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GSK3 regulates VRN1 to control flowering time in wheat[FA]

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The precise control of flowering time is important for the regional adaptability and productivity of many crops. Various categories of genes related to flowering have been isolated and characterized functionally in wheat (*Triticum aestivum*) in response to vernalization to adjust flowering initiation. Before vernalization, the inhibitory histone modification H3K27me3 is enriched in the promoter and the first intron of the vernalization gene *VRN1*. In addition, GLYCINE-RICH RNA-BINDING PROTEIN 2 (TaGRP2) binds directly o the "critical region" of the first intron of *VRN1*; the P2 motif of the *VRN1* promoter and the I4 motif in its first intron form a loop structure, and together restrict the transcription of *VRN1*. During vernalization, the inhibitory H3K27me3 modification of *VRN1* is converted into the active H3K4me3 modification, and phosphorylated VERNALIZATION-RELATED 2 (VER2) enters the nucleus from the cytoplasm and interacts directly with the *O*-GlcNAcylation of TaGRP2 (a process likely to have been mediated by TaOGT1), relieving the inhibition of *VRN1* transcription by TaGRP2. Furthermore, *VRN1* produces the long noncoding RNA *VAS*, which recruits the TaRF2b–TaRF2a heterodimer to bind directly to the Sp1 motif of the *VRN1* promoter, enhancing its transcription. In addition, VRN1 can form a complex with TaVrt2 that binds directly to the CArG box of *VRN1* to increase *VRN1* expression (Xiao et al., 2022). In addition, the brassinosteroid (BR) signaling pathway is known to influence flowering time, as many BR-deficient and -insensitive mutants exhibit late flowering (Domagalska et al., 2007; Li et al., 2010; Chen et al., 2020), but the molecular mechanism underlying BR-mediated flowering remains largely unclear. Here, we demonstrate that GLYCOGEN SYNTHASE KINASE 3 (GSK3) physically interacts with VRN1 and regulates its accumulation to mediate flowering in wheat.

We recently reported that the gain-of-function mutant of *gsk3* harbors a "G" to "A" nucleotide substitution in *GSK3* (*TraesCS3D02G137200*), resulting in an amino acid change (E286K) in the conserved TREE motif. This mutant displays a dwarf phenotype associated with the Green Revolution gene *Rht-B1b* (Dong et al., 2023). Moreover, a mutation of *TaSG-D1*, which is the same gene as *GSK3*, from Indian dwarf wheat (*Triticum sphaerococcum*) causes a round-grain phenotype (Cheng et al., 2020). In this study, we observed that the *gsk3* mutant shows late heading and flowering (Figure 1A–C), as mentioned in a previous report (Cheng et al., 2020). Our earlier transcriptome analysis (Yang et al., 2021) showed that *GSK3* is highly expressed in the shoot apex and leaves of wheat variety YZ4110 at the W2.0 and W3.5 stages (Figure S1). This result raises the interesting question of how GSK3 regulates the heading and flowering dates of wheat.

To further explore the late heading and late flowering of *gsk3*, we measured *GSK3* transcripts and examined the phenotypes of transgenic wheat lines harboring *GSK3* and its mutant form of *gsk3* on the winter wheat cultivar Kenong199 (KN199) background. The *pUbi::GSK3* transgenic lines with different *GSK3* transcript levels displayed similar heading and flowering times to the transgenic null lines (TNLs), whereas the *pUbi::gsk3* transgenic lines with different *gsk3* transcript levels showed a 2-d delay in heading and a 5-d delay in flowering time (Figure S2), indicating that the "E286K" substitution in GSK3 has important effects on flowering.

To explore the role of GSK3 in wheat flowering in more detail, we analyzed the subcellular localization of GSK3 and gsk3. Both localized to the nucleus and cytoplasm (Figure S3), indicating that the "E286K" substitution in GSK3 does not affect its localization. Generally, GSK3 functions by phosphorylating its downstream substrates and influencing their stability, localization, and activity (Li et al., 2021), prompting the question whether GSK3 regulates flowering by affecting the stability of proteins related to the flowering pathway. Hence, we chose several proteins in the flowering pathway in wheat to screen for new GSK3-interacting proteins through luciferase complementation imaging (LCI) assays in *Nicotiana benthamiana* leaves. GSK3 strongly interacted with VRN1-5A (Figure 1D), VRN1-5B (Figure S4A), and VRN1-5D (Figure S4B), the key regulators of wheat flowering in the vernalization pathway (Yan et al., 2003). Furthermore, bimolecular fluorescence complementation (BiFC) (Figure S5A), pull-down (Figure S5B), and co-immunoprecipitation assays (Figure 1E) revealed that GSK3 interacts with VRN1-5A *in vitro* and *in vivo*.

