Winter cereals require prolonged cold to transition from vegetative to reproductive development. This process, referred to as vernalization, has been extensively studied in Arabidopsis (Arabidopsis thaliana). In Arabidopsis, a key flowering repressor called FLOWERING LOCUS C (FLC) quantitatively controls the vernalization requirement. By contrast, in cereals, the vernalization response is mainly regulated by the VERNALIZATION genes, VRN1 and VRN2. Here, we characterize ODDSOC2, a recently identified FLC ortholog in monocots, knowing that it belongs to the FLC lineage. By studying its expression in a diverse set of Brachypodium accessions, we find that it is a good predictor of the vernalization requirement. Analyses of transgenics demonstrated that BdODDSOC2 functions as a vernalization-regulated flowering repressor. In most Brachypodium accessions, BdODDSOC2 is down-regulated by cold, and in one of the winter accessions in which this down-regulation was evident, BdODDSOC2 responded to cold before BdVRN1. When stably down-regulated, the mechanism is associated with spreading H3K27me3 modifications at the BdODDSOC2 chromatin. Finally, homoeolog-specific gene expression analyses identify TaAGL33 and its splice variant TaAGL22 as the FLC orthologs in wheat (Triticum aestivum) behaving most similar to Brachypodium ODDSOC2. Overall, our study suggests that ODDSOC2 is not only phylogenetically related to FLC in eudicots but also functions as a flowering repressor in the vernalization pathway of Brachypodium and likely other temperate grasses. These insights could prove useful in breeding efforts to refine the vernalization requirement of temperate cereals and adapt varieties to changing climates.

Optimal adaptation to the environment is a vital factor in the survival of all living organisms. Plants have evolved various mechanisms to sense environmental signals, which help them to synchronize their development to environmental changes. Among environmental cues, seasonal temperature and photoperiod variation play a decisive role in determining the optimal time to flower. Many plants adapted to temperate climates flower only after a prolonged exposure to cold to prevent the premature flowering during warm autumn days, a process referred to as vernalization (Chouard, 1960; Kim et al., 2009). A better understanding of this process can have a significant impact on crop yield as temperate winter cereals grow vegetatively without vernalization and transition to their reproductive state only after their vernalization requirement is saturated.

The process of vernalization has been traditionally studied in economically important temperate cereals like wheat (Triticum aestivum) and barley (Hordeum vulgare; Chouard, 1960), yet its regulation has been most extensively investigated in Arabidopsis (Arabidopsis thaliana). In winter-annual Arabidopsis ecotypes, FLOWERING LOCUS C (FLC) is a central repressor of flowering (Michaels and Amasino, 1999; Sheldon et al., 2000), which represses the flowering pathway integrators FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1; Michaels et al., 2005). Prolonged cold results in epigenetic silencing of FLC, which releases repression of FT and SOC1 to enable flowering after return to warm conditions (Bastow et al., 2004; De Lucia et al., 2008). By contrast, in temperate cereals such as wheat and barley, vernalization is mainly governed by two key genes, namely VERNALIZATION1 (VRN1) and VRN2, which regulate flowering integrator VRN3 (Dennis and Peacock, 2009;
Greenup et al., 2009). VRN1, a MADS-box transcription factor related to Arabidopsis APETAL1 (API), is a promoter of flowering. In varieties that require vernalization, VRN1 is up-regulated in response to cold, while in spring varieties VRN1 is expressed without vernalization, which reduces or eliminates their vernalization requirement (Danyluk et al., 2003; Preston and Kellogg, 2006; Trevaskis et al., 2003; Yan et al., 2003; Preston and Kellogg, 2008). Analogous to the epigenetic regulation of FLC, VRN1 up-regulation in winter varieties is associated with low levels of the repressed chromatin state H3K27me3 and high levels of the active chromatin mark H3K4me3 (Oliver et al., 2009). The subsequent stable high expression level of VRN1 results in the down-regulation of the flowering repressor VRN2 after prolonged cold (Distelfeld et al., 2009; Hemming et al., 2008; Trevaskis et al., 2006; Yan et al., 2004b). However, more recently it was shown that VRN1 is not essential for flowering or for the down-regulation of VRN2 during vernalization, suggesting that additional genes control VRN2 down-regulation when exposed to cold (Chen and Dubcovsky, 2012). Finally, down-regulation of VRN2 releases VRN3, a homolog of FT in Arabidopsis, which induces flowering after cold and in response to long days (Distelfeld et al., 2009; Trevaskis et al., 2007; Turner et al., 2005; Yan et al., 2006).

One reason why it is thought that the vernalization response evolved independently in Arabidopsis and temperate cereals has been the difficulty of identifying FLC-like genes in monocots by sequence homology searches (Alexandre and Hennig, 2008; Hemming and Trevaskis, 2011; Kim et al., 2009). However, we recently identified three paralogous FLC gene lineages, i.e. ODDSOC1, ODDSOC2, and MADS37 in Pooidae (Ruelens et al., 2013). In Brachypodium distachyon (hereafter referred as Brachypodium), BdODDSOC2 and BdMADS37 expressions levels are down-regulated in response to cold analogous to FLC in Arabidopsis, while BdODDSOC1 expression does not change in response to cold in the spring accession Bd21 (Ruelens et al., 2013). The cold response of BdODDSOC2 in Brachypodium Bd21 is similar to barley Golden Promise (Greenup et al., 2010), and it has been shown that overexpression of HvODDSOC2 delays flowering and reduces spike growth, stem, and leaf length. However, HvODDSOC2 RNAi transgenic lines did not show a phenotypic effect in the spring accession Golden Promise. Recently, it was reported that VRN1 can bind to the promoter of ODDSOC2 in Hordeum and ODDSOC2 expression is up-regulated in VRN1 mutants in wheat, though not before or during vernalization (Greenup et al., 2010; Deng et al., 2015).

Natural variation in the vernalization requirement allows plants to adapt to local climate conditions as the presence and duration of the cold season represents a crucial cue influencing the optimal flowering time (Kim et al., 2009). In Arabidopsis, natural variation in flowering time through vernalization is correlated with allelic variation at the FLC and FRIGIDA (FRI) loci (Shindo et al., 2005). Moreover, loss-of-function mutations at the FLC locus have been linked to the early flowering phenotype of many Arabidopsis spring accessions. The FLC locus of these accessions typically contains independent insertions of a transposon in the first regulatory intron resulting in weak or nonfunctional FLC alleles (Gazzani et al., 2003; Michaels et al., 2003b). However, in economically important crops like wheat and barley, mainly allelic variation at the VRN1 and VRN2 loci govern the natural variation in vernalization-mediated flowering (Trevaskis et al., 2003; Yan et al., 2003, 2004b). For instance, natural allelic variation at the VRN1 locus is associated with differences in the vernalization requirement between spring and winter cereals (Trevaskis et al., 2003; Yan et al., 2003, 2004a). Furthermore, spring varieties often also have mutations or deletions in the CCT domain of VRN2 (Dubcovsky et al., 2005; Yan et al., 2004b). Similar to cereals, different natural accessions of Brachypodium have a widely varying vernalization requirement; however, the genetic determinants for this variation are not well defined (Schwartz et al., 2010; Tyler et al., 2014). Likewise, whether FLC-like genes in monocots contribute to the variation in vernalization requirement has not been explored.

