LEAFY regulates spikelet number per spike and floret development in wheat

By

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THESIS

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## DEDICATION

First, to the wonderful man who is my husband. He supported me when I first decided to go back to school at a community college. Cheered me on when I transferred to a university. And wasn't surprised, years later, when I decided I wanted a graduate degree. During our twenty years together, I worked full time, studied part time, was blessed with two miracle babies, and built a beautiful life with him by my side. It all would not have been possible without his patience and encouragement. Thank you, Mark. I love you.

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## ABSTRACT

In wheat, the transition of the inflorescence meristem to a terminal spikelet (IM>TS) determines the spikelet number per spike (SNS), an important grain yield component. In this study, we demonstrate that the plant-specific transcription factor LEAFY (LFY) physically and genetically interacts with WHEAT ORTHOLOG OF APO1 (WAPO1) and that both genes act cooperatively to increase SNS. Loss-of-function mutations in either or both genes result in significant and similar reductions in SNS. We also show that this trait is modulated by significant genetic interactions between *LFY* and the *SQUAMOSA* MADS-box genes *VRN1* and *FUL2*, which promote the IM>TS transition. Single-molecule fluorescence *in-situ* hybridization revealed a down-regulation of *LFY* and up-regulation of the *SQUAMOSA* MADS-box genes in the distal part of the developing spike during the IM>TS transition, supporting the opposite roles of these genes in the regulation of SNS in wheat. This transition coincides with the partial overlap of *LFY* and *WAPO1* transcription domains in the most developed spikelets. Understanding the genetic network regulating SNS is a necessary first step to engineer this important agronomic trait.

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## INTRODUCTION

Every year, trillions of wheat spikes mature worldwide, carrying the grains that provide one-fifth of the calories and proteins consumed by the human population (FAOSTAT, 2017). Therefore, increasing the maximum number of grains produced by each spike can contribute to the need to increase wheat productivity to feed a growing human population.

Wheat spikes, as other grass inflorescences, are comprised of specialized reproductive organs called spikelets, which are short indeterminate branches. Each spikelet has two proximal sterile bracts (glumes) followed by a variable number of florets. Individual florets include a lemma, which is also a bract, subtending the floral organs (palea, two lodicules, three stamens and a pistil) (Preston *et al.*, 2009; Debernardi *et al.*, 2020a). The wheat inflorescence meristem (IM) produces multiple lateral spikelet meristems (SM) in a distichous order before transitioning to a terminal spikelet. This transition determines the spikelet number per spike (SNS) and the maximum number of grains that can be formed in the spike. Therefore, the regulation of the timing of the transition of the IM into a terminal spikelet (henceforth, IM>TS) is critical for determining the maximum potential number of grains per spike.

The timing of the IM>TS transition in wheat is affected by multiple environmental conditions including drought, salt stress, heat, and reduced nutrients, all of which result in reduced SNS (Frank and Bauer, 1982; Frank *et al.*, 1987; Maas and Grieve, 1990). However, differences in SNS also have a strong genetic component, with broad sense heritability ranging from $H^2$ = 0.84 in irrigated fields to $H^2$ = 0.59 in water-stressed environments (Zhang *et al.*, 2018). This high heritability has facilitated the identification of several wheat genes involved in

the regulation of SNS. *VERNALIZATION1*(*VRN1*), *FRUITFULL2* (*FUL2*), and *FUL3*, the wheat homologs of Arabidopsis *SQUAMOSA* MADS-box genes *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*) and *FUL*, were found to be essential for spikelet development and for the regulation of the IM>TS transition (Li *et al.*, 2019). Loss-of-function mutations in *vrn1* or *ful2* result in normal plants with significant increases in SNS. However, in the *vrn1 ful2* double mutant, the IM remains indeterminate, and lateral spikelets are converted into tiller-like organs with vestigial floral organs. These vestigial floral organs disappear in the *vrn1 ful2 ful3* higher-order mutant, in which spikelets revert to vegetative tillers subtended by leaves (Li *et al.*, 2019).

Genes that regulate *VRN1* expression have been shown to affect SNS. *FT1*, the wheat homolog of Arabidopsis florigen *FLOWERING LOCUS T* (*FT*), binds directly to the *VRN1* promoter as part of a floral activation complex, and functions as a transcriptional activator (Li and Dubcovsky, 2008, Li *et al.*, 2015). Mutants (or knock-down transgenic plants) of *FT1* (Lv *et al.*, 2014) or its closest paralog *FT2* (Shaw *et al.*, 2019) show reduced or delayed expression of *VRN1*, which is associated with significant increases in SNS. In contrast, overexpression of these genes results in a precocious IM>TS transition and spikes with very few spikelets (Lv *et al.*, 2014, Shaw *et al.*, 2019). Mutations in *PPD1* that reduce or delay *FT1* expression result in SNS increases (Shaw *et al.*, 2013), whereas mutations in *ELF3* that result in the upregulation of *FT1* and *VRN1* expression reduce SNS (Alvarez *et al.*, 2016). *bZIPC1* encodes a protein that physically interacts with FT2, and its mutants also show a large decrease in SNS (Glenn *et al.*, 2023).

However, the underpinning mechanism by which the recently cloned gene *WHEAT ORTHOLOG OF APO1* (*WAPO1*) (Kuzay *et al.*, 2019, Kuzay *et al.*, 2022) regulates SNS has not yet been elucidated. *WAPO1* is orthologous to the *Oryza sativa* (rice) gene *ABERRANT PANICLE*

*ORGANIZATION1* (*APO1*), and to the Arabidopsis gene *UNUSUAL FLORAL ORGANS* (*UFO*), which are both involved in floral development (Levin and Meyerowitz, 1995, Ikeda *et al.*, 2007, Rieu *et al.*, 2023a). In addition to floral defects, loss-of-function mutations in *WAPO1* or *APO1* result in significant reductions in SNS in wheat (Kuzay *et al.*, 2022) and in the number of branches in the rice panicle (Ikeda *et al.*, 2005), respectively. These results indicate that both *WAPO1* and *APO1* prevent a precocious transition of the IM into a terminal spikelet.

In Arabidopsis, UFO physically interacts with the plant-specific transcription factor LEAFY (LFY) (Lee *et al.*, 1997, Chae *et al.*, 2008, Rieu *et al.*, 2023b), and the interaction is conserved between the rice homologs APO1 and APO2 (Ikeda-Kawakatsu *et al.*, 2012). The Arabidopsis LFY protein activates class-A MADS-box genes *AP1* (Parcy *et al.*, 1998, Wagner *et al.*, 1999) and *CAL* (William *et al.*, 2004), which are homologous to the wheat *VRN1* and *FUL2* genes. Since *VRN1*, *FUL2* (Li *et al.*, 2019) and *WAPO1* (Kuzay *et al.*, 2022) are all involved in the regulation of SNS, we investigated the role of *LFY* on wheat spike development.

In this study, we demonstrate that LFY physically interacts with WAPO1 and that plants carrying loss-of-function mutations in either or both genes result in similar floral abnormalities and similar reductions in SNS. We also show significant genetic interactions for SNS between *LFY* and the meristem identity gene *VRN1*, which together with its closest paralog *FUL2* promote the IM>TS transition. Finally, we use single-molecule fluorescence *in-situ* hybridization (smFISH) to visualize the spatio-temporal expression profiles of these genes and other floral genes. These studies reveal a ten-fold increase in the ratio between the *SQUAMOSA* MADS-box genes (*VRN1 + FUL2*) and *LFY* in the distal part of the spike at the time of the IM>TS transition, supporting the opposite role of these genes in the regulation of SNS.

## RESULTS

### Induced loss-of-function mutations in *LFY* reduce SNS and alter floral morphology

Using our sequenced Kronos mutant population (Krasileva *et al.*, 2017), we selected truncation mutations K2613 for *LFY-A* (henceforth *lfy-A*) and K350 for *LFY-B* (henceforth *lfy-B*). The *lfy-A* mutant has a G>A change in the acceptor splice site of the second intron, which results in mis-splicing of the third exon, a shift in the reading frame, and a premature stop codon (Fig. 1A). The *lfy-B* mutant has a premature stop codon at position 249 (Q249*) that truncates 37 % of the protein, including most of the conserved C-terminal domain (Maizel *et al.*, 2005) (Fig. 1A). Primers used to track these mutations are described in data S1. The mutants were backcrossed to Kronos to reduce background mutations and intercrossed with each other to select sister lines homozygous for the different mutation combinations, including the wildtype (WT), *lfy-A*, *lfy-B*, and the *lfy-A lfy-B* combined mutant, designated hereafter as *lfy*.

