LEAFY interacts with WHEAT ORTHOLOG OF APO1 to regulate spikelet number per spike in wheat

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Short Title: *LFY* regulates wheat spike development

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Summary Statement

The transcription factor *LEAFY* plays a central role in the regulation of the number of spikelets per spike in wheat.

Abstract

In wheat, the transition of the inflorescence meristem (IM) to a terminal spikelet determines the spikelet number per spike (SNS) and the maximum number of grains. In this study, we demonstrate that this transition is repressed by the plant-specific transcription factor *LEAFY* (*LFY*) and promoted by the MADS-box genes *VRN1* and *FUL2*, affecting SNS in opposite directions. LFY physically interacts with WHEAT ORTHOLOG OF APO1 (WAPO1), and loss-of-function mutations in either or both genes result in significant and similar reductions in SNS. These results indicate that both genes are required to maintain IM indeterminacy. The spatio-temporal distribution of these genes during spike development characterized by single-molecule fluorescence *in-situ* hybridization provides insights on their roles in spike and spikelet development. Finally, we identify natural *LFY-B* alleles that affect SNS, determine their interactions with natural alleles of *WAPO-A1*, and select an allele combination that maximizes SNS for wheat improvement.

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 (*1*). Therefore, increasing the maximum number of grains that can be produced by each spike can contribute to the urgent need to increase wheat productivity to feed a growing human population.

Wheat spikes, as other grass inflorescences, are comprised of specialized organs called spikelets, which are short indeterminate branches. Each spikelet has two proximal sterile bracts (glumes) followed by a variable number of florets, each subtended by a bract (lemma) with an axillary flower (*2, 3*). 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 limits the maximum number of grains that can be formed in the spike. Therefore, the regulation of the timing of the transition between the IM and the terminal spikelet (henceforth, IM>TS) is critical for the determination of 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 (*4-6*). However, differences in SNS also have a strong genetic component, with broad sense heritability for this trait ranging from $H^2 = 0.84$ in irrigated fields to $H^2 = 0.59$ in water stressed environments (*7*). This high heritability has facilitated the identification of several wheat genes involved in the regulation of the IM>TS transition. *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 (*8*). Loss-of-function mutations in *vrn1* or *ful2* result in normal plants with significant increases in SNS. However, in the *vrn1 ful2* 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 (*8*).

Genes that regulate *VRN1* expression have been shown to affect SNS indirectly. *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 (*9,*

10). Mutants (or knock-down transgenic plants) of *FT1* (*11*) or its closest paralog *FT2* (*12*) show reduced or delayed expression of *VRN1* which is associated with significant increases in SNS, whereas overexpression of these genes result in a precocious IM>TS transition and spikes with very few spikelets (*11, 12*). Similarly, mutations in *PPD1* that reduce or delay *FT1* expression result in SNS increases (*13*), whereas mutations in *ELF3* that result in the upregulation of *FT1* and *VRN1* expression reduce SNS (*14*). *bZIPC1* encodes a protein that physically interacts with FT2, and its mutants also show a large decrease in SNS (*15*).

However, the underpinning mechanism by which the recently cloned gene *WHEAT ORTHOLOG OF APO1* (*WAPO1*) (*16, 17*) 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 (*18-20*). In addition to floral defects, loss-of-function mutations in *WAPO1* or *APO1* result in significant reductions in SNS in wheat (*16*) and number of panicle branches in rice (*21*), respectively. These results indicate that both *WAPO1* and *APO1* promote IM indeterminacy and prevent a precocious transition of the IM into a terminal spikelet.

In Arabidopsis, UFO physically interacts with the plant-specific transcription factor LEAFY (LFY) (*22-24*), an interaction that is conserved between the rice homologs APO1 and APO2 (*25*). The Arabidopsis LFY protein activates class-A MADS-box genes *AP1* (*26, 27*) and *CAL* (*28*), which are homologous to the wheat *VRN1* and *FUL2*. Since *VRN1*, *FUL2* (*8*) and *WAPO1* (*16*) 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 reductions in SNS. We also show that *LFY*'s delay of the IM>TS transition is opposed by the *VRN1* and *FUL2* MADS-box genes, which are required for spikelet development and promote this transition. The genetic results presented in this study are complemented by detailed spatio-temporal expression profiles of these genes using single-molecule fluorescence *in-situ* hybridization (smFISH). Finally, we characterize the effect of natural variation in *LFY* on SNS, describe its interactions with natural variants of *WAPO1*, and identify optimal allele combinations that maximize SNS.

Results

Natural variants in *LFY* affects SNS in wheat

To test if *LFY* was associated with natural variation in SNS in polyploid wheat, we explored a set of wheat Nested Association Mapping (NAM) populations previously characterized for this trait (*7*). We did not find QTLs for SNS associated with the A or D genome copies of *LFY* (*LFY-A = TraesCS2A02G443100* and *LFY-D = TraesCS2D02G442200*), but we found one associated with marker IWB35244 closely linked to the *LFY-B* (*TraesCS2B02G464200*) homeolog in the Berkut x PBW343 population. Lines carrying the Berkut allele showed significantly higher SNS than those with the PBW343 allele across three field trials (data S1). To validate this result, we generated heterogeneous inbred families (HIFs) from two F_5 plants heterozygous for the *LFY-B* region. Sister $F_{5:2}$ lines homozygous for the Berkut allele showed 1.6 to 1.8 more spikelets per spike than those homozygous for the PBW343 allele ($P < 0.001$, Fig. 1A, data S2), confirming the significant effect of this chromosome region on spike development.

To characterize wheat natural variation in *LFY-B*, we performed a haplotype analysis for an 813 kb region of chromosome arm 2BL including *LFY-B* (see Materials and Methods). The 146 SNPs identified in this region defined five haplotypes designated as H1 to H5 (data S3). We then determined the haplotypes present in the sequenced wheat genomes (*29*), and used them to obtain the *LFY-B* sequences associated with each haplotype (data S4). The H3 haplotype was not present among the sequenced wheat genomes, so we sequenced *LFY-B* from hexaploid wheat cultivar RAC875 and deposited the sequence in GenBank (OR753911).

Comparison of the *LFY-B* sequences associated with the different haplotypes revealed a proline to leucine change at position 34 (P34L), an arginine to serine change at position 80 (R80S), a proline deletion in a stretch of eight prolines in positions 26 to 33 (henceforth 8P to 7P), and a 220-bp insertion 872 to 653 bp upstream from the start codon (P+ = presence and P- = absence of the insertion, Fig. 1B). The R80S polymorphism is located within the N-terminal conserved Sterile Alpha Motif oligomerization domain (SAM, Fig. 1C) (*30, 31*), whereas the 8P to 7P and P34L polymorphisms are outside the conserved region (Fig. 1C).

The *LFY-B* allele P-,34P,80R,8P associated with the H3 haplotype (Fig. 1B) is likely the ancestral combination because it is the one present in other grass species (fig. S1). The *LFY-B* alleles associated with the H1 and H2 haplotypes differ from H3 by the presence of the 34L

polymorphism (P-,34L,80R,8P). The 34L polymorphism was also present in the *LFY-B* alleles associated with the H4 and H5 haplotypes, but in H4 it was combined with the proline deletion (P-,34L,80R,7P) and in H5 with the 80S polymorphism and the promoter insertion (P+,34L,80S,8P) (Fig. 1B). This promoter insertion was associated with a 17 % decrease in *LFY* expression in the developing spike at the lemma primordia stage (P= 0.036, Fig. 1D, data S2).

