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A Flowering locus C homolog is a vernalization-regulated repressor in Brachypodium and is cold-regulated in wheat

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Running Title: An *FLC* homolog in *Brachypodium* and Wheat

Summary

Our study reveals that *ODDSOC2/TaAGL33* functions in vernalization-responsive flowering of Poaceae in a similar but also unique manner compared to its *Arabidopsis* homolog, *FLOWERING LOCUS C*.

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Abstract

Winter cereals require prolonged cold to transition from vegetative to reproductive development. This process, referred to as vernalization, has been extensively studied in *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, for the first time 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 downregulated by cold and in one of the winter accessions in which this downregulation was evident, *BdODDSOC2* responded to cold before *BdVRN1.* When stably downregulated, the mechanism is associated with spreading H3K27me3 modifications at the *BdODDSOC2* chromatin. Finally, homeolog-specific gene expression analyses identifies *TaAGL33* and its splice variant *TaAGL22* as the *FLC* orthologs in *Triticum* 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 novel insights could prove useful in breeding efforts to refine the vernalization requirement of temperate cereals and adapt varieties to changing climates.

Introduction

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 and barley (Chouard, 1960), yet its regulation has been most extensively investigated in *Arabidopsis*. 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* in order 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 APETALA1* (*AP1*), is a promoter of flowering. In varieties that require vernalization, *VRN1* is upregulated 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* upregulation 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 downregulation 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 downregulation of *VRN2* during vernalization, suggesting that additional genes control *VRN2* downregulation when exposed to cold (Chen and Dubcovsky, 2012). Finally, downregulation 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 Pooideae (Ruelens et al., 2013). In *Brachypodium distachyon* (hereafter referred as *Brachypodium*), *BdODDSOC2* and *BdMADS37* expressions levels are downregulated 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 *Hordeum vulgare cv.* 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 upregulated in *VRN1* mutants in *Triticum,* 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., 2004b, Yan et al., 2003). 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., 2004a, Yan et al., 2003). 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 distachyon*. 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* pre-vernalization expression levels. During cold, *BdODDSOC2* is downregulated in most accessions and this response occurs hours before *BdVRN1*. We show that stable downregulation is associated with the spreading of histone methylation across the *ODDSOC2* locus. Consistent with a conserved role of *ODDSOC2* genes among cereals, the *Triticum*

aestivum 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 1). 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 weeks treatment of cold of three-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 1) (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 accessions from southern latitudes possess a short vernalization requirement.

To assess to what extent *BdODDSOC2* downregulation in response to cold is conserved among *Brachypodium* accessions, we examined the expression level of *BdODDSOC2* before vernalization (three week old plants, 23°C, 16h light/ 8h dark) and following four weeks of vernalization in 25 different accessions (4°C, 8h light/ 16h dark). Our results show that in 18 out of 25 accessions *BdODDSOC2* expression levels decrease in response to cold (Fig. 1A). In 11 accessions, this expression is significantly

downregulated (*Student's t*-test, $p<0.05$, Supplemental Table 1). In seven accessions *BdODDSOC2* levels increase (Fig. 1A), however, only two accessions, Bd1-1 and BdTR9K, exhibited a significant increase following four weeks at 4°C compared to pre-vernalization (*Student's t*-test, $p<0.05$, Supplemental Table 1). *BdVRN1* expression increased in all accessions in response to cold and 19 accessions exhibited a significant increase (*Student's t*-test, $p<0.05$, Supplemental Table 1). 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 six different accessions (Fig. 1B). In Bd3-1 and Adi10, *BdODDSOC2* expression during vernalization did not significantly change compared to non-vernalization. Expression levels of *BdODDSOC2* in BdTR2B, BdTR2G, Kah-1 and BdTR5I exhibited clear downregulation, although expression during vernalization in Kah-1 did not significantly change compared to non-vernalization. In the accessions Bd3-1 and Adi10, for which *ODDSOC2* did not change following four weeks of vernalization, a longer cold period also did not lead to cold induced downregulation.

Previous studies in *Arabidopsis* showed a significant correlation between the expression level of *FLC* in non-vernalized 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 non-vernalized plants or four 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 four weeks of cold could not predict the vernalization requirement ($R^2=0.044$, $p=0.32$). This suggests that mRNA transcript downregulation 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 which is amenable to *Agrobacterium*-mediated transformation (Vogel and Hill, 2008). Bd21-3 is a facultative winter accession that flowers without vernalization after 125 days (16h light/ 8h dark photoperiod), but exposure to two weeks of cold significantly accelerates flowering by 64 days (Fig. 2A). To assess how *BdODDSOC2* responds to cold in Bd21-3, we examined its expression using qRT-PCR prior to vernalization (NV), following two weeks of vernalization (2wV) and after one 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* prior to vernalization in Bd21-3, which requires two 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 *Zea mays UBIQUITIN* promoter, for which we obtained 17 overexpression lines. Ten lines with maximum seed yield were chosen for detailed characterization (see Methods). In all ten overexpression lines, the *BdODDSOC2* transcript level was increased as determined by semiquantitative RT-PCR (Supplemental Fig. 1). In order to test whether *BdODDSOC2* affected flowering time, T_2 overexpression transgenic lines were grown and phenotyped together with non-transgenic controls and wild-type Bd21-3 plants in two different conditions (see Methods). Non-transgenic controls (NC) were plants that went through transformation

but did not carry the transgene in T_0. In non-vernalized conditions (NV), all *BdODDSOC2* overexpression plants carrying the transgene flowered at the same time as the control plants (Fig. 2C, Supplemental Fig. 2A). By contrast, when vernalized (V), eight out of ten *BdODDSOC2* overexpression lines exhibited a significant delay in flowering time. Out of these eight lines, line 3, 11, 11.1, 16 and 17 flowered on average 62 days 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 days later (Supplemental Fig. 2B). The two lines showing no delay in flowering, lines 10 and 12 (Supplemental Fig. 2B), did show the least increase in *BdODDSOC2* expression level, suggesting flowering time is affected by *BdODDSOC2* in a dosage-sensitive manner (Supplemental Fig. 1A). 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. 2E). No difference in leaf number could be detected between transgenic lines and control plants grown in NV condition (Fig 2G, Supplemental Fig. 2G). 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 non-vernalized conditions for plants that ectopically express a hairpin construct in Bd21-3 intended to knock down endogenous *BdODDSOC2* transcripts. Semiquantitative RT-PCR revealed that out of ten lines, eight lines exhibited a reduction in *BdODDSOC2* expression levels (Supplemental Fig. 1B), while lines 14 and 22 did not show a measurable *BdODDSOC2* knockdown. When grown in non-vernalized conditions five RNAi lines exhibited significantly earlier flowering compared to control plants (Fig. 2E, Supplemental Fig. 2C). Out of these five lines, RNAi lines 4 and 5 flowered 20 and 27 days earlier respectively, while lines 3, 8 and 19 flowered on average 12 days earlier. When vernalized, *BdODDSOC2* RNAi-knockdown lines flowered at the same time as their

non-transgenic control or wild type plants (Fig. 2F, Supplemental Fig. 2C). This might be explained by the fact that two 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 condition (Fig. 2I, Supplemental Fig. 2F). Yet leaf number was similar in transgenic RNAi and control plants under vernalized conditions (V) (Fig. 2J, Supplemental Fig. 2H). Transgenic RNAi plants did not exhibit any morphological or developmental changes except for those described here. To conclude, *BdODDSOC2* RNAi lines exhibit early 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 hours 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 six weeks of cold (Supplemental Table 1). Our results revealed that expression of *BdODDSOC2* is already significantly down after six hours of cold and remained significantly down after 12, 24 and 48 hours of cold (Fig. 3A). By contrast, expression of *BdVRN1* was not altered after 6 or 12 hours of cold (Fig. 3B), *BdVRN1* responded to cold only after 24 hours of cold and expression remained significantly higher after 48 hours 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 downregulation of *BdODDSOC2* or sets the *ODDSOC2* initial expression level (Deng et al., 2015, Woods et al., 2016).

