USDA-ARS
U.S. Wheat and Barley Scab Initiative
FY18 Performance Report
Due date: September 23, 2019

Cover Page

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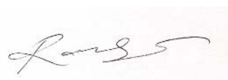
Phone:	848-932-6350
Fiscal Year:	2018
USDA-ARS Agreement ID:	58-2050-8-012
USDA-ARS Agreement Title:	Genetic Engineering Barley for Fusarium Head Blight Resistance.
FY18 USDA-ARS Award Amount:	$ 48,095
Recipient Organization:	Rutgers, The State University of New Jersey Division of Grant and Contract Accounting ASB 111, 3 Rutgers Plaza New Brunswick, NJ 08901·8559
DUNS Number:	00-191-2864
EIN:	22-6001086
Recipient Identifying Number or Account Number:	824460-800
Agency PI:	Phil Bregitzer
Project/Grant Reporting Period:	8/1/18 - 7/31/19
Reporting Period End Date:	07/31/19

USWBSI Individual Project(s)

USWBSI Research Category*	Project Title	ARS Award Amount
GDER	CRISPR-Gene Editing Barley to Improve Fusarium Head Blight Resistance.	$ 24,048
GDER	A Centralized Barley Transformation Facility for the FHB Community.	$ 24,047
	FY18 Total ARS Award Amount	$ 48,095



Rong Di 9/23/2019
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

FY18 Performance Report
PI: Di, Rong
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Project 1: *CRISPR-Gene Editing Barley to Improve Fusarium Head Blight Resistance.*

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

The major goals of this project are to develop the CRISPR-gene editing platform for barley and to edit three barley FHB susceptibility genes to improve FHB resistance. Our specific objectives for this project are: (1) Construction of barley *HvHSK, Hv2OGO* and *HvEIN2* transient and integrating CRISPR-editing vectors, (2) Production of *HvHSK-, Hv2OGO-* and *HvEIN2*-edited barley plants, and (3) Evaluation of *HvHSK, Hv2OGO* and *HvEIN2* mutant barley plants for FHB resistance.

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

1) major activities

We initially proposed to CRISPR-edit Conlon cultivar (with seeds provided by Dr. R. Horsley, later seeds derived from a single seed/plant were provided by Dr. P. Bregitzer) as its regeneration protocol has been developed. We then added ND Genesis (original seeds provided by Dr. R. Horsley, NDSU) as it is a popular cultivar, and two-rowed as Conlon.

We have been mainly working on cloning the FHB susceptibility genes from both Conlon and ND Genesis, constructing the CRISPR-gene editing vectors and developing Conlon and ND Genesis tissue culture and transformation protocols.

2) specific objectives

(1) Construction of barley *HvHSK, Hv2OGO* and *HvEIN2* transient and integrating CRISPR-editing vectors:

In order to construct CRISPR-editing vectors, we need the genomic DNA (gDNA) sequence information. We have conducted RNAseq analysis of cv. Conlon. With all the *HSK* genes that we have checked from Arabidopsis, Brachypodium, grape and basil, this gene does not have an intron. We compared our Conlon RNAseq data for the potential *HvHSK* gene to the predicated protein sequence of cv. Haruna Nijo in NCBI, designed primers and cloned the 3' half of the Conlon *HvHSK* gDNA. We also cloned the 3' half of the *HvHSK* gDNA from ND Genesis and found it matches 100% to that of Conlon.

Using Morex genome information in NCBI and our RNAseq data, we cloned *Hv2OGO* cDNAs from both Conlon and ND Genesis and found them to be essentially 100% matching to the Morex *2OGO* sequence. We also cloned the complete gDNA of *Hv2OGO* from ND Genesis. Similarly, we cloned the complete gDNA for Conlon *HvEIN2* gene, and the complete cDNA of ND Genesis *HvEIN2* which is 100% homologous to Conlon *HvEIN2* cDNA.