Comparisons between the homozygous sister lines in a growth chamber, revealed a highly significant decrease (37%, $P < 0.001$) in SNS in the combined *lfy* mutant relative to the wildtype (WT, Fig. 1B-C, data S2). Smaller but still significant decreases in SNS were detected for the single *lfy-A* (12%) and *lfy-B* (8%) mutants (Fig. S1, data S3), which indicates that modification of *LFY* gene dosage can be used to fine tune SNS in wheat. No significant differences in heading time or leaf number were detected between the combined *lfy* mutant and the WT (Fig. 1D-E, data S2), suggesting a limited effect of LFY on the timing of the

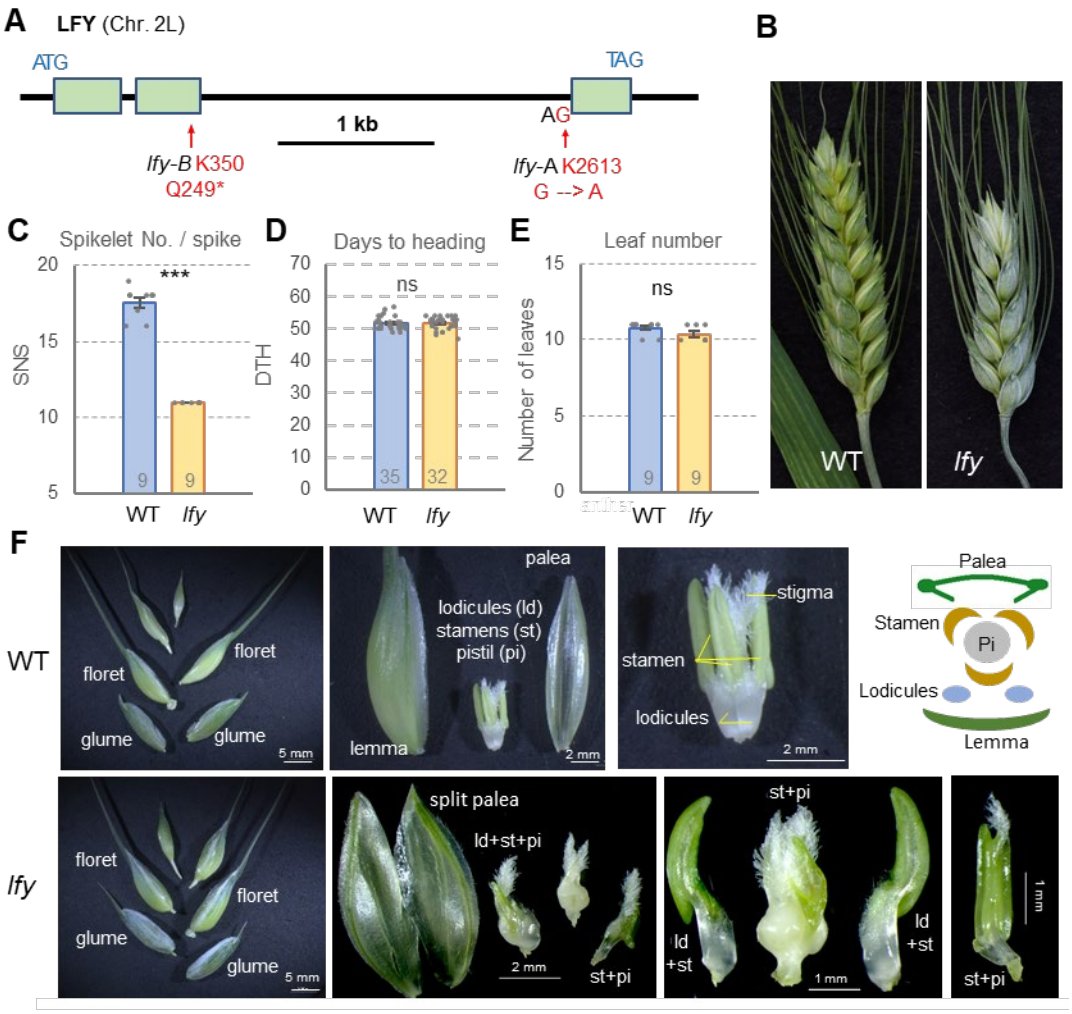


**Figure 1. Characterization of LFY loss-of-function mutants**. (A) LFY gene structure and location of selected mutations in *lfy-A* and *lfy-B*. (B) Representative wildtype (WT) and *lfy* combined mutant. Comparison between WT and *lfy* for (C) spikelet number per spike, (D) days to heading, and (E) leaf number. Numbers inside the bars indicate number of biological replicates (separate plants). (F) Top: wild type spikelet, floret and schema of a normal wheat floret. Bottom: most frequent floral abnormalities observed in the *lfy* mutant. Raw data for bar graphs C to E and frequencies for floral abnormalities in F are available in data S2.

transition between the vegetative and reproductive meristems.

In addition to its effects on SNS, the *lfy* mutant showed severe alterations in floral organs (Fig. 1F). We quantified the frequency of the defects in 27 first and 27 second florets

from spikelets located in the basal, central, and distal part of the spike (data S2). Glumes and lemmas were normal, but 18.5% of the paleas were split in two (Fig. 1F). Forty percent of the paleas were fused with lodicules or, less frequently, with stamens (data S2). Lodicules were also fused to stamens or membranous structures. The average number of normal stamens was reduced to 1.7 (data S2), and one fifth of the florets showed additional abnormal stamens and fusions with lodicules, membranous structures or pistils. Only 9% of the florets showed single pistils (mostly those with three normal anthers) and the rest showed more than one pistil and frequent homeotic conversions between stamens and pistils (Fig. 1F, data S2).

## Overexpression of *LFY* partially rescues the reduced SNS phenotype of the *lfy* mutant

To test if *LFY* function was sufficient to rescue the mutant phenotypes, we generated transgenic plants expressing the *LFY-A1* coding region driven by the constitutive maize *UBIQUITIN* promoter, either without a tag or fused to a C-terminal HA tag. Transgenic lines for five independent *UBI:LFY* events and five independent *UBI:LFY-HA* events, all showed significantly higher *LFY* transcript levels in the leaves than non-transgenic sister lines and wildtype Kronos, which showed no expression of endogenous *LFY* in this tissue (Fig. S2, data S4).

We then crossed both the *UBI:LFY* and *UBI:LFY-HA* transgenic plants with the *lfy* mutant, and in the progeny selected sister lines homozygous for combined *lfy* mutations or for wildtype alleles (WT), each with or without the transgenes. Among the plants without the transgene, the combined *lfy* mutants showed reduced SNS (6.6 spikelets, $P < 0.001$), as in previous experiments. No significant differences in SNS were observed between *UBI:LFY* and *UBI:LFY-HA*

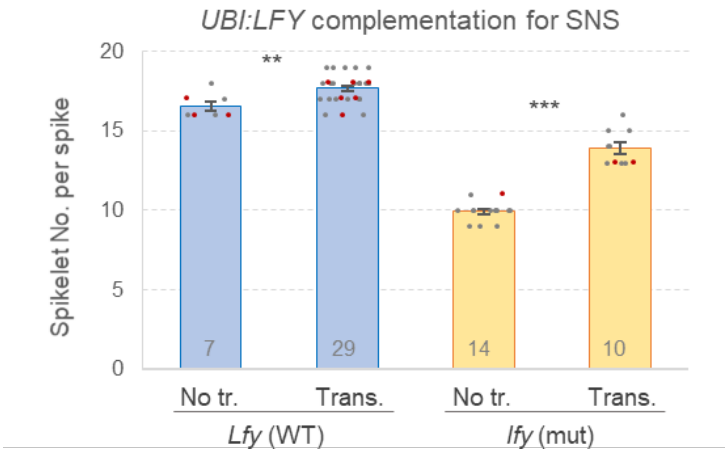


**Figure 2**. **Effect of *UBI:LFY* and *UBI:LFY-HA* on spikelet number per spike (SNS).** No significant differences were detected between *UBI:LFY* (grey dots) and *UBI:LFY-HA* (red dots) so data were analyzed together. The effect of the transgenes on SNS was evaluated in both the wildtype (WT) and *lfy* mutant backgrounds. Numbers within the bars indicate the number of independent biological replicates. *P* values correspond to two-tail *t*-tests between transgenic and non-transgenic (No tr.) lines in the same *LFY* background. ns = not significant, ** = $P < 0.01$, and *** = $P < 0.001$. Raw data are available in data S5.

plants, so data was combined for statistical analyses. In the presence of the wildtype *LFY* alleles, transgenic plants showed 1.1 more spikelets per spike than non-transgenic controls ($P = 0.0045$, Fig. 2, data S5). The effect was larger in the *lfy* mutant, where transgenic plants showed four more spikelets per spike than the controls ($P < 0.001$, Fig. 2).

The frequent floral abnormalities in the *lfy* mutant, combined with the reduced SNS, resulted in a low number of grains per plant (2.7 ± 0.7 grains per plant, data S5), which usually germinated well. The low fertility of the *lfy* mutants was partially restored by the presence of the transgene (23 ± 10 grains per plant, $P = 0.0003$) (data S5). However, the comparison of sister lines carrying the wildtype *Lfy* allele showed that the presence of the transgene was associated with reduced fertility (34 ± 7 grains per plant) relative to the wildtype (94 ± 25 grains

per plant, *P* = 0.019) (data S5). These results indicate that the ectopic expression of *LFY* under the *UBI* promoter partially complements the *lfy*-reduced SNS and fertility.

