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A TaKNOX1–TaAPO1–Rht1 feedback regulatory module orchestrates spikelet number and yield potential in wheat

Dear Editors,

The Green Revolution dramatically improved lodging resistance and harvest index in wheat through the widespread adoption of dwarfing alleles such as *Rht-B1b* and *Rht-D1b*. However, subsequent productivity gains have relied primarily on agronomic intensification, particularly increased spike density per unit area, a strategy that requires heavy nitrogen fertilizer use and has caused substantial environmental costs (Wu et al., 2020; Liu et al., 2022; Zhang et al., 2025). Achieving sustainable crop production therefore requires enhancing the intrinsic yield capacity of individual spikes, notably by increasing spikelet number per spike (SPS). Although genes such as *ABERRANT PANICLE ORGANIZATION 1* (*APO1*) and *FRIZZY PANICLE* are known to regulate meristem and inflorescence architecture in grasses (Huang et al., 2021; Li et al., 2021), knowledge of the genetic and molecular mechanisms controlling SPS in wheat remains limited (Zhang et al., 2023).

Here, we performed a genome-wide association study of wheat SPS across two environments using 3,816,482 high-quality SNPs. We identified 84 significant SNPs ($-\log_{10}P \geq 6$), of which 96.4% were located on chromosome 4B (Figure 1A and Supplemental Figure 1A–1E; Supplemental Table 1). Linkage disequilibrium analysis revealed that four significant SNPs, chr4B:49,408,745, chr4B:50,114,749, chr4B:50,310,339, and chr4B:51,281,956, formed a linkage disequilibrium block spanning 1.87 Mb (Figures 1B and 1C). This block contained 12 genes, of which only *TraesCS4B03G0124400* and *TraesCS4B03G0126000* harbored nonsynonymous SNPs in their coding sequences; the remaining 10 genes lacked such polymorphisms (Supplemental Table 2). Of these two genes, *TraesCS4B03G0124400* expression was undetectable, whereas *TraesCS4B03G0126000* was highly expressed in stems and young spikes (Figure 1D and Supplemental Figure 1F; Waddington et al., 1983). Moreover, *TraesCS4B03G0126000* belonged to the class I KNOTTED1-LIKE HOMEOBOX (KNOX) transcription factor family, which is known to regulate plant meristem maintenance (Matsuoka et al., 1995; Chen et al., 2022; Byrne et al., 2024). The coding-region SNP in *TraesCS4B03G0126000 (*chr4B:51,281,956) explained 16.30% of the SPS variance in the population. Thus, we prioritized *TraesCS4B03G0126000* as the probable candidate underlying natural variation in SPS and named it *TaKNOX-B1*.

We found that the putative causal SNP was a G-to-A transition in the second exon, resulting in an amino acid substitution from leucine to phenylalanine at position 84 ($Leu^{84}Phe$; L84F). Structural modeling predicted that this substitution enabled a new interaction with Phe^{169} in the second KNOX domain (K2), most likely altering protein conformation (Figure 1E and Supplemental Figure 2). Haplotype analysis confirmed that accessions carrying the derived *TaKNOX-B1-Hap2* allele (Phe^{84}) exhibited significantly higher SPS than those carrying the ancestral *Hap1* allele (Leu^{84}) (Figure 1E; Supplemental Table 3). Fixation index (F_{ST}) and nucleotide diversity (π) analyses comparing landraces and modern cultivars showed that the genomic region harboring *TaKNOX-B1* had undergone significant selection (Supplemental Figure 3). Hap2 frequency increased substantially among accessions released in modern breeding eras, indicating positive breeding selection (Figure 1F). *In situ* hybridization showed that *TaKNOX-B1* was specifically expressed in spikelet meristems at the glume primordium present stage (W3) (Supplemental Figure 4). The L84F substitution co-segregated with SPS and grain number per spike (GNS) in $F_{2:3}$ lines, supporting a functional association between this allele and spikelet/grain number (Supplemental Figure 5). The genetic function of *TaKNOX-B1* was confirmed using the ethyl methane sulfonate (EMS) mutants of the tetraploid wheat cv. Kronos and the Chinese common wheat cv. Jing411 (Supplemental Figures 6–8). CRISPR–Cas9 editing of *TaKNOX-B1* in the Chinese cv. KN199 further confirmed its role in positively regulating SPS, GNS, and plant height in a dose-dependent manner (Figures 1G and 1H and Supplemental Figures 9 and 10). Micro-synteny and gene structure analyses highlighted the genomic conservation of *TaKNOX1* orthologous regions across grass species (Supplemental Figure 11).

