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 plasmid constructs for barley transformation;

(2) transformed ~2000 (70 plates) barley embryos of Gold Promise (GP) by agrobacterium-mediation and ~1000 (36 plates) GP embryos and ~700 (22 plates) Excelsior Gold (EG; an elite two-row barley cultivar from the Cornell University) embryos by Biolistic-bombardment.

(3) Bombarded >1,000 EG embryos with the mlo-targeting RNP plus Fhb7 donor DNA.

(4) Screening of 247 T_0 and 384 plants of 6 T_1 families for transgenes.

(5) We have constructed a binary vector carrying two separate T-DNA cassettes (2T) with the *Fhb7* gene in one and transformation selection marker HygR in another and transformed into GP.

Objective 2.

(1) Preparation of conidia spores from *F. graminearum* GZ3639 and GZ3639-infected corn kernels as inoculums.

(2) Establishment of detached leaf assay method in barley.

(3) Conduction of detached leaf assays on 39 T_0 and 147 plants of 5 T_1 families; and reverse transcription PCR (RT-PCR) assay of the *Fhb7* transgene.

(4) Field and greenhouse evaluation of Fhb7 insertion lines for FHB resistance.

Objective 3.

(1) Development of 12 primers (six from mlo sites and six from Fhb7 sites) for 18 combinations to detect targeted insertions of Fhb7 in the *mlo* locus by amplifying the Fhb7-mlo junctions and screening 39 T_0 plants for targeted insertion.

(2) Illumina sequencing five Fhb7 insertion lines for identification of insertion junctions.

What were the significant results?

Objective 1.

(1) Two plasmid constructs were developed: an all-in-one construct including CRISPR/Cas9 and Fhb7 donor for *Agrobacterium*-mediated transformation and an optimized CRIPSR/Cas construct for bombardment together with 5'-phosphorylated and phosphorothioate linkage -protected PCR product of the *Fhb7* gene. *Agrobacterium*-mediated transformation produced 247 seedlings based on Hygromycin selection, and PCR screening of these seedlings identified 39 transgenic T_0 plants. Of the 384 seedlings screened, a total of 284 seedlings were found carrying the transgene, and the Fhb7 transgene is segregating in the T_1 populations screened into 3 (present):1 (absent), indicative of single copy of transgene insertion.

(2) From the Cas9 ribonucleoprotein (RNP) bombardment of EG embryos, 304 seedlings were regenerated without transformation selection, suggesting that EG has good potential of regeneration for barley transformation.

(3) Twenty-one regenerated plants were obtained through *Agrobacterium*-mediated transformation of the 2T construct. From three T1 families screened, marker-free Fhb7 transgenic plants were obtained.

Objective 2.

(1) RT-PCR showed that *Fhb7* transgene was expressed in leaf and spike.

(2) Detached leaf assay of wild-type GP side-by-side with Chinese Spring (CS) (FHB susceptible) and RWG52, which carries Fhb7 in the CS background, showed that 72 hours after inoculation, fungus is growing on CS leaves (level 1) but not RWG52 leaves (level 0) and fungus is growing on the GP leaves and led to chlorosis (yellowing; level 2), indicating that barley is much more susceptible to *F. graminearum* compared to wheat. Detached leaf assay in T_1 transgenic seedlings showed that most of the seedlings had a level 1 reaction, but a small number of seedlings had a level 0 reaction. Because DON causes leaf photobleaching, the degree of leaf yellowing may reflect the levels of DON accumulation. Thus, results from detached leaf assay suggest that Fhb7 functions in barley: degrading DON and suppressing the growth *of F. graminearum*.

(3) In collaboration with Dr. Brian Steffenson, we evaluated FHB resistance in two lines, #24-80 and #24-81, together with EG as a control, in the field condition using mixed *F. graminearum* strains 2024 summer. Compared to EG, the FHB incidence and severity were reduced in #24-80 by 26% and 27.9% ($p = 0.11934$) and in #24-81 by 60% and 76.9% ($p = 0.0042$). We also tested these lines in the greenhouse condition using *F. graminearum* strain GZ3639-infected corn kernels in 2024 summer and 2025 spring. In the 2024 season, the FHB severity was reduced from 14% in EG to 6% in #24-80 ($p = 9.3E-6$) and 9% in #24-81 ($p = 0.03086$). In the 2025 season, FHB incidence, severity and index were reduced by 20% ($p = 0.0413$), 33% ($p = 0.0082$), and 45% ($p = 0.00081$) in #24-80 and by -0.6% ($p = 0.92174$), 19% ($p = 0.04307$), and 19% ($p = 0.14803$) in #24-81, respectively, as compared with wildtype EG. These results indicate that Fhb7 confers FHB resistance in barley.

Objective 3.

(1) Of the 18 primer combinations evaluated at different annealing temperatures on both orientations, nine primer combinations gave clear background on the wild-type GP. These primers were used to screen the 39 T_0 transgenic plants for targeted insertion of *Fhb7* in the *mlo* locus, but no positive amplifications were detected in the T_0 population.

(2) The PCR assay of the plants regenerated from RNP-bombarded EG calluses showed that only one plant has a junction between mlo and Fhb7, indicating high frequency of off-target insertions. Considering the usefulness of the junction information in marker development and challenges in isolation of the insertion junctions, we sequenced genomes of five insertion lines, including #24-80 and #24-81, to ~10x coverage of the genomes and obtained 2.1 billion paired end (PE) reads. We are analyzing the sequences to identify the insertion junctions. The nature of the short reads makes the process challenging, but the sequences will be used for petitioning exemption of transgenic regulation, which will clear the road for deploying the insertion lines in barley breeding to improve FHB resistance.

List key outcomes or other achievements.

1. Plasmid constructs are up to request.
2. Marker-free Fhb7 insertion lines as new germplasm are up to request.
3. An improved procedures for biolistic transformation of immature barley embryos.
4. A procedure for evaluating FHB in barley in the greenhouse condition.

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

This project provided opportunities for training and professional development of two graduate students, one undergraduate student, one research scientist and a technician. Ph.D. student **Mohd Kyum**, previously trained in maize genetics, performed biolistic transformation, tissue culture, transgene screen, and detached leaf assay. Ph.D. student **Wei Jiang,** previously trained in Oil seeds genetics, worked on molecular biology (cloning), tissue culture, and plant pathology (preparation Fusarium conidia spores and FHB inoculation) in the project. Research assistant **Xi Chen**, previously trained in rice developmental biology, worked on the project in PCR screening of Biolistic transformed plants in the project. Undergraduate student **William Hummel**, majored in plant science, screened the T1 populations of GP transformed with 2T construct for marker-free Fhb7 transgenic plants and performed bioinformatics analysis of the Illumina sequences for the insertion junctions. Technician **Yanhang Zhang** worked on tissue culture and greenhouse management.

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

The results were disseminated conference presentations.