United States Department of Agriculture

Biotechnology Risk Assessment Grants Program

Annual Project Director's Meeting



USDA APHIS-BRS
Riverdale, Maryland
June 5, 2014

United States Department of Agriculture
National Institute of Food and Agriculture

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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, 2012, and 2013.

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 In the Food, Conservation, and Energy Act of 2008 (i.e., 2008 Farm Bill), the authority was not repealed, so the BRAG program continued its role in supporting risk assessment research related to biotechnology.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 5, 2014
USDA-APHIS-BRS Headquarters
Oklahoma Memorial Conference Center
4700 River Road
Riverdale, MD 20737

Thursday, June 5, 2014

8:30 - 9:00 AM	**Arrival and Poster Setup**
9:00 - 9:35 AM	Welcome Shing Kwok - USDA-NIFA Janet Bucknall, Associate Deputy Administrator – USDA-BRS
9:35 - 9:55 AM	APHIS: Plant Pest Risk Assessment John Turner - USDA-APHIS
9:55 - 10:15 AM	EPA: Research Needs and Considerations for Plant-incorporated Protectants and Microbial Pest Control Agents Chris Wozniak - EPA
10:15 - 10:35 AM	Environmental Assessment of GE Animals at Center for Veterinary Medicine (CVM) – Food and Drug Administration (FDA) Evgenij Evdokimov – FDA
10:35 - 10:50 AM	**Break**
10:50 - 11:10 AM	The BRAG Program in the Context of USDA Efforts to Strengthen Coexistence Office of Pest Management Policy, Biotechnology Coordinator Michael Schechtman - USDA-ARS, DC
11:10 -11:30 AM	Fostering Coexistence: Industry-driven Field and Landscape Research on Pollen-mediated Gene Flow in Genetically Engineered Alfalfa Doug Walsh- Washington State University

11:30 - 11:50 AM	Environmental Risk Assessment of Perennial Grasses Genetically Engineered for Abiotic Stress Tolerance Hong Lou – Clemson University
11:50 - 1:00 PM	**Lunch - On Your Own**
1:00 - 1:20 PM	Developing a Framework for Assessing the Risks of in planta RNAi on Non-target Arthropods Xuguo Zhou – University of Kentucky
1:20 - 1:40 PM	Evaluating Environmental Impacts of Maturing Transgenic American Chestnut Trees and their Nut Crop Relative to Chestnut Trees Produced by Conventional Breeding William Powell – State University of New York College of Environmental Science and Forestry (SUNY-ESF)
1:40 - 2:00 PM	Gene Flow Networks and Potential Invasiveness of Perennial Biofuel Grasses (Miscanthus) Catherine Bonin – Iowa State University
2:00 - 2:20 PM	Genomic Approaches for Bt Resistance Risk Assessment and Improvement of Regulatory Triggers Fred Gould – North Carolina State University
2:20 - 2:40 PM	**Break**
2:40 - 3:30 PM	Discussion
3:30 - 5:00 PM	Poster Session

POSTERS

1	Auer, Carol	University of Connecticut	Perennial Grasses for Bioenergy: Pollen Aerobiology, Biocontainment, and Plant Genetics
2	Brunet, Johanne	USDA-ARS, Wisconsin	Linking Pollinator Behavior to Gene Flow to Reduce Gene Flow Risk Over the Landscape
3	Carriere, Yves	University of Arizona	Risk Assessment for Insect Resistance to Pyramided Bt Cotton
4	Douches, David	Michigan State University	Assessing the Impact of Gene Replacement and Genetic Modification Methods in a Crop Species at the Whole Genome Level
5	Arora, Arinder	Biomedical Research Institute of New Mexico (BRINM)	Antibody-Based Paratransgenics for Pierce's Disease: Advanced Methods for Transmission Blocking and Environmental Monitoring
6	Egan, Scott	University of Notre Dame	Monitoring the Dispersal of Genetically Engineered Organisms and Their Byproducts Using Light Transmission Spectroscopy
7	Coates, Brad	Iowa State University	Risks from Field-Evolved Resistance to Bt Corn by Western Corn Rootworm
8	Gu, Xingyou	South Dakota State	Silencing of Naturally Occurring Genes Controlling Seed Dormancy to Reduce Fitness of Transgene-Contaminated Weedy Rice
9	Handler, Alfred	USDA-ARS, Florida	Recombinase-Based Transformation Vectors for Improved Transgenic Strain Development and Ecological Safety in Tephritid Pest Species
10	Lauth, Xavier	Center for Aquaculture Technologies	Validation of a Maternally Mediated Sterilization Platform for Reproductive Containment of GE Fish with Initial Application to Tilapia
11	Li, Yi	University of Connecticut	Reducing Root Suckering of Transgenic Poplar
12	Lovett, Brian	University of Maryland	Assessing the Impact of Genetically Modified Metarhizium Anisopliae
13	Mogren, Chrissy	USDA-ARS, South Dakota	An Adaptive Framework for Non-Target Risk Assessment of RNAI-Based, Insect Resistant GM Crops
14	Mandel, Jennifer	University of Memphis	Assessing the Risk of Transgene Escape Via Pollen Flow in Carrot
15	Paterson, Andrew	University of Georgia	Transmission Genetics of Sorghum to Johnsongrass Gene Transfer
16	Scott, Maxwell	North Carolina State University	An Investigation into the Potential Risks of Release of Transgenic New World Screwworm Fly Cochliomyia Hominivorax
17	Stewart, Charles Neal	University of Tennessee	1) Pollen-Mediated Gene Flow in Switchgrass 2) Switchgrass Bioconfinement: Delayed Flowering, Selective Male- And Seed-Sterility, And Conditional Total Bioconfinement
18	Wang, Ping	Cornell University	Molecular Genetic Basis of Insect Resistance to Bt-Crops
19	Yang, Bing	Iowa State University	Genome-Wide Assessment of Off-Target Effect and Removal of Transgenes Associated with TALEN-Based Gene Editing in Plant

CONTENTS

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

Auer, C.; Meyer, T.

University of Connecticut
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 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: 2012/09 TO 2013/08
Target Audience: The target audience is scientists, government risk assessors, land managers, biotechnology companies, crop breeders and related individuals. Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? Training opportunities have been provided to one Postdoctoral Researcher and one PhD student. Professional development has included scientific meetings, workshops and presentations. How have the results been disseminated to communities of interest? One paper describing the model of pollen dispersal has been published in a scientific journal. Posters have been presented at various regional and national conferences. The PI (C. Auer) presented the results to university audiences in Ecuador and an international conference on plant gene flow. This conference was organized by the PI and others to educate scientists and government decision-makers in Ecuador with regards to ecological risk assessment for transgenic crops. What do you plan to do during the next reporting period to accomplish the goals? Work will continue on all research projects to achieve the goals described in the grant proposal.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? In the second year of the project we made progress towards all three major goals. We published a scientific paper summarizing our work on switchgrass pollen biology and a model of pollen dispersal. The objective of this study was to model switchgrass pollen dispersal using a Lagrangian approach informed by data on pollen longevity and size. With outdoor exposure, the viability of pollen from three cultivars declined over 60 min, but rare events showed pollen longevity of up to 100 minutes. To model pollen dispersal, wind fields were measured in two locations to create case studies representing light wind conditions with buoyant turbulence or stronger winds with pressure-driven, nonturbulent

conditions. In the first case study, switchgrass pollen entrained in light wind conditions with buoyancy-driven turbulence moved up to approximately 3.5 km from the source with a maximum flight time of 6000 s. In the second case study, pollen released in stronger winds with pressure-driven conditions moved up to approximately 6.5 km with a maximum flight time of approximately 1300 s. In both cases, the majority of pollen grains were deposited close to the source. These case studies provide information helpful for predicting pollen-mediated transgene flow, isolating field trials, creating containment and coexistence strategies, and conserving valued switchgrass populations in coastal areas and prairies. The second project is working to characterize switchgrass ecotypes in the Northeastern US. Over 200 switchgrass plants have been collected from inland roadside habitats and coastal grassland areas. Switchgrass DNA has been extracted and amplified using 18 microsatellite (SSR) markers. Data analysis is continuing, but preliminary results suggest that the two populations are largely genetically distinct from each other. However, there is evidence for a small amount of migration of coastal plants to inland roadside sites, and a few coastal plants appear to be introduced cultivars (non-native genotypes). Completion of this project and publication are expected in the coming year. The third project is evaluating the ability of a forest windbreak to act as a containment mechanism or barrier to pollen-mediated gene flow. Switchgrass fields were established in 2012 in a open position (control field) or in a plot surrounded by forest trees. In the summer of 2013, data from both fields was collected on pollen source strength, plant phenology, wind patterns and containment of pollen by the forest windbreak. Final biomass and plant trait data will be collected at the end of the growing season (October, 2013). Data analysis is underway leading to a scientific publication in 2014.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08

1. Type: Journal Articles Status: Published Year Published: 2013 Citation: Ecker, G, Meyer, T and C Auer. 2013. Pollen Longevity and Dispersion Models for Switchgrass. Crop Science 53:1120?1127
2. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Auer, C. Plant distribution, gene flow and the question of transgenic crops. Universidad de San Francisco de Quito, Ecuador May 13, 2013
3. Type: Conference Papers and Presentations Status: Other Year Published: 2012 Citation: Auer, C. Ecological risk assessment and gene flow in grasses. Escuela Politecnica de Ejercito, Ecuador October 3, 2012
4. Type: Conference Papers and Presentations Status: Other Year Published: 2012 Citation: Auer, C. Plant distribution, gene flow and the question of transgenic crops. Pontifica Universidad Catolica del Ecuador, Quito, Ecuador November 12, 2012
5. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Lienin, P, Meyer, T and C Auer. The effect of forest borders on wind dynamics, switchgrass pollen aerobiology and growth. Connecticut Natural Resources Conference, Storrs, Connecticut February 18, 2013
6. Type: Conference Papers and Presentations Status: Other Year Published: 2012 Citation: Auer, C. Plant distribution, gene flow and the question of transgenic crops. Universidad Estatal de Bolivar, Guaranda, Ecuador November 24, 2012
7. Type: Conference Papers and Presentations Status: Other Year Published: 2012 Citation: Auer, C. Plant distribution, gene flow and the question of transgenic crops. Universidad

Tecnologica Indoamerica, Quito, Ecuador December 3, 2012
8. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Ecker, G, Meyer, T and C Auer. Native and cultivated switchgrass: distribution patterns and pollen aerobiology. US Deparment of Agriculture, Project directors meeting, Biotechnology Risk Assessment Grants Program June 14, 2013 - June 15, 2013
9. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Auer, C. An?lisis de riesgo ecol?gico e investigaci?n: Ejemplos de dos graminas. Conferencia: Flujo genetica en cultivos agricolas y sus implicaciones para la Bioseguridad. May 6-7, 2013, Quito, Ecuador May 7, 2013 - Present

LINKING POLLINATOR BEHAVIOR TO GENE FLOW TO REDUCE GENE FLOW RISK OVER THE LANDSCAPE

Brunet, J.; Clayton, MU, K.

US Department of Agriculture
Peoria, Illinois

Award Number: 2013-33522-20999

NON-TECHNICAL SUMMARY: The types and acreages planted to genetically-engineered (GE) crops keep increasing. Despite the large number of insect-pollinated crops and the fact that GE varieties are available or being developed for many of these crops, the understanding of how insect pollinators affect the movement of genes via pollen (gene flow) is lagging in comparison to how wind pollination influences gene movement. A better understanding of the factors that affect gene flow in insect-pollinated systems would improve our predictions of gene flow risk and promote the development of management strategies to reduce gene flow. This would increase the potential for coexistence of agricultural crops and reduce the risk of increased invasiveness and decreased genetic diversity of wild populations. A main goal of this research is to link pollinator behavior to gene flow in order to better predict gene flow in insect-pollinated crops. Because pollinator type and landscape attributes have been shown to influence pollinator movements and gene flow, we examine the impact of these factors on the movement of three pollinator types in alfalfa patches of two different sizes or patches planted at different distances from one another. Analyses of these data will highlight the factors that affect pollinator movements. This information will be used to simulate pollinator movements and combined with pollen deposition curves or seed curves, will help better predict pollen movement or gene flow, respectively. The model will be tested using gene flow data obtained experimentally for distinct pollinators in specific landscape settings. A better understanding of the factors that affect gene flow in insect-pollinated systems will not only help us better predict the risk of gene flow, but will also help identify the critical factors mediating gene flow and thus serve as a foundation for improving seed purity Best Management Practices (BMPs). Our approach will help facilitate coexistence among different insect-pollinated production systems in the US.

OBJECTIVES: The overall goal of this research project is to develop and validate a model of gene flow by insect pollinators at the landscape level. The model links pollinator foraging behavior to gene flow. The specific objectives include (1) to determine the features of the landscape that affect pollinator movements; (2) to determine whether differences exist among pollinators in their movements between flowers while foraging; (3) to examine how pollinator type affect pollen deposition and seed curves; (4) to develop a simulation-based model of pollinator movements for distinct pollinators in different landscapes using the empirical data collected in (1-2) ; (5) to combine data on pollen deposition and seed curves to the pollinator movement model to capture pollen and gene flow; and (6) to validate the model using empirically collected gene flow data in distinct landscapes.