Given that the *TaKNOX1* mutation reduced plant height, a phenotype reminiscent of *Rht-1* dwarfing alleles, we examined whether TaKNOX1 functionally interacted with Rht-B1b, the Green Revolution dwarfing allele. *In vitro* protein–protein interaction assays revealed that Rht-B1b physically associated with TaKNOX-B1 but not with the TaKNOX-$B1^{Phe84}$ variant. We found that both the K1 and K2 domains of the TaKNOX-B1 protein were indispensable for this interaction, as deletion of either domain abolished binding. TaKNOX-B1 also interacted with Rht-D1b, whereas TaKNOX-$B1^{Phe84}$ did not (Supplemental Figures 12–14). The protein interaction was confirmed *in planta* by split-firefly luciferase complementation and co-immunoprecipitation assays (Figures 1I and 1J). Importantly, the transcriptional activity of TaKNOX-B1 was strongly repressed by Rht-B1b, whereas the TaKNOX-$B1^{Phe84}$ variant was refractory to such repression (Supplemental Figure 12), supporting Rht-B1b as a direct inhibitor of TaKNOX-B1 transactivation.

To identify downstream targets of TaKNOX1, we conducted transcriptome profiling of W3-stage spikes from wild-type plants and *TaKNOX1* mutants. Principal component analysis clearly separated

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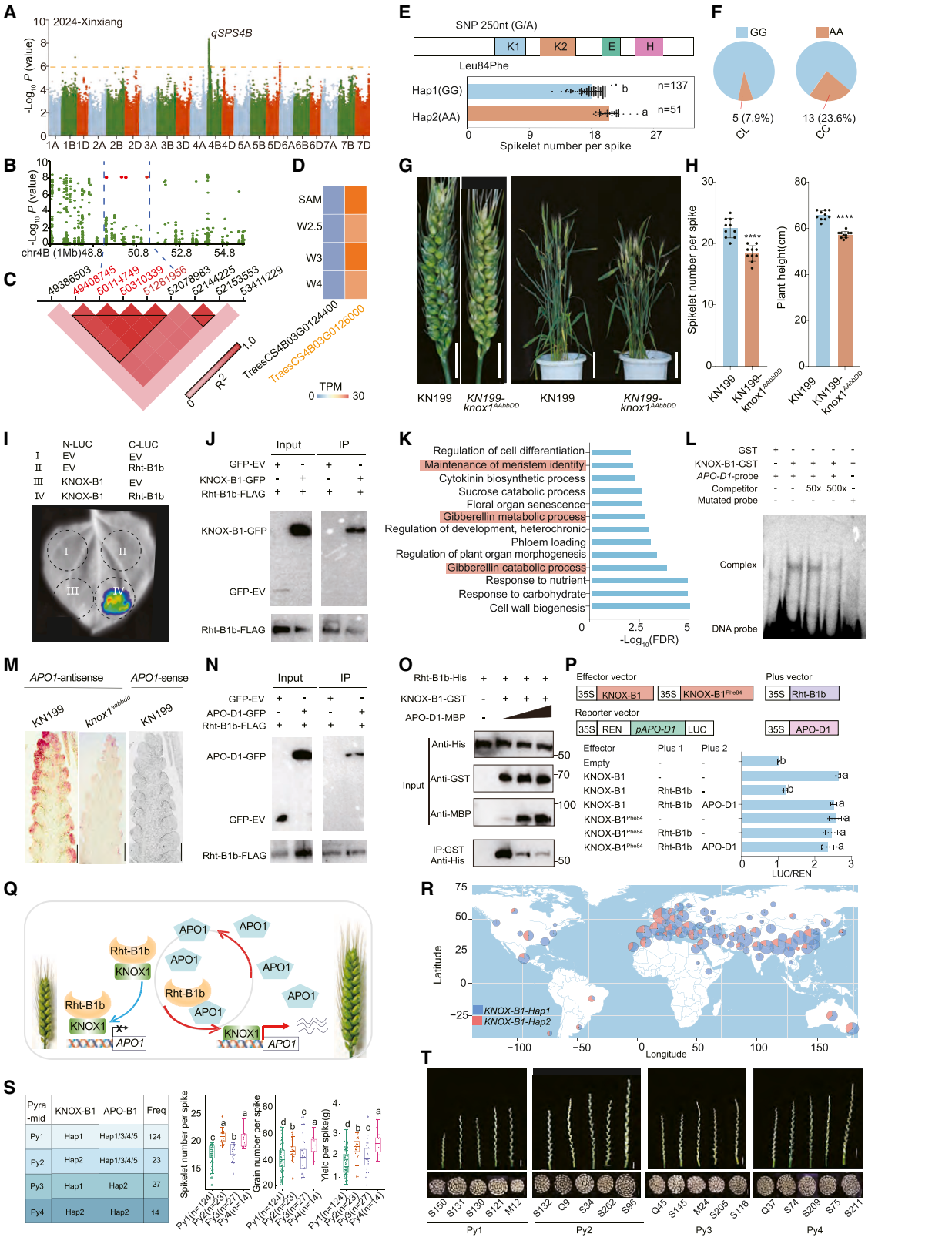