We tested whether VRN1 is a substrate of GSK3 phosphorylation by performing *in vitro* VRN1 phosphorylation assays using equivalent amounts of GSK3 or gsk3 proteins. Indeed, VRN1 was phosphorylated by both GSK3 and gsk3 (Figure S6), confirming that the "E286K" substitution in GSK3 enhances its stability, but does not affect its activity (Cheng et al., 2020; Dong et al., 2023). We investigated whether GSK3 regulates the transcript abundance of *VRN1* via qRT-PCR. The mRNA levels of *VRN1-5A* were not significantly different between *gsk3* and YZ4110 (Figure S7A) or among the TNL, *pUbi::GSK3*, and *pUbi::gsk3* transgenic lines (Figure S7B).

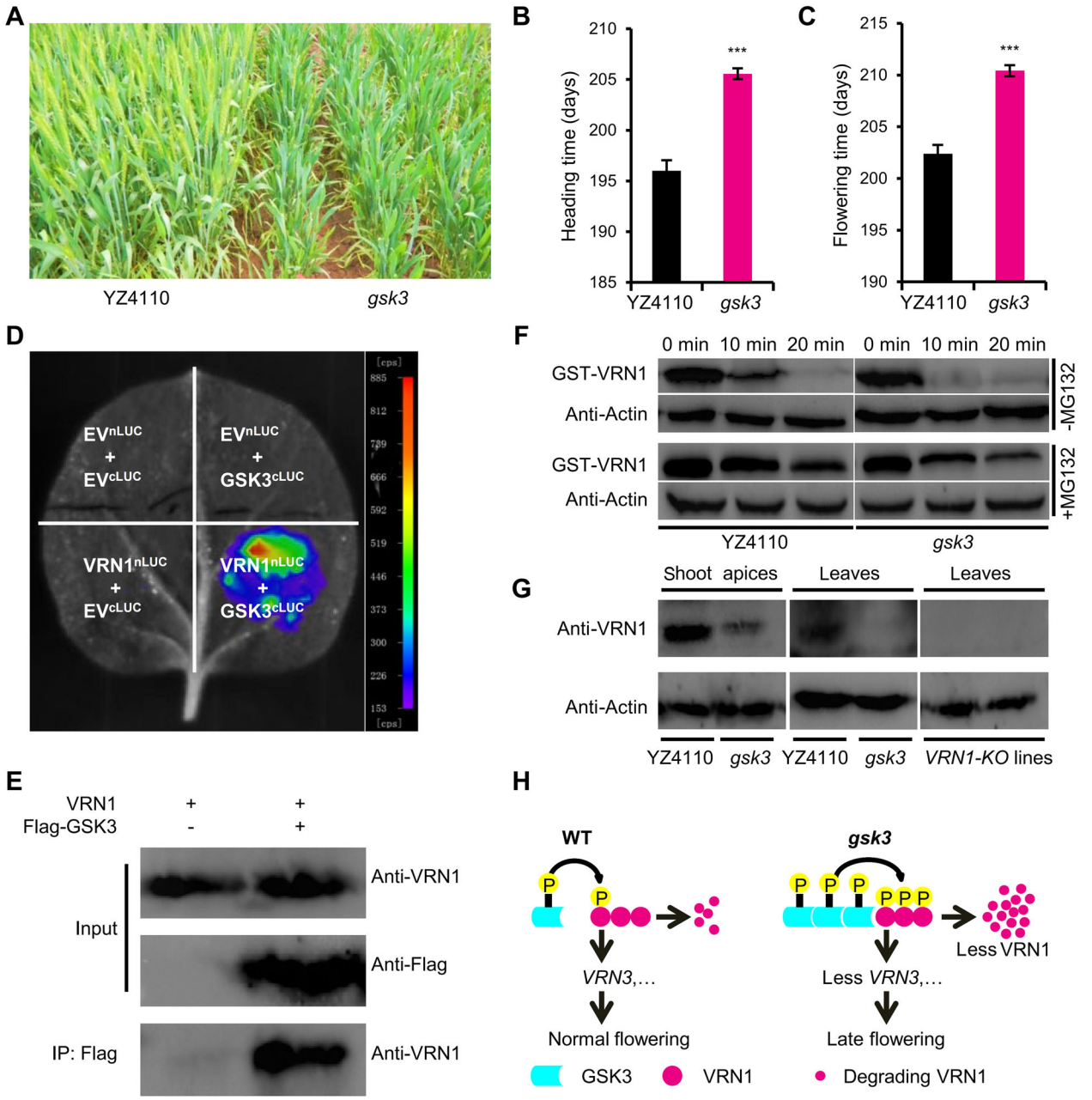

Figure 1. GSK3 regulates heading date and flowering date in wheat

(A) Morphology of wild-type (WT; YZ4110) and *gsk3* plants at the heading stage. **(B, C)** Means ± *SD* ($n = 10$) for the heading and flowering times of YZ4110 and *gsk3* are shown. ***$P < 0.001$ (two-tailed Student's *t* test). **(D)** LCI assay. **(E)** Co-immunoprecipitation assay. **(F)** The stability of VRN1-5A-GST in cell-free wild-type (YZ4110) and *gsk3* plant extracts grown with or without 50 μM of the proteasome inhibitor MG132. **(G)** Immunoblotting to detect the accumulation of VRN1 in the shoot apices and leaves of YZ4110 and *gsk3* during the late double ridge stage. The specificity of the anti-VRN1 antibody was validated using leaves of T_5 generation *VRN1-KO* plants. Anti-actin antibody served as an internal reference in **(F)** and **(G)**. **(H)** Proposed model for the roles of GSK3 and VRN1 in regulating flowering time in wheat.

We examined VRN1 protein accumulation in YZ4110 and *gsk3* by transforming YZ4110 and *gsk3* leaf protoplasts with *35 S::VRN1-5A-GFP*, *35 S::VRN1-5B-GFP*, and *35 S::VRN1-5D-GFP*. VRN1 protein levels were higher in YZ4110 than in *gsk3* (Figure S8), suggesting that GSK3 may affect the stability of VRN1. To further test whether GSK3 regulates VRN1 protein abundance, we performed a cell-free protein degradation assay to monitor the contents of GST-VRN1 in the absence or presence of the 26S proteasome inhibitor MG132. In the absence of MG132, the rate of GST-VRN1 degradation was much slower in YZ4110 versus *gsk3* protein extracts, whereas in the presence of MG132, the abundance of GST-VRN1 was comparable in YZ4110 and *gsk3* protein extracts (Figure 1F). Furthermore, we compared VRN1

protein abundance in the shoot apices and leaves of YZ4110 and *gsk3* at the late double ridge stage via immunoblotting using an anti-VRN1 polyclonal antibody. The VRN1 protein showed reduced abundance in *gsk3* compared with YZ4110 and was not detectable in the *VRN1-KO* lines generated by CRISPR/Cas9, which displayed severely late heading and flowering (Figures 1G, S9). These results indicated that the over-accumulated gsk3 protein readily promotes the degradation of VRN1 in the gain-of-function *gsk3* mutant.