***BdODDSOC2* downregulation is stably maintained after vernalization in a winter accession BdTR3C, but not in a spring accession Bd21**

To assess whether downregulation of *BdODDSOC2* and upregulation 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 downregulated in response to cold (Ruelens et al., 2013) and compared this to the winter accession BdTR3C. We collected samples before cold (NV), after two weeks of cold for Bd21, six weeks of cold for BdTR3C and after 1, 2, 3, 4, 5, 6, and 7 weeks postvernalization. Our results show that expression of *BdODDSOC2* is significantly downregulated after two (Bd21) and six (BdTR3C) weeks of vernalization in both accessions, but this downregulation is stably maintained only in the winter accession BdTR3C (Fig. 3C-D). However, in the spring accession Bd21, expression of *BdODDSOC2* increased again when plants were returned to warm temperatures for one week (PV) (Fig. 3C). As expected, expression of *BdVRN1* was significantly higher after vernalization (2wV and 6wV) and also remained significantly higher during postvernalization in both *Brachypodium* accessions (Bd21 and BdTR3C) (Fig. 3D, 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* downregulation is stably maintained long after vernalization in the winter accession BdTR3C but not in the spring accession Bd21.

Downregulation of *BdODDSOC2* is associated with H3K27me3 enrichment at *BdODDSOC2* chromatin

To investigate the mechanism related to the downregulation of *ODSSCO2*, we performed chromatin immunoprecipitation experiments (ChIP). In *Arabidopsis*, stable downregulation of *FLC* is associated with H3K27me3 (histone 3 lysine 27 trimethylation) enrichment at the *FLC* chromatin (Bastow et al., 2004, Sung and Amasino, 2004). To determine whether downregulation 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 two weeks for Bd21 and six weeks of vernalization for BdTR3C and one week after returning the plants to warm conditions (PV). 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. 3I, K). However, H3K27me3 enrichment was significant mainly 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 one week after return to warm conditions (PV), but only locally in the spring accession Bd21 (region upB, 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 downregulation of *BdODDSOC2* in BdTR3C.

In *Hordeum*, *HvVRN1* upregulation 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 *VERNALIZATION1/2/3*

To understand possible cross-regulation and to identify downstream genes acting in the same pathways as *ODDSOC2,* we investigated expression of candidate targets in the most severely affected lines. In *Hordeum*, overexpression of *ODDSOC2* downregulates *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 (line 3 and 11.1) and RNAi (line 4 and 5) lines at three different time points: before cold (NV), after two weeks of vernalization (V) and after two weeks of postvernalization (PV). 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.6 A-B), the expression levels of the aforementioned genes were not detectably affected (Supplemental Fig.6 C-G).

FLC homologs in *Triticum aestivum*

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 *Triticum.* Six gene sequences were previously identified as *FLC* orthologs in wheat (Ruelens et al., 2013) (Supplemental Fig. 3). 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 are in principle present as three homeologous 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. 3). 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 the current 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 homeolog of *TaAGL33-B* on the D subgenome, hence is referred to hereafter as *TaAGL33-D*. In summary, the six previously identified genes are collapsing to four *FLC-like* genes in *Triticum aestivum* 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 ten wheat varieties with high genetic differentiation as measured by F_{ST} between winter habit and spring habit (Table 1) (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 days to non-flowering (>111 days). Winter accessions (Xiaoyan54, Toisondor, Mendel, Grafton, Kingdom) did flower only after eight weeks of vernalization (Table 1). 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 days earlier when vernalized for eight weeks in comparison to without vernalization (Table 1).

To study the expression of *FLC*-related homologs, plant samples were collected from three weeks old seedlings prior to cold exposure (NV), after 2, 4, 6 and 8 weeks of cold (2wV, 4wV, 6wV and 8wV) and 2 and 4 weeks after return to warm temperatures (2wPV, 4wPV). As a control, we first characterized the expression of *VRN1-A, VRN2* and *VRN3*. As expected, we found that *VRN1-A* expression is upregulated upon cold exposure and *VRN3* starts to peak only after return to warm and long day conditions in all studied *Triticum* accessions (Fig. 5A, Supplemental Fig. 4). Interestingly, our results show that *VRN2* was downregulated during the first two or four weeks of cold in most studied *Triticum* varieties as expected, but after longer cold exposure *VRN2* expression increased higher than pre-vernalization levels in seven of the studied *Triticum* varieties (Supplemental Fig. 4B). Although this upregulation is unexpected it has to be noted that in our study plants were vernalized under a 12h-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 homeolog-aspecific expression patterns for the *BdODDSOC1* homolog *TaAGL42*, the *BdODDSOC2* homologs *TaAGL41*, *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 downregulated after cold only in spring varieties (Fig. 5A). Furthermore, we found that *TaAGL41* expression decreased during cold exposure, but downregulation was strongest in spring accessions that also exhibited high expression of *TaAGL41* prior to vernalization (Fig. 5A, Supplemental Fig. 5A). As *TaAGL33* and its variant *TaAGL22* exhibited a similar homeolog-aspecific expression pattern as *BdODDSOC2* (data not

462 shown), we designed homeolog-specific primers for these genes and performed
463 subgenome-specific expression analyses (Fig. 5A, Supplemental Fig. 5B-G). Consistent
464 with *TaAGL33* and *TaAGL22* sharing a promoter region, expression between variants
465 originating from the same subgenome was very similar. Moreover, the transcript levels
466 of *TaAGL33* (*A, B, D*) and *TaAGL22* (*A, B, D*) were stably downregulated by cold in the
467 majority of accessions (Fig. 5A, Supplemental Fig. 5B-G). Expression of some
468 *TaAGL33* and *TaAGL22* homeologs did increase during the first two weeks of cold but
469 later decreased during cold treatment. The expression prior to vernalization and the
470 subsequent downregulation varied per subgenome, indicating that each of the
471 subgenomes carries slightly different alleles. When relative expression of *TaAGL22* and
472 *TaAGL33* under non-vernalized conditions is compared between winter and spring
473 varieties a similar pattern is present as in *Brachypodium* in that winter varieties
474 consistently show a higher expression level than spring varieties (Fig. 5B)**.** Notably,
475 *TaAGL22-D* and *TaAGL33-D* exhibited a clearly significant expression difference
476 between winter and spring wheat varieties prior to vernalization (Fig. 5B). Furthermore,
477 *TaAGL33-D* expression showed the strongest downregulation during vernalization,
478 which is maintained in post vernalization (Fig. 5C). This observation may suggest that
479 the *TaAGL33* homeolog from the D subgenome acquired highest functional significance
480 in vernalization-dependent regulation of flowering time. Similar to our observations in
481 *Brachypodium*, the expression in response to cold first changes for *TaAGL33-A* and
482 only afterwards for *TaVRN1* across the winter accessions (Fig. 5, Supplemental Fig. 4A
483 and 5B-D), emphasizing that prior to the onset of cold *ODDSOC2*/*TaAGL33* is not
484 bound by *VRN1*. This is in line with the observation that *vrn1* mutants do not affect
485 *ODDSOC2* before vernalization (Greenup et al., 2010).
486

487 ## Discussion

488 Despite *FLC* being one of the most studied plant genes, orthologs of this flowering
489 repressor have only been recently identified in monocots (Ruelens et al., 2013). Now
490 with the perspective that it is a gene closely related to *FLC*, we go beyond previous
491 functional characterization of *ODDSOC2* in barley by studying this gene in relation to
492 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 downregulation 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 homeolog 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 downregulation 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 non-functional 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 non-vernalized 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, i.e. the duration of cold saturating the vernalization requirement. However, some *Brachypodium* accessions, including accessions needing a long exposure to cold, exhibit relatively low pre-vernalization *BdODDSOC2* 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, i.e. higher *ODDSOC2* transcript levels before cold result in a longer cold period needed to saturate

