAL BIOFUEL GRASSES (MISCANTHUS)

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Award Number: 2012-33522-19961

NON-TECHNICAL SUMMARY: With the planned introduction of transgenic perennial grasses such as *Miscanthus* as biofuel cultivars, data on the extent and consequences of gene flow via pollen and seeds are needed for environmental risk assessment. Because these crops are relatively new, and because experimental transgenes must be confined during early field trials, it is essential to understand the baseline ecology and genetics of the cultivars and their weedy relatives. In addition, more information is needed to assess the long-term consequences of releasing novel, transgenic cultivars. *Miscanthus* species, which are leading candidates for bioenergy production, are non-native cultivars that are widely grown as ornamentals and have naturalized to become invasive in some areas of the USA. Surprisingly little is known about the population ecology of naturalized populations or their ability to hybridize with bioenergy and ornamental cultivars. The future trajectory of how quickly these taxa will spread is not known, and the current distribution and abundance of feral populations may be in a state of flux and could be affected by the large-scale cultivation of bioenergy cultivars. Ecological information about the potential for *Miscanthus* to become weedy when planted for bioenergy is urgently needed by USDA's Biotechnology Regulatory Services. Industry-sponsored field trials with transgenic *Miscanthus* are ongoing and deregulation could be proposed in the next few years. Seeded, nontransgenic cultivars also could be released soon. Non-sterile *Miscanthus* cultivars with improved agronomic traits are being developed, including transgenic lines field-tested in 2011. Biofuel cultivars may become naturalized and could hybridize with feral and ornamental *M. sinensis* and *M. sacchariflorus*. However, little is known about the ecology and distribution of free-living populations of *Miscanthus*, which we refer to as "feral" or "naturalized" interchangeably. We plan to address the following information gaps regarding feral populations by using field surveys, common garden experiments, and mathematical models that integrate key life-cycle data for these plants, with parallel approaches in Ohio and Iowa: 1) Gene flow characterization, including population genetic structure, ability to hybridize, pollen-limited seed production, and pollen dispersal distances, and 2) Fitness comparisons among feral, cultivar, and hybrid biotypes, including studies of seed germination, seed dormancy, and seed longevity; ability to start new populations in seed addition experiments; and clonal growth and competitive ability. **OUTCOMES** - Our findings will be useful for establishing isolation distances for field trials, managing volunteer plants from field trials, and evaluating larger-scale ecological consequences, if any, of gene flow from biofuel crops to feral populations.

OBJECTIVES: *Miscanthus* offers many advantages as a biofuel crop, but quantitative studies are needed to examine gene flow and the potential for new cultivars to become invasive,

including the potential for transgenic traits to exacerbate weed problems if naturalized plants become too abundant. Industry-sponsored field trials with transgenic *Miscanthus* are ongoing and deregulation could be proposed in the next few years. Seeded, nontransgenic cultivars also could be released soon. Non-sterile *Miscanthus* cultivars with improved agronomic traits are being developed, including transgenic lines field-tested in 2011. Biofuel cultivars may become naturalized and could hybridize with feral and ornamental *M. sinensis* and *M. sacchariflorus*. In the short term, research is needed to provide regulatory agencies with findings that are relevant to the design of small- and medium-scale field trials prior to deregulation. This includes data on sexually compatible, naturalized populations, pollen and seed dispersal, and fitness characteristics of feral and hybrid progeny. The proposed research will help fill this gap by examining gene flow, population dynamics, and the relative competitive ability of *Miscanthus* cultivars, naturalized biotypes, and their hybrids in a variety of locations and environmental conditions. Fitness traits will be measured in both cropping and non-cropping (marginal lands) ecosystems. **EXPECTED OUTPUTS:** Our findings will be useful for establishing isolation distances for field trials, managing volunteers from field trials, and evaluating larger-scale ecological consequences, if any, of gene flow from biofuel crops to feral populations. Information and expertise needed by APHIS-BRS for regulatory oversight of the safe development of transgenic perennial grasses for biofuel production. Specifically, we plan to disseminate our results via scientific presentations, peer-reviewed publications, and information for farmers and natural research managers.