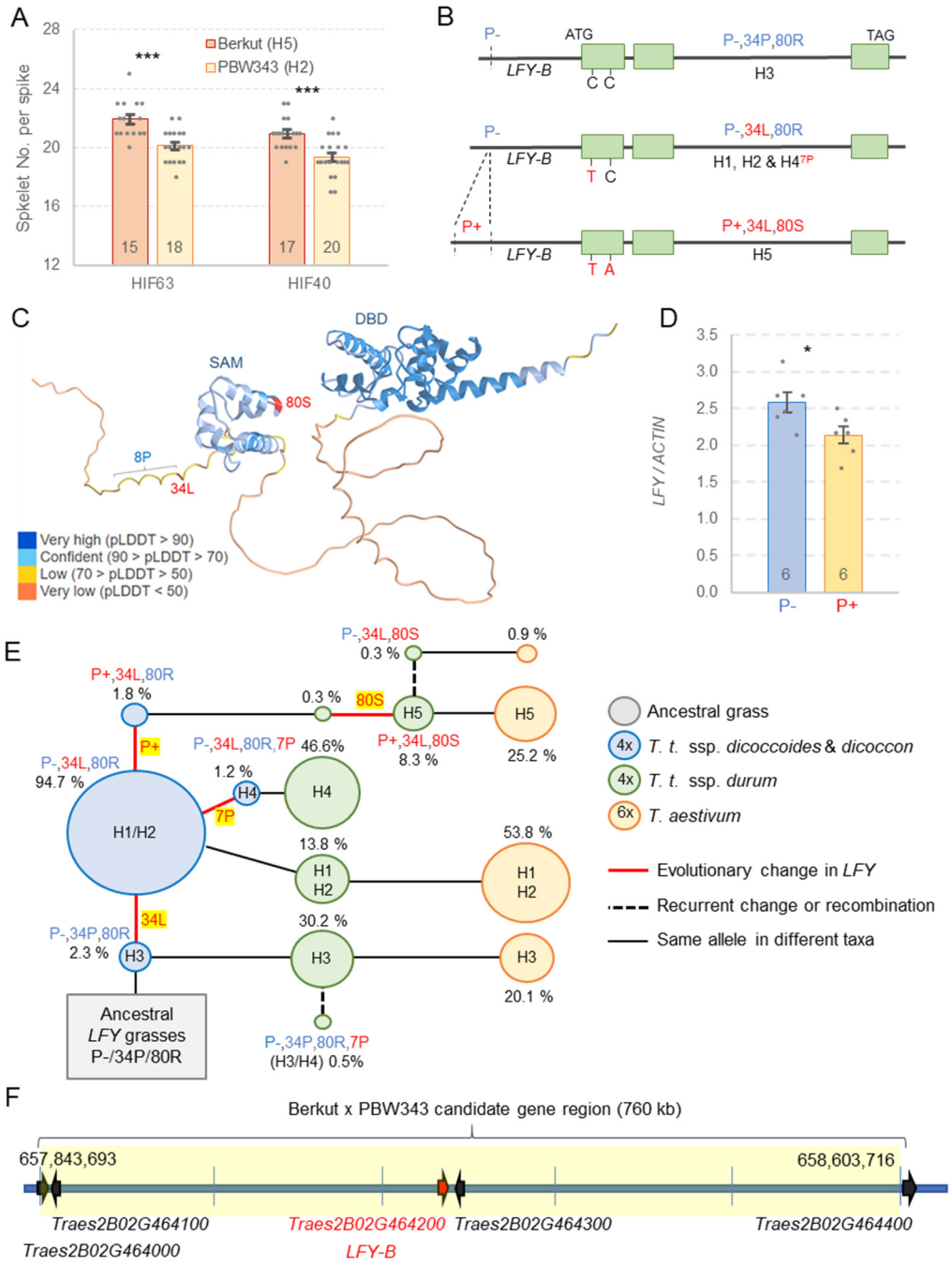

Fig. 1. Natural variation in *LFY-B* in wheat. (**A**) Differences in SNS between sister lines homozygous for the *LFY-B* alleles from PBW343 and Berkut in $F_{5:2}$ heterogeneous inbred families (HIFs). (**B**) *LFY-B* alleles differ in a promoter insertion (P+), amino acid changes P34L and R80S and a proline deletion (8P to 7P, only in H4). Derived alleles are indicated in red. (**C**) Location of the natural polymorphisms (in red) in an Alpha-fold simulation of the wheat LFY protein for haplotype H5. Colors indicate confidence levels (pLDDT = Local Distance Difference Test), SAM = Sterile Alpha Motif oligomerization domain and DBD = DNA binding domain. (**D**) Quantitative Reverse Transcription PCR (qRT-PCR) comparison of *LFY* expression between HIF sister lines homozygous for the presence (P+) or absence (P-) of the 220-bp promoter insertion in developing spikes at the lemma primordia stage. For A and D, the number of biological replications is indicated at the base of the bars. Error bars indicate SEM and probability values are from two tailed *t*-tests. * = $P < 0.05$, *** = $P < 0.001$. Raw data and statistics for (A) and (D) are available in data S2. (**E**) Changes in *LFY-B* allele frequencies in ancestral tetraploid (blue circles), modern tetraploid (green circles) and modern hexaploid (orange circles) wheat accessions. The likely origins of the different polymorphisms are marked in red bold lines and highlighted. (**F**) High-density map of the *LFY-B* region based on recombination events described in data S7.

To estimate the frequency of the different *LFY-B* alleles, we designed markers for the promoter insertion, the two non-synonymous changes (P34L and R80S) and the proline deletion (primers in data S5), and used them to characterize 171 wild and 618 cultivated wheat accessions (data S6). The ancestral *LFY-B* allele P-,34P,80R (H3) was found in 30.2% of the *T. turgidum* ssp. *durum* and 20.1 % of the *T. aestivum* accessions; but surprisingly in only 2.3 % of the ancestral tetraploid species *T. turgidum* ssp. *dicoccoides* and *T. turgidum* ssp. *dicoccon* (Fig. 1E and data S6). The derived *LFY-B* allele P-,34L,80R was the most abundant in all wheat taxa (Fig. 1E), suggesting that the 34L change was an early event in wheat evolution that was favored by selection. We did not find the R80S polymorphism among the ancestral tetraploid accessions, but found it in 8.3% of the modern durum and in 25.2 % of the common wheat varieties, which suggests positive selection in modern hexaploid wheat breeding programs. The promoter insertion was frequently associated with the 80S polymorphism in both durum (97.0 %) and common wheat (96.7 %), but was also found in combination with other alleles at very low frequencies (Fig. 1E), suggesting either recurrent changes or recombination among *LFY* alleles. Finally, we detected the 8P to 7P deletion associated with the H4 haplotype in few ancestral tetraploids (1.2 %), at a higher frequency in modern durum (46.6 %) accessions, and absent in hexaploid wheat (Fig. 1E).

We then generated a high-density map to confirm the linkage between the SNS QTL and the *LFY-B* polymorphisms in the Berkut (H5) x PBW343 (H2) HIF population. To identify

recombination events in the region, we screened 770 segregating chromosomes with markers flanking a 3 Mb region encompassing *LFY-B*, identified lines with recombination events, and genotyped these lines with six new markers (primers in data S5) to select the closest recombination events to *LFY-B* (data S7). Progeny tests from the three lines with the closest recombination events showed significant differences in SNS between sister lines (data S7), indicating that *LFY-B* and the QTL for SNS were linked within a 760 kb region (data S7).

The candidate gene region includes only two complete and two partial high-confidence annotated genes in addition to *LFY-B* (Fig. 1F, data S8). Among these genes, *TraesCS2B02G464000* and *TraesCS2B02G464100* encode proteins with kinase domains that are expressed at very low levels during spike development (*32*). *TraesCS2B02G464300* is highly expressed in the developing spike (*32*), but encodes a 50S ribosomal protein L11 that has no amino acid polymorphisms between the parental lines. Finally, only the promoter region of *TraesCS2B02G464400* is located within the candidate gene region, and it does not show major polymorphisms. Taken together, these results suggest that *LFY-B* is the most likely causal gene for the SNS QTL detected in the Berkut x PBW343 population. This hypothesis is supported by the significant effect of *LFY* loss-of-function mutations on SNS described below.

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

Using our sequenced Kronos mutant population (*33*), 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 the mis-splicing of the third exon, a shift in the reading frame, and a premature stop codon (Fig. 2A). 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 (*34*) (Fig. 2A). Primers used to track these mutations are described in data S5. 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 (henceforth, *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. 2B-C, data S9), and smaller but still significant decreases in the single *lfy-A* (12%) and

lfy-B (8%) mutants (fig. S2, data S10). No significant differences in heading time or leaf number were detected between *lfy* and the WT (Fig. 2D-E, data S9), suggesting a limited effect of *LFY* on the timing of the transition between the vegetative and reproductive meristems. Based on its effect on SNS, we infer that *LFY* delays the IM>TS transition.

In addition to its effects on SNS, the *lfy* mutant also showed severe alterations in floral organs. These alterations included missing or altered lodicules, mosaic lodicule-stamen organs, reduced number of stamens, and increased number of carpels. Thirteen out of the 14 flowers checked showed one stamen and three carpels suggesting homeotic conversions of stamens to carpels (Fig. 2F). The frequent floral abnormalities in the *lfy* mutant, combined with the reduced SNS, resulted in low spike fertility.

