Project FY22-GD-011: Genotype-independent Transformation in Barley

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1. **What are the major goals and objectives of the research project?**
   1) To obtain transgenic barley plants using the GRF-GIF chimera.
   2) To transfer the *FHB7* gene to barley.
   3) To develop genotype-independent transformation protocol in barley.

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) Modified tissue culture media with enriched nutrients.

   We conduct factorial experiments varying cytokinin/auxin ratios and additional supplements (e.g., antioxidants, osmoprotectants) to enhance shoot regeneration. Quantify regeneration efficiency by calculating the percentage of embryogenic calli producing viable shoots.

   2) Enhanced T-DNA delivery efficiency

   A ternary transformation system including an additional helper plasmid encoding virulence (vir) genes and a mutated virG to improve T-DNA delivery. Moreover, the *NahG* gene was incorporated into the helper vector to suppress plant defense responses by reducing salicylic acid accumulation. T-DNA delivery efficiency was assessed using the RUBY visible marker system.

   3) Developed and tested combinations of development genes on transformation

   A series of altruistic plasmids to improve barley efficiency were developed, including WUSCHEL (WUS), BABY BOOM (BBM), GRF-GIF combinations, and LEAFY COTYLEDON1 (LEC1). The transformation efficiency was evaluated with stable transformation.

   4) Developing flexible system to remove developmental regulators in the transgenic plants

   Overexpression of developmental regulators improved transformation efficiency, but it also caused undesirable effects on plant growth. Therefore, these development genes should better be removed after transgenic plants are obtained. We first tested the heat shock protein (HSP) promoter-controlled Cre/loxP system. However, high-temperature induction can damage barley callus tissue and reduce subsequent regeneration capacity. Therefore, we employed a chemical inducer controlled Cre/loxP to efficiently excise or deactivate morphogenes post-transformation. The excision efficiency was assess using the RUBY marker.

   5) Transferred *FHB7* to Bowman

   The wheat *FHB7* gene was cloned into pCAMBIA1300 and pANIC5A under the control of the native promoter and a ubiquitin promoter, respectively. *Agrobacterium* strain Agl1 was used for barley transformation.

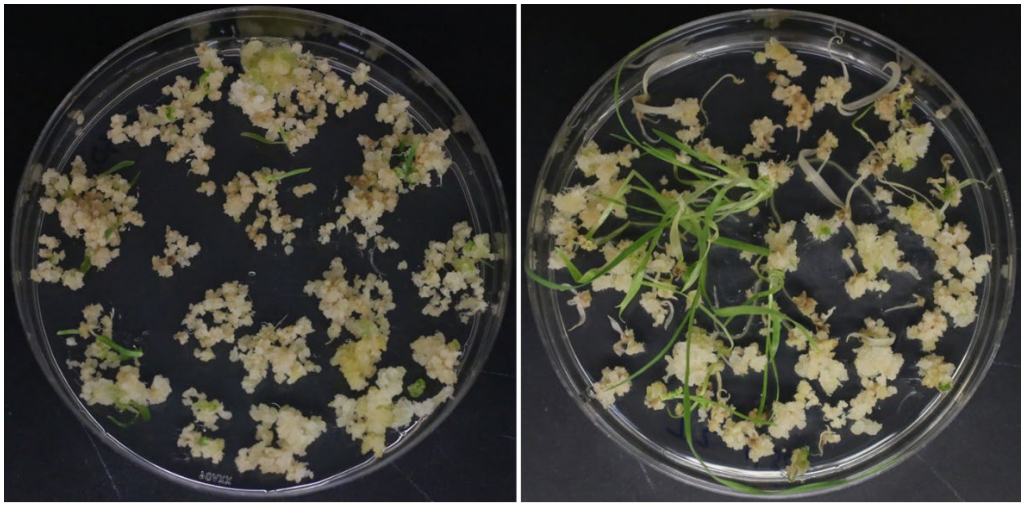
6) Assessing the FHB resistance with FHB7-transgenic plants

The homozygous T1 plants overexpressing FHB7 were collected to evaluate disease responses. Using the grain spawn method, FHB severity was assessed under filed conditions in a randomized complete block with three replicates. After counting the number of infected kernels from at least 50 spikes for both wild type and transgenic, the severity will be expressed as a percentage of the total kernels checked.