## Wheat LFY and WAPO1 show physical and genetic interactions

Since *LFY* and *WAPO1* mutants were both associated with similar reductions in SNS (Kuzay *et al.*, 2022), and their homologous proteins interact with each other in Arabidopsis (Chae *et al.*, 2008) and rice (Ikeda-Kawakatsu *et al.*, 2012), we tested the ability of LFY and WAPO1 proteins to interact physically with each other in wheat. We used co-immunoprecipitation (Co-IP) to test the interaction between LFY-A and two WAPO-A1 natural alleles that differ in the presence of a cysteine or a phenylalanine at position 47. The *WAPO-A1*-47F allele was previously associated with higher SNS than the *WAPO-A1*-47C allele (Kuzay *et al.*, 2022). We co-transformed wheat leaf protoplasts with *UBI:LFY-HA* combined with either *UBI:WAPO1-47C-MYC* or *UBI:WAPO1-47F-MYC*. After immunoprecipitation with anti-MYC beads, we detected LFY-HA using an anti-HA antibody in both the WAPO1-47C-MYC and WAPO1-47F-MYC precipitates (Fig. 3A). These results indicate that LFY can interact with either WAPO-A1 variant in wheat.

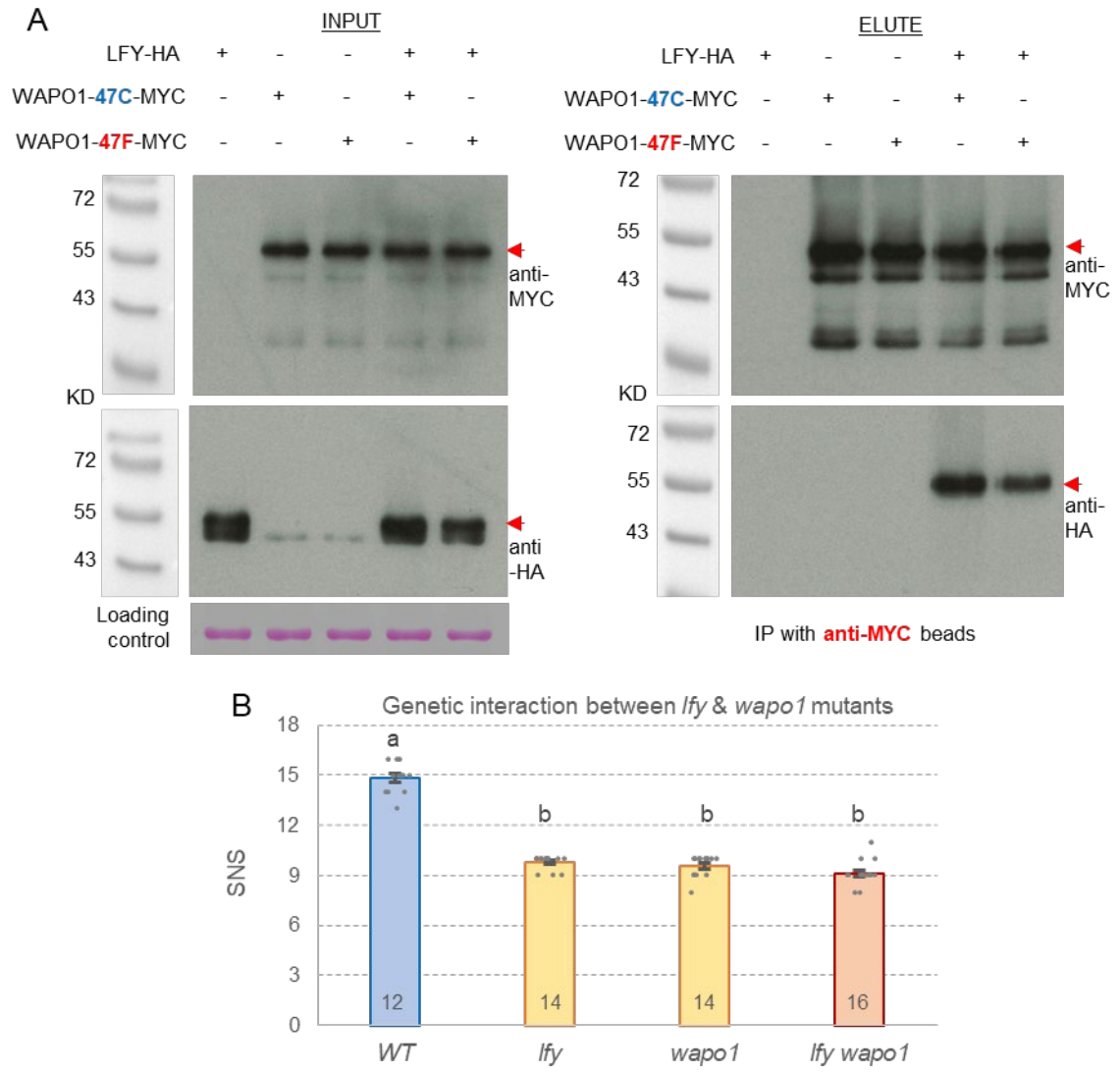


**Figure 3**. **Physical and genetic interactions between WAPO1 and LFY.** (**A**)The interaction between LFY and both WAPO1 alleles (47F and 47C) was tested in tetraploid wheat leaf protoplasts by co-immunoprecipitation (co-IP). (Left) Input and loading control. (Right) Elute showing LFY-HA signal using an anti-HA antibody in both the WAPO1-47C-MYC and WAPO1-47F-MYC precipitates with anti-MYC beads. (**B**) Genetic interaction between loss-of-function *lfy* and *wapo1* mutants. Different letters above the bars indicate significant differences in Tukey tests ($P < 0.05$), and numbers inside the bars the number of biological replications. Error bars are SEM. Raw data and statistics are available in data S6.

To test if the physical interaction between LFY and WAPO1 was reflected in a genetic interaction for SNS, we intercrossed the *lfy* and *wapo1* mutants. Due to the highly reduced fertility of the homozygous *lfy* mutants (data S5), we first selected lines homozygous for the *lfy-A* mutant allele and heterozygous for *lfy-B* among $F_2$ and $F_3$ progenies. We then screened a large $F_4$ segregating population and selected the four homozygous classes (WT, *lfy*, *wapo1*, and combined *lfy wapo1*) using molecular markers (primers in data S1). Plants homozygous for mutations in both homeologs of *LFY* (*lfy*) or *WAPO1* (*wapo1*) showed large and similar reductions in SNS relative to the wildtype (34% and 35% reduction, respectively, Fig. 3B). Interestingly, the combined *lfy wapo1* mutant showed a reduction of 38%, which was not significantly different from the reductions observed in the single mutants (Fig. 3B, data S6). The genetic interaction for SNS between *LFY* and *WAPO1* was highly significant in a factorial ANOVA (data S6). These results suggest that LFY and WAPO1 proteins are both required to regulate SNS and that they likely work cooperatively in the regulation of this trait.

## *LFY* and *WAPO1* show dynamic expression profiles during wheat spike development

A previous RNA-seq study including different tissues at different developmental stages in Chinese Spring (CS), detected *LFY* and *WAPO1* transcripts in developing spikes and elongating stems (Choulet *et al.*, 2014)(Fig. S3A). A separate RNA-seq study including five spike developmental stages in tetraploid wheat Kronos (VanGessel *et al.*, 2022), showed transcripts for both genes present at all five stages. *LFY* transcript levels were more abundant than those of *WAPO1*, and both genes showed lower transcript levels in the apical region at the vegetative stage than at the double-ridge to floret primordia stages (Fig. S3, data S7). These studies indicate that *LFY* and *WAPO1* are present at the same stages of spike development. To refine

the localization of *LFY* and *WAPO1* transcripts within the developing spike, we examined their dynamic spatial patterns using smFISH (Fig. 4). For all the smFISH studies, we only compared hybridization signals across developmental stages for individual genes because comparisons among genes are affected by probe sensitivity and can be misleading.

During the transition between the vegetative and reproductive phases (W1.5 in Waddington scale (Waddington *et al.*, 1983)), *LFY* transcripts were concentrated in bands radiating from the axis of the elongating shoot apical meristem (SAM) towards the lateral primordia (Fig. 4A). Only few *LFY* transcripts were detected at the tip of the IM at this or later spike developmental stages (Fig. 4C-D). At the late double-ridge stage (W2.5), in the less developed lateral meristems present at the bottom (Fig. 4B) and top (Fig. 4C) of the developing spike, *LFY* expression was stronger at the leaf ridge (also known as lower ridge) than at the spikelet or upper ridge (blue arrows). In the more mature spikelet meristems (SMs) at the center of the developing spike, *LFY* expression was abundant except for the central-distal regions of the SMs (Fig. 4B, blue arrows), suggesting that low *LFY* levels may favor spikelet development. Some SMs showed a more uniform distribution of *LFY*, but those may be the result of off-centered sections.

