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Analysis of a Series of Mutants Highlights Complex Regulation of Fusarium Head Blight Resistance Conferred by *Fhb1* Locus in Wheat

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Fusarium head blight (FHB), which is mainly caused by *Fusarium graminearum*, is one of the devastating wheat diseases that threaten global wheat production (Rawat et al. 2016; Li et al. 2019; Su et al. 2019; Wang et al. 2020). Improvement of FHB resistance has become an urgent need for securing worldwide wheat production. *Fhb1*, a quantitative trait locus located on chromosome 3BS and exploited in Chinese variety *cv* Sumai 3 (SM3), provides the most stable and major effect on FHB resistance in wheat. *Fhb1*-mediated FHB resistance is reported to be conferred by either a pore-forming toxin-like (PFT) gene *TaPFT* (Rawat et al. 2016) or a putative histidine-rich calcium-binding protein (TaHRC or TaHis, hereafter referred as TaHRC) encoding gene (Li et al. 2019; Su et al. 2019). At the *Fhb1* locus, *TaPFT*, which only has one homoeolog on chromosome 3BS, was suggested to confer wheat FHB resistance (Rawat et al. 2016). Later, *TaHRC* was identified to be the key determinant of resistance to FHB at the *Fhb1* locus by two different laboratories (Li et al. 2019; Su et al. 2019). However, the two groups reached contrasting conclusions regarding this gene's function. This discrepancy may hinder further efforts in using the *Fhb1* locus for improving FHB resistance and thus necessitates further investigation. Besides, given that common wheat is a hexaploid with three subgenomes (AABBDD), the roles of the other two homoeologs, *TaHRC-3A* and *TaHRC-3D*, in FHB resistance or susceptibility remain unclear.

To clarify the roles of *TaPFT* and *TaHRC* at the *Fhb1* locus in FHB resistance in the same genetic background of SM3, we first generated a series of mutant lines including *Tapft*, *Tahrc-3B*, *Tapft*/*Tahrc-3B* double mutant lines, and different mutant lines of three *TaHRC* homoeologs in SM3, respectively. *TaPFT* was a single-copy gene in SM3 (Rawat et al. 2016), and we designed a guide RNA (gRNA) targeting its first exon (Figure 1A). Furthermore, of the three *TaHRC* homoeologs in SM3, *TaHRC-3B* is 42-bp longer than *TaHRC-3A* and *TaHRC-3D* at the N-terminus of the encoding region due to a rare deletion spanning the start codon, which was supposed to result in either gain of function (Li et al. 2019) or loss of function of *TaHRC* (Su et al. 2019) and thus increased FHB resistance (Figure S1). Therefore, we designed a *TaHRC-3B* specific target sequence (Target 1) in this region, which was also used together with the target of *TaPFT* to simultaneously edit both genes. (Figure 1A,B; Figure S1). Moreover, we designed another gRNA (Target 2) targeting the conserved region for simultaneously editing of the three *TaHRC* homoeologs (Figure 1A; Figure S1; Table S1). We successfully obtained two *Tapft*, two *Tahrc-3B*, and two *Tapft*/*Tahrc-3B* mutant lines, and seven null mutant lines of *TaHRC* homoeologs including single, double, or triple mutant lines in SM3, respectively (Table S2). Following segregation, we generated a series of transgene-free homozygous mutants in T_1 progenies derived from the T_0 mutant lines (Table S3). The editing profiles of the representative lines were indicated in Figure 1C.

Lei Yan and Jinfu Tian have contribute equally to this work.

