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

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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 worked with the USWBSI coordinators and established the "Barley Genetic Engineering Facility" at Rutgers University. A barley transformation request form has been uploaded to the USWBSI webpage. The description of the Facility has been published on the USWBSI Newsletter April 2023 Edition.

We have started working with other barley researchers to develop tissue culture protocols specific for their barley cultivars and to transform Genesis barley with several transgenes. (2) We have spent a significant portion of FY23-24 to systematically evaluate every step of the protocols for barley transformation and regeneration from immature embryo explant to improve the tissue culture efficiency for cultivars of Genesis, Morex and Thunder. We have initiated studies to assess the efficacy of the morphogenes *HvBBM* and *HvWUS* for the improvement of barley embryogenic callus induction and regeneration. These morphogenes are known to improve transformation and regeneration efficiency in other monocot species. (3) We have developed the dual tRNA-based, multiplexing CRISPR platform to improve the efficiency and specificity of editing barley genes for enhancing FHB resistance. We have transformed cultivars Genesis and Morex with our CRISPR-gene editing vectors and with other transgenes to improve barley FHB resistance.

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. Genesis with the following overexpression constructs: #1, pB835, the base vector overexpressing GFP; #2, pATLTP4.4, overexpressing Arabidopsis lipid transfer protein (LTP) (AT5G55450) and GFP fusion protein; #3, pTaLTP3, overexpressing wheat LTP3 (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 barley *HvVIPP1*, a protein involved in chloroplast thylakoid membrane maintenance, shown previously to provide FHB resistance.

We have bombarded cultivar Genesis calli with these four constructs. The transformed embryogenic calli are currently being selected on hygromycin-containing media; several shoots have been regenerated and these will be rooted to provide mature, seed-bearing plants.

We have extended our service to other barley researchers through the USWBSI website and our USWBSI colleagues. Particularly, we have provided Dr. P. Hayes' (Oregon State University) collaborator, Dr. Raj Nandety (USDA-ARS, North Dakota), with guidance on constructing CRISPR-editing vectors for their target genes. 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 transformed Morex with pRD549, a barley dtRNA (dual tRNA)-based CRISPR-KO vector, to disrupt the *HvUGT* promoter. We are currently evaluating the regenerated plantlets for mutations.

(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. There have been attempts from others and ourselves 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, to date, no routine procedures using this approach for barley have been published; we have not been able to establish successful regeneration from transformed leaf tissues. We have also tried very hard to develop the anther/microspore cultures for Genesis, Thunder and Morex, which have been unsuccessful.

Therefore, we have decided to focus on embryogenic calli, which provide the best material for both the gene gun and for *Agrobacterium* transformation to produce non-chimeric transgenic barley plants. If transient CRISPR-gene editing vectors are used for gene editing, embryogenic calli will be more likely than leaf tissues to regenerate transgene-free, gene mutated barley plants, because integrating morphogenes which by virtual are transgenes, are more needed for leaf tissues to enter embryogenesis and to regenerate.

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 the 1-month at 4 ºC/1-month at 12 ºC/2-3 weeks at 26 ºC growing period for Genesis and Morex, and 2-month at 4 ºC/1.5-month at 12 ºC/2-3 weeks at 26 ºC growing period for Thunder. Using and testing at least 100 starting explants for each cultivar, we have adjusted the concentrations of auxin (2,4-D), cytokinin (BAP) and the selective hygromycin, replaced sucrose with maltose as the carbon source in the initial tissue culture stages. 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 in 8 weeks. When *Agrobacterium tumefaciens* is used to mediate transformation of the embryogenic calli, two more weeks are needed for the selection and regeneration of transgenic barley. These results are significant, because they help relieve the bottleneck of tissue culture 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, we have cloned barley *HvBBM* and *HvWUS* from Morex and constructed transient overexpression vectors with these two morphogenes driven by 3XUbi and NOS promoters respectively. We have tested these two transient morphogene vectors on the transformation of Genesis, Thunder and Morex embryogenic calli. Our early results using these constructs are encouraging and show that the presence of *HvBBM* and *HvWUS* promotes the regeneration of multiple shoots from calli derived from a single immature seed.

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

With the improved transformation and regeneration protocol, we have selected and regenerated several RD549 Genesis and RD554 Morex plants which are being molecularly characterized for the integration of the transgenes and the induced mutations in *HvEIN2* and *HvUGT* promoter.

List key outcomes or other achievements.

The key outcomes of our FY23-24 research activities are the immensely improved barley transformation and regeneration protocol for Genesis, Thunder and Morex cultivars using embryogenic calli derived from immature seeds.

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

This project provided the funding and training for AD, who is a full-time technician and a graduate student working on the molecular characterization of regenerated barley plants. This project provided funding for another full-time technician YC who works on the tissue culturing of barley. This project has also provided training for two undergraduate students AP and FG 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 Forum in December 2023 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.