CRISPR/Cas9mediated restoration of Tamyb10 to create pre harvest sproutingresistant red wheat

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Brief Communication

CRISPR/Cas9-mediated restoration of *Tamyb10* to create pre-harvest sprouting-resistant red wheat

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Wheat pre-harvest sprouting (PHS) reduces yield and grain quality and occurs in almost every wheat-growing region around the world (Vetch *et al.*, 2019). In general, red-grained wheat varieties are more tolerant to PHS than white-grained varieties (Himi *et al.*, 2011). Moreover, the red pigment of the grain coat contains proanthocyanidins, whose antioxidant activity and free radical scavenging ability have health-promoting properties. Therefore, producing elite red wheat varieties is an important objective in breeding high-yield and high-quality wheat.

R2R3-MYB is one of the largest families of transcription factors in plants, which play crucial roles in regulating plant development, metabolism, and stress responses. The R2R3-MYB transcription factor Tamyb10 of hexaploid wheat activates flavonoid biosynthesis genes to specify red grain colour and influences PHS (Himi *et al.*, 2011). In most white wheat varieties, the *Tamyb10-A1a*, *Tamyb10-B1a*, and *Tamyb10-D1a* genes have large insertions or deletions, which disrupt the IRTKAL/IRC motif and regulatory function (Himi *et al.*, 2011). Among the *Tamyb10* genes, the *Tamyb10-B1a* allele has a 19-bp deletion in nearly 88.6% of bread wheat lines; this deletion causes a frameshift in the open-reading frame and disrupts the resulting protein (Dong *et al.*, 2015; Himi *et al.*, 2011). Given that CRISPR/Cas9-induced mutations are typically +1/−1-bp indels at the specific target sites (Zhang *et al.*, 2014, 2016), we can revert the frameshift mutations within the *Tamyb10-B1a* allele (caused by a 19-bp deletion) to in-frame mutations (18-bp or other multiples of three bases).

Here, we first sequenced the *Tamyb10-B1a* locus in the spring wheat cultivar Fielder and confirmed the 19-bp deletion in the third exon of *Tamyb10-B1a* in this white wheat variety (Figure 1a; Figure S1). To restore functionality of the *Tamyb10-B1a* allele, a single gRNA was designed to target the sequences flanking the 19-bp deletion site (Figure 1a; Appendix S1). Then, the *pWMB110-SpCas9-sgRNA* was constructed and transformed into Fielder via *Agrobacterium*-mediated transformation (Wang *et al.*, 2022). Fifteen putative transgenic plants were produced, from which five *Tamyb10-B1a*-edited plants were selected (Figure 1b). Among the T_0 edited lines, four plants with a 1-bp insertion upstream of the 19-bp deletion site were identified. These 1-bp insertions restored the reading frame by converting the frameshift mutation in the *Tamyb10-B1a* allele (19-bp deletion) to in-frame mutation (18-bp deletion; Figure 1b). Furthermore, no mutation events were found across all potential off-target sites (Table S1).

To investigate whether the target mutations observed were heritable, we tracked the inheritance of in-frame *Tamyb10-B1a* variants (NF243-3, NF243-12, and NF243-15). The CRISPR/Cas9-induced mutations in the T_0 plants were stably transmitted to the T_1 generation without the occurrence of new mutations (Figure 1c; Table S2). Sequence alignment analysis showed that, compared with the wild-type Tamyb10-B1b protein, the protein sequences of the in-frame *Tamyb10-B1a* variants carried a minor deletion of six amino acids, thereby restoring the intact IRTKAL/IRC motif (Figure 1d; Figure S2). Homozygous mutants with the in-frame *Tamyb10-B1a* sequence were selected to investigate grain pericarp and PHS resistance phenotypes.

As expected, the coloration of the grains harvested from T_1 lines harbouring the in-frame *Tamyb10-B1a* variants changed from white to red (Figure 1e). The accumulation of anthocyanins in these lines was assessed using a spectral imaging chamber. Compared with the wild-type Fielder seeds, the in-frame *Tamyb10-B1a* lines displayed an elevated anthocyanin index (Figure 1e),

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suggesting that restoring the *Tamyb10-B1a* allele caused accumulation of anthocyanidins in wheat grains. Furthermore, the main anthocyanin and proanthocyanidin content in in-frame *Tamyb10-B1a* seeds was higher than in wild-type seeds (Figure 1f; Figure S3). Our RNA-seq and qRT-PCR data showed that the expression of flavonoid biosynthetic genes increased in the in-frame *Tamyb10-B1a* lines compared with the wild type (Figure 1g,h; Table S3). The expression levels of *Tamyb10-B1a* were

