Interactions between SQUAMOSA and SHORT VEGETATIVE PHASE MADS-box proteins regulate meristem transitions during wheat spike development

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Abstract

Inflorescence architecture is an important determinant of crop productivity. The number of spikelets produced by the wheat inflorescence meristem (IM) before its transition to a terminal spikelet (TS) influences the maximum number of grains per spike. Wheat MADS-box genes VERNALIZATION 1 (VRN1) and FRUITFULL 2 (FUL2) (in the SQUAMOSA-clade) are essential to promote the transition from IM to TS and for spikelet development. Here we show that SQUAMOSA genes contribute to spikelet identity by repressing MADS-box genes VEGETATIVE TO REPRODUCTIVE TRANSITION 2 (VRT2), SHORT VEGETATIVE PHASE 1 (SVP1), and SVP3 in the SVP clade. Constitutive expression of VRT2 resulted in leafy glumes and lemmas, reversion of spikelets to spikes, and downregulation of MADS-box genes involved in floret development, whereas the vrt2 mutant reduced vegetative characteristics in spikelets of squamosa mutants. Interestingly, the vrt2 svp1 mutant showed similar phenotypes to squamosa mutants regarding heading time, plant height, and spikelets per spike, but it exhibited unusual axillary inflorescences in the elongating stem. We propose that SQUAMOSA–SVP interactions are important to promote heading, formation of the TS, and stem elongation during the early reproductive phase, and that downregulation of SVP genes is then necessary for normal spikelet and floral development. Manipulating SVP and SQUAMOSA genes can contribute to engineering spike architectures with improved productivity.
IN A NUTSHELL

Background: More than 750,000,000 tons of wheat grains are produced globally each year. These grains are produced within an inflorescence known as the spike, which has multiple lateral secondary inflorescences called spikelets. The number of spikelets, and consequently the number of grains per spike, is determined when the terminal spikelet is formed. We previously showed that developmental genes of the SQUAMOSA family are essential to promote the formation of the terminal spikelet and for spikelet development. Therefore, combined SQUAMOSA mutants have more spikelets but they are transformed into vegetative organs. Here we show that another group of developmental genes of the SVP family contributes to spikelet number through their interactions with the SQUAMOSA genes without having negative effects on spikelet characteristics.

Question: How do developmental genes of the SQUAMOSA family control spikelet number and spikelet development in wheat?

Findings: By comparing the expression of genes in SQUAMOSA mutants vs. normal wheat plants during spike development, we discovered three genes of the SVP family that were expressed at very high levels in the SQUAMOSA mutants. Combined SVP mutants showed a higher number of spikelets per spike, delayed heading and shorter plants. When one SVP mutant was combined with the SQUAMOSA mutants, it reduced the vegetative characteristics of the spikelets. Interestingly, the SVP mutant exhibited axillary inflorescences in the elongating stem (like a maize cob) that are not observed in normal wheat. We propose that SVP genes control the formation of axillary spikes and spikelets. We also propose that SQUAMOSA–SVP interactions are important to promote heading, formation of the terminal spikelet, and stem elongation in early reproductive development, and that down regulation of SVP genes is then necessary for normal spikelet and floral development.

Next steps: The induced mutations in the SVP genes, and some recently discovered natural mutations in these genes, increase the number of spikelets per spike. These mutants are useful tools for wheat breeders to increase the number of grains per spike and wheat productivity. We are currently introgressing these variants into commercial wheat varieties to test their effects on total grain yield.

Introduction

Each year more than 750,000,000 tons of wheat (Triticum aestivum L.) grains are produced around the world, providing one-fifth of the calories and protein consumed by the human population (FAOSTAT, 2017). These wheat grains are produced in an inflorescence called the spike, which is generated by the inflorescence meristem (IM). The IM first produces multiple axillary meristems, called spikelet meristems (SMs), each subtended by a suppressed leaf ridge. Then, the SMs differentiate into sessile spikelets on the spike axis (rachis) and the IM transitions into a terminal spikelet (TS), resulting in a determinate inflorescence.

The spikelet is the basic unit of the grass inflorescence (Kellogg, 2001) and, in wheat, it comprises two basal sterile bracts (glumes) and an indeterminate number of florets. Each floret has a bract called lemma with an axillary floral meristem (FM) that generates a two-keeled structure called palea, two scales called lodicules, three stamens, and a terminal ovary (Clifford, 1987). In wheat, the SM produces an indeterminate number of FMs on an axis called racilla, with only the most basal florets surviving to set grains (Sakuma et al., 2019).

Variation in the activity and maturation rate of meristems has profound effects on inflorescence architecture and crop productivity (Park et al., 2014; Liu et al., 2021b). In wheat, the timing of the transition from the IM to TS determines the number of spikelets per spike, which, together with the number of fertile florets per spikelet, determines the maximum number of grains that a spike can produce. Because these are important components of grain yield, a better understanding of their regulatory mechanisms will be useful to engineer more productive wheat plants.

Significant progress has been made in understanding the pathways regulating grass inflorescence development, particularly in rice (Oryza sativa L.) and maize (Zea mays L.). A complex gene network involving several members of the MADS-box gene family regulates the identity shifts of different meristems in these species (Callens et al., 2018; Wu et al., 2018; Chongloi et al., 2019). During floral development, MADS-box proteins act as tetrameric complexes, and different protein combinations result in the specification of different organ identities, as has been documented in the ABCDE model of flower development in Arabidopsis (Arabidopsis thaliana (L.) Heynh.) (Theissen et al., 2016) and rice (Wu et al., 2018), which is defined by the five classes of homeotic genes involved, named A, B, C, D and E.

In wheat, there is currently limited knowledge of the role of these genes in spike development. We have recently shown that wheat MADS-box meristem identity genes VRN1 and FUL2 from the SQUAMOSA-clade are essential for the transition of the IM to a TS (Li et al., 2019). In the vrn1 ful2 loss-of-function mutant, the inflorescence remains indeterminate and fails to produce TS, whereas in the single vrn1 or ful2 mutants the transition to TS is
delayed, leading to an increased number of spikelets per spike.

In addition, SQUAMOSA genes are essential for spikelet identity specification. In the vrn1 ful2 mutant, the axillary meristems in the spike develop into vegetative structures resembling tillers, some of which have residual flower organs. When the loss-of-function mutations in FUL3 homeologs (the third member of the SQUAMOSA-clade) were combined in a vrn1 ful2 ful3 mutant, the spike axillary meristems generated fully vegetative tillers and the leaf ridges were derepressed and formed leaves (Li et al., 2019). These results demonstrated that VRN1, FUL2, and FUL3 have redundant and essential roles in SM identity, spikelet development, and repression of the lower leaf ridge.

The wheat SQUAMOSA genes also regulate the initiation of reproductive development and affect both heading time and plant height. VRN1 is a key gene affecting flowering in wheat (Yan et al., 2003). Spring wheat varieties carrying dominant Vrn1 alleles do not have a vernalization requirement, whereas winter wheat varieties with the functional but recessive vrn1 allele require several weeks of vernalization to acquire flowering competence (Yan et al., 2003; Fu et al., 2005; Kippes et al., 2018). The transition from a vegetative meristem (VM) to an IM is delayed in the vrn1-null mutant, further delayed in vrn1 ful2 and the most greatly delayed in vrn1 ful2 ful3, which indicates redundant roles of these three genes in the regulation of the initiation of the reproductive phase (Li et al., 2019). Functional redundancy was also observed for plant height, with the vrn1 ful2 ful3 mutant being shorter than any other mutant combinations (Li et al., 2019).

In this study, we aimed to identify the gene network regulated by VRN1 and FUL2 during the early stages of spike development in wheat. In particular, we wanted to identify the specific genes responsible for the reversion of spikelets to tillers in the vrn1 ful2 mutant. By comparing the developing spike transcriptomes of vrn1 ful2 (spikelets transformed into tillers) and vrn1 mutants (normal spikes), we identified three MADS-box genes of the SHORT VEGETATIVE PHASE (SVP) clade that were upregulated in the vrn1 ful2 mutant. These genes include SVP1, VEGETATIVE TO REPRODUCTIVE TRANSITION 2 (VRT2, synonymous SVP2; Kane et al., 2005), and SVP3 (Schilling et al., 2020). Gene names, synonyms, accession numbers, and orthologs in rice of the MADS-box genes analyzed in this study are provided in Supplemental Table S1.

Here, we show the complementary and overlapping roles of SQUAMOSA and SVP genes in the early reproductive phase, and their antagonistic effects during spikelet and initial floral development. We also show that constitutive expression of VRT2 promotes leafy glumes and lemmas, likely by the downregulation of multiple MADS-box genes of the SEPALLATA (SEP) clade, which are known to be involved in floral development. Finally, we describe a complex network of interactions among wheat proteins from the SQUAMOSA, SVP, and SEP clades. The SQUAMOSA genes promote the downregulation of SVP genes, and this facilitates the interactions between SQUAMOSA and SEP proteins that are critical for normal spikelet and floral development.

Results

Quant-Seq analysis of developing wheat spikes in vrn1 and vrn1 ful2 mutants identifies genes regulated by VRN1 and FUL2

During early spike development, the IM in the vrn1 mutant produces axillary meristems that acquire SM identity and develop into spikelets, whereas in the vrn1 ful2 mutant, the IM produces axillary VMs that later develop into tillers subtended by a bract (Li et al., 2019). To identify the genes and pathways that repress the vegetative program and activate the spikelet identity program, we compared the transcriptomes of developing apices of these two mutants at four developmental stages covering the early steps of spike development: vegetative (VEG), double ridge (DR), post double ridge (PDR) and TS (Figure 1A). The average number of unique reads per sample and other transcriptome statistics are summarized in Supplemental Table S2.