Figure 1. The TaKNOX1-TaAPO1-Rht1 feedback module enhances grain number and yield potential in wheat

(A) Genome-wide association study (GWAS) analysis best linear unbiased estimates (BLUEs) for spikelet number per spike (SPS) in 188 wheat accessions grown in Xinxiang in 2024. The dotted line indicates the significance threshold of $-\log_{10} P = 6$.

(legend continued on next page)

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the transcriptional profiles of the two genotypes (Supplemental Figures 15A and 15B). Gene Ontology enrichment of genes downregulated in the mutants highlighted terms related to "maintenance of meristem identity" and "gibberellin metabolic process," suggesting that TaKNOX1 affects meristem maintenance and gibberellin (GA) metabolism (Figure 1K and Supplemental Figure 15C; Supplemental Tables 4–7). Notably, the downregulated genes included the F-box gene *TaAPO1* (Supplemental Figure 15D), which is known to have a conserved role in regulating inflorescence meristem activity and spikelet number in cereals (Kuzay et al., 2019; Huang et al., 2021; Hake et al., 2022; Wittern et al., 2022; Jiang et al., 2026). *TaAPO1* exhibited spikelet-meristem-specific expression that closely mirrored that of *TaKNOX1*. Mutation of *TaAPO1* similarly reduced SPS and plant height in a dose-dependent manner (Supplemental Figures 16–18), and transcriptomic analysis of *TaAPO1* mutants showed downregulation of GA pathway genes, suggesting that the two genes may operate in the same pathway (Supplemental Figure 19; Supplemental Tables 8–11). Indeed, yeast one-hybrid and electrophoretic mobility shift assays demonstrated direct binding of TaKNOX-B1 to the canonical TGAC motif in the *TaAPO1-D1* promoter (Figure 1L and Supplemental Figure 20A). Subsequent *in vitro* and *in planta* assays confirmed that TaKNOX-B1 directly activated *TaAPO1-D1* expression (Figure 1M and Supplemental Figures 20B and 20C). Moreover, multiple TGAC motifs were found in the promoters of *TaAPO1-D1* and its homologs, and these motifs were conserved across Poaceae, suggesting that KNOX1–APO1 regulatory relationships may be conserved in grass species (Supplemental Figure 21).

We found that the TaAPO1-D1 protein also interacted with Rht-B1b through multiple independent assays (Figure 1N and Supplemental Figure 22). Strikingly, TaAPO1 competed with TaKNOX1 for binding to Rht-B1b, as the presence of TaAPO1 weakened the TaKNOX-B1–Rht-B1b interaction (Figure 1O). Consistently, Rht-B1b repressed TaKNOX-B1-mediated activation of *TaAPO1-D1* expression, and this repression was alleviated when TaAPO1-D1 was co-expressed with Rht-B1b (Figure 1P). In contrast, the transcriptional activity of the TaKNOX-B1^{Phe84} variant remained high in the presence of either Rht-B1b or TaAPO1-D1, indicating that the mutation-induced conformational change in TaKNOX-B1 altered its protein-interaction properties. Together, these findings support a feedback loop in which TaKNOX1 activates *TaAPO1* expression; the resulting TaAPO1 competes with TaKNOX1 for Rht-B1b binding, thereby liberating TaKNOX1 from repression, which in turn promotes *TaAPO1* expression (Figure 1Q). This positive regulatory circuit enhances the activities of both TaKNOX1 and TaAPO1, ultimately increasing SPS and grain yield.