CRISPR has been used to knock-out several genes in barley. All of these studies were carried out with the cultivar of Golden Promise, and none of the genes was related

to improve FHB resistance. However, these studies have demonstrated that wheat U6, rice U6 and U3 and barley U3 promoters seemed to effectively drive the expression of all gRNAs in Golden Promise. There has been no publication on CRISPR-gene editing in Conlon or ND Genesis barley and we are uncertain which small RNA promoter would function efficiently. Therefore, we constructed several CRISPR-editing vectors using barley (Hv), rice (Os) and wheat (Ta) U3 or U6 promoter. The gRNA target site is defined by the restriction enzyme immediately upstream of the PAM site for Cas9 nuclease. The monocot (mo) codon-optimized or humanized (h) Cas9 cassette is driven either by the maize ubiquitin/intron promoter (ZmUbi) or the rice ubiquitin promoter (OsUBQ). The transient vector is in the backbone of either pGEM3Zf(+) or pBS vector. The integrating vector is in the backbone of either pCAMBIA1300 (with hygromycin selection for plants) or pCAMBIA2300 (with kanymycin selection for plants). They are listed here.
pRD330 [PHvU3::HvHSK-SacII::PZmUbi::Cas9-mo in pGEM3Zf(+)]
pRD388 (PHvU3::HvHSK-SacII::PZmUbi::Cas9-mo in pCAMBIA1300)
pRD380 (POsU3::Hv2OGO-NdeI::POsUBQ::Cas9-h in pBS)
pRD383 (POsU3::Hv2OGO-NdeI::POsUBQ::Cas9-h in pCAMBIA1300)
pRD279 [PHvU3::HvEIN2-SphI::PZmUbi::Cas9-mo in pGEM3Zf(+)]
pRD281 (PHvU3::HvEIN2-SphI::PZmUbi::Cas9-mo in pCAMBIA1300)
pRD282 (PHvU3::HvEIN2-SphI::PZmUbi::Cas9-mo in pCAMBIA2300)
pRD395 [PTaU6::HvEIN2-SphI::PZmUbi::Cas9-mo in pGEM3Zf(+)]
pRD403 (PTaU6::HvEIN2-SphI::PZmUbi::Cas9-mo in pCAMBIA1300)

(2) Production of *HvHSK-, Hv2OGO-* and *HvEIN2-*edited barley plants:

Based on the Conlon regeneration and transformation method published by Dr. L. Dahleen and Dr. P. Bregitzer, we have modified the barley tissue culture protocol for Conlon and ND Genesis. Since we need to transform the six-rowed Morex for Project 2, we use the same protocol for these three barley cultivars. Basically, we use immature or mature barley embryos as explants and transform them by gene gun or *Agrobacterium.* Without transformation and hygromycin selection, we have been able to regenerate mostly single, sometimes multiple shoots from a single immature embryo of Conlon, ND Genesis and Morex 2 (initial seeds from Dr. Bregitzer) (Fig. 1). All regenerated shoots can be induced to produce roots, transferred to soil and produce seeds. We have used the same tissue culture protocol for mature embryos of Conlon (all mature Conlon seeds are from the commercial source, Johnny's Seeds) and ND Genesis, and obtained similar results, with mostly single shoot regenerated from a single embryo.

We have used both gene gun and *Agrobacterium* EHA105 to transform barley. Here are some of the results. Fig. 2A shows the regeneration of T_0 Conlon plantlets from gene gun-transformed mature Conlon embryos with pRD383 (targeting *Hv2OGO*). Four regenerated and rooted plants have been transferred to soil. gDNA from each plantlet and the non-transformed, wild type (wt) plant was isolated, the 425 bp fragment spanning the *Nde*I-target site was PCR-amplified. The PCR products were purified and subjected to *Nde*I restriction enzymatic digestion. This restriction fragment length polymorphism (RFLP) analysis will show two digested fragments if the plant is wt or the mutation did not occur on *Nde*I. If one *Hv2OGO* allele of the diploidic barley is mutated at the target

site and the *Nde*I site is destroyed, there will be two populations of PCR products, one of which will be digested by *Nde*I, the other will not. If both alleles are mutated, there will be only one PCR product that is not digested by *Nde*I. As shown in Fig. 2B, plantlets 383-1, 2, 3 not only have the *Nde*I-digested 262 bp and 163 bp bands as the wt plantlet, they also have the undigested 425 bp band, indicating that these three might be *Hv2OGO* monoallelic mutants. Plantlet 383-4 has only the digested 262 bp and 163 bp fragments, indicating that it is likely not mutated. It is also possible that mutation occurred at or around the target site, but did not destroy the *Nde*I sequence. The uncut fragments from RD383-2, 3 were purified and sequenced. The results show that the *Nde*I target site (underlined in Fig. 2C) was not mutated, indicating that it is either because *Nde*I did not digest the DNA fragments completely or the sequencing reaction did not occur on the potentially *Nde*I- mutated molecules. Nevertheless, the sequencing result shows that mutations have occurred downstream of the *Nde*I target site, all resulting in amino acid changes in these two plants. We have also used gene gun to transform mature Conlon embryos with pRD388 and pRD403. Some plantlets have been selected on hygromycin-containing media and currently being induced to produce roots (Fig. 3).