At W3.25, when developing spikes have several SMs similar to the final SNS, we detected the presence of *FRIZZY PANICLE* (*FZP*, *TraesCS2A02G116900*) in the youngest lateral meristems immediately below the IM (Fig. S4, data S8). *FZP* was not detected in the distal part of the spike at earlier spike developmental stages (W2.5), and was observed at the axils of the

developing glumes of more mature spikelets, similar to previously reported results in rice

(Komatsu *et al.*, 2003). Taken together, these results indicate that the presence of *FZP* in the

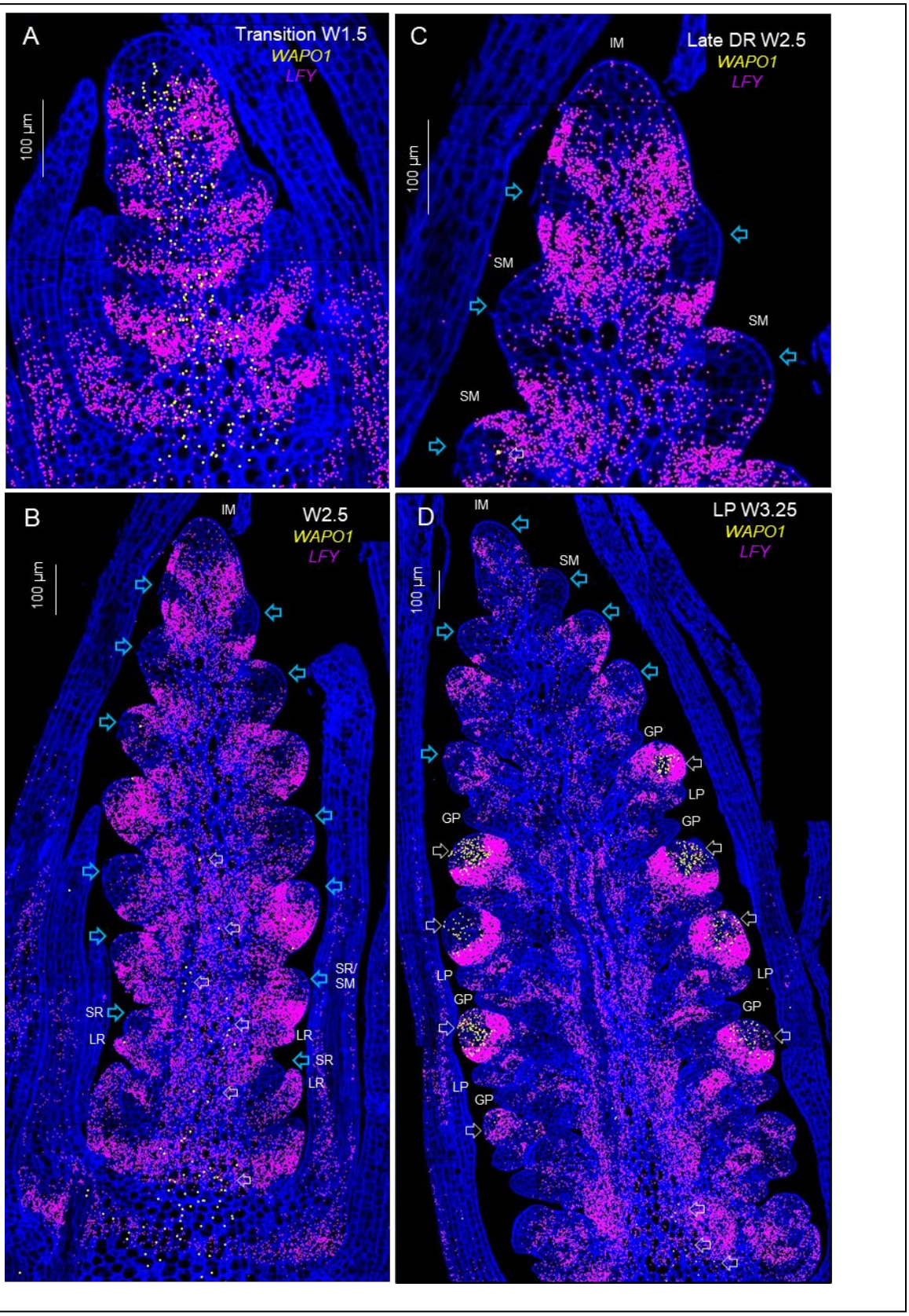

**Figure 4**. **Single-molecule fluorescence in-situ hybridization (smFISH) of *LFY* and *WAPO1* expression during wheat spike development.** Cell walls stained with calcofluor are presented in dark blue. Additional images without *LFY* are available in Fig. S5 to facilitate *WAPO1* visualization. (**A**) Elongated shoot apical meristem transitioning from a vegetative to an inflorescence meristem (IM, W1.5). (**B**) Late double-ridge stage (W2.5). (**C**) Detail of the IM region from (B). (**D**) Lemma primordia stage (W3.25). Blue arrows indicate regions of the spikelet meristems (SMs) where *LFY* (pink dots) is excluded or expressed at lower levels. LR = leaf ridge, SR = spikelet ridge, GP = glume primordium, LP = lemma primordium. Scale bars are 100 µm. W numbers = Waddington scale of wheat spike development (Waddington *et al.*, 1983).

youngest lateral meristems is an early indication of the IM>TS transition.

At the W3.25 stage, the more mature spikelets at the center of the developing spike showed glume and lemma primordia (Fig. 4D). In these spikelets, *LFY* transcripts were highly expressed within a narrow region like a bird's nest located distal to the lemma primordia, which delimited a distal region of the developing spikelet with low *LFY* and high *WAPO1* hybridization signal (Fig. 4D).

*WAPO1* transcripts were detected at the axis of the developing spike at W1.5 in a region overlapping with *LFY* (Fig. 4A and S5). However, at later stages (W2.5 and W3.25), *WAPO1* expression was restricted to the base and center of the developing spike, likely in the differentiating vascular tissue (Fig. 4B and D). To facilitate the visualization of the relatively less abundant *WAPO1* transcripts, we generated additional supplementary images, including *WAPO1* only (Fig. S5). At the lemma primordia stage (W3.25), *WAPO1* was also expressed at the distal part of the more developed spikelets in a region where *LFY* transcript levels were low (Fig.

4D and S5C-D). In summary, the different but partially overlapping expression domains of *LFY* and *WAPO1* in the developing spikelets generate an area of contact, which we hypothesize favors their protein interactions and provides important spatial information for normal floral development.

## Spatio-temporal expression profiles of *LFY* and *SQUAMOSA* MADS-box genes

In Arabidopsis, LFY activates the meristem identity genes *AP1* (Parcy *et al.*, 1998, Wagner *et al.*, 1999) and *CAL* (William *et al.*, 2004), so we first tested using qRT-PCR if the homologous wheat *VRN1* and *FUL2* genes (data S9) were also regulated by *LFY* (Fig. S6, data S10). We found no significant differences between *lfy* and the wildtype control for *VRN1* or *FUL2* transcript levels at W2.0, W3.0, or W4.0 (Fig. S6). Analyses of previously published RNAseq data for Kronos spike development (VanGessel *et al.*, 2022) showed that *VRN1* is induced earlier and is expressed at higher levels than the other two *SQUAMOSA* genes, with *FUL2* expressed at higher levels than *FUL3* (Fig. S7A). In the same RNAseq study, *LFY* was expressed at low levels in the vegetative meristem (W1.0) and increased rapidly during W2.0 and W3.0 (Fig. S7A).

We then compared the smFISH spatial and temporal expression profiles of *VRN1* and *FUL2* during spike development. At the late vegetative stage (W1.0), the hybridization signal of *VRN1* was relatively low and *FUL2* was not detected in the apical meristem. The signal for both genes increased during the early transition to the reproductive stage, although *FUL2* remained low (Fig. 5B, W1.5). These results were consistent with the RNAseq data (Fig. S7A). At later stages (W2.5 and W3.25), *VRN1* and *FUL2* were both highly expressed in the IM and young lateral SMs (Fig. 5C-D). In the more developed spikelets, located at the center of the developing

spike, *VRN1* and *FUL2* expression was stronger at the glume and lemma primordia than in the distal region (Fig. 5D, W3.25), which overlapped with *WAPO1* expression domain (Fig. 4D). *FUL3* showed a similar spatial expression profile as *FUL2* and it is presented separately (Fig. S8)

because of its lower expression levels in the RNAseq data (Fig. S7A) and limited impact on SNS (Li *et al.*, 2019).