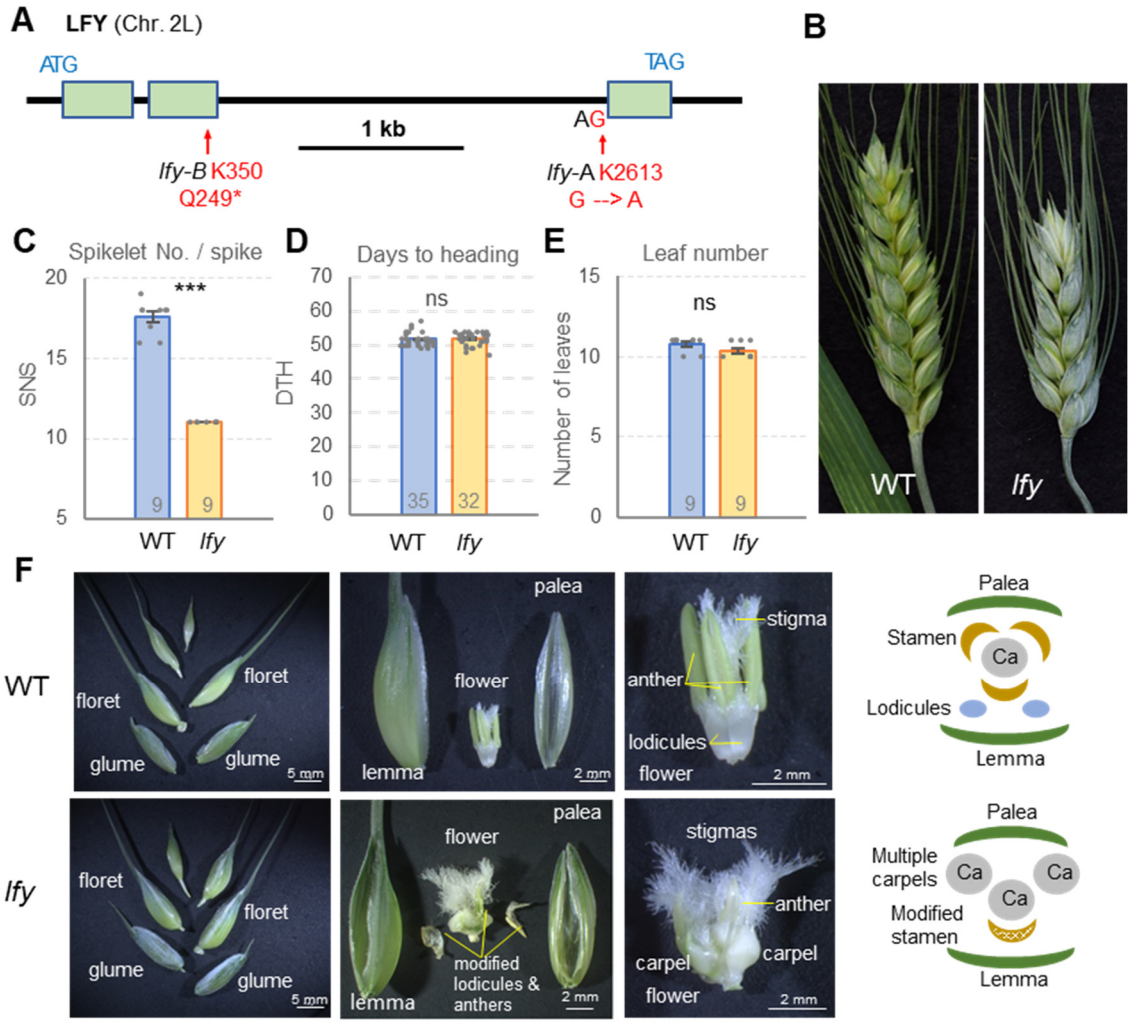

Fig. 2. Characterization of *LFY* loss-of-function mutants. (**A**) *LFY* gene structure and location of the selected mutations in *lfy-A* and *lfy-B*. (**B**) Representative wildtype (WT) and *lfy* combined mutant. Comparison between WT and *lfy* for (**C**) spikelet number per spike, (**D**) days to heading, and (**E**) leaf number. Numbers inside the bars indicate number of biological replicates (separate plants). Raw data and statistics for C-E are available in data S9. (**F**) Floral defects observed in the *lfy* mutant. A schema of the most frequent changes is presented on the right.

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*, 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, where expression of endogenous *LFY* was not detected (fig. S3, data S11).

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 *lfy* mutations or for wildtype alleles (WT), each with or without the transgenes. Among the plants without the transgene, the *lfy* allele was associated with a significant reduction in the number of spikelets per spike (6.6 spikelets, $P < 0.001$), as in previous experiments. No significant differences in SNS were observed between *UBI:LFY* and *UBI:LFY-HA* 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. 3, data S12). The effect was larger in the *lfy* mutant, where transgenic plants showed 4 more spikelets per spike than the controls ($P < 0.001$, Fig. 3).

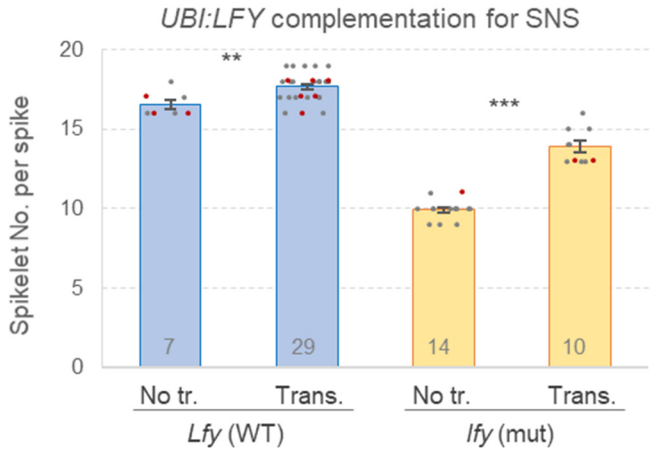

Fig. 3. **Effect of *UBI:LFY* and *UBI:LFY-HA* on spikelet number per spike (SNS).** No significant differences were detected between *UBI:LFY* (grey dots) and *UBI:LFY-HA* (red dots) so data were analyzed together. The effect of the transgenes on SNS was evaluated in both the wildtype (WT) and *lfy* 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 is available in data S12.

The low fertility of the *lfy* mutants (2.7 ± 0.7 grains per plant) was partially restored by the presence of the transgene (23 ± 10 grains per plant, $P = 0.0003$) (data S12). However, the comparison of sister lines carrying the wildtype *Lfy* allele, showed that the presence of the transgene itself was associated with reduced fertility (34 ± 7 grains per plant) relative to the wildtype, (94 ± 25 grains per plant, $P = 0.019$) (data S12). These results indicate that the ectopic expression of *LFY* under the *UBI* promoter is not sufficient to restore full fertility, and that a natural *LFY* promoter would be likely required to fully complement the *lfy* mutant.

Taken together, the results from the high-density genetic mapping, the *lfy* mutants and the *UBI:LFY* transgenic plants, indicate that *LFY* is the causal gene of the 2BL QTL for SNS detected in the Berkut x PBW343 segregating population.

LFY interacts with WAPO1 in wheat leaf protoplasts

Since *LFY* and *WAPO1* mutants were both associated with similar reductions in SNS (*16*), and their homologous proteins interact with each other in Arabidopsis (*22*), we tested the ability of LFY and WAPO1 to interact physically with each other in wheat. We used 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 (47F is associated with higher SNS than 47C (*16*)). 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. 4). These results indicate that LFY can interact with either WAPO1 variant in wheat.

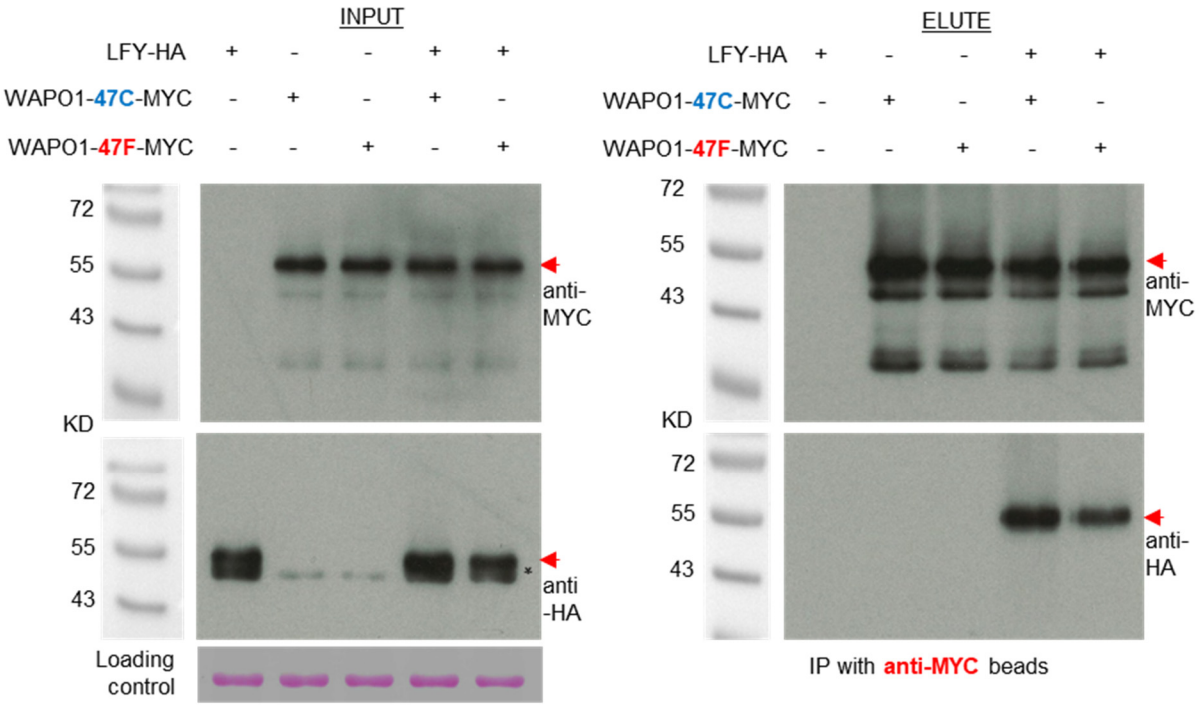

Fig. 4. **Physical interaction between WAPO1 and LFY proteins.** The interaction between LFY and both WAPO1 alleles (47F and 47C) was tested in tetraploid wheat leaf protoplasts by co-immunoprecipitation (co-IP). (Left) Input and loading control. (Right) Elute showing LFY-HA signal using an anti-HA antibody in both the WAPO1-47C-MYC and WAPO1-47F-MYC precipitates with anti-MYC beads.