To better understand the function of these genes, we functionally characterized ODDSOC2, one of the FLC homologs in grasses, in the model Brachypodium. Like FLC, BdODDSOC2 functions as a vernalization-responsive repressor of flowering. In addition, natural variation in the vernalization requirement among different Brachypodium accessions can be attributed to BdODDSOC2 prevernalization expression levels. During cold, BdODDSOC2 is down-regulated in most accessions, and this response occurs hours before BdVRN1. We show that stable down-regulation is associated with the spreading of histone methylation across the ODDSOC2 locus. Consistent with a conserved role of ODDSOC2 genes among cereals, the wheat ODDSOC2 ortholog TaAGL33 exhibits an expression pattern similar to FLC in response to vernalization.

RESULTS

BdODDSOC2 Expression Linked to Vernalization Requirement among Brachypodium Accessions

To evaluate the role of BdODDSOC2 in the vernalization response, we studied its expression in response to cold in a set of Brachypodium inbred lines derived from plants originating from different climatic regions (Supplemental Table S1). Although previous studies already investigated the vernalization requirement of numerous Brachypodium accessions (Ream et al., 2014; Schwartz et al., 2010), we first assessed the vernalization requirement of the selected accessions in our laboratory conditions. Vernalization requirement was analyzed by determining the number of days until flowering following a 0-, 2-, 4-, 6-, 10-, or 12-week treatment of cold of 3-week-old plants. Subsequently, we assigned each accession a vernalization requirement as the minimum number of weeks of vernalization that results in the largest decrease in days to flowering. This
analysis corroborates previous results reporting a large variation in vernalization requirement among *Brachypodium* accessions (Supplemental Table S1; Schwartz et al., 2010). Furthermore, vernalization requirement shows the expected positive correlation with the latitude of origin (Pearson, \( r = 0.504, P = 0.007 \)), indicating that in general accessions with a long vernalization requirement originate from more northern latitudes, while accesses from southern latitudes possess a short vernalization requirement.

To assess to what extent *BdODDSOC2* down-regulation in response to cold is conserved among *Brachypodium* accessions, we examined the expression level of *BdODDSOC2* before vernalization (3-week-old plants, 23°C, 16 h light/8 h dark) and following 4 weeks of vernalization in 25 different accessions (4°C, 8 h light/16 h dark). Our results show that in 18 of 25 accessions, *BdODDSOC2* expression levels decrease in response to cold (Fig. 1A). In 11 accessions, this expression is significantly down-regulated (Student’s *t* test, \( P < 0.05 \); Supplemental Table S1). In seven accessions, *BdODDSOC2* levels increase (Fig. 1A); however, only two accessions, Bd1-1 and BdTR9K, exhibited a significant increase following 4 weeks at 4°C compared to prevernalization (Student’s *t* test, \( P < 0.05 \); Supplemental Table S1). *BdVRN1* expression increased in all accesses in response to cold, and 19 accessions exhibited a significant increase (Student’s *t* test, \( P < 0.05 \); Supplemental Table S1). To study the response of *BdODDSOC2* to cold in more detail, we monitored *BdODDSOC2* expression levels during 0, 2, 4, 6, 10 weeks of cold in 6 different accessions (Fig. 1B). In Bd3-1 and Adi10, *BdODDSOC2* expression during vernalization did not significantly change compared to nonvernalization. Expression levels of *BdODDSOC2* in BdTR2B, BdTR2G, Kah-1, and BdTR51 exhibited clear down-regulation, although expression during vernalization in Kah-1 did not significantly change compared to nonvernalization. In the accessions Bd3-1 and Adi10, for which *ODDSOC2* did not change following 4 weeks of vernalization, a longer cold period also did not lead to cold-induced down-regulation.

Previous studies in *Arabidopsis* showed a significant correlation between the expression level of *FLC* in nonvernalized plants and the vernalization response (Sheldon et al., 2000; Shindo et al., 2005). Therefore, we analyzed whether this correlation is also present in *Brachypodium*. Specifically, we performed regression analyses between the expression level of *ODDSOC2* in nonvernalized plants or 4-week vernalized plants and vernalization requirement. These analyses show that vernalization requirement is significantly correlated with the expression level of *BdODDSOC2* before vernalization (Fig. 1C). Moreover, *BdODDSOC2* transcript level before vernalization is a good predictor of the length of vernalization (\( R^2 = 0.50, P < 0.001 \)). However, expression levels of *BdODDSOC2* in plants exposed to 4 weeks of cold could not predict the vernalization requirement (\( R^2 = 0.044, P = 0.32 \)). This suggests that mRNA transcript down-regulation of *BdODDSOC2* by itself cannot explain the vernalization length requirement. We also performed a regression analyses between vernalization requirement and *BdVRN1* expression, which in wheat plays a pivotal role in the vernalization response and explains part of the variation in vernalization requirement. However, in *Brachypodium* the expression level of *BdVRN1* before cold is not significantly correlated with the vernalization requirement (\( R^2 = 0.13, P = 0.067 \)). Our analyses did detect a correlation between the expression level of *BdVRN1* of vernalized plants and vernalization requirement, albeit only marginally significant and poorly predictive (\( R^2 = 0.15, P = 0.047 \); Fig. 1C).

**BdODDSOC2 Overexpression Plants Exhibit Delayed Flowering and Are Unresponsive to Vernalization**

To investigate *BdODDSOC2*’s role during vernalization, we first overexpressed *BdODDSOC2* cDNA in *Brachypodium* accession Bd21-3 that is amenable to *Agrobacterium*-mediated transformation (Vogel and Hill, 2008). Bd21-3 is a facultative winter accession that flowers without vernalization after 125 d (16-h light/8-h dark photoperiod), but exposure to 2 weeks of cold significantly accelerates flowering by 64 d (Fig. 2A). To assess how *BdODDSOC2* responds to cold in Bd21-3, we examined its expression using qRT-PCR prior to vernalization (NV), following 2 weeks of vernalization (2wV), and after 1 week return to warm conditions (1wPV). Our results show that *BdODDSOC2* mRNA levels are not significantly altered following vernalization (Fig. 2B). However, relative expression of *BdODDSOC2* NV in Bd21-3, which requires 2 weeks of cold to saturate its vernalization requirement, is lower compared to most accessions requiring a longer cold exposure (Fig. 1C, red dot).

We generated and transformed a construct expressing *BdODDSOC2* cDNA from the constitutively active maize (**Zea mays**) *UBIQUITIN* promoter, for which we obtained 17 overexpression lines. Ten lines with maximum seed yield were chosen for detailed characterization (see “Materials and Methods”). In all 10 overexpression lines, the *BdODDSOC2* transcript level was increased as determined by semiquantitative RT-PCR (Supplemental Fig. S1). To test whether *BdODDSOC2* affected flowering time, T2 overexpression transgenic lines were grown and phenotyped together with nontransgenic controls and wild-type Bd21-3 plants in two different conditions (see “Materials and Methods”). Nontransgenic controls (NCs) were plants that went through transformation but did not carry the transgene in T0. In NV, all *BdODDSOC2* overexpression plants carrying the transgene flowered at the same time as the control plants (Fig. 2C; Supplemental Fig. S2A). By contrast, when vernalized (V), 8 of 10 *BdODDSOC2* overexpression lines exhibited a significant delay in flowering time. Of these eight lines, lines 3, 11, 11.1, 16, and 17 flowered on average 62 d later than control plants, which is similar to the delay in flowering of wild-type Bd21-3 plants without undergoing vernalization (Fig. 2D).
Lines 8, 14, and 17.1 flowered on average 41 d later (Supplemental Fig. S2B). The two lines showing no delay in flowering, lines 10 and 12 (Supplemental Fig. S2B), showed the least increase in BdODDSOC2 expression level, suggesting flowering time is affected by BdODDSOC2 in a dosage-sensitive manner (Supplemental Fig. S1A). In agreement with their phenotype, late-flowering lines also had significantly more total numbers of leaves compared to control plants in the vernalized condition (Fig. 2H; Supplemental Fig. S2E). No difference in leaf number could be detected between transgenic lines and control plants grown in NV conditions (Fig. 2G; Supplemental Fig. S2G).