In the comparisons between developing spikes of vrn1 and vrn1 ful2 mutants at the VEG and DR stages, we found 187 differentially expressed genes (DEGs; 86 downregulated and 101 upregulated) and 209 DEGs (63 downregulated and 146 upregulated), respectively (Figure 1B; Supplemental Data Set S1). These numbers greatly increased in the PDR and TS stages to 1,574 and 1,753 DEGs, respectively (Figure 1B).

We then performed a cluster analysis of the 1,399 upregulated and 1,268 downregulated nonredundant DEGs (Figure 1C) based on their expression profiles across the four developmental stages. This analysis resulted in five clusters for each of the two sets, which included at least 10% of the up- or downregulated genes (Figure 1, D and E; Supplemental Data Set S1). Clusters 4 and 5 included genes that were upregulated in vrn1 but not in vrn1 ful2 at PDR (Cluster 4) or TS (Cluster 5). The gene ontology (GO) analysis of these clusters revealed an enrichment of genes involved in early reproductive development in Cluster 4 and in flower development in Cluster 5, including four genes of the SEP clade (Figure 1, D and F). These results are consistent with the reproductive fate of the SM in vrn1 relative to vrn1 ful2, where they develop into tiller-like structures. Additional genes from these clusters with known roles in inflorescence development are described in Supplemental Table S3.

We observed the opposite profiles in Clusters 9 and 10, which included genes upregulated in vrn1 ful2 but not in vrn1 between DR and PDR (Cluster 9) or at TS (Cluster 10) (Figure 1, E and G). The GO analysis of these clusters revealed an enrichment for genes involved in photosynthesis (Figure 1E), which is consistent with the vegetative fate of the vrn1 ful2 spike axillary meristems. These clusters also include florigen antagonists CENTRODIALIS 2 (CEN2), CEN4, and CEN5 (Figure 1G). Cluster 8 showed a peak at the DR stage and included several genes previously shown to be
Figure 1 DEGs in developing spikes of vrn1 and vrn1 ful2 mutants. A, Representative images of vegetative (VEG) and reproductive apices (DR = double ridge; PDR = post double ridge; TS = terminal spikelet) from vrn1 (upper row) and vrn1 ful2 (lower row) collected for Quant-seq analysis. Bars = 100 μm. B, Number of DEGs between vrn1 and vrn1 ful2 identified at each stage. C, Venn diagrams showing the nonredundant DEGs in comparisons among the different developmental stages. Downregulated (left) and upregulated (right). D and E, Mean normalized expression patterns for clusters of nonredundant DEGs in vrn1 and vrn1 ful2 samples. Red lines represent the mean of all DEGs in the cluster, light gray lines show individual DEGs. Significantly enriched GO terms were identified only for Clusters 1, 3, 4, 5, 9, and 10, and are presented below the curves. F, Heat map showing mean normalized expression of selected genes for Clusters 4 and 5. G, Heat map showing the relative expression patterns of selected genes for Clusters 7, 8, 9, and 10. In the scale, 0–2 indicates mean normalized expression. A full list of DEGs for the 10 clusters is presented in Supplemental Data Set S1, and a description of DEGs with known functions in inflorescence development in Supplemental Table S3.
Identification and combination of loss-of-function mutants for VRT2 and SVP1 in tetraploid wheat (Triticum turgidum L. subsp. durum (Desf.) Husn.)

We selected truncation mutations for the A and B genome homeologs of VRT2 and SVP1, which are summarized in Figure 2, A and B, respectively (for more detail see "Materials and methods"). To generate the VRT2 loss-of-function mutant, designated hereafter as vrt2, we combined the premature stop codon mutation Q125* in the A-genome homeolog (vrt-A2) with a splice site mutation in the B-genome homeolog (vrt-B2; Figure 2A). This vrt-B2 mutation results in splice variants with premature stop codons or a large deletion in the middle of the protein (Supplemental Figure S2). To generate the SVP1 loss-of-function mutant, designated hereafter assvp1, we intercrossed an svp-A1 mutant carrying a splice site mutation that generates splice variants with premature stop codons (Supplemental Figure S2) with an svp-B1 mutant carrying the premature stop codon Q99* (Figure 2B). We generated PCR markers for each of these four mutations to trace them in the different crosses and backcrosses (Supplemental Table S4).

The selected vrt2 and svp1 mutants are likely loss-of-function mutants (or severely hypomorphic mutants) because the encoded proteins have truncations that eliminate more than half of the conserved K domain or, for one of the alternative splice forms of svp-A1, a protein with a large deletion including parts of the MADS and K domains (Figure 2B; Supplemental Figure S2). Figure 2C presents the crosses and backcrosses used to generate vrt2, svp1, vrt2 svp1, and the higher-order mutants described in other sections of this study.

The vrt2 and svp1 mutations delay heading time, reduce plant height, and increase number of spikelets per spike

Plants homozygous for vrt2 or svp1 mutations were shorter, flowered later, and had higher SNS than the wild-type (WT), and all these effects were magnified in the vrt2 svp1 mutant (Figure 3, A and B). Statistical analyses of these traits showed that vrt2 and svp1 mutants headed significantly later than WT in two independent experiments (2.9–4.8 days, Figure 3C; Supplemental Figure S3, A and B; Supplemental Data Set S2), indicating a small but consistent effect of both genes as promoters of heading time. In vrt2 svp1, the delay in heading time (29 days) was much larger than the sum of the individual gene effects, reflecting a highly significant interaction between these two genes (Supplemental Table S5). Mutations in the individual A- and B-genome homeologs of each gene showed no significant differences in heading time (Supplemental Figure S3).

The late heading time of the vrt2 svp1 mutant was correlated with a highly significant increase in leaf number (4.4 more leaves than WT, \( P < 0.001 \), Figure 3D), which
indicates that part of the delay in heading time was caused by a delayed transition of the shoot apical meristem (SAM) from the vegetative to the reproductive stage. Using qRT-PCR, we detected reduced expression levels of the flowering promoting genes VRN1 and FT1 and higher levels of the flowering repressor VRN2 in the fifth leaf of the vrt2 svp1 mutants compared with the WT (Supplemental Figure S4; Supplemental Data Set S2).

Both vrt2 and svp1 produced significantly more spikelets per spike than the WT under controlled environmental conditions, with larger differences between the VRT2 alleles (3.6–4.9 spikelets) than between the SVP1 alleles (2.1–2.9 spikelets; Figure 3E; Supplemental Figure S3, C and D). The increase in SNS in the vrt2 svp1 mutant relative to the WT (13 spikelets or ~70% increase) was larger than the added differences of the individual mutants, indicating a highly significant interaction (Supplemental Table S5). Mutants for the A- and B-genome homeologs of both genes showed significant effects on SNS under controlled environments, and these effects were validated for vrt2 in the field (Supplemental Figure S3, C, D, and G; Supplemental Data Set S2).

We also observed significant reductions in plant height in vrt2, svp1, and vrt2 svp1 mutants (Figure 3F), and detected a significant interaction between the two genes ($P = 0.0032$, Supplemental Data Set S2). The effect of vrt2 on plant
height (–17.5 cm) was stronger than that of svp1 (–6.8 cm, Figure 3F), and both were determined mainly by reductions in peduncle length (Supplemental Figure S3, E and F; Supplemental Table S5). In a field experiment, the effect of vrt2 on plant height was even stronger than in the controlled environment experiments (–28.4 cm; Supplemental Figure S3, H and I; Supplemental Data Set S2).

In summary, these results indicate that VRT2 and SVP1 have overlapping functions during early reproductive development in wheat, accelerating the transitions from VM to IM and from IM to TS, and promoting elongation of the peduncle.

The vrt2 svp1 mutant has axillary spikes at the nodes of the elongating stems

A surprising characteristic of the vrt2 svp1 mutant was the presence of axillary spikelets or spikes subtended by leaves in the nodes of the elongating stem below the peduncle (Figure 4A). Although axillary inflorescences are common in some species from other grass subfamilies including the Bambusoideae, Andropogoneae, and Panicoideae (Stapleton, 1997; Vegetti, 1999), WT wheat does not have axillary spikelets, spikes, or tillers in the nodes of the elongating stem (Figure 4, B–D).

We observed a gradient in the development of the axillary buds, with those in node –1 closest to the spike developing into a single spikelet (Figure 4E), those in node –2 into one or two spikelets, and those in node –3 into normal spikes with multiple spikelets (Figure 4, I, J, and N). The single axillary spikelets in node –1 were initially enclosed by one bract (Figure 4I). Upon removal of these bracts, we observed normally developing spikes (Figure 4J), which were delayed in their development relative to the corresponding apical spikes at the same time point (Figure 4A). These unusual axillary spikelets and spikes were also observed in some nodes of vrt2 (Figure 4K) and svp1 (Figure 4L) individual mutants, but at lower frequencies (Figure 4M) and were usually less developed than in the vrt2 svp1 mutant. In vrt2 svp1 mature plants, some axillary spikes were able to emerge from the subtending leaves (Figure 4N, yellow arrow).

Expression patterns of VRT2 and SVP1 in wheat inflorescences correlate with mutant phenotypes

To further characterize VRT2 and SVP1 genes, we performed in situ hybridization at different stages of spike development.
We also included VRN1 and FUL2 in our in situ hybridization analyses to compare the expression patterns of SVP and SQUAMOSA genes. In Kronos, VRN1 and FUL2 expressions were detected in leaf primordia and in the SAM. During inflorescence development, both genes were expressed in the IM, in the emerging SM and in the subtending vegetative ridge (Supplemental Figure S5) supporting their roles in spikelet development, repression of the vegetative ridge, and determinacy of the IM (Li et al., 2019). Similar expression patterns were observed in diploid Triticum monococcum L. (Supplemental Figure S5, D and I).

