USDA-ARS/
U.S. Wheat and Barley Scab Initiative
FY16 Final Performance Report
Due date: July 28, 2017

Cover Page

Principle Investigator (PI):	Phil Bregitzer
Institution:	USDA-ARS
E-mail:	Phil.Bregitzer@ARS.USDA.GOV
Phone:	208-397-4162 x116
Fiscal Year:	2016
USDA-ARS Agreement ID:	N/A
USDA-ARS Agreement Title:	Down with DON: Stable Expression of Proven Genes in a Marker-free Background.
FY16 USDA-ARS Award Amount:	$ 51,246

USWBSI Individual Project(s)

USWBSI Research Category*	Project Title	ARS Award Amount
BAR-CP	Small RNAs in Response to RNAi Down Regulation in F. graminearum.	$ 26,246
GDER	Down with DON: Stable Expression of RNAi Contructs in a Marker-free Plant.	$ 25,000
	FY16 Total ARS Award Amount	**$** 51,246

Phil Bregitzer 7/28/2017

Principal Investigator Date

* MGMT – FHB Management
FST – Food Safety & Toxicology
GDER – Gene Discovery & Engineering Resistance
PBG – Pathogen Biology & Genetics
EC-HQ – Executive Committee-Headquarters
BAR-CP – Barley Coordinated Project
DUR-CP – Durum Coordinated Project
HWW-CP – Hard Winter Wheat Coordinated Project
VDHR – Variety Development & Uniform Nurseries – Sub categories are below:
SPR – Spring Wheat Region
NWW – Northern Soft Winter Wheat Region
SWW – Southern Soft Red Winter Wheat Region

Project 1: *Small RNAs in Response to RNAi Down Regulation in F. graminearum.*

1. **What are the major goals and objectives of the project?**
   To enhance understanding of the process of RNA interference (RNAi) in *F. graminearum* in support of transgenic efforts to enhance FHB resistance (project 2). Key objectives are vector construction, fungal transformation with RNAi constructs that encode inverted repeats (IRs) that will produce double stranded (ds) RNA targeting fungal genes, and characterizations of fungal phenotypes and the small RNA (sRNA) profiles that result from degradation of dsRNA.

2. **What was accomplished under these goals?** *Address items 1-4) below for each goal or objective.*

   1) Major activities:
      - sRNA profiles were determined previously for fungal strains transformed with RNAi vectors targeting full-length *TRI6* (which has a regulatory role in mycotoxin synthesis) transcripts. In FY16, a set of RNAi vectors encoding IRs targeting shorter (200-350 bp) regions of *TRI6* were assessed for their sRNA profile and effect on fungal phenotype. These shorter IRs are expected to inform and facilitate optimal vector design and construction, and minimize the potential for off-targeting (*i.e.*, affecting non-targeted genes), thus maximizing specificity.
      - To facilitate comparisons between experiments and to reduce the number of mutant strains required for analysis, vectors were created to enable insertion of RNAi vectors into two specific spots (outside of known genes or promoters) via homologous recombination (HR). Transgenic *F. graminearum* containing RNAi vectors inserted randomly vs. in either HR1 or HR2 were assessed for variability in sRNA profiles and effects on fungal phenotype.
      - Candidate target genes *OAH* and *FDB2* were tested for their effect on fungal phenotype using mutant knockout strains created by HR. *OAH* produces oxalate in other fungal species, and has been implicated as a factor in pathogenicity of other fungal plant pathogens. *FDB2* functions to detoxify BOA, which is a defense compound produced by plants including members of the *Triticieae*.

   2) Specific objectives:

      These activities addressed objective 1 (construct and transform *Fg* with fungal vectors with variable IRs and different HR sites, targeting single genes as well as multiple genes), but this objective will not be fully achieved until FY17, when multiple gene targets will be addressed. Similarly, objective 2 (characterize transformants for their sRNA profile resulting from IR expression) is completed for the available materials but additional materials will be generated. Objective 3 (explore novel candidate pathogenicity factors for FHB and for pyramiding IR traits) was achieved for the two potential targets that were proposed for investigation.