APPROACH: Our approach is unique in developing a gene flow model that links pollinator foraging behavior to gene flow. Our model also examines how pollinator movements vary among pollinators and how such movements are influenced by features of the agricultural landscape. We will first examine the impact of three specific landscape features on pollinators foraging on alfalfa plants. These landscape features include patch size, patch isolation distance and plant density. For each treatment, we will plant plots of alfalfa of two different sizes or at different densities or at different distances from one another. The impact of plant density, patch size and isolation distances will be examined on distances and directions traveled between consecutive racemes, number of flowers visited per raceme, residence time (number of flowers visited per patch) and tripping rate and contrasted between the three types of pollinators, honeybees, leafcutting bees and bumble bees. For each treatment, patch density, patch size or isolation distance, we will set up 5 replicates for each of two factors. The 5 replicates will be planted in a linear arrangement and data will be gathered from the 3 middle patches to minimize edge effects. Analyses of these experimental data will determine which variables are important in describing and affecting pollinator movements. We will use Mixed model Analysis of Variance to examine the impact of bee type or patch type on distance traveled or number of flowers visited per raceme. We will use serial angular correlations to determine whether there is directionality of movement within a foraging bout. We will use a Uniformity test to test if there is an overall preferred direction for a given pollinator type or for a given patch type. Finally, we will use permutation tests for homogeneity to identify differences in the distribution of directions among pollinator types or patch types. The variables shown to impact pollinator movements will be included in our simulation model. As we develop the model, we may realize that more data are needed for some variables or that some other aspect of pollinator behavior should be measured and we will thereby adjust the experiments accordingly. We will combine pollinator movements with pollen deposition and seed curves to better describe pollen movement and gene flow. These pollen deposition curves and seed curves will be obtained for each of the three pollinators, honey bees, bumble bees and leaf cutting bees. We will then test the predictions derived from our model at both a small and a large scale using gene flow experiments. These results will provide an opportunity to test how well our model predicts longer distance events and to refine our models if needed; the essential idea will be to use goodness of fit procedures to compare the observed data with the predicted data. If there is significant lack of fit, then parameters in the combined dispersal model will be re-estimated to provide better fit. By linking pollinator behavior to gene flow, our model highlights the mechanisms behind gene flow. Our model has strong predictive power and the potential to be applied to a variety of circumstances and to a variety of crops and plant species. We will present our results at meetings and publish them in peer-reviewed publications. Our effort will include outreach events that discuss and educates the public about pollinators, their use in agriculture, their role in moving pollen between fields and methods to increase their preservation and to limit their between-field movements and hence limit transgene escape. After our model is developed and tested, we will promote its applications to different agricultural landscapes, pollinators and insect-pollinated crops.

RISK ASSESSMENT FOR INSECT RESISTANCE TO PYRAMIDED BT COTTON

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Tucson, Arizona

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: 2012/09 TO 2013/08
Target Audience: Scientists through publications. Our publications were extensively covered by the press (national and international) Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? One full-time research specialist is working on this project. Two part-time research specialists have also contributed to the project. Two graduate students are working on the project. Three undergraduate students are involved. How have the results been disseminated to communities of interest? Publications. What do you plan to do during the next reporting period to accomplish the goals? Continue on all objectives.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? All Objectives are ongoing. Below is a summary of activity and findings during funding period. Pyramided Bacillus thuringiensis (Bt) crops produce two or more toxin targeting the same pest. While these crops can reduce the risk of resistance evolution under ideal conditions, little is currently known about some of the critical assumptions

underlying success of the refuge strategy for these crops. One key assumption favoring success of two-toxin plants is that they kill insects selected for resistance to one toxin, which is called "redundant killing." We tested this assumption for a major pest, Helicoverpa zea, on transgenic cotton producing Bt toxins Cry1Ac and Cry2Ab. Cotton producing these two toxins is the main type of pyramided Bt cotton currently grown in the U.S. Selection with Cry1Ac increased survival on two-toxin cotton, which contradicts the key assumption of redundant killing. The concentration of Cry1Ac and Cry2Ab declined during the growing season, which would tend to exacerbate this problem. Analysis of results from selection experiments with several species of lepidopteran pests indicates that some cross-resistance typically occurs between Cry1A and Cry2A toxins. Incorporation of these empirical data into simulation models shows that the observed deviations from ideal conditions could reduce the benefits of the pyramid strategy for pests like H. zea, which have inherently low susceptibility to Bt toxins and have been exposed extensively to one of the toxins in the pyramid before two-toxin plants are adopted. Our results indicate that for such pests, the pyramid strategy could be improved by incorporating empirical data on deviations from ideal assumptions about redundant killing and cross-resistance. To investigate factors affecting the evolution of resistance to Bt crops in the field, we reviewed two decades of resistance monitoring data from eight countries tracking pest responses to six toxins in Bt corn and Bt cotton. The review demonstrate that reduced efficacy of Bt crops caused by field-evolved resistance has now been reported for some populations of 5 of 13 major pest species examined, compared with only one such species as of 2005. Analyses of empirical data and field outcomes support predictions from simulation models that pest resistance to Bt crops can be delayed substantially by toxin concentrations high enough to make inheritance of resistance recessive, low initial frequency of resistance alleles, and abundant refuges of non-Bt host plants. The results imply that proactive evaluation of the inheritance and initial frequency of resistance are useful for predicting the risk of resistance and enhancing strategies to sustain the effectiveness of Bt crops. Results also indicate that it is very important at this time to develop realistic, empirically-based strategies to manage the evolution of resistance in pests targeted by Bt crops. Knowledge of the molecular genetic basis of resistance can yield efficient tools for monitoring and managing resistance to Bt crops, thereby facilitating regulatory decisions. During the last year we have made significant progress to elucidate the mechanism of Bt resistance to Cry1Ac in our laboratory-selected strains of H. zea. We have: 1) developed a PCR-RFLP technique to differentiate the resistant and susceptible cadherin alleles based on the five amino acid mutations identified in 2012; 2) set up 10 families of single pair cross between a susceptible strain (BE-S) and our resistant strain (GA-R); and 3) identified two discriminating doses of Cry1Ac that will be used to treat the backcross progeny of the 10 single-pair families. From now on, we plan to: 1) use the two discriminating doses of Cry1Ac to treat the backcross progeny of the 10 single-pair families; 2) analyze the genotypes of each survivors and each dead individuals using the PCR-RFLP technique developed; and 3) determine if the H. zea resistance to Cry1Ac is linked to the cadherin mutations using the genotyping data obtained in 2).

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08

1. Type: Journal Articles Status: Published Year Published: 2013 Citation: Brevault, T., Heuberger, S., Zhang, M., Ellers-Kirk, C., Ni, X., Masson, L., Li, X., Tabashnik, B. E., and Carriere, Y. 2013. Potential shortfall of pyramided Bt cotton for resistance management. Proceedings of the National Academy of Sciences USA. 110: 5806-5811.

2. Type: Journal Articles Status: Published Year Published: 2013 Citation: Tabashnik, B. E., Brevault, T., and Carriere, Y. 2013. Insect Resistance to Bt Crops: Lessons from the first billion acres. Nature Biotechnology. 31: 510-520.

ASSESSING THE IMPACT OF GENE REPLACEMENT AND GENETIC MODIFICATION METHODS IN A CROP SPECIES AT THE WHOLE GENOME LEVEL

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Award Number: 2013-33522-21090

NON-TECHNICAL SUMMARY: New methods to engineer crop plants are emerging that have the potential to provide unparalleled specificity in genome modification. However, quantitative data in crop species of potential off-target effects in the genome following targeted genome modification are essential for informed risk assessments of engineered plants. This research is focused on collecting quantitative, whole genome sequence and expression data as well as replicated field trial phenotypic data on a panel of 64 potato lines engineered using three distinct genome modification methods - Agrobacterium transformation and two targeted gene editing methods (CRISPR/Cas and TALENs). For comparison, data will also be gathered from potato lines mutagenized with a conventional chemical mutagen. Importantly, the three genome modification strategies and conventional mutagenesis will create plants with the same phenotype, namely herbicide resistance due to mutations introduced into the acetolactate synthase gene. This proposal addresses the BRAG priority area "Comparison between Transformation-associated Genomic Variation and Genomic Variation Introduced by Non-genetic Engineering Approaches in Plants". This study is relevant to environmental risk assessment and the federal regulatory process.

OBJECTIVES: This proposal addresses the BRAG priority area "Comparison between transformation-associated genomic variation and genomic variation introduced by non-genetic engineering approaches in plants" as well as "Comparison of the types and frequencies of nucleic acid changes introduced into plant genomes via genetic insertion techniques versus other plant breeding techniques". This proposed study is relevant to environmental risk assessment and the federal regulatory process and should provide data that will quantify the potential of off-target effects that may occur from newer genetic engineering technologies in comparison to current methods for induced mutation. Our specific objectives are to: Objective 1: Generate independent herbicide resistant lines using Agrobacterium-mediated transformation, CRISPR/Cas and TALEN-mediated gene modification for a total of 48 independent lines. In parallel, we will generate 16 independent herbicide resistant lines using ethyl methanesulfonate (EMS). Objective 2: Perform whole genome sequencing in the 48 genetically modified lines and 16 EMS lines to assess off-target effects on the DNA sequence. Objective 3: Perform whole transcriptome gene profiling in the set of 64 lines to assess the impact of genetic modification or induced variation on expression patterns at the whole transcriptome level. Objective 4: Assess agronomic phenotypes of the 48 genetically modified and 16 EMS lines in the field to determine phenotypic effects relevant to agriculture.

APPROACH: Objective 1 A. TALEN gene replacement. We have chosen to modify the potato acetolactate synthase gene (ALS), which encodes a protein involved in branched-chain amino acid biosynthesis. ALS has been successfully modified by the Voytas lab using ZFNs; the modifications introduced were amino acid substitutions that confer resistance to sulfonylurea and imidazolinone herbicides (35, 56). In potato, we propose to introduce similar mutations using TALENs (Zhang et al 2012). Protocols for engineering TALENs are robust (7), and we have already been assembled TALENs to target the native potato ALS gene. B. CRISPR/Cas-mediated gene replacement. Despite being a newcomer to the genome engineering field, the CRISPR/Cas system for making targeted double-strand breaks is rapidly being used to create a wide variety of DNA sequence modifications in living cells. One type of CRISPR/Cas system consists of a single protein (Cas9) that cleaves DNA at a specific target site {Jinek, 2012}. Target specificity is determined by two RNAs: the CRISPR RNA (crRNA), which base pairs with the DNA target, and the tracer RNA (tracrRNA), which helps in RNA processing. In bacteria and archaea, the crRNA is complementary to viruses, plasmids and invading DNA molecules, and cleavage of the DNA target inactivates the invaders. For purposes of genome engineering, the crRNA and tracrRNA may be combined into a single chimeric RNA molecule (chiRNA). By changing the DNA binding sequence of the chiRNA, any DNA target can be recognized and cleaved. Using this system, targeted DSBs were efficiently created in vivo at genomic loci in zebrafish, mouse and human cells, enabling precise genome modifications {Cong, 2013 ;Mali, 2013; Hwang, 2013}. C. Generation of genetically modified potato lines expressing herbicide resistance via Agrobacterium-mediated transformation Genome alterations in the genetically modified and EMS lines relative to the wild-type 523-S9-1 will be assessed by direct genome sequencing using next generation sequencing methods. DNA will be isolated from leaf tissue using the Qiagen Plant DNA extraction kit, fragmented to 500 bp and Illumina TruSeq libraries constructed. Libraries will be sequenced (100 bp, paired end reads) on the Illumina HiSeq2000 sequencer at the MSU Research Technology Support Facility. Sequences will be filtered for quality and aligned to the DM reference genome (54) using the Bowtie short read aligner (34). Reads that fail to map to the reference DM genome may represent novel sequences present in . Non-mapping reads will be rigorously cleaned, assembled using Velvet (64), screened for contamination (e.g., bacteria, viruses) and used to construct an artificial chromosome. Both target and non-target effects will be assessed using the whole genome shotgun sequence. In total, five types of alterations in the genome will be assessed in the EMS and genetically modified lines relative to the wild type 523-S9-1: Single Nucleotide Polymorphisms (SNPs), insertions/deletions (InDels), copy number variation (CNV), and rearrangements. Using the Bowtie alignments, SNPs and small indels (<3 bp) relative to the wild type 523-S9-1 will be called using the SAMTools (36) variant calling pipeline with filtering to minimize false positives. CNVs will be determined using the CNVAnnotater software package (1) which uses statistical approaches to determine if read coverage is aberrant in a region such that this would represent copy number variation. Larger structural variation will be determined through examination of the read pair mapping. Objective 3 Expression profiling of Agrobacterium-transformed, gene replacement, and EMS lines Gene expression profiles of leaf tissue of wild type, Agrobacterium-transformed, gene replacement and EMS lines will be assessed using whole transcriptome sequencing, known as RNA-Seq (60). Leaf tissue will be harvested from young expanding leaves from plants growing in parallel in the greenhouse and RNA isolated using the Qiagen RNAeasy kit. cDNA will be synthesized from RNA samples

using the Illumina TruSeq RNA-seq kit and 50 bp reads generated on the Illumina HiSeq2000 sequencer. Three biological replicates will be performed to address any variation attributable to biological variation. Reads will be quality filtered, mapped to the DM genome (54) using TopHat (58) with parameters optimized for the DM genome and expression values for each gene model (FPKM) determined using Cufflinks (57). Differential expression between wild-type, genetically modified, and EMS lines will be determined using the CuffDiff program (57) within Cufflinks to determine effects on gene transcription at the genome level by either our protoplast regeneration and/or genetic modification methods. Buell has extensive experience in next generation sequence analysis in potato (2, 38,54) and we foresee no barriers in completing this objective. Objective 4 Phenotypic characterization of genetically modified and EMS lines. The goal of the multi-year, multi-location field trials is to provide two years of data for a comprehensive comparison of plant phenotypic variability attributed to the three gene modification methods. Conducting trials from virus-free plants and seed tubers is necessary for unbiased phenotypic comparisons. Vegetative propagation is the standard practice of maintaining and propagating a non-inbred vegetatively propagated crop and all lines obtained from the three methods and EMS as well as the wild type 523-S9-1.