In agreement with the expression profile observed in the Quant-Seq analysis for the SVP-clade genes (Figure 1G), the in situ hybridizations with VRT2 and SVP1 revealed a progressive decrease of signal in the IM and SM with spike development (Figure 5, A–C and E–G). When spikelets reached the stamen primordia stage, we observed a strong signal in the stamen primordia for SVP1 (central spikelets develop earlier in wheat; Figure 5C), but not for VRT2 (Figure 5C). This latter result is consistent with the expression pattern reported for the SVP1 homolog OsMAD22 in rice (Pelucchi et al., 2002; Sentoku et al., 2005).

Both SVP1 and VRT2 showed hybridization signal in leaf primordia below the developing spikes, with a stronger signal at the base of these organs. Similar expression profiles were detected in Kronos (Figure 5, A–C and E–G) and T. monococcum (Supplemental Figure S6). In contrast to WT Kronos, the vrn1 ful2 mutant showed ectopic expression of VRT2 and SVP1 at later stages of spike development, a result consistent with the Quant-Seq data (Figure 1G). This ectopic expression was concentrated in the spike axillary organ primordia that then develop into tiller-like structures (Figure 5, I–L).

Similar to VRT2 and SVP1, the CEN2, CEN4, and CEN5 genes showed higher expression levels after DR in vrn1 ful2 than in vrn1 in the Quant-Seq analysis (Figure 1G).
Therefore, we investigated the expression profiles of CEN2 in WT and vrn1 ful2 mutant by in situ hybridization. We selected CEN2 because it was expressed at higher levels than CEN4 and CEN5 in the developing spikes. In the WT Kronos, CEN2 showed strong hybridization signals at the base of the leaves of the early developing spikes, similar to VRT2 and SVP1 (Supplemental Figure S7, A–D). In the vrn1 ful2 mutant, we observed ectopic expression of CEN2 in the spike axillary organ primordia, similar to what we observed for VRT2 and SVP1 (Figure 5, I–L; Supplemental Figure S7E), suggesting that both SVP and CEN genes may contribute to the leafy characteristics of the spike axillary meristems in this mutant.

Constitutive expression of VRT2 alters spike development

To test whether the ectopic expression of SVP-like genes observed in the vrn1 ful2 mutant contributes to the vegetative characteristic of its inflorescences, we generated transgenic plants constitutively expressing VRT-A′′2 (cloned from T. monococcum A′′ genome) under the maize UBIQUITIN promoter (hereafter referred to as UBIpro:VRT2). We characterized three independent transgenic events (T#2, T#4, and T#8) that displayed varying degrees of phenotypic effects (Figures 6 and 7). The intensity of these phenotypic defects was partially correlated with VRT2 transcript levels in their developing spike at the TS stage. Transgenic lines T#2 and...
T#4 showed higher transcript levels than T#8, and all three had transcript levels significantly higher (P < 0.0001) than the nontransgenic sister lines (WT, Figure 8A).

The T#8 plants showed no significant differences in heading time, whereas the T#4 and T#2 plants headed 4.3 and 10.7 days later than the WT, respectively (P < 0.0001, Figure 6A). Interestingly, in this experiment, T#8 showed small but opposite effects to T#4 and T#2 for both stem length (Figure 6B) and SNS (Figure 6C). Relative to the WT, T#8 showed a significantly longer stem (2.6 cm, P = 0.008) and reduced SNS (1 spikelet, P = 0.0015), while both T#4 and T#2 showed shorter stems (12.2 and 11.8 cm shorter, P < 0.001, Figure 6B) and higher SNS (2.9 and 3.7 more spikelets, respectively, P < 0.001, Figure 6C). The increase in SNS in T#4 and T#2 resulted in significant increases in spikelet density (Figure 6D). All three transgenic lines showed longer glumes and lemmas than the WT, but the differences were significant (P < 0.001) only for T#4 and T#2 (Figure 6, E and F). T#2 exhibited the most severe morphological alterations (Figure 7, A–E), including very long glumes and lemmas, and replacement of basal spikelets by branches with multiple spikelets (also with elongated glumes and lemmas, Figure 7, C–E).

We explored the ability of the weakest UBIpro:VRT2 transgenic line (T#8) to complement the morphological changes observed in vrt2 in the F2 progeny of a cross between T#8 and vrt2 (Figure 2C). In the absence of the transgene, the vrt2 mutant headed 3.2 d later than the WT, but those differences disappeared in the presence of the transgene, indicating full complementation (Supplemental Figure S8A). The differences in peduncle length between the WT and vrt2 mutant (19.6 cm) were significantly reduced in the presence of UBIpro:VRT2 (11.4 cm, Supplemental Figure S8B), indicating partial complementation. However, there was no complementation for the differences in SNS, with similar increases in SNS in the vrt2 mutant relative to the WT in the transgenic and nontransgenic backgrounds (Supplemental Figure S8C).

To understand better the effect of UBIpro:VRT2 on the regulation of spikelet development, we used qRT-PCR to compare the transcript levels of several MADS-box flowering regulators between the three UBIpro:VRT2 transgenic and the nontransgenic sister line at the TS stage (Figure 8). We observed significant reductions in the transcript levels of A-class gene VRN1, B-class gene PI1, C-class gene AG1, and E-class genes SEP1-2, SEP1-4, SEP3-1, and SEP3-2 (Figure 8, B–E) in the strongest T#2 transgenic wheat line. The downregulation of these flowering regulators was correlated with the spike phenotypic changes in the different transgenic events, and was significant in the T#4 transgenic line only for SEP1-4, and not significant for all the studied genes for T#8 (Figure 8, B–E).

The ful2 mutant enhances spikelet defects in weak UBIpro:VRT2 transgenic plants

Strong constitutive expression of VRT2 resulted in spikelets with leaf-like glumes and lemmas similar to those observed in the partial mutants carrying one functional copy of VRN-A1 in the heterozygous state (Vrn1) and no functional copies of FUL2 (ful2), which was previously designated as Vrn1 ful2 (Li et al., 2019). Based on these results, we hypothesized that VRT2 and FUL2 may have opposite effects on spikelet development. To test if the combination of ful2 and UBIpro:VRT2 would enhance the spike and spikelet defects of the individual lines, we crossed the weak UBIpro:VRT2 T#8 with ful2.

The differences in stem length were highly significant for ful2 but not for UBIpro:VRT2 (Figure 9A; Supplemental Data Set S2). In contrast, SNS, glume length, and lemma length were significantly affected by both ful2 and UBIpro:VRT2, and highly significant interactions were detected for all three traits (Supplemental Data Set S2; Figure 9, B–D). Plants combining UBIpro:VRT2 T#8 and ful2 showed long leaf-like glumes and lemmas, similar to those of the strong transgenic lines T#4 and T#2 and a large increase in floret number (Figure 9E), a phenotype reported previously in Vrn1 ful2 mutant plants (Li et al., 2019). Dissection of basal spikelets showed that some florets were replaced by spikelets and that the rachilla ended in a TS (resembling a determinate branch) in seven of the eight plants analyzed (Figure 9, F–H). Taken together, these results suggest that VRT2 and FUL2 have antagonistic effects on spikelet development.

The vrt2 mutant reduces spikelet developmental defects in the Vrn1 ful2 mutant

Because VRT2 constitutive expression exacerbated the spikelet defects observed in the ful2 mutant, and VRT2 and SVP1 are ectopically expressed in vrn1 ful2 “spikelets” (Figure 5, I–L), we hypothesized that the loss-of-function of VRT2 could reduce some of the vegetative characteristics of the vrn1 ful2 “spikelets.” To test this hypothesis, we crossed vrt2 with Vrn1 ful2 and selected two pairs of sister lines, the first one homozygous for vrn1 (vt2 vrn1 ful2 and vrn1 ful2) and the second one with one functional copy of Vrn-A1 (vt2 Vrn1 ful2 and Vrn1 ful2).

The vrn1 ful2 mutant plants were taller than the vrt2 vrn1 ful2 mutant plants (Supplemental Figure S9, A–B), and most of its shoots produced spikes with axillary spikelets replaced by tillers (Supplemental Figure S9C), as described in a previous study (Li et al., 2019). In contrast, 84% of the inflorescences in the vrt2 vrn1 ful2 mutant failed to emerge (Supplemental Figure S9D). Spikes that emerged from the nontransgenic sister lines (WT, Figure 8A). Scanning electron microscope (SEM) images of the developing inflorescences at the PDR stage showed that, in both vrt2 vrn1 ful2 and vrn1 ful2, the spike axillary meristems resembled VMs, some of them bearing axillary buds in the first leaf-like primordia (Supplemental Figure S10, A–D). In the vrn1 ful2 SEM images, the visible buds were undifferentiated...
Dissection of vrn1 ful2 mature tiller-like organs revealed modified floral organs (Li et al., 2019), suggesting that these buds have the potential to eventually differentiate. Interestingly, in the SEM images of vrt2 vrn1 ful2, we observed more developed floret organ primordia (orange arrows) in the axils of some basal leaf-like primordia, indicating that vrt2 mutation may reduce “spikelet” defects of vrn1 ful2 mutants.

To better visualize the effect of the vrt2 mutation on spikelet development, we compared sister lines Vrn1 ful2 and vrt2 Vrn1 ful2 with one functional copy of Vrn-A1 (Figure 10; Supplemental Figures S10 and S11). SEM images of the early developing inflorescences showed no clear differences, with both genotypes bearing axillary SMs showing normal floret organ primordia (Supplemental Figure S10, E–H). However, clear morphological differences between these genotypes occur at later developmental stages.