APPROACH: We plan to address the following information gaps regarding feral populations by using field surveys, common garden experiments, and mathematical models that integrate key life-cycle data for these plants, with parallel approaches in Ohio and Iowa: 1) Gene flow characterization, including population genetic structure, ability to hybridize, pollen-limited seed production, and pollen dispersal distances, and 2) Fitness comparisons among feral, cultivar, and hybrid biotypes, including studies of seed germination, seed dormancy, and seed longevity; ability to start new populations in seed addition experiments; and clonal growth and competitive ability. Analyses of population genetic structure will provide a foundation for understanding recent and historical gene flow among feral populations of both *M. sinensis* and *M. sacchariflorus*. We will gain insights into whether feral populations of each species form a cohesive group or include distinctive populations that may have unique origins and phenotypic characteristics. Our results also will serve as a baseline for comparisons between current populations with those that become established near biofuel cultivars in the future, when non-sterile *Miscanthus* is cultivated on a much larger scale. If some cultivars and crop-feral hybrids consistently have lower fitness than feral biotypes, or similar fitness, they are not expected to become invasive under conditions similar to this study. However, if current or future cultivars have higher fitness and greater projected population growth rates, further research could be carried out to learn more about their expected effects on managed and unmanaged habitats, including models that include increased propagule pressure over very large areas where *Miscanthus* could be cultivated.

MOLECULAR GENETIC BASIS OF INSECT RESISTANCE TO BT-CROPS

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Award Number: 2012-33522-19791

NON-TECHNICAL SUMMARY: Since 1996, genetically engineered crops (GE-crops) with insecticidal genes from the soil bacterium *Bacillus thuringiensis* (Bt) (Bt-crops) have been rapidly adopted in the US and the acreage of Bt-crops worldwide has reached near 60 million hectares with proven economic and environmental benefits. However, the widespread adoption of Bt-crops greatly increases the selection pressure for development of insect resistance to Bt toxins, which is the primary risk to the long-term future of Bt-based biotechnology and environmentally sustainable pest management programs. To date, cases of insect resistance to Bt toxins in the field or greenhouses have been reported in six Lepidoptera and one Coleoptera species, and increasing frequencies of Bt-resistant individuals in populations of four insect pests have been observed in regions where Bt-cotton has been planted. Therefore, there is an urgent need to assess and manage the risk of development of Bt-resistance in insects, which critically relies on understanding the genetic basis of Bt resistance evolved in insects in agricultural systems. Identification of the genes and genetic mechanisms conferring insect resistance to Bt-crops is particularly important for effective and efficient monitoring of Bt resistance in insect populations and for development of regulatory framework for insect-resistant GE-crops. However, molecular genetic basis of Bt-resistance has not been identified in any insects that have developed resistance in an agricultural setting, conferring resistance to Bt-crops. *T. ni* is one of the seven insect species that has developed resistance to Bt in agricultural systems upon selection pressure with Bt products. It is a significant pest of agriculture with an exceptionally broad and diverse range of host plants, including at least 160 plants in 36 families. The Bt-resistance mechanism selected in *T. ni* confers resistance not only to Bt toxins on artificial diet but also to Bt-broccoli and commercial Bt-cotton varieties. Therefore, the Bt-resistant strains of *T. ni* provide us with a unique and timely opportunity to identify the molecular genetic basis of insect resistance to Bt-crops. This project is designed to understand the molecular genetic basis of resistance to Bt toxin Cry1Ac in *T. ni* in order to generate new information and provide tools important for assessment and management of the risk of development of insect resistance to Bt-crops in agriculture. Knowledge obtained from this project will assist federal regulatory agencies in making science-based decisions about the effects of introducing into the environment genetically engineered organisms.

OBJECTIVES: Development of Bt-resistance in insect populations associated with the planting of Bt-crops has been reported in five insect pests since the introduction of Bt-crops in agriculture. Moreover, the frequencies of individuals resistant to Bt toxins in populations of four insect pests have been observed to be drastically increased in areas where Bt-cotton has been extensively planted. Evidently, the risk of resistance development in insect populations in response to the widespread adoption of Bt-crops requires urgent attention, and assessment of

resistance development is a very important component of the regulatory framework for insect-resistant GE-crops. We have used the Bt-resistant cabbage looper, *Trichoplusia ni*, populations evolved in commercial greenhouses to establish a unique biological system to study the mechanism of Bt-resistance which is selected in agriculture and confers high-level of resistance to commercial Bt-crops. We have recently identified that the biochemical basis for Cry1Ac resistance in *T. ni* is alteration of APN (aminopeptidase N) expression by a trans-regulatory yet to be known mechanism, and confirmed that the genetic basis for the resistance is different from those currently known in some laboratory-selected insects. With the unique *T. ni* strains established and the research foundation built, we have an unique opportunity to study the molecular genetic basis of Bt resistance evolved in an agricultural system and to identify molecular markers for assessment and management of the risk of insect resistance to Bt-crops, which will contribute important information for regulators to make science-based decisions on GE-crops in agriculture. In this project, we will focus on understanding the molecular genetic basis of resistance in *T. ni* to Bt toxin Cry1Ac, which is the primary insecticidal toxin in current commercial Bt-crops to target Lepidoptera pests, by 1) cloning the ABC transporter gene ABCC2 and determining the association of ABCC2 with Cry1Ac-resistance, 2) identifying genes and molecular markers associated with Cry1Ac-resistance, and 3) identifying mutations and altered expression of midgut genes associated with Cry1Ac-resistance. Understanding on the molecular genetic basis of Cry1Ac resistance in *T. ni* acquired from this project will contribute knowledge towards assessment and management of the risk of Bt-resistance in agriculture.