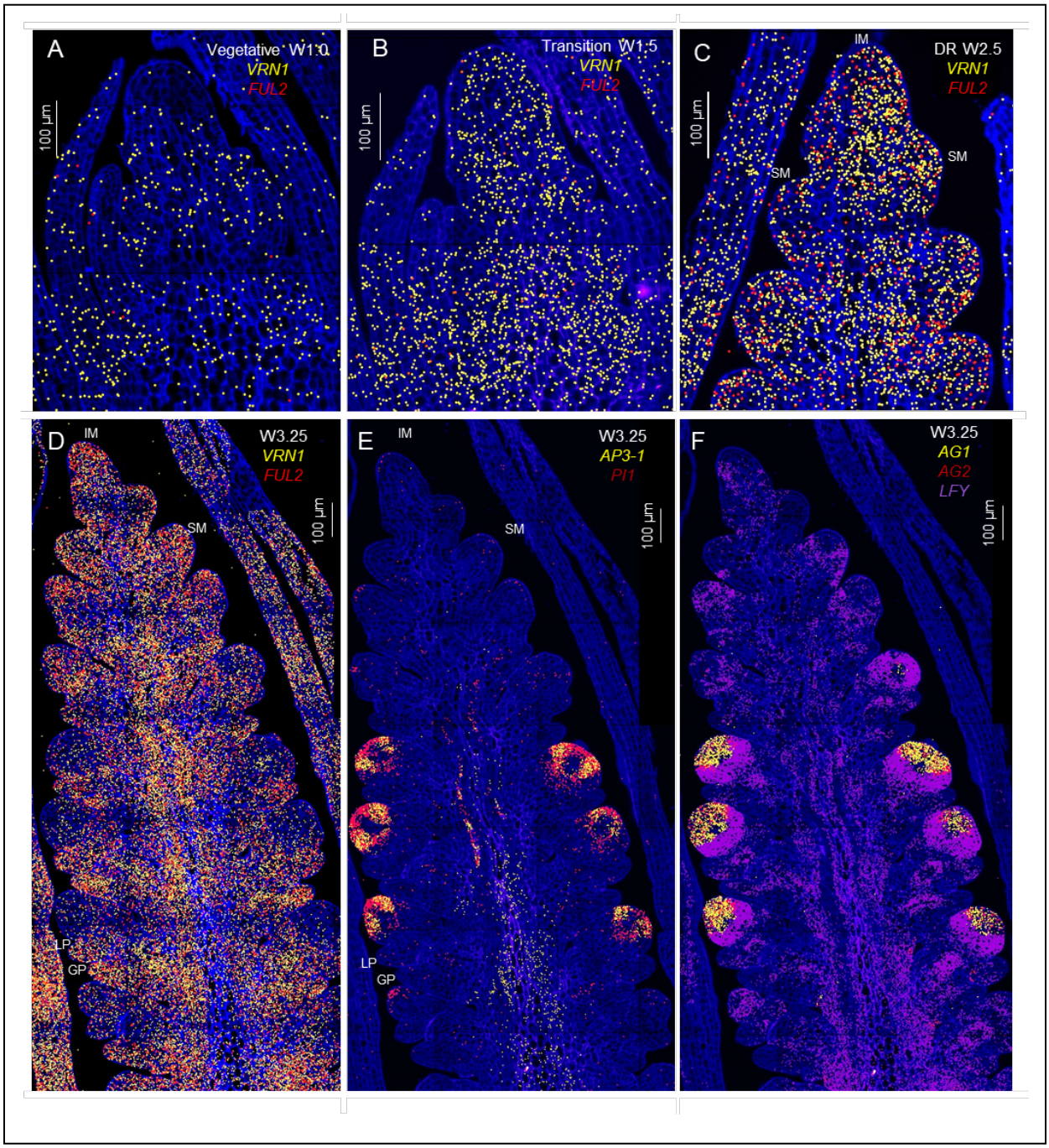

**Figure 5**. **Transcription profiles of *LFY*, *WAPO1* and MADS-box floral genes during wheat spike development.** Cell walls stained with calcofluor are presented in dark blue. (A-B) Relative distribution of *VRN1* (yellow), *FUL2* (red) and *LFY* (violet). (**A**) Late vegetative SAM W1.0 (no *FUL2*). (**B**) Transitioning SAM W1.5 (limited *FUL2*). (**C**) *VRN1* and *FUL2* at the late double ridge stage (W2.5) (D-F) Lemma primordia stage (W3.25). (**D**) *VRN1* (yellow) and *FUL2*. (**E**) Class-B MADS-box genes *AP3-1* (yellow) and PI1 (red). (**F**) Class-C MADS-box genes *AG1* (yellow) and *AG2* (red) relative to *LFY* (violet). IM = inflorescence meristem, SM = spikelet meristem, GP = glume primordium, LP = Lemma primordium. Quantification of the *VRN1*, *FUL2* and *LFY* signals in the IM at W2.5 and W3.25 is available in data S8. Gene identifications based on CS RefSeq v1.1 and rice orthologs are provided in data S9.

To quantify *VRN1*, *FUL2* and *LFY* expression changes in the distal part of the developing spike between the late double ridge stage (W2.5) and the start of the transition to a terminal spikelet (W3.25), we calculated their hybridization signal per 100 µm$^2$ (signal density). In both the IM and the IM plus the two youngest lateral meristems (IM+2LM), we observed a 3- to 4-fold increase in *FUL2* signal density (Fig. S7B) and a 53 to 69 % increase in *VRN1* between W2.5 and W3.25, but only the differences for *FUL2* were significant (Fig. S7C, data S8). Similar results were obtained when the *VRN1* and *FUL2* hybridization signals were normalized using the *CDC20* signal (data S8). In the same tissue sections, we detected a 78% decrease in *LFY* signal density (Fig. S7D), and the differences were significant or highly significant depending on the normalization method used (data S8).

Analyses of the ratios between the *SQUAMOSA* and *LFY* signals showed that the *FUL2/LFY* ratio increased more than 20-fold between W2.5 and W3.25 in both the IM and IM+2L (Fig. S7E, data S8). Similarly, *VRN1/LFY* ratios increased 8-fold between the same

developmental stages (Fig. S7E, data S8). The *SQUAMOSA/LFY* ratios are independent of the normalization method used, and since they are determined in the same tissue sections, they provide the best evidence of a significant increase in the expression of the *SQUAMOSA* genes relative to *LFY* in the distal part of the developing spike at the time of the IM>TS transition.

**Spatio-temporal expression profiles of floral organ identity genes**

We also characterized the spatial distribution of MADS-box genes involved in floral organ development (data S9). The hybridization signals of class-B (*AP3-1* and *PI1*, Fig. 5E), class-C (*AG1*and *AG2*, Fig. 5F) and class-E (*SEP3-1* and *SEP3-2*, Fig. S9) floral organ identity genes were concentrated in a distal region of the developing spikelets that mostly overlapped with the expression of *WAPO1* (Fig. 5D). *SEP1-2*, *SEP1-4*, and *SEP1-6* were expressed outside of the region where the two *SEP3* were expressed (Fig. S9), suggesting functional divergence between the *SEP1* and *SEP3* genes in wheat.

Finally, we used qRT-PCR to characterize the effect of the *lfy* mutation on the expression of the floral organ identity genes in the wheat developing spike at W4.0. The *lfy* mutant showed a significant downregulation of *AP3-1* and *PI1* (Fig. S10A), *AG1* (Fig. S10B), *SEP3-1* and *SEP3-2* (Fig. S10C) relative to the wildtype (data S11). Taken together these results indicate that *LFY* plays an important role in the direct or indirect regulation of the floral organ identity genes.

**Genetic interactions between *LFY* and class-A MADS-box genes**

Given the opposite effects of *LFY* and the MADS-box genes *VRN1* and *FUL2* on SNS, we examined their genetic interactions for this trait. We crossed a plant homozygous for *lfy-A* and heterozygous for *lfy-B* with mutants homozygous for *vrn1* and *ful2*-A but heterozygous for *ful2*-

B and, in the progeny, selected sister plants homozygous for the four gene combinations for each gene (WT, *lfy*, *vrn1*, *lfy vrn1* and WT, *lfy*, *ful2*, *lfy ful2*).

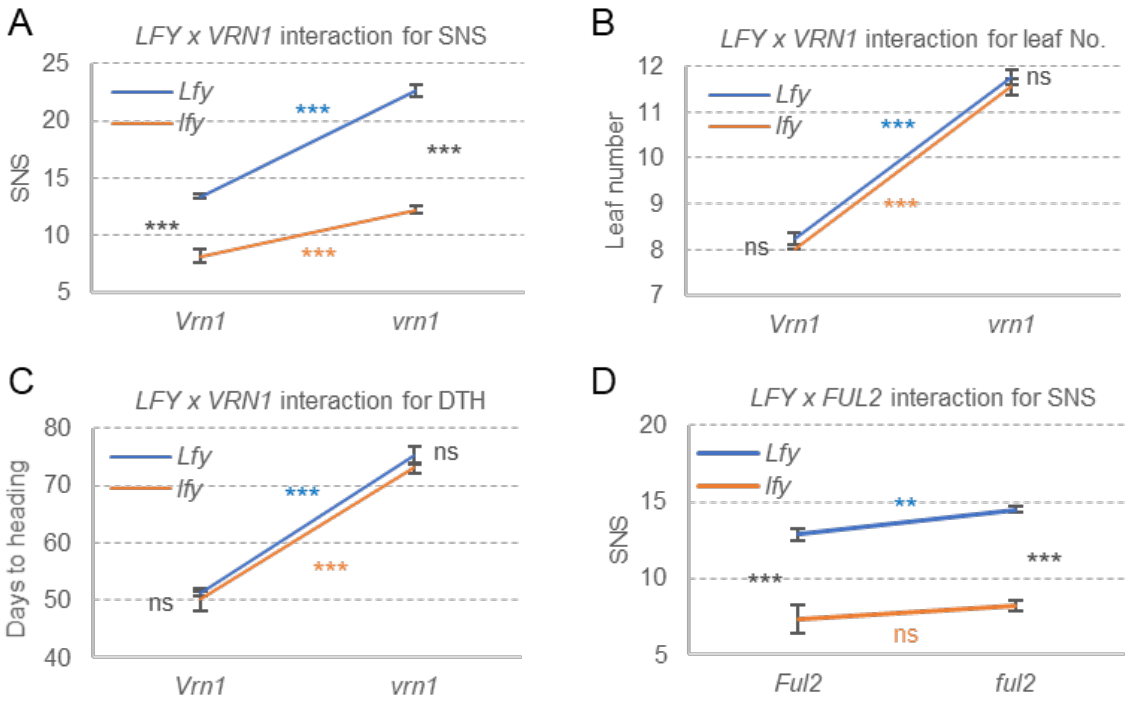


**Figure 6. Genetic interactions between *LFY* and *SQUAMOSA* MADS-box genes *VRN1* and *FUL2*.** (**A-C**) Interactions graphs between *LFY* and *VRN1* for (**A**) spikelet number per spike (SNS) (total N = 34), (**B**) leaf number (total N = 34), and (**C**) days to heading (total N = 34). (**D**) Interaction between *LFY* and *FUL2* for SNS (total N = 23). In these interaction graphs, parallel lines indicate additive effects and non-parallel lines reflect interactions. *P* values indicate the significance of the four possible contrasts in the ANOVAs described in data S12. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Error bars are SEM. Raw data and statistics are available in data S12.