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

For the LFY-WAPO1 interaction described above to be biologically relevant, the spatial and temporal expression of the two proteins needs to have some overlap. 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 (*35*). A separate RNA-seq study including five spike developmental stages in tetraploid wheat Kronos (*32*), showed transcripts for both genes present at the different stages. *LFY* transcript levels were more abundant than those of *WAPO1*, and both genes showed lower transcript levels at the vegetative stage than at the double-ridge to floret primordia stages (fig. S4, data S13). These studies indicate that *LFY* and *WAPO1* are present at the same stages of spike development. To refine the position of *LFY* and *WAPO1* transcripts within the developing spike, we examined their dynamic spatial patterns using smFISH (Fig. 5)

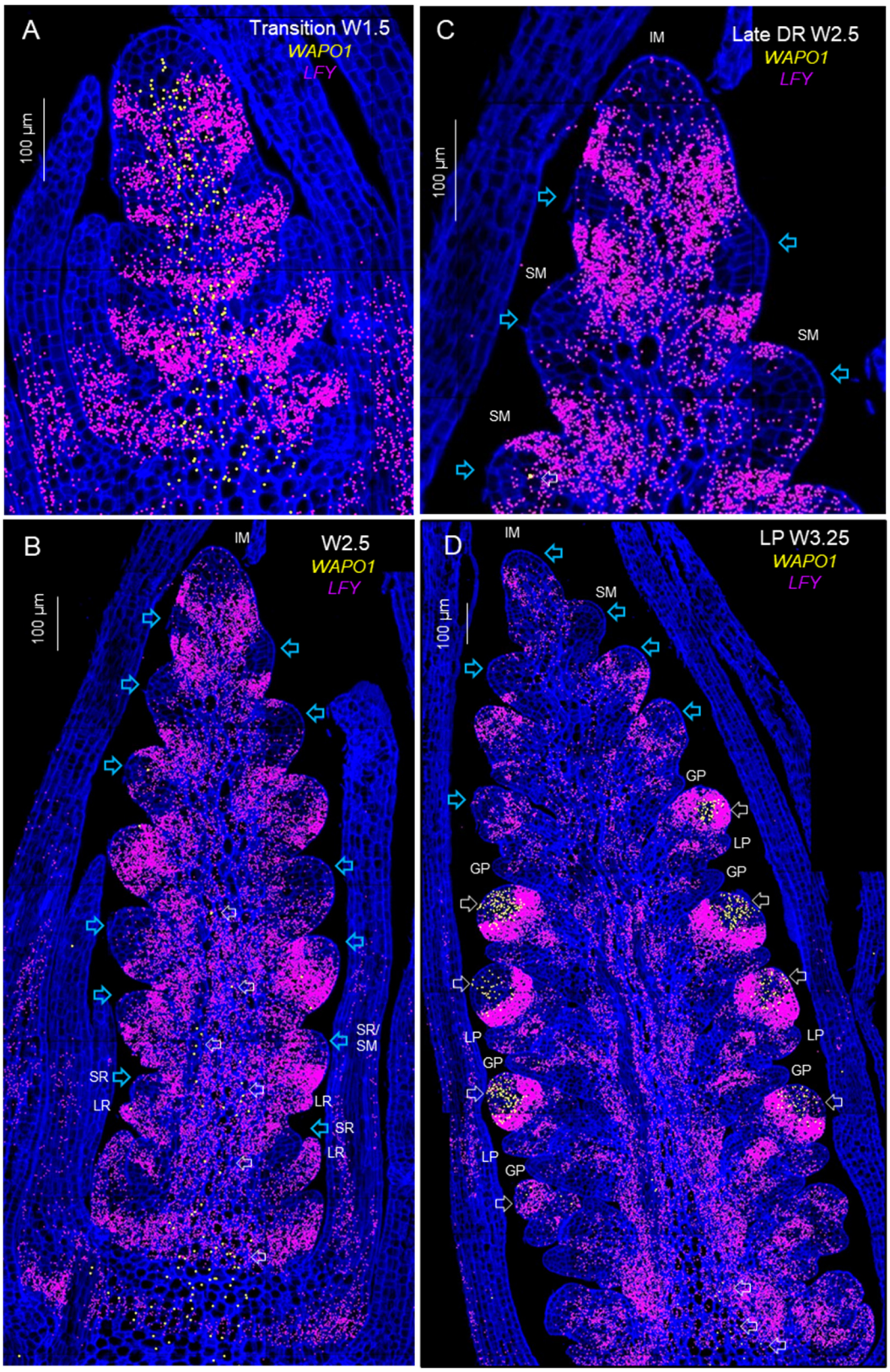

Fig. 5. **Single-molecule fluorescence in-situ hybridization (smFISH) of *LFY* and *WAPO1* expression during wheat spike development.** Cell walls stained with calcofluor are presented in dark blue. Additional figures without

LFY are available in fig. S6 to facilitate *WAPO1* visualization. (**A**) Elongated shoot apical meristem transitioning 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). In B and D, *WAPO1* (yellow dots, white arrows) was detected at the central region at the base of the spike. 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 correspond to the Waddington scale of wheat spike development (*36*)

During the transition between the vegetative and reproductive phases (W1.5), *LFY* transcripts were concentrated in bands radiating from the axis of the elongating shoot apical meristem (SAM) towards the lateral primordia (Fig. 5A). Few *LFY* transcripts were detected at the tip of the IM at this or later spike developmental stages (Fig. 5C-D). At the late double-ridge stage (W2.5), in the less developed lateral meristems present at the bottom (Fig. 5B) and top (Fig. 5C) 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 (Fig. 5B, blue arrows). Some SMs showed a more uniform distribution of *LFY*, but those may be the result of off-centered sections.

The number of *LFY* transcripts per 100 μm^2 (transcript density) in the IM or the IM plus the first two lateral meristems (henceforth, IM+2LM) was significantly lower (78%) at the lemma primordia stage (W3.25) than at the previous late double ridge stage (W2.5) (Fig. 5D, data S14). A similar result was obtained when *LFY* transcript levels were normalized using the number of transcripts detected for the cell division cycle gene CDC20 detected in the same sections and selected areas (data S14).

We inferred that the IM and youngest lateral meristems in the previous sections (Fig. 5D, W3.25) were transitioning to a terminal spikelet, because we detected the presence of *FRIZZY PANICLE* (*FZP*, *TraesCS2A02G116900*) at the two youngest lateral meristems in the same section (fig. S5, data S14). *FZP* was not detected in the distal part of the spike at earlier spike developmental stages and was observed only in more mature spikelets at the axils of the developing glumes, similar to previously reported results in rice (*37*). Taken together, these results indicate that the reduction in *LFY* transcript levels and the presence of *FZP* in the lateral meristems are early indications of the IM>TS transition.

At W3.25, the more mature spikelets at the center of the developing spike showed glume and lemma primordia (Fig. 5D). In these spikelets, *LFY* transcripts were highly expressed within a narrow region similar to a bird's nest located distal to the lemma primordia, delimiting a distal region of low *LFY* expression. This distal region largely overlapped with the *WAPO1* expression domains (Fig. 5D, white arrows).

At W1.5, *WAPO1* transcripts were detected at the axis of the developing spike (Fig. 5A and S6), in a region that overlapped with *LFY*. 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. 5B and D). To facilitate the visualization of the relatively less abundant *WAPO1* transcripts, we generated additional supplementary images including only *WAPO1* transcripts (fig. S6). At the lemma primordia stage (W3.25), *WAPO1* was also expressed at the distal part of the more developed spikelets, where *LFY* transcript levels were low (Fig. 5D and S6C-D). In summary, the complementary expression domains of *LFY* and *WAPO1* in the developing spikelets generate an overlapping area of contact, which we hypothesize favors their protein interactions and provides important spatial information for normal floral development.

Spatio-temporal expression profiles of meristem and floral organ identity genes

In Arabidopsis LFY activates the meristem identity genes *AP1* (*26, 27*) and *CAL* (*28*), so we first tested if the homologous *VRN1* and *FUL2* genes (data S15) were also regulated by *LFY* in wheat using qRT-PCR (fig. S7, data S16). 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. S7). We then compared the expression profiles of *VRN1* and *FUL2* relative to *LFY* using smFISH. At the late vegetative stage (W1.0), *VRN1* and *LFY* were expressed at low levels, and *FUL2* was not detected (Fig. 6A). During the transition to the reproductive stage (W1.5), transcript levels of *FUL2* were very low (orange arrows), whereas those for *LFY* and *VRN1* showed a significant increase, with *VRN1* showing a more uniform distribution than *LFY* (Fig. 6B). At later stages (W2.5 and W3.25), both *VRN1* and *FUL2* were highly expressed in the IM and young lateral SMs (Fig. 6C-D).