Other differences, developmental or morphological, were not detected in the analyzed transgenic lines compared to their null-siblings. These results show that BdODDSOC2 overexpression plants did not respond to vernalization and flowered at a similar time as wild-type Bd21-3 grown without vernalization.

RNAi-Mediated Knockdown of BdODDSOC2 Results in Accelerated Flowering

We also analyzed flowering time in both vernalized and NV for plants that ectopically express a hairpin construct in Bd21-3 intended to knock down endogenous BdODDSOC2 transcripts. Semiquantitative RT-PCR revealed that of 10 lines, 8 lines exhibited a reduction in BdODDSOC2 expression levels (Supplemental Fig. S1B), while lines 14 and 22 did not show a measurable BdODDSOC2 knockdown. When grown in NV, five RNAi lines exhibited significantly earlier flowering compared to control plants (Fig. 2E; Supplemental Fig. S2C). Of these five lines, RNAi lines 4 and 5 flowered 20 and 27 d earlier respectively, while lines 3, 8, and 19 flowered on average 12 d earlier. When vernalized, BdODDSOC2 RNAi-knockdown lines flowered at the same time as their nontransgenic control or wild-type plants (Fig. 2F; Supplemental Fig. S2C). This might be
explained by the fact that 2 weeks of vernalization almost completely saturates the vernalization requirement of Bd21-3, masking any further, especially partial, acceleration of flowering (Schwartz et al., 2010). Surprisingly, although not all RNAi-mediated knockdown lines show early flowering in NV conditions, except for line 3, all lines have a smaller number of leaves in comparison with the controls in NV conditions (Fig. 2I; Supplemental Fig. S2F). Yet leaf number was similar in transgenic RNAi and control plants under vernalized conditions (V; Fig. 2J; Supplemental Fig. S2H). Transgenic RNAi plants did not exhibit any morphological or developmental changes except for those described here. To conclude, BdODDSOC2 RNAi lines exhibit early

Figure 2. BdODDSOC2 as a repressor of flowering. A, Days to heading for wild-type Bd21-3 without and with vernalization (NV and V). B, BdODDSOC2 expression in NV, 2wV, and 1wPV. C and D, Days to heading for BdODDSOC2 overexpression without vernalization (NV; C) and with 2 weeks vernalization treatment (V; D). E and F, Days to heading for BdODDSOC2 RNAi lines in NV (E) and with vernalization (F). G and H, Final leaf number for BdODDSOC2 overexpression (NV; G) and with vernalization (H). I and J, Final leaf number for BdODDSOC2 RNAi (NV; I) and with V (J). Asterisks indicate P value of Student’s test: *P < 0.05, **P < 0.01, and ***P < 0.001. The tests were conducted to compare transgenic lines with both NC and wild-type Bd21-3. Error bars represent SE of four biological replicates for BdODDSOC2 expression and for phenotyping SE of 4 to 15 biological replicates.
flowering when grown without vernalization, while flowering is similar to wild-type Bd21-3 in the vernalized condition.

**BdODDSOC2 Responds Earlier Than BdVRN1 in a Vernalization-Responsive Accession BdTR3C**

To investigate the early vernalization response mechanism, we analyzed the expression level of *BdODDSOC2* and *BdVRN1* after 0, 6, 12, 24, and 48 h of cold in the vernalization-sensitive winter accession *BdTR3C*. We used this accession because *Bd21-3* is a facultative winter accession and does not have mandatory cold requirement, while *BdTR3C* is a winter accession requiring 6 weeks of cold (Supplemental Table S1). Our results revealed that expression of *BdODDSOC2* is already significantly down after 6 hours of cold and remained significantly down after 12, 24, and 48 h of cold (Fig. 3A). By contrast, expression of *BdVRN1* was not altered after 6 or 12 h of cold (Fig. 3B), *BdVRN1* responded to cold only after 24 h of cold, and expression remained significantly higher after 48 h of cold (Fig. 3B). This suggests that *BdODDSOC2*’s response to cold is earlier than *BdVRN1* in a vernalization-responsive accession (*BdTR3C*). While it is known for *Brachypodium* and barley that VRN1 is a negative regulator of *ODDSOC2*, our observation makes it less likely that *BdVRN1* is causing the early transcriptional down-regulation of *BdODDSOC2* or sets the *ODDSOC2* initial expression level (Deng et al., 2015; Woods et al., 2016).

**BdODDSOC2 Down-Regulation Is Stably Maintained after Vernalization in a Winter Accession BdTR3C, But Not in a Spring Accession Bd21**

To assess whether down-regulation of *BdODDSOC2* and up-regulation of *BdVRN1* in response to vernalization is maintained after returning the plants to warm conditions in a winter compared to a spring accession, we performed expression analysis of *BdODDSOC2* and *BdVRN1* in the spring accession *Bd21*, in which *BdODDSOC2* is down-regulated in response to cold (Ruelens et al., 2013) and compared this to the winter accession *BdTR3C*. We collected samples before cold (NV), after 2 weeks of cold for *Bd21*, 6 weeks of cold for *BdTR3C*, and after 1, 2, 3, 4, 5, 6, and 7 weeks post-vernvalization. Our results show that expression of *BdODDSOC2* is significantly down-regulated after 2 (*Bd21*) and 6 (*BdTR3C*) weeks of vernalization in both accessions, but this down-regulation is stably maintained only in the winter accession *BdTR3C* (Fig. 3, C and D). However, in the spring accession *Bd21*, expression of *BdODDSOC2* increased again when plants were 1wPV (Fig. 3C). As expected, expression of *BdVRN1* was significantly higher after vernalization (2wV and 6wV) and also remained significantly higher during post-vernvalization in both *Brachypodium* accessions (*Bd21* and *BdTR3C*; Fig. 3, D and F). Interestingly, expression levels of *BdVRN1* in postvernalization stages (1wPV to 7wPV) were low in comparison to *BdVRN1* expression level during cold in *Bd21* (Fig. 3D). Overall, these results suggest that *BdODDSOC2* down-regulation is stably maintained long after vernalization in the winter accession *BdTR3C* but not in the spring accession *Bd21*.

**Down-Regulation of BdODDSOC2 Is Associated with H3K27me3 Enrichment at BdODDSOC2 Chromatin**

To investigate the mechanism related to the down-regulation of *ODDSOC2*, we performed chromatin immunoprecipitation experiments (ChIP). In Arabidopsis, stable down-regulation of *FLC* is associated with H3K27me3 (histone 3 Lys 27 trimethylation) enrichment at the *FLC* chromatin (Bastow et al., 2004; Sung and Amasino, 2004). To determine whether down-regulation of *BdODDSOC2* in response to vernalization is associated with H3K27me3 modifications, we analyzed H3K27me3 modification in the spring *Brachypodium* accession *Bd21* and the winter accession *BdTR3C* before cold (NV), after 2 weeks for *Bd21* and 6 weeks of vernalization for *BdTR3C*, and 1wPV. H3K27me3 modification is associated with repression of transcription (Barski et al., 2007; Zhang et al., 2007). Our results show that H3K27me3 levels were high at *BdODDSOC2* chromatin in response to vernalization in *Bd21* and *BdTR3C* (Fig. 3, I and K). However, H3K27me3 enrichment was significantly near the transcription start site or nucleation region in the spring accession *Bd21* (region upB, A, B, C, D), while in the winter accession *BdTR3C*, significant H3K27me3 enrichment spanned the entire *BdODDSOC2* locus during vernalization (region upB, A, B, C, D, E, F, G, H). In addition, H3K27me3 modification was also maintained at *BdODDSOC2* chromatin 1wPV, but only locally in the spring accession *Bd21* (region upB, A, B, C) and across the locus in the winter accession *BdTR3C* (region upB, A, B, C, E, G, H). These results suggest that enrichment of H3K27me3 across the *BdODDSOC2* locus is responsible for stable down-regulation of *BdODDSOC2* in *BdTR3C*.