The vrt2 Vrn1 ful2 mutant headed 3.5 days later (Supplemental Figure S11A), had shorter stems (Supplemental Figure S11B, not significant [NS]), and produced on average 4.7 more spikelets per spike (Supplemental Figure S11C) than Vrn1 ful2. The spikes of Vrn1 ful2 showed long glumes and lemmas (Figure 10B; Supplemental Figure S11, D and E), an unusually high number of florets (Figure 10E) and branches replacing 80% of the basal spikelets (Figure 10, G–H; Supplemental Figure S11F). In contrast, the spikes of vrt2 Vrn1 ful2 showed significantly shorter glumes and lemmas (Figure 10D; Supplemental Figure S11, D and E), a more normal number of florets (Figure 10F), and a reduced proportion of basal spikelets replaced by “branches” (30%, Supplemental Figure S11F). In summary, the spikes and spikelets of vrt2 Vrn1 ful2 appeared more normal than in Vrn1 ful2 (Figure 10, E–F), despite the presence of lemmas still showing some leafy characteristics (Figure 10I).

Figure 8 Relative expression of wheat flowering genes in developing spikes at the TS stage of UBIpro:VRT2 transgenic lines T#8, T#4, and T#2 and sister lines without the transgene (WT). A, VRT2 (transgenic plus endogenous transcripts). B, A-class MADS-box genes VRN1 and FUL2. C, B-class MADS-box genes PI1 (~OsMADS4) and AP3-1 (~OsMADS16). D, C-class MADS-box genes AG1 (~OsMADS58) and AG2 (~OsMADS3). E, E-class MADS-box genes SEP1-2 (~OsMADS1), SEP1-4 (~OsMADS5), SEP1-6 (~OsMADS34), SEP3-1 (~OsMADS7) and SEP3-2 (~OsMADS8). A–E, Graphs are based on four biological replicates (each replicate is a pool of 6–8 developing spikes at the TS stage). *P < 0.05, **P < 0.01, ***P < 0.001 in Dunnett’s test versus the WT control (Supplemental Data Set S2). Expression was determined by qRT-PCR using ACTIN as endogenous controls and normalization relative to the WT (WT = 1). Box plot features are explained in the “Statistical analyses” section of “Materials and methods.”
A qRT-PCR comparison of some of the DEGs identified in the Quant-Seq data showed significantly higher transcript levels of SEPALATA genes SEP1-2 and SEP3-1 and reduced expression of CEN2, CEN5, and TB1-2 in developing spikes was validated in the vrt2 svp1 mutants (Supplemental Figure S12A). These expression profiles are consistent with the changes observed between vrn1 and vrn1 ful2 apices (Figure 1, F and G). Taken together, the genetic interactions and the qRT-PCR results indicate that VRT2 is partially responsible for the spikelet defects of the Vrn1 ful2 mutant, possibly by regulating the expression of some of the DEGs identified in the Quant-Seq analyses.

Wheat SQUAMOSA proteins interact with SVP and SEP MADS-box proteins

Because the previous mutant analyses revealed genetic interactions among SQUAMOSA, SVP, and SEP genes, we decided to explore the pairwise physical interactions among the proteins encoded by these genes using yeast two-hybrid (Y2H) assays. After confirming that none of these wheat proteins caused autointeraction in Y2H assays (Supplemental Figure S13), we tested the interactions within the clades. Individual members of the SVP-clade did not interact with each other, and only SVP1 was able to form homodimers (Figure 11; Supplemental Figure S14). Among the SQUAMOSA proteins, FUL2 and FUL3 showed strong and weak homodimerization, respectively (Supplemental Figure S14). FUL2 interacted with both VRN1 and FUL3, whereas the latter two did not interact with each other (Supplemental Figure S14). Pairwise interactions between proteins from the two clades revealed that VRT2 and SVP1 interacted with all three SQUAMOSA proteins, whereas SVP3 interacted only with FUL2. The interactions of SVP1 with all three SQUAMOSA proteins were of similar strength but the VRT2 interaction was weakest with VRN1 and strongest with FUL2 (Supplemental Figure S15).

Rice SEP proteins of the LOFSEP subclade (OsMADS1, OsMADS5, and OsMADS34) are critical for spikelet and floret organ identity and have been shown to interact with SQUAMOSA proteins (Wu et al., 2018). In wheat, we observed positive Y2H interactions for all nine possible pairwise combinations of the three LOFSEP proteins with the three SQUAMOSA proteins (Figure 11). The interactions for all three LOFSEP proteins were strongest with FUL2, intermediate with FUL3 and weakest with VRN1 (Supplemental Figure S15). In contrast, there were fewer positive Y2H interactions between proteins of the LOFSEP and SVP clades. Among the nine possible pairwise combinations, we only detected a strong interaction between SEP1-2 and SVP1 and a weak interaction between SEP1-6 and SVP3. In summary, wheat proteins from the SQUAMOSA-clade interacted with most of the proteins from the SVP and LOFSEP clades in yeast, whereas the latter two showed limited interaction with each other (Figure 11).

We also used bimolecular fluorescence complementation (BiFC) to validate the positive Y2H interactions in wheat protoplasts (Supplemental Table S6). We observed fluorescent signals in the nucleus (and sometimes in the cytoplasm) for 9 of the 15 tested interactions (Supplemental Table S6; Supplemental Figure S16, A–I), whereas the six
interactions of SEP1-4 and SEP1-6 with the SQUAMOSA proteins showed no nuclear fluorescence (Supplemental Figure S16, J–O). Some of the positive and negative interactions showed fluorescing protein aggregates outside the nucleus (Supplemental Figure S16, G and H, see footnote 1). We did not detect fluorescent nuclear signals or aggregates for the negative controls using the C-terminal part of the Yellow Fluorescent Protein (YFP-C) paired with individual proteins of all three clades fused to YFP-N (N-terminal part of YFP) (Supplemental Figure S16, P–W). The lack of nuclear fluorescence between the three SQUAMOSA proteins with SEP1-4 and SEP1-6 (Supplemental Table S6) served as additional negative controls for proteins of the same families.

Wheat SVP and LOFSEP proteins compete for interactions with SQUAMOSA in yeast
Because both SVP and LOFSEP proteins interact with SQUAMOSA proteins and VRT2 ectopic expression results in spikelet and floret defects, we then tested whether the presence of the SVP proteins could interfere with the interaction between the three SQUAMOSA proteins with SEP1-4 and SEP1-6 (Supplemental Table S6) served as additional negative controls for proteins of the same families. The expression of VRT2 as the competing protein in Y3H assays significantly reduced the α-gal activity of the three strong FUL3–LOFSEP interactions (12.0% in SEP1-2, 20.6% in SEP1-4, and 22.0% in SEP1-6, $P < 0.01$). Among the weak interactions, the presence of VRT2 only had a significant effect on the FUL3–SEP1-2 interaction (93.8% reduction, $P < 0.0001$, Figure 12). Taken together, these results indicate that the presence of wheat VRT2 can interfere with some of the wheat SQUAMOSA–LOFSEP interactions in yeast.

Discussion
Roles of SQUAMOSA genes in the transcriptome of developing spikes and spikelets in wheat
By comparing the transcriptomes of developing spikes from vrn1 ful2 (in which spikelets are replaced by tillers) and vrn1 (normal spikelets) at four developmental stages, we identified genes and pathways regulating spike and spikelet development (Figure 1). The dramatic increase in the number of DEGs between the DR and PDR stages suggests that this developmental interval is critical for the establishment of the different developmental fates of the axillary spike meristems in the vrn1 and vrn1 ful2 mutants. The lower number of DEGs in the VEG and DR stages of spike development correlates with the similar morphology of the early developing spikelets of vrn1 and vrn1 ful2 mutants up to the DR stage (Li et al., 2019). Similarly, the dramatic morphological differences observed in the spike axillary meristems at the PDR and TS stages between these two genotypes, correlate with the higher number of DEGs (Figure 1).

The large morphological differences at the later stages of spike development are reflected in the DEGs in Clusters 4, 5, 9, and 10. Genes in Cluster 4 are enriched in genes involved in the regulation of the early stages of spikelet development and include several known regulators of axillary branch and...
SM development and determinacy (Supplemental Table S3). Cluster 5 DEGs include multiple class-B, class-C, and class-E (except SEP1-6 = OsMADS34) MADS-box floral genes that are upregulated in vrn1 at TS, reflecting the progression of the axillary meristems into spikelets and florets in the WT but not in vrn1 ful2 (Figure 1F). In contrast, transcript levels of the floral antagonists CEN2, CEN4, and CEN5 are strongly upregulated in the vrn1 ful2 mutant in Clusters 9 and 10 (Figure 1G), which are also enriched in genes with photosynthetic functions reflecting the leaf-like structures generated by the spike axillary meristems in this mutant (Figure 1E).

Genes in Cluster 8 peak at the DR stage and include several genes previously shown to control the number of spikelets per inflorescence (Supplemental Table S3) and SEP1-6, the only wheat gene from the LOFSEP-clade that is upregulated in the vrn1 ful2 mutant (Figure 1G). Finally, cluster 7 includes the three SVP-clade genes investigated in this study (Figure 1G), and genes known to either extend the activity of the IM or delay the transition of IM to SM identity (Supplemental Table S3). In summary, the complete list of DEGs presented in Supplemental Data Set S1 represents a valuable genomics resource for researchers interested in genes and gene networks that act downstream of VRN1 and FUL2 and play important roles in the early stages of spike and spikelet development. Supplemental Table S3 highlights a subset of these genes, which have already been found to play important roles in inflorescence and floret development in grasses.

