Project FY22-BA-018: Functional Validation of the Barley Fhb1 Ortholog in Susceptibility to FHB

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1. **What are the major goals and objectives of the research project?**
   1) to develop targeted gene knockouts in barley using CRISPR-mediated mutagenesis,
   2) to obtain the transgene-free barley mutants with resistance to FHB,
   3) to test the disease response of barley mutants under greenhouse and field conditions.

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) Identification of the barley ortholog of FHB1

      Using the proteins sequence of TaHRC (CBH32655.1), BLAST search within the barley genome of Morex identified a putative sarcoplasmic reticulum histidine-rich calcium-binding protein with high similarity (HORVU3Hr1G006250.2) on 3H, which was named as HvHRC. The BLAST result was listed below.

      Score = 215 bits (548), Expect = $2e^{-56}$, Method: Compositional matrix adjust. Identities = 236/260 (90%), Positives = 244/260 (93%), Gaps = 1/260 (0%)

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TaHRC  1   MDAKKFLQMVEDKKKRLLEKKEAPLKWQQKLEAAIKATEEKEKKLKSKKHRRRSYSSSES  60
           MDAKKFLQMVEDKKKRLLEKKEAPLKWQQKLEAAIKATEEKEKKLKS+KHRRRSYSS  ES
HvHRC  1   MDAKKFLQMVEDKKKRLLEKKEAPLKWQQKLEAAIKATEEKEKKLKSRKHRRRSYSSLES  60

TaHRC  61  DSESESDSDRKHRKRKDRKRHKRHGHSDSDGARRRKHRSKRRSSGSSDESDSDEYDGESE  120
           DSESESDSDRKHRKRKDKRH ++ HGHSDSDGARRRKHRSKRRSSSDESDSD+Y+   ESE
HvHRC  61  DSESESDSDRKHRKRKDSKRHRKHGHSDSDGARRRKHRSKRRHSDSSDESDSDDYNSESE  120

TaHRC  121 EERRRKKHSHRRKHRRHSSRSESDASDYSSDDDERRSTRKDHSRSRRRRHRSSDDESEEK  180
           EERRKKHSHRRKHRRHSSRSESDASDYSSDDD  ERRSTRKDHSRSRRRRHRSSDDESE+K
HvHRC  121 EERPRKKHSHRRKHRRHSSRSESDASDYSSDDDERRSTRKDHSRSRRRRHRSSDDESEDK  180

TaHRC  181 IRSRHRKRHHRSSDEDKPSDSDNHKRHRSRSMSLDDGAAGEPDKMNDGKGSHKSRHHRRH  240
           IRSR ++      HRSSDEDKPSDSDNHKRHRSRSMSLDDGAAGEPDKMNDGKGSHKSRHH
HvHRC  181 IRSR-HRKRHRSSDEDKPSDSDNHKRHRSRSMSLDDGAAGEPDKMNDGKGSHKSRHHHHR  239

TaHRC  241 HHHHDHRVNSAEPSDGKQLV  260
             HHHDH      SAEPSDGKQLV
HvHRC  240 RHHHDHHGKSAEPSDGKQLV  259
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   2) CRISPR-mediated mutagenesis of HvHRC
      Barley variety Bowman was used for CRISPR-mediated mutagenesis. *Agrobacterium tumefaciens* strain AGL1 was be used to deliver the gRNA and Cas9 expression cassettes to the barley genome. Immature embryos of Bowman were isolated for transformation. To eliminate excessive Agrobacteria during regeneration, the antibiotic Timentin was added to tissue culture media. For better mutagenesis efficiency, two target sites were chosen for gene editing of *HvHRC* by using the versatile CRISPR vector pHUE411.

   3) Phenotype screening with the derived mutants

The homozygous mutants at T1 were collected to evaluate disease responses in FHB nursery. After counting the number of infected spikelets from at least 50 spikes for both wild type and homozygous mutant, the severity was expressed as a percentage of the total spikelets checked.

4) DON analysis
Grains from least 10 Infected spikes were grounded for quantification of DON using GC-MS.

What were the significant results?