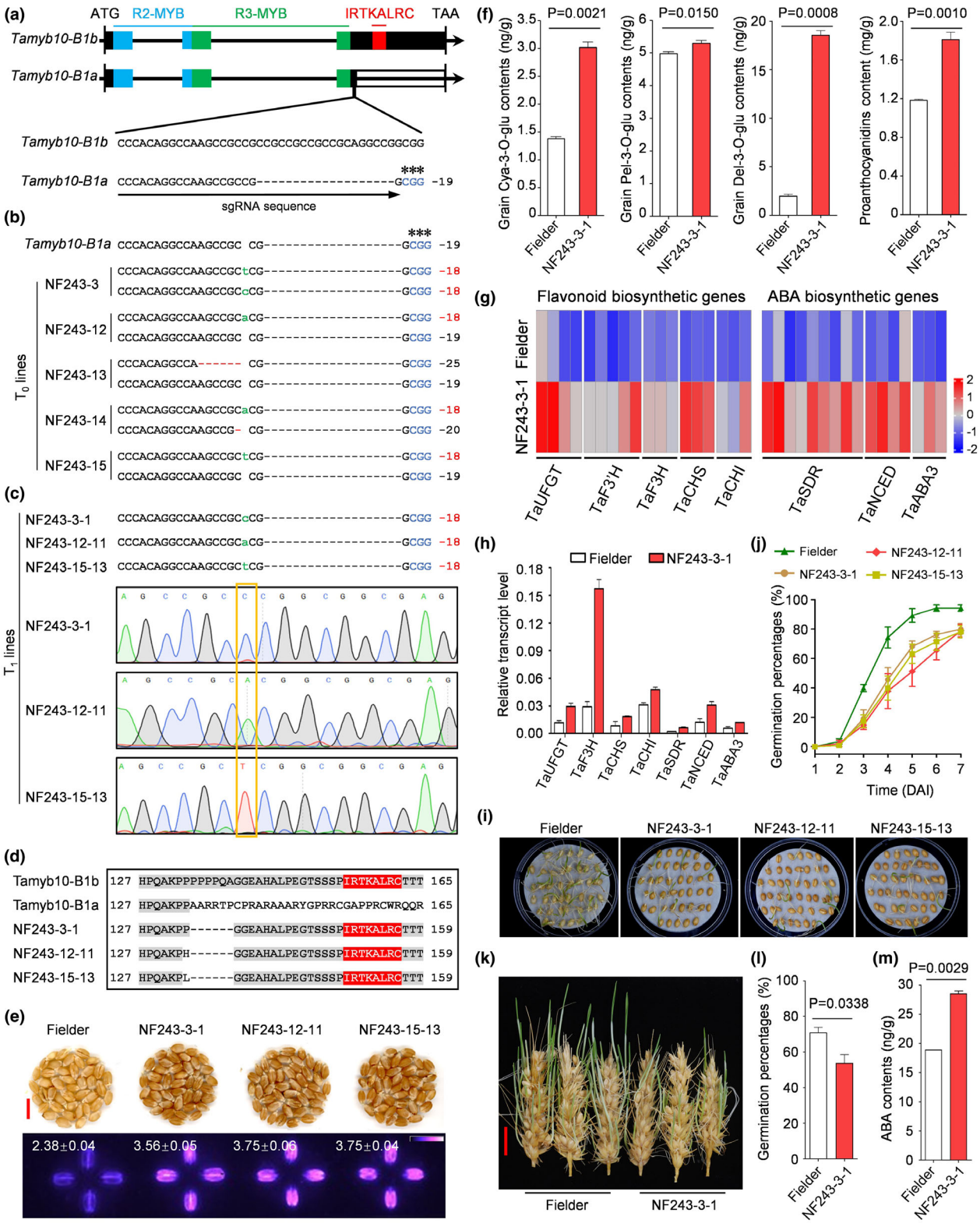

Figure 1 Development of elite PHS-resistant red wheat through CRISPR/Cas9-mediated functional recovery of the *Tamyb10-B1a* allele. (a) Gene structures of *Tamyb10-B1b* in red wheat and *Tamyb10-B1a* in white wheat. The 19-bp deletion and protospacer adjacent motif (PAM) in *Tamyb10-B1a* are indicated by black dashes and asterisks, respectively. (b) Mutations in T_0 plants harbouring in-frame and frameshift *Tamyb10-B1a* variants. Numbers on the right indicate deletion length compared with *Tamyb10-B1b*. (c) Genetic analysis and corresponding sequencing chromatograms of in-frame T_1 *Tamyb10-B1a* mutants. (d) Multiple sequence alignment of the deduced IRTKAL/IRC motifs of *Tamyb10-B1b*, *Tamyb10-B1a*, and in-frame *Tamyb10-B1a* variants. (e) Red pigment of grains (upper panel) and pseudo-colour images of anthocyanins in grains (lower panel) of the in-frame *Tamyb10-B1a* mutants and wild-type Fielder. The numerical values in pseudo-colour images represent the anthocyanin index. Scale bar, 1 cm. (f) Contents of three main anthocyanidin types and proanthocyanidins in the in-frame *Tamyb10-B1a* mutant and Fielder. (g, h) Transcript levels of genes in the flavonoid and ABA biosynthetic pathways were determined by RNA-seq and qRT-PCR. (i, j) The germination phenotypes and percentages of the in-frame *Tamyb10-B1a* mutants and Fielder. DAI, days after imbibition. (k) Germination performance in whole spikes of the in-frame *Tamyb10-B1a* variants and Fielder. Scale bar, 2.5 cm. (l) Germination percentages in whole spikes were calculated for the in-frame *Tamyb10-B1a* mutant and Fielder 10 days after imbibition. (m) Measurement of ABA content in Fielder and in-frame *Tamyb10-B1a* seeds. Data are presented as mean ± SD.

not affected after editing (Figure S4). These findings confirmed that *Tamyb10-B1a* allele function was successfully restored.

Tamyb10, a gene with pleiotropic effects, is also associated with PHS tolerance of grains (Lang *et al*., 2021). Our data revealed that seed germination in the in-frame *Tamyb10-B1a* lines was delayed compared with wild-type threshed seeds (Figure 1i). The germination percentage (GP) of threshed seeds in the in-frame *Tamyb10-B1a* lines was significantly reduced about 16.18%–32.63% at 5–7 days after imbibition compared with wild-type seeds (Figure 1j). Likewise, the GP of in-frame *Tamyb10-B1a* mutant seeds also decreased by 24.23% in whole spikes 10 days after imbibition compared with the wild type (Figure 1k,l).