Localization of SVP and SQUAMOSA genes in wheat inflorescences

Our in situ experiments detected similar hybridization profiles in SVP and SQUAMOSA genes at the early stages of spike development. However, at PDR and TS stages expression of VRT2 and SVP1 was no longer detected in the IM or the early differentiating SMs (Figure 5, C and G), whereas expression of VRN1 and FUL2 persisted in these tissues (Supplemental Figure S5). Similar profiles have been reported for SQUAMOSA genes in previous studies in wheat and barley (Hordeum vulgare) (Preston and Kellogg, 2007, 2008; Alonso-Peral et al., 2011). The overlapping expression domains of SVP and SQUAMOSA genes during early stages of inflorescence development are consistent with the positive synergistic interaction between genes from these families observed in this study.

We detected ectopic expression of VRT2 and SVP1 within the developing spike axillary organs in the vrn1 ful2 mutant but not in WT Kronos, confirming that SQUAMOSA genes are required to repress SVP gene expression at later stages of spike development in the WT. Ectopic expression of VRT2 in glumes and lemmas was also detected by in situ hybridization in developing spikes of Triticum turgidum subsp. polonicum, which carries a VRT-A2 allele with a shorter first intron (Liu et al., 2021a). Liu et al. (2021a) proposed that the structural changes in the first intron may disrupt the binding of the protein encoded by MULTIFLORET SPIKELET 1 (MFS1, TraesCS1A02G314200, an APETALA2/EThYLENE-RESPONSIVE FACTOR), resulting in ectopic expression of VRT-A2 and elongated glumes. Interestingly, MFS1 was downregulated in the apices of the vrn1 ful2 mutant at PDR and TS stages (Cluster 4), which may provide a molecular link to the ectopic expression of SVP genes in this mutant.

Wheat genes from the SVP clade accelerate heading time

In Arabidopsis, SVP acts as a flowering repressor (Hartmann et al., 2000) but the related AGL24 acts as a flowering promoter (Michaels et al., 2003), suggesting a great degree of flexibility of genes from this clade to regulate flowering time. In pepper (Capsicum annuum L.) and tomato (Solanum lycopersicum L.), the SVP homologs CafJOINTLESS and JOINTLESS both function as flowering promoters (Cohen et al., 2012; Thouet et al., 2012), similar to the VRT2 and SVP1 genes in wheat. The late heading vrt2 svp1 mutant showed a significant increase in leaf number, suggesting a delayed transition of the SAM from the vegetative to the reproductive stage. This delay was associated with a significant decrease in the transcript levels of flowering promoter genes VRN1 and FT1 and a significant increase of the flowering repressor VRN2 in the leaves of the vrt2 svp1 mutant (Supplemental Figure S4). These three genes are part of a positive feedback loop that promotes wheat flowering by increasing the transcript levels of FT1 (Distelfeld et al., 2009a). In Arabidopsis and rice, it was demonstrated that FT1 homologs encode a mobile protein that is transported from the leaves to the SAM (Corbesier et al., 2007; Tanaka et al., 2015).

Changes in VRT2 expression levels or in its spatiotemporal expression profiles can revert the function of this gene from a flowering promoter to a flowering repressor. The weak
SVP and SQUAMOSA genes contribute to stem elongation

Both SQUAMOSA and SVP genes contribute to stem elongation in wheat. Mutants for all three SQUAMOSA genes have shorter stems, with the vrn1 ful2 ful3 mutant being shorter than any other mutant combination (Li et al., 2019). Significant reductions in plant height have also been reported for the mutants of the SQUAMOSA orthologs in rice (osmads14 and osmads15), suggesting a conserved function in grasses (Wu et al., 2017). Reduced plant height was also observed in the vrt2 and sup1 mutants in tetraploid wheat (Figure 3F) and in transgenic rice plants with reduced transcript levels of OsMADS55 and OsMADS47 (Lee et al., 2008), the rice orthologs of wheat VRT2 and SVP3.

In contrast, constitutive expression of SVP genes B1M1 in barley (~SVP3) (Trevaskis et al., 2007) and OsMADS55 in rice (Lee et al., 2008) has been shown to promote stem elongation. We also observed a significant increase in stem length associated with the weak UBIpro:VRT2 transgenic T#8 (Figure 6B). Increased expression of VRT-A2 in the natural mutant T. turgidum subsp. polonicum was also associated with increased stem elongation (Adamski et al., 2021; Liu et al., 2021a). Because both SVP and SQUAMOSA genes promote stem elongation, we speculate that interactions between them may explain the drastic reduction in stem elongation in the vrt2 vrn1 ful2 mutant.

Mutations in SVP and SQUAMOSA genes alter inflorescence architecture

An unexpected phenotype of the vrt2 sup1 mutant was the development of axillary spikelets or spikes in the nodes of the elongating stem (Figure 4, E–J and N), which indicated that both VRT2 and SVP1 function redundantly as repressors of axillary meristems in the nodes of the elongating stem. Axillary spikes were reported before in wheat but the causal genes were not identified (Wang et al., 2016). The vrt2 sup1 axillary spikes were located in the same position as the ears in a maize plant, or the axillary inflorescences or “paracladia” in species from the Bambusoideae, Panicoideae, and Andropogoneae subfamilies (Stapleton, 1997; Vegetti, 1999). Andropogoneae species can develop large axillary

UBIpro:VRT2 T#8 transgene accelerated flowering 1.3 days (Supplemental Figure S8A), whereas the strong T#2 event delayed heading up to 10 days (Figure 6A). A similar delay was observed when the barley B1M1 (~SVP3) gene was constitutively expressed under the ZmUBIQUITIN promoter (Trevaskis et al., 2007). The effect of the constitutive expression of VRT2 on heading time is likely modulated by genetic background and environment, since constitutive expression of UBIpro:VRT2 in winter wheat accelerated flowering in unvernalized plants but not in fully vernalized plants (Xie et al., 2019).

One possible interpretation of the contrasting roles of VRT2 on heading time in mutants and transgenic plants is that changes in VRT2 protein abundance and distribution in the transgenic plants may affect the composition and stability of different MADS-box protein complexes resulting in multiple pleiotropic effects. The altered balance of these multiple effects across a complex and interconnected regulatory network can lead to different outcomes depending on the timing, location and levels of VRT2 expression. This hypothesis is based on interactions of VRT2 with all SQUAMOSA proteins (Figure 11), its ability to compete with other MADS-box proteins for interactions with SQUAMOSA proteins (Figure 12) and its regulatory effects on the expression of multiple floral genes (Figure 8). A similar hypothesis may explain the opposite effects of VRT2 on plant height and SNS in the vrt2 mutant and the strongest transgenic UBIpro:VRT2 line.

Figure 12 Wheat VRT2 competes with LOFSEP proteins for interactions with SQUAMOSA proteins in yeast. A–C, Y3H assays were used to test the effect of VRT2 as a competitor, where (A) SEP1-2 (~OsMADS1), (B) SEP1-4 (~OsMADS5), and (C) SEP1-6 (OsMADS34) were expressed as DNA-binding domain fusions, and SQUAMOSA proteins VRN1/FUL2/FUL3 were expressed as activation domain fusions. The $\gamma$-gal activity of the protein interactions in the absence of the competitor is shown in green box plots and in the presence of the competitor in orange box plots. Relative $\gamma$-gal activity values for each interaction are the average of 12 replicates. **$P < 0.01$ and ***$P < 0.001$ (paired t tests, Supplemental Data Set S2). The insets show the $\gamma$-gal activity values for each interaction using different scales. Box-plot features are explained in the “Statistical analyses” section of “Material and methods.”
inflorences or small ones consisting of one or few spikelets (Vegetti, 1999), a variation similar to the one we observed in the vrt2 svp1 mutant (Figure 4, E–I). It would be interesting to investigate if SVP genes in other grasses can also regulate the development of axillary inflorences.

SVP MADS-box genes also play critical roles in inflorescence development by regulating meristem transitions in both monocot and eudicot plants. In tomato and pepper SVP genes JOINTLESS and CalJOINTLESS play important roles in the regulation of inflorescence architecture and are required to maintain the inflorescence state by suppressing the sympodial vegetative program (Szymkowiak and Irish, 2006; Cohen et al., 2012). Without JOINTLESS function, after one or two flowers are formed from the initial tomato IM, subsequent growth from that apex is vegetative (Szymkowiak and Irish, 2006).

In wheat, SVP and SQUAMOSA genes showed opposite effects on the regulation of the number of florets per spikelet but overlapping effects on the regulation of SNS. The number of florets per spikelet increased in the ful2 UBIpro::VRT2 plants relative to the individual mutant or transgenic plants (Figure 9) but decreased in vrt2 Vrn1 ful2 relative to Vrn1 ful2 (Figure 10, E–F and I). The single ful2 mutant also produced a higher number of florets per spikelet than Kronos WT indicating that this gene negatively regulates the number of florets per spikelet, an effect that was not observed for vrn1 or ful3 (Li et al., 2019). In contrast, ectopic expression of VRT2 prolonged the activity of the SM and promoted the production of additional FM. These results suggest that dynamic changes in the relative abundance of SQUAMOSA and SVP genes are critical for the normal progression of floret meristems within spikelets.

In contrast with their antagonistic roles in the regulation of the SM activity, SQUAMOSA and SVP genes showed synergistic roles in promoting the transition of the IM to a TS. Individual SVP mutants (Figure 3E) and SQUAMOSA mutants show a delayed IM transition resulting in significant increases in SNS relative to the WT (Li et al., 2019). A synergistic effect of the SQUAMOSA and SVP-clade mutants was also evident in vrt2 Vrn1 ful2 relative to Vrn1 ful2 (Supplemental Figure S11C) and in the wheat vrt2 vrn1 ful2 mutant, in which spikes remained undeveloped and eventually died within the sheaths (Supplemental Figure S9H).