3) significant results

We have conducted RNAseq on Conlon barley and shown the gDNA sequences of *HvHSK*, *Hv2OGO* and *HvEIN2* are the same for Conlon and ND Genesis. Based on these gDNA sequences, we have constructed several CRISPR-editing vectors with barley, rice or wheat U3 or U6 promoter, all of which can be used to transform and edit these three genes in both cultivars.

We are able to regenerate barley plants from immature embryos of Conlon, ND Genesis and Morex cultivars, and from mature embryos of Conlon. We have successfully transformed Conlon barley with our *Hv2OGO*-targeting CRISPR vector (pRD383) by gene gun. Two of the regenerated RD383 plants have been shown to be mutated in the *Hv2OGO* gene.

4) key outcomes or other achievements

Plant transformation and regeneration take time. Our *Hv2OGO*-edited barley plants have been transferred to grow in soil. The other transformed tissues are being selected in tissue culture media. Hence the goal in our objective #3 "Evaluation of *HvHSK, Hv2OGO* and *HvEIN2* mutant barley plants for FHB resistance" is not achieved yet. We are currently growing Conlon and ND Genesis plants for more immature embryos for transformation.

Prior and during the course of this project, we have used CRISPR-editing technology to mutate the Arabidopsis orthologous FHB susceptibility genes, *At2OGO, AtHSK* and *AtEIN2*. Our results have shown that the Arabidopsis *At2OGO-, AtHSK*- and *AtEIN2*-knock out (KO) plants are resistant to *F. graminearum*. Moreover, we complement-transformed *At2OGO*-KO and *AtEIN2*-KO Arabidopsis plants with barley *Hv2OGO* and *HvEIN2* cDNAs. The *At2OGO*-KO/*Hv2OGO* and *AtEIN2*-KO/*HvEIN2* Arabidopsis

plants regained the susceptibility to *F. graminearum*. These results indicate that both *Hv2OGO* and *HvEIN2* genes are involved in *F. graminearum* susceptibility, providing the molecular basis for knocking out these two genes in barley to produce FHB resistant barley plants. A manuscript describing the *At2OGO*-editing and *Hv2OGO* complementation is being prepared for publication.

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

This project has provided training for a Ph. D student YCL, who has mastered the CRISPR-gene editing technology. YCL worked on gene editing in Arabidopsis and complementation assay with barley orthologous genes. This project has also provided training in molecular biology, CRISPR-gene editing and plant tissue culture for two other graduate students (DCS and JP), two undergraduate students (BF and JC) and an hourly laboratory technician (YC).

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

Studies and results on the involvement of barley *Hv2OGO* and *HvEIN2* in FHB susceptibility and the feasibility of CRISPR-editing to improve FHB resistance have been presented in the form of YCL's Ph. D thesis defense seminar and as part of the PI's lectures to the students majored in Biotechnology and Plant Biology at Rutgers University. Our findings have also been presented to the participants at the annual meeting of our Multistate Project "NC1183: Mycotoxins: Biosecurity, Food Safety and Biofuels Byproducts" on September 12, 2019.

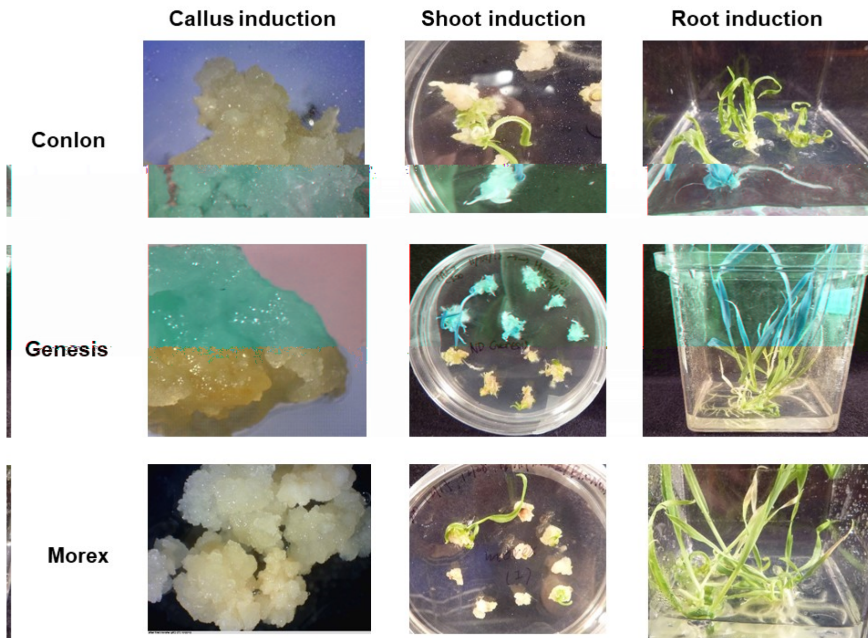

Fig. 1 Regeneration of barley cv. ND Conlon, ND Genesis and Morex 2 from immature embryos.

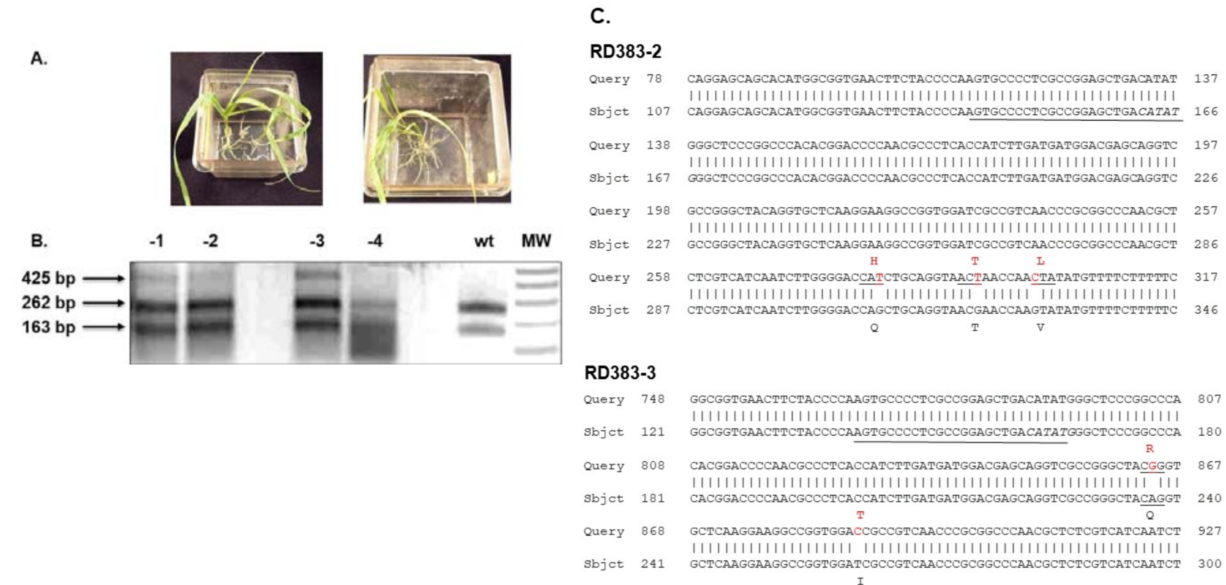

Fig. 2. Production of *Hv2OGO*-edited Conlon mutant plants. A. Regeneration of gene gun-transformed Conlon from mature embryos with pRD383. B. RFLP by *Nde*I on RD383-1, 2, 3, 4 and wt plants. C. Sequencing of RD383-2, 3 425 bp fragmnets. *Hv2OGO*-*Nde*I target site is underlined. Nucleotide and amino acid mutations are marked.

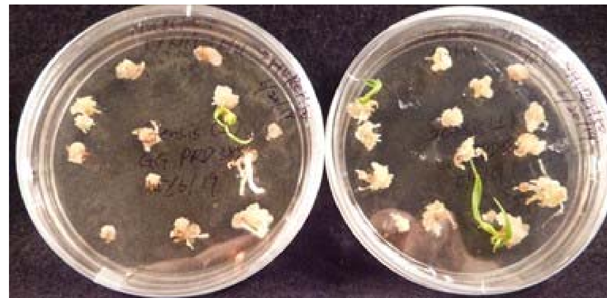

Fig. 3. Regeneration of gene gun-transformed ND Genesis from immature embryos with pRD388.

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Project 2: *A Centralized Barley Transformation Facility for the FHB Community.*

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

(1) Editing the *HvUGT* (*UDP-glucosyltransferase*) promoter in the FHB susceptible barley cv. Morex to study the *HvUGT* gene expression kinetics, (2) Editing the *HvNud* gene (encoding an ethylene response factor transcription factor) in two-rowed and hulled Conlon and ND Genesis barley to study the effect of hull type on the FHB development, (3) Editing the *HvVrs1* gene (encoding a transcription factor) in the six-rowed cultivar Morex to study the effect of row type on the FHB development, and (4) Developing barley anther culture for CRISPR-gene editing and barley engineering.

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

1) major activities

In this project, I proposed to build the centralized barley transformation facility for the FHB community. As shown above under Project 1 "*CRISPR-Gene Editing Barley to Improve Fusarium Head Blight Resistance*", within a year, we have been able to develop the tissue culture protocol and regenerate barley plants from immature embryos of Conlon, ND Genesis and Morex cultivars, and from mature embryos of Conlon. We have developed our own CRISPR-gene editing systems for barley. The barley *Hv2OGO* gene has been mutated in cultivar Conlon. Therefore, we are ready to mutate or knock-out other barley genes, namely the *HvUGT* promoter, *HvNud* and *HvVrs1*, that are associated with FHB resistance. With the support of Dr. G. Muehlbauer (University of Minnesota) and Dr. B. Steffenson (University of Minnesota), we will be able to investigate the roles of these genes in barley FHB resistance.

In the past year, we have been mainly cloned the *HvUGT* promoter partial sequences from Morex, *HvNud* from Conlon and ND Genesis and *HvVrs1* from Conlon, ND Genesis and Morex. We are in the process of constructing the CRISPR-editing vectors for these genes.

2) specific objectives

(1) Editing the *HvUGT* (*UDP-glucosyltransferase*) promoter in the FHB susceptible barley cv. Morex to study the *HvUGT* gene expression kinetics:

The *HvUGT* promoter has been cloned from cv. Morex by the group of Dr. G. Muehlbauer. The 3000 bp DNA sequence information has been provided to us. We have identified two potential target sites, the *Mfe*I site that is 888 bp upstream of the start codon of *HvUGT*, and the *Nco*I site which is 165 bp upstream of the start codon. We have PCR-cloned and sequencing-confirmed the 420 bp and 405 bp fragments spanning these two CRISPR target sites. We are in the process of constructing the CRISPR-editing vectors. Collaborating with Dr. G. Muehlbauer, we are going to edit the *HvUGT* promoter in Morex.

(2) Editing the *HvNud* gene (encoding an ethylene response factor transcription factor) in two-rowed and hulled Conlon and ND Genesis barley to study the effect of hull type on the FHB development:

We have cloned and sequenced the complete *HvNud* gDNA including an intron from ND Conlon and ND Genesis. An identical *Pvu*II target site has been selected. We are in the process of constructing the CRISPR-editing vector. We will collaborate with Dr. B. Steffenson on this objective.

(3) Editing the *HvVrs1* gene (encoding a transcription factor) in the six-rowed cultivar Morex to study the effect of row type on the FHB development:

We have cloned and sequenced the complete *HvVrs1* gene including introns from Conlon, ND Genesis and Morex. Sequence alignment showed that the *HvVrs1* amino acid sequences are identical for Conlon and ND Genesis. However, there is a single nucleotide deletion at the 3' end of the Morex *HvVrs1*, leading to dysfunctional *HvVrs1*.

It would be interesting to test if CRISPR-editing can be used to restore *HvVrs1* in Morex and change it to two-rowed and evaluate the row type's role in FHB susceptibility. We will collaborate with Dr. B. Steffenson on this objective. This test can pave the way for future gene replacement and gene analysis in barley.

(4) Developing barley anther culture for CRISPR-gene editing and barley engineering: Even though we have been able to regenerate gene-edited barley plants, the transformation efficiency is approximately 1-3% using embryos as explants. We intend to develop barley anther cultures and use cultured anther tissues as explants for transformation. We have received support from Dr. P. Hayes at OSU to develop anther cultures initially with Conlon, ND Genesis and Morex barley. As high frequency of double haploid (DH) homozygous transgenic plants has been reported at the T_0 generation, using anther culture for CRISPR-gene editing would greatly enhance the efficiency of producing gene-edited barley plants.

Conlon, ND Genesis and Morex 2 (single seed-derived plant, seeds provided by Dr. P. Br..) plants are being grown in the greenhouse. Pollens will be collected to start the anther culture.

3) significant results

We have cloned and sequenced parts of the *HvUGT* promoter and the complete *HvNud* and *HvVrs1* genes. Their gDNA sequences enable us to build the CRISPR-editing vectors.

4) key outcomes or other achievements

From our research results under Project 1, we have learned that immature and mature embryos of Conlon, ND Genesis and Morex can be directly transformed by gene gun (or *Agrobacterium*), barley plants can be regenerated, however, with a transformation efficiency of 1-3% similar to that for most plants. Barley anther cultures may provide different type of explant for transformation and gene editing.

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

This project has provided training for a Ph. D student YCL, who has mastered the CRISPR-gene editing technology. YCL worked on gene editing in *Arabidopsis* and complementation assay with barley orthologous genes. This project has also provided training in molecular biology, CRISPR-gene editing and plant tissue culture for two other graduate students (DCS and JP), two undergraduate students (BF and JC) and an hourly laboratory technician (YC).

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

Studies and results on the involvement of barley *Hv2OGO* and *HvEIN2* in FHB susceptibility and the feasibility of CRISPR-editing to improve FHB resistance have been presented in the form of YCL's Ph. D defense seminar and as part of the PI's lectures to the students majored in Biotechnology and Plant Biology at Rutgers University. Our findings have also been presented to the participants in the Multistate Project "NC1183: Mycotoxins: Biosecurity, Food Safety and Biofuels Byproducts" in September 2019.

Training of Next Generation Scientists

Instructions: Please answer the following questions as it pertains to the FY18 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 FY18 award period?**

    N/A

    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 FY18 award period?**

    Yes

    If yes, how many?

    1

3. **Have any post docs who worked for you during the FY18 award period and were supported by funding from your USWBSI grant taken faculty positions with universities?**

    N/A

    If yes, how many?

4. **Have any post docs who worked for you during the FY18 award period and were supported by funding from your USWBSI grant gone on to take positions with private ag-related companies or federal agencies?**

    N/A

    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 FY18 award period. All columns must be completed for each listed germplasm/cultivar. Use the key below the table for Grain Class abbreviations.

NOTE: 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

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Publications, Conference Papers, and Presentations

Instructions: Refer to the FY18-FPR_Instructions for detailed instructions for listing publications/presentations about your work that resulted from all of the projects included in the FY18 grant. Only include citations for publications submitted or presentations given during your award period (8/1/18 - 7/31/19). If you did not have any publications or presentations, state 'Nothing to Report' directly above the Journal publications section.

NOTE: Directly below each reference/citation, you must indicate the Status (i.e. published, submitted, etc.) and whether acknowledgement of Federal support was indicated in publication/ presentation. See example below for a poster presentation with an abstract:

> Conley, E.J., and J.A. Anderson. 2018. Accuracy of Genome-Wide Prediction for Fusarium Head Blight Associated Traits in a Spring Wheat Breeding Program. In: Proceedings of the XXIV International Plant & Animal Genome Conference, San Diego, CA.
> Status: Abstract Published and Poster Presented
> Acknowledgement of Federal Support: YES (poster), NO (abstract)

Journal publications.

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

Yee Chen Low. 2019. Ph.D. Dissertation to fulfill the requirement of the Plant Biology Graduate Program at Rutgers University. "Applications of Biotechnology for Crop Enhancement in Disease Resistance and Nutrition".
Status: Published
Acknowledgement of Federal Support: Yes

Other publications, conference papers and presentations.

Yee Chen Low. Sept. 20, 2019. Ph.D. Thesis defense seminar at Rutgers University, "Applications of Biotechnology for Crop Enhancement in Disease Resistance and Nutrition".
Status: Presented
Acknowledgement of Federal Support: Yes

R. Di, Y.C. Low and M.A. Lawton. Sept. 12, 2019. Presentation at the annual meeting of our Multistate Project "NC1183: Mycotoxins: Biosecurity, Food Safety and Biofuels Byproducts" at Virginia Tech, "CRISPR-editing susceptibility genes to improve FHB resistance".
Status: Presented
Acknowledgement of Federal Support: Yes