524 the vernalization requirement. Although previous studies have provided evidence that
525 *VRN1* can regulate *ODDSOC2* in barley and *Brachypodium* (Deng et al., 2015, Woods
526 et al., 2016), it is unlikely that *BdODDSOC2* expression before cold is set by *BdVRN1*
527 since the latter is only transiently expressed before cold in a 16h-photoperiod and
528 *BdODDSOC2* responds earlier than *BdVRN1* to cold. In this manner, *ODDSOC2* could
529 contribute to an adaptive mechanism by which distinct populations have evolved a
530 different vernalization length. However, it remains unclear how *BdODDSOC2*
531 expression before vernalization is set and whether this involves *FRI*-like genes like in
532 *Arabidopsis* (Michaels and Amasino, 1999).
533
534 Downregulation of *FLC* by vernalization has been extensively studied in *Arabidopsis*
535 where it is represents a universal mechanism to relieve the plant from *FLC* repression
536 after vernalization (Sheldon et al., 2000, Shindo et al., 2006). Previously, it has been
537 shown that *ODDSOC2* is downregulated by cold in *Brachypodium* accession Bd21 and
538 *Hordeum vulgare* (Greenup et al., 2010, Ruelens et al., 2013). Conversely, our results
539 show that *BdODDSOC2* downregulation is not widespread across *Brachypodium*
540 accessions with only 11 out of 25 accessions showing significant downregulation in
541 response to cold. By contrast, *BdVRN1* is upregulated in all accession and significantly
542 increased during vernalization in 19 accessions. We hypothesized that the lack of a
543 uniform *BdODDSOC2* response to cold might be linked to their vernalization
544 requirement, however neither the *BdODDSOC2* response to cold nor its expression
545 level in vernalized plants could be linked to either spring or winter habit. Possibly,
546 *ODDSOC2* functions differently in those accessions in which its expression is not
547 affected or upregulated by cold, or *BdODDSOC2* repression might be achieved through
548 post-transcriptional mechanisms like *FLM* in *Arabidopsis* (Pose et al., 2013, Lee et al.,
549 2013). Alternatively, differences in the response of *BdODDSOC2* to cold among
550 accessions suggest that the molecular mechanism of its function is different from that of
551 *FLC* in *Arabidopsis*. Indeed, while *FLC* acts directly upon floral integrators *FT* and
552 *SOC1*, we found no evidence that *BdODDSOC2* regulates *BdVRN3* or *BdSOC1*.
553

554 An important aspect of *FLC*'s function in *Arabidopsis* is the ability to "remember" winter,
555 which is molecularly achieved through the addition of mitotically stable repressive
556 H3K27me3 histone marks at the *FLC* locus during vernalization (Angel et al., 2011,
557 Bastow et al., 2004, Sung and Amasino, 2004). Similar to *FLC,* downregulation of
558 *BdODDSOC2* in Bd21 and BdTR3C in response to cold is associated with an increase
559 in H3K27me3 at the *BdODDSOC2* locus indicating that *BdODDSOC2* in these
560 accessions is epigenetically silenced. By contrast, in *Hordeum* downregulation of
561 *ODDSOC2* during cold could not be associated with alteration of H3K27me3 levels
562 although in this case only a single region at the transcription start site was analyzed
563 (Greenup et al., 2010). However, to truly "remember" winter, it is important that when
564 the plants are returned to warm, these histone marks are retained or even spread
565 throughout the locus. Only in the case of BdTR3C, H3K27me3 marks were retained at
566 the *BdODDSOC2* locus following cold exposure suggesting the vernalized state is
567 maintained at the *BdODDSOC2* chromatin. Previously, it has been shown in *Hordeum*
568 that upregulation of *VRN1* is associated with an increase in H3K4me3 and decrease in
569 H3K27me3 at the *VRN1* chromatin (Oliver et al., 2009). Likewise in BdTR3C,
570 H3K27me3 marks decreased during cold exposure and were retained post-vernalization
571 indicating epigenetic upregulation of *VRN1* is conserved throughout Pooideae.
572 Together, these results suggest that both *ODDSOC2* and *VRN1* might be involved in
573 remembering winter in *Brachypodium*. However, the fact that not all *Brachypodium*
574 winter accessions exhibit downregulation of *ODDSOC2* indicates that the epigenetic
575 regulation of winter memory cannot be –even partially– through *ODDSOC2* in all
576 accessions. Furthermore, in the spring accession KU104-2 of *Triticum monococcum*,
577 *TmODDSOC2* repression in vernalized plants is only maintained when *TmVRN1* is
578 present (Greenup et al., 2010). Future research in winter accessions of *Brachypodium*
579 will help further elucidate the role of *VRN1* and *ODDSOC2* in the memory of
580 vernalization in Pooideae.
581
582 Complementary with the overall expression pattern of *ODDSOC2* in *Brachypodium*, we
583 show that *BdODDSOC2* acts as a repressor of flowering through vernalization. Plants
584 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 results of the limitations of RNAi knockdown and a more pronounced effect could possibly be demonstrated using a full knockout, e.g. 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 *Hordeum vulgare cv.* Golden Promise *HvVRN1* can bind to the promoter of *HvODDSOC2* (Deng et al., 2015, Woods et al., 2016). In addition, winter *Hordeum* with low basal *VRN1* expression exhibit 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 similar to non-vernalized 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 *Triticum aestivum* accessions. We show that specifically *TaAGL33* and its splice variant *TaAGL22* are stably downregulated in response to prolonged cold in the majority of *Triticum* accessions. Additionally, in winter *Triticum* 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 subgenome. As the introduction of the D genome resulted in a broader radiation of hexaploid *Triticum aestivum* into temperate regions than its tetraploid progenitor, which is mostly cultivated in semi-arid 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 five 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 Pooideae, 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, *OsMADS51*, functions as 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 towards the Pooideae. 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 Pooideae (Ruelens et al., 2013). Other examples of flowering regulators switching between a repressing or promoting

function include *BvFT1* and *BvFT2* in beet and *AGL24* and *SVP* in core eudicots (Hartmann et al., 2000, Michaels et al., 2003a, Pin 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 *AP1, and its paralogs FUL and AGL79,* in *Arabidopsis,* is derived from an ancestral AP1/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 *ODDSOC2* exactly represses flowering.

Material 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 1. 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 hours 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 16h-8h light-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

678 Lumilux cool white lights). After three weeks, plants were transferred for vernalization to
679 a Conviron at 4°C and a short-day photoperiod of 8h light and 16h dark. We chose to
680 vernalize during vegetative growth (three-week-old plants) as this represents a realistic
681 growth scenario for winter-annuals (Chouard, 1960). Following this cold treatment,
682 plants were returned to the growth chamber at 23°C with a 16h-8h light-dark cycle.
683 Flowering time was measured as the number of days from return to warm conditions
684 until the spike was approximately 1cm emerged from the plant. For non-vernalized
685 plants, days-to-flowering was between three weeks of age and spike emergence.
686 Accessions were classified into different vernalization classes based on how effective a
687 specific length of cold (weeks: 0, 2, 4, 6, 10, 12) could accelerate flowering
688 (Supplemental Table 1). For expression analysis of *BdODDSOC2* and *BDVRN1* in NV,
689 V, PV and extended PV (1wPV to 7wPV) in Bd21 and BdTR3C, short-day photoperiod
690 of 8h light and 16h dark were used. Primers used for downstream target of
691 *BdODDSOC2* are listed in Supplemental Table 3.
692
693 **Generation of *BdODDSOC2* overexpression and RNAi transgenic plants**
694 Generation of *BdODDSOC2* overexpression construct was done by introducing full
695 length *BdODDSOC2* cDNA (from accession Bd21-3) into Gateway cloning vector
696 pIPKb002 (Invitrogen). To clone RNAi hairpin construct, gateway cloning vector
697 pIPKb007 was used (Himmelbach et al., 2007). In both vectors transgene expression is
698 driven by the *Zea mays* (maize) *UBIQUITIN* promoter. *BdODDSOC2* overexpression
699 and RNAi construct were transformed in *Brachypodium* accession Bd21-3 using
700 *Agrobacterium tumefaciens*-mediated transformation. We obtained 17 *BdODDSOC2*
701 overexpression lines. Ten lines with maximum seed yield were chosen for functional
702 characterization. Out of these ten, eight lines were generated from independent
703 transformation events while lines 11.1 and 17.1 derived from distinct shoots from the
704 same callus of event 11 and 17 respectively. For *BdODDSOC2* RNAi, we generated 23
705 RNAi-mediated knockdown lines, from which 10 lines with maximum seed yield were
706 selected for further study. Out of ten, nine lines were derived from independent
707 transformation events, but line 2.1 was generated from the same callus as line 2, but
708 from a different shoot.