In barley, *HvVRN1* up-regulation in response to vernalization is associated with a decrease in H3K27me3 and an increase in H3K4me3 modification at *HvVRN1* (Oliver et al., 2009). Similarly, we analyzed H3K27me3 histone modification at *BdVRN1* in *Bd21* and *BdTR3C*. *BdVRN1* chromatin showed a significant decrease in H3K27me3 modifications during vernalization (region upA, C) and postvernalization (region upA, A, C) in the winter accession *BdTR3C* (Fig. 3L). By contrast, our results did not reveal an effect of vernalization on H3K27me3 histone marks at *BdVRN1* chromatin in the spring accession *Bd21* (Fig. 3J).

**Altered Expression of BdODDSOC2 Does Not Affect mRNA Levels of VRN1/2/3**

To understand possible cross-regulation and identify downstream genes acting in the same pathways as
ODDSOC2, we investigated expression of candidate targets in the most severely affected lines. In barley, overexpression of ODDSO2 down-regulates FPF1 (Greenup et al., 2010), while FLC in Arabidopsis is known to repress FT and SOC1 (Michaels et al., 2005). We quantified the transcript levels of their Brachypodium orthologs using qRT-PCR in the two strongest overexpression (lines 3 and 11.1) and RNAi (lines 4 and 5) lines at three different time points: NV, after 2 weeks of vernalization (V), and 2wPV. In addition to BdVRN3, BdFPF1, and BdSOC1, we also evaluated whether expression of BdVRN1 and BdVRN2 is affected in BdODDSOC2 transgenic lines. Despite significantly higher and lower transcript levels of BdODDSOC2 in overexpression and RNAi lines, respectively (Supplemental Fig. S6, A and B), the expression levels of the aforementioned genes were not detectably affected (Supplemental Fig. S6, C–G).

Figure 3. Expression of BdODDSOC2 and BdVRN1 in response to vernalization and H3K27me3 histone modifications at BdODDSOC2 and BdVRN1 in Bd21 and BdTR3C. A and B, Expression of BdODDSOC2 and BdVRN1 NV after 0, 6, 12, 24, and 48 h of cold in BdTR3C. C and D, Expression of BdODDSOC2 and BdVRN1 in Bd21. E and F, BdODDSOC2 and BdVRN1 in BdTR3C (NV), after 2 (Bd21) and 6 weeks (BdTR3C) of cold, and after 1wPV, 2wPV, 3wPV, 4wPV, 5wPV, 6wPV, and 7wPV. G and H, Schematic representation of BdODDSOC2 and BdVRN1 locus. I and J, Relative enrichment of H3K27me3 at BdODDSOC2 and BdVRN1 in Bd21. K and L, H3K27me3 at BdODDSOC2 and BdVRN1 in BdTR3C. Asterisks indicate P value of Student’s t test: *P < 0.05, **P < 0.01, and ***P < 0.001. The t tests were conducted to compare nonvernalized with vernalized and postvernalized plants. Error bars represent SE of four biological replicates for BdODDSOC2 and BdVRN1 expression and for H3K27me3 ChIP SE of two to five biological replicates.
FLC Homologs in Wheat

After exploring the role of BdODDSOC2 in Brachypodium, we further studied the role of FLC homologs in the vernalization response of the economically important crop wheat. Six gene sequences were previously identified as FLC orthologs in wheat (Ruelens et al., 2013; Supplemental Fig. S3). These genes were blasted against the available wheat genome sequence of Chinese Spring, the sequenced reference line (International Wheat Genome Sequencing Consortium, 2014; Choulet et al., 2014). Due to the hexaploid nature of wheat (AABBDD genome), each of those genes is in principle present as three homoeologous genes. Our search for wheat FLCs showed that TaAGL12, belonging to the MADS37 clade, is the sole FLC homolog located on chromosome 7. All other genes are located on chromosome 3, including the ODDSOC1 homolog TaAGL42 and the four ODDSOC2 homologs TaAGL41, TaAGL33, TaAGL22, and TaMADS2 (Fig. 4), as identified earlier (Ruelens et al., 2013; Supplemental Fig. S3). Sequence alignments further revealed that TaAGL42 is located downstream of TaAGL41. TaAGL42 fragments are present in the A and D subgenome but seem to be absent in the B subgenome of this genome sequence. TaAGL41 is located immediately downstream of TaAGL33-B and TaAGL22-B in a tandem duplication on the B subgenome (Fig. 4). Curiously, TaAGL33 and TaAGL22, which were previously isolated from wheat cDNA and annotated as separate genes (Zhao et al., 2006), share their promoter and MADS-box domain, yet both variants have different exons coding for the I- K- and C-domains of the protein. These exons of the two variants are located 18 kb apart from each other. The fact that two gene variants share the MADS box has to our knowledge not been previously observed in the MADS-box gene family (Fig. 4, inset). Additional searches could not find another MADS-box in the region (Fig. 4, inset). The presence of both transcripts, including the same MADS box and the respectively different exons coding for the I- K- and C-domains (Fig. 4, inset), was verified with cDNA amplification (data not shown). As their encoded proteins consist of the same DNA-binding MADS-domain, both proteins likely target the same genes. However, their highly diverged I- and K-domains, which determine protein-protein interaction specificity, could result in the formation of different protein complexes with distinct regulatory functions. Consequently, we refer to TaAGL22 and TaAGL33 as variants from the same transcriptional unit. TaMADS2 appears to be identical to the homoeolog of TaAGL33-B on the D subgenome and hence is referred to hereafter as TaAGL33-D. In summary, the six previously identified genes are collapsing to four FLC-like genes in wheat each on three subgenomes, with exception of TaAGL42, which is only present on the A and D subgenomes.

Expression of ODDSOC2-Like Genes in a Diverse Set of Wheat Cultivars

To assess the role of wheat FLC homologs in vernalization, we studied their expression in 10 wheat varieties with high genetic differentiation as measured by FST between winter habit and spring habit (Table I; regions as described in Cavanagh et al., 2013). Since in wheat, VRN1 is most clearly associated to vernalization requirement and PPD is involved in the response to photoperiod after the vernalization requirement is fulfilled, the varieties used were balanced for those loci. Vernalization requirements were established as days to flowering after exposure to 0, 4, and 8 weeks of cold. As expected, days to flowering...
varied greatly from 30 d to nonflowering (>111 d). Winter accessions (Xiaoyan54, Toisondor, Mendel, Grafton, Kingdom) did flower only after 8 weeks of vernalization (Table I). By contrast, spring accessions (Azhurnaya, Faller, Westonia, Taifun) flowered approximately at the same time regardless vernalization or the duration of vernalization. The facultative accession (Chinese Spring) flowered 31 d earlier when vernalized for 8 weeks in comparison to without vernalization (Table I).