A factorial ANOVA including the four homozygous *VRN1* combinations showed highly significant effects on SNS for both *VRN1* and *LFY*, and a highly significant interaction between these two genes ($P < 0.0001$, Fig. 6A, data S12). The effect of *LFY* on SNS was stronger in the

*vrn1* mutant (10.3 spikelets) than in the presence of the functional *Vrn1* allele (5.2 spikelets). In contrast, the effect of *VRN1* on SNS was stronger in the presence of the functional *LFY* allele (9.1 spikelets) than in the presence of the *lfy* combined mutant (4.0 spikelets, Fig. 6A). *VRN1* also showed highly significant effects on the number of leaves and heading time, similar to previous studies (Li *et al.*, 2019), while *LFY* showed no-significant differences for these traits in the presence of the *Vrn1* or *vrn1* alleles. No significant interactions were detected for these two traits (Fig. 6B-C, data S12).

The effect of *FUL2* on SNS was smaller than that of *VRN1* (Fig. 6D) and the interaction with *LFY* was not significant (data S12). However, the trends were like those observed in the interactions between *LFY* and *VRN1*: a stronger effect of *LFY* on SNS in the presence of the mutant *ful2* allele (6.3 spikelets) than in the presence of the wildtype *Ful2* allele (5.6 spikelets), and a stronger effect of *FUL2* in the presence of wildtype *LFY* allele (1.6 spikelets) than in the presence of the *lfy* mutant (0.9 spikelets). In summary, these interactions suggest the existence of a cross-talk regulating SNS between the *SQUAMOSA* and *LFY* pathways.

## DISCUSSION

### Similarities and differences in *LFY* function between Arabidopsis and wheat

The most conserved functions of *LFY* across the flowering plants are those associated with the regulation of organ identity in the three inner floral whorls, which include pistils, stamens and petals in eudicot or lodicules in grass species (Yoshida, 2012). *LFY* mutations have limited effects on bracts (lemmas in grasses) or on the outermost floral whorls, including sepals in Arabidopsis and paleas in grasses. However, the first floret of the basal spikelet frequently

showed a palea divided in two organs (Fig. 1F), suggesting an interaction between *LFY* and genes expressed in the base of the wheat spike in the regulation of palea development.

In the grass species, similar defects in the inner floral organs have been observed for wheat *lfy* mutants (Fig. 1F and data S2, this study), rice *apo2* mutants (Ikeda-Kawakatsu *et al.*, 2012), barley *multiovary 5* mutants (Selva *et al.*, 2021), and maize *zfl1 zfl2* mutants (Bomblies *et al.*, 2003). These defects include fused organs, reduced number and altered morphology of lodicules (including transformation into bracts and fusions with stamens), reduced number of stamens and increased number of pistils. Fused lodicules with stamens and homeotic conversions of stamens into pistils were observed frequently in the wheat *lfy* mutant (Fig. 1F). The Arabidopsis strong *lfy* mutants fail to develop flowers, but weak *lfy* mutants show petals transformed into small sepals or mosaic organs, reduced stamen numbers and increased pistil numbers (Huala and Sussex, 1992, Weigel *et al.*, 1992), similar to the grass species.

Despite the conserved roles of *LFY* in floral organ development, there are also important differences in *LFY* functions between Arabidopsis and grasses. First, strong *lfy* mutations in Arabidopsis result in the replacement of most flowers by shoots subtended by cauline leaves and the few observed late flowers have intermediate inflorescence characteristics (Schultz and Haughn, 1991, Weigel *et al.*, 1992). In contrast, florets initiate normally in the *lfy* mutants in wheat (Fig. 1F) and other grasses (Bomblies *et al.*, 2003, Ikeda-Kawakatsu *et al.*, 2012), and defects appear only later at the inner floral whorls. These results indicate that *LFY* is required to confer the initial floral meristem identity in Arabidopsis but not in the grass species.

This difference likely contributes to the opposite functions of *LFY* in inflorescence development in these species. In Arabidopsis, constitutive expression of *LFY* (*35S:LFY*) results in

conversion of both apical and axillary meristems into terminal flowers, demonstrating that *LFY* is a limiting factor defining when and where flowers are produced (Weigel and Nilsson, 1995). In contrast, constitutive expression of *LFY* in wheat increases the number of lateral spikelets (Fig. 2). Mutations in *LFY* also result in contrasting effects in eudicots and grasses inflorescence development. Weak *lfy* mutants delay the formation of flowers and increase the number of secondary branches in Arabidopsis and other eudicot species (Coen *et al.*, 1990, Schultz and Haughn, 1991, Weigel *et al.*, 1992, Souer *et al.*, 1998, Molinero-Rosales *et al.*, 1999). In contrast, *LFY* loss-of-function mutations result in significant reductions in SNS in wheat (Fig. 1B-E), and in the number of branches in rice panicles (Ikeda-Kawakatsu *et al.*, 2012) and maize male inflorescences (Bomblies *et al.*, 2003). These results indicate that in grasses, *LFY* promotes the formation of branches (or spikelets in wheat), whereas in Arabidopsis it promotes the formation of flowers decreasing the number of secondary branches.

In summary, *LFY* has a similar role on floral organ development in Arabidopsis and grasses, but plays different roles in floral meristem identity and inflorescence development.

## Interactions between LFY and WAPO1 are critical for their functions

LFY and WAPO1 proteins physically interact with each other in wheat (Fig. 3A), barley (Selva *et al.*, 2021), rice (Ikeda-Kawakatsu *et al.*, 2012) and Arabidopsis (Chae *et al.*, 2008). In Arabidopsis, UFO interaction with LFY, modifies its DNA binding specificity and alters the set of genes directly regulated by LFY (Rieu *et al.*, 2023b), including the class-B genes *AP3-1* and *PI.* In addition to the class-B genes, *ufo* mutations in other eudicot species show altered expression of class-C genes (snapdragon), class-E genes (cucumber) and both class-C and -E genes (petunia, reviewed in (Rieu *et al.*, 2023a)). The simultaneous regulation of class-B, -C and -E MADS-box

genes seems to be conserved in the temperate grasses, where *lfy* mutations in wheat (Fig. S10) and barley (Selva *et al.*, 2021), and *wapo1* mutants in wheat (Kuzay *et al.*, 2022) have been all associated with the downregulation of class-B (*AP3-1* and *PI1*), class-C (*AG1*) and class-E (*SEP3*) floral organ identity genes. This result explains the similar floral defects observed in the wheat *lfy* (Fig. 1F) and *wapo1* mutants (Kuzay *et al.*, 2022), and indicates that both LFY and WAPO1 are required for the correct regulation of these floral organ identity genes.

In Arabidopsis, *ufo* mutants show only small (but still significant) increases in the number of secondary branches (Levin and Meyerowitz, 1995). By contrast, the *wapo1* mutant in wheat shows similar reductions in SNS as the *lfy* and *lfy wapo1* combined mutants (Fig. 3B). Similar reductions in the number of panicle branches are also observed in the *apo1*, *apo2* and combined *apo1 apo2* mutants in rice (Ikeda-Kawakatsu *et al.*, 2012). These results suggest that the physical interaction between LFY and WAPO1 likely plays a more prominent role in the regulation of inflorescence architecture in grasses than in Arabidopsis.

## Interactions between *LFY* and *SQUAMOSA* genes modulate their opposite effects on the IM development

MADS-box transcription factors act as master regulators of developmental switches and organ specification, with meristem identity genes from the *SQUAMOSA* clade playing essential roles in the initiation of flower development. In the wheat *vrn1 ful2 ful3* combined mutant, lateral spikelets are completely transformed into vegetative tillers subtended by leaves (Li *et al.*, 2019), whereas in the Arabidopsis *ap1 cal ful* triple mutant flowers are transformed into leafy shoots (Ferrándiz *et al.*, 2000). In Arabidopsis, AP1 orchestrates floral initiation by directly regulating hundreds of genes (Kaufmann *et al.*, 2010), a function that is favored by AP1's ability

to modulate chromatin accessibility of its target genes (Pajoro *et al.*, 2014). This ability is shared by LFY, which is also a pioneer transcription factor that can regulate genes by contacting its binding motifs even in closed chromatin (Jin *et al.*, 2021, Lai *et al.*, 2021).

In Arabidopsis, LFY positively regulates the expression of *AP1* and *CAL* (Parcy *et al.*, 1998, Wagner *et al.*, 1999, William *et al.*, 2004), thereby indirectly regulating their downstream targets. *LFY* also directly regulates hundreds of genes independently of *AP1* and *CAL* (Goslin *et al.*, 2017). However, induction of *LFY* in *ap1 cal* mutants is insufficient to rescue the limited and late formation of flowers observed in this mutant. Instead, induction of *LFY* in *ap1 cal* mutants results in a lower proportion of plants with flowers than in the control without *LFY* induction (Goslin *et al.*, 2017). This last result indicates that, in the absence of *AP1* and *CAL, LFY* can inhibit flower formation in Arabidopsis, similar to its function in the grass species. These results also suggest that LFY directs regulation of the *SQUAMOSA* genes in Arabidopsis but not in wheat (Fig. S6), likely contributing to the opposite functions of *LFY* in the regulation of IM development between these species.

