Project FY22-TS-001: Transfer Fhb7 to Barley Through CRISPR-mediated Targeted Gene Insertion

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1. **What are the major goals and objectives of the research project?**
   The Objectives of this project are: (1) Generate transgenic barley expressing both the CRISPR/Cas9 and the *Fhb7* donor; (2) Evaluate the *Fhb7* function in transgenic barley; and (3) Screen the transgenic plants for targeted *Fhb7* insertion events.

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

What were the major activities?

Objective 1. (1) Development of a new plasmid construct (pWL2202) containing two separate T-DNA cassettes for transformation of Gold Promise (GP) barley by agrobacterium-mediation; (2) transformation of ~600 (36 plates) GP embryos with pWL2202; (3) development of two plasmids carrying two new alleles of *Fhb7* donor DNA, *Fhb7-M10* and *Fhb7-V29P-M10*; and (4) Biolistic transformation of ~300 Excelsior Gold (EG; an elite two-row barley cultivar from the Cornell University) embryos with the Mlo-targeting RNP plus *Fhb7-M10* donor DNA.

Objective 2. (1) Field test of *Fhb7* transgenic plants of EG for FHB resistance and (2) establishment of greenhouse FHB inoculation system for barley.

Objective 3. (1) Screening of 35 T_0 and 151 T1 GP transgenic plants for Fhb7 and the transformation marker and (2) screening of over 300 plants of 10 EG T_1 families for *Fhb7* insertion and *Mlo* editing.

What were the significant results?

Objective 1. (1) A binary plasmid construct, *i.e.*, pWL2202, was developed containing two separate T-DNA cassettes: one for the transformation selection marker gene *HygR* for hygromycin resistance and another for the *Fhb7* gene, with expectation that *HygR* and *Fhb7* will be inserted to different locations in the barley genome after transformation and segregate for marker-free Fhb7 transgenic lines in the T_1 generation. In the Fhb7 cassette, maize ubiquitin promoter *Ubi* is upstream and tandem with the native *Fhb7* promoter. Thus, the *Fhb7* gene is driven by two promoters for overexpression in the construct. (2) This double T-DNA construct was used for *Agrobacterium*-mediated transformation of GP. Approximately 600 embryos were transformed, from which 35 transgenic plants were generated, 23 of which carry the Fhb7. (3) Recent research showed that ammino acid substitution can significantly enhance the Fhb7 enzyme activity, including V29P and M10 alleles. Accordingly, we constructed two plasmids for these new *Fhb7* alleles. In the first plasmid, we replaced 20 amino acids (60 bp sequences) from position 56 to 76 with counterpart of the CsGST83044 protein. In the second plasmid, we also introduce V29P substitution in addition to the M10 substitutions. (4) While characterizing the EG Fhb7 insertion lines, we have performed Biolistic transformation of Cas9 RNP plus *Fhb7-M10* of EG embryos. In the first experiment,

~300 embryos were bombarded, all bombarded embryo generated calluses, from which 35 seedlings are under regeneration.

Objective 2. (1) In collaboration with Dr. Brian Steffenson of University of Minnesota, we are testing the EG *Fhb7* transgenic plants in the field with FHB-infected corn seeds as inoculum. Two EG Fhb7 (T_2) insertion lines with pedigree #24-80 and #24-81 were submitted together with wild-type EG. While EG showed severity of 3.3%, #24-80 and #24-81 had severity of 2.2% (p = 0.11934) and 0.8% (p = 0.00531), respectively. Genotyping the remaining seeds of #24-80 showed that it is still segregating, which may attribute to higher severity. Although this result is preliminary as the first field trial, it shows that Fhb7 functions in barley, corroborating previous results from the detached leaf assay. The seeds will be harvested and submitted to the USWBSI DON lab for DON determination. (2) Considering the limitations of the field test, we are establishing a greenhouse FHB inoculation system for barley using FHB-infected corn seeds as inoculum. With the protocol from Steffenson's group, we successfully prepared corn inoculum using *F. graminearum* GZ3639. At the booting stage, 10 FHB-infected corn seeds are place in a 6" pot containing a single barley plant, and the pots are placed in home-made chamber (lamber frame covered by plastic membrane). The chamber was sprayed for five minutes at 5:00 pm, kept closed overnight, and re-open at 8:00 am in the morning. The FHB infection symptom in EG showed 10 days after inoculation. This system will be used for evaluation of the *Fhb7* insertion lines of EG and GP backgrounds.

Objective 3. (1) Transformation of the double T-DNA construct pWL2202 regenerated 35 hygromycin-resistant T_0 plants. PCR genotyping showed that 23 of them contain the *Fhb7* transgene cassette. PCR genotyping 151 plants of seven T_1 families for *HygR* and *Fhb7* identified 15 *HygR*-free *Fhb7* transgenic plants from four T1 families. (2) We screened over 300 plants of seven EG T_1 families for *Fhb7* insertion and Mlo editing. *Fhb7* insertion was detected in all seven families. In two families, we detected obvious editing of Mlo as no amplification of the fragment containing the target site. An insertion junction was amplified by PCR from one of these lines, sequencing the PCR product using the *Fhb7*-specific primer revealed the *Mlo* sequence, confirming the target insertion although the sequencing quality needs to be improved.

List key outcomes or other achievements.

Plasmid constructs and seeds of *Fhb7* transgenic plants are up to request.

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

This project has been providing opportunities for training postdoc, undergraduate, and technician. **Dr. Ming Ma**, a postdoc scientist, worked on the molecular biology and pathologic aspects of the project, including molecular cloning, PCR screening of the transgenic plants, Biolistic transformation, preparation corn seeds inoculum, and establishment of the greenhouse FHB inoculation. **William Hummel**, a sophomore undergraduate majoring in plant science, was trained in molecular biology by DNA isolation

and PCR screening of the *HygR*-free *Fhb7* transgenic plants under Dr. Ma Ming's supervision. **Yanhang Zhang**, a lab technician, was trained in tissue culture and barley transformation by Agrobacterium-mediation.

4. **How have the results been disseminated to communities of interest?**
   The results from this Transformational Science category project have been presented in the general session of the National FHB Forum 2023.

5. **What do you plan to do during the next reporting period to accomplish the goals and objectives?**
   Month 1-3: Field test of the *Fhb7* transgenic GP is scheduled 2023 summer in collaboration with Dr. Steffenson.
   Months 4-12: Complete the evaluation of Fhb7 function in transgenic barley, i.e., resistance phenotyping, in the greenhouse condition and DON level determination.
   Months 1-8: Continue screening of all the transgenic populations for targeted insertion events.
   Months 1-12: Summarize the results to prepare a manuscript and the final report.