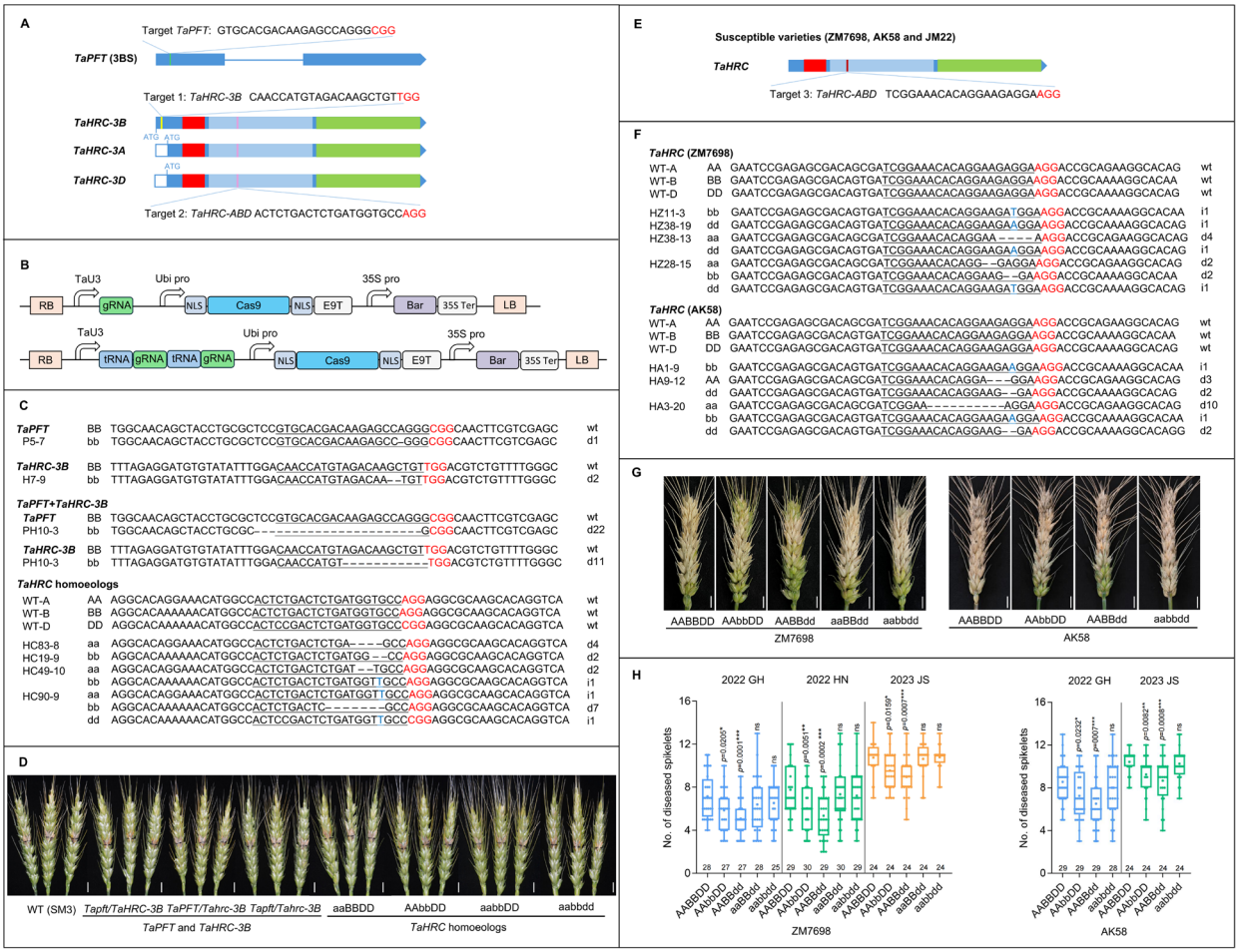

FIGURE 1 | Systemically evaluating the roles of *TaPFT* and *TaHRC* homoeologs at *Fhb1* locus in wheat FHB resistance through genome editing. (A) The gene structures and target sequences of *TaPFT, TaHRC-3B* (Target 1) and *TaHRC* homeologs (Target 2) in SM3. Exons are shown as blue boxes. Target sites are shown and PAM sites (5′-NGG-3′) are highlighted in red. The red, light blue and green boxes represent the CC, IDR1 and IDR2 regions, respectively (He et al. 2024). (B) Schematics of the linearized CRISPR/Cas9 constructs for editing of *TaPFT, TaHRC-3B, TaHRC* homoeologs and both *TaPFT* and *TaHRC*, respectively. (C) The representative sequences of homozygous lines of *Tapft, Tahrc-3B* and *Tapft/Tahrc-3B* single and double mutants, as well as different mutants of *TaHRC* homoeologs detected in the T_1 generation of SM3. Target sites are underlined. PAM sites (5′-NGG-3′) are highlighted in red. Insertions are highlighted in blue. Each base deleted was represented as "–". "wt", wild type; "d", deletion; "i", insertion. (D) Disease symptoms in spikes of *Tapft, Tahrc-3B* and *Tapft/Tahrc-3B* double mutants, as well as different mutants of *TaHRC* homoeologs of SM3 at 21 dpi. The scale bar represents 1 cm. (E) The location of the target sequence (Target 3) of *TaHRC* homoeologs in three FHB-susceptible elite Chinese wheat varieties ZM7698, AK58 and JM22, respectively. (F) The representative sequences of homozygous lines of *TaHRC* homoeologs detected in the T_1 generation of ZM7698 and AK58, respectively. (G) Disease symptoms in spikes of different *Tahrc* null mutant lines of ZM7698 and AK58 at 21 dpi. The scale bar represents 1 cm. (H) The numbers of diseased spikelets per head in wild type and different *Tahrc* mutant lines of ZM7698 at 21 dpi in the greenhouse (GH) in 2022, in the field in Henan (HN) in 2022 and in the field in Jiangsu (JS) in 2023, and of AK58 in the greenhouse (GH) in 2022 and in the field in Jiangsu (JS) in 2023, respectively. The number below each box indicates the numbers of inoculated spikes in each lines. Box-and-whisker plots show the medians, upper and lower quartiles (box edges) of the data points, and 1.5× the interquartile range (whiskers). *, ** and *** indicate the significance at the 0.05, 0.01 and 0.001 levels, respectively (Student's *t* test). ns, indicates not significant.