Haplotype analysis showed that *TaKNOX-B1-Hap2*, which carries the Phe^{84} substitution, was an elite allele associated with significantly higher GNS, thousand-grain weight, and yield per spike (Supplemental Figure 23; Supplemental Table 12). Its increased frequency during breeding (Figure 1F) and global prevalence (Figure 1R) further support its agronomic value. Similarly, *TaAPO-B1-Hap2* was associated with superior agronomic performance. This effect may be attributable, at least in part, to a D143N mutation that is predicted to stabilize the TaAPO1 three-dimensional structure by forming a novel

(B and C) Manhattan plot and linkage disequilibrium (LD) heatmap of the genomic region surrounding the associated peak on chromosome 4B for SPS. Blue dashed lines indicate the candidate genomic region in the regional association plot, and the zoomed-in region shows the positions of significant SNPs, with chr4B:51,281,956 highlighted in red. Black dashed lines indicate the candidate genomic region in the LD heatmap.
(D) Expression patterns during the spikelet meristem formation stage (SAM-W4) of two genes carrying nonsynonymous exonic SNPs.
(E) Protein domain structure (top) and comparison of SPS between accessions carrying *TaKNOX-B1-Hap2* and *TaKNOX-B1-Hap1* (bottom). K1, KNOX1 domain; K2, KNOX2 domain; E, ELK domain; H, homeobox domain. Data were analyzed using Duncan's multiple range test.
(F) Pie charts showing the number and percentage of the two SNP haplotypes (GG and AA) in wheat accessions. CL, Chinese landraces; CC, Chinese cultivars.
(G and H) Spike and plant height phenotypes of *TaKNOX-B1* CRISPR–Cas9-edited mutant plants **(G)** and corresponding quantification **(H)**. Scale bars, 1 cm for spikes and 10 cm for plant height. Data are mean ± SEM (n = 10) and were analyzed using two-tailed Student's t-test.
(I) Split firefly luciferase complementation (SFLC) assay showing the interaction between N-terminal fragment of Luciferase-tagged TaKNOX-B1 and C-terminal fragment of Luciferase-tagged Rht-B1b in tobacco leaves.
(J) Co-immunoprecipitation (Co-IP) assays of FLAG-Rht-B1b and GFP-TaKNOX-B1 after immunoprecipitation with anti-FLAG magnetic beads.
(K) Gene Ontology (GO) enrichment analysis of genes downregulated in *taknox1* mutants compared with the wild type. P values were adjusted by the Benjamini–Hochberg correction, and only significant categories (false discovery rate [FDR] < 0.05) are displayed. GO terms related to gibberellin metabolism are highlighted.
(L) Electrophoretic mobility shift assay (EMSA) showing binding of TaKNOX-B1 to probes from the *TaAPO-D1* promoter. Competition with the labeled probes was tested by adding excess of unlabeled probes as indicated.
(M) *In situ* hybridization showing *TaAPO-D1* expression in spikelet meristems and its loss in *TaKNOX1* mutants (middle panel).
(N) Co-immunoprecipitation assays of FLAG-Rht-B1b and GFP-TaAPO-D1 after immunoprecipitation with anti-FLAG magnetic beads.
(O) GST pull-down assay using TaKNOX-B1-GST as bait and Rht-B1b-His as prey. The addition of increasing amounts of TaAPO-D1-MBP (triangle) reduced the amount of Rht-B1b-His recovered with TaKNOX-B1-GST, indicating that TaAPO-D1 competes with Rht-B1b for binding to TaKNOX-B1.
(P) Transient transactivation assay assessing the activity of the TaKNOX-B1–Rht-B1b complex on the *TaAPO-D1* promoter in the presence or absence of TaAPO-D1. Renilla luciferase (REN) was used as an internal control, and relative firefly luciferase (LUC)/REN ratios are shown. Data are the mean ± SEM (n = 6). Statistical analyses were performed using Duncan's multiple range test. Shared lowercase letters indicate no significant difference (p > 0.05).
(Q) Proposed feedback model for the TaKNOX1–TaAPO1–Rht-B1b regulatory pathway.
(R) Global distribution of *TaKNOX-B1-Hap1/2* in a worldwide collection of 1,641 wheat accessions from SNP Hub (https://wheat.cau.edu.cn/WheatUnion).
(S) Comparison of SPS, GNS, thousand-grain weight (TGW), and yield per spike (YPS) between accessions carrying different haplotype combinations of *TaKNOX-B1* and *TaAPO-B1*. Data are the mean ± SEM, with different letters denoting significant differences (p < 0.05) according to Duncan's multiple range test. Py, pyramided haplotypes.
(T) Spike morphology and YPS of representative accessions carrying various combined *TaKNOX-B1* and *TaAPO-B1* haplotypes. Scale bar, 1 cm.