What were the significant results?

1) Barley transformation efficiency and duration was significantly increased.

Through modified tissue culture media and ternary transformation system, the transformation efficiency was improved from 8% at 80 days after isolation of embryo (DAI) to 30% at 35 DAI, 50% at 45 DAI (Figure 6), and 80% at 70 days.



35 days after embryo isolation 45 days after embryo isolation

Figure 6. Barley transformation using modified media and helper plasmid.

2) Applied developmental regulators into barley transformation

We successfully combined WUSCHEL (WUS), BABY BOOM (BBM), GRF-GIF fusion, and LEAFY COTYLEDON1 (LEC1) into a single altruistic plasmid. This approach achieved transformation efficiencies of up to 30% within 30 days (Figure 7A). However, constitutive expression of these developmental regulators resulted in undesirable traits, including low rooting rates (Figure 7B), bushy growth (Figure 7C), severe seed infertility, and reduced plant stature (Figure 7D).

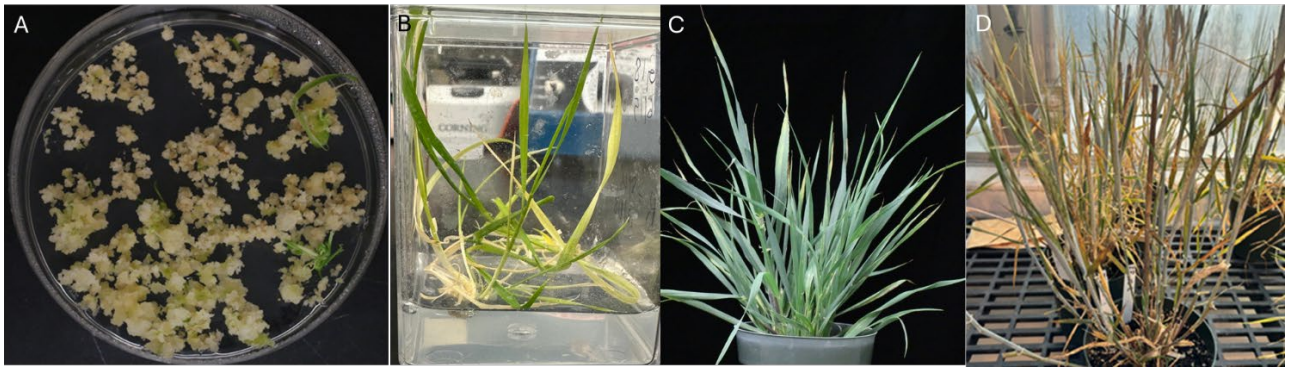


Figure 7. Application of developmental genes in barley transformation. Although developmental genes increased transformation efficiency (A), the resulting transgenic plants

exhibited poor rooting efficiency (B). Rooted transgenic plants displayed a bushy growth habit (C), along with a high number of sterile kernels, delayed flowering, and reduced plant height (D).

3) Designed Cre/loxP system to remove developmental regulators in the transgenic plants

To remove developmental regulators from transgenic plants, we initially employed a heat shock protein (HSP) promoter-controlled Cre/loxP system. However, heat shock treatment caused embryo browning and loss of vigor (Figure 8A). As an alternative, we used a chemically inducible promoter to control Cre/loxP, which successfully excised the developmental genes while preserving callus viability and cell division capacity (Figure 8B). In the absence of the inducer, the RUBY-tagged altruistic T-DNA was visibly expressed. One week after adding the inducer to the medium, RUBY pigmentation significantly faded (Figure 8B), allowing for the selection of non-RUBY calli for subsequent plant regeneration.