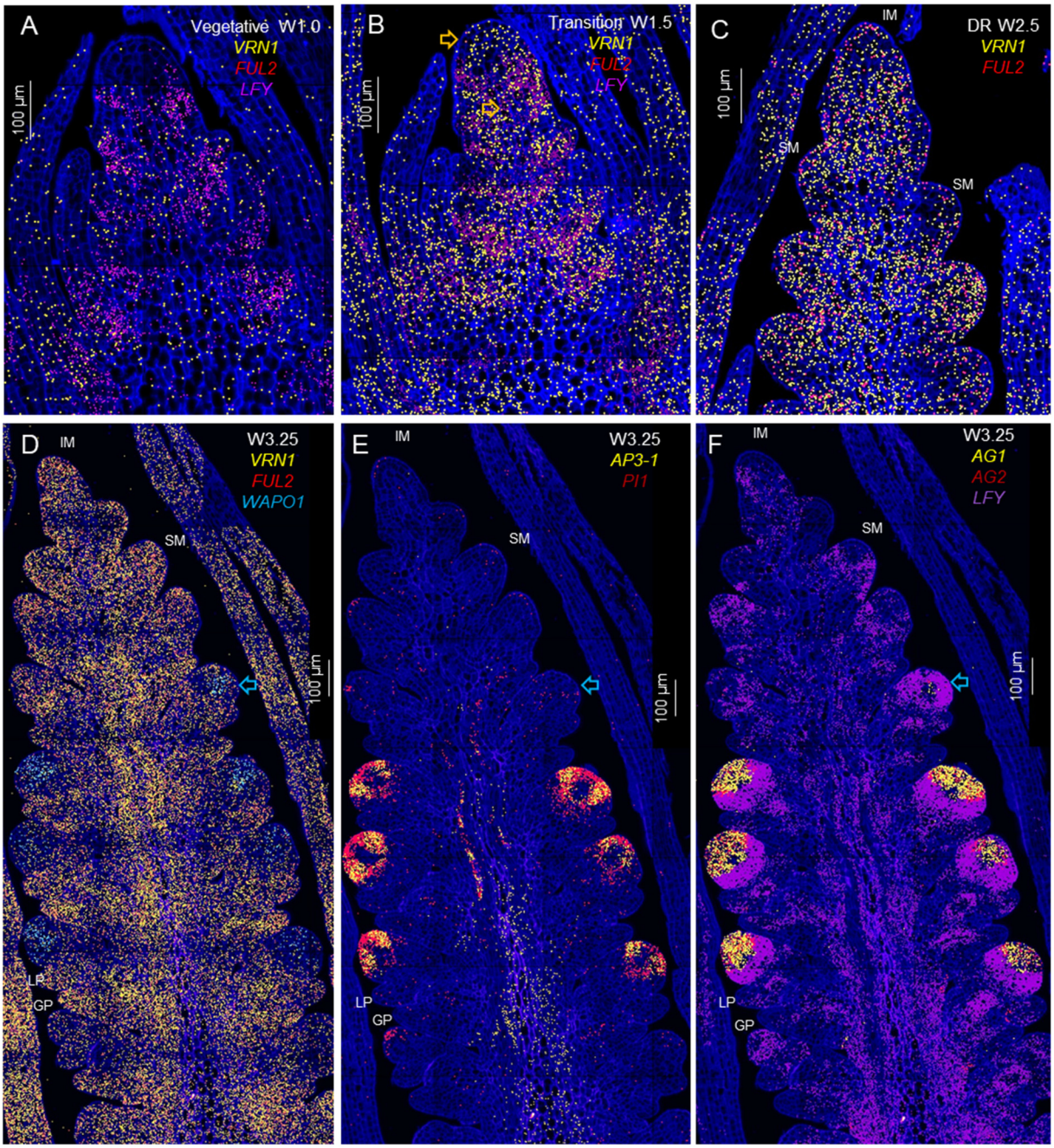

Fig. 6. **Transcription profiles of *LFY*, *WAPO1* and MADS-Box floral genes during wheat spike development.** Cell walls stained with calcofluor are presented in dark blue. (A-B) Relative distribution of *VRN1* (yellow), *FUL2* (red) and *LFY* (violet). (**A**) Late vegetative SAM W1.0 (no *FUL2*). (**B**) Transitioning SAM W1.5 (limited *FUL2*, orange arrows). (**C**) *VRN1* and *FUL2* at the late double ridge stage (W2.5) (D-F) Lemma primordia stage (W3.25). (**D**) *VRN1* (yellow), *FUL2* (red) and *WAPO1* (light blue). (**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). The blue arrow indicates a young spikelet where *WAPO1* is expressed earlier than the class-B and class-C MADS-box genes. IM =

inflorescence meristem, SM = spikelet meristem, GP = glume primordium, LP = Lemma primordium. Gene identifications based on CS RefSeq v1.1 and rice orthologs are provided in data S15.

A quantitative analysis of the IM and IM+2LM at the time of the transition to a terminal spikelet (W3.25) revealed a 3- to 4-fold significant increase in *FUL2* transcript density relative to the previous W2.5 stage (data S14). *VRN1* transcript density also showed a 53 to 69 % increase in the same region at W3.25 relative to W2.5, but the differences were not significant (data S14). Similar results were obtained when the *VRN1* and *FUL2* transcripts were normalized using *CDC20* (data S14). These increases in *VRN1* and *FUL2* transcript density were observed in the same tissue sections where we detected the 78% decrease in *LFY* transcript density described above. Analyses of the ratios between the *SQUAMOSA* and *LFY* gene densities (which are independent of the normalization method, data S14) showed that *FUL2* transcript densities increased from ~4% of the *LFY* at W2.5 to 80 to 114% at W3.25. Similarly, *VRN1* transcripts increased from 14 to 15% of *LFY* at W2.5 to 114 to 123% of *LFY* at W3.25 (data S14). In summary, the IM and youngest lateral meristems, showed a significant increase in the ratio between *SQUAMOSA* and *LFY* genes at the IM>TS transition time relative to previous developmental stages.

In the more mature spikelets located at the center of the developing spike (W3.25), *VRN1* and *FUL2* expression was stronger at the glume and lemma primordia than in the distal region that overlapped with *WAPO1* expression (Fig. 6D). *FUL3* showed a similar spatial expression profile as *FUL2* and it is presented separately (fig. S8) because of its lower expression levels and limited impact on SNS (*8*).

We also characterized the spatial distribution of MADS-box genes involved in floral organ development (data S15). The transcripts of class-B (*AP3-1* and *PI1*, Fig. 6E), class-C (*AG1*and *AG2*, Fig. 6F) 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. 6D). *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 these two groups of genes. 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 S17).

LFY and *WAPO1* show significant genetic interactions for SNS

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 S12), 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 S5). Plants homozygous for mutations in both *LFY* (*lfy*) or in both *WAPO1* (*wapo1*) homeologs showed large and similar reductions in SNS relative to the wildtype (34% and 35% reduction, respectively, Fig. 7A). 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. 7A, data S18). These results suggest that both LFY and WAPO1 proteins are required to suppress a precocious IM>TS transition.

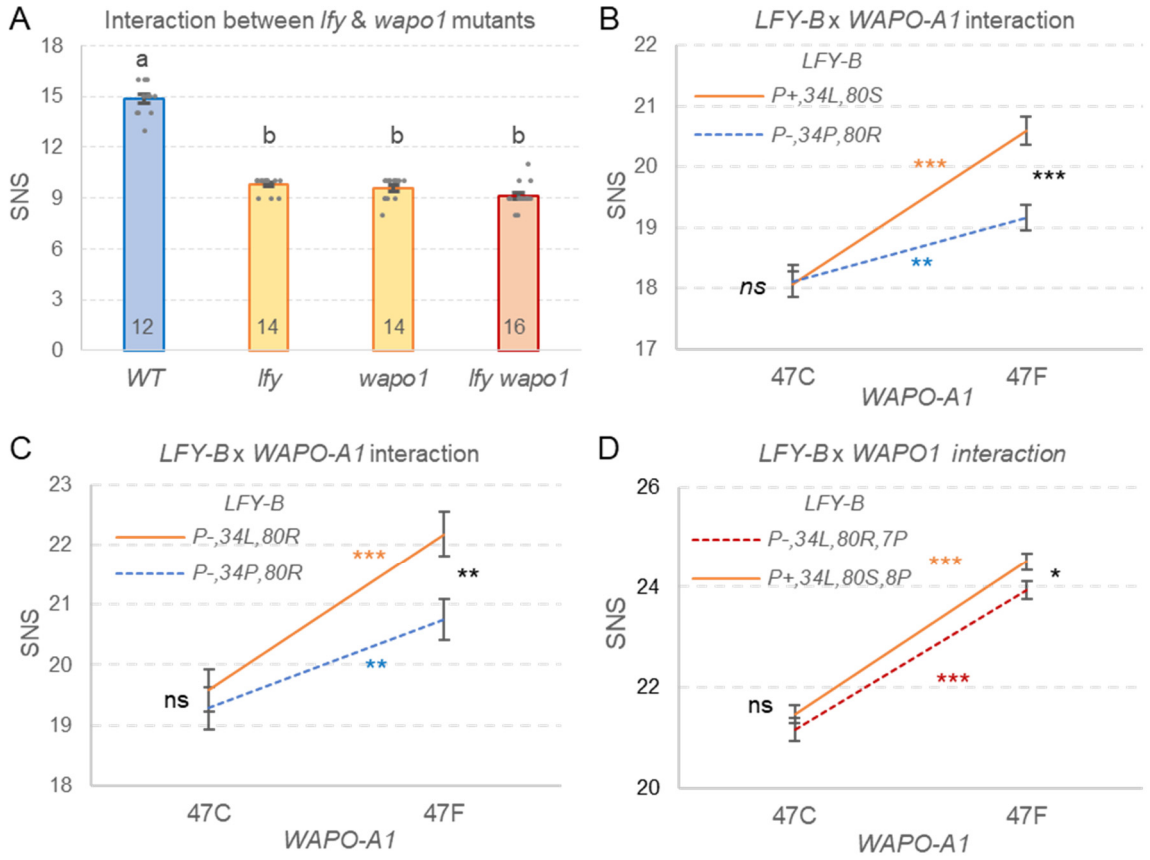

Fig. 7. Epistatic interactions for SNS between *LFY* and *WAPO1*. (**A**) Interaction between loss-of-function *lfy* and *wapo1* mutants. Different letters above the bars indicate significant differences in Tukey tests ($P < 0.05$), and numbers inside the bars the number of biological replications (**B-D**) Interactions between *WAPO-A1* and *LFY-B* natural alleles. (**B**) Berkut x RAC875 RILs evaluated in 6 environments, total N = 390 (*7*). (**C**) RSI5 x RAC875. Results from the combined ANOVA of 3 experiments using experiment as block, total N = 118. (**D**) Combined analysis of tetraploid populations Kronos x PI 519639 and Kronos/Gredho/Kronos-47F evaluated in the field. *P* values indicate the significance of the four possible contrasts in the ANOVAs described in data S19. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Error bars are SEM. Raw data and statistics are available for (A) in data S18 and for (B-D) in data S19.

