Transcription factor VRT2 reinitiates vernalization when interrupted by warm temperatures in a temperate grass model

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Abstract

Vernalization-responsive plants use cold weather, or low temperature, as a cue to monitoring the passing of winter. Winter cereals can remember the extent of coldness they have experienced, even when winter is punctuated by warm days. However, in a seemingly unnatural process called "devernalization," hot temperatures can erase winter memory. Previous studies in bread wheat (*Triticum aestivum*) have implicated the MADS-box transcription factor VEGETATIVE TO REPRODUCTIVE TRANSITION 2 (VRT2) in vernalization based on transcriptional behavior and ectopic expression. Here, we characterized 3 *BdVRT2* loss-of-function alleles in the temperate model grass *Brachypodium distachyon*. In addition to extended vernalization requirements, mutants showed delayed flowering relative to wild-type plants when exposed only briefly to warm temperatures after partial vernalization, with flowering being unaffected when vernalization was saturating. Together, these data suggest a role for BdVRT2 in both vernalization and in its reinitiation when interrupted by warm temperatures. In controlled constant conditions, *BdVRT2* transcription was not strongly affected by vernalization or devernalization. Yet, by monitoring *BdVRT2* expression in seasonally varying and fluctuating conditions in an unheated greenhouse, we observed strong upregulation, suggesting that its transcription is regulated by fluctuating vernalizing–devernalizing conditions. Our data suggest that devernalization by hot temperatures is not a peculiarity of domesticated cereal crops but is the extreme of the reversibility of vernalization by warm temperatures and has broader biological relevance across temperate grasses.

Introduction

Timing the transition to flowering with favorable seasonal conditions is essential for maximum reproductive success and is a key trait that must be modified when plants are adapting to new environments or cultivation zones (Jung and Müller 2009; Díaz et al. 2012). In several temperate cereals (Pooideae, Poaceae), flowering is encouraged by different external conditions, including exposure to prolonged cold temperatures (vernalization) followed by warm spring temperatures. Although impressive advances have been made in understanding the molecular genetic mechanisms involved in temperature-regulated flowering time (Hemming et al. 2012; Ford et al. 2016; Ejaz and von Korff 2017; Kiss et al. 2017; Xu and Chong 2018; Dixon et al. 2019; Mayer et al. 2019), how low- and high-temperature signals are integrated into this pathway is still not well understood. This knowledge gap has implications for predicting how plants will respond to climate change, as well as our ability to engineer crops for sustainable and equitable agriculture.

Many temperate plants utilize vernalization as a signal to distinguish autumn and spring, as both seasons can share similar warm temperature conditions. In non-vernalized autumn plants,

warm ambient temperatures prevent precocious flowering, whereas similar temperatures promote reproductive development in vernalized spring plants (Rawson and Richards 1993; Hemming et al. 2012; Ejaz and von Korff 2017). Mechanistically, the vernalization response involves an epigenetic "memory" of winter, and in many plants, the occasional warm winter day will not interfere with saturation of the response. However, the vernalized state of a plant can be, at least in some species, partially or fully reversed if a period of warm to hot (15 to >30 °C), non-vernalizing temperatures is experienced before the vernalization response is saturated or once vernalization is complete (Purvis and Gregory 1952; Chintraruck and Ketellapper 1969; Bouché et al. 2015). Such "devernalization" indicates to the plant that conditions are unpredictable, thus acting to reset the memory until the arrival of a more convincing winter. As global warming increases the likelihood of unseasonal events, devernalization might equally be adaptive during summer–fall frosts to prevent precocious flowering, but maladaptive with winter warming to cause delayed flowering in the spring.