As VRN1 activates the expression of the floral pathway integrator gene, *VRN3*, encoding a RAF kinase inhibitor similar to FLOWERING LOCUS T (FT) in *Arabidopsis thaliana* (Yan et al., 2006), we measured the expression of *VRN3*. As expected, *VRN3* expression was significantly reduced in *gsk3*, which has lower VRN1 protein levels (Figure S10A). We also analyzed *VRN3* expression in the TNL, *pUbi::GSK3*, and *pUbi::gsk3* transgenic lines and determined that *VRN3* expression was significantly reduced in *pUbi::gsk3* lines (Figure S10B), which is consistent with their flowering phenotypes. These results support the notion that the decreased VRN1 protein abundance in *gsk3* downregulates *VRN3* expression, leading to late flowering.

Taken together, our data revealed that GSK3 physically interacts with and phosphorylates VRN1 to regulate flowering date in wheat. Based on the current and previously published results, we propose that the gain-of-function mutant protein gsk3 over-accumulates compared with GSK3 (Dong et al., 2023) and promotes the degradation of VRN1, resulting in the late flowering phenotype of *gsk3* (Figure 1H). Our study provides direct evidence that GSK3, the core kinase in the BR signaling pathway, regulates flowering in wheat by modulating the protein stability of VRN1.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

X.L., X.K., and G.C. designed the experiments. G.C., D.L., L. Z., and C.X. performed the experiments. G.C. and X.K. wrote the manuscript. All authors read and approved the paper.

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SUPPORTING INFORMATION

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Figure S1. Relative abundance of *GSK3* transcripts
Figure S2. Morphological analysis of TNL, *pUbi::GSK3*, and *pUbi::gsk3* transgenic plants
Figure S3 Subcellular localization of GSK3 and gsk3 in leaf protoplasts of YZ4110
Figure S4 GSK3 interacts with VRN1-5B **(A)** and VRN1-5D **(B)** in LCI assays
Figure S5. GSK3 interacts with VRN1-5A in BiFC (**A**) and pull-down (**B**) assays
Figure S6. GSK3 phosphorylates VRN1-5A *in vitro*
Figure S7. Analysis of *VRN1-5A* transcript abundance via qRT-PCR
Figure S8. VRN1-5A-GFP, VRN1-5B-GFP, and VRN1-5D-GFP accumulation in YZ4110 and *gsk3* leaf protoplasts
Figure S9 Genotypes and phenotypes of *VRN1-KO* lines
Figure S10. Relative abundance of *VRN3* transcripts
Table S1. Primers used in this study