hydrogen bond with S152. Consistent with this possibility, TaAPO1 carrying D143N showed stronger interaction with Rht-B1b in yeast two-hybrid assays (Supplemental Figure 24; Supplemental Table 13). Moreover, accessions carrying both elite haplotypes of *TaKNOX-B1* and *TaAPO-B1* showed additional yield benefits, outperforming those carrying only a single elite haplotype (Figures 1S and 1T; Supplemental Table 14).

Together, these findings establish that, in wheat, the conserved inflorescence regulator APO1 (TaAPO1) is transcriptionally activated by the KNOX1-family transcription factor TaKNOX1. Importantly, both proteins physically interact with the mutant DELLA protein Rht-B1b and appear to compete for this interaction. This tripartite interaction expands the previously reported unidirectional DELLA–KNOX repression module (Su et al., 2021) into a self-reinforcing positive feedback loop that may amplify the output of both TaKNOX1 and TaAPO1 during spikelet development. This circuit is supported at the genetic level by the synergistic yield increase observed when elite haplotypes of the two genes were combined. Our findings therefore identify *TaKNOX1* and *TaAPO1* as promising targets for marker-assisted selection and genome editing aimed at designing wheat varieties with improved spike architecture and yield potential. Future work should elucidate the full scope of the TaKNOX1 regulatory network, evaluate the stability and epistatic interactions of the TaKNOX1–TaAPO1–Rht-B1b module across diverse genetic and environmental backgrounds, and functionally validate key SNPs via precise prime-editing replacement. It will also be important to determine whether the corresponding elite alleles carry trade-offs in traits such as nutrient-use efficiency or stress resilience. Addressing these questions will strengthen the predictive power of this pathway and guide the rational design of next-generation wheat ideotypes that combine high yield with enhanced robustness.

DATA AND CODE AVAILABILITY

RNA-seq data generated in this study have been deposited in the National Genomics Data Center, China National Center for Bioinformation (https://ngdc.cncb.ac.cn) under accession number PRJCA053990. The gene IDs used in this article (see https://wheatgenome.org/projects/reference-genome-project/refseq-v2.1) are as follows: *TaAPO-A1* (*TraesCS7A03G1166400*), *TaAPO-B1* (*TraesCS7B03G1034000*), *TaAPO-D1* (*TraesCS7D03G1112600*), *TaKNOX-A1* (*TraesCS4A03G0671200*), *TaKNOX-B1* (*TraesCS4B03G0126000*), *TaKNOX-D1* (*TraesCS4D03G0106600*), *Rht-B1b* (*TraesCS4B03G0093100*), and *Rht-D1b* (*TraesCS4D03G0067100*).

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AUTHOR CONTRIBUTIONS

L.M., A.L., X.F., and S.G. designed the research, analyzed the data, and supervised the project. Z.D., Q.C., S.L., and W.L. developed the genetic populations and materials. Z.D., Q.C., S.W., W.L., and S.L. performed the experiments. Y.C. performed the genome-wide association study and transcriptome analysis. W.X. performed the protein structure analysis. Z.D., Q.C., S.L., W.L., D.C., X.Z., G.C., Z.W., T.C., T.L., Z.Z., and Z.Y. performed the field trials in Beijing. D.M. L., X.L., D.L., S.G., A.L., L.M., and X.F. drafted and edited the manuscript.

SUPPLEMENTAL INFORMATION

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