The abscisic acid (ABA) content is closely related to PHS tolerance. Therefore, we quantified the levels of endogenous ABA in wild-type and *Tamyb10-B1a* variant seeds. As shown in Figure 1m, ABA levels were 51.4% higher in seeds in the in-frame *Tamyb10-B1a* lines compared to the wild type. In addition, both the RNA-seq and qRT-PCR results showed that the expression of ABA biosynthetic genes increased in the in-frame *Tamyb10-B1a* mutants (Figure 1g,h; Table S3). Furthermore, greater TTC staining was seen in Fielder seeds than in the in-frame *Tamyb10-B1a* mutant seeds, suggesting that seed coat permeability was reduced in the variants (Figure S5).

In summary, we successfully converted a white wheat variety into a red one and improved its PHS tolerance through CRISPR/Cas9-mediated functional restoration of the *Tamyb10-B1a* allele. It is worth noting that aside from red grains and elevated PHS resistance, the in-frame *Tamyb10-B1a* variants did not differ from the wild type in other major agronomic traits (Figure S6). In addition, the phenotypes of the edited in-frame *Tamyb10-B1a* variants were stable after the *Cas9* transgene was segregated out (Figure S7). Compared with targeted gene knock-in by homology-directed genome editing, our strategy efficiently recovers gene function without donor DNA and would greatly accelerate the breeding of new red-grained and PHS-resistant wheat varieties with elite agronomic traits. The present study also presents an alternative strategy for crop improvement by editing genes that were lost through domestication via the restoration of reading frames.

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Conflict of interest

The authors have not declared a conflict of interest.

Author contributions

C.-J.Z. and Y.Z. designed the study; Y.Z., Y.L., Y.F., Y.W., P.L., J.X., Y.H., and K.W. performed the experiments; Y.Z., Y.L., S.C., X.Y., F.W., J.G., J.-K.Z., K.W., and C.-J.Z. analysed the data; Y.Z., Y.L., K.W., and C.-J.Z. wrote the article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 - S7 Supplementary Figures.
Table S1 - S4 Supplementary Tables.
Appendix S1 Methods.