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

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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.

PROGRESS: 2012/09 TO 2013/08
Target Audience: 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: Entomologists with interest in control of vector-borne disease Agriculturalists in several international communities (Europe, Asia, Africa and North America) who are developing filed-based strategies to enhance output Public health officials tasked with development of novel methods for control of vector-borne human diseases Biotechnologists with a focus on environmental applications Medical personnel, such as infectious disease specialists, with particular focus on vector-borne disease. Global health officials with interest in global food security and agricultural output (i.e. the Gates Foundation). Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? Postdoctoral associate Kendra Pesko traveled to London for 5 weeks to work at Queen Mary University of London with Angray Kang in developing mixed library approach for screening of Pierce's disease antigens against antibodies from an immunized mouse. How have the results been disseminated to communities of interest? The approach and preliminary results were presented at the Proceedings of the 2013 Pierce's Disease Research Symposium in Sacramento in December, as part of an oral presentation "Symbiotic Control of Pierce's Disease Using the Registered Agent Pantoea and Microencapsulation Delivery" given by Arinder Arora, and a poster presentation "Paratransgenic control of Pierce's disease." What do you plan to do during the next reporting period to accomplish the goals? During the next period, we will inoculate more mice with heat killed X. fastidiosa in order to produce different single chain antibodies and fresh materials with which to generate single chain libraries. We will build PCR generated libraries from these spleens, and screen them against our mixed library of antigen sequences using in vitro transcription and translation approach and mixing of the libraries. After pulling down with Streptactin beads (for antibodies) and spycatcher protein (for antigens), we will extract RNA from antibody-antigen complexes and use antigen or antibody specific primers to amplify from these pulled down RNAs. We will use cloning and sequencing, or deep sequencing to examine enrichment of each target after pull down with antigen or antibody tags. In this way, we will identify antibody sequences after pull down with antigen tags that were attached to X. fastidiosa antigens. Any potential X. fastidiosa antibody sequences will be cloned into Pet 32A and expressed as single chains. Monomeric red fluorescent protein will then be inserted between the heavy and light chains and used to generate recombinant single chain antibodies. These antibodies will be tested for efficacy against X. fastidiosa. Antibodies that prove effective in attaching to X. fastidiosa will then be transformed into P. agglomerans and used in close-cage settings. Simultaneously we will insert monomeric red fluorescent protein into the single chain antibody 4XfMopB3 which has been previously shown to interact with X. fastidiosa (Azizi et al. 2012). This antibody will then be transformed into P. agglomerans and optimized for secretion using the HlyA/B/D system mentioned above. We will then test the efficacy of this target in closed-cage settings to disrupt transmission of X. fastidiosa by H. vitripennis.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? We have conducted an extensive literature search to find key surface-exposed epitopes of Xylella fastidiosa. We have obtained the sequences for twelve of these targets from the X. fastidiosa Temecula genome (PRJNA57869) and submitted

these sequences to websites that predict the signal peptide (SignalP 4.1 server, http://www.cbs.dtu.dk/services/SignalP/) and the PRED TMBB website, which hosts a hidden Markov model based algorithm that can predict the transmembrane regions of proteins for Gram negative bacteria (http://biophysics.biol.uoa.gr/PRED-TMBB). We have designed primers to amplify only these regions, and then add SPYtag, T7 promoter, and Kozak sequences to the antigen targets. We used these PCR generated products to perform in vitro transcription and translation, resulting in antigen protein sequences linked to mRNA. These libraries will be used to screen against antibody libraries generated in a similar way from spleens of X. fastidiosa-exposed mice, as described (Azizi et al. 2012). We have used PCR to generate a library of single chain antibody sequences from the spleens of X. fastidiosa-exposed mice, and performed in vitro transcription and translation with these products. After pull down with both SpyCatcher protein and Streptavidin, and RNA extraction, we were able to recover sequences from the antigens. The single chain antibody libraries must be further explored for potential binding to antigens, however this initial attempt failed to produce useable targets, thus we are planning to re-inoculate mice with X. fastidiosa in the months to come in order to produce fresh spleen RNA as a starting material. We have optimized an electroporation transformation protocol for Pantoea agglomerans, and used it to transform P. agglomerans with constructs containing several antimicrobial peptides (amps) along with a hemolysin system to secrete the amps. This hemolysin system adapted from E. coli, Hly A/B/D, may also be of use for secreting the recombinant REDantibodies. Using P. agglomerans transformed with an enhanced GFP containing plasmid, we have shown uptake in close-cage settings and colonization of the anterior cibarium of H. vitripennis (Arora et al. in prep). This model system allows visualization of bacterial localization. P. agglomerans was fed to H. vitripennis using an artificial feeding system, developed to test acquisition of microbes by H. vitripennis. This same technique will be used to monitor uptake of P. agglomerans transformed with REDantibody by H. vitripennis.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08

1. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Arora, A. Symbiotic Control of Pierce's Disease Using the Registered Agent Pantoea and Microencapsulation Delivery. Oral presentation at the Proceedings of the 2013 Pierce?s Disease Research Symposium in Sacramento, December 2013
2. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Arora A, Forshaw A, Pesko K, Quintero-Hern?ndez V, Kang A, Miller T, Durvasula R.Paratransgenic control of Pierce?s disease.Poster presentation at the Proceedings of the 2013 Pierce?s Disease Research Symposium in Sacramento, December 2013

MONITORING THE DISPERSAL OF GENETICALLY ENGINEERED ORGANISMS AND THEIR BYPRODUCTS USING LIGHT TRANSMISSION SPECTROSCOPY

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Award Number: 2013-33522-21007

NON-TECHNICAL SUMMARY: Understanding and monitoring the distribution and dispersal of genetically engineered organisms and their byproducts is a critical component of the safe and responsible use of transgenic technology. However, we currently lack the ability to rapidly and adequately track the movement of genetically engineered organisms and their byproducts in the environment, even though research has demonstrated that they can escape their intended ranges. Our multidisciplinary team, which includes evolutionary biologists, ecologists, physicists, and biogeochemists, will address this critical challenge by working to increase our ability to detect genetically engineered organisms and their byproducts in the environment. We will adapt a novel technology called Light Transmission Spectroscopy, which has the ability to identify and accurately measure in real-time the size, shape, and number of small particles suspended in fluid at the nanometer scale (1 nanometer equals 1 billionth of a meter). Light Transmission Spectroscopy has demonstrated high sensitivity and greater size resolution than competing technologies, which we will use to address two specific and pressing needs in genetically engineered detection: the potential dispersal of genetically engineered fish and the byproducts of genetically engineered maize. To detect genetically engineered fish dispersal, we will combine Light Transmission Spectroscopy with another developing technology, environmental DNA (eDNA). eDNA is a species surveillance tool that recognizes a unique advantage of aquatic environmental sampling: Water often contains microscopic bits of tissue in suspension, including the scales of fish, the exoskeletons of insects and the sloughed cells and tissues of aquatic species. These tissue fragments can be filtered from water samples, and then a standard DNA extraction is performed on the filtered matter. Then, we will develop the Light Transmission Spectroscopy device to detect the DNA of a specific species. To detect the byproducts of genetically engineered maize, we will take a similar approach. Here, the byproduct is the Bt toxin produced by genetically engineered maize. The Bt toxin is intended to kill insect pests feeding on the maize, but can leach into nearby streams. The Bt toxin can also be sampled from aquatic environments by filtering. Then, we will develop the Light Transmission Spectroscopy device to detect the Bt toxin. Overall, Light Transmission Spectroscopy exhibits the potential to be a field ready device that can generate rapid and highly accurate detection results, even when a target is at low densities

OBJECTIVES: To increase our ability to detect genetically engineered organisms and their byproducts in the environment, even at low concentrations, and increase our knowledge and understanding about the distribution and dispersal of genetically engineered organisms and their

byproducts in the environment using a newly-developed portable detection system this is rapid and accurate and works at locations in the field where real time information is critical.

APPROACH: To detect GE fish dispersal, we will combine LTS with another developing technology, environmental DNA (eDNA). eDNA is a species surveillance tool that recognizes a unique advantage of aquatic environmental sampling: Water often contains microscopic bits of tissue in suspension, including the scales of fish, the exoskeletons of insects and the sloughed cells and tissues of aquatic species. These tissue fragments can be filtered from water samples, and then a standard DNA extraction is performed on the filtered matter. We will then take species-specific fragments of synthetic single-stranded DNA and bind them to the surface of very small nanoparticles. When we mix these tagged nanoparticles with the DNA of a target species, it binds to the target DNA and the particle grows in size. This growth is detectable by the LTS device and indicates the detection of the target species. To detect the byproducts of GE maize, we will take a similar approach. Here, the byproduct is the Bt toxin produced by GE maize, which is intended to kill insect pests, but can leach into nearby streams. The Bt toxin is a protein and can also be sampled from aquatic samples by filtering. Then we can use another target-specific molecule called an antibody, which will only bind to the Bt toxin and no other protein. Again, there will be a change in the size of the antibody indicating the presence of the Bt toxin and this change in size is detectable by LTS. We will used a series of experiments to test this technology at different concentrations of both targets (Bt toxin and GE fish eDNA) using leaching experiments into lab aquaria, an artificial stream facility at the Cary Institute for Ecosystem Studies, and the Notre Dame Linked Experimental Ecosystem Facility.

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.

KEYWORDS: gene editing; sex selection; gene targeting; monosexing; infertility

PROGRESS: 2012/08 TO 2013/07
Target Audience: Livestock genetics companies that need to protect their genetics from environmental escape. This could include pigs, cattle, goats, sheep and aquaculture. Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? To date, the Y-chromosome had not been targeted in livestock. This unique challenge provided required a great deal of troubleshooting and process optimization that benefited scientists and management alike. How have the results been disseminated to communities of interest? To date, results have only been disseminated at the USDA NIFA BRAG panel in June of 2013 via a poster presentation. Ultimately, the data will be compiled and published in the scientific literature. What do you plan to do during the next reporting period to accomplish the goals? Y-chromosome targeting. Promoter/transgene reporter constructs are currently being tested in mice for restriction of transgene products within the source spermatid. The best performing promoter/transgene pair will be placed on SSCY and the targeted cells will be cloned to observe distribution of transgene expression in swine spermatids. The anticipated cloning date is January of 2014. Reversible sterilization. The knockout cells will be cloned in November of 2013 with parturition expected in March of 2014. The animals will be raised for phenotypic assessment.

IMPACT: 2012/08 TO 2013/07
What was accomplished under these goals? Y-chromosome targeting. Our initial objective was to target expression cassettes to two loci on the swine Y chromosome (SSCY). A third locus was added, and transgene targeting experiments were performed for each loci. A variety of transgenes were targeted to each loci using two template types, linear DNA with short homology arms (60-70bp) and supercoiled DNA with long homology arms (600-800bp). Each strategy was successful and 1-10 percent of isolated clones was positive for the targeted allele. This represents the first successful targeting of the Y-chromosome in livestock. Reversible sterilization. The Kisspeptin receptor (KissR) was chosen as a genetic target for reversible sterilization. TALENs were assembled to cleave within exon 3 of porcine KissR. After identification of TALENs with high activity (>30% rate of non-homologous end joining) a oligonucleotide template was developed to introduce a pre-mature stop codon and unique HindIII restriction site. Gene knockout experiments were conducted by co-introduction of TALENs with the oligonucleotide template. A total of 96 colonies were propagated and cryopreserved. In total 40 of 96 (42%) and 17 of 96 (18%) were heterozygous and homozygous for the knockout allele respectively. Pools of heterozygosity and homozygosity positive clones were generated for cloning in late 2013.

PUBLICATIONS (not previously reported): 2012/08 TO 2013/07
No publications reported this period.

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.