Devernalization has been reported in a range of species including Arabidopsis (*Arabidopsis thaliana*; Chintraruck and Ketellapper

1969; Shindo et al. 2006; Bouché et al. 2015) and kohlrabi (*Brassica oleracea*); Gongylodes (Brassicaceae) (Wiebe et al. 1992), leek (*Allium porrum*) (Wiebe 1994), and onion (*Allium cepa*) (Amaryllidaceae) (Khokhar et al. 2007); root chicory (*Cichorium intybus*) (Périlleux et al. 2013; Mathieu et al. 2020) and lettuce (*Lactuca serriola*) (Asteraceae) (Marks and Prince 1979, 1982); sugar beet (Durrant and Jaggard 1988) and bread wheat (*Triticum aestivum*) (Slafer 1995; Dixon et al. 2019); and rye (*Secale cereale*) (grasses, Poaceae) (Gregory and Purvis 1936; Purvis and Gregory 1952). Evidence suggests that a response to vernalization evolved independently in each of these plant families (Preston and Sandve 2013), thus making it likely that devernalization responsiveness has multiple evolutionary origins, too. Despite these independent origins, it appears that factors regulating vernalization are commonly targeted during devernalization. In Arabidopsis, for example, reactivation of the major vernalization-dependent floral repressor *FLOWERING LOCUS C* (*FLC*) occurs as a result of devernalization treatment, via decreased histone methylation marks at the promoter (Bouché et al. 2015; Shirakawa et al. 2021). Similarly, in wheat and barley (*Hordeum vulgare*) exposure to warm temperatures after non-saturating vernalization leads to the reactivation of vernalization-dependent repressors of the floral transition: *VERNALIZATION 2* (*VRN2*) and/or the monocot *FLC* homolog *ODDSOC2* (Dixon et al. 2019). These changes are also associated with lower levels of the floral promoters *VRN1*, which is a *FRUITFULL* (*FUL*)-like MADS-box gene and one of the florigen activation complex genes *FLOWERING LOCUS T* (*FT*), manifesting in delayed flowering.

Outside of the wheat tribe (Triticeae), devernalization responsiveness has not been described for grasses, posing the question as to the importance of this trait within vernalization-responsive, undomesticated members of this economically important family. Moreover, while some progress has been made in elucidating the molecular genetic basis of devernalization, much is still to be learned. Here, we show that the model temperate grass species Brachypodium (*Brachypodium distachyon*), that is sister to the "core" Pooideae cereal clade, can be devernalized by moderate to high temperatures after incomplete vernalization. We find that an ortholog of the wheat flowering promoter *VEGETATIVE TO REPRODUCTIVE TRANSITION 2* (*VRT2*) tempers devernalization and contributes to setting the vernalization requirement. In wheat, *VRT2* has been linked to vernalization based on its expression level being responsive to cold (Kane et al. 2005; Xie et al. 2021). However, functional analyses are incomplete for the wheat gene, with a loss-of-function line suggesting it is a weak flowering promoter in a non-vernalization-responsive "spring" variety (Li et al. 2021) and overexpression experiments showing mixed impacts on flowering time (Adamski et al. 2021; Li et al. 2021; Xie et al. 2021). Here, we characterize loss-of-function lines in a facultative vernalization-responsive "winter" accession of Brachypodium, which allows us to functionally link *VRT2* to both vernalization and devernalization.

Results

Vernalization in Brachypodium is reversed by moderate to high temperatures

While devernalization has been described for Triticeae crops, the relevance for undomesticated Pooideae grasses remains unclear. We therefore wondered whether and to what extent the process of devernalization is also present in wild-type Brachypodium. To investigate this, we compared the flowering time of Brachypodium accessions Bd21-3 exposed to a variety of temperature conditions as seeds (Fig. 1). Bd21-3 has a facultative vernalization response, with a delay in flowering of approximately 2 mo when not vernalized and grown in 16 h long-day conditions (Ream et al. 2014; Sharma et al. 2017) (Fig. 1, A and C; V vs. VI). The 1-wk partially vernalized plants were devernalized by warm temperature (24 °C) compared with moderate (18 °C) temperature, based on delayed days to emergence of leaves and flowering (Fig. 1, A and B). Significantly delayed flowering in line with non-vernalized plants (Fig. 1, A and C; VI) was also evident after 2 wk of non-saturating vernalization followed by a heat break at 32 °C (Fig. 1, A and C; IV vs. V). Taken together with other observations of devernalization responsiveness (Purvis and Gregory 1952; Chintraruck and Ketellapper 1969; Périlleux et al. 2013; Bouché et al. 2015), this behavior contrasts with the intuition that warm temperatures always accelerate flowering time (e.g. McClung et al. 2016), and highlights the more complex and widespread interaction of temperature on plant development.