1) We have obtained transgenic plants (n > 30) using Golden Promise. The transgene was confirmed with PCR analysis (Figure 1A). DNA sequencing was used to identify mutations. Double peaks around the target sites observed in sequencing chromatograms are indicative of gene mutations. To determine the mutated alleles accurately, the PCR products was cloned into T-vectors for sequencing. We found various small indels at each target site and deletion of ~80-bp flanked by the two targets. Homozygous mutants at M1 with the transgene being segregated out will be subjected to pathogen infection. The preliminary results indicated at least two homozygous mutants with deletion of 80-bp were obtained (Figure 1B).

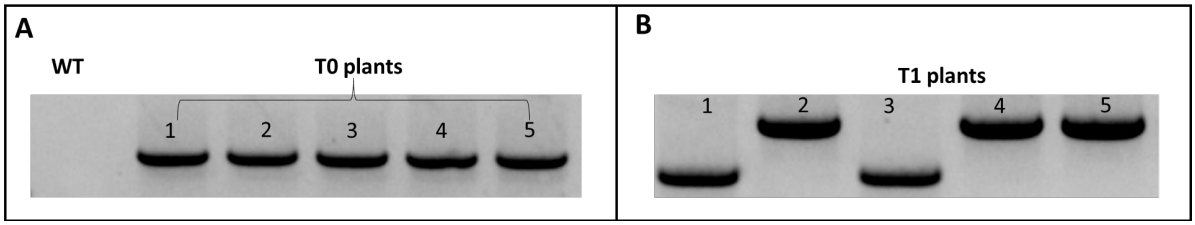

Figure 1. Transgenic plants with Golden Promise. The transgene was amplified in transgenic plants only (A). Deletion of 80 bp flanked by the two targets was identified in two of the T1 plants (B).

2) We also obtained transgenic plants (n > 20) with cv Bowman. The transgene was identified by PCR analysis (Figure 2A), and a heterozygous mutant (Plant 5) carried an 80-bp deletion in *HvHRC* (Figure 2B). The deletion was confirmed by DNA sequencing after the PCR product was cloned into T-vector (Figure 3). Similarly, small indels were identified as well at each target (Figure 4).

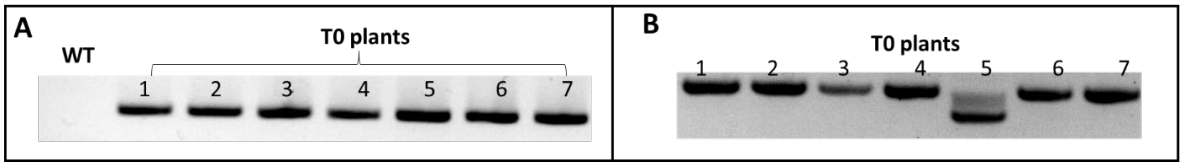

Figure 2. Transgenic plants with Bowman. The transgene was amplified in transgenic plants only (A). Deletion of 80 bp flanked by the two targets was identified in one of the T0 plant (B).

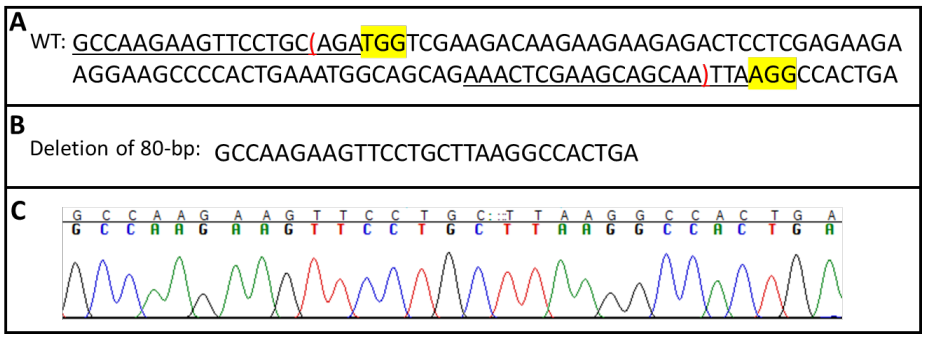

Figure 3. Confirmation of the deletion of 80-bp in T0 plant. The underlined sequences are two targets for gene editing. PAM sequences were highlighted in yellow (A). The deleted sequence in the mutant (B) was flanked by the red bracket (A), which was confirmed by DNA sequencing (C).