We then investigated the genetic interactions for SNS between natural *LFY-B* alleles (Fig. 1B) and *WAPO-A1* alleles 47C and 47F in three different segregating populations (Fig. 7B-D, data S19). In the Berkut x RAC875 RIL population, which was evaluated in 6 environments (*7*), we observed significant effects on SNS for both *WAPO-A1* ($P < 0.001$) and *LFY-B* ($P = 0.0028$), as well as a significant interaction between the two genes ($P = 0.0013$). The effect of *LFY* on SNS was significant only in the presence of the *WAPO-A1* allele 47F, and among the latter, the plants carrying the *LFY-B* allele P+,34L,80S showed 1.4 more spikelets per spike than the plants with the P-,34P,80R allele ($P < 0.001$, Fig. 7B). The effect of *WAPO-A1* was significant in the presence of both *LFY-B* alleles, but was stronger in the presence of the *LFY-B* allele P+,34L,80S (2.5 spikelets/spike) than in the presence of P-,34P,80R (1.0 spikelets per spike, Fig. 7B).

The second population, RSI5 x RAC875, segregated for the *LFY-B* polymorphism L34P (both lines are P-/80R/8P) and for the *WAPO-A1* polymorphism C47F. This population showed significant effects on SNS for both *WAPO-A1* ($P < 0.001$) and *LFY-B* ($P = 0.0165$). The interaction was not statistically significant ($P = 0.1142$) but, as in the previous population, *LFY-B* showed a stronger effect on SNS in the presence of the *WAPO-A1* 47F allele (1.4 spikelets/spike, $P = 0.0056$) than the 47C allele (0.3 spikelets/spike, $P = 0.5481$, Fig. 7C).

Finally, we studied the effect of the *LFY-B* alleles P+,80S,8P and P-,80R,7P on SNS in a fixed 34L background. We combined the analyses of two tetraploid wheat populations where the durum line Kronos (H4, P-,34L,80R,7P and *WAPO-A1* 47C) was crossed with two different tetraploid lines carrying the P+,34L,80S,8P allele (H5). The *WAPO-A1* 47F allele was present in one of the parental lines and was introgressed from Kronos-47F (PI 698810) to develop the second segregating population (see Materials and Methods). The P+,80S,8P allele was associated with a significant increase in SNS ($P = 0.0205$), which was stronger in the presence of the

WAPO-A1 allele 47F (0.6 spikelets/spike, $P = 0.0190$) than in the presence of 47C, where no significant differences were detected ($P = 0.2892$, Fig. 7D). The differences between *WAPO-A1* 47F and 47C alleles were significant in the presence of both *LFY-B* alleles ($P < 0.0001$, Fig. 7D).

We speculate that the 7P polymorphism associated with P-,34L,80R may have a small positive effect on SNS, reducing the difference in SNS with the also favorable allele P+,34L,80S,8P (0.6 spikelets per spike) relative to the differences between this last allele and P-,34L,80R,8P in the Berkut x PBW343 population (1.7 spikelets/spike, data S19). This hypothesis would be consistent with the rapid increase of the frequency of the 7P polymorphism among the commercial tetraploid wheat varieties (Fig. 1E).

In summary, in all three populations the positive effects of the derived *LFY-B* alleles on SNS relative to the ancestral allele (P-,34P,80R) were significant only in the presence of the *WAPO-A1* 47F allele (high SNS).

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

Class-A MADS-box genes *VRN1* and *FUL2* promote the IM>TS transition whereas *LFY* represses or delays this transition. Given the opposite effects of these genes in the IM>TS transition, we examined their epistatic interactions for SNS. We crossed a plant homozygous for *lfy-A* and heterozygous for *lfy-B* (to maintain fertility) 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 (WT, *lfy*, *vrn1*, and combined *lfy vrn1*).

A factorial ANOVA including the four homozygous gene combinations showed highly significant effects on SNS for both *VRN1* and *LFY*, and a highly significant interaction ($P < 0.0001$, Fig. 8A, data S20). 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). By 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 *lfy* (4.0 spikelets, Fig. 8A). *VRN1* also showed highly significant effects on the number of leaves and heading time, similar to previous studies (*8*), 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. 8B-C, data S20).

The effect of *FUL2* on SNS was smaller than the effect of *VRN1* (Fig. 8D) and the interaction with *LFY* was not significant (data S20). However, the trends were similar to 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 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).

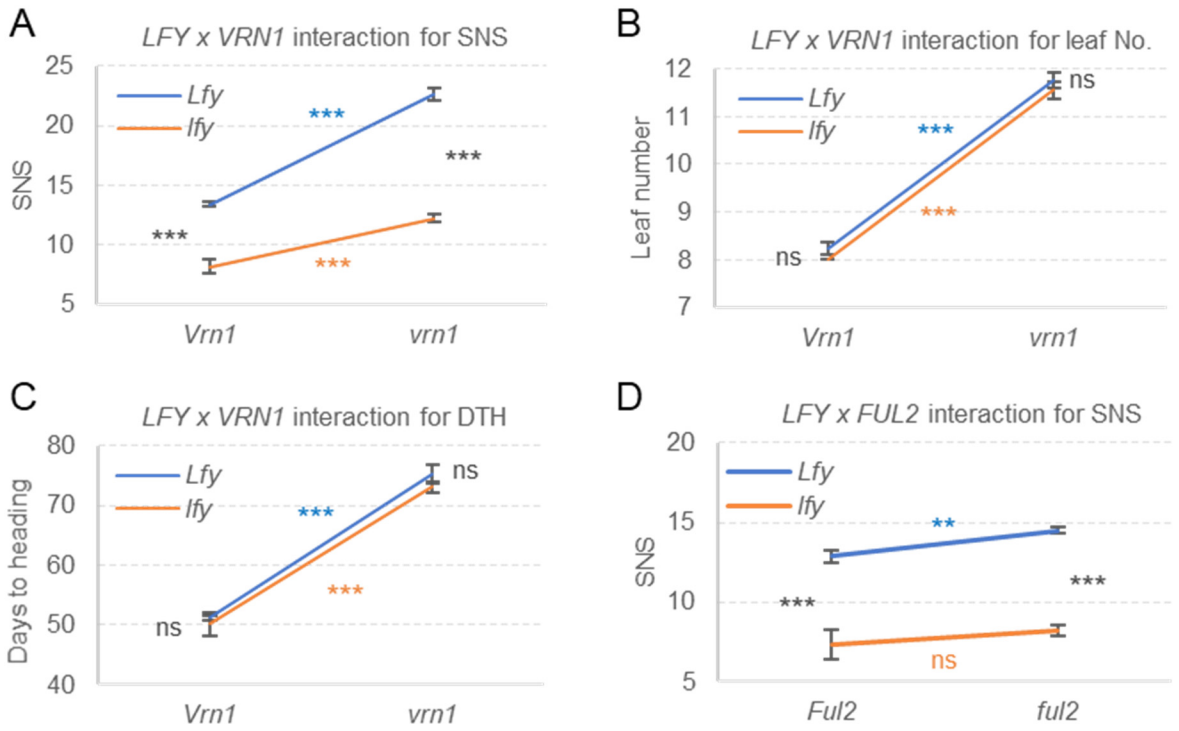

Fig. 8. Genetic interactions between *LFY* and both *VRN1* and *FUL2*. (**A-C**) Interactions between *LFY* and *VRN1* for (**A**) spikelet number per spike (SNS) (total N = 34), (**B**) leaf number (total N = 34), and (**C**) days to heading (total N = 34). (**D**) Interaction between *LFY* and *FUL2* for SNS (total N = 23). *P* values indicate the significance of the four possible contrasts in the ANOVAs described in data S20. 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 S20.