   3) Significant results:

      The sRNA profiles in mutants with IRs targeting full length *TRI6* had been previously observed to result in a discontinuous distribution, where sRNAs homologous to certain

regions of *TRI6* were very abundant but absent for other regions. The sRNA profile from shorter IRs were nearly identical to the profile from the full length IR for the targeted section. All constructs reduced pathogenicity and DON, but some were noticeably less effective than the full length IRs. Although the sRNAs mapped to consistent positions from strain to strain, their total amounts varied significantly. Versus random integration of IRs, HR-based insertion did not interrupt native genes. The potential for more consistent expression of inserted genes among strains was investigated by comparing the numbers of specific sRNAs produced among strains with integrations achieved randomly or via IR, but no advantage of the IR strains was seen. *OAH* knockouts did not have reduced pathogenicity. Following the determination that only wild *Hordeum* species produce BOA, work was discontinued on barley but as the strains were already produced, *FDB2* knockouts were tested on 15 wheat varieties. Infection severity was unchanged, but substantial differences among varieties were observed for DON production. Preliminary data showed that exogenous application of BOA reduced infection severity of wild type and *FDB2* knockout strains.

4) Key outcomes or other achievements:

The sRNA profile data derived from replicated experiments, which is unique in the fungal literature. This allowed us to see although the particular sRNAs produced were consistent, the total amounts of sRNAs were not. The observed variability in total sRNA production produced by HR insertion indicated that this was not likely the result of differences in IR expression among strains. However, the source of this variability has not yet been determined. Data on RNAi construct efficacy will be used to inform future barley transformation efforts: the short IR construct with the best DON reduction will be incorporated into a barley transformation vector.

The two main advantages expected for HR insertion would be 1) less expression variability among strains of inserted genes; and 2) no or less disruption of native gene function. The latter characteristic was demonstrated: all mutants with genes inserted by IR had the genes in the expected places, whereas tow of six mutants generated by standard transformation techniques had one or more genes interrupted by transgene insertions. Because of the observed variability in sRNA amounts among strains, the consistency of expression was tested by inserting the mCherry marker into HR1. Preliminary results suggest greater expression stability. These HR vectors are a unique resource that is expected to be valuable for transgenic experiments in *F. graminearum*. They are available for others to use, will be described in the new FgMutantDB (see project 2).

The investigation of candidate target *OAH* yielded negative results. This is new information, and although negative, this gene now is known not to be a useful target for RNAi, and recording this data in FgMutantDB will result in this information being widely shared. It was disappointing to realize that the *FDB2* target would not be applicable to barley, but we have been able to pass this information on to Juliet Marshall, who is taking this work forward on wheat. Also, the discovery of reduced pathogenicity afforded by exogenous BOA application suggest a potential control method applicable to both barley and wheat.

3. What opportunities for training and professional development has the project provided?

All work involving the *F. graminearum* system is the responsibility of Tom Baldwin, post-doc with specific expertise in fungal genetics, and this work has enabled him to learn from and contribute to the community of FHB researchers. His work provides training in the most up-to-date concepts, procedures, and analytical techniques relevant to this field of genetic research. In addition, the communication of the PI's expertise in barley genetics and transformation have expanded Dr. Baldwin's beyond fungal genetics. In addition, we employed a student assistant on this project, Cole Morrison, and involved him in these experiments. He now has a a position with the Idaho State Dept. of Agriculture, where he will be dealing with invasive species.

4. How have the results been disseminated to communities of interest?

Poster presentations at the 2016 USWBSI Forum; at the 2017 Asilomar 29th annual Fungal Genetics conference; in the form of a manuscript describing the RNAi work against *TRI6* (under review); and via personal communication with various members of the barley and fungal genetics communities.

Project 2: *Down with DON: Stable Expression of RNAi Contructs in a Marker-free Plant.*

1. What are the major goals and objectives of the project?

This project is concentrated on the development of transgene delivery systems based on *Ds*-mediated transposition and recombinase-mediated cassette exchange (RMCE), and the delivery of transgenes that will reduce FHB and the accumulation of DON in barley.

2. What was accomplished under these goals? *Address items 1-4) below for each goal or objective.*

1) Major activities:
   - New barley *Ds* (transposition competent) direct-transformation vectors have been constructed and are being tested for introduction into barley of dsRNA against *TRI6.*
   - Two EXCH vectors have been built for use with recombinase mediated cassette exchange
   - *Fusarium graminearum* bioassay system used to identify relative efficiencies of *TRI6* silencing and DON reduction conferred by dsRNA corresponding to different regions of *TRI6*.
   - Plants with TAG sites that have moved from the original site of insertion have been identified.