To evaluate the FHB resistance, the transgene-free homozygous lines were grown in two different environments, with the wheat spikes inoculated by *Fusarium* and examined for pathogen spread at 21 days post inoculation (dpi). The evaluations in both the greenhouse and the field showed that all the *Tapft*, *Tahrc-3B*, and *Tapft/Tahrc-3B* null mutant lines exhibited high FHB resistance similar to the wild type (WT) SM3 (Figure 1D and S2), indicating that *TaPFT* at the *Fhb1* locus is not necessary for FHB resistance, consistent with the recent report by Shi et al. (2025). Furthermore, the seven mutant lines of *TaHRC* homoeologs, including one *aaBBDD*, two *AAbbDD*, two *aabbDD*, and two *aabbdd* lines generated by Target 2 (Figure 1C; Table S3), also showed no significant differences in FHB resistance compared to WT SM3 (Figure 1D; Figure S2), implying that the loss of function of *TaHRC-3B* due to a naturally occurred causal mutation may account for increased FHB resistance in SM3 (Su et al. 2019).

To investigate whether genome editing of *TaHRC* may be employed to enhance the FHB resistance in FHB-susceptible wheat varieties, we generated a series of mutant lines of three *TaHRC* homoeologs in three varieties including Zhengmai 7698 (ZM7698), Aikang 58 (AK58) and Jimai 22 (JM22), respectively, by designing another common gRNA (Target 3) (Figure 1E;

Figure S1). In total, we identified six, four and five independent T_0 *Tahrc* mutant lines in ZM7698, AK58 and JM22, respectively (Table S2). Following segregation in T_1 progenies, we obtained different types of transgene-free *Tahrc* mutant lines, including two *AAbbDD*, two *AABBdd*, one *aaBBdd* and two *aabbdd* lines in ZM7698, and one *AAbbDD*, one *AABBdd* and two *aabbdd* lines in AK58, and two *AAbbDD*, one *AABBdd* and two *aabbdd* lines in JM22, respectively (Figure 1F; Figure S3; Table S3).