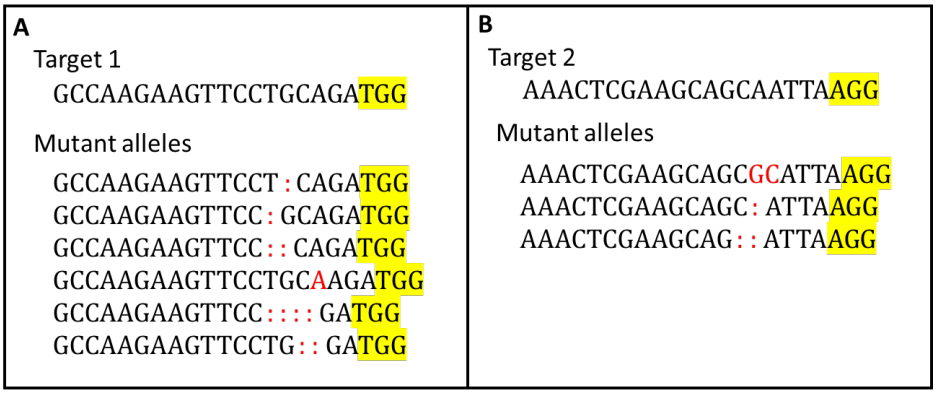

Figure 4. Various small indels caused by gene editing at target 1 (A) and target 2 (B). PAM sequence was highlighted in yellow.

3) Preliminary field test using four independent knockout lines indicated that *Hvhrc* loss-of-function mutation enhanced FHB resistance, and three of the lines exhibited lower DON levels than those in WT, as suggested by Figure 5 below.

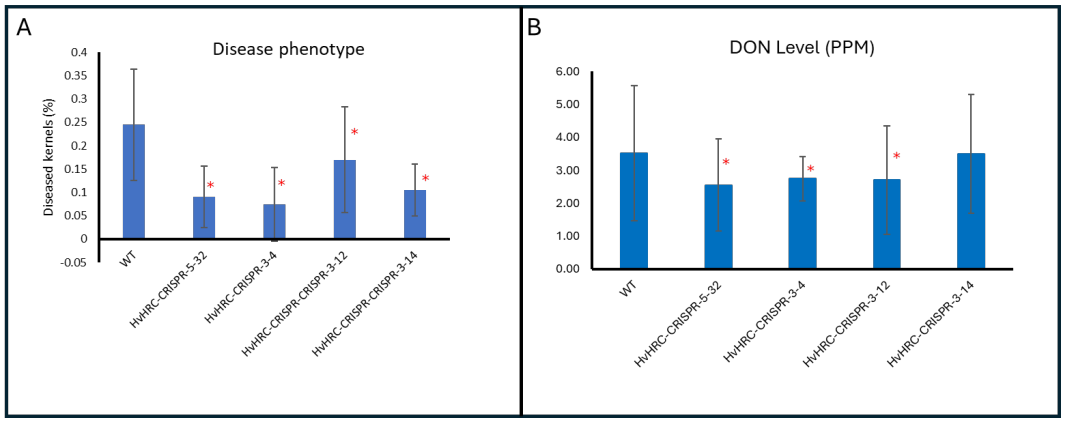

Figure 5. Phenotype analysis of *HvHRC*-edited lines. Percentage of diseased kernels (A) and DON levels (B) were significantly decreased in the CRISPR mutants in Bowman. Asterisk indicates the difference is significant ($P < 0.05$) in comparison with wild type (WT).

List key outcomes or other achievements.

1) We developed a stable protocol for barley transformation in Golden Promise and Bowman.

2) We improved barley transformation efficiency in recalcitrant genotypes, such as Conlon and Larker.
3) The barley ortholog of *FHB1* (*HvHRC*) in Golden Promise and Bowman was knocked out.
4) Homozygous mutant plants in Bowman and Conlon were obtained.
5) Preliminary field test using four independent knockout lines indicated that *Hvhrc* loss-of-function mutation enhanced FHB resistance, and three of the lines exhibited lower DON levels than those in WT.

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

One postdoc and one Ph. D. student 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) Improve transformation efficiency with barley development genes.
3) Include more lines for field trials in the nursey.
4) Combine *FHB7* with *FHB1* in barley to boost barley FHB resistance.