Interactions between SQUAMOSA and SVP homologs have been reported also in Arabidopsis. Although no obvious changes in inflorescence architecture were observed in the svp agl24 double mutant (Gregis et al., 2006), inflorescences of the Arabidopsis svp agl24 ap1 triple mutant failed to produce FMs and continuously produced IMs (Gregis et al., 2008). Another MADS-box mutant combination in Arabidopsis including svp agl24 soc1 sep4, resulted in inflorescences with a striking increase in branching that was not observed in other combinations of these mutants (Li et al., 2013). These four genes redundantly regulate inflorescence branching in Arabidopsis by repressing the expression of TERMINAL FLOWER-LIKE 1 (TFL1) in the emerging FMs, a mechanism that only occurs in the presence of AP1 activity and that seems to be conserved in rice (Liu et al., 2013).

Interactions between MADS-box and TFL1/CEN homologous genes also regulate inflorescence architecture in pea. WT pea (Pisum sativum) plants have compound inflorences with lateral branches carrying multiple flowers, but in the pea veg1 mutant the inflorescence lateral meristems produced vegetative shoots instead of secondary inflorescences bearing flowers (Berbel et al., 2012). The mutated gene in veg1 is a homolog of Arabidopsis AGL79, which encodes a SQUAMOSA MADS-box protein more distantly related to the wheat VRN1/FUL2/FUL3 proteins than Arabidopsis AP1/CAL/FUL. The pea CEN homolog DET, which is expressed only in the IM in the WT, was also expressed in the lateral meristems in the veg1 mutant. Interestingly, the det veg1 double mutant was able to produce flowers, indicating a role of DET in the transformation of the flowering branches into vegetative shoots in the pea veg1 mutant (Berbel et al., 2012).

Our results also point to an interaction between SVP, SQUAMOSA, and CEN genes in wheat. The CEN genes showed reduced expression levels in developing spikes of vrt2 Vrn1 ful2 relative to Vrn1 ful2 and vrt2 svp1 relative to WT (Supplemental Figure S12, A and B), which suggests that VRT2 promotes CEN transcription and provides a molecular link to the CEN upregulation in the vrn1 ful2 mutant (Figure 1G). This hypothesis is also supported by similar in situ profiles of CEN2, VRT2, and SVP1, both in WT Kronos and vrn1 ful2 (Supplemental Figure S7). Similarly, Arabidopsis AGL24 and SOC1 were shown to bind to TFL1 regulatory regions to induce its expression in the SAM (Azpeitia et al., 2021). We hypothesize that the ectopic expression of CEN genes in the developing axillary organs in the spikes of vrn1 ful2 may have contributed to their vegetative characteristics. We also hypothesize that their reduced expression in the spikelets of vrt2 Vrn1 ful2 relative to Vrn1 ful2 contributed to the reduced vegetative characteristic and floret number (Figure 10).

The effect of CEN2 on inflorescence architecture is supported by the positive correlation reported between CEN-D2 transcript levels and both SNS and floret number in UBIpro::CEN-D2 transgenic wheat plants (Wang et al., 2017), and by the effect of the cen-H2 mutants on SNS in barley (Bi et al., 2019). A mechanism for the regulation of inflorescence architecture involving competition between FT-like (florigen) and CEN/TFL1 (anti-florigen) proteins has been described in Arabidopsis and rice (Kaneko-Suzuki et al., 2018; Zhu et al., 2020). In rice, CEN/TFL1 proteins compete with FT-like florigen proteins for binding to 14-3-3 proteins in the formation of floral activation complexes that regulate SQUAMOSA genes and inflorescence development (Kaneko-Suzuki et al., 2018). Because vrn1 ful2 mutants have both lower FT1 expression in leaves and higher CENs expression in developing spikes (Li et al., 2019), the balance between these two groups of proteins in the inflorescence is likely altered. Given the known interaction of wheat FT1–14-3-3C–FDL2 complex with the VRN1
promoter (Li et al., 2015), a florigen/anti-florigen competition mechanism represents an interesting area for future research in wheat spike development.

Ectopic expression of SVP genes results in glumes and florets with leafy characteristics

Despite large changes in inflorescence architecture, the spikelets and florets of the wheat vrt2 svp1 mutant looked normal. Similarly, the early flowering svp agl24 double mutant in Arabidopsis showed only mild floral defects including reduced number of organs and partial homeotic transformation in the first whorl (Gregis et al., 2006). In contrast, ectopic expression of VRT2 in tetraploid transgenic lines (Figures 6, E and F) or in natural mutants such as T. turgidum subsp. polonicum (Adamski et al., 2021; Liu et al., 2021a) results in glumes and florets with vegetative characteristics. Ectopic expression of SVP genes has been associated with vegetative characteristic also in spikelet organs in barley (Trevaskis et al., 2007), rice (Sentoku et al., 2005), and maize plants carrying the dominant Tunicate1 (ZMM19, ~SVP1) pod corn mutation (Han et al., 2012; Wingen et al., 2012).

Ectopic expression of ZMM19, OsMADS22, and OsMADS47 (~SVP2) in Arabidopsis leads to leaf-like sepals and evergreen flowers similar to those observed with 35Spro:SVP and 35Spro:AGL24, suggesting a conserved function (He et al., 2004; Fornara et al., 2008). Increases in sepal size have been also observed in transgenic tomato plants with reduced expression of the SQUAMOSA gene LeMADS-MC (Vrebalov et al., 2002) or with mutations in the SEP gene Ej2 (Soyk et al., 2017). These results suggest that leaf-like sepals in eudicots can be induced by ectopic expression of SVP-clade genes or by mutations in SQUAMOSA- or SEP-clade genes. This result parallels the leaf-like glumes observed in wheat plants transformed with UBIpro:VRT2 and in the vrn1 ful2 mutant. We also observed strong interactions between SQUAMOSA and SVP genes on spikelet development in plants combining ful2 and UBIpro:VRT2 alleles (Figure 9; Supplemental Data Set S2).

We currently do not know whether the SVP-clade genes actively induce vegetative characteristics or if they have an effect on the repression of floral organs that leads to the regression to a “default” vegetative developmental program. We show in this study that constitutive expression of VRT2
results in the downregulation of MADS-box A-, B-, C-, and most E-class genes, a function conserved in Arabidopsis (Gregis et al., 2009; Liu et al., 2009). Similar results were observed in the apices of wheat lines with the vrt2 allele from T. turgidum subsp. polonicum (Liu et al., 2021a). Moreover, we showed that higher expression levels of SEP1-2 in vrt2 vrn1 ful2 relative to vrn1 ful2, was associated with more normal glumes and lemma and a reduced proportion of spikelets with branches (Supplemental Figure S1I). A role of the SEP genes in the development of normal glumes and lemma was also demonstrated in the leaf-like lemmas of spikelets and floral development (Theissen et al., 2016). Our Y3H results suggest that the failure to downregulate the SVP genes in the vrt2 vrn1 ful2 mutant may result in competition of the SVP proteins with branches. SEP proteins play a pivotal role in the sequential transition between MADS-box protein complexes favoring early reproductive development and those favoring the development of floral organs. A similar function as protein interaction hubs between the flower induction pathway (e.g., SVP, AGL24, and SOC1) and floral organ identity proteins has been proposed in Arabidopsis for the SQUAMOSA proteins AP1 and FUL (de Folter et al., 2005).

In summary, this study shows that SVP and SQUAMOSA genes have synergistic effects on the acceleration of the transitions of the apical meristems (SAM to IM to TS) and stem elongation, but antagonistic effects on the regulation of axillary meristems in spikes and spikelets, with SQUAMOSA genes promoting the transition to floral organs and SVP genes having a regressive effect. Our results also show that it is possible to rationally manipulate the dosage or activity of these MADS complexes to optimize wheat spike architecture. Although mutations in both SQUAMOSA and SVP genes result in increases in SNS, the reduced pleiotropic effects of the SVP genes can facilitate their deployment in practical breeding applications. Our results for the separate vrt2 and svp1 mutants, and their individual homeologs, show that these effects can be readily fine-tuned in a polyploid species like wheat.

Materials and methods
Quant-Seq of vrn1 and vrn1 ful2 developing spikes
We collected SAMs from vrn1 and vrn1 ful2 mutants at four developmental stages: VEG, DR, PDR, and TS (Figure 1A). These four stages correspond to W1, W2.5, W3.25, and W3.5 stages in the Waddington Scale of wheat spike development (Waddington et al., 1983). We performed Quant-Seq analysis using four biological replicates for each of the four developmental stages, with each replicate including pools of 6 apices for PDR and TS stages, 9 apices for DR, and 12 apices for VEG stages. Sequencing of the 32 samples (2 genotypes × 4 developmental stages × 4 biological replicates) using Hi-seq (100-bp reads not paired) yielded an average of 7,335,215 unique reads per sample after filtering for duplicates, with an average read length of 74.3 bp (after trimming) and an average quality of 36.4 (Supplemental Table S2). BioProject numbers are available in the “Materials and methods” section “Accession Numbers”. Plant growth conditions are described in the section entitled “Growth conditions and phenotyping.”