To study the expression of FLC-related homologs, plant samples were collected from 3-week-old seedlings NV; 2wV, 4wV, 6wV, and 8wV; and 2wPV and 4wPV. As a control, we first characterized the expression of VRN1-A, VRN2, and VRN3. As expected, we found that VRN1-A expression is up-regulated upon cold exposure, and VRN3 starts to peak only after return to warm- and long-day conditions in all studied wheat accessions (Fig. 5A; Supplemental Fig. S4). Interestingly, our results show that VRN2 was downregulated during the first 2 or 4 weeks of cold in most studied wheat varieties as expected, but after longer cold exposure VRN2 expression increased higher than prevernalization levels in seven of the studied wheat varieties (Supplemental Fig. S4B). Although this up-regulation is unexpected, it has to be noted that in our study plants were vernalized under a 12-h photoperiod to mimic the short days of winter in temperate regions. By contrast, in previous studies in wheat and barley where VRN2 expression was found downregulated in response to cold, expression was determined in long-day conditions (Yan et al., 2004b; Trevaskis et al., 2006). This expression pattern, however, is similar to BdVRN2L behavior in Brachypodium, where it is induced upon cold exposure and functions as a repressor of flowering (Ream et al., 2014; Woods et al., 2016), which underlines Brachypodium as a potential model system to further elucidate the evolution and the molecular mechanisms of the vernalization response.

We first studied the homoeolog-aspecific expression patterns for the BdODDSOC1 homolog TaAGL42, the BdODDSOC2 homologs TaAGL41 and TaAGL33 (and TaAGL22), and the BdMADS37 homolog TaAGL12 (Fig. 5A). We were not able to detect any TaAGL12 expression in leaves at all studied time points. TaAGL42 expression was down-regulated after cold only in spring varieties (Fig. 5A). Furthermore, we found that TaAGL41 expression decreased during cold exposure, but down-regulation was strongest in spring accessions that also exhibited high expression of TaAGL41 NV (Fig. 5A; Supplemental Fig. S5A). As TaAGL33 and its variant TaAGL22 exhibited a similar homoeolog-aspecific expression pattern as BdODDSOC2 (data not shown), we designed homoeolog-specific primers for these genes and performed subgenome-specific expression analyses (Fig. 5A; Supplemental Fig. S5, B–G). Consistent with TaAGL33 and TaAGL22 sharing a promoter region, expression between variants originating from the same subgenome was very similar. Moreover, the transcript levels of TaAGL33 (A, B, D) and TaAGL22 (A, B, D) were stably downregulated by cold in the majority of accessions (Fig. 5A; Supplemental Fig. S5, B–G). Expression of some TaAGL33 and TaAGL22 homoeologs did increase during the first 2 weeks of cold but later decreased during cold treatment. The expression NV and the subsequent down-regulation varied per subgenome, indicating that each of the subgenomes carries slightly different alleles. When relative expression of TaAGL22 and TaAGL33 under NV is compared between winter and spring varieties, a similar pattern is present as in Brachypodium in that winter varieties consistently show a higher expression level than spring varieties (Fig. 5B). Notably, TaAGL22-D and TaAGL33-D exhibited a clearly significant expression difference between winter and spring wheat varieties NV (Fig. 5B). Furthermore, TaAGL33-D expression showed the strongest down-regulation during vernalization, which is maintained in postvernalization (Fig. 5C). This observation may suggest that the TaAGL33 homoeolog from the D subgenome acquired highest functional significance in vernalization-dependent regulation of flowering time. Similar to our observations in Brachypodium, the expression in response to cold first changes for TaAGL33-A and only afterward for TaVRN1 across the winter accessions (Fig. 5; Supplemental Fig. S4A and S5, B–D), emphasizing that prior to the onset of

Table I. Vernalization requirement of wheat varieties used in this study

<table>
<thead>
<tr>
<th>Cultivar Name</th>
<th>Origin</th>
<th>VRN-A1 Allele</th>
<th>PPD-D1 Allele</th>
<th>DTF without V</th>
<th>DTF after 4wV</th>
<th>DTF after 8wV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azhumaya</td>
<td>Ukraine</td>
<td>Spring</td>
<td>Insensitive</td>
<td>38.8</td>
<td>36.4</td>
<td>33.8</td>
</tr>
<tr>
<td>Faller</td>
<td>USA</td>
<td>Spring</td>
<td>Sensitive</td>
<td>42.2</td>
<td>41.0</td>
<td>34.6</td>
</tr>
<tr>
<td>Westonia</td>
<td>Australia</td>
<td>Spring</td>
<td>Insensitive</td>
<td>45.0</td>
<td>42.8</td>
<td>42.3</td>
</tr>
<tr>
<td>Taifun</td>
<td>Germany</td>
<td>Spring</td>
<td>Sensitive</td>
<td>47.4</td>
<td>44.8</td>
<td>43.4</td>
</tr>
<tr>
<td>Chinese Spring</td>
<td>China</td>
<td>Spring</td>
<td>Sensitive</td>
<td>70.0</td>
<td>66.2</td>
<td>41.6</td>
</tr>
<tr>
<td>Xiaoyan54</td>
<td>China</td>
<td>Winter</td>
<td>Insensitive</td>
<td>DNF</td>
<td>DNF</td>
<td>31.0</td>
</tr>
<tr>
<td>Toisondor</td>
<td>France</td>
<td>Winter</td>
<td>Insensitive</td>
<td>DNF</td>
<td>DNF</td>
<td>51.2</td>
</tr>
<tr>
<td>Mendel</td>
<td>France</td>
<td>Winter</td>
<td>Sensitive</td>
<td>DNF</td>
<td>DNF</td>
<td>55.0</td>
</tr>
<tr>
<td>Grafton</td>
<td>Great Britain</td>
<td>Winter</td>
<td>Sensitive</td>
<td>DNF</td>
<td>DNF</td>
<td>60.4</td>
</tr>
<tr>
<td>Kingdom</td>
<td>Great Britain</td>
<td>Winter</td>
<td>Sensitive</td>
<td>DNF</td>
<td>DNF</td>
<td>DNF</td>
</tr>
</tbody>
</table>
cold ODDSOC2/TaAGL33 is not bound by VRN1. This is in line with the observation that vrn1 mutants do not affect ODDSOC2 before vernalization (Greenup et al., 2010).

**DISCUSSION**

Despite FLC being one of the most studied plant genes, orthologs of this flowering repressor have only been recently identified in monocots (Ruelens et al., 2013). Now with the perspective that it is a gene closely related to FLC, we go beyond previous functional characterization of ODDSOC2 in barley by studying this gene in relation to vernalization in Brachypodium (Greenup et al., 2010). We add a quantitative relation between the vernalization length and ODDSOC2 expression before cold, a loss of function phenotype, its early regulation before VRN1, and stable down-regulation associated with H3K27me3 spreading at the level of chromatin. In wheat, TaAGL33 transcripts, considered to be Type 1 MADS-box genes, had been observed to respond to winter conditions (Winfield et al., 2009). We have added homoeolog-specific expression and resolved the relation to the TaAGL22 transcript, alternatively generated from the same locus. Based on previous and our data, we postulate that ODDSOC2 functions as a flowering repressor in the vernalization pathway of Pooideae similar to its ortholog FLC in Brassicaceae. Here, we further discuss the similarities and differences between these two homologs.