2) Specific objectives:
   These activities addressed objectives 1a (construction of *Ds* delivery vectors; achieved previously but we were unhappy with the performance of the original vectors) and 1b (construction of EXCH vectors for barley); objective 2 (construct fungal RNAi vectors targeting *TRI5, TRI6, AND LAEA*, and test them in *F. graminearum*); objective 3 (introduce dsRNA sequences effective against *Fg* into barley *Ds* and EXCH vectors); objective 4 (produce transgenic Conlon plants with *Ds*-bordered *Ds*-vectors or TAG sites); and objective 5 (initiate transposition of *Ds*-bordered sequences by crossing to *AcT* plants), all of which were scheduled to be addressed during FY16 and FY17. In addition, objective 6 (select plants with *Ds*-vectors or TAG sites segregated from *AcT* and the original insertion site) was addressed.

3) Significant results:
   The original *Ds*-delivery vector had poor expression of *hyg* (resistance to hygromycin), making difficult the recovery of transformed plants. The *Ds*-*Ds* transposition-competent cassette now is in a backbone with *hyg* driven by a stronger promoter for monocots, rice ubiquitin 2. This vector has been used to recover GUS-positive tissues, indicating that it is functional and ready for use as a vector for RNAi cassettes designed to downregulate FHB genes.

   The two EXCH vectors will enable iterative exchanges and enable stacking multiple genes at a single site. One vector contains a cassette that encodes the *bar* selectable marker necessary to effect site-specific recombination with the TAG site (which contains *hyg*); contains alternate 'B' recognition sites for use with different enzymes for future

cassette exchanges; and a site to enable loading genes of interest. The second vector encodes the *hyg* selectable marker necessary to effect alternate site-specific recombination (that is, a second recombination step that can introduce a second gene; contains alternate 'P' recognition sites for use with different enzymes for future cassette exchanges; and a site to enable loading various genes of interest.

Results from the *F. graminerium* model, used for rapid assessment of RNAi vectors prior to initiating barley transformation, showed that silencing *TRI6* drastically reduced DON production on barley and wheat and reduced pathogenicity on wheat. DON is not thought to be a major pathogenicity factor on barley. The greatest reduction (to nearly zero DON) was achieved using an RNAi vector producing dsRNA targeting most of the *TRI6* gene. Targeting shorter regions (200-350 bp, which would enable simpler, smaller RNAi vectors) varied in their effectiveness, so the choice of the region targeted is important.

Plants with transposed TAG sites have been identified. The rationale behind *Ds*-mediated delivery of TAG sites is that this will de-link the TAG site from undesirable sequences (vector DNA, selectable markers, and rearrangements) at the original insertion site. Plants with transpositions were identified following PCR screens of several thousand progeny from crossed of plants with the TAG site and plants expressing *Ac* transposase (which catalyzes transposition. The location of some of the transposed TAG sites was determined by a chromosome walking technique. Most moved to closely-linked sites, which complicated the selection of plants where the TAG site had segregated from the original insertion site. However, one has the TAG site segregated away from the original insertion site. Homozygous progeny of this plant will be identified and used to investigate functionality of the site-specific recombination system in barley.

4) Key outcomes or other achievements:
The development of the EXCH vectors, and the re-designed, improved *Ds*-delivery vector, provide the complete suite of transformation vectors necessary to achieve our stated objectives. In addition, these vectors can serve as a resource for other barley or wheat transformation projects, and will be made available to others on request. The results of testing RNAi vectors for their effects on *F. graminearum* will guide rational design of RNAi vectors for plant transformation. Identifying plants with transposed TAG sites enables testing functionality of RMCE in barley; if functional, this will be a significant step towards precise transgene insertion and our ability to stack multiple genes in a pre-determined location.

A notable achievement was the creation of FgMutantDB, a crowd-source, Google Docs-based database that compiles information on *F. graminearum* mutants, *e.g.* their characteristics, and the laboratory/PI that created and/or is maintaining them.. This resource is useful for encouraging researcher interactions and enables reporting of negative data to the community. In addition, FgMutantDB cross-references and shares information with genomic databases (FungiDB, Ensemble, and PHI-base) allowing curation across multiple databases at once. This tool will also help facilitate the current transition away from the obsolete BROAD annotation numbers, thus ensuring researchers are unified when referencing genes and mutants.

3. What opportunities for training and professional development has the project provided?

All work involving the *F. graminearum* system is the responsibility of Tom Baldwin, post-doc with specific expertise in fungal genetics, and this work has enabled him to learn from and contribute to the community of FHB researchers. His work provides training in the most up-to-date concepts, procedures, and analytical techniques relevant to this field of genetic research. In addition, the communication of the PI's expertise in barley genetics and transformation have expanded Dr. Baldwin's beyond fungal genetics. In addition, we employed a student assistant, Cole Morrison, on this project, and involved him in these experiments.