We processed the raw reads using DOE JGI BBTools (https://sourceforge.net/projects/bbmap/) program bbduk.sh to remove Illuma adapter contamination and low-quality reads (for trim left = 21 qtrim = r trimq = 10). Processed reads were mapped to the International Wheat Genome Sequencing Consortium (IWGSC) RefSeq version 1.0 genome assembly, using the STAR aligner (Dobin et al., 2013). We used parameters –outSAMtype BAM SortedByCoordinate –outSAMunmapped Within –outSAMattributes Standard –quantMode TranscriptsPerSAM GeneCounts to generate Binary Sequence Alignment/Map (BAM) files for each sample. We used the high confidence gene models from IWGSC
Refseq version1.0 (IWGSC_v1.1_HC_20170706.gff) in combination with the BAM files in the R program (featureCounts.R) which uses the Rsubread package (Liao et al., 2019) to calculate the overlap between reads and features. We used the “readExtensions” option that allows a read to be counted as belonging to a gene when the gene was a defined number of bases 5' of the read (we used 500 bp).

The raw t test values between read counts of vrn1 and vrn1 ful2 were corrected for false discovery rate (FDR) using the R function p.adjust (method = “BH,” aka “FDR”) (Benjamini and Hochberg, 1995; R Core Team, 2020). DEGs between vrn1 and vrn1 ful2 mutants for each stage were defined as those with a fold change in transcript levels ≥2 and FDR ≤0.05. We then generated a list of nonredundant downregulated and upregulated DEGs across the four stages and performed a cluster analysis based on their expression profiles using the MultiExperiment Viewer software (www.tm4.org). For this analysis, the expression levels of each DEG were normalized to the average expression value of each DEG across genotypes and stages (mean normalized expression), and then clustered using K-means with a minimum limit of 10% of total genes per cluster. We then used the clustered lists as queries for BLAST analyses against a rice gene database available from Phytozome (Osativa_323_V7.0.cds_primaryTranscriptOnly.fa) to obtain a functional annotation for the DEGs (Supplemental Data Set S1). Finally, the lists containing the best rice blast hits were used to perform GO enrichment analysis using AgriGO web tool (http://bioinfo.cau.edu.cn/agriGO/).

Identification of loss-of-function mutations in VRT2
The sequenced ethyl methanesulfonate mutagenized populations of the tetraploid wheat variety Kronos and hexaploid variety Cadenza (Krasileva et al., 2017) were screened for mutations using BLASTN with the sequences of VRT2 (TraesCS7A02G175200 and TraesCS7B02G080300) and SVP1 (TraesCS6A02G313800 and TraesCS6B02G343900) as queries. For VRT-A2, we detected 46 mutations that generated amino acid changes but we found no truncation mutations in the Kronos mutant population. Therefore, we screened the mutant population of the hexaploid wheat Cadenza, where we identified a mutation that generated a Q125* premature stop codon (mutant line Ca0424), which is predicted to eliminate 47% of the VRT2 protein, including part of the K-box and C terminal domains (Figure 2A, above gene model).

The Kronos mutant line K3404 carries a mutation in the donor splice site of the fourth intron of the VRT-B2 gene, designated hereafter as vrt-B2 (Figure 2a, below gene model). Sequencing of the RT-PCR products from K3404 revealed three vrt-B2 alternatively spliced forms, all resulting in severe truncations (Supplemental Figure S2). The first alternatively spliced form showed a 5-bp insertion as a result of the utilization of the next available GT splicing site in intron 4. This resulted in a reading frame shift and a premature stop codon that is predicted to eliminate half of the protein including 30% of the conserved K domain. The second alternatively spliced form had an insertion of the last 4 bp of intron four between exons four and five that generated a reading frame shift and an early stop codon. Similar to the first alternative splice form, this change is also predicted to eliminate half of the protein and 30% of the K domain. Finally, the third alternatively spliced form was missing exons 3, 4, and 5. Although exons 6, 7, and 8 retained the correct reading frame, the deletion resulted in the elimination of 80% of the K domain (Supplemental Figure S2).

Because Kronos × Cadenza crosses result in hybrid necrosis, we crossed Ca0424 to an F2 plant from the cross between the hexaploid Insignia and the tetraploid Kronos as a bridge cross. We then intercrossed the F1 with the vrt-B2 mutant K3404 to combine both mutations (Figure 2C). To reduce the background mutations, we backcrossed the F1 plant from the cross between vrt-A2 and vrt-B2 3 times to Kronos, and from the segregating BC1F2 plants we selected a double homozygous mutant vrt-A2 vrt-B2, which was designated vrt2.

To test the genetic interaction between vrt2 and members of the MADS-box genes from the SQUAMOSA-clade (VRN1 and FUL2), we combined vrt2 with loss-of-function mutations at vrn1 ful2 in the same Kronos background (Li et al., 2019). Because the vrn1 ful2 line is sterile, we used a line heterozygous for VRN-A1 and FUL-B2 for crossing. In the progeny, we selected two pairs of isogenic lines, one with no functional copies of VRN1 (urt2 vrn1 ful2/vrn1 ful2) and one heterozygous for Vrn-A1 and homozygous for all the other truncation mutations (urt2 Vrn1/ful2 Vrn1 ful2). All these mutant lines were developed in a Kronos background with no functional copies of VRN2 to avoid the extremely late heading of the vrn1 mutant in the presence of VRN2, which is a strong flowering repressor in wheat (Distelfeld et al., 2009b). We self-pollinated the F1 plant and from the F2 plants we selected lines homozygous for vrn1 ful2 and either homozygous for vrt2 or for the WT alleles (Figure 2C). Because the vrt2 vrn1 ful2 mutant failed to form spikelets, we also selected lines Vrn1 ful2 with and without vrt2 to study its effect on spike morphology.

Identification of loss-of-function mutations in SVP1
For SVP-A1, we identified the Kronos line K4488 that carries a mutation in the spike donor site in the third intron, designated as svp-A1 (Figure 2B). The sequencing of SVP-A1 RT-PCR products from K4488 revealed two alternatively spliced forms (Supplemental Figure S2). The first one lacks the third exon, which alters the reading frame and generates a premature stop codon that eliminates >60% of the SVP-A1 protein. Because this deletion includes the complete K domain, the resulting protein is likely not functional. The second alternatively spliced form lacks both the second and third exons, which results in the loss of 47 amino acids but does not alter the reading frame. Since this predicted deletion includes the end of the MADS domain and the beginning of the K domain, the resulting protein is likely not functional.

For SVP-B1, we identified Kronos line K0679 with a mutation that generates a premature stop codon in the third exon (Q99*), designated as svp-B1 (Figure 2B). We crossed
both mutants separately 2 times to the parental Kronos to reduce background mutations (BC) and then combined them by crossing and selection in BC:F2 to generate the double mutant designated sup1. Finally, we intercrossed sup1 and vrt2, self-pollinated the F1, and selected F2 plants homozygous for the four mutations (vrt-A2 vrt-B2 sup-A1 sup-B1) which were designated as vrt2 sup1 (Figure 2C).

**In situ hybridization**

We performed in situ RNA hybridization following the protocol described previously (Zhong et al., 2021). Tissues were obtained from diploid *T. monococcum* (accession PI 167615), tetraploid Kronos WT, and Kronos vrt1 ful2 mutant. We amplified DNA fragments of 300–400 bp covering the end of the coding region and the 3′–untranslated region (UTR) from *T. monococcum* with gene-specific primers appended with T7 or T3 promoter or from Kronos with gene-specific primers, and then inserted them into pGEM-T easy vectors (Promega, Madison, WI, USA). The probes were synthesized using T7 or T3 RNA Polymerase (Promega) and labeled with Digoxigenin-11-UTP (Roche). Images were taken using a Zeiss AxioCam M2 microscope with an AxioCam512 color camera, or a Zeiss SteREO Discovery.V20 microscope with an AxioCam506 color camera. Primers used to amplify the hybridization probes are described in Supplemental Table S4.

**SEM**

Apices from the different genotypes and developmental stages were dissected and fixed for a minimum of 24 h in Formaldehyde Alcohol Acetic Acid (FAA) (50% ethanol, 5% (v/v) acetic acid, and 3.7% (v/v) formaldehyde), and then dehydrated through a graded ethanol series to absolute ethanol. Samples were critical point dried in liquid CO2 (tousi- mis 931 Series critical point dryer), mounted on aluminum stubs, sputter-coated with gold (Bio-Rad SEM Coating Station in Davis, CA (38° 32’ N, 121° 46’ W). We used 1-m rows with 20 plants each as experimental units, organized in a completely randomized design. The experiment included 20 replications for vrt2 and the WT sister lines, and 10 replications for vrt-A2 and vrt-B2. Plants in the field were evaluated for heading time, SNS, and total plant height (measured from the soil to the top of the main spike excluding awns).

**Statistical analyses**

Effects of individual homeologs (A and B genome) or of individual genes (e.g. VRT2 and SVP1) and their interactions were computed using 2 × 2 factorial analysis of variance (ANOVA) with homeologs or genes as factors and alleles as levels. Simple effects were evaluated using orthogonal contrasts. Means of the individual genotypes were compared with the WT using Dunnett’s test. Homogeneity of variances was tested with the Levene’s test and normality of residuals with the Shapiro–Wilk test. When necessary, we transformed data to meet the assumptions of the ANOVA. All statistical analyses were performed using SAS version 9.4. Results of all statistical analyses are presented in Supplemental Data Set S2. The distribution of the data within each genotype is presented with box plots including individual data points generated with Excel. The middle line of the box represents the median and the x represents the mean. The bottom line of the box represents the first quartile and the top line the third quartile. The whiskers extend from the ends of the box to the minimum value and maximum value. A data point was considered an outlier if it exceeded a distance of 1.5 times the interquartile range. The whiskers extend from the ends of the box to the minimum value and maximum value. A data point was considered an outlier if it exceeded a distance of 1.5 times the interquartile range. The number of plants analyzed is indicated in each graph.