In Brassicaceae, the need for vernalization is overcome through the down-regulation of flowering repressor FLC in response to cold (Sheldon et al., 2000; Wang et al., 2009). Moreover, winter Arabidopsis accessions have dominant FLC alleles, while many rapid cycling accessions have nonfunctional or weak FLC alleles, suggesting FLC expression levels determine the vernalization response in a quantitative way (Gazzani et al., 2003; Michaels et al., 2003b; Sheldon et al., 2000; Shindo et al., 2005). Indeed, the expression level of FLC...
in nonvernalized plants among naturally occurring accessions shows a correlation with their respective vernalization requirement (Shindo et al., 2005). Also in barley and wheat, ODDSOC2/TaAGL33 expression is higher in winter varieties and low in spring varieties (Greenup et al., 2010; Winfield et al., 2009; Trevaskis et al., 2003). Similarly, we observe that ODDSOC2 expression before cold in different Brachypodium accessions is correlated with vernalization length, that is the duration of cold saturating the vernalization requirement. However, some Brachypodium accessions, including accessions needing long exposure to cold, exhibit relatively low prevernalization expression levels, suggesting additional factors mediate vernalization length, which may be more or less important depending on the accession. The overall strong correlation between ODDSOC2 levels before cold and vernalization length does suggest that BdODDSOC2 expression before cold possibly plays a role in setting the length of the vernalization requirement in a rheostatic manner, that is higher ODDSOC2 transcript levels before cold result in a longer cold period needed to saturate the vernalization requirement. Although previous studies have provided evidence that VRN1 can regulate ODDSOC2 in barley and Brachypodium (Deng et al., 2015; Woods et al., 2016), it is unlikely that BdODDSOC2 expression before cold is set by BdVRN1, since the latter is only transiently expressed before cold in a 16-h photoperiod and BdODDSOC2 responds earlier than BdVRN1 to cold. In this manner, ODDSOC2 could contribute to an adaptive mechanism by which distinct populations have evolved a different vernalization length. However, it remains unclear how BdODDSOC2 expression before vernalization is set and whether this involves FRI-like genes like in Arabidopsis (Michaels and Amasino, 1999).

Down-regulation of FLC by vernalization has been extensively studied in Arabidopsis where it represents a universal mechanism to relieve the plant from FLC repression after vernalization (Sheldon et al., 2000; Shindo et al., 2006). Previously, it has been shown that ODDSOC2 is down-regulated by cold in Brachypodium accession Bd21 and barley (Greenup et al., 2010; Ruelens et al., 2013). Conversely, our results show that BdODDSOC2 down-regulation is not widespread across Brachypodium accessions with only 11 of 25 accessions showing significant down-regulation in response to cold. By contrast, BdVRN1 is up-regulated in all accessions and significantly increased during vernalization in 19 accessions. We hypothesized that the lack of a uniform BdODDSOC2 response to cold might be linked to their vernalization requirement; however, neither the BdODDSOC2 response to cold nor its expression level in vernalized plants could be linked to either spring or winter habit. Possibly, ODDSOC2 functions differently in those accessions in which its expression is not affected or up-regulated by cold, or BdODDSOC2 repression might be achieved through posttranscriptional mechanisms like FLM in Arabidopsis (Pose et al., 2013; Lee et al., 2013). Alternatively, differences in the response of BdODDSOC2 to cold among accessions suggest that the molecular mechanism of its function is different from that of FLC in Arabidopsis. Indeed, while FLC acts directly upon floral integrators FT and SOC1, we found no evidence that BdODDSOC2 regulates BdVRN3 or BdSOC1.

An important aspect of FLC’s function in Arabidopsis is the ability to “remember” winter, which is molecularly achieved through the addition of mitotically stable repressive H3K27me3 histone marks at the FLC locus during vernalization (Angel et al., 2011; Bastow et al., 2004; Sung and Amasino, 2004). Similar to FLC, down-regulation of BdODDSOC2 in Bd21 and BdTR3C in response to cold is associated with an increase in H3K27me3 at the BdODDSOC2 locus, indicating that BdODDSOC2 in these accessions is epigenetically silenced. By contrast, in barley down-regulation of ODDSOC2 during cold could not be associated with alteration of H3K27me3 levels, although in this case only a single region at the transcription start site was analyzed (Greenup et al., 2010). However, to truly “remember” winter, it is important that when the plants are returned to warm, these histone marks are retained or even spread throughout the locus. Only in the case of BdTR3C, H3K27me3 marks were retained at the BdODDSOC2 locus following cold exposure, suggesting the vernalized state is maintained at the BdODDSOC2 chromatin. Previously, it has been shown in barley that up-regulation of VRN1 is associated with an increase in H3K4me3 and decrease in H3K27me3 at the VRN1 chromatin (Oliver et al., 2009). Likewise in BdTR3C, H3K27me3 marks decreased during cold exposure and were retained postvernalization, indicating epigenetic up-regulation of VRN1 is conserved throughout Pooidae. Together, these results suggest that both ODDSOC2 and VRN1 might be involved in remembering winter in Brachypodium. However, the fact that not all Brachypodium winter accessions exhibit down-regulation of ODDSOC2 indicates that the epigenetic regulation of winter memory cannot be, even partially, through ODDSOC2 in all accessions. Furthermore, in the spring accession KU104-2 of Triticum monococcum, TmODDSOC2 repression in vernalized plants is maintained only when TmVRN1 is present (Greenup et al., 2010). Future research in winter accessions of Brachypodium will help further elucidate the role of VRN1 and ODDSOC2 in the memory of vernalization in Pooidae.

Complementary with the overall expression pattern of ODDSOC2 in Brachypodium, we show that BdODDSOC2 acts as a represser of flowering through vernalization. Plants overexpressing BdODDSOC2 exhibited delayed flowering when vernalized. Since overexpression plants flowered at the same time as wild-type Bd21-3 plants without vernalization, BdODDSOC2 overexpression plants are rendered unresponsive to vernalization. BdODDSOC2 RNAi lines flowered earlier than control plants when grown without vernalization treatment. In contrast to the overexpression phenotype, these early flowering lines
are still vernalization responsive. This partial phenotype could be the result of the limitations of RNAi knockdown, and a more pronounced effect could possibly be demonstrated using a full knockout, for example via the CRISPR-Cas9 system. Alternatively, the partial effect might indicate that additional pathways, likely involving other vernalization regulators, control the vernalization response in *Brachypodium*. A previous study already demonstrated that the functions of *VRN1* and *VRN3* are conserved between *Brachypodium* and cereals, suggesting that the *VRN1-VRN3* regulatory interaction of the vernalization pathway is likewise conserved (Ream et al., 2014). Moreover, it was shown that *BdVRN1* knockdown in *Bd21-3* resulted in increased ODDSOC2 levels and in barley Golden Promise, *HvVRN1* can bind to the promoter of *HvODDSOC2* (Deng et al., 2015; Woods et al., 2016). In addition, winter barley with low basal *VRN1* expression exhibits high expression of ODDSOC2 (Deng et al., 2015). These data suggest that ODDSOC2 is downstream of *VRN1*, which together with the fact that altered *BdODDSOC2* levels do not affect *BdVRN3* transcript levels indicate that a *VRN1-ODDSOC2* interaction might regulate vernalization-induced flowering parallel to the floral integrator *VRN3*. Another line of evidence for a pathway parallel to *VRN1-VRN3* is the observation that *BdODDSOC2* overexpression plants flower similarly to nonvernalized wild-type plants. This observation is very different from Arabidopsis where overexpression of *FLC* delays flowering even without vernalization (Michaels and Amasino, 1999). However, in Arabidopsis, *FLC* represses two central flowering integrators preventing parallel pathways to induce flowering. As *BdODDSOC2* does not target *BdVRN3*, the photoperiod pathway possibly takes over. This might be specific to *Bd21-3*, which has a strong photoperiod pathway that could override the repression by ODDSOC2 (Ream et al., 2014). Such a mechanism might represent a fail-safe system, allowing flowering following an unusually mild winter.