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 carpels, anthers and petals or lodicules in eudicot and grass species, respectively (*38*). *LFY* mutations have limited

effects on bracts (lemmas in grasses) or on the outermost floral whorls, including sepals in Arabidopsis and paleas in grasses (Fig. 2F). In the grass species, similar defects in the inner floral organs have been reported for wheat *lfy* mutants (Fig. 2), rice *apo2* mutants (*25*), barley *multiovary 5* mutants (*39*), and maize *zfl1 zfl2* mutants (*40*). These defects include reduced number and altered morphology of lodicules (including transformation into bracts and fusions with stamens), reduced number of stamens and increased number of carpels. One stamen and three carpels were observed frequently in the wheat *lfy* mutant (Fig. 2F), suggesting homeotic conversions from stamens to carpels. The Arabidopsis strong *lfy* mutants fail to develop flowers, but weak *lfy* mutants also show petals transformed into small sepals or mosaic organs, reduced stamen numbers and increased carpel numbers (*41, 42*), similar to the grass species.

In spite of 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 (*42, 43*). By contrast, flowers initiate normally in the *lfy* mutants in wheat (Fig. 2F) and other grasses (*25, 40*), 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.

The previous difference, likely affects 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 (*44*). By contrast, constitutive expression of *LFY* in wheat increases the number of lateral spikelets, likely by delaying the IM>TS transition (Fig. 3). Mutations in *LFY* also result in opposite effects in eudicots and grasses. Weak *lfy* mutants delay the formation of flowers and increase the number of secondary branches in Arabidopsis and other eudicot species (*42, 43, 45-47*). In contrast, *LFY* loss-of-function mutations result in significant reductions in SNS in wheat (Fig. 2), and in the number of branches in rice panicles (*25*) and maize male inflorescences (*40*). These results indicate that in grasses, *LFY* promotes indeterminate growth and prevents the precocious formation of terminal spikelets, whereas in Arabidopsis it promotes the formation of flowers.

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 meristem determinacy.

Interactions between LFY and WAPO1 are critical for their functions

LFY and WAPO1 proteins physically interact with each other in wheat (Fig. 4), barley (*39*), rice (*25*) and Arabidopsis (*22*). In Arabidopsis, UFO interaction with LFY modifies its DNA binding specificity and alters the set of genes directly regulated by LFY (*24*), 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 (*20*)). The simultaneous regulation of class-B, -C and -E MADS-box genes seems to be also common in the temperate grasses, where *lfy* mutations in wheat (fig. S10) and barley (*39*), and *wapo1* mutants in wheat (*16*) have been 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 *lfy* (Fig. 2) and *wapo1* mutants (*16*) in these species, 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 (*19*). By contrast, the *wapo1* mutant in wheat shows similar reductions in SNS as the *lfy* and *lfy wapo1* combined mutants (Fig. 7A). Similar reductions in the number of panicle branches are also observed in the *apo1*, *apo2* and combined *apo1 apo2* mutants in rice (*25*). These results suggest that the physical interaction between LFY and WAPO1 likely plays a more prominent role in the regulation of IM determinacy in grasses than in Arabidopsis.

Interactions between *LFY* and *SQUAMOSA* genes modulate their opposite effects on IM determinacy

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 (*8*), whereas in the Arabidopsis *ap1 cal ful* triple mutant flowers are transformed into leafy shoots (*48*). In Arabidopsis, AP1 orchestrates floral initiation by directly regulating hundreds of genes (*49*), a

function that is favored by AP1's ability to modulate chromatin accessibility of its target genes (*50*). 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 (*51, 52*).

In Arabidopsis, *LFY* positively regulates the expression of *AP1* and *CAL* (*26-28*) and, therefore, indirectly regulates their direct targets. *LFY* also directly regulates hundreds of genes independently of *AP1* and *CAL* (*53*). 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 (*53*). 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 the *LFY* direct regulation of the *SQUAMOSA* genes in Arabidopsis but not in wheat (fig. S7) likely contributes to the opposite functions of *LFY* in the regulation of the IM determinacy between these species.

Approximately 200 genes have been identified in Arabidopsis as high-confidence direct targets of both LFY and AP1 (*54*). 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 (*53*). These common gene targets can contribute to the epistatic 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 between *LFY* and *VRN1* in this study (Fig. 8A). 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 S20). These results indicate that the interaction between *LFY* and *SQUAMOSA* explain 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. 5B-C), a pattern reported previously by *in situ* hybridization (*55*). This spatial differentiation continues in the young SMs, where *LFY* is abundant in the proximal region but rare in the distal region (Fig. 5B-C). A similar pattern was also reported in the SMs of young barley spikes (*56*) and in the primary and secondary branch meristems in the developing rice panicles (*57, 58*). These results suggest a conserved *LFY* spatial pattern in developing grass inflorescences.

In wheat, the SM regions with low *LFY* expression show high levels of *VRN1* and *FUL2* transcripts (Fig. 6C-D) and, therefore, high *SQUAMOSA / LFY* ratios. We hypothesize that this change in the balance between promoting and repressing pioneer transcription factors is critical to mark the regions where the lateral spikelets will develop. This hypothesis is also supported by the drastic changes in the transcript densities of these genes in the IM and two youngest lateral meristems at the time of the IM>TS transition (determined by the presence of *FZP*, fig. S5). 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 (data S14).

Changes in gene dosage for *LFY* 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. S2), 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 (data S14) (*8*). Interestingly, four recently cloned genes affecting SNS in wheat including *WAPO1* (*16*), *FT-A2* (*12, 59*), *bZIPC1* (*15*) and *SPL17* (*60*) 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 a central role in the determination of SNS in wheat.

The smFISH results provided valuable insights on the potential roles of *LFY* and the *SQUAMOSA* genes on the regulation of the IM>TS transition, but also revealed unexpected results for *WAPO1.* Given the strong genetic interactions for SNS between *WAPO1* and *LFY* (Fig. 7A), we were expecting to find both genes colocalized in the IM close to the IM>TS

transition. *WAPO1* and *LFY* transcripts overlapped in the IM at the initial stages of spike development (W1.5, Fig. 5), but at later stages, *WAPO1* transcripts were restricted to the basal - central region of the developing spike (Fig. 5 and S6). Non-mutually exclusive hypotheses to explain this unexpected result include: *i*) expression of *WAPO1* in the IM below our current detection levels, *ii*) persistence of the WAPO1 protein in the IM, and *iii*) transport of the WAPO1 protein from the base of the developing spike to the IM through the differentiating vascular tissue.

Spikelet development

The smFISH also provided insights on 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 (Fig. 5D). *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. 6 and S9), suggesting that LFY and WAPO1 may provide important spatial information. We speculate that the adjacent expression domains of *LFY* and *WAPO1* in the developing spikelets generate a curved region of contact (Fig. 5D), where proteins encoded by these two genes may have a higher opportunity to interact with each other. Based on the published Arabidopsis results (*24*), we also speculate that within this dynamic overlap region WAPO1 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 downregulated in both the *lfy* (fig. S10) and *wapo1* mutants (*16*), and by the common floral defects observed in the *lfy* and *wapo1* mutants. In summary, these results suggest that the spatial information provided by LFY and WAPO1 is critical for the normal development of the floral organs in the multiple florets formed within each of the wheat spikelets.

Epistatic interactions among genes controlling SNS have important practical implications

The strong effect of the *vrn1* mutants on SNS suggest that this gene could be a possible target for SNS improvement. However, the strong pleiotropic effects of these mutations on heading time (*61*) limit their utilization for SNS improvement in wheat breeding programs. To avoid these pleiotropic effects recent efforts have been concentrated on the identification of genes with natural variants affecting SNS but with limited effects on heading time.

A recent example is the natural D10A mutation identified in the FT-A2 protein in wheat, which showed positive effects on SNS, grain number per spikes, and grain weight per spike, with limited effects on heading time. The derived *FT-A2* allele associated with increased SNS (A10) showed evidence of positive selection in hexaploid wheat supporting its potential value for breeding applications (*59*). FT2 physically interacts with bZIPC1, which also showed differences in SNS associated with induced and natural variation, and evidence of positive selection in modern wheat varieties (*15*). Genetic interactions for SNS were detected between natural variants for *FT-A2* and *bZIPC-B1*, with the alleles resulting in the optimum combination for SNS showing simultaneous frequency increases in modern wheat varieties (*15*).

In this study, we observed increases in the frequency of the *LFY-B* allele P+,34L,80S in hexaploid wheat (Fig. 1E) that parallel those described previously for the *WAPO-A1* allele 47F (*16, 17*). These two alleles, when combined, showed a favorable effect on SNS (Fig. 7B) with limited pleiotropic effects on heading time. Taken together, the significant *FT-A2*/*bZIPC-B1* and *LFY-B/WAPO-A1* interactions for SNS, and the simultaneous increases in the frequencies of the optimal allele combinations suggest that alleles with significant genetic and physical interactions are likely to be affected simultaneously by natural or human selection.

Wheat breeders usually select for higher grain yield, larger spikes, and/or spikes with more grains, exerting a strong selection pressure on genes controlling the IM>TS transition. This pressure has resulted in gradual increases in the frequencies of alleles or allele combinations that favor SNS increases. However, the acceleration of current rates of yield improvement and the engineering of optimal allele combinations of genes controlling different grain yield components requires a better understanding of the epistatic interactions among the genes controlling these traits. This study highlights the value of combining developmental and genetic studies to identify optimal allele combinations to maximize yield components in a crop species that is critical for our global food security.