Phenotyping of transgenic lines

Transgenic plants were grown same as plants for expression analysis. T_1 plants were screened for the segregation of transgene. Phenotyping was done with T_2 plants. Plants which carried the transgene in T_2 were only used for the analysis. Seeds from plants without the transgene in T_0 were used as non-transgenic control plants. Wild-type Bd21-3 plants were used as a second control to compare days to heading 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 *ZmUBI* promoter forward primer (F: 5'-TTCCGCAGACGGGATCGATCTAGG-3') and *BdODDSOC2* reverse primer (R: 5'-ATCAGTGTTTGCCCACCACCC-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 inflorescence.

RNA extraction and qRT-PCR of *Brachypodium*

Samples for total RNA isolation consisted of all above-ground tissues which were snap frozen in liquid nitrogen and stored at -80°C. RNA was extracted using TRIzol according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). To remove any genomic DNA, total RNA was DNase treated with TURBO DNA-free (Ambion, Austin, USA). RNA quality and quantity was analyzed by spectrophotometer. cDNA was synthesized from 1µg total RNA by reverse transcription using AMV reverse transcriptase (Promega, Madison, USA). qRT–PCR was performed using 10ng of cDNA on a StepOne Plus (Applied Biosystems, Foster City, USA) with Fast SYBR Green Master Mix following the manufacturer's protocol (Applied Biosystems, Foster City, USA). Relative expression levels were calculated using the delta-delta Ct method using *UBC18* (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 non-vernalized Bd3-1 sample. For expression analysis in Bd21-3, NV plant sample was used as the reference.

The following primers were used for qRT-PCR:

UBC18: F: 5′-GTCGACTTCCCCGAGCATTA-3′,

R: 5′-ATAGGCGCCGGGTTGAG-3′

BdODDSOC2: F: 5′-AAATCCAAGATATTGGCAAAACG-3′,

R: 5′-CCTTAGGCTCACTGGAGTTCTCA-3′

BdVRN1: F: 5’-ACCAAGGGCAAGCTCTACGA-3’,

R: 5’-GTAGCGCTCATAGCGTTCAAGA-3’

Chromatin Immunoprecipitation (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 8h light and 16h dark for NV, V and PV. S-adenosylmethionine decarboxylase (*SamDC*) 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 3.

Semiquantitative RT-PCR

Three leaves were collected from T_0 transgenic plants. RNA isolation and cDNA preparation was performed as mentioned previously. 10ng of cDNA was used to perform Semiquantitative RT-PCR. *Actin* was used as the endogenous control to compare expression of transgene. To detect *BdODDSOC2* level in overexpression lines 32 cycles were used for PCR amplification. For *BdODDSOC2* RNAi lines, PCR reaction was done with 35 cycles. Primers used for semi qRT-PCR were as follow.

BdODDSOC2: F: 5′-CAGGTGCGCTTCTCCAAG-3′,

R: 5′-CTGTCCCTTAGGCTCACTGG-3′

ACTIN: F: 5′-GATGGATCCTCCAATCCAGACACTGTA-3′,

R: 5′-GTATTGTGTTGGACTCTGGTGATGGTGT-3′

Statistics

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

771

772 **Wheat vernalization and expression experiment**

773 Seedlings of ten wheat lines, with differentiation in winter and spring growth habit, were
774 grown for three weeks at 21°C in long-day conditions (16-h light/8-h dark). After three
775 weeks, seedlings were exposed to either no vernalization or to vernalization treatments
776 of four or eight weeks in a climate chamber at 4°C (12-h light/12-h dark). Afterwards
777 seedlings were transferred to the greenhouse under long days (16-h light/8-h dark, with
778 supplementary lighting when natural light levels dropped below 200 µE). The
779 vernalization treatments were staggered in reverse order, such that all lines entered the
780 greenhouse at the same time. Flowering time was measured as the number of days
781 from return to warm conditions until the spike had emerged. For non-vernalized plants,
782 days to flowering was between three weeks of age and spike emergence.

783

784 **RNA extraction and qRT-PCR for *Triticum* genes**

785 Total RNA was isolated using the RNeasy® Plant Mini Kit (Qiagen). After TURBO DNA-
786 free™ treatment, 500ng of RNA was used for cDNA synthesis reaction using RNA to
787 cDNA EcoDry™ Premix (TAKARA Clontech). Gene expression was carried out in two
788 steps. First, sequences were preamplified in a sequence-specific manner using 1,5µl
789 cDNA (25ng/µl), as described in the PreAmp with Fluidigm® PreAmp Master Mix and
790 TaqMan® Assays (PN 100-5876 B1) protocols (www.lifetechnologies.com). In a second
791 step, high-throughput quantitative PCR was carried out using a 1:5 diluted pre-
792 amplification product, following the 96.96 IFC Using Standard TaqMan® Assays (PN
793 68000130 D1) protocol (www.fluidigm.com/documents), using the BioMark™ System for
794 fluorescence detection.

795

796 Cycling threshold values were calculated with Fluidigm® Real-Time PCR Analysis
797 Software v4.1.2. For further qRT-PCR data analysis, the expression data were
798 normalized using the delta-delta Ct method against the control gene using the
799 Biogazelle qBase+ v2.6.1 software. Transcript levels for *ODDSOC2* homologs were
800 monitored in leaves of 3-week-old seedlings, after four and eight weeks of vernalization
801 and two and four weeks post vernalization using quantitative real-time reverse

802 transcription qRT-PCR with TaqMan® assays. The (homeolog-specific) primer and
803 probe sequences for each target gene and the stably expressed control gene 68 kDa
804 protein HP68 (Ta.2776) control gene (Paolacci et al., 2009), are listed in Supplemental
805 Table 2 .
806
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813
814 **Conflict of interest:** KG, KK and PR authored a patent relating to this work
815 (PCT/EP2014/063985).
816
817 ## Figure Legends
818 **Figure 1. *BdODDSOC2* response to cold in a set of *Brachypodium distachyon***
819 **accessions. A.** Heat map showing the response of *BdODDSOC2* and *BdVRN1* gene
820 expression in different *Brachypodium distachyon* accessions following a four week cold
821 period (4wV) relative to the expression prior to vernalization (NV) (log_{10} fold change).
822 Vern. R. depicts the vernalization requirement in weeks (w) as assessed in this study.
823 **B.** Expression pattern of *BdODDSOC2* during prolonged cold in selected *Brachypodium*
824 accessions. Bars represent the standard error of mean of 3-5 biological replicates.
825 Asterisks are indicated when p-value < 0.05 of a student's *t*-test between NV (0 weeks
826 of cold) and vernalized samples. **C**. Relationship between vernalization requirement and
827 expression of *BdODDSOC2* in non-vernalized plants and *BdVRN1* of plants grown
828 during a four week cold period. Each dot represents the mean of *BdODDSOC2* or
829 *BdVRN1* relative expression of 2-5 biological replicates. R^2 and p depicts the square of
830 the Pearson correlation coefficient and its associated p-value respectively. The solid
831 lines represent linear regression trend lines. NV: non-vernalized three week old plants,