To explore the extent by which our results in Brachypodium can be translated to wheat, an economically important crop, we performed expression analyses of chypodium can be translated to wheat, an economically important crop, we performed expression analyses of vernalization regulators, control the vernalization response in Brachypodium. A previous study already demonstrated that the functions of *VRN1* and *VRN3* are conserved between Brachypodium and cereals, suggesting that the *VRN1-VRN3* regulatory interaction of the vernalization pathway is likewise conserved (Ream et al., 2014). Moreover, it was shown that *BdVRN1* knockdown in *Bd21-3* resulted in increased ODDSOC2 levels and in barley Golden Promise, *HvVRN1* can bind to the promoter of *HvODDSOC2* (Deng et al., 2015; Woods et al., 2016). In addition, winter barley with low basal *VRN1* expression exhibits high expression of ODDSOC2 (Deng et al., 2015). These data suggest that ODDSOC2 is downstream of *VRN1*, which together with the fact that altered *BdODDSOC2* levels do not affect *BdVRN3* transcript levels indicate that a *VRN1-ODDSOC2* interaction might regulate vernalization-induced flowering parallel to the floral integrator *VRN3*. Another line of evidence for a pathway parallel to *VRN1-VRN3* is the observation that *BdODDSOC2* overexpression plants flower similarly to nonvernalized wild-type plants. This observation is very different from Arabidopsis where overexpression of *FLC* delays flowering even without vernalization (Michaels and Amasino, 1999). However, in Arabidopsis, *FLC* represses two central flowering integrators preventing parallel pathways to induce flowering. As *BdODDSOC2* does not target *BdVRN3*, the photoperiod pathway possibly takes over. This might be specific to *Bd21-3*, which has a strong photoperiod pathway that could override the repression by ODDSOC2 (Ream et al., 2014). Such a mechanism might represent a fail-safe system, allowing flowering following an unusually mild winter.

To explore the extent by which our results in Brachypodium can be translated to wheat, an economically important crop, we performed expression analyses of *ODDSOC2* homologs in different wheat accessions. We show that specifically, *TaAGL33* and its splice variant *TaAGL22* are stably down-regulated in response to prolonged cold in the majority of wheat accessions. Additionally, in winter wheat accessions *TaAGL33* and *TaAGL22* expression is high before cold while their expression is low in spring accessions, suggesting that these genes function similar to *ODDSOC2* in Brachypodium. This pattern was most pronounced for *TaAGL22* and *TaAGL33* transcribed from the D sub-genome. As the introduction of the D genome resulted in a broader radiation of hexaploid wheat into temperate regions than its tetraploid progenitor, which is mostly cultivated in semiarid regions (Dubcovsky and Dvorak, 2007), it is interesting to speculate that the introduction of *TaAGL22-D* and *TaAGL33-D* might have contributed in part to the increased adaptability to more temperate climates. Similar results were obtained from genome-wide expression analysis of the floral transition in wheat, in which a *TaAGL33* transcript was higher in two winter wheat varieties than in a spring accession. Expression of *TaAGL33* declined after 5 weeks, already suggesting *TaAGL33* is a repressor of flowering (Winfield et al., 2009).

Together with previous results from barley (Greenup et al., 2010), our results suggest that *ODDSOC2*’s role as a repressor of flowering is conserved among core Pooidaeae, which include important cereal crops such as wheat, oats, and rye. In a larger evolutionary context, it is interesting to note that *ODDSOC2*’s homolog in rice (*Oryza sativa*), *OsMADS51*, functions as a short-day promoter of flowering (Kim et al., 2007). Given their close relationship to core eudicot *FLC*-like genes, it is most parsimonious to assume that the promoter function of *OsMADS51* evolved independently after the Oryzoideae branched off from the stem lineage toward the Pooidaeae. This model in which a promoter function evolved from an ancestral repressor function does not have to be surprising, as the *FLC* clade itself is sister to the *APETALA1* (AP1) clade, which includes flowering promoters *AP1* in Arabidopsis and *VRN1* in Pooidaeae (Ruelens et al., 2013). Other examples of flowering regulators switching between a repressing or promoting function include *BeFT1* and *BeFT2* in beet (*Beta vulgaris*) and *AGL24* and *SVP* in core eudicots (Hartmann et al., 2000; Michaels et al., 2003a; Fin et al., 2010). Since *VRN1* also confers vernalization responsiveness and given the *FLC/ODDSOC2-VRN1/AP1* phylogenetic sister relationship (Ruelens et al., 2013), one could speculate that the absence of a vernalization role for *API*, and its paralogs *FUL* and *AGL79* in Arabidopsis, is derived from an ancestral *API*/VRN1 gene functioning in vernalization-mediated flowering before the monocot-dicot split, similar to *VRN1* in Poaceae.

In conclusion, our findings suggest a key role for *ODDSOC2* in the vernalization response of Brachypodium and identify *TaAGL33* as a good candidate for performing a similar function in wheat. We highlight the previously underestimated similarities between *FLC* in Arabidopsis and *ODDSOC2/TaAGL33* in monocots, which sheds light on the evolution of the vernalization mediated flowering. The provided knowledge can possibly be utilized in the future for crop improvement in order to adapt the vernalization requirement of elite varieties to changing climates. Key future questions to be addressed are whether the molecular mechanisms regulating *ODDSOC2* are also similar to *FLC* in Arabidopsis and how exactly *ODDSOC2* represses flowering.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions of Expression Study in a Set of Brachypodium Accessions**

Information on the accessions used in this study is given in Supplemental Table S1. Seeds of these accessions were obtained from the USDA germplasm, Dr. David Garvin, or Dr. Luis Mur after which seeds were propagated in house.
(Filiz et al., 2009; Mur et al., 2011; Vogel et al., 2006). Seeds were first incubated during 24 h at 23°C in the dark on a petri dish with filter paper and 2 mL of distilled water to ensure synchronous germination. Imbibed seeds were sown in Slim Rootrainers pots filled with a 50:50 mix of soil and vermiculite and placed in a growth chamber at 23°C with a 16-h-light/8-h-dark cycle and a light intensity of ~60 μmol m⁻² s⁻¹ at ground level produced by four fluorescent tubes (two Philips Master TL-D 36W/830 and two Osram L 36W 840 G13 Luxol cool-white lights). After 3 weeks, plants were transferred for vernalization to a Conviron at 4°C and a short-day photoperiod of 8 h light and 16 h dark. We chose to vernalize during vegetative growth (3-week-old plants), as this represents a realistic growth scenario for winter annuals (Choudar, 1960). Following this cold treatment, plants were returned to the growth chamber at 23°C with a 16-h-light/8-h-dark cycle. Flowering time was measured as the number of days from return to warm conditions until the spike approximately 2 cm emerged from the plant. For nonvernalized plants, days to flowering (Supplemental Table S1) was calculated from the day the seed was planted until the appearance of the first inflorescence.

Generation of BdODDSOC2 Overexpression and RNAi Transgenic Plants

Generation of BdODDSOC2 overexpression construct was done by introducing full-length BdODDSOC2 cDNA (from accession Bd21-3) into Gateway cloning vector pIPKB002 (Invitrogen). To clone RNAi hairpin construct, gateway cloning vector pIPKB007 was used (Himmelbach et al., 2007). In both vectors, transgene expression is driven by the maize (Zea mays) UBQUITIN promoter. BdODDSOC2 overexpression and RNAi construct were transformed in Brachypodium accession Bd21-3 using Agrobacterium tumefaciens-mediated transformation. We obtained 17 BdODDSOC2 overexpression lines. Ten lines with maximum seed yield were chosen for functional characterization. Of these 10, eight lines were generated from independent transformation events, while lines 11 and 17 derived from distinct shoots from the same callus of event 10, eight lines were generated from independent transformation events, while line 21 was generated from the same callus as line 2 but from a different shoot.

