Project FY22-GD-003: A Barley Genetic Engineering Facility for FHB Research Community

---

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

The overall goals of this project are to: (1) establish a barley genetic engineering facility to provide a no-cost transformation service for the *Fusarium* head blight (FHB) research community; (2) continue to improve the transformation and regeneration protocols for different barley cultivars; (3) develop and apply CRISPR-gene editing technology to discover genes involved in FHB susceptibility, and engineer FHB resistance in barley cultivars grown in the U.S.

2. What was accomplished under these goals or objectives? *(For each major goal/objective, address these three items below.)*

What were the major activities?

(1) We have continued to work with the USWBSI coordinators to promote the "Barley Genetic Engineering Facility" services established at Rutgers University. Specifically, Dr. R. Di presented an invited talk at the 2024 National Fusarium Forum (Dec. 8-10, 2024)), and at the USDA NC1183 Multistate Project (Mycotoxins in a Changing World) Annual meeting (May 31, 2024), and promoted the services offered by the Facility at Rutgers.

We have started working with other barley researchers to develop tissue culture protocols specific for their barley cultivars. We have transformed barley cultivar Genesis with several transgenes, and cultivar Thunder with a marker gene.

(2) We have systematically evaluated and optimized every step of the protocols for barley transformation and subsequent regeneration from immature embryo explants to improve the tissue culture efficiency for cultivars of ND Genesis, Morex and Thunder. We have evaluated the efficacy of *HvBBM* and *HvWUS* morphogenes for improving induction and regeneration of barley embryogenic callus.

(3) We have developed our own dual tRNA-based, multiplexing CRISPR platform to improve the efficiency and specificity of barley gene editing with the goal of enhancing FHB resistance. We have also adopted the pYPQ vectors into our pipeline to allow multiplexed barley gene-editing to be carried out.

What were the significant results?

(1) For the activities in the "Barley Genetic Engineering Facility", we have worked with Dr. John McLaughlin at Rutgers University since 2023 to produce transgenic barley cv. ND Genesis with the following overexpression constructs: #1, pB835, the base vector overexpressing GFP; #2, pATLTP4.4, overexpressing Arabidopsis lipid transfer protein (AtLTP) (AT5G55450) and GFP fusion protein; #3, pTaLTP3, overexpressing wheat TaLTP3 (AY226580) and GFP fusion protein. Dr. McLaughlin's previous research (funded by USWBSI) has shown that LTP can confer Fusarium disease resistance. Additionally, from Dr. McLaughlin, we have obtained #4, pJM1 overexpressing wheat TaLTP9-1B and #5, pJM4 overexpressing barley *HvVIPP1*, a protein involved in chloroplast thylakoid membrane maintenance. Both pJM1 and pJM4 have been previously shown to provide resistance to FHB and several turfgrass pathogenic fungi.

We have bombarded cultivar ND Genesis calli with these constructs. We have regenerated transgenic ND Genesis plants transformed with pJM4 by the gene gun method.

The seeds from these plants are being harvested. Hygromycin resistant calli of ND Genesis transformed by pJM1 via gene gun have been obtained. Currently shoots are being regenerated from these calli.

We have extended our service to other barley researchers through the USWBSI website and our USWBSI colleagues. Particularly, we have provided Dr. P. Hayes' (retired from Oregon State University) collaborator, Dr. Raj Nandety (USDA-ARS, North Dakota), with guidance on constructing CRISPR-editing vectors for their target genes. Similarly, we have provided consultation services to Dr. Nidhi Rawat (University of Maryland) and Dr. Guixia Gui (USDA-ARS) on vector constructions for barley transformation. We have been working with Dr. Gary Muehlbauer (University of Minnesota) to study and manipulate the dynamics of *HvUGT* gene espression in response to *F. graminearum* infection. We have produced edited Morex with pRD549, a barley dtRNA (dual tRNA)-based CRISPR-KO vector, to disrupt the *HvUGT* promoter. Additional details of these specific activities are described below in (3).

In addition, we have produced a transgenic Thunder plant using the plant expression vector pCAMBIA1300, which can serve as a vector-control plant for phenotypic comparisons.

(2) The ultimate bottleneck for barley genetic engineering is the efficiency of transformation and regeneration of barley tissues. We have been working with the two-rowed Genesis and Thunder and the six-rowed Morex, all U.S. cultivars. We and others have attempted to use young barley leaf tissue as the explant for transformation, in combination with the use of morphogenes to promote regeneration from transformed cells. However, these attempts have been largely unsuccessful.