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Competing Interests

No competing interests declared

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Author contributions

FP, JZ and HL performed most of the experimental work and contributed to data analysis. FP provided the first version of the paper except for the natural variation section that was provided by JZ. HL led the smFISH experiment. GFB contributed the Alpha-fold figures. CL contributed the transgenic plants, and supervised HL. TL and AJ contributed to several experiments. DW and JMD supervised FP, helped to characterize the mutants and transgenic lines, and contributed to the design of several experiments. JD designed and coordinated the project, obtained funding, supervised the research team, performed statistical analyses, and wrote the final version of the manuscript. All authors contributed to the writing and revision of the manuscript.

Data Availability

All data generated during this study and the corresponding statistical analyses are included the supporting Excel file. The sequence of *LFY* for the H3 haplotype has been deposited in GenBank

as accession OR753911. Kronos mutants K2613 for *lfy-A* and K350 for *lfy-B* are available from the authors upon request without any restrictions for use and from the Germplasm Resources Unit (GRU) at the John Innes Centre. Images for the spike sections used in the smFISH and hybridization coordinates for the genes presented in this study are available at https://dubcovskylab.ucdavis.edu/content/spatial-transcriptomic.

Materials and Methods

Plant Materials

We first tested if natural variation in the chromosome regions including *LFY-A* and *LFY-B* were associated with differences in spikelet number per spike (SNS), to determine the agronomic potential of a study including these genes. We reanalyzed a set of nested association mapping populations that were previously characterized for this trait in three field experiments under full and limited irrigation at the University of California, Davis between 2014 and 2016 (*7*). We only detected significant differences in SNS associated with the *LFY-B* region in one of the populations (Berkut x PBW343). To validate this association, we developed heterogeneous inbred families (HIFs) from two F_5 lines (SSD40 and SSD63) from this population that were still heterozygous for the *LFY-B* region, and evaluated them for SNS.

To confirm the linkage between *LFY-B* and the QTL for SNS, we screened 385 $F_{5:3}$ lines (770 chromosomes) segregating for the *LFY-B* region for recombination events between flanking markers 2B-657.1 (2B: 657,072,439) and 2B-660.1 (660,114,463, data S5). We genotyped the recombinant lines with six additional markers developed within this region (data S5) and identified three $F_{5:2}$ lines heterozygous for *LFY-B* with close recombination events at both sides of *LFY-B* (data S7). From the progeny of these lines, we selected 10 $F_{7:2}$ plants homozygous for the recombinant chromosome and 10 sister plants homozygous for the non-recombinant chromosome, carrying the alternative *LFY-B* alleles. We evaluated the $F_{7:3}$ progeny of these 20 lines as single 1m rows in the field in 2021, at the UC Davis Experimental Station.

Natural variation and haplotype analysis

We performed a haplotype analysis for an 813.4 kb region of chromosome 2B including *LFY-B* (657,790,878 to 658,604,020 in CS RefSeq v1.1) using exome capture data from 56 common and

pasta wheat accessions (https://wheat.triticeaetoolbox.org/downloads/download-vcf.pl) generated by the WheatCAP project (Exome_Capture_2017_WheatCAP_UCD). To obtain the complete *LFY* sequences associated to the different haplotypes, we assigned the different wheat sequenced genomes (*29, 62-64*) to the haplotypes identified in the previous analysis.
The previous analysis revealed four polymorphisms among the different *LFY* alleles, for which we developed molecular markers. We used these markers to screen a wheat germplasm collection including 85 *T. turgidum* ssp. *dicoccoides*, 86 *T. turgidum* ssp. *dicoccon*, 384 *T. turgidum* ssp. *durum*, and 234 *T. aestivum* accessions, which are part of a photoperiod insensitive spring wheat panel from North America previously evaluated for multiple agronomic traits (*7*).

Epistatic interactions between natural variants of *LFY-B* and *WAPO-A1*

To evaluate the combined effect of the natural variants in *LFY-B* and *WAPO-A1*, we characterized two hexaploid and two tetraploid populations segregating for both genes. For *WAPO-A1*, we focused on the C47F amino acid change which was previously shown to be associated with significant differences in SNS (*16*). The first hexaploid population, Berkut x RAC875, included 75 RILs that were previously evaluated for SNS in six different environments (*7*). The second hexaploid population, RSI5 x RAC875, was evaluated in three separate experiments, first as F_2, then as $F_{2:2}$ plants derived from F_2 plants heterozygous for *LFY-B* and *WAPO-A1*, and finally as $F_{3:2}$ plants derived from F_3 plants heterozygous for both genes. Plants homozygous for the four possible *LFY-B/WAPO-A1* allele combinations were selected with molecular markers and evaluated for SNS. Average SNS was calculated from two spikes per plant from the two taller tillers.

For the first tetraploid population, we crossed Kronos x Gredho (which carried the desired *LFY-B* allele) and then backcrossed it to a Kronos line in which we have previously introgressed the *WAPO-A1* 47F allele from hexaploid wheat (*16*). We backcrossed this plant four times to the Kronos-47F line, selecting in each generation plants heterozygous for both genes using molecular markers. Finally, we generated BC_4F_2 seeds from a single BC_4F_1 plant heterozygous for both genes and planted it in the field at UC Davis in November 2022 as single plants. The second population was generated from the cross between Kronos and an unusual durum line from Syria (PI 519639) which carried the desired *LFY-B / WAPO-A1* allele combination. The F_1 was backcrossed once to Kronos, and one BC_1F_3 plant heterozygous for both alleles was used to

generate $BC_1F_{3:2}$ plants that were grown in the UC Davis field for SNS evaluation. We genotyped both populations using molecular markers, and evaluated the mature plants homozygous for the four different *LFY-B/ WAPO-A1* combinations in 2023 for SNS using three spikes per plant.

Ethyl methanesulfonate (EMS) induced mutants

We screened the sequenced tetraploid wheat variety Kronos population (*33*) 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_{1-2}. 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 S5.

The *lfy* mutant was intercrossed with Kronos lines developed in previous studies homozygous for loss-of-function EMS mutations in the A and B genomes of *VRN1* (*65*) and *FUL2* (*8*), and for CRISPR generated loss-of-function mutations in *WAPO1* (*16*), to generate higher order mutant combinations. 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.

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 plant level. Plants were grown under inductive 16-hour 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, and that time the spikelet number per spike (SNS) was measured 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 S5. 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 wheat variety Kronos using *Agrobacterium*-mediated transformation (EHA105) at the UC Davis Plant Transformation Facility as described before (*66*). The presence of the *LFY* transgene was determined with primers LFY-Genotyping-R5 and UBI-F2 listed in data S5.

LFY transcript levels were determined by qRT-PCR using primers LFY_qPCR_F and LFY_qPCR_R and *ACTIN* as endogenous control (data S5). 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 (*67*). 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 $2^{(ACTIN\ C_T - TARGET\ C_T)}$ ± 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 (*15*).

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 S15), we targeted the homoeolog expressed at higher levels in a Kronos transcriptome including different stages of spike development (*32*), and provided Resolve BioSciences their respective homeologs to be excluded in their quality control for 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 (*15*). 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 (*68*), 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 S5), 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.

Supplementary figures

fig. S1. Alignment of LFY proteins from multiple grass species.

fig. S2. Effect of individual and combined *LFY* mutants on spikelet number per spike (SNS).

fig. S3. *LFY* transcript levels in transgenic plants overexpressing *LFY*.

fig. S4. Expression analysis of *LFY* and *WAPO1*.

fig. S5. Single-molecule fluorescence *in situ* hybridization for *FRIZZY PANICLE* (*FZP*).

fig. S6. Single-molecule fluorescence *in situ* hybridization for *WAPO1*.

fig. S7. Effect of *lfy* mutations on *VRN1* and *FUL2* expression.

fig. S8. Single-molecule fluorescence *in situ* hybridization for *FUL3*.

fig. S9. Single-molecule fluorescence in situ hybridization for *SEPALLATA* genes.

fig. S10. Effect of *lfy* mutations on the expression of floral organ identity genes.

fig. S11. Simplified working model of the regulation of SNS in wheat.

Supplementary Data

data S1. SNS QTL linked to *LFY-B* in the Berkut x PBW343 segregating RIL population.

data S2. Supporting data for Fig. 1A and D.

data S3. Haplotype analysis of the *LFY-B* region.

data S4. *LFY-B* haplotypes in wheat varieties sequenced in the pangenome project.

data S5. Primers used in this study.

data S6. Frequency of the different *LFY-B* alleles in tetraploid and hexaploid wheat.

data S7. Supporting data Fig. 1F.

data S8. Genes annotated in the candidate gene region for the SNS QTL.

data S9. Supporting data for Fig. 2C-E.

data S10. Supporting data for fig. S2.

data S11. Supporting Data for fig. S3.

data S12. Supporting data for Fig. 3.

data S13. Supporting data for fig. S4.

data S14. *LFY* transcripts density at the distal region of the developing spike.

data S15. Genes used in the sm-FISH experiments.

data S16. Supporting data for fig. S7.

data S16. Supporting data for fig. S10.

data S18. Supporting data for Fig. 7A.

data S19. Supporting data for Fig. 7B-D.

data S20. Supporting data for Fig. 8.

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