APPROACH: Our studies have revealed that the resistance to Cry1Ac evolved in *T. ni* is conferred by a mechanism which poses a serious threat to the long-term efficacy of Bt-based biotechnology for insect pest management. The high-level resistance to Cry1Ac in *T. ni* is conferred by down-regulation of the APN1 gene expression in the midgut by a trans-regulatory yet to be understood mechanism and the resistance gene has been mapped to the ABCC2 locus. To identify and validate the gene(s) associated with the resistance, we will approach the question "what is the gene and the genetic mechanism conferring the Cry1Ac-resistance in *T. ni*" using three independent but complementary approaches to reach three objectives. (1) We will identify the ABC transporter gene ABCC2 and characterize its association with Cry1Ac-resistance, to answer the question "Is a mutation and/or altered expression of ABCC2 associated with the resistance". To reach this objective, we will clone the full-length cDNA of ABCC2 from both the susceptible and resistant strains to identify ABCC2 mutations which result in protein sequence changes, determine the ABCC2 expression in the midgut of susceptible and resistant larvae at mRNA and protein levels to identify quantitative alteration of ABCC2 expression, and determine the association of ABCC2 with the resistance, if alteration of ABCC2 in sequence or level of expression is identified in the resistant strain. (2) We will identify genes and molecular markers associated with Cry1Ac-resistance using a genomic approach, to answer the question "What genes or molecular markers are associated with the resistance". To reach this objective, we will take advantage of the Next Generation Sequencing (NGS) technologies to identify genes or DNA markers associated with Cry1Ac-resistance using a genomic DNA sequencing based approach with the focus on genes in the ABCC2 locus region. (3) We will identify mutations and altered expression of midgut genes associated with Cry1Ac-resistance using a transcriptomic approach, to answer the question "What gene mutations and/or altered expressions are associated with the

resistance". To reach this objective, we will use NGS technologies to perform deep sequencing of the midgut transcriptomes from the susceptible, resistant, F1 and Cry1Ac-selected and non-selected backcross progenies to identify mutations/SNPs and altered expression of the midgut genes and their association with the resistance.

Questions for Discussion

1. Strategies for coexistence - open
2. Is deregulation of transgenic becoming more difficult (requiring longer time and more data for deregulation)?
3. Why are there not many transgenic crop plants deregulated in the US in recent years?
4. What are the main concerns for deregulation of transgenic trees?
5. If someone develops a transgenic plant that is deer browsing resistant (can make deer sick but not lethal), how likely will the transgenic plant be deregulated?
6. What are current statuses of herbicide resistant transgenic turf/lawn grasses?
7. Will the USDA or any other regulatory agency govern the commercialization of crop varieties that are developed by inserting foreign genetic material that has no components from pest species?

Appendix: Appropriate Acknowledgment of Your NIFA Award

The Biotechnology Risk Assessment Grant (BRAG) program plays an essential role in fulfilling the mission of the National Institute of Food and Agriculture and the Agricultural Research Service. Proper acknowledgment of your USDA BRAG funding in published manuscripts, presentations, press releases, and other communications is critical for the success of our USDA's programs. This includes proper acknowledgment of the Program and agencies, as well as that of the Department and grant number (Please note that the '####-#####-#####' below refers to your award number and not your proposal number).

We expect you to use the following language to acknowledge NIFA support, as appropriate:

"This project was supported by Biotechnology Risk Assessment Grant Program competitive grant no. ####-#####-##### from the USDA National Institute of Food and Agriculture and Agricultural Research Service."

We also expect that you will use our agency's identifier in all of your slide and poster presentations resulting from your BRAG award. The identifier is sent to you twice annually for at least 2 years after the termination date of your grant.



**United States
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Please alert us of significant findings, publications, news releases, and other media coverage of your work. With your permission, we may highlight your project in a national impact story or news release. If your research is featured on the cover of a scientific journal, we can showcase the cover as well.

Examples of these publications can be found at: www.nifa.usda.gov/newsroom/newsroom.html.

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