832 4wV: during a vernalization period of four weeks. The red dot in the upper panel
833 highlights *BdODDSOC2* expression in *Brachypodium* Bd21-3.
834
835 **Figure 2: *BdODDSOC2* as a repressor of flowering. A.** Days to heading for wild type
836 Bd21-3 without and with vernalization (NV and V), **B.** *BdODDSOC2* expression in NV,
837 2wV and 1wPV. **C-D.** Days to heading for *BdODDSOC2* overexpression without
838 vernalization (NV, **C.**) and with two weeks vernalization treatment (V, **D.**). **E-F.** Days to
839 heading for *BdODDSOC2* RNAi lines in NV (**E**.) and with vernalization (**F**.). **G-H.** Final
840 leaf number for *BdODDSOC2* overexpression (NV, **G**.) and with vernalization (**H**.). **I-J**
841 Final leaf number for *BdODDSOC2* RNAi (NV, **I**.) and with V (**J**.). Asterisks indicate p-
842 value of Student's *t*-test: * p<0.05,** p<0.01, ***p<0.001. The *t*-tests were conducted to
843 compare transgenic lines with both non-transgenic control (NC) and wild type Bd21-3.
844 Error bars represent s.e.m. of four biological replicates for *BdODDSOC2* expression
845 and for phenotyping s.e.m of 4-15 biological replicates.
846
847 **Figure 3**: **Expression of *BdODDSOC2* and *BdVRN1* in response to vernalization**
848 **and H3K27me3 histone modifications at *BdODDSOC2* and *BdVRN1* in Bd21 and**
849 **BdTR3C**. **A-B.** Expression of *BdODDSOC2* and *BdVRN1* before cold (NV), after 0, 6,
850 12, 24 and 48 hours of cold in BdTR3C. **C-D.** Expression of *BdODDSOC2* and *BdVRN1*
851 in Bd21 **E-F.** *BdODDSOC2* and *BdVRN1* in BdTR3C, before cold (NV), after two (Bd21)
852 and six weeks (BdTR3C) of cold and after 1, 2, 3, 4, 5, 6, 7 weeks of return to warm
853 temperatures (1wPV, 2wPV, 3wPV, 4wPV, 5wPV, 6wPV, 7wPV). **G-H.** Schematic
854 representation of *BdODDSOC2* and *BdVRN1* locus. **I-J.** Relative enrichment of
855 H3K27me3 at *BdODDSOC2* and *BdVRN1* in Bd21. **K-L.** H3K27me3 at *BdODDSOC2*
856 and *BdVRN1* in BdTR3C. Asterisks indicate p-value of Student's *t*-test: * p<0.05,**
857 p<0.01, ***p<0.001. The *t*-tests were conducted to compare non-vernalized with
858 vernalized and postvernalized plants. Error bars represent s.e.m. of four biological
859 replicates for *BdODDSOC2* and *BdVRN1* expression and for H3K27me3 ChIP s.e.m of
860 2-5 biological replicates.
861

Figure 4: *FLC*-like genes in *Triticum aestivum.* Genomic localization of *ODDSOC1* (*TaAGL42*) and *ODDSOC2* (*TaAGL22*, *TaAGL33*, *TaAGL41*) homologs**.** *MADS37* ortholog*, TaAGL12,* is not shown. *Inset*. Schematic representation of the locus encoding both *TaAGL22* and *TaAGL33* on pseudochromosome 3B.

Figure 5: Expression of *FLC* homologs in response to vernalization in *Triticum* varieties. A. Sparkline plots showing the expression pattern of *VRN* and *FLC*-like genes in 10 different *Triticum* accession in response to vernalization. *VRN1-A, VRN2, VRN3* expression is shown prior to vernalization (NV), after two, four, six and eight weeks of vernalization (2wV, 4wV, 6wV, 8wV) and two and four weeks post vernalization (2wPV, 4wPV). Expression of *ODDSOC1/2* genes was assayed prior to vernalization (NV), after four and eight weeks of vernalization (4wV, 8wV) and two and four weeks post vernalization (2wPV, 4wPV). Individual plots with standard error of mean are shown in Supplemental Figure 4 and 5. N/A (Not Available) refers to samples for which expression patterns were not reproducible. **B.** Box plots comparing the relative expression levels of *TaAGL22* and *TaAGL33* homeologs in NV between winter and spring *Triticum* varieties. Student's *t*-test was used to compare between groups and resulting p-values are shown above each plot. **C.** *TaAGL33-D* expression after four and eight weeks of vernalization (4wV, 8wV) and two and four weeks post vernalization (2wPV, 4wPV).

Table 1. Vernalization requirement of *Triticum* varieties used in this study.

Supplemental information

Supplemental Figure 1. Expression of *BdODDSOC2* in transgenic lines using semiquantitative PCR.

Supplemental Figure 2. Days to heading and leaf number for *BdODDSOC2* overexpression and RNAi lines**.**

Supplemental Figure 3: Phylogenetic tree representing the relationships between angiosperm *FLC* genes using *SQUAMOSA*-like genes as the outgroup.

Supplemental Figure 4. Expression of *VRN1-A* homologs prior to vernalization.

Supplemental Figure 5. Expression of wheat *ODDSOC2* homologs in response to vernalization in different *Triticum aestivum* varieties.

Supplemental Figure 6. Expression analysis of putative targets of *BdODDSOC2* in overexpression (line 3 and 11.1) and RNAi (line 4 and 5) in NV, V and PV.

Supplemental Table 1. *Brachypodium* accessions used in this study and vernalization requirement assessed by this study.

Supplemental Table 2. List of primer/probe for target genes in *Triticum*.

Supplemental Table 3. List of primers used in the H3K27me3 ChIP and downstream target of *BdODDSOC2.*

Supplemental Figure 1. Expression of *BdODDSOC2* in transgenic lines using semiquantitative PCR. **A.** *BdODDSOC2* overexpression lines. **B.** *BdODDSOC2* RNAi lines.

Supplemental Figure 2. Days to heading and leaf number for *BdODDSOC2* overexpression and RNAi lines**. A-B.** Days to heading for *BdODDSOC2* overexpression without vernalization (NV), with vernalization (V). **C-D.** Days to heading for *BdODDSOC2* RNAi NV, with V. **E-F.** Final leaf number for *BdODDSOC2* overexpression NV, with V. **G-H.** Final leaf number for *BdODDSOC2* RNAi in NV, with V. Asterisks indicate p-value of Student's *t*-test: * $p<0.05$,** $p<0.01$, ***$p<0.001$. The *t*-tests were conducted to compare transgenic lines with both non-transgenic control (NC) and wild type Bd21-3. Error bars represent s.e.m. of four biological replicates for *BdODDSOC2* expression and for phenotyping s.e.m of 4-15 biological replicates.

Supplemental Figure 3: Phylogenetic tree representing the relationships between angiosperm *FLC* genes using *SQUAMOSA*-like genes as the outgroup. Numbers next to each node indicate bootstrap support values of 100 non-parametric bootstraps. Phylogenetic analysis was performed with PhyML 3.0. Dataset from (24) supplemented with additional sequences using BLAST.

Supplemental Figure 4. Expression of *VRN1-A* homologs prior to vernalization (NV), after two, four, six and eight weeks of vernalization (2wV, 4wV, 6wV, 8wV), and two and four weeks post vernalization (2wPV, 4wPV) in different *Triticum aestivum* varieties. Error bar represent standard error of mean of four biological replicates.

Supplemental Figure 5. Expression of wheat *ODDSOC2* homologs in response to vernalization in different *Triticum aestivum* varieties. **A.** *TaAGL41*, **B.** *TaAGL33-A,* **C.** *TaAGL33-B,* **D.** *TaAGL33-D*, **E.** *TaAGL22-A*, **F.** *TaAGL22-B*, **G.** *TaAGL22-D,* prior to vernalization (NV), after four and eight weeks of vernalization 4wV, 8wV and two and four weeks of post vernalization 2wPV, 4wPV. Error bar represent standard error of the mean of 2-4 biological replicates.

Supplemental Figure 6. Expression analysis of putative targets of *BdODDSOC2* in overexpression (line 3 and 11.1) and RNAi (line 4 and 5) in NV, V and PV. **A.** *BdODDSOC2* in overexpression lines, **B.** *BdODDSOC2* in RNAi lines, **C.** *BdVRN1,* **D.** *BdVRN2*, **E.** *BdVRN3*, **F.** *BdFPF1*, **G.** *BdSOC1,* prior to vernalization (NV), after two weeks of vernalization (V) and two weeks of post vernalization (PV). Asterisks indicate p-value of *Student's* t-test: * $p<0.05$,** $p<0.01$, ***$p<0.001$. t-test was conducted by comparing transgenic lines with non-transgenic control (NC). Error bar represent standard error of the mean of 3-4 biological replicates.