4. How have the results been disseminated to communities of interest?

Poster presentations at the 2016 USWBSI Forum; at the 2017 Asilomar conference; in the form of a manuscript describing the RNAi work against *TRI6* (under review); and via personal communication with various members of the barley and fungal genetics communities. The development and deployment of FgMutantDB has involved substantial personal communication with curators of other fungal databases and *Fusarium* researchers. Publication will follow.

Training of Next Generation Scientists

Instructions: Please answer the following questions as it pertains to the FY16 award period. The term "support" below includes any level of benefit to the student, ranging from full stipend plus tuition to the situation where the student's stipend was paid from other funds, but who learned how to rate scab in a misted nursery paid for by the USWBSI, and anything in between.

1. **Did any graduate students in your research program supported by funding from your USWBSI grant earn their MS degree during the FY16 award period?** No

   If yes, how many?

2. **Did any graduate students in your research program supported by funding from your USWBSI grant earn their Ph.D. degree during the FY16 award period?** No

   If yes, how many?

3. **Have any post docs who worked for you during the FY16 award period and were supported by funding from your USWBSI grant taken faculty positions with universities?** No

   If yes, how many?

4. **Have any post docs who worked for you during the FY16 award period and were supported by funding from your USWBSI grant gone on to take positions with private ag-related companies or federal agencies?** No

   If yes, how many?

Release of Germplasm/Cultivars

Instructions: In the table below, list all germplasm and/or cultivars released with full or partial support through the USWBSI during the FY16 award period. All columns must be completed for each listed germplasm/cultivar. Use the key below the table for Grain Class abbreviations. *Leave blank if you have nothing to report or if your grant did NOT include any VDHR-related projects.*

Name of Germplasm/Cultivar	**Grain Class**	**FHB Resistance** (S, MS, MR, R, where R represents your most resistant check)	**FHB Rating** (0-9)	**Year Released**

Add rows if needed.

NOTE: List the associated release notice or publication under the appropriate sub-section in the 'Publications' section of the FPR.

Abbreviations for Grain Classes

Barley - BAR
Durum - DUR
Hard Red Winter - HRW
Hard White Winter - HWW
Hard Red Spring - HRS
Soft Red Winter - SRW
Soft White Winter - SWW

Publications, Conference Papers, and Presentations

Instructions: Refer to the FY16-FPR_Instructions for detailed instructions for listing publications/presentations about your work that resulted from all of the projects included in the FY16 grant. Only include citations for publications submitted or presentations given during your award period (5/3/2016 - 5/2/2017). If you did not have any publications or presentations, state 'Nothing to Report' directly above the Journal publications section.

Journal publications.

Baldwin, TT, Islamovic E, Esvelt Klos K, Schwarz P, Gillespie J, Bregitzer P. (in review). Discontinuous and repeatable peaks of small interfering RNAs mapping to *TRI6* associated with deoxynivalenol reductions by RNA interference in *Fusarium graminearum.*
Status: under review
Acknowledgement of Federal Support: YES

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Baldwin, T. and Bregitzer P. 2017 Full length and five short segments of *TRI6* RNA interference constructs that reduce deoxynivalenol production in *Fusarium graminearum* reveal consistent patterns of siRNA and different silencing efficiencies. 29th Fungal Genetics Conference, March 14-19, Genetics Society of America, Asilomar, CA, p. 221
Status: Abstract published and poster presented.
Acknowledgement of Federal support: YES (poster) NO (abstract)

Baldwin, T. and Bregitzer P. 2016 Insight into the mechanism of the TRI6 RNA interference ablating deoxynivalenol production in *Fusarium graminearum* with patterns of siRNA production. In: S. Canty, A. Clark, K. Wolfe and D. Van Sanford (Eds.), *Proceedings of the 2016 National Fusarium Head Blight Forum* (p. 45). East Lansing, MI/Lexington, KY: U.S.
Status: Abstract published and poster presented.
Acknowledgement of Federal support: YES (poster) YES (abstract)

Bregitzer, Phil. 2016. "Tissue Culture Induced Variability: Critical Issues that Impact the Evaluation and Use of Transgenic Parents." In: S. Canty, A. Clark, K. Wolfe and D. Van Sanford (Eds.), *Proceedings of the 2016 National Fusarium Head Blight Forum* (pp. 69-77). East Lansing, MI/Lexington, KY: U.S.
Status: Paper published.
Acknowledgement of Federal support: YES (poster)