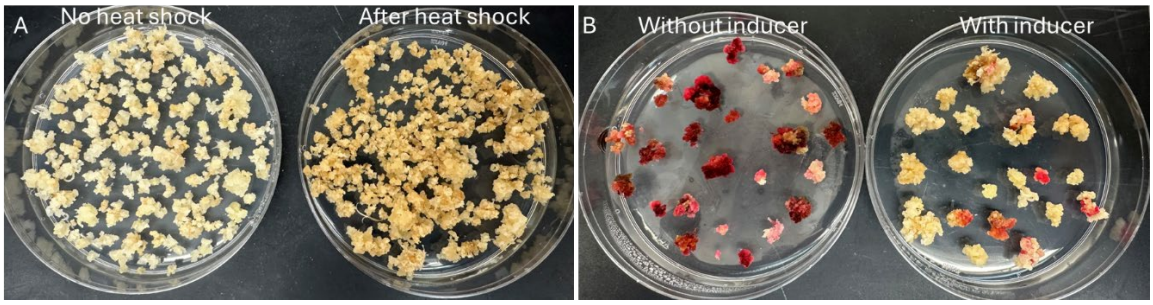


Figure 8. Removal of development regulators using a Cre/loxP system controlled by the heat shock protein promoter (A) and a chemical inducible promoter (B).

4) Obtained FHB7-transgenic Bowman plants

A total of 38 independent lines have been regenerated from 185 induced calli (Figure 9), and the transgene was confirmed with PCR and DNA sequencing.

Figure 9. Partial of the transgenic plants with the barley variety Bowman.

5) Field test in 2023 and 2024 indicated that transfer of FHB7 enhanced FHB resistance.

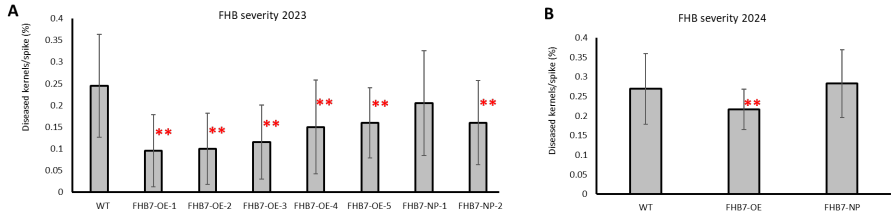


Figure 10. Field test for FHB resistance with FHB7-transgenic lines in 2023 (A) and 2024 (B).

Percentage of diseased kernels were significantly decreased in transgenic Bowman plants overexpressing FHB7. WT, wild type; OE, overexpression of FHB7 using a constitutive promoter; NP, FHB driven by the native promoter. Tucky-test: *P<0.05, **P<0.01.

List key outcomes or other achievements.

1) We developed a stable protocol for barley transformation in Golden Promise, Bowman, and several recalcitrant genotypes.
2) We significantly improved transformation efficiency in barley.
3) Homozygous *FHB7*-transgenic plants exhibited enhanced resistance to FHB.
4) The ternary transformation system we developed has been successfully applied to wheat, durum, and corn, establishing a stable and flexible transformation platform for cereal crops.
5) We have supported colleagues and collaborators with transformation services. Gene-edited and transgenic barley or wheat plants have been delivered to both national and international collaborators, including Rober Bruggeman (WSU), Gongshe Hu (USDA), Justin Faris (USDA), Shaobin Zhong (NDSU/USDA), Guixia Hao (USDA), and Thorsten Schnurbusch (IPK, Germany). Additional transformation-based collaborations are currently under discussion.

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

One postdoc, one Ph. D. student, and two undergraduate students were trained with barley transformation and FHB inoculation through the project.

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

The results have been disseminated through publications and presentations at different workshops and seminars and communications with collaborators.

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

1) Identify more homogeneous mutants for DON analysis.
2) Finalize the removal system for development genes in transgenic plants.
3) Include more lines for field trials in the nursey.
4) Combine *FHB7* with *FHB1* in barley to boost barley FHB resistance.