Supplemental Table 1. *Brachypodium* accessions used in this study and vernalization requirement assessed by this study.

Supplemental Table 2. List of primer/probe for target genes in *Triticum*.

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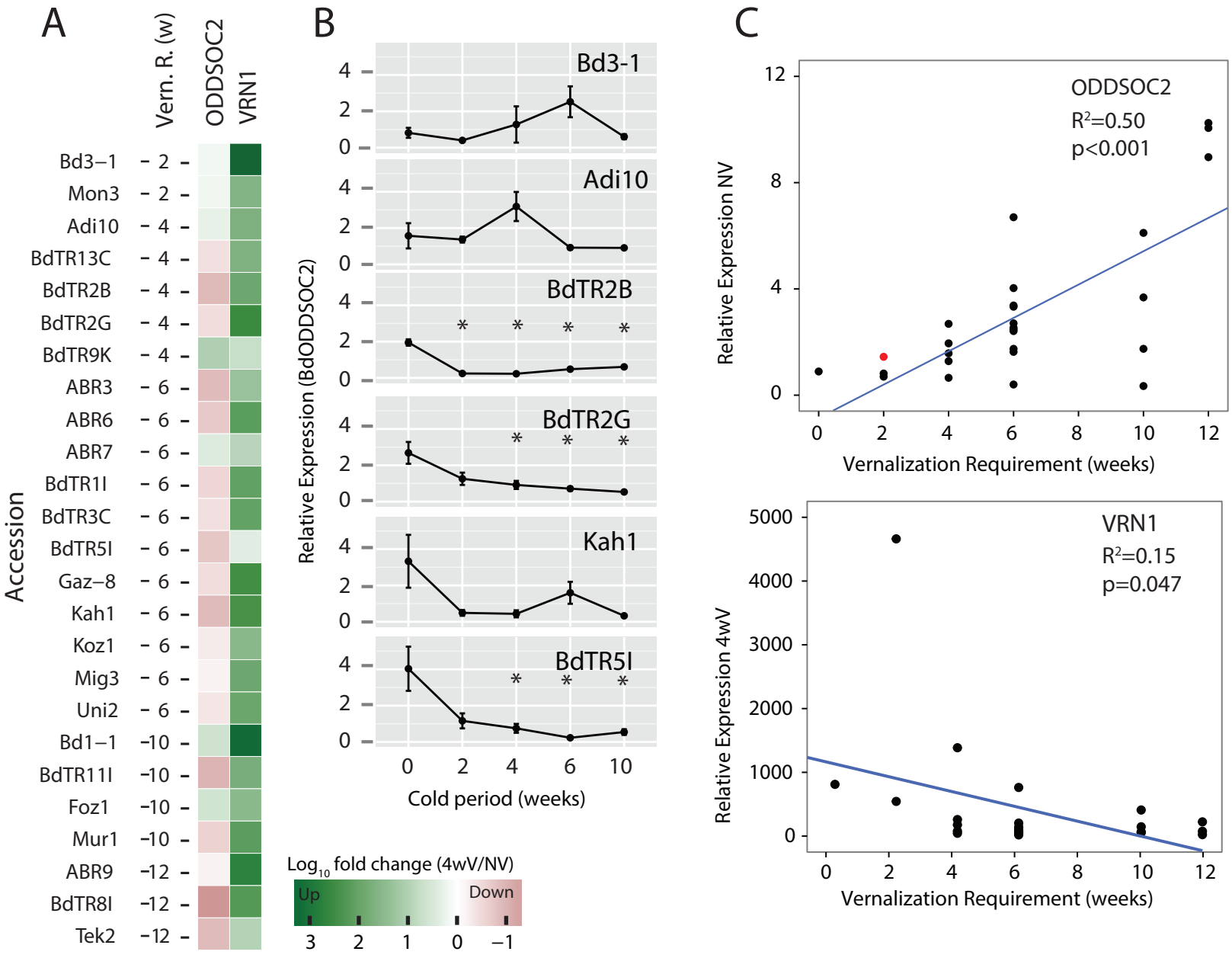

Figure 1. *BdODDSOC2* response to cold in a set of *Brachypodium distachyon* accessions. A. Heat map showing the response of *BdODDSOC2* and *BdVRN1* gene expression in different *Brachypodium distachyon* accessions following a four week cold period (4wV) relative to the expression prior to vernalization (NV) (log10 fold change). Vern. R. depicts the vernalization requirement in weeks (w) as assessed in this study. **B.** Expression pattern of *BdODDSOC2* during prolonged cold in selected Brachypodium accessions. Bars represent the standard error of mean of 3-5 biological replicates. Asterisks are indicated when p-value < 0.05 of a student's t-test between NV (0 weeks of cold) and vernalized samples. **C.** Relationship between vernalization requirement and expression of *BdODDSOC2* in non-vernalized plants and *BdVRN1* of plants grown during a four week cold period. Each dot represents the mean of *BdODDSOC2* or *BdVRN1* relative expression of 2-5 biological replicates. R^2 and p depicts the square of the Pearson correlation coefficient and its associated p-value respectively. The solid lines represent linear regression trend lines. NV: non-vernalized three week old plants, 4wV: during a vernalization period of four weeks. The red dot in the upper panel highlights *BdODDSOC2* expression in *Brachypodium* Bd21-3.