Therefore, we have focused on using embryogenic calli, which provide the optimal material for both the gene gun and for *Agrobacterium* transformation to produce non-chimeric transgenic barley plants.

We have extensively tested, assessed and modified every step of our existing tissue culture protocol, including germinating and growing plants in the greenhouse to provide immature embryos as explants, embryogenic callus induction, transformation of embryogenic calli, selection of transformed embryogenic calli and regeneration of transgenic barley plants. We have established regimens consisting of: 1-month at 4 ºC/1-month at 12 ºC/2-3 weeks at 26 ºC growing period for Genesis and Morex; and 2-months at 4 ºC/1.5-month at 12 ºC/2-3 weeks at 26 ºC growing period for Thunder. We have adjusted the concentrations of auxin (2,4-D), cytokinin (BAP) and the selective agent, hygromycin. By applying these regimens, we have been able to produce embryogenic calli for all three cultivars within a 3-week period. After gene gun bombardment of the embryogenic calli, we have been able to produce transgenic barley plantlets within 8 weeks. When *Agrobacterium tumefaciens* is used to transform embryogenic calli, two additional weeks are needed for the selection and regeneration of transgenic barley. These results are significant, because they help relieve the tissue culture bottleneck that has been preventing researchers from applying molecular tools to improve barley cultivars (especially those that are commercially grown) through gene editing and related methods.

In addition to these improvements in tissue culture, transformation and regeneration, we have constructed transient *HvBBM* and *HvWUS* overexpression vectors (pRD635 and pRD636) driven by 3XUbi and NOS promoters respectively. Our results have shown that these two barley morphogenes promote the regeneration of multiple shoots from calli derived from a single immature seed of ND Genesis, Morex and Thunder.

(3) To continue our efforts to apply CRISPR-gene editing technology to produce gene edited FHB resistant barley plants and to study FHB resistance mechanisms, we have focused on our dtRNA-based CRISPR vectors pRD549, to disrupt the promoter of the *HvUGT* gene in Morex, and pRD554, to knock-out (KO) the *HvEIN2* gene in Genesis.

Using these improved transformation and regeneration protocols, we have selected and regenerated several RD549 Morex plants. Our results confirmed that we were able to produce 3 RD549 T_0 mutants, termed 549-1, 3, 4. The T_1 seeds of these mutants have been selected and are currently being grown in the greenhouse. These plants are currently being evaluated for the heritability of mutations in the *HvUGT* promoter. The other RD549 T_0 plants are also being evaluated.

In an effort to compare the gene-editing efficiency of the dtRNA-based CRISPR-editing vector and the pYPQ (constructed by Dr. Y.P. Qi at University of Maryland) vectors, we constructed pRD664 to KO the *HvUGT* promoter and pRD667 to KO the *HvHRC* gene (encoding a histidine-rich calcium-binding protein) in pYPQ vector background. It has been shown that a deletion mutation in wheat *TaHRC* confers FHB resistance in wheat. We have produced several putative transgenic Morex plants following transformation with *Agrobacterium tumefaciens* strain Agl1, carrying pRD664. We have also produced putative transgenic ND Genesis calli following transformation with pRD667 using both the gene gun and *A. tumefaciens* Agl1.

List key outcomes or other achievements.

The key outcomes of our FY23-24 research activities are a substantially improved barley transformation and regeneration protocol for Genesis, Thunder and Morex cultivars using embryogenic calli derived from immature seeds. Additionally, *HvUGT* mutant plants have been produced.

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

This project provided partial funding for a full-time technician YC who works on the tissue culturing and transformation of barley. This project has also provided training for an undergraduate students AP and a graduate student ICKF in plant tissue culture and genetic engineering.

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

We have presented our progress at the National Fusarium Head Blight Forum in December 2024 as listed below.

We have also presented our findings in other meetings and the courses that Dr. Di teaches at Rutgers in the undergraduate and graduate programs in Biotechnology and Plant Science.

5. What do you plan to do during the next reporting period to accomplish the goals and objectives?

We plan to (1) continue to advocate our barley transformation service to the barley researchers nationwide, (2) modify our barley transformation and regeneration protocol for barley cultivars chosen by other researchers, (3) continue to transform Genesis and Morex with our own CRISPR-editing vectors and those provided by the researchers, and (4) characterize those barley plantlets that have been regenerated.

During the past year, we had been contacted by several researchers with requests to transform barley with their vectors. However, due to the current unstable funding situation, some USDA-ARS researchers were not able to provide us with their vectors. Therefore, we plan to periodically contact these researchers so that we can assist them to resume their research programs.