Phenotyping of Transgenic Lines

Transgenic plants were grown the same as plants for expression analysis. T1 plants were screened for the segregation of transgene. Phenotyping was done with T2 plants. Plants that carried the transgene in T2 were used for the analysis. Seeds from plants without the transgene in T0 were used as non-transgenic control plants. Bd-type Bd21-3 plants were used as a second control to compare days to flowering and final leaf number. Final leaf number referred to the total number of leaves including those on tillers on the entire plant on the day flowering became visible.

All transgenic plants were genotyped by PCR using ZmUbi1 promoter forward primer (F: 5′-TTCCGGAGACCGGATGATCAGTCTAGG-3′) and BdODDSOC2 reverse primer (R: 5′-ATCAGTGTGGTTGACCAACCC-3′). Plants that carried the transgene were used for phenotyping. Days to heading were calculated from the day the seed was planted until the appearance of the first inshorecence.

RNA Extraction and qRT-PCR of Brachypodium

Samples for total RNA isolation consisted of all above-ground tissues that were snap frozen in liquid nitrogen and stored at −80°C. RNA was extracted using TRIzol according to the manufacturer’s instructions (Invitrogen). To remove any genomic DNA, total RNA was DNase treated with TURBO DNA-free (Ambion). RNA quality and quantity were analyzed by spectrophotometer. cDNA was synthesized from 1 μg total RNA by reverse transcription using AMV reverse transcriptase (Promega). qRT-PCR was performed using 10 ng of cDNA on a StepOne Plus (Applied Biosystems) with Fast SYBR Green Master Mix following the manufacturer’s protocol (Applied Biosystems). Relative expression levels were calculated using the ∆∆ Ct method using UBIC8 (ubiquitin-conjugating enzyme 18) as a reference gene. The reference sample used depends on the experiment. BdODDSOC2 and BdVRN1 expression levels in the natural variation experiment are relative to a nonvernalized Bd3-1 sample. For expression analysis in Bd21-3, the NV plant sample was used as the reference.

The following primers were used for qRT-PCR: UBIC8, F: 5′-GTGACCTT-CCCGAGACTTAA-3′ and R: 5′-ATAGCCGCGGTTGAG-3′; BdODDSOC2, F: 5′-AAATCCACAGATTGGCAAAAGC-3′ and R: 5′-CTTACTGCTAC-TGGATTTCA-3′; BdVRN1, F: 5′-ACCAAGGCCAAGCTCTACGAAGG-3′ and R: 5′-GTTGCGCTTGATCACCTAGA-3′.

ChIP

ChIP was performed as previously described (Kaufmann et al., 2010) using whole plant tissue (without root). Plants were grown under a short-day photoperiod of 8 h light and 16 h dark for NV, V, and PV. The Sadenosyl-Met decarboxylase (Sadenosyl-Met decarboxylase) gene was used as a reference gene, as this gene was reported as most stable in plants grown under various environmental stress (Hong et al., 2008). The anti-h3K27me3 antibody was obtained from Millipore (Cat. #07-449). Sequences of primers used in ChIP are listed in Supplemental Table S3.

Semiquantitative RT-PCR

Three leaves were collected from transgenic plants. RNA isolation and cDNA preparation was performed as mentioned previously. Then 10 ng of cDNA was used to perform semiquantitative RT-PCR. Actin was used as the endogenous control to compare expression of transgene. To detect BdODDSOC2 expression in overexpression lines, 32 cycles were used for PCR amplification. For BdODDSOC2 RNAi lines, PCR reaction was done with 35 cycles. Primers used for semiquantitative RT-PCR were as follows: BdODDSOC2, F: 5′-CAACGTGGGCTCTTCAACAGG-3′ and R: 5′-CTTACCTTGACTACGTGGG-3′; BdODDSOC2 RNAi, F: 5′-GATGGTGCCTCAATTCCAGACAC-3′ and R: 5′-GATTTGCTTTGACCTCGTGTATGTTG-3′.

Statistics

Student’s t tests were performed in excel or R. Linear regressions and Pearson correlation tests were performed in R.

Wheat Vernalization and Expression Experiment

Seedlings of 10 wheat (Triticum aestivum) lines, with differentiation in winter and spring growth habit, were grown for 3 weeks at 21°C in long-day conditions (16 h light/8 h dark). After 3 weeks, seedlings were exposed to either no vernalization or to vernalization treatments of 4 or 8 weeks in a climate chamber at 4°C (12 h light/12 h dark). Afterward, seedlings were transferred to the greenhouse under long days (16 h light/8 h dark, with supplementary lighting when natural light levels dropped to <200 μE). The vernalization treatments were staggered in reverse order, such that all lines entered the greenhouse at the same time. Flowering time was measured as the number of days from return to warm conditions until the spike had emerged. For nonvernalized plants, days to flowering was between three weeks of age and spike emergence.

RNA Extraction and qRT-PCR for Wheat Genes

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). After TURBO DNA-free treatment, 500 ng of RNA was used for cDNA synthesis reaction using RNA to cDNA EcoDry Premix (TAKARA Clontech). Gene expression was carried out in two steps. First, sequences were preamplified in a sequence-specific manner using 1.3 μL cDNA (25 ng/μL) as described in the PreAmp with Fluidigm PreAmp Master Mix and TaqMan Assays (PN 100-5876 B1) protocols (www.lifetechnologies.com). In a second step, high-throughput quantitative PCR was carried out using a 1:5 diluted preamplification product, following the 96.96 iPC Using Standard TaqMan Assays (PN 6800130D1) protocol (www.fluidigm.com/documents), using the BioMark System for fluorescence detection.

Cycling threshold values were calculated with Fluidigm Real-Time PCR Analysis Software v4.1.2. For further qRT-PCR data analysis, the expression data were normalized using the ∆∆ Ct method against the control gene using the Biogazelle qBase+ v2.6.1 software. Transcript levels for ODDSOC2 homologs
were monitored in leaves of 3-week-old seedlings, after 4 and 8 weeks of vernalization and 2 and 4 weeks postvernalization using qRT-PCR with TaqMan assays. The (homoeolog-specific) primer and probe sequences for each target gene and the stably expressed control gene 68-kD protein HP68 (Ta.2776) control gene (Paolacci et al., 2009) are listed in Supplemental Table S2.

Supplemental Data
The following supplemental materials are available.

Supplemental Figure S1. Expression of BdODDSC02 in transgenic lines using semiquantitative PCR.

Supplemental Figure S2. Days to heading and leaf number for BdODDSC02 overexpression and RNAi lines.

Supplemental Figure S3. Phylogenetic tree representing the relationships between angiosperm FLC genes using SQUAMOSA-like genes as the outgroup.

Supplemental Figure S4. Expression of VRN1-A homologs NV.

Supplemental Figure S5. Expression of wheat ODDSC02 homologs in response to vernalization in different wheat varieties.

Supplemental Figure S6. Expression analysis of putative targets of BdODDSC02 in overexpression (lines 3 and 11.1) and RNAi lines (lines 4 and 5) in NV, V, and PV.

Supplemental Table S1. Brachypodium accessions used in this study and vernalization requirement assessed by this study.

Supplemental Table S2. List of primer/probe for target genes in wheat.

Supplemental Table S3. List of primers used in the H3K2me3 ChIP and downstream target of BdODDSC02.

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