Approximately 200 genes have been identified in Arabidopsis as high-confidence direct targets of both LFY and AP1 (Winter *et al.*, 2015). Although many of the shared genes directly regulated by the induction of *AP1* or *LFY* show changes in expression with identical directionality, some of them are regulated in opposite directions, including several key regulators of floral initiation (Goslin *et al.*, 2017). These common gene targets can contribute to the epistatic genetic interactions between *LFY* and *AP1* and to their ability to coordinate the transcriptional programs required for flower initiation and early flower development in Arabidopsis.

Genes directly regulated by both *LFY* and *SQUAMOSA* genes are likely to exist also in wheat and may contribute to the significant genetic interaction for SNS observed in this study between *LFY* and *VRN1* (Fig. 6A). The net effect of this interaction, 5.2 spikelets per spike, was smaller than the maximum differences of 14.4 spikelets observed between the plants carrying the *vrn1 Lfy* combination (22.6 spikelets/spike) and those carrying the *Vrn1 lfy* combination (8.2 spikelets/spike, data S12). These results indicate that the interaction between *LFY* and *SQUAMOSA* explains only part of the variation in SNS.

In summary, *LFY* and *VRN1* affect SNS in opposite directions and the magnitude of their effects is partially modulated by their genetic interaction.

## Spatio-temporal expression patterns "illuminate" wheat spike and spikelet development

### *IM>TS transition*

From the beginning of the wheat spike development, *LFY* expression is not uniform, with higher expression levels at the lower or leaf ridge than at the upper or spikelet ridge (Fig. 4B-C), a pattern reported previously in wheat by *in situ* hybridization (Shitsukawa *et al.*, 2006). This spatial differentiation continues in the early spike development (W2.5), where *LFY* is abundant in the proximal region of the young SMs but rare in their distal region (Fig. 4B-C). A similar pattern was also reported in the SMs of young barley spikes (Zhong *et al.*, 2021) and in the primary and secondary branch meristems in the developing rice panicles (Kyozuka *et al.*, 1998, Miao *et al.*, 2022). These results suggest a conserved *LFY* spatial pattern in developing grass inflorescences and highlight the importance of reduced *LFY* levels in initiating spikelet development.

In wheat, the SM regions with low *LFY* expression show high levels of *VRN1* and *FUL2* transcripts (Fig. 5C-D) and, therefore, high *SQUAMOSA / LFY* ratios. We hypothesize that this change in the balance between promoting and repressing pioneer transcription factors is critical for marking the regions where the lateral spikelets will develop. This hypothesis is also supported by the drastic changes in the smFISH signal densities of these genes in the IM and two youngest lateral meristems between the early stages of spike development (W2.5) and the time of the IM>TS transition (determined by the presence of *FZP*, W3.25 Fig. S4). The combined transcript densities of *VRN1* and *FUL2* changed from being less than one-fifth of the *LFY* transcripts at W2.5 to be approximately two-fold higher than *LFY* at W3.25 (Fig. S7 and data S8).

Changes in gene dosage for *LFY* (Fig. S1) and the *SQUAMOSA* genes result in changes in SNS, confirming the importance of their relative transcript levels on spike development. The single *lfy-A* and *lfy-B* mutants show intermediate reductions in SNS relative to the combined *lfy* mutant (Fig. S1), whereas mutations in *FUL2* result in smaller increases in SNS than mutations in *VRN1*, which is expressed at higher levels than *FUL2* in the IM (Fig. S7A and data S8) (Li *et al.*, 2019). Interestingly, four recently cloned genes affecting SNS in wheat including *WAPO1* (Kuzay *et al.*, 2022), *FT-A2* (Shaw *et al.*, 2019, Glenn *et al.*, 2022), *bZIPC1* (Glenn *et al.*, 2023) and *SPL17* (Liu *et al.*, 2023) show potential connections with the regulation of *VRN1* or *LFY* (Fig. S11). In summary, we propose that the modulation of *VRN1* or *LFY* transcript levels plays an important role in the determination of SNS in wheat.

The smFISH results provided valuable insights into the potential roles of *LFY* and the *SQUAMOSA* genes on the regulation of the IM>TS transition, but also revealed unexpected results for *WAPO1* that generated new and interesting questions. Given the strong genetic

interactions for SNS between *WAPO1* and *LFY* (Fig. 3B), we initially expected both genes to be expressed together in the IM close to the time of the IM>TS transition, but that was not observed in our smFISH experiments. *WAPO1* and *LFY* hybridization signals overlapped in the IM at the initial stages of spike development (W1.5, Fig. 4). Still, at later stages, the *WAPO1* signal was restricted to the basal-central region of the developing spike or the distal part of the developing spikelets at the lemma primordia stage (Fig. S5B-D).

To explain how *WAPO1* regulates SNS without being expressed together with *LFY* in the IM at the time of the IM>TS transition (W3.25), we propose two alternative hypotheses: *i*) WAPO1 protein is a mobile signal that can be transported from the base of the developing spike to the IM through the differentiating vascular tissue, and *ii*) the overlapping expression domains of *WAPO1* and *LFY* in the central spikelets at W3.25 (Fig. 4) can promote directly or indirectly a mobile signal that is transported to the IM and triggers the IM>TS transition.

### *Spikelet development*

The smFISH studies also provided insights into the dynamic spatial distribution of *LFY*, *WAPO1*, and floral organ identity genes during spikelet development. In the more developed central spikelets at W3.25, *LFY* was highly expressed in a narrow nest-shaped region distal to the lemma primordia, delimiting a distal region with low *LFY* expression in the developing spikelet (Fig. 4D). *WAPO1* and the floral organ identity genes *AP3-1*, *PI1*, *AG1*, *AG2, SEP3.1*, and *SEP3.2* were preferentially expressed in this spikelet distal region (Fig. 5 and S9), suggesting that LFY and WAPO1 may provide important spatial information. We speculate that the overlapping expression domains of *LFY* and *WAPO1* in the developing spikelets generate a curved region of contact (Fig. 4D), where proteins encoded by these two genes may have a higher opportunity to

interact with each other. Based on published Arabidopsis results (Rieu *et al.*, 2023b), we also speculate that within this dynamic overlapping region, the WAPO1-LFY interaction can redirect LFY to a different set of gene targets than the ones regulated by LFY alone, providing important spatial information for the correct regulation of the floral organ identity genes.

The importance of both LFY and WAPO1 for the correct regulation of these floral organ identity genes in wheat is supported by their significant downregulation in both *lfy* (Fig. S10) and *wapo1* mutants (Kuzay *et al.*, 2022) and by the common floral defects observed in the *lfy* and *wapo1* mutants. In summary, these results suggest that the dynamic spatial information provided by LFY and WAPO1 is critical for the normal development of the floral organs present within the multiple florets formed within each of the wheat spikelets.

In summary, this study shows that *LFY* plays an essential role in wheat spike and floral organ development, and that changes in *LFY* dosage can be used to manipulate SNS in wheat. It also shows that the IM>TS transition, which is critical in the determination of SNS, is marked by opposite changes in the transcript levels of *LFY* and the *SQUAMOSA* MADs-box genes in the distal part of the developing spike. Finally, this study shows that the effects of *LFY* on SNS are modulated by its interactions with *WAPO1* and *VRN1*, providing valuable information on the genetic network regulating this important agronomic trait, in a crop that is central to global food security.

## MATERIALS AND METHODS

### Ethyl methanesulfonate (EMS) induced *LFY* mutants and their interactions with *VRN1*, *FUL2* and *WAPO1.*

We screened the sequenced tetraploid wheat variety Kronos population (Krasileva *et al.*, 2017) by BLASTN to identify loss-of-function mutations in the *LFY-A1* and *LFY-B1* homeologs. To reduce background mutations, the *lfy-B* mutant was backcrossed twice to Kronos and then to the *lfy-A* mutant. The double mutant was backcrossed once to Kronos, and in the progeny homozygous sister lines were selected for the four possible homozygous combinations, including the *lfy-A lfy-B* combined mutant (henceforth, *lfy*). This line is $BC_1$ for *lfy-A* and $BC_2$ for *lfy-B* so it is referred to as $BC_{1\text{-}2}$. Genome specific markers for the *lfy-A* and *lfy-B* mutations were designed and used to genotype plants during backcrossing and combination with other mutations described below. Primers are listed in data S1.

To study the interactions between *LFY* and other spike development genes, we intercrossed the *lfy* combined mutant with previously developed Kronos lines homozygous for loss-of-function EMS or CRISPR mutations in both genomes of *VRN1* (Chen and Dubcovsky, 2012), *FUL2* (Li *et al.*, 2019), or *WAPO1* (Kuzay *et al.*, 2022). These lines had at least two backcrosses to the parental line Kronos to reduce the number of background mutations. For these crosses, we used a line heterozygous for one of *LFY* mutation to restore fertility, and molecular markers to select the four possible homozygous combinations in the $F_2$ progeny of each cross.