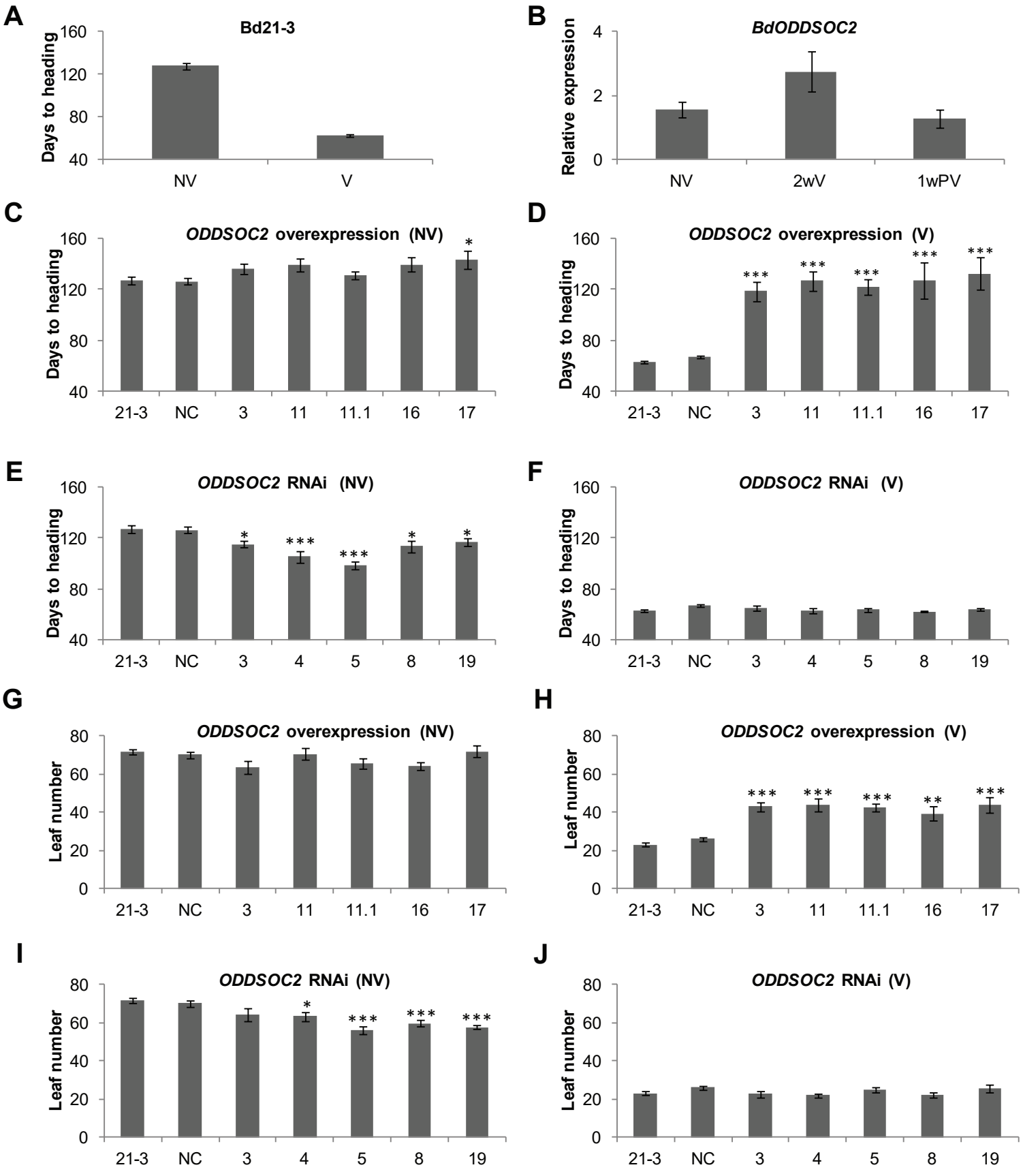

Figure 2: *BdODDSOC2* as a repressor of flowering. A. Days to heading for wild type Bd21-3 without and with vernalization (NV and V), **B.** *BdODDSOC2* expression in NV, 2wV and 1wPV, **C-D.** Days to heading for *BdODDSOC2* overexpression without vernalization (NV, **C.**) and with two weeks vernalization treatment (V, **D.**). **E-F.** Days to heading for *BdODDSOC2* RNAi lines in NV (**E.**) and with vernalization (**F.**). **G-H.** Final leaf number for *BdODDSOC2* overexpression (NV, **G.**) and with vernalization (**H.**). **I-J.** Final leaf number for *BdODDSOC2* RNAi (NV, **I.**) and with V (**J.**). Asterisks indicate p-value of Student's t-test: * $p<0.05$,** $p<0.01$, ***$p<0.001$. The t-tests were conducted to compare transgenic lines with both non-transgenic control (NC) and wild type Bd21-3. Error bars represent s.e.m. of four biological replicates for *BdODDSOC2* expression and for phenotyping s.e.m of 4-15 biological replicates.

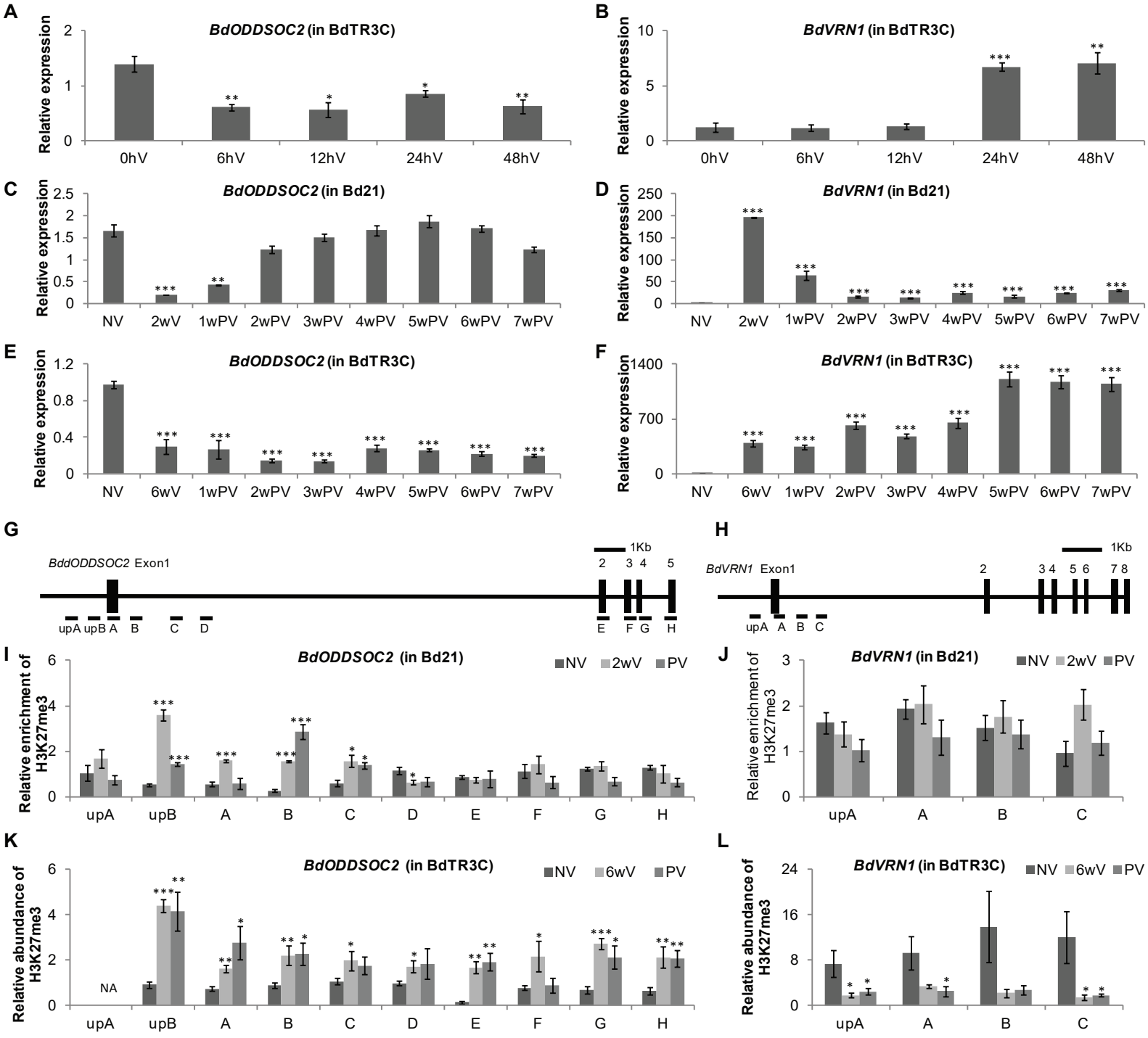

Figure 3: Expression of *BdODDSOC2* and *BdVRN1* in response to vernalization and H3K27me3 histone modifications at *BdODDSOC2* and *BdVRN1* in Bd21 and BdTR3C. A-B. Expression of *BdODDSOC2* and *BdVRN1* before cold (NV), after 6, 12, 24 and 48 hours of cold in BdTR3C. **C-D.** Expression of *BdODDSOC2* and *BdVRN1* in Bd21 **E-F.** *BdODDSOC2* and *BdVRN1* in BdTR3C, before cold (NV), after two (Bd21) and six weeks (BdTR3C) of cold and after 1, 2, 3, 4, 5, 6, 7 weeks of return to warm temperatures (1wPV, 2wPV, 3wPV, 4wPV, 5wPV, 6wPV, 7wPV). **G-H.** Schematic representation of *BdODDSOC2* and *BdVRN1* locus. **I-J.** Relative enrichment of H3K27me3 at *BdODDSOC2* and *BdVRN1* in Bd21. **K-L.** H3K27me3 at *BdODDSOC2* and *BdVRN1* in BdTR3C. Asterisks indicate p-value of Student's t-test: * p<0.05,** p<0.01, ***p<0.001. The t-tests were conducted to compare non-vernalized with vernalized and postvernalized plants. Error bars represent s.e.m. of four biological replicates for *BdODDSOC2* and *BdVRN1* expression and for H3K27me3 ChIP s.e.m of 2-5 biological replicates.