**cDNA preparation and qRT-PCR analysis**

To quantify transcript levels of different flowering genes in transgenic plants overexpressing VRT2, we extracted total RNA from pools of 6–8 SAMs at TS stage from four

**Growth conditions and phenotyping**

We grew the plants used for the Quant-Seq experiment and for phenotypic evaluation of mutants and transgenic plants in PGR15 CONVIRON growth chambers under long-day photoperiod (16-h light/8-h dark) and temperatures of 22°C during the day and 18°C during the night. The light intensity of the sodium halide lights was ~330 μmol m−2 s−1. Plants were germinated in Petri dishes at 4°C for 3–5 days. After the first leaf emerged, we transplanted the seedlings into Sun Gro professional growing mix (Sunshine Mix #1) in one-gallon pots, and recorded days to heading from this day until emergency of half of the main spike from the flag leaf. Length measurements were taken at maturity for the complete plants and for each of the internodes and peduncle separately.

We also evaluated the vrt2 mutant in a field experiment (sown November 22, 2019) at the UC Experimental Field Station in Davis, CA (38° 32’ N, 121° 46’ W). We used 1-m rows with 20 plants each as experimental units, organized in a completely randomized design. The experiment included 20 replications for vrt2 and the WT sister lines, and 10 replications for vrt-A2 and vrt-B2. Plants in the field were evaluated for heading time, SNS, and total plant height (measured from the soil to the top of the main spike excluding awns).
biological replicates using the Spectrum Plant Total RNA Kit (Sigma-Aldrich St Louis, MO, USA). Samples were collected 4–5 h after the lights were turned on in the morning. The cDNA was synthetized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814) from 2 μg RNA treated with RNase-free DNase (Promega). The cDNA was then diluted 20-fold in water and 5 μl of the dilution was used for the qRT-PCR analysis. The Quantitative PCR was performed using the 7500 Fast Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) with 2× VeriQuest Fast SYBR Green qPCR Master Mix (Affymetrix, 75690). The relative transcript level was determined for each sample and normalized using ACTIN as an endogenous control. The normalization was performed as described previously (Livak and Schmittgen, 2001). Melting curve analyses at the end of the process and "no template controls" were performed to ensure product-specific amplification without primer-dimer artifacts. Primer sequences are given in Supplemental Table S4.

Y2H assay and BiFC
We used the GAL4-based Y2H system to investigate protein interactions. We amplified the full-length cDNAs of the different genes from the SQUAMOSA- (VRN1, FUL2, and FUL3), SEP- (VRT2, SEP1, SEP3), and SEP clades (SEP1-2, SEP1-4, and SEP1-6) and cloned them into the gateway pDONR/Zeo Vector (Catalog number: 12535035) using the primers listed in Supplemental Table S4. We then cloned these genes into the Takara Bio Y2H vectors pGADT7 (activation-domain vector) and pGBK7T (DNA-binding domain vector) by either restriction enzyme-based cloning or In-Fusion HD vector) and pGBKT7 (DNA-binding domain vector) by either restriction enzyme-based cloning or In-Fusion HD Cloning method (638910n-Fusion HD Cloning Plus Takara). Primer sequences used to generate Y2H and Y3H constructs are listed in Supplemental Table S4. Both bait and prey vectors were transformed into yeast AH109 Gold strain (Saccharomyces cerevisiae from TaKaRa/Clontech). The cotransformants were plated on selective solid Synthetic Dropout agar medium without leucine (L) and tryptophan (W) (SD-L-W). Positive transformants were re-plated on Synthetic Dropout medium lacking L, W, histidine (H), and adenine (A) to test for interaction (SD-L-W-H-A). We cotransformed each bait vector with pGADT7 and each prey vector with pGBK7T to test for autoactivation.

For the BiFC (or split YFP) assays, we cloned the same genes into modified Gateway-compatible vectors UBIpro-NYFP-GW and UBIpro-CYFP-GW by recombination reactions (UBIpro = the maize UBIQUITIN promoter, 1,986-bp upstream of the ATG). These vectors generated fusion proteins with YFP-N-terminal fragment or YFP-C-terminal-fragment at the N-terminus and the proteins being tested at the C-terminus. Wheat protoplasts were prepared, transfected, and visualized as described in (Shan et al., 2014).

Y3H assays
The pBridge Y3H system (Clontech CATALOG No. 630404) was used to test whether the wheat VRT2 protein can interfere with the interactions between SEP and SQUAMOSA proteins. This vector can express two proteins, a DNA-binding domain fusion, and a second protein (Bridge protein) that is controlled by pMET25, an inducible promoter responsive to methionine levels in the medium. The Bridge protein is only expressed in the absence of methionine and inhibited by the addition of 1 mM methionine. For each pBridge vector, one of the LOFSEPs (SEP1-2, SEP1-4, or SEP1-6) genes was fused to the DNA-binding domain, and the VRT2 gene was inserted downstream of the MET25 promoter. The same prey vectors generated for VRN1, FUL2, and FUL3 in Y2H assays were used in Y3H assays. Each pBridge vector was then paired with one prey vector and co-transformed into yeast Gold. Transformants containing both vectors were selected on SD-L-W medium. Protein interactions were quantified using quantitative α-galactosidase assays as described before (Li et al., 2011). All constructs used in Y2H and Y3H assays have the GAL4 DNA binding (bait) and activation domains (prey) at the N-terminus, and the proteins being tested at the C-terminus of the fusion protein.

Accession numbers
The T. monococcum VRT-A”tm2 sequence used for the constitutive expression construct is deposited in GenBank under accession number MW218446. The Quant-Seq datasets for the vrn1 and vrn1ful2 mutants have been deposited in GenBank under the following project numbers (each including four biological replicates): PRJNA681065 (vrn1, VEG samples), PRJNA681067 (vrn1, DR), PRJNA681097 (vrn1, post DR) PRJNA681099 (vrn1, TS), PRJNA681036 (vrn1 ful2, VEG samples), PRJNA680890 (vrn1 ful2, DR), PRJNA681027 (vrn1 ful2, post DR), and PRJNA681032 (vrn1 ful2, TS). Seed stocks have been deposited in the National Small Grain Collection for the following Kronos mutants: vrn1 vrn2 (PI 698812), ful2 vrn2 (PI 698814), ful3 vrn2 (PI 698815), vrt2 (PI 698811), and sup1 (PI 698813). All other data and genetic materials are available from the authors upon request.

Supplemental data
The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic relationship among SEP proteins in wheat, barley, rice, and Arabidopsis.

Supplemental Figure S2. Effects of vrt2 and sup1 mutations on the encoded proteins.

Supplemental Figure S3. Effects of mutations in the A and B genome homeologs of VRT2 and SVP1.

Supplemental Figure S4. Transcript levels of flowering genes FT1, VRN1, and VRN2 in the fifth leaf of single mutants vrt2 and sup1, combined mutant vrt2 sup1 and WT control.

Supplemental Figure S5. In situ hybridizations of VRN1 and FUL2 in wheat spikes at different developmental stages.

Supplemental Figure S6. In situ hybridizations of VRT2 and SVP1 in T. monococcum spikes at different developmental stages.
**Supplemental Figure S7.** In situ hybridizations of CEN2 expression in Kronos spikes at different developmental stages.

**Supplemental Figure S8.** Complementation of the vrt2 mutation by the weak UBIpro:VRT2 transgenic line T#8.

**Supplemental Figure S9.** Phenotypic comparison between vrn1 ful2 and vrt2 vrn1 ful2 mutants (in a vrn2 mutant background).

**Supplemental Figure S10.** SEM images of developing spikes.

**Supplemental Figure S11.** Effect of the vrt2 mutation in the partial mutant Vrn1 ful2 (the underline indicates a plant heterozygous for Vrn-A1 vrn-A1 and homozygous vrn-B1 vrn-B1).

**Supplemental Figure S12.** Effect of VRT2 and SVP1 on the transcript levels of SEP, CEN, and TB1 genes in developing spikes.

**Supplemental Figure S13.** Auto-activation tests for the bait and prey vectors used in Y2H assays.

**Supplemental Figure S14.** Y2H interactions among proteins within the SQUAMOSA- and SVP-clades.

**Supplemental Figure S15.** Y2H interactions between wheat MADS-box proteins of the SQUAMOSA, SVP, and SEP classes.

**Supplemental Figure S16.** BiFC between proteins of the SQUAMOSA-clade and proteins of both the SVP and SEP clades in wheat protoplasts.

**Supplemental Table S1.** Nomenclature, accession numbers, and synonyms of wheat genes used in this study and their rice orthologs.

**Supplemental Table S2.** Summary statistics for the Quant-Seq samples.

**Supplemental Table S3.** DEGs between vrn1 and vrn1 ful2 at four different developmental stages of spike development.

**Supplemental Table S4.** Primers used for gene cloning, genotyping of mutations and transgenic plants, qRT-PCR, generation of Y2H and Y3H constructs, and in situ hybridization.

**Supplemental Table S5.** Effects of vrt2 and sup1 mutations on heading time, SNS, stem length, and leaf number in growth chambers under LD conditions.

**Supplemental Table S6.** Summary of Y2H interactions and BiFC interactions tested among the SQUAMOSA, SVP, and SEP MADS-box proteins.

**Supplemental Data Set S1.** DEGs in Clusters 1–10.

**Supplemental Data Set S2.** Statistical analysis tables.

**Supplemental File S1.** Sequence alignment used to produce the phylogenetic tree in Supplemental Figure S1.

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**Conflict of interest statement.** None declared.

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