## Plant growth and phenotypic characterization

Plants were stratified for 2-days at 4 °C in the dark and then planted in growth chambers (PGR15, Conviron, Manitoba, Canada). Lights were set to 350 µmol $m^{-2}$ $s^{-1}$ at canopy level. Plants were grown under inductive 16-h long days with temperatures set during the day to 22°C and during the night to 17°C. Relative humidity in growth chambers was maintained at 60-70% throughout the duration of the experiments. Heading time was recorded as the number of days from germination to full emergence of the spike from the leaf sheath. Spikelet number per spike (SNS) was measured at maturity from the main tiller.

## Generation of the wheat transgenic lines overexpressing *LFY*

We cloned the *LFY-A* coding regions by PCR from cDNA derived from Kronos developing spikes using primer LFY-A-GW-F combined with either LFY-A-GW-R1 (with no tag) or LFY-A-GW-R2 (with an HA tag) listed in data S1. We then recombined it into pDONR/zeo entry vector using Life Technologies BP Clonase II following the manufacturer's protocol. The pDONR/zeo vector containing the *LFY-A* coding region was next recombined into the Japan Tobacco pLC41 vector downstream of the maize *UBIQUITIN* promoter using Life Technologies LR Clonase II to generate two constructs: one expressing *LFY* with a C-terminal 3xHA tag, and the other without a tag. Clones were verified by Sanger sequencing at each cloning step. The two T-DNA binary constructs were transformed into the wheat variety Kronos using *Agrobacterium*-mediated transformation (EHA105) at the UC Davis Plant Transformation Facility as described before (Debernardi *et al.*, 2020b). The presence of the *LFY* transgene was determined with primers LFY-Genotyping-R5 and UBI-F2 listed in data S1.

*LFY* transcript levels were determined by qRT-PCR using primers LFY_qPCR_F and LFY_qPCR_R and *ACTIN* as endogenous control (data S1). For qRT-PCR experiments, RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) or as previously described by (Ream *et al.*, 2014). One µg of RNA was treated with RQ1 RNase-Free DNase (Promega, M6101) first and then used for cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The qRT-PCR experiments were performed using Quantinova SYBR Green PCR kit (Qiagen, 208052) in a 7500 Fast Real-Time PCR system (Applied Biosystems). Transcript levels for all genes are expressed as linearized fold-*ACTIN* levels calculated by the formula $2^{(ACTIN\ CT\ -\ TARGET\ CT)}$ ± standard error (SE) of the mean.

## Single-molecule fluorescence *in-situ* hybridization (smFISH)

We used the Molecular Cartography™ technology from Resolve BioSciences combinatorial smFISH. Wheat shoot apical meristems were collected from the vegetative to the spike lemma primordia stage. The samples were immediately fixed in 4% PFA after harvest, dehydrated, and embedded in paraffin. Sections from the central plane of the developing spikes (10µm-thick) were placed on the slides and dried overnight at 37°C, followed by a 10-minute bake at 50°C. The sections were then deparaffinized, permeabilized, and refixed according to the user guide. After complete dehydration, the sections were mounted using SlowFade-Gold Antifade reagent, covered with a thin glass coverslip, and sent to Resolve BioSciences on dry ice for analysis as described in our previous study (Glenn *et al.*, 2023).

Probes were designed using Resolve BioSciences' proprietary design algorithm and gene annotations from Chinese Spring RefSeqv1.1. To identify potential off-target sites, searches

were confined to the coding regions. Each target sequence underwent a single scan for all k-mers, favoring regions with rare k-mers as seeds for full probe design. For each of the wheat genes selected for smFISH probe design (data S9), we selected the homoeolog expressed at higher levels in a Kronos transcriptome including different stages of spike development (VanGessel *et al.*, 2022), and provided Resolve BioSciences their respective homeologs to be excluded in their quality control for primer specificity performed against all the coding sequences of the wheat genome (Ref Seq v1.1). Therefore, probes are not genome-specific and may detect both homoeologs for each gene (Catalogue No. PGGS, all these probes are part of kit number K7128).

Imaging and image processing was performed as described before (Glenn *et al.*, 2023). Final image analysis was performed in ImageJ using the Polylux tool plugin from Resolve BioSciences to examine specific Molecular Cartography™ signals.

## Co-immunoprecipitation (Co-IP) assay and western blotting

To test the physical interaction between WAPO and LFY, we performed Co-IP experiments in wheat leaf protoplasts using a method described previously (Zhang *et al.*, 2023), with minor modifications. The *WAPO1* coding region was initially synthesized by Genewiz into the pUC57 vector, amplified with primers WAPO1_BP_F and WAPO1_BP_R (data S1), cloned into pDONR/zeo vector using Life Technologies BP Clonase II, and recombined into the Japan Tobacco pLC41 vector downstream of the maize *UBIQUITIN* promoter with a C-terminal 4xMYC tag (for transgenic experiments). Next, we switched both *UBI:WAPO1-MYC* and *UBI:LFY-HA* from the pLC41 binary vector to the smaller pUC19 vector to enhance the transfection efficiency of the protoplasts. The *UBI:WAPO-MYC* and *UBI-LFY-HA* DNA fragments were cleaved

using restriction enzymes *Hind*III and *Spe*I, gel purified, and then ligated with the *Hind*III-*Xba*I linearized pUC19 vector (*Spe*I and *Xba*I create compatible ends). Both constructs were verified by restrictions and Sanger sequencing.

We transformed Kronos leaf protoplasts with 50 µg of each of the *UBI:LFY-HA* and *UBI:WAPO1-MYC* plasmids in 50 ml tubes containing 2 ml of protoplast (roughly 0.5 x $10^6$ cell per mL). As negative controls, we performed separate transformations including only one of the two plasmids. After transformation, protoplasts were resuspended in 5 ml W5 buffer and incubated in a 6-well plate at room temperature overnight. Total protein was extracted with 1 ml of IP lysis buffer (25 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40 substitute, 5% glycerol and 1 x Protease Inhibitors). Part of the protein extract was set aside as input control (50 µl), and the rest was used for co-immunoprecipitation using Pierce Anti-HA Magnetic Beads (ThermoFisher Cat. 88836) by gentle agitation on a tube rotator for 30 min at room temperature with additional 1x proteinase inhibitors. Proteins were eluted by boiling the beads in 50 µl 1x Laemmli sample buffer for 10 min.

For Western Blot, half of the Co-IP elution and 50 µg of input for each sample were loaded onto a 12% SDS-PAGE gel. After protein was transferred to a PVDF membrane using the Bio-Rad Trans-Blot Turbo Transfer System (Cat. 1704150), the membrane was blocked with 1x TBST buffer containing 5% non-fat milk for 1 h at room temperature. Anti-cMyc-peroxidase monoclonal antibody (Roche 11814150001) was added at a dilution of 1:10,000 and was incubated at room temperature for 1h. After four 10 min washes using 1x TBST buffer, the signals were developed using SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher, Cat. 34096). After imaging, the membrane was stripped with mild stripping

buffer (1.5% glycine, 0.1% SDS, 1% Tween 20, pH 2.2), re-blocked for 1 h at room temperature, and then probed with anti-HA-peroxidase at a dilution of 1:2500 (Roche 12013819001) for 1 h at room temperature.

## FUTURE DIRECTION

For my future studies of wheat spike developmental, I would like to perform comparative studies with the other grass species, to gain new insights into the conserved and different mechanisms that control the diversity of grass inflorescences. Another area I would like to explore is the connections between the *LFY* and *SQUAMOSA* genes I characterized in my thesis with the SQUAMOSA promoter-binding protein-like (SPL). Several studies in wheat, rice, and Arabidopsis, have shown that the SPL proteins play a vital role in plant growth and development, functioning as regulators of grain size and shape, plant architecture, and floral transition (Li *et al*., 2020). There are several SPL genes in plants with variable counts in different species. In 2023, Liu *et al*., published a large-scale genotype-phenotype study on SPL17 in wheat. Their findings revealed that SPL17 regulates spikelet and floret meristem development. Miao *et al*., 2022 found SPL7, SPL14, and SPL17 to be directly regulated by APO2 (rice LFY) during the reproductive stage, specifically during the transition to the inflorescence meristem and branch meristem. SPL13 has also been shown to regulate inflorescence architecture and development (Li *et al*., 2020). I am interested in understanding how the wheat SPL proteins interact with LFY and the SQUAMOSA genes, and how these interactions contribute to wheat spike development.

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## Supplementary Materials

Supplementary Figures S1 to S11 are included in a separate PDF.

Supplementary data S1 to S12 (listed below) are included in Excel file Supplementary_Data.xlsx

**data S1**. Primers used in this study.

**data S2**. Supporting data for Fig. 1C-F.

**data S3**. Supporting data for Fig. S1.

**data S4**. Supporting data for Fig. S2.

**data S5**. Supporting data for Fig. 2.

**data S6**. Supporting data for Fig. 3B.

**data S7**. Supporting data for Fig. S3.

**data S8**. Supporting data for Fig. S7.

**data S9**. Genes used in the smFISH experiments.

**data S10**. Supporting data for Fig. S6.

**data S11**. Supporting data for Fig. S10.

**data S12**. Supporting data for Fig. 6.