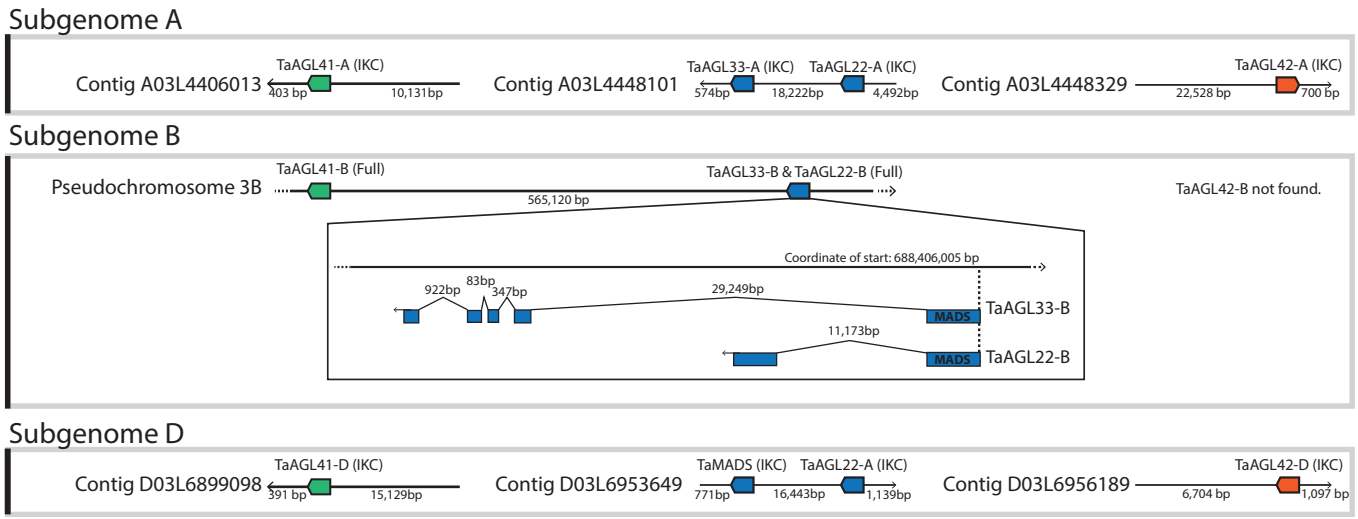

Figure 4: FLC-like genes in *Triticum aestivum.* Genomic localization of *ODDSOC1* (*TaAGL42*) and *ODDSOC2* (*TaAGL22, TaAGL33, TaAGL41*) homologs. *MADS37* ortholog, *TaAGL12*, is not shown. Inset. Schematic representation of the locus encoding both *TaAGL22* and *TaAGL33* on pseudochromosome 3B.

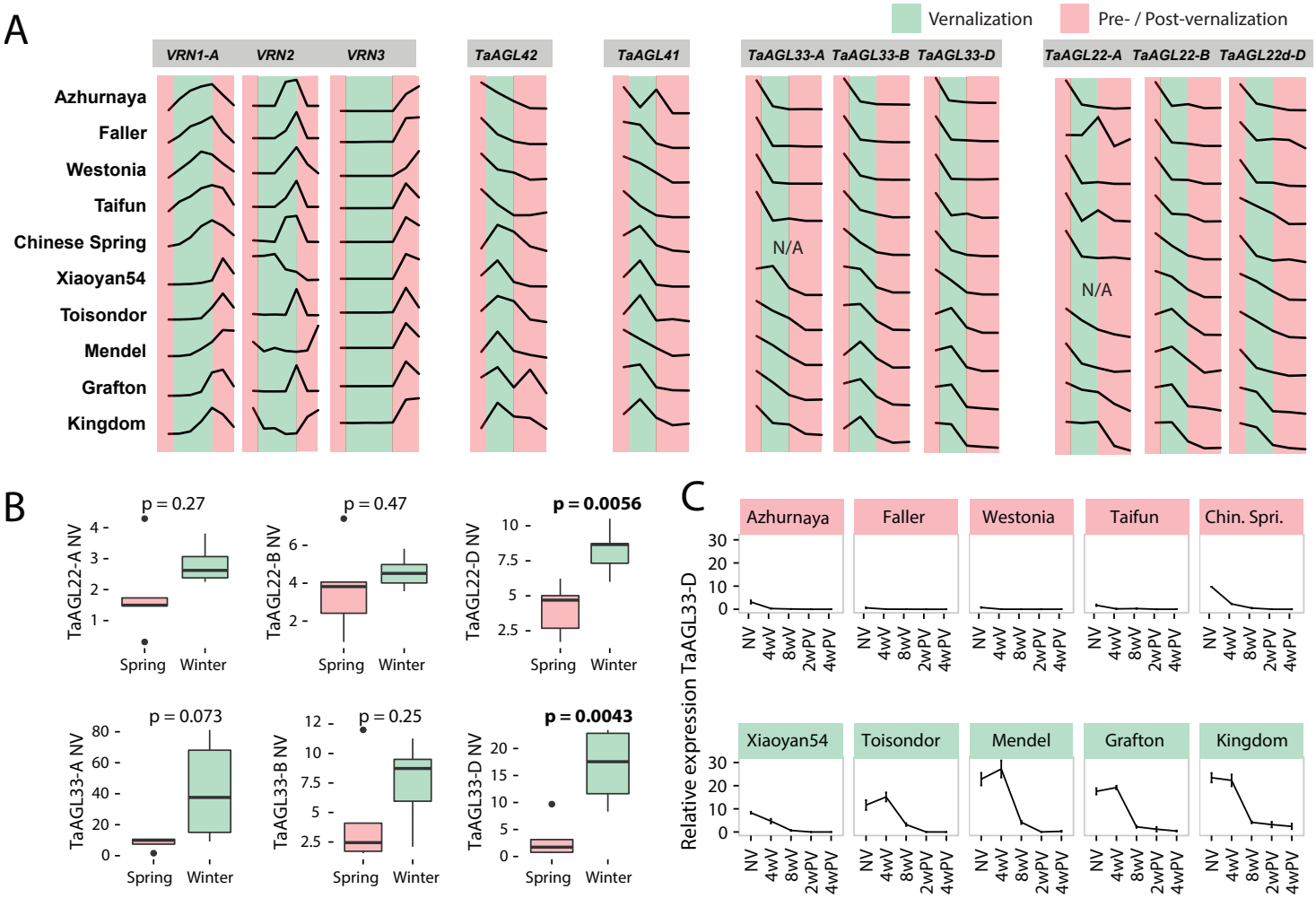

Figure 5: Expression of *FLC* homologs in response to vernalization in Triticum varieties. A. Sparkline plots showing the expression pattern of *VRN* and *FLC*-like genes in 10 different *Triticum* accession in response to vernalization. *VRN1-A, VRN2, VRN3* expression is shown prior to vernalization (NV), after two, four, six and eight weeks of vernalization (2wV, 4wV, 6wV, 8wV) and two and four weeks post vernalization (2wPV, 4wPV). Expression of *ODDSOC1/2* genes was assayed prior to vernalization (NV), after four and eight weeks of vernalization (4wV, 8wV) and two and four weeks post vernalization (2wPV, 4wPV). Individual plots with standard error of mean are shown in Supplemental Figure 4 and 5. N/A (Not Available) refers to samples for which expression patterns were not reproducible. **B.** Box plots comparing the relative expression levels of *TaAGL22* and *TaAGL33* homeologs in NV between winter and spring *Triticum* varieties. Student's t-test was used to compare between groups and resulting p-values are shown above each plot. **C.** *TaAGL33-D* expression after four and eight weeks of vernalization (4wV, 8wV) and two and four weeks post vernalization (2wPV, 4wPV).

Table 1. Vernalization requirement of Triticum varieties used in this study

Cultivar name	Origin	VRN-A1 allele	PPD-D1 allele	DTF without V	DTF after 4wV	DTF after 8wV
Azhurnaya	Ukraine	Spring	insensitive	38.8	36.4	33.8
Faller	USA	Spring	sensitive	42.2	41	34.6
Westonia	Australia	Spring	insensitive	45	42.8	42.3
Taifun	Germany	Spring	sensitive	47.4	44.8	43.4
Chinese Spring	China	Spring	sensitive	70	66.2	41.6
Xiaoyan54	China	Winter	insensitive	DNF	DNF	31
Toisondor	France	Winter	insensitive	DNF	DNF	51.2
Mendel	France	Winter	sensitive	DNF	DNF	55
Grafton	Great Britain	Winter	sensitive	DNF	DNF	60.4
Kingdom	Great Britain	Winter	sensitive	DNF	DNF	DNF

DNF and DTF abbreviate for did not flower (>111 days) and days to flowering respectively.

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