USDA-ARS
U.S. Wheat and Barley Scab Initiative
FY19 Performance Report
Due date: September 30, 2020

Cover Page

Cooperating Principle Investigator (CPI):	Rong Di
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Phone:	848-932-6350
Fiscal Year:	2019
USDA-ARS Agreement ID:	58-2050-8-012
USDA-ARS Agreement Title:	Genetic Engineering Barley for Fusarium Head Blight Resistance
FY19 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:	Kathy Esvelt Klos
Project/Grant Reporting Period:	8/1/19 - 7/31/20
Reporting Period End Date:	7/31/2020

USWBSI Individual Project(s)

USWBSI Research Category*	Project Title	ARS Award Amount
GDER	A Centralized Barley Transformation Facility for the FHB Community	$ 24,048
GDER	CRISPR-Gene Editing Barley to Improve Fusarium Head Blight Resistance	$ 24,047
	FY19 Total ARS Award Amount	**$ 48,095**

9/29/2020

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 Nurlines – Sub categories are below:
SPR – Spring Wheat Region
NWW – Northern Soft Winter Wheat Region
SWW – Southern Soft Red Winter Wheat Region

FY19 Performance Report
CPI: Di, Rong
NACA Agreement #: 58-2050-8-012
Reporting Period: 8/1/19 - 7/31/20

Project 1: *A Centralized Barley Transformation Facility for the FHB Community*

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

In this project, I proposed to build the centralized barley transformation facility for the FHB community. The specific objectives are: (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 aGenesis 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 or objectives? *(For each major goal/objective, address items a-b) below.)*

a) What were the major activities?

Within the first year of this project (2018-2019), we have been able to develop the tissue culture protocol and regenerate barley plants from immature embryos of Conlon, Genesis and Morex cultivars, and from mature embryos of Conlon. We have developed our own CRISPR-gene editing systems for barley. With the support of Dr. G. Muehlbauer (University of Minnesota) and Dr. B. Steffenson (University of Minnesota), we intend to investigate the roles of the *HvUGT* promoter, *HvNud* and *HvVrs1* genes in barley FHB resistance.

(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 have constructed the CRISPR-editing vector pRD424 (P_{TaU6}:*HvUGTP-Nco*I24//P_{ZmUbi}:Cas9-Mo in pCAMBIA1300) to target the *HvUGT* promoter *Nco*I site, and pRD438 (P_{TaU6}:*HvUGTP-Mfe*I20//P_{ZmUbi}:Cas9-Mo in pCAMBIA1300) to target the *HvUGT* promoter *Mfe*I site.

We have used gene gun to bombard the immature embryos of Morex2 (a single seed line provided by Dr. Phil Bregitzer, ARS) with pRD438 and regenerated 5 individual plants (Fig. 1). The successful integration of the transgene was confirmed in all 5 plants by PCR-amplification of the scaffold RNA, the 22-bp spacer and the 5' end of the ZmUbi promoter, cloning of the PCR products and by Sanger sequencing. We are in the process of evaluating the possible mutations in the *HvUGT* promoter *Mfe*I site induced by pRD438 CRISPR vector in these plants.

pRD438 pRD424

Fig. 1 Transformation and regeneration of pRD438- and pRD424- bombarded Morex2 immature embryos. Calli were selected on 15 mg/L hygromycin-containing media. Roots were induced on 5 mg/L hygromycin-containing medium from regenerated shoots.

We have also regenerated one Morex2 plant by bombarding the immature embryos with pRD424 targeting the *HvUGT* promoter *Nco*I site. We are currently characterizing this plant for the transgene integration and possible mutations.

Once these plants are confirmed of mutations, we will collaborate with Dr. G. Muehlbauer at U. of Minnesota to study their effects on the function of the *HvUGT* gene in Morex barley.

(2) Editing the *HvNud* gene (encoding an ethylene response factor transcription factor) in two-rowed and hulled Conlon and 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 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, Genesis and Morex. Sequence alignment showed that the *HvVrs1* amino acid sequences are identical for Conlon and 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, 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.