In contrast to high devernalizing temperatures, reports on Triticeae cereals suggest that lower non-chilling temperatures can actually stabilize the vernalized state (Friend and Purvis 1963). To determine whether the same is true for Bd21-3, seeds were moved to 18 °C post-vernalization for 1 wk before devernalization at 32 °C, and the flowering time was compared with plants that did not receive the 18 °C temperature (Fig. 1, A and C; III vs. IV). As predicted, the 18 °C treatment significantly reduced the delay in flowering time imposed by devernalization of germinating seeds, albeit not to the extent of vernalized control plants (Fig. 1, A and C; V). In contrast to several non-grass taxa but similar to winter rye (Purvis and Gregory 1952; Périlleux et al. 2013), we also observed that Bd21-3 devernalization was no longer possible after 4 wk of saturating seed vernalization (Fig. 1, A and D; I vs. II).

BdVRT2 gene expression is upregulated in natural conditions

Homologs of *BdVRT2* are suggested to be involved in vernalization-dependent flowering in cereals based on their gene expression (Kane et al. 2005; Sasani et al. 2009; Xie et al. 2021) and regulate temperature-dependent flowering in Arabidopsis (Lee et al. 2007; Li et al. 2008). Early studies in wheat reported that *TaVRT2* expression was downregulated by vernalization and that *TaVRT2* might be a floral repressor that negatively regulates *TaVRN1* (Kane et al. 2005, 2007). Later studies showed that *VRT2* homologs were upregulated by vernalization in wheat and barley, casting doubts on that hypothesis (Trevaskis et al. 2007; Dubcovsky et al. 2008). To investigate whether *BdVRT2* expression is regulated by vernalization in Brachypodium, a gene expression time course was conducted (Fig. 2). Wild-type Bd21-3 plants were grown under long days (16 h/8 h) at 22 °C for 3 wk, before being moved to short days (8 h/16 h) at 4 °C to simulate winter. The vernalization treatment here contrasts with the previous experiment as vernalization is occurring in juvenile plants, as opposed to seeds. This method was chosen to obtain more material to assess gene responses to vernalization as vernalization is equally effective in both plants and seeds once cells are mitotically active. Additionally, the transcripts activated across the different tissue types are generally the same (Finnegan and Dennis 2007).