PROGRESS: 2012/09 TO 2013/08
Target Audience: The target audience included agricultural companies, biotechnology companies, extension specialists, farmers, members of the scientific community, regional agronomists, regulators, and the general public. Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? Two M.S. students and one Ph.D. student have participated in research conducted during the first year of the project. Additionally, several undergraduate students have assisted with laboratory and field experiments. One M.S. student presented results from this research at the North Central Branch meeting of the Entomological Society of America. How have the results been disseminated to communities of interest? Data from this research contributed to three presentations at scientific conferences, and two presentations at the annual meeting of the USDA's North Central Communications Committee 46. In Iowa, results contributed to two outreach videos for pesticide applicator training, eighteen outreach presentations, and five outreach publications. Slides based on this research were made available to Dr. Erin Hodgson, Iowa State University Extension Entomologist, and to Iowa State University field agronomists for use in their meetings with clientele. In Nebraska, results were presented to the Independent Crop Consultant Association. L. Meinke was interviewed by National Public Radio, which aired during July 2013, "As biotech seed falters, insecticide use surges in Corn Belt". A. Gassmann was interviewed by Canadian Broadcasting Company, which Aired during May, 2013, "Rootworms and the future of genetically modified farming". What do you plan to do during the next reporting period to accomplish the goals? Research will continue under all objectives. A M.S. student and a Ph.D. student recruited during the first year will conduct research under this grant during year two. For objective one, bioassay and on-farm studies will be conducted as they were during year one. For objective two, experiments will be run to measure fitness costs and inheritance of resistance in diapausing and non-diapausing strains of western corn rootworm. Inbred lines will be initiated during year two, to enable QTL mapping of resistance traits as described under objective three. For objective four, analysis will be conducted for RNA-Seq data obtained during year one, and additional non-diapausing Bt-resistant strains will be used to analyze patterns of gene expression.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? Bioassays are currently being conducted on western corn rootworm populations collected from 15 fields in Iowa and Nebraska with high levels of injury to Bt corn. Also, field trials are underway in six fields in Iowa and Nebraska that suffered severe injury to Bt corn from western corn rootworm during 2012. Experiments are underway to measure fitness costs and inheritance of resistance in four non-diapausing strains of western corn rootworm that were derived from populations sampled in fields with Bt resistance. The first set of exposure experiments to provide material for RNA-Seq has been completed. Neonate larvae

(≤ 24 hours old) from a Bt-resistant strain and a susceptible strain were fed Cry3Bb1 corn and non-Bt corn. Neonate larvae from each strain were allowed to feed for either four or eight hours, flash-frozen in liquid nitrogen and stored at -80ºC. RNA was extracted from 30 larvae per sample, from a total of 24 samples (2 WCR strains x 2 corn types x 2 time points x 3 replicates). All of the RNA preparations passed quality-control checks and were sent to the University of Nebraska Medical Center DNA sequencing core facility. Construction of sequencing libraries at the core facility is underway.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
No publications reported this period.

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.

PROGRESS: 2012/09 TO 2013/08
Target Audience: We are in the midst of our data collection and analysis. We have presented one symposium talk about the work at the Entomological Society of America metting. We gave a poster presentation at the BRAG meeting last spring. We have also helped with training other NCSU staff on the use of RADtag next generation sequencing and analysis Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? The postdoc on this project, Megan Fritz, has learned a huge amount about quantitative genetics and bioinformatics. She has done a great job of helping other students, staff, and faculty to understand more about RADtag analysis by developing a journal club on the subject. She has also trained two undergraduates in the lab on molecular techniques. How have the results been disseminated to communities of interest? One symposium talk at the ESA meeting. One poster presentation at the BRAG annual meeting What do you plan to do during the next reporting period to accomplish the goals? We hope to finish analysis of the lab populations and move on to analysis of the field-collected samples.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? The first step in achieving our goals was to use the RADtag approach to determine if we could "prove the principle" that one could find markers associated with Bt resistance alleles in strains of Heliothis virescens where we knew that two specific genes had been selected for during strain adaptation to Bt toxins. The strains used were YHD2 and KCB. For YHD2 we knew from past research that the genes seleted for were a cadherin genes and an ABC transporter. The first thing we did was to use Sanger sequencing to determine the genetic diversity of these to genes in the selected and control strain. As expected there was much lower diversity in the selected strain. Our next objective was to use RADtag sequencing to deterimine if we could find markers that had also decreased in sequence diversity follwoing selection, and then use our genetic map to localize those markers. We have run

RADtag analysis of the control and selected strains and we found 300 RADtags for which there was decreased diversity. We are now in the process of mapping these RADtags based on a cross of the selected strain to an unrelated H. virescens strain from another lab. The molecular analysis is about half way done. We should have results in a few more weeks.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
No publications reported this period.

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

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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 asseed 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.

SILENCING OF NATURALLY OCCURRING GENES CONTROLLING SEED DORMANCY TO REDUCE FITNESS OF TRANSGENE-CONTAMINATED WEEDY RICE

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Award Number: 2013-33522-21097

NON-TECHNICAL SUMMARY: Cross-pollination between genetically modified crops and local wild relatives, or gene flow, may create fitness-enahncedweedsthat combine the wild with transgenic characteristics to exacerbate weed problems in agriculture. Seed dormancy, a key adaptive trait controlled by genes, disseminates germination across seasons, making weeds persist in agro-ecosystems. This project aims to develop a transgenic mitigating (TM) strategy to reduce the risk of gene flow by linking to a primary transgene with RNA interference (RNAi) structures to silence seed dormancy genes prevailing in weeds. Because of the linkage with reduced dormancy or increased germination uniformity, transgene-containing weeds would be less persistent in the soil seed bank and also relatively easy to eliminate by agronomic practices. The seed dormancy genes SD7-1, SD7-2 and SD12 cloned from weedy "red" rice will be used to design the silencing structures, which will be ligated with the herbicide resistance gene Bar on the same vectors to transform a rice cultivar. Transgenic lines will be selected to cross with isogenic lines for the dormancy genes to determine the silencing effects under controlled conditions. Selected transgenic lines will be also crossed with weedy rice to evaluate fitness of the transgene-containing weed genotypes under field conditions across generations. Expected outcomes include new knowledge about the efficacy of the TM approach inthe rice crop system, techniques that could be extended to or modified for the other crop systems to prevent the rising of fitness-enhanced weeds, and purified transgenic materials that will be disseminated for in-depth analysis of gene flow.

OBJECTIVES: The goal of this project is to develop a transgenic mitigation (TM)strategy by silencing naturally occurring seed dormancygenes prevailing in wild/weed populationsto lower the risk of "geneflow" from genetically modified (GM) crops in agro-ecosystems. The objectives of this project are: 1) To develop transgenic lines of rice using a built-in construct that contain an herbicide-resistant gene and RNA interference (RNAi) structure(s) silencing one to three seed dormancy genes cloned from weedy red rice; 2) To determine effects of the built-in constructs on germination uniformity, herbicide resistance, and other adaptive traits in a nearly isogenic background under controlled conditions; and, 3) To evaluate fitness of the transgene-containing weed genotypes under field conditions across generations.

APPROACH: The seed dormancy genes SD7-1, SD7-2 and SD12 cloned from weedy "red" rice will be used to design Inverted Repeat Sequences (IRSs) as silencing structures. One or more IRSs will be ligated with the herbicide resistance gene Bar on the same vectors and recombinants with the Bar+IRS tandem constructs used to transform a rice cultivar. Transgenic lines will be

selected to cross with isogenic lines for the dormancy gene(s) to precisely determine effects of the Bar+IRS constructs on germination uniformity, herbicide resistance, and other adaptive traits in the hybrid F1 and F2 populations. The transgenic lines with a high level of silencing effect will be crossed with accessions of weedy red rice to evaluate fitness of the transgene-containing genotypes under field conditions.

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.

PROGRESS: 2012/09 TO 2013/08
Target Audience: The target audience for this research is primarily other scientists involved in functional genomics research and the development of transgenic insect strains for population control. Additionally are scientists and administrators involved in methods development for biologically-based population control, including USDA-APHIS-PPQ and FAO/IAEA. Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? The project has provided ongoing training and profesional developmenbt for a technician and post-doctoral research associate dedicated to the project. How have the results been disseminated to communities of interest? The results of this project have been disseminated by the cited publications and by seminar and symposium presentations by the Project Directors and research associate. What do you plan to do during the next reporting period to accomplish the goals? During the next reporting period the new Cre/loxP RMCE target site strains for Anastrepha ludens and Drosophila suzukii will be tested for successful RMCE, and optimal target site strains will be identified for A. suspensa, and tested for RMCE with antioxidant genes expected to enhance fitness and reproduction in males sterilzed for the Sterile Insect Technique. Testing of a new RMCE system based on FLP/FRT recombination will begin for tephritid fruit fly species.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? The Cre/lox recombination system was previously tested for RMCE in Drosophila and the tephritid pest species, Anastrepha suspensa, which allowed a direct functional comparison of the artificial promoter, 3xP3. To test this system in other pest species, a Cre/lox RMCE target site vector was transformed into the Mexican fruit fly, A. ludens, and the spotted winged drosophilid, Drosophila suzukii. This also demonstrated the first germ-line transformation for D. suzukii, which is a recent pest species of small fruits. The testing of RMCE in these species is currently in progress. To identify optimal genomic sites for targeted transgene integrations, 12 new Cre/lox RMCE target site strains have been created for A. suspensa that are being evaluated for transgene expression, strain fitness and mating competitiveness. Optimal strains will then have the transformation vector stabilized, and then used for targeted transformation with recently isolated antioxidant genes whose expression will be compared to their endogenous homologs.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
1. Type: Journal Articles Status: Published Year Published: 2013 Citation: Schetelig, M.F., Handler, A.M. (2013) A functional comparison of the 3xP3 promoter by recombinase-mediated cassette exchange in Drosophila and a tephritid fly, Anastrepha suspensa. G3: Genes, Genomes, Genetics (Bethesda). 2013 Mar 11, doi:pii: g3.112.005488v1. 10.1534/g3.112.005488.
2. Type: Journal Articles Status: Published Year Published: 2013 Citation: Schetelig, M.F., Handler, A.M. (2013) Germline transformation of the spotted wing drosophilid, Drosophila suzukii, with a piggyBac transposon vector. Genetica. 141:189-93.

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 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 apopotic 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 contruct MSCs for testing in tilapia. At least two MSCs will be used to generate transgenic lines, in an effort to identify an MSC driving apotosis 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 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: 2012/09 TO 2013/08
Target Audience: Dr. Lauth presented our results via oral poster session at the 5th Strategic Conference of Zebrafish Investigators: “Maternal sterility for genetic containment of GM and other exotic farmed fish” in Pacific Grove, CA, January 19 – 23, 2013. Dr. Lauth also gave a comprehensive review of the “Maternally-Induced Sterility in Fish” to an international audience at the World Aquaculture Society (WAS) meeting in Nashville, Tennessee, February 21-25, 2013. Changes/Problems: The initial project proposed to breed an established line of Growth Hormone (GH) transgenic tilapia with our MSC transgenic line to demonstrate that the sterility phenotype can easily be transferred to a new line and stacked to another phenotype (objective 4). Unfortunately, the GH transgenic line has been lost. As a replacement we elected to produce transgenic lines of tilapia bearing a GFP reporter construct initially investigated in zebrafish (ZPC5:tbax:tnos3’UTR). The new GFP line will also serve to assay sterility in early embryonic stages. This does not represent a major change from the initial objective of the grant. Another unexpected difficulty resulted from the absence of F1 female in many lines. To ensure females are produced in each line to evaluate the sterility phenotype, we used estrogenic hormone to produce F2 females. What opportunities for training and professional development has the project provided? Nothing Reported How have the results been disseminated to communities of interest? Nothing Reported What do you plan to do during the next reporting period to accomplish the goals? We will score the sterility phenotype at the physiological, cellular and molecular level in the progeny of transgenic females. Gonads will be sectioned and stained to study the overall cellular architecture and number of germ cells. We will develop a quantitative real time PCR assay to measure the expression level of the germ cell specific genes vasa, testis specific gene Amh and ovarie specific gene Cyp19a1a and compared expression in dissected gonads. 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.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? Objective 3: Two tilapia constructs (MSCzar1, MSCzpc5) and one zebrafish construct (MSCzpc3) were microinjected into approximately 800 tilapia embryos. 112 putative founders were identified and 32 reached sexual maturity (14 males and 18 females). The remaining 80 putative founder will be tested for germ line transmission in early 2014. We screened all 14 putative founder male and identified 6 that transmitted the transgene to their progeny. From this result we estimate that at least another 10 founder males will be detected which will bring us close to our goal of establishing 20 germ-line transgenic male founders. We screened in excess of 1000 progeny from founder males with wild type females and observed germ line transmission rates ranging from 0.4% to 17%. From these

crosses, a total of 40 F1s transgenic were selected and raised to sexual maturity. We produced F2 progeny from F1 males and this progeny is fertile. We also produced progeny from F1 females and are currently rearing this progeny to assay for maternally induced sterility. Objective 4: we microinjected the reporter construct ZPC5:eGFP:nos 3'UTR in tilapia and established 4 germ-line transgenic tilapia (F2 are being raised). Female transgenic in these lines should produce embryos with GFP labeled PGCs and will serve as tools to measure the direct effect of the MSC transgene on PGCs ontogeny. We will produce double transgenic tilapia and expect that MSC&GFP-females will produce embryos with reduced PGCs number relative to control sibling GFP-females.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
Type: Conference Papers and Presentations Status: Published Year Published: 2012 Citation: Laible, G. (2012). Conference VIII. Transgenic Res, 21, 901-925.

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 form 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: 2012/09 TO 2013/08
Target Audience: Scientists and students at university/college and research institutions working in similar research areas; biotechnology industry. Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? This project has provided opportunities to train three 3 Ph.D. students,four visiting scientists and one postdoctoral scientist on molecularcontainment technologies, evaluation techniques, and research presentationskills. How have the results been disseminated to communities of interest? We have presented some of the results and findings from this project at5 meetings in the format of posters and oral presentations (such as the Annual Meeting for the American Society of Plant Biologists-Northeast Section; UMass Plant Biology Symposium; UConn Graduate Research Forum; UConn Plant Science Graduate Student Seminar). We have also presented some data and findings from this project at 7 invited presentations (such as, The First EITA Conference on Agricultural

Science and Technology, Biosystems Engineering: Precision Agriculture: Challenges and Future Directions. Cornell University, Ithaca, NY, U.S.A., June 27-28, 2013; Chinese Academy of Sciences-Jiangsu Institute of Botany, Nanjing, July 8, 2013; Chinese National Academy of Forestry, Beijing, July 12, 2013; International Symposium on Molecular Biology of Fruit Trees, Wuhan, China, Oct 18-20, 2013; The 12th Chinese National Congress for Horticulturists. Chengdu, China, Oct 21, 2013.) What do you plan to do during the next reporting period to accomplish the goals? Greenhouse and field evaluations of the poplar transgenic plants, particularly their ability to repress suckering as described in our proposal will be our focusin the next reporting period. We will conduct field evaluations for 2 to 3 years under field conditions.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? Major objectives accomplished: Objective 1) Construction of root sucker repressing genes. We have completed the construction of all proposed genes. These genes are 1) root specific promoter-CKX gene, 2) root specific promoter-ARR15 gene, 3) root specific promoter-iaaM gene and 4) Arr5 promoter-GUS gene. All of these genes werecloned into a Ti-plasmid vector. Objective 2) Production of transgenic poplar plants using the sucker repressing genes. We have completed all proposed poplar transformation with more than 50 transgenic lines for each construct. In addition to poplar, wealso transformed tobacco with the proposed gene constructs. Objective 3) Evaluation of effectiveness of the root sucker repressing genes in poplar. We are evaluating the transgenic tobacco and poplar plants. We have observed that a number of root specific CKX transgenic poplar plants display significant reduction in root suckers under greenhouse conditions. We have not yet observed the same phenomenon in the ARR15 or iaaM overexpressing plants since they are much younger than the CKX plants. We have propagated some transgenic plant lines and planted them in field in 2013 with a permit from USDA for field release. Additional transgenic poplar lines are grown in the greenhouse and will be planted in the field. Objective 4) Characterization of growth and development patterns of the transgenic poplar that express the root sucker repressing genes. We are evaluating any alterations in growth and development patterns of all transgenic poplar and tobacco plants under both greenhouse and field conditions. So far, we have not observed significant changes in growth rates of above organs in these plants although root growth in some transgenic plant lines has been enhanced. The transgenic plants generally have more and longer roots and we expect that these plants could be more tolerant to some environmental stresses such as drought. Preliminary observations also suggest that some transgenic plant lines may respond to some environmental factors such as salt and light differently than the wild type controls. Our characterization of transgenic tobacco plants have generated some interesting results, such as reduced cell programmed death in response to environmental stresses. Manuscripts reporting these findings will be submitted for publication soon.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
1. Type: Journal Articles Status: Published Year Published: 2014 Citation: Jing Ding, Hui Duan, Ziniu Deng, Degang Zhao, Ganjun Yi, Richard McAvoy, and Yi Li (2014): Molecular Strategies for Addressing Gene Flow Problems and Their Potential Applications in Abiotic Stress Tolerant Transgenic Plants. Critical Reviews in Plant Sciences, 33:190?204,
2. Type: Journal Articles Status: Published Year Published: 2013 Citation: Li, Y. (2013). Gene

deletor: a new tool to address gene flow and food safety concerns over transgenic crop plants. Front. Biol. (7): 565-576

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.

PROGRESS: 2012/09 TO 2013/08
Target Audience: We targeted the EPA, APHIS, Monsanto, the scientific community, and the general public during this reporting period. We worked with EPA and APHIS as we developed an overview manuscript that was inspired by the grant we wrote (but no funding from the RNAi project was used to produce the manuscript). We discussed risk of pesticidal RNAi with scientists of Monsanto to compare our approaches and optimize methodology. Risk of RNAi was presented to the scientific community at the Entomological Society of America Annual Meeting: Mogren and Lundgren. 2013. Considerations for risk management of RNAi technology and pollinators. Entomological Society of America annual meeting, Austin TX, November 09-14, 2014 The research concepts were presented at several field days (Fuller Farms, Emporia, KS; Burleigh County Soil Health Clinic, Bismarck, ND; USDA-ARS North Central Agricultural Research Laboratory Soil Health Clinic, Brookings, SD; MT, ND, and SD Honeybee Producer State Meetings. Changes/Problems: The growing season in South Dakota was unusually cold, which reduced our insect collections from the field sites. We will repeat the field collections in summer 2014 in order to ensure a meaningful sample size will be used for the quantitative food web. All other milestones should be met according to schedule. What opportunities for training and professional development has the project provided? Nothing Reported How have the results been disseminated to communities of interest? The results of this summer's field collections are still be analyzed, so the actual experimental results were not disseminated. The research plans were disseminated through extension presentations at grower field days, stakeholder regional meetings, and direct communications with scientists and regulators. We also presented an

overview poster of the risks posed by RNAi-based crops at the Entomological Society of America annual meeting in Austin, TX What do you plan to do during the next reporting period to accomplish the goals? The development of the laboratory assay systems are underway for winter 2013-14, and non-target insect colonies are in development.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? In order to identify arthropods that may be at risk for exposure to siRNA in the field, insect specimens were collected from non-genetically modified, organic or refuge corn fields on ten farms in ten different counties across the eastern half of South Dakota. Insecticidal sprays and seed treatments were not in use in nine of the ten fields, and both no-tillage and conventional-tillage fields were sampled. Collections were conducted during two separate sampling periods: an early-season period (corresponding with degree-day model estimates for European corn borer population peaks), and a mid-season period (corresponding with the peak of anthesis). The early-season sampling period took place on 27 Jun – 2 Jul, and the anthesis sampling period took place on 29 Jul – 1 Aug. Samples were collected using three techniques to collect foliar, epigeal and belowground arthropods. Foliar arthropods were collected in whole-plant counts (n = 50 per sampling period per site), using beat sheets and dissection of all plant tissues. Epigeal arthropods were collected by 5-min visual searches within metal quadrat frames pressed into the soil (50 cm × 50 cm; n = 5 per sampling period per site). Belowground arthropods were collected in soil cores (10 cm diameter × 10 cm deep; n = 7 per sampling period per site), and extracted using Berlese-Tullgren funnels. Samples are being sorted and identified to the lowest possible taxon, and specimens from the fifty most abundant species will be included in molecular gut-content assays. Major taxa collected include aphids, thrips, springtails, ants, lady beetles and lacewings. DNA primers that selectively amplify corn DNA were previously developed and are being tested against a wide array of non-target, agrobiont organisms in preparation for gut-content assays during the winter.Genetically modified crops are currently being developed to utilize RNA interference (RNAi) to combat specific insect pests. However, RNAi presents unique challenges for risk assessment: the short interfering RNA (siRNA) molecules used by this technology cannot be directly traced in vivo using current molecular tools (unlike Cry1 endotoxins, which can be tracked using ELISA). Risk-assessment protocols must therefore focus on tracking potential routes of exposure to identify animals that could be at risk. The first objective of this project is assess the potential for exposure via trophic pathways using PCR gut-content analysis. Uptake of DNA from corn indicates potential for uptake of siRNA from transgenic corn.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
Type: Journal Articles Status: Published Year Published: 2013 Citation: Lundgren, J. G. and J. J. Duan. 2013. RNAi-based insecticidal crops: A novel paradigm in risk to non-target species. Bioscience 63(8): 657-665.

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: 2012/09 TO 2013/08
Target Audience: Plant biotechnologists in academia, industry and regulatory agencies, turfgrass breeders, turfgrass industry. Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? Training activities: 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, molecular analysis and performance assessment of transgenic plants, as well as participated in field trial studies. Graduate students, 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. How have the results been disseminated to communities of interest? The research data obtained so far have been reported in peer-reviewed journal papers and book chapters. They have also been disseminated through invited seminars in academic institutions and private industries, and by oral presentations and posters in international scientific conferences. What do you plan to do during the next reporting period to accomplish the goals? Nothing Reported

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? Impact of the project: 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. The main objective of this research project is to assess potential environmental risk associated with genetically engineered abiotic stress tolerance in transgenic perennial species, turfgrass by manipulating multiple molecular pathways that are involved in plant response to environmental stimuli. Specifically,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. We have conducted the following research to achieve our goals. 1. Using transgenic approaches, we first produced independent creeping bentgrass transgenic lines expressing various transgenes. These lines were evaluated in the greenhouse, and shown to exhibit enhanced performance under diverse environmental adversities. These studies clearly demonstrated that manipulation of different moelcular pathways in transgenic plants dramatically changed plant response to environmental stresses, leading to enhanced plant performance. These results allow us to meet the first goal proposed in our project and provide the basis for further research to achieve the rest three goals we proposed. 2. To study environmental impact of these genetically engineered plants, various transgenic lines were vegetatively propagated for use in a field trial

study under an USDA-APHIS permit. Currently, transgenic and wild-type control plants have been well established in the field, and the studies on how transgene expression impacts weediness and invasiveness of genetically engineered perennials and how transgenic perennials interact with environment are being conducted. We expect that in the following year, we would be able to collect data to address these questions and achieve the three other goals proposed in this project.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08

1. Type: Book Chapters Status: Awaiting Publication Year Published: 2013 Citation: Saski, C., Luo, H. (2013) Switchgrass genomic resources development and genome sequencing initiatives. In: Compendium of Bioenergy Plants ? Switchgrass. Luo, H., and Wu, Y. (eds), CRC Press, Tailor & Francis Group (in press).
2. Type: Book Chapters Status: Awaiting Publication Year Published: 2013 Citation: Li, D., Zhou, M., Li, Z., Luo, H. (2013) MicroRNAs and their potential applications in switchgrass improvements. In: Compendium of Bioenergy Plants ? Switchgrass. Luo, H., and Wu, Y. (eds), CRC Press, Tailor & Francis Group (in press).
3. Type: Journal Articles Status: Published Year Published: 2013 Citation: Zhou, M., Luo, H. (2013) MicroRNA-mediated gene regulation: potential applications for plant genetic engineering. Plant Molecular Biology 83:59-75.
4. Type: Journal Articles Status: Published Year Published: 2013 Citation: Zhou, M., Li, D., Li, Z., Hu, Q., Yang, C., Zhu, L., Luo, H. (2013) Constitutive expression of a miR319 gene alters plant development and enhances salt and drought tolerance in transgenic creeping bentgrass (Agrostis stolonifera L.). Plant Physiology 16:1375-1391.
5. Type: Journal Articles Status: Published Year Published: 2013 Citation: Li, Z., Hu, Q., Zhou, M., Vandenbrink, J., Li, D., Menchyk, N., Reighard, S.R., Norris, A., Liu, H., Sun, D., Luo, H. (2013) Heterologous expression of OsSIZ1, a rice SUMO E3 ligase enhances broad abiotic stress tolerance in transgenic creeping bentgrass. Plant Biotechnology Journal 11:432-445.
6. Type: Journal Articles Status: Published Year Published: 2012 Citation: Teng, S., Luo, H., Wang, L. (2012) Predicting protein sumoylation sites from sequence features. Amino Acids 43:447-55.
7. Type: Conference Papers and Presentations Status: Published Year Published: 2013 Citation: Luo, H. (2013) Grass is greener on the other side ? biotechnology for turfgrass genetic improvement. 2013 In Vitro Biology Meeting. June 15-19, Providence, RI. Oral presentation.

ASSESSING THE RISK OF TRANSGENE ESCAPE VIA POLLEN FLOW IN CARROT

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Award Number: 2013-33522-21006

NON-TECHNICAL SUMMARY: Gene flow between genetically modified (GM) crops and their wild relatives has the potential to enhance weediness and/or invasiveness of wild species. In the United States, invasive species have major negative impacts on natural ecosystems, leading to billions of dollars per year in economic and environmental damages, and invasive species are often responsible for the displacement and/or extirpation of native species. Given that crop plants and their sexually-compatible wild relatives often overlap in terms of geographic proximity and flowering time, the likelihood of gene escape can be quite high. The placement of a transgene into the organellar (chloroplast or mitochondrial) genome of a plant has been suggested as a means of reducing the risk of pollen-mediated transgene escape from GM crops to wild relatives because these genomes are usually transmitted solely through seed and not pollen (that is, the chloroplast and mitochondria are maternally transmitted). Carrot lines carrying transgenes conferring a variety of beneficial traits are being developed and organellar placement of such transgenes has been proposed to reduce the risk of transgene escape via pollen to wild and weedy carrot. Maternal transmission of organellar genomes is, however, far from universal, and mathematical models have suggested that even low levels of transmission in pollen may be sufficient for the establishment and spread of transgenes in the wild. In carrot, we have found preliminary evidence that the chloroplast and/or mitochondrial genomes may sometimes be paternally transmitted thus allowing a transgene to potentially escape via pollen flow to its highly inter-fertile weedy relative, wild carrot (Queen Anne's Lace). We plan to use multiple approaches to assess the likelihood and degree of paternal transmission of organellar genomes in carrot. The proposed research will greatly improve our understanding of the potential for the paternal transmission of organellar genomes to allow transgene escape via crop-wild hybridization. Beyond providing insight into specific risks associated with crop-wild gene flow in carrot, this project will also inform risk assessment efforts in other crop systems.

OBJECTIVES: The specific goals of this project are to: (1) characterize the level of heteroplasmy in natural populations of wild carrot and evaluate the potential for paternal leakage and cytoplasmic gene flow; (2) directly measure the frequency of paternal leakage and heteroplasmy in controlled crosses between cultivated and wild carrot; (3) investigate historical levels of crop-wild gene flow across the range of carrot cultivation, thereby providing baseline estimates of gene flow for use in mathematical models aimed at assessing the risk of transgene escape; (4) implement and expand upon predictive models of the spread of cytoplasmic transgenes in wild populations.

APPROACH: In general terms, the methods we will employ during this research project will include laboratory, field, and greenhouse methods and protocols. We will use a PCR-RFLP

method previously designed and described by us to perform a large-scale population genetic analysis of cytoplasmic diversity within and among wild carrot populations. These data will provide information about gene flow for our mathematical modeling efforts. Additionally, in order to assess the risk of transgene escape by pollen flow in carrot, we will employ a Taqman qPCR strategy, also designed by us, to document organellar paternal leakage in natural populations of wild carrot. We will also generate controlled crosses between wild and crop carrot in order to assess the degree of paternal leakage again utilizing our qPCR protocol. We will also use nuclear and cytoplasmic genetic markers to investigate historical levels of crop-wild gene flow across the range of carrot cultivation, thereby providing baseline estimates of gene flow for use in mathematical models aimed at assessing the risk of transgene escape.

PROGRESS: 2013/09 TO 2016/08
Target Audience: The target audiences for this research were ecological geneticists and biotechnology regulators. This research was also relevant for seed producers who wish to minimize crop-to-crop transgene dispersal. Changes/Problems: The PD has moved to the University of Memphis (Assistant Professor of Biological Sciences). There has been no modification of the scientific work or plan for this grant. The grant is being transferred to the University of Memphis. What opportunities for training and professional development has the project provided? Since this project had just begun in September 2013, and the project is being transferred to the University of Memphis where the PD is now an Assistant Professor, no training and professional developments have occurred at this time. These opportunities will occur as the project continues in the PD's new institution. How have the results been disseminated to communities of interest? Since this project had just begun in September, and the project is being transferred to the University of Memphis where the PD is now an Assistant Professor, no results have been disseminated at this time. Results will be disseminated to communities of interest as the project continues in the PD's new institution. What do you plan to do during the next reporting period to accomplish the goals? Nothing Reported

IMPACT: 2013/09 TO 2016/08
What was accomplished under these goals? A series of plant collections from wild populations of Daucus carota (Queen Anne’s Lace) have been made along the eastern coast of the United States. When possible, both leaves and seeds were collected to facilitate the goals of Objectives 1 and 2 of this grant: characterize the level of heteroplasmy in natural populations of wild carrot and use indirect methods to evaluate the potential for paternal leakage and cytoplasmic gene flow, and characterize the level of heteroplasmy in natural populations of wild carrot and use indirect methods to evaluate the potential for paternal leakage and cytoplasmic gene flow. DNA extraction of these wild individuals is underway. In addition, the assays for quantifying heteroplasmy in D. carota have been designed, ordered, obtained, and calibration experiments of the assays have begun. With respect to Objectives 3 and 4, no progress has been made to date. Work on these objectives will continue at the PD's new institution.

PUBLICATIONS (not previously reported): 2013/09 TO 2016/08
No publications reported this period.

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 worlds 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.

PROGRESS: 2012/09 TO 2013/08
Target Audience: The PI presented early results at a ‘convening’ organized by the Bill and Melinda Gates Foundation in October of 2012, and at the BRAG PI meeting in June of 2013, in each case addressing other scientists, program personnel, and decision-makers.
Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? Several postdoctoral scientists (particularly Uzay Sezen, Changsoo Kim, and Tae-ho Lee) and graduate students (particularly Hui Guo and Wenqian Kong) funded by a variety of sources have been engaged in genetic mapping, phenotyping, and data analysis. Many technicians and part-time student workers in the PIs’ lab also assisted with phenotyping. How have the results been disseminated to communities of interest? Presentations at BMGF and BRAG meetings (see description of the target audience(s) reached). What do you plan to do during the next reporting period to accomplish the goals? We consider that we are on track, indeed a little ahead of schedule, based on the proposed timeline. Notification of a probable award was early enough that we were able to plant experimental populations in time for the 2012 growing season, which permitted us to gain some time relative to the proposed timetable.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? Genetic mapping of the S. bicolor x S. halepense tetraploid F2 population is in progress, with a framework of about 100 SSRs mapped. A first batch of reduced-representation sequencing (for genotyping-by-sequencing) was performed, but additional sequence is needed to make definitive genotype calls (genome reduction may have been insufficient, protocols are being revised). Extensive phenotyping was performed both in Georgia and in Kansas on F3 progeny rows from the F2 plants, including the spectrum of morphological and physiological traits proposed as well as regrowth after overwintering.

Substantial differences between the locations, particularly regarding winter survival (>90% in GA, about 24% in KS) are expected to be valuable for genetic analysis. In the Georgia location, we have also added a measurement of biomass produced from regrowth after overwintering based on a harvest on 31 July (approximately the midpoint of the growing season) and intend to take a second measurement at the end of the growing season, to provide a further assessment of perenniality (the ability to sustain similar levels of biomass over multiple harvests may be a valuable complement to 'regrowth' measurements that primarily assessed winter survival).

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
No publications reported this period.

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 resisance 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 liter 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.

PROGRESS: 2012/09 TO 2013/08
Target Audience: Target Audiences: Regulators, researchers, and the general public are the target audiences. Presentations: SUNY-ESF Alumni Tours of chestnut plantings at the Lafayette Road Experiment Station. Included BRAG research plots.10/6/12 Fifth International Chestnut Symposium, Shepherdstown, WV. 9/4/12 -9/8/13: Developing transgenic American chestnut (Castanea dentata) to enhance blight resistance, W.A. Powell, C.A. Maynard, S.A. Merkle, C.J. Nairn, A.E. Newhouse, K.M. Baier, B. Zhang, L.C. Northern, K.M. D'Amico, A.D. Oakes, L.D. McGuigan The American Chestnut Foundation's Board and Annual Meeting, Ashville, NC. 10/18/12 – 10/21/12. Four posters presented: Enhancing blight resistance in American chestnut using an oxalate oxidase gene from wheat. W.A. Powell, A.E. Newhouse, B. Zhang, K.M. Baier, L.D. McGuigan , A.D. Oakes, H. Liang, W. Rottmann,, & C.A. Maynard Detached Leaf Assay: Quick Screen for Blight Susceptibility in Transgenic American Chestnuts. Andrew E Newhouse, Charles A Maynard, and William A Powell Assessing Ectomycorrhizal Associations on Transgenic American Chestnut Compared to the Wild-Type, a Conventionally-Bred Hybrid, and Related Fagaceae Species. Katherine M D'Amico, Charles A Maynard, Thomas R Horton, William A Powell Introducing FT1, an Early Flowering Gene, into American Chestnut as a Tool for Accelerated Breeding. L.C. Northern, A.D. Oakes, K.M. Baier, A.E. Newhouse, C.A. Maynard, and W.A. Powell National Geographic "de-extinction" workshop, Washington DC, 10/22/12-10/25/12. New York State American Chestnut Research and Restoration Program at SUNY-ESF. Note: This presentation lead to the TEDx talk later. The New Chapter of The American Chestnut Foundation Annual meeting, which we also hosted at SUNY-ESF, Syracuse. 10/26/12 – 10/27/12. American Chestnut Research and Restoration Project at SUNY-ESF. Gave the first announcement that we achieved enhanced blight resistance in our transgenic American chestnut trees. Invited speaker, Asa Gray Biological Seminar Series, Utica College, NY. 12/3/12. American Chestnut Research and Restoration Project at SUNY-ESF. Approximately 40 attending. Invited speaker, TEDx DeExtinction, National Geographic, Washington DC. 3/14/13 – 3/15/13. Reviving the American forest with the American chestnut. This TEDx talk was with an audience of over 300 in the auditorium and a live feed to over 3000 viewers on the web. The TEDx talk is on YouTube at
http://www.youtube.com/watch?v=WYHQDLCmgyg&noredirect=1 and so far has over 4275 views to date. Invited speaker at annual meeting of the Pennsylvania chapter of The American Chestnut Foundation. Hershey, PA. 4/5/13- 4/7/13. American Chestnut Research and Restoration Project at SUNY-ESF. Approximately 40 attending. Invited Speaker, SUNY Cortland biology lecture series. Cortland, NY. 4/8/13. "Where there be mountains, there be chestnuts." Approximately 30 attended. CNY Biotechnology Symposium 2013. Syracuse, NY. 5/17/13. Chaired the Agricultural Biotechnology Session. Invited Speaker: Enhancing blight resistance in American chestnut. Approximately 30 attending. Invited speaker at the IUFRO Tree Biotechnology 2013 conference. Asheville, NC. 5/26/13 – 6/1/13. Transgenics or cisgenics, which will save the American chestnut? BRAG Annual Project Director's Meeting. 6/14/13. Poster: American chestnut restoration introduces a new paradigm of transgene introgression to save a keystone species. WA Powell, AE Newhouse, K D'Amico, S. Tourtellot, T. Horton, D.

Parry, T. Tschaplinski, and CA Maynard. News articles in The Wall Street Journal 8/20/12; Nature 10/3/12; The Economist 5/4/13; The Atlantic 5/29//13; Der Spiegel 12/8/13
Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? In addition to one undergraduate working one the BRAG research over the summer, two Skaneateles high school students helped with objective one and learned how to do DNA and RNA extractions, agaorse geles, PCR and RT-qPCR. Here is a link to an article about the students: Skaneateles high school students get hands on experience in SUNY ESF lab. http://www.eaglenewsonline.com/news/2013/oct/02/skaneateles-high-school-student

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? Objective 1: Determine the stability of transgene expression over time and between generations. PCR and RT-qPCR was performed on four-year-old transgenic trees and controls. All the transgenic trees retained their transgenes and all continued to express these transgenes. The level of gene expression varied a small but significant amount among the trees tested, probably due to environmental effects. 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. Transgenic pollen was produced from Hinchee 1 and Darling 4 events prior to the start of this grant and used in controlled pollinations. The nuts were harvested under this grant and sent for analysis at Oak Ridge National Labs. Non-transformed controls were also analyzed. There were no significant differences in most of the metabolites tested between the transgenic nut and controls. The couple metabolites that did differ were most likely due to the nuts beginning to germinate. This analysis is being repeated this year with nuts that haven't started germination. Also, leaf and stem samples have been sent for analysis and we are waiting on the results. 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. Within Year Comparison: In 2012 we looked at the growth rate of four lepidopteran folivores (gypsy moth, forest tent caterpillar, Polyphemus moth, and fall webworm) on transgenic and conventionally bred cultivars in our chestnut panel. Species selected spanned the growing season, representing early, mid, and late season feeding guilds. All tested species performed as well or even slightly better on transgenic cultivars than on those conventionally bred although these differences were not statistically significant. The growth of Polyphemus moth, a mid-season feeding species was significantly higher on 'Hinchee', which has both an OxO expressing transgene and a construct coding for an antimicrobial peptide. Among Years: We contrasted the relative growth rates for gypsy moth in three consecutive years (2011-2013) on the same trees and found little interannual variation in performance. Larvae grew faster on the transgenic varieties in each year (10%, 8%, and 4% in 2011, 2012 and 2013, respectively) but these differences were small and only significant in 2011. Poorest performance was always on the conventionally bred American chestnut variety 'Zoar', followed by the pure Chinese cultivar 'Cropper'. Objective 4: Examine and compare decomposition of leaf liter between the transgenic events, empty-vector transgenic controls, isogenic non-transgenic controls, and the standard panel of conventionally-bred chestnuts. Leaf litter bags of transgenic and non-transgenic leaves were set out in the spring of 2013. These will be collected in one year to determine decomposition. 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. Initial measurements specific leaf areas of: Hinchee 1, Zoar, GR 68-B1, K-L-BC-1, and Darling 4; data has been entered, triple checked and is ready for analysis. Initial measurements of all BRAG seedlings for height, length and diameter (Year 4 measurements) (NOTE: year 1-3 measurements included only length and diameter); data was entered, triple checked and is ready for analysis. Note: Much of the preliminary work has been accomplished for objectives 4 and 5 and the graduate students are just coming on board this fall to begin the data analysis and continue data collection.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
No publications reported this period.

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

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Raleigh, North Carolina

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 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 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

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: 2012/09 TO 2013/08
Target Audience: Research scientists Regulatory agencies such as APHIS Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? Training and professional development was provided for three graduate students and two postdoctoral researchers. How have the results been disseminated to communities of interest? CONTRIBUTED switchgrass PAPERS AT PROFESSIONAL MEETINGS: (* designates presenter) Chang*, H., A.A. Snow, E. Mutegi, E. Lewis, M.N. Miriti, and E.A. Heaton. Hybridization between cultivated and wild switchgrass (Panicum virgatum) as a function of distance from cultivar field trials: implications for Biosafety procedures. Botanical Society of America Annual Meeting, New Orleans, LA, July 27-31, 2013. Palik*, D.J., A.A. Snow, A.L. Stottlemyer, M.N. Miriti, and E.A. Heaton. Responses of cultivated and wild switchgrass (Panicum virgatum) to competition: implications for new biofuel cultivars. Botanical Society of America Annual Meeting, New Orleans, LA, July 27-31, 2013. OTHER PRESENTATIONS THAT INCLUDED OUR FINDINGS: 1. Heaton E.A., Schulte L.A, & Wilson D.M.* (2013) Integrating Food and Fuel: How to Manage a 2G Crop Portfolio. BioFuelNet Canada Annual Meeting, June 22-24, 2013. Montreal, CA. 2. Heaton E.A. (2013) Dedicated Energy Crops. Community College Teacher Continuing Education Webinar, Ames, IA. www.agenergy.ia.org. 3. Heaton E.A., Schulte L.A. & Milster F. 2013. Integrating food and fuel production in the Corn Belt. Kohn Lecture Series, University of Iowa, Iowa City, IA. 4. Heaton E.A. 2013. Energy Crops in Iowa. Graduate Program in Sustainable Agriculture Colloquium, Iowa State University, Ames, IA. 5. Heaton E.A., Schulte-Moore L.A., Helmers M., Liebman M. & Milster F. (December 6, 2013) Producing food, feed and energy: How can agriculture do it all? 25th Annual Integrated Crop Management Conference. Iowa State University, Ames, IA; 182 participants. 6. Heaton E.A. (August 14, 2013) Bioenergy crop research. Congressional Aide field tour. Boone, IA, 53 participants. 7. Heaton E.A. (August 17, 2013) Bioeconomy Media Tour. Boone, IA, 18 participants. 8. Heaton E.A., Boersma N.N.* Schulte L.A. & Wilson D.* (March 19, 3013) Perennial energy crops in Iowa. 3rd Annual Biomass Workshop. Iowa City, IA. 30 participants. 9. Heaton E.A., Schulte L.A. & Wilson D.* (March 12, 3013) Managing for bioenergy – integrating food and fuel production. Ag Education Day, Emmetsburg, IA, 130 participants. 10. Heaton E.A. (February 28, 2013) Perennial bioenergy crops benefit soil and water. University of Illinois Soil and Water Workshop, Taylorville, IL, 75 participants. What do you plan to do during the next reporting period to accomplish the goals? We plan to complete several publications in the next reporting period. The field work is now complete, and all that remains are DNA analyses, data analysis, and writing.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? We investigated 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. We also compared 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). Our results have been presented at professional meetings and we are working on manuscripts for publication.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
Type: Journal Articles Status: Published Year Published: 2013 Citation: 2013 ? Mutegi, E., A. L. Stottlemyer, A. A. Snow, and P. M. Sweeney. Genetic structure of remnant populations and cultivars of switchgrass (Panicum virgatum) in the context of prairie conservation and restoration. Restoration Ecology DOI: 10.1111/rec.12070

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.

PROGRESS: 2011/09 TO 2012/08
Target Audience: Nothing Reported Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? This project has provided training for a post doc, a graduate student and three undergrads How have the results been disseminated to communities of interest? Results were presented at conferences What do you plan to do during the next reporting period to accomplish the goals? We will be analyzing the results of the results of the microarray analysis. a new winter wheat plot was set in the same facility using the same design. this plot will be used to corroborate any genetic and biological results found from the microarray analysis. As well as this, insect foraging of the wheat and sampling of mortality of local insect fauna will be observed with this plot. From these field trials, we expect to characterize the impact the application of genetically modified Metarhizium to managed and unmanaged systems. The microarray will provide unprecedented insight into the genetic consequences of introducing a modified fungus into the rhizosphere both on the root and in the soil surrounding it. We expect that these methods will set a standard for future scrutiny of modified organisms applied to soil systems. The results of this study should also provide an in depth comparison between the activity of the microbial communities in winter wheat and turf rhizospheres

IMPACT: 2011/09 TO 2012/08
What was accomplished under these goals? A field trial was set up at the University of Maryland Beltsville Facility, examining the effect of Metarhizium robertsii on the rhizosphere and bulk soil surrounding both turf and winter wheat (Triticum aestivum). Winter wheat seeds were treated with gum arabic with or without GFP expressing Metarhizium conidia prior to sowing, and the turf plots were drench inoculated with conidial suspensions in water. The treatment of the sections of the winter wheat plot was randomized with 20 seeds in each the 9 sections. Sections were surrounded by 1 meter, barren buffer zones. Weekly, plots were weeded by hand until the wheat reached maturity. Turf and winter wheat soil samples were taken from the rhizosphere, bulk soil. DNA from the soil samples were immediately extracted using a FastDNA SPIN Kit for Soil, and sent to the Institute for Environmental Genomics at the University of Oklahoma. Here, they are currently being analyzed using the latest GeoChip microarray, which is customized for analyzing the activity of the functional community of soil microbes.

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

POLLEN-MEDIATED GENE FLOW IN SWITCHGRASS

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Knoxville, Tennessee

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: 2012/09 TO 2013/08
Target Audience: The target audience for our project is three-fold. First, regulators interested in establishing and enforcing guidelines germane to the risk of outcrossing (via pollen flow and successful pollination and fertilization) of future plantings of transgenic switchgrass with compatible wild relatives are interested in our results. Second, conservation biologists from federal and non-federal entities (including NGOs) are interested in invasiveness traits in agronomic x wild hybrids, as well as the relatedness of agronomic cultivars and wild

populations. Third, the private sector, including seed companies and biofuel industry, is interested in our findings, as future efforts that would include further transgenic switchgrass development are tied to our work. Efforts undertaken in this reporting period include the PD's participation in the annual PD meeting, and a poster presentation at the Switchgrass II meeting in Madison, Wisconsin. Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? Postdoc attendance at the Switchgrass II conference in Madison, Wisconsin occurred. This allowed our postdoc to present our current findings and network with switchgrass researchers. How have the results been disseminated to communities of interest? Poster presentations at pertinent venues (e.g. Switchgrass II conference, Madison, Wisconsin). What do you plan to do during the next reporting period to accomplish the goals? Nothing Reported

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? Switchgrass Pollen Flow Work on switchgrass pollen flow continues. Pollen traps that collected pollen in 2012 were screened and orange fluorescent protein (OFP) pollen grains were quantified. Summed OFP pollen counts (to 100 m) declined as a function of distance from source patch (Poisson regression, $F_{1,8}=351.76$, $P<0.0001$). When distances are modeled as an exponential distribution ($\lambda=0.0266$), the probability of pollen traveling > 100 m = 0.07. Results here indicate that the vast majority of pollen does not travel < 100 m; however, depending on the density of pollen-producing switchgrass plants, a considerable amount of pollen is capable of traveling > 100 m. An additional year of data on this is currently being pursued. Of the seeds collected from receptor plants during the study period, very few seeds (N=27) germinated. Seedling counts declined as a function of distance from the pollen source patch (Poisson regression, $F_{1,22}=9.67$, $P=0.005$). OFP was PCR-confirmed in ~50% of seedlings. An additional year of data on this is currently being pursued. 'Field-to-field' Pollen Flow/Hybridization The 'field-to-field' pollen flow/hybridization portion of the study continued. During this project period, SNPs potentially distinguishing cv. Alamo and cv. Kanlow (EG1102) at our study sites, resulting from Next Generation Illumina sequencing, have been identified. Quantitative PCR (qPCR) screening work is underway to further ascertain their applicability. Breeding Studies Breeding studies continue. Attempted crosses included (1) (agonomic) cv. Alamo X congener (Panicum dichotomiflorum), and (2) cv. Alamo X wild switchgrass native to TN. The limited crosses of cv. Alamo with P. dichotomiflorum were unsuccessful. Crosses of cv. Alamo with native switchgrass were successful and resulted in ample seed. Seeds were cold-stored through the winter, and were sown in Spring 2013. Data collected from germinants included three variables associated with invasiveness: (1) time to germination, (2) seedling height at 1 month, and (3) a standard water use efficiency metric. Hybrid offspring did not exhibit faster germination times. Water use efficiency of hybrids was not enhanced. These results indicate that hybrids resulting from introgression would not be invasive. Heights of 1-month-old hybrid seedlings were taller than those of agronomic and wild seedlings; this trait could convey competitive advantages. An additional year of data is currently being pursued.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
1. Type: Journal Articles Status: Published Year Published: 2013 Citation: Nageswara-Rao, M., J.R. Soneji, C. Kwit, and C.N. Stewart. 2013. Advances in biotechnology and genomics of

switchgrass. Biotechnology for Biofuels 6: 77.
2. Type: Journal Articles Status: Published Year Published: 2013 Citation: Nageswara-Rao, M., C.N. Stewart, and C. Kwit. 2013. Genetic diversity and structure of natural and agronomic switchgrass (Panicum virgatum L.) populations. Genetic Resources and Crop Evolution 60: 1057-1068.
3. Type: Book Chapters Status: Published Year Published: 2013 Citation: Kwit, C., M. Nageswara-Rao, and C.N. Stewart. In Press. Switchgrass (Panicum virgatum L.) as a bioenergy crop: advantages, concerns, and future prospects. In H. Lou and Y. Wu, eds. Compendium of Bioenergy
4. Type: Conference Papers and Presentations Status: Published Year Published: 2013 Citation: Nageswara-Rao, M., M. Hanson, S. Agarwal, C.N. Stewart, and C. Kwit. 2013. Population genetics and diversity analysis of natural and agronomic switchgrass populations. Switchgrass II, Madison, Wisconsin, USA. (poster)
5. Type: Conference Papers and Presentations Status: Published Year Published: 2013 Citation: Hanson, M., M. Nageswara-Rao, S. Agarwal, C.N. Stewart, and C. Kwit. 2013. Diversity and genetic structure of switchgrass populations. Exhibition of Undergraduate Research and Creative Achievement (EUR?CA), Knoxville, Tennessee, USA. (poster)

SWITCHGRASS BIOCONFINEMENT: DELAYED FLOWERING, SELECTIVE MALE- AND SEED-STERILITY, AND CONDITIONAL TOTAL BIOCONFINEMENT

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University of Tennessee
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Award Number**:** 2013-33522-20997

NON-TECHNICAL SUMMARY: Genes that are engineered into crops typically serve the purpose of adding value to the crop or product. There remains a concern among regulators of biotechnology, some scientists and citizens, about gene flow from biotech crops into other non-engineered plants, including free-living wild plants and weeds. In a crop such as switchgrass, where wild switchgrass exists in the areas of cultivation, the gene flow concern can be addressed by engineering plants so that they do not flower, or that pollen, seed, or both are non-viable. We will test three different systems to achieve these endpoints. The first uses a gene that delays flowering 'microRNA 156,' that also increases biomass production. The second system utilizes an enzyme to degrade DNA that will be targeted to be produced specifically in pollen or seed. This DNA chopper--the restriction enzyme 'EcoRI'--has proven to render male sterility in a test plant system and thus, it will be applied to switchgrass. The third system to limit gene flow is to produce a switchable system--a 'synthetic circuit' in engineered plants. This circuit will allow plants to flower and set seed when someone wants them to reproduce, say, when breeding to produce seed to sell to farmers, but be activated to limit gene flow in production fields. The systems could be combined to increase effectiveness of gene flow limitation. Taken together these systems will advance our technological toolkit and knowledge to limit gene flow in engineered crops.

OBJECTIVES: The overall goal of the project is to develop systems to bioconfine transgenes into the intended host of switchgrass. Specific objectives of the project include the following: 1. Delayed or no flowering. Switchgrass plants engineered for moderate overexpression of microRNA156 (miR156), which renders delayed or no flowering, will be field-tested. 2. Male- and seed-sterility. A restriction endonuclease, EcoRI, will be specifically expressed in pollen or seed tissues and the efficacy for ablating target cells will be assessed, first in rice as a grass model and then translated to switchgrass. 3. Conditional total transgene bioconfinement. A novel system in which EcoRI-based seed sterility is repressible by chemical treatment for breeding (in trans) will be developed and assessed in rice and then translated to switchgrass.

APPROACH: We will use a combination of methods from the computational-based genetic engineering and synthetic biology to field experiments. The three technologies focus on 1) delayed-to-no flowering, 2) male- and seed-specific sterility approaches, and 3) conditional total bioconfinement. The total bioconfinement system is a default-on system that can be toggled to the off position for breeding and on for deployment. Success in one or more of these approaches will translate to important steps for bioconfinement of transgenes for switchgrass and other crops. The proposed research is based on recent discoveries and preliminary biotechnological

data demonstrating that certain key components for bioconfinement are effective and show promise for additional research. While the delayed-to-no flowering transgenic system in switchgrass is most advanced in its development, we need to better understand its limits of seed production and transcriptomic effects. Male sterility and seed sterility are based on new technologies that are designed for switchgrass. Conditional total bioconfinement based on toggling is the "grail" for bioconfinement, since it would enable breeding and seed production by companies, yet, if effective, provide complete transgene bioconfinement in production fields. All research will follow NIH Guidelines and USDA APHIS regulations.

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-resitance 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.

PROGRESS: 2012/09 TO 2013/08
Target Audience: The target audience of this project includes researchers and technologists in academia and industry in the areas of insect pathology, agricultural biotechnology and Bt-resistance management. Audience of this project also includes insect pest management professionals and growers, students and postdoctoral trainees in relevant areas, relevant government agencies, and the general public. In this reporting period, target audience was reached through presentations of our research project and results from the project in academic meetings, including 4 presentations in international and national academic conferences, 1 presentation in this USDA funding agency workshop, 1 presentation in an international agricultural biotechnology symposium, 1 presentation in a graduate student research symposium, and 1 public outreach event to deliver information about Bt-crops and Bt-resistance in insects to the general public (number of visitors was over a thousand). This project also provided training opportunities to 1 postdoctoral researcher, 3 graduate students and 1 undergraduate student, and trainees obtained knowledge from the research. Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? This project provided opportunity for training to 1 postdoctoral associate, 3 PhD students and 1 undergraduate student. The project is also an excellent opportunity for professional development for the PI and a research support specialist on the research team. How have the results been disseminated to communities of interest? The results from our research were disseminated to the communities of interest by our presentations in international and national academic conferences, workshops and symposia, as well as out reach events to reach the general public. What do you plan to do during the next reporting period to accomplish the goals? The project has progressed as proposed in this reporting period. We will continue this project as planned in the proposal.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? In this first year of the project, a full time postdoctoral researcher was successfully recruited to join the project, starting in April of 2013. Overall, the project progressed well as planned in the proposal. To accomplish the objectives of this project, we have obtained the cDNA sequence of the ABCC2 gene from T. ni and a fragment of its genomic DNA sequence. We have obtained data for the expression of the ABCC2 gene at both mRNA and protein level, and our preliminary analysis has indicated that the ABCC2 gene is not differentially expressed between the susceptible and Bt Cry1Ac resistant T. ni strains, but is genetically linked with the resistance. We have also identified single nucleotide polymorphisms (SNPs) in the ABCC2 gene between the susceptible and resistant T. ni strains, and further full length cDNA cloning from individual T. ni larvae and structural analysis and genetic linkage of the SNPs in the ABCC2 gene with the Cry1Ac resistance is currently on going. Using the Next Generation Sequencing technology, we have so far identified several thousands of SNPs between the susceptible and resistant T. ni strains. We are continuing our sequencing experiments for the project. At present, we have sequenced >40 RNA-seq libraries from genetically structured T. ni families with multiple biological replications and have started to identify association of genes and their expression with Bt resistance.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08

1. Type: Conference Papers and Presentations Status: Other Year Published: 2012 Citation: Wang, P. 2012. Proteomic approaches to understand resistance to Bt toxins in Trichoplusia ni. The 2nd International Symposium on Insect Midgut Biology, Guangzhou, China. Sept. 24-28, 2012.
2. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Song, X. and Wang, P. 2013. Biochemical analysis of resistance of Bacillus thuringiensis toxins in cabbage looper, Trichoplusia ni. Annual Entomology Department Symposium, Cornell University, Ithaca, NY. Jan. 18, 2013.
3. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Wang, P. 2013. What mechanisms of resistance to transgenic Bt-crops may be selected in insect populations in the field? The 1st Emerging Information and Technology Association Conference on Agricultural Science and Technology, Biosystems Engineering -- Precision Agriculture: Challenges and future directions. Cornell University, Ithaca, NY. June 27-28, 2013.
4. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Tetreau, G., Song, X., Chen, Y-R., Gao, S., Fei, Z., Blissard, G. and Wang, P. 2013. Midgut transcriptome of the cabbage looper, Trichoplusia ni. Annual Meeting of the Entomological Society of America, Austin, TX. Nov. 10-13, 2013.
5. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Wang, P. 2013. ABC transporter-associated resistance to Bacillus thuringiensis toxins in insects. Annual Meeting of the Entomological Society of America, Austin, TX. Nov. 10-13, 2013.
6. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Wang, P. 2013. Variation of the midgut cadherin in the cabbage looper, Trichoplusia ni. Annual Meeting of the Entomological Society of America, Austin, TX. Nov. 10-13, 2013.
7. Type: Conference Papers and Presentations Status: Other Year Published: 2013 Citation: Wang, P. 2013. Molecular genetic basis of insect resistance to Bt-crops. NIFA and BRAG annual project director?s meeting. Riverdale, MD. Jun 14, 2013.

GENOME-WIDE ASSESSMENT OF OFF-TARGET EFFECT AND REMOVAL OF TRANSGENES ASSOCIATED WITH TALEN-BASED GENE EDITING IN PLANT

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Award Number: 2013-33522-21091

NON-TECHNICAL SUMMARY: This proposal will address the risks associated with TALEN-based genetic engineering in crop plants. The hypotheses underlying this work are that TALENs, fusion proteins of TAL effectors and the DNA cleavage domain of endonuclease FokI, are capable of introducing precise, targeted genomic modifications in crops and that the TALEN constructs can be completely removed through genetic segregation. Our published and preliminary data demonstrate, as a proof-of-concept, that TALENs can create site-specific gene changes and intended phenotypes in rice and that the transgenes are undetectable by PCR in the modified genomes after genetic segregation. Thus, our experiments are designed to assess the broad applicability of TALEN technology to polyploid switchgrass and to use the rice model to thoroughly assess potential unintended genotypic and phenotypic effects of TALENs, as well as the unequivocal removal of TALEN transgenes from the modified genomes. We will use whole-genome sequencing to compare the genome of wild-type rice with those of rice mutants that have undergone two rounds of TALEN-mediated gene editing, to identify and quantify potential risks associated with any promiscuous activity of TALENs and residues of TALEN transgenes in the modified rice. Finally, developing a better assessment of the TALEN-associated risks will provide a broad foundation for future crop engineering efforts directed at genomic modification in rice, provide comprehensive insight into the applicability of TALEN technology to other crop and bioenergy plants, and assist federal regulatory agencies in determining whether TALEN technology merits either an exemption from regulatory oversight or a less rigorous regulatory process.

OBJECTIVES: Establish a pipeline of TALEN-based gene editing and assess its robustness and general applicability in crop plants by focusing on rice and switchgrass. Assess potential off-target mutations caused by TALENs in modified rice genomes by comparing seven modified genomes against the parental reference genome. Assess the removal of the TALEN transgenes from the modified rice genome through genetic segregation at the genomic level. Analyze potential phenotypic variations in rice plants associated with TALEN-based gene editing by investigating a number of morphological traits (plant height, tiller number, dry biomass, etc.).

APPROACH: 1. Assess the efficiency of TALEN technology in rice with multiple target genes. we will mainly use TALENs to target the coding regions of the 15 rice SWEET genes. 2. Assess the feasibility and efficiency of TALEN-mediated genome editing in switchgrass. we propose to use the TALEN technology to produce transgenic tetraploid switchgrass, in which the switchgrass homologs of the CBF2 (C-repeat binding factor 2), MYB 15 (MYB transcription

factor), FT (Flowering locus T) and AP1 (APETALLA 1) genes are inactivated. 3. Assess the off-target effect of TALENs in modified rice genomes by comparing seven target genomes with the parental reference genome. Whole-genome sequencing technology will be deployed to resequence seven modified rice lines and the parental Kitaake and perform comparative analysis of their genomes to identify any potential off-target modifications caused by the two independent pairs of TALENs. 4. Assessment of T-DNA removal from the modified rice genomes through genetic segregation at a genomic level. Comparative analysis of the seven modified rice genomes and the parental genome will be performed to establish with a high degree of confidence whether the whole T-DNA region or any residual sequence from the T-DNA or binary vector exists in the modified genomes. 5. Phenotypic analysis of TALEN-modified rice plants. Experiments will include disease assays to assess modified plants for intended disease resistance, and biomass and yield assays to assess the modified plants for growth and production.

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: 2012/09 TO 2013/08
Target Audience: Nothing Reported Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? This proposal represents a collaborative effort among scientists from the University of Kentucky (PI Zhou) and the University of Nebraska (Co-PI Siegfried). Richard Hellmich (USDA-ARS) has been providing monarch butterfly eggs for the development of RNAi toxicity assay. Funding from this proposal currently supports two post-doctoral associates at the University of Kentucky and at the University of Nebraska, respectively, and a temporary laboratory technician at the University of Kentucky. How have the results been disseminated to communities of interest? Nothing Reported What do you plan to do during the next reporting period to accomplish the goals? We are on track to complete all the stated objectives which will provide answers to questions directly pertaining to the development of a risk assessment framework for the RNAi crops. The newly established in vivo RNAi toxicity assay for each surrogate species will allow us to define the test conditions (worst case scenario), and determine the appropriate endpoint measurements for the risk assessment of RNAi crops. The research will directly assist in the formulation of ecological risk assessment framework for RNAi transgenic crops by establishing in vivo RNAi toxicity assays for relevant surrogate species representing a diverse community of non-target arthropods within the RNAi maize agroecosystem.

IMPACT: 2012/09 TO 2013/08
What was accomplished under these goals? We have 1) developed in vivo RNAi toxicity assay for the incidental pollen feeders, honeybee, Apis mellifera, and monarch butterfly, Danaus plexippus, bio-control agents, ladybugs, Harmonia axyridis and Hippodamia convergens, and

decomposers, springtails, Folsomia candida and Sinella curviseta; 2) refined the worst case scenario for the RNAi toxicity test; and 3) investigated risk assessment endpoints and test duration for respective surrogate species. Sequence similarity, dose, and exposure are the key factors that define the worst case scenario for the RNAi-toxicity test. However, during the development of in vivo RNAi toxicity assay for the surrogate species, sequence identity, especially identical sequences of at least 21 nucleotides (20mers) between target and non-target arthropods of arthropod-active genes, might dictate the specificity of transgenic RNAi maize. Interestingly, different surrogate species, even within the same taxonomic group, respond to dsRNA-mediated RNAi treatment differently. Our preliminary results showed that ladybugs are more sensitive toward dsRNA-mediated RNAi treatment than honeybees. However, among the two ladybug species, Hippodamia convergens is significantly more responsive toward dsRNAs than Harmonia axyridis, and displayed strong cross-reaction using the Western corn rootworm dsRNA. The validity of in vivo RNAi toxicity assay was examined at the transcriptional level using a readily available qRT-PCR protocol and at the translational level using a commercially available V-ATPase polyclonal antibody. In addition to mortality, we also documented other life history traits, including body weight, development time, and fecundity, as the potential endpoints for the ecological risk assessment of RNAi crops, especially under sublethal conditions of exposure.

PUBLICATIONS (not previously reported): 2012/09 TO 2013/08
No publications reported this period.

QUESTIONS FOR DISCUSSION

1. CFR 340 is grounded in pest science and many parts of the regulations are not pertinent to today's biotechnology. For example, by using the gene gun and common bacterial-based antibiotic resistance genes and plant genes, genetically engineered items are not regulated by BRS. And we don't know how synthetic biology/genome editing in plants will be regulated in the US. When will USDA APHIS BRS revise its regulations to make them more relevant to gene/plant functionality and synthetic biology?

2. What is the Regulator's assessment towards the regulation of TALENs and CRISPRs technology?

3. Does the FDA have the authority to regulate food animal breeding and if so, what are the specific statutes that grant that authority?

4. Since the FDA purports to regulate product not process, do they have the authority to regulate the non-meiotic introgression of naturally occurring alleles that could also be meiotically introgressed by traditional crossbreeding?

5. Please describe the "regulated articles letter of inquiry" process at USDA and address the following questions:
    a. What is the ecological risk assessment process within the agency for GE plants described in a letter of inquiry?
    b. What criteria must be met for a decision of 'no plant pest risk' ?
    c. What is the process for determining CBI in the letters of inquiry? How is the request for CBI balanced with transparency to the general public and stakeholders?

6. The Institutional Biosafety Committee (IBC) is responsible for oversight of projects involving GE plants at universities and other institutions. At present, the IBC expects faculty working with transgenic crops to comply with the USDA application/review/field inspection process for experimental field trials. How will this new regulatory process be

communicated to the IBC and Research Compliance administrators in universities and other institutions?

a. Does the agency require the applicant to create analytical methods for detection of these novel plants ?
b. If issues arise, will the agency make information available to seed producers, food processors, land managers, or other stakeholders to conduct tests?

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 Department of Agriculture

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.