We have adapted several previously published barley anther culture protocols and developed our own microspore culture method. In brief, barley cv. Genesis and Morex2 plants were germinated at 4 ºC for 1 month, grown at 16 ºC for 1 month and then grown in the greenhouse to harvest spikes when the microspores were at the mid- to late-uninucleate stage. The spikes were pre-treated in 0.3 M mannitol for 4 days, and the microspores were isolated by blending with a PowerGen125 micro-blender, filtering and centrifugation. The microspores were then cultured in FHGI medium containing macro and micro salts, vitamins and 10 mg/L phenylacetic acid and 1 mg/L BAP. We have been able to obtain large amount of microspores from Genesis and Morex2 (Fig. 2). Microcalli are currently being induced from these microspores. We have also used gene gun to deliver several different CRISPR-gene editing vectors into microspores of Genesis (pRD383, pRD388 and pRD403) and Morex2 (pRD424, pRD438). Bombarded microspores are also being cultured to induce calli.

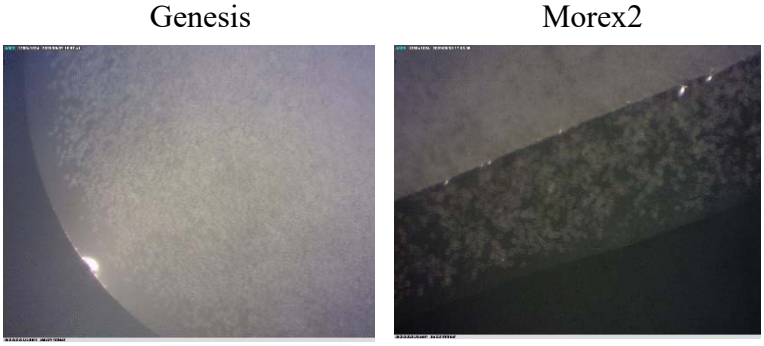

Fig. 2 Isolation and culturing of microspores from Genesis and Morex2.

b) What were the significant results?

Our significant results in the past year (2019-2020) include the production of transgenic Morex2 plants by gene gun bombardment with pRD438 and pRD424. Even though the transformation rate was conventionally low at 1-5% of the bombarded immature embryos, all the shoots that regenerated on 15 mg/L hygromycin selective medium produced roots on 5 mg/L hygromycin-containing medium and were confirmed as transgenic plants.

We have also started the anther/microspore cultures of barley cv. Genesis and Morex2, which should speed up the CRISPR-gene editing process and the production of gene-edited barley plants.

c) List key outcomes or other achievements.

The key outcomes are (1) CRISPR-gene editing vectors have been constructed, and (2) transgenic barley plants have been produced. The relatively efficient barley transformation and regeneration system has been developed.

3. Was this research impacted by the COVID-19 pandemic (i.e. university shutdowns, reduced or lack of support personnel, etc.)? If yes, please explain how this research was impacted or is continuing to be impacted.

Yes, the COVID-19 pandemic has greatly impacted this research. Rutgers shut down the whole university in the middle of March 2020. I requested and was granted to keep my lab and research programs open, initially at 25% capacity, then at 50% capacity in the summer to now at 100%. The severe reduction in research capacity has negatively impacted our progress by limiting the time the researchers could spend in the lab and the greenhouse, the delivery of necessary supplies and chemicals, the lengthened time of DNA sequencing completion etc. As a result, I requested and was approved a no-cost-extension of the project to now end on 1/31/2021. However, compared to most labs on our campus, my programs have continued to make satisfactory progresses in all of the projects.

4. 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. YCL obtained her Ph. D degree in the Fall of 2019. This project provided the funding and the training of YCL to continue working with me as a postdoc researcher. 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).

5. 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 and September 2020.

Project 2: *CRISPR-Gene Editing Barley to Improve Fusarium Head Blight Resistance*

1. What are the major goals and objectives of the research 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 or objectives? *(For each major goal/objective, address items a-b) below.)*

a) What were the 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 (Conlon1), as its regeneration protocol has been developed. We then added Genesis (original seeds provided by Dr. R. Horsley, NDSU) as it is a popular cultivar, and two-rowed as Conlon.

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

The existing genomic information of barley is from cv. Morex. We conducted our own RNAseq analysis on cv. Conlon and deposited the data in NCBI SRA database (accession number SRR10059574) under BioProject PRJNA563590. Using Morex genome information in NCBI and our RNAseq data, we cloned *Hv2OGO* cDNAs from both Conlon and Genesis and found them to be essentially 100% matching to the Morex *2OGO* sequence. We also cloned the complete gDNA of *Hv2OGO* from Genesis. Similarly, we cloned the complete gDNAs and cDNAs of *HvEIN2* and *HvHSK* genes from both Conlon and Genesis. Their sequences are 99.9% similar between two cultivars.

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 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:

As mentioned above, we have developed our own barley tissue culture and transformation protocol using immature embryos of Conlon1, Genesis and Morex2 by both gene gun and Agrobacterium (EHA105 strain) transformation.

We have so far produced many transgenic barley plants. They are: 11 Conlon1 plants with pRD383 by gene gun (383-C lines), 10 Conlon1 plants with pRD383 by EHA105 (383-CA lines), 1 Genesis plant with pRD383 by gene gun (383-G lines) and 7 Genesis plants with pRD383 by EHA105 (383-GA lines); 3 Conlon1 plants with pRD388 by gene gun (388-C lines), 8 Conlon1 plants with pRD388 by EHA105 (388-CA lines), 2 Genesis plants with pRD388 by gene gun (388-G lines) and 10 Genesis plants with pRD388 by EHA105 (388-GA lines); 8 Conlon1 plants with pRD403 by EHA105 (403-CA lines). All of these plants have been confirmed as transgenic with the integration of the transgene by PCR-amplification of the gRNA and the spacer with the gDNA templates. Some of the representative regenerated tissues and plantlets are shown in Fig. 3.

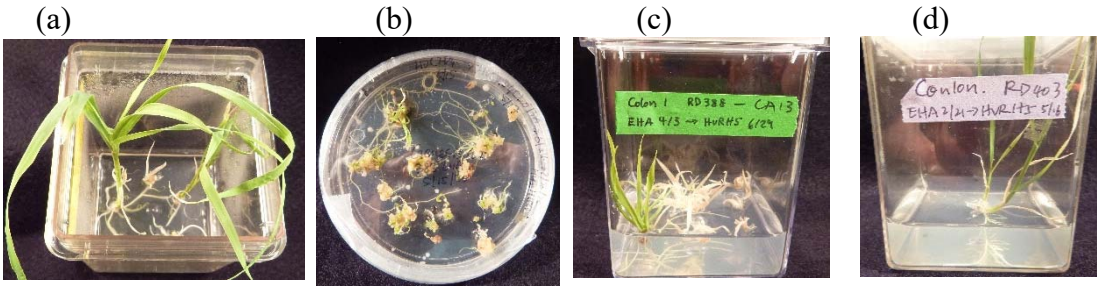

Fig. 3 Transformation and regeneration of pRD383, pRD388 and pRD403 transgenic barley plants. (a) pRD383 Conlon1 by gene gun, (b) pRD383 Genesis by EHA105, (c) pRD388 Conlon1 by EHA105, and (d) pRD403 Conlon1 by EHA105.

We then PCR-amplified the gDNA fragments spanning the *Hv2OGO-Nde*I site for the potentially pRD383-edited plants, the gDNA fragments spanning the *HvHSK-Sac*II site for the potentially pRD388-edited plants, and the gDNA fragments spanning the *HvEIN2-Sph*I site for the potentially pRD403-edited plants. We have cloned some of the PCR fragments and sequenced them. Our results showed that CRISPR-gene editing has resulted in 383-C1 with two amino acid changes, 383-C2 with one amino acid change at the *Hv2OGO* target site, 383-CA2 with one amino acid change at the *Hv2OGO* target site. We have also produced 388-C6 with two amino acid mutations slightly off the target on the *HvHSK* gene. We are in the process of characterizing this large number of transgenic barley plants produced in the past year. Some of the transgenic gene-edited barley plants have produced seeds that will be evaluated for the inheritance of the mutations.

b) What were the significant results?

We have successfully transformed and regenerated many Conlon1 and Genesis barley plants with our CRISPR-gene editing vectors pRD383 to target *Hv2OGO*, pRD388 to target *HvHSK* and pRD403 to target *HvEIN2* genes. Some of the regenerated transgenic barley plants have been confirmed to be mutants. This indicates that our CRISPR-gene editing platform works and that our barley transformation and regeneration system is efficient.

c) List key outcomes or other achievements.

Some gene-mutated barley plants have been produced.

3. Was this research impacted by the COVID-19 pandemic (i.e. university shutdowns, reduced or lack of support personnel, etc.)? If yes, please explain how this research was impacted or is continuing to be impacted.

Yes, the COVID-19 pandemic has greatly impacted this research. Rutgers shut down the whole university in the middle of March 2020. I requested and was granted to keep my lab and research programs open, initially at 25% capacity, then at 50% capacity in the summer to now at 100%. The severe reduction in research capacity has negatively impacted our progress by limiting the time the researchers could spend in the lab and the greenhouse, the delivery of necessary supplies and chemicals, the lengthened time of DNA sequencing completion etc. As a result, I requested and was approved a no-cost-extension of the project to now end on 1/31/2021. However, compared to most labs on our campus, my programs have continued to make satisfactory progresses in all of the projects.

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We have published our results on the involvement of the *2OGO* gene in FHB susceptibility: Low, Y., M. A. Lawton and **R. Di**. 2020. Validation of barley *2OGO* gene as a functional orthologue of Arabidopsis *DMR6* gene in *Fusarium* head blight susceptibility. Sci. Reports. 10:9935. DOI:10.1038/s41598-020-67006-5.

Training of Next Generation Scientists

Instructions: Please answer the following questions as it pertains to the FY19 award period (8/1/19 - 7/31/20). 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 FY19 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 FY19 award period?**
   Yes

   If yes, how many? 1

3. **Have any post docs who worked for you during the FY19 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 FY19 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 FY19 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

Publications, Conference Papers, and Presentations

Instructions: Refer to the FY19-FPR_Instructions for detailed more instructions for listing publications/presentations about your work that resulted from all of the projects included in the FY19 grant award. Only citations for publications published (submitted or accepted) or presentations presented during the **award period (8/1/19 - 7/31/20**) should be included. If you did not publish/submit or present anything, state 'Nothing to Report' directly above the Journal publications section.

NOTE: Directly below each citation, you **must** indicate the Status (i.e. published, submitted, etc.) and whether acknowledgement of Federal support was indicated in the publication/ presentation.

Journal publications.

Low, Y., M. A. Lawton and **R. Di**. 2020. Validation of barley *2OGO* gene as a functional orthologue of Arabidopsis *DMR6* gene in *Fusarium* head blight susceptibility. Sci. Reports. 10:9935. DOI:10.1038/s41598-020-67006-5.
Status: Published
Acknowledgement of Federal Support: YES

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

Other publications, conference papers and presentations.

(1) Di, R., Y. Low and M. A. Lawton. 2019. CRISPR-editing susceptibility genes to improve FHB resistance. NC1183 Multistate Project, Mycotoxins: Biosecurity, Food Safety and Biofuels Byproducts, Sept. 12, 2019, Blacksburg, VA.
Status: Oral presentation
Acknowledgement of Federal Support: YES

(2) Di, R., Y. Low and M. A. Lawton. 2020. CRISPR-editing susceptibility genes to improve FHB resistance. NC1183 Multistate Project, Mycotoxins: Biosecurity, Food Safety and Biofuels Byproducts, Sept. 15, 2020, University of Nebraska-Lincoln, via Zoom.
Status: Oral presentation
Acknowledgement of Federal Support: YES