We then evaluated the performances of these *Tahrc* mutant lines of different varieties in FHB resistance in different environments. For the *Tahrc* mutant lines of ZM7698, albeit not high FHB resistance as observed in SM3, the numbers of diseased spikelets of the two single mutant lines, *AAbbDD* and *AABBdd*, were significantly decreased compared to that of the ZM7698 WT across three environments, indicating that both *AAbbDD* and *AABBdd* lines exhibited improved FHB resistance (Figure 1G,H). Notably, the *AABBdd* lines showed further decline of diseased spikelets compared to *AAbbDD* lines (Figure 1G,H). However, we also observed that the double (*aaBBdd*) and triple (*aabbdd*) mutant lines of ZM7698 did not show significant differences in the number of diseased spikelets compared to WT ZM7698 (Figure 1G,H). Similar to the above results, the *AAbbDD* and *AABBdd* lines of AK58 also exhibited significant decreases in diseased spikelets relative to WT control, and the *AABBdd* line showed superior FHB resistance over the *AAbbDD* line across two environments (Figure 1G,H). These results reinforce that knocking out *TaHRC-3D* could more potently enhance FHB resistance. It is worth noting that the expression levels of *TaHRC-3B* and *TaHRC-3D*, especially *TaHRC-3D*, in ZM7698 and AK58, are much higher than those of *TaHRC-3A* in wheat spikelets at different time points after inoculation with *F. graminearum*, respectively, suggesting that both of them, especially *TaHRC-3D*, may play more crucial roles in FHB susceptibility (Figure S4). Similar to ZM7698, simultaneous knockout of all three *TaHRC* homoeologs in AK58 did not give rise to FHB resistance either (Figure 1G,H). Therefore, we speculate that the regulation of FHB resistance by *TaHRC* is very complex and only certain null mutations of specific homoeologs in some genetic backgrounds, such as *Tahrc-3B* in SM3, the *Tahrc-3B* and *Tahrc-3D* mutants in ZM7698 and AK58 backgrounds demonstrated in this study, as well as *Tahrc-3B* in wheat variety *cv* Bobwhite in previous studies (Su et al. 2019; Chen et al. 2022), could confer improved FHB resistance. In addition, the mutations of *TaHRC* homeologs had no negative effects on major agronomic traits in ZM7698 and AK58 (Figures S5–S8).

Unexpectedly, we also observed that the *Tahrc* mutant lines of JM22, including *AAbbDD*, *AABBdd* and *aabbdd*, did not exhibit significant differences in the number of diseased spikelets compared to WT JM22 (Figure S9), implying that different genes/networks may be involved in wheat FHB susceptibility or the existence of inhibitors in different genetic backgrounds (Zheng et al. 2022). This adds another layer of complexity to the regulation of FHB resistance conferred by the *Fhb1* locus in wheat.

In summary, through systematically and rigorously evaluating a relatively large number of edited null mutants, we here demonstrate that *Tahrc-3B* rather than *TaPFT* is responsible for *Fhb1*-mediated resistance to FHB in SM3. Compared to *Tahrc-3B*, *Tahrc-3D* increases FHB resistance in the tested two elite varieties, providing a practical and valuable strategy for bolstering FHB resistance in some elite wheat cultivars through genome editing. Overall, our work uncovers high complexity underlying the regulation of FHB resistance conferred by *Fhb1* locus, whose exact nature and mode of action thus need to be explored in future, and may benefit from the different sets of null mutant lines generated in our work.

Author Contributions

L.X. conceived the project. L.Y., J.T., J.L., S.L., C.Z., and Y.H. performed the experiments. L.Y. and J.T. wrote the manuscript. L.X. revised the manuscript. All the authors read the final version of this manuscript.

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Data Availability Statement

The data that supports the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** pbi70318-sup-0001-DataS1.docx.