Analysis of *BdVRT2* expression showed relatively minor changes in these controlled conditions. While some changes are significant when compared with plants grown without vernalization, the changes are so small that it is unclear whether they are likely to be functional. This therefore suggests that neither vernalization,
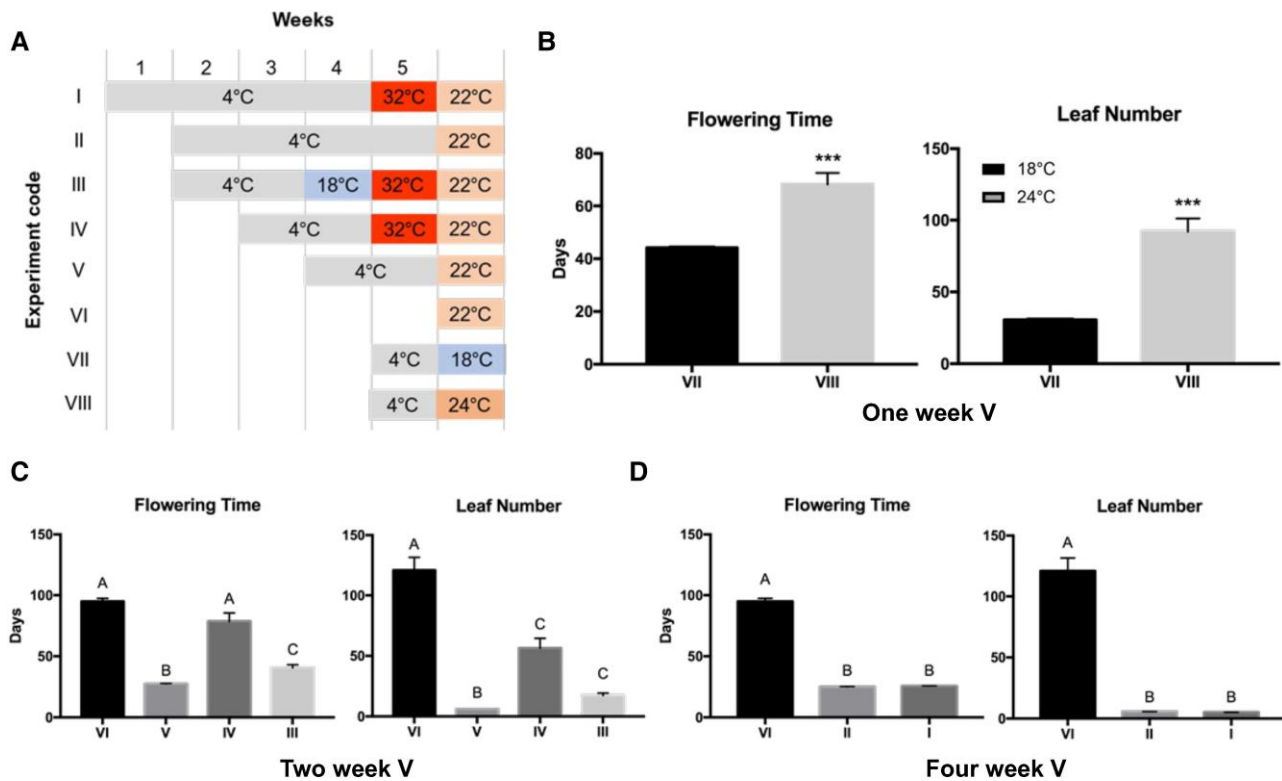
Figure 1. The devernalization response of *Brachypodium distachyon* 21-3. **A)** Experimental setup of devernalization treatments. Wild-type Bd21-3 was vernalized at 4 °C as seeds before being moved to various temperature treatments. Temperatures in the final column remained constant for the remainder of the experiment. **B)** Devernalization phenotypes from 1 wk of vernalization followed by moderate (18 or 24 °C) temperatures. **C)** Devernalization phenotypes from 2 wk of vernalization. **D)** Devernalization phenotypes from 4 wk of vernalization. DV, devernalization; V, vernalization. Flowering time is the date of the first sign of heading. Leaf number is total leaf number at flowering time. Error bars represent standard error of the mean (SEM) ($n = 25$). The letters above the bars represent post hoc test results of ANOVA **C** and **D)**. The asterisks are results of a Mann–Whitney *U* test **B)**. Data that do not share a letter are significantly different ($P < 0.01$). ***$P < 0.001$.

nor a change in photoperiod (as vernalization was in short days), nor warm conditions after partial vernalization strongly affect *BdVRT2* gene expression. We reasoned that this could be because temperature regulation could mainly occur at the protein level, similar to its ortholog *SHORT VEGETATIVE PHASE* in Arabidopsis, which is heat labile (Lee et al. 2013; Jin et al. 2022).

To further investigate Bd21-3 gene expression in Brachypodium, we also grew plants in an unheated greenhouse in Leuven, Belgium, and monitored hourly temperature and expression of *BdVRT2* over the course of winter and spring until flowering (Fig. 2, C and D). *BdVRT2* expression now strongly increased at the start of the winter season and subsequently plateaued (Fig. 2D), suggesting that its transcriptional regulation either responds to fluctuating environmental conditions (Fig. 2C) or to a gradual decrease in the photoperiod. While photoperiod has been described to regulate *VRT2* in wheat and barley (Kane et al. 2005), in the above controlled experiment, a switch between long days, short days in combination with cold or extended long days post-vernalization did not affect *BdVRT2* gene expression to the extent observed in the unheated greenhouse experiment. Therefore, it remains undetermined whether the key determinant of changing *BdVRT2* transcript expression is variable temperature, gradually changing photoperiod, humidity, or their interaction.

BdVRT2 knock-outs show delayed flowering in warm temperatures after vernalization

To functionally test whether *BdVRT2* gene function is affected by temperature in temperate grasses, we investigated flowering in

a Bd21-3 accession T-DNA line of *BdVRT2* (*bdvrt2*-T1), with an insertion in the first intron and barely detectable expression levels of *BdVRT2* (Supplementary Fig. S1). The 2-wk vernalized *bdvrt2* T-DNA insertion mutants were significantly delayed in flowering relative to null sibling controls (NS-T) in a temperature-dependent manner (Fig. 3A). Mutants grown at 18 °C post-vernalization flowered significantly later than NS-T plants, with a concomitant increase in leaf and tiller number (Supplementary Fig. S2). With a 6 °C increase in temperature from 18 to 24 °C, flowering time of *bdvrt2* mutants was further delayed by an average of 61.2 d with more leaves and tillers (Fig. 3A; Supplementary Fig. S2). By contrast, in the null siblings, increased temperature had no significant effect on days to flowering, but did increase leaf and tiller number at flowering, which is comparable to previous reports in Brachypodium (Boden et al. 2013; An et al. 2015). To confirm these findings using independent loss-of-function lines, we generated 2 *bdvrt2* Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) lines (bdvrt2-C1 and bdvrt2-C2) and again compared flowering to null siblings (NS-C) obtained through segregation of the CRISPR lesion mutants. Under the same conditions, both CRISPR lines flowered later than controls as well as the T-DNA line, suggesting that the former lines have stronger loss-of-function alleles than *bdvrt2*-T1 (Fig. 3A). Together, these mutant phenotypes provide strong evidence that *BdVRT2* plays a role in promoting flowering under warm long-day conditions following vernalization. The *bdvrt2* mutants appear to be more sensitive to devernalizing conditions, at least at the moderate temperature of 24 °C (Fig. 3A).
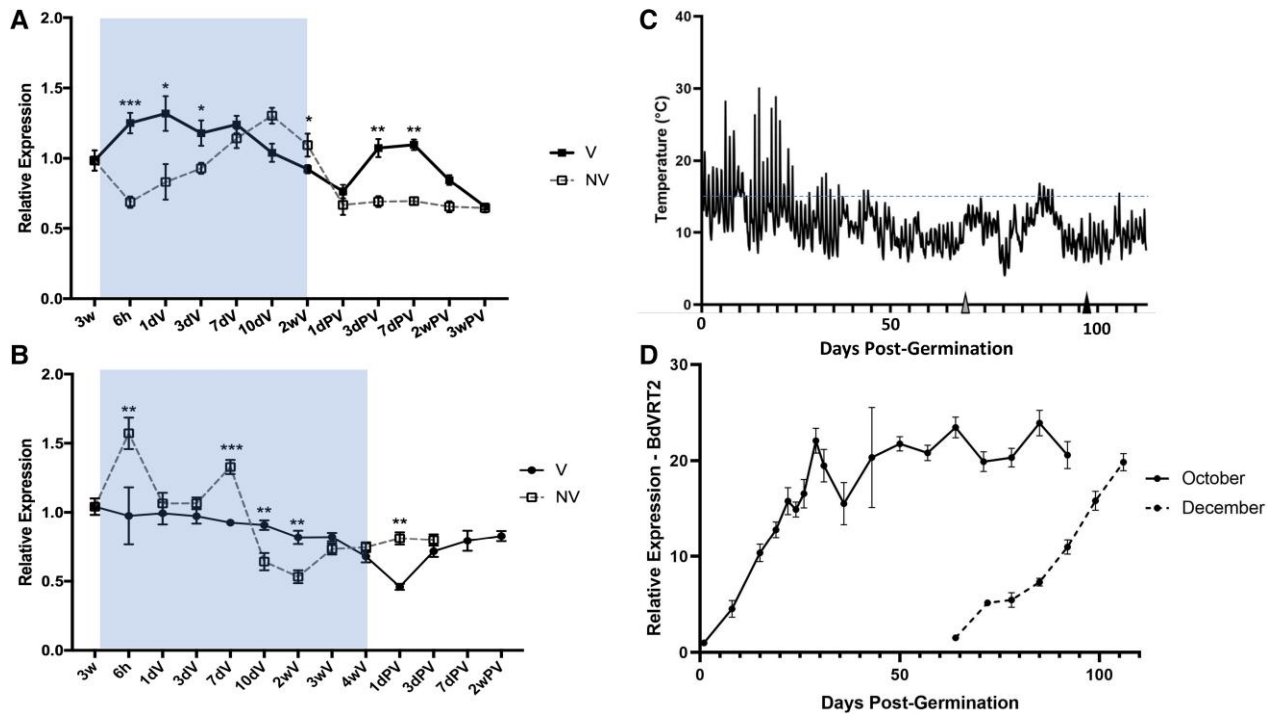
Figure 2. *BdVRT2* expression is strongly upregulated under natural conditions. *BdVRT2* relative expression in a temperature-controlled growth chamber during 2 (**A**) and 4 (**B**) weeks of vernalization. Bd21-3 plants were grown at 22 °C under a long-day photoperiod (16/8 h) for 3 wk before being moved to 4 °C under short days (8/16 h). The samples were compared with non-vernalized control plants, which remained at 22 °C. Blue boxes delimit the period of vernalization. **C)** Temperature conditions from October to January in an unheated, naturally lit greenhouse. Gray and black triangles indicate the start of the December- and October-sown time course, respectively. The blue-dashed line denotes the reported upper limit of plant vernalization temperatures. **D)** Relative expression of *BdVRT2* from plants sown in October or December under the conditions in **C**). Error bars are SEM. Asterisks indicate the *P*-value from a Bonferroni-corrected Kruskal–Wallis test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. wk, week; h, hour; d, day; V, vernalization at 4 °C; PV, post-vernalization.
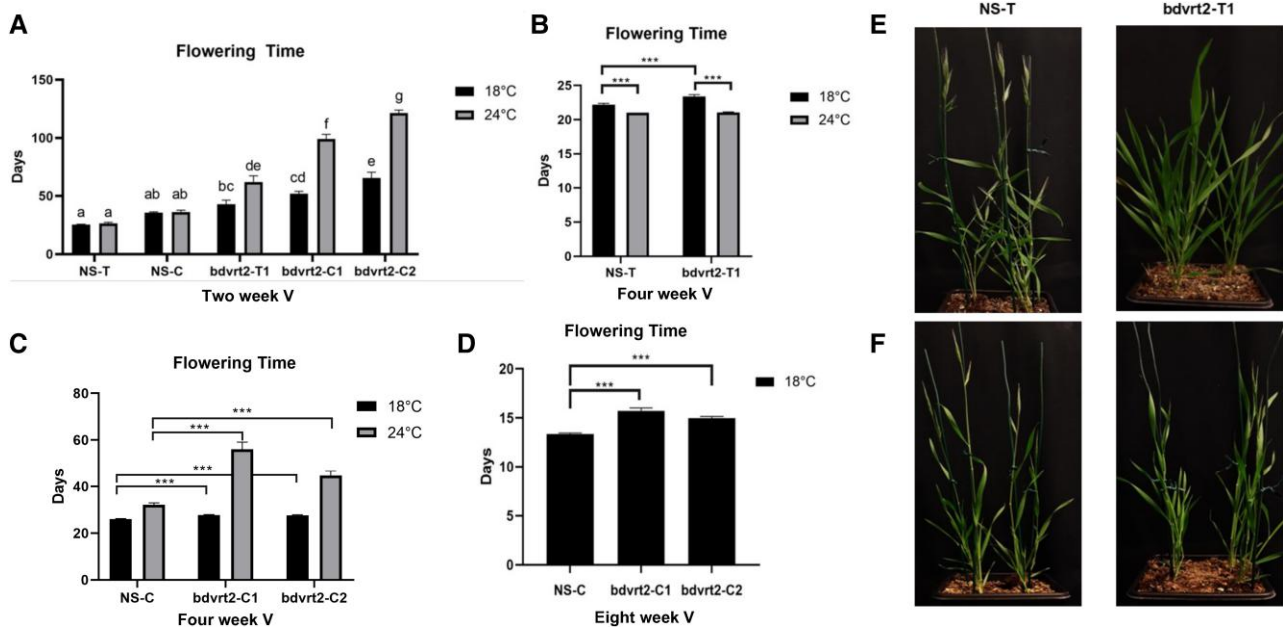

Figure 3. Effect of temperature on *bdvrt2* mutants under incomplete or complete vernalization. **A** and **E**). Mutants exhibit delayed flowering in a moderate temperature-dependent manner under incomplete 2-wk vernalization. **B** and **F**). When given 4 wk of vernalization, the flowering delay between NS and *bdvrt2-T1* vanishes. **C)** Flowering is still delayed in CRISPR lines treated with 4 wk of cold temperature. **D)** An 8-wk vernalization period greatly reduces the flowering delay between NS and *bdvrt2-C* mutants; however, a small delay remains. **E** and **F)** Images show exemplar phenotypes under 24 °C. The asterisks indicate the adjusted *P*-value from a Bonferroni-corrected Kruskal–Wallis test: ***$P < 0.0001$. Data bars in **A**) that do not share common letters are significantly different ($P < 0.05$) based on Tukey HSD test. Error bars represent SEM. V, vernalized; T, T-DNA; C, CRISPR.

BdVRT2 contributes to establishing the length of the vernalization response

Given that the moderate temperature-dependent delay in *bdvrt2* mutant flowering was generally absent in control plants, we hypothesized that *bdvrt2* has a role in vernalization and/or devernalization. Although devernalization is a possible explanation, the typical temperature range in which this process is reported to occur is high (e.g. >27 °C), potentially failing to explain the observed late flowering of *bdvrt2* mutants at moderate (18 and 24 °C) temperatures. In the null siblings, no ambient temperature effect was observed, suggesting that their vernalization response was already saturated at the end of 2 wk of low temperatures (Fig. 3, A and E). By contrast, the temperature-dependent delay on *bdvrt2* mutant flowering suggested that their vernalization was incomplete (Fig. 3, A and E). To confirm this, we repeated the above experiment, increasing seed vernalization to 4 wk to ensure that the vernalization requirement was satisfied for all plants. As predicted, after what was presumably full vernalization, the extreme delay in 24 °C relative to the 18 °C flowering of the *bdvrt2* T-DNA mutants was alleviated (Fig. 3, B and F). In fact, both control and mutant plants flowered slightly but significantly earlier with warmer temperatures. This observation has not previously been reported for Brachypodium possibly due to daylength differences between studies (Boden et al. 2013; An et al. 2015), but is congruent with research in cereal grasses (Fischer 1985; Rawson and Richards 1993; Porter and Gawith 1999; Karsai et al. 2013). We applied the same 4-wk vernalization treatment to the homozygous CRISPR lines to confirm these findings. However, these lines retained their late flowering phenotype in 24 °C relative to 18 °C plants (Fig. 3C), again suggesting that the CRISPR edits cause complete null alleles that take longer than both control and T-DNA mutant plants to saturate their vernalization response. As such, we further extended the vernalization treatment for the CRISPR lines to 8 wk (Fig. 3D). Although the significant delay in flowering remained for the CRISPR lines at 18 °C (Fig. 3D), the difference with controls was only 2 to 3 d. Together, these results demonstrate that *BdVRT2* contributes to the saturation point of the vernalization requirement in Brachypodium Bd21-3, a trait that has been found to vary across naturally occurring accessions of Brachypodium and Arabidopsis in response to different seasonal environments (Stinchcombe et al. 2005).

BdVRT2 restarts vernalization after devernalizing temperatures

We have shown that exposure to moderate to hot temperatures after a period of low temperatures can delay flowering time in Brachypodium. However, a more natural situation is where intermittent warm periods disrupt cool, vernalizing periods. Previous reports in winter rye have shown that disrupting the vernalization process can delay flowering time, despite the total sum of vernalizing days being equal to the vernalization requirement (Purvis and Gregory 1952). To determine if an 18 °C disruption to seed vernalization accelerates flowering in Bd21-3, and if *bdvrt2* mutants are differently impacted, we vernalized mutant and null sibling seeds for 1 wk at 4 °C before moving them to 18 °C for 1, 2, or 4 d (Fig. 4). The seeds were then returned to 4 °C so that the total amount of time at vernalizing temperatures was 4 wk. With a disruption of 2 or 4 d at 18 °C, mutants exhibited delayed flowering compared with NS after 2 and 4 d, respectively (Fig. 4), of disrupted vernalization. This provides further evidence that *BdVRT2* is important in the vernalization process, but specifically to restart vernalization after devernalizing temperatures
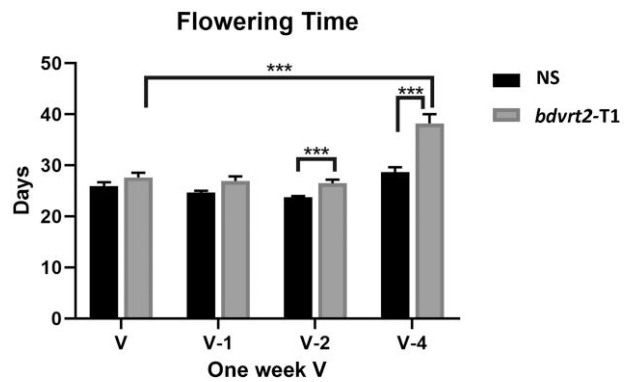

Figure 4. Influence of disrupted vernalization on flowering time. Null siblings (NS) and *bdvrt2-T1* mutants were vernalized for 1 wk at 4 °C before being moved to 18 °C for 1 (v-1), 2 (v-2) or 4 d (v-4). The seeds were then returned to 4 °C to complete a total of 4 wk under vernalizing conditions. The seeds vernalized for 4 wk without a disruption were used as a control (V). Asterisks indicate the adjusted *P*-value from a Bonferroni-corrected Kruskal–Wallis test: ***$P < 0.001$. Error bars represent SEM ($n = 20$).

have been experienced. These results also emphasize that the phenotype of *bdvrt2* mutants is a devernalization phenotype, and not a moderate temperature response.

Key floral promoters and repressors are differentially expressed in *bdvrt2* mutants

To obtain a global view on Brachypodium devernalization at the transcriptomic level, we performed 2 RNA-seq experiments. Comparing 3-wk-old *bdvrt2-T1* mutants to NS plants after 3 d at 4 °C revealed 254 differentially expressed genes (DEGs), whereas the same genotypic comparisons after the 2-wk incomplete seed vernalization followed by devernalization revealed 80 and 155 DEGs at 18 and 24 °C, respectively (Fig. 5A). Altogether 14 DEGs were common to all 3 treatments, one of which was *BdVRT2*, which was reduced in the mutant line as expected (Fig. 5B). We also identified the Polycomb Repressive Complex 2 (PRC2) gene *BdSUPPRESSOR OF ZESTE12* (*BdSUZ12*) (Fig. 5B), which was upregulated under all conditions in the mutant, as confirmed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) (Fig. 6). *BdSUZ12* is homologous to *EMBRYONIC FLOWER2* (*EMF2*) in Arabidopsis, a major regulator of plant development, particularly during the floral transition, where it negatively regulates floral homeotic genes like *AGAMOUS* and *APETALA1* (Chen et al. 1997; Yoshida et al. 2001; Kim et al. 2010). Additionally, we identified an RNA-binding protein *BdGRP2*, an ortholog of wheat *GLYCINE-RICH PROTEIN2* (*TaGRP2*) that negatively regulates *TaVRN1* before vernalization (Xiao et al. 2014). *BdGRP2* is downregulated in *bdvrt2* mutants (Figs. 5B and 6), suggesting that its role in Brachypodium is different to that in wheat, perhaps acting to target a negative regulator of flowering or affecting *BdVRN1* mRNA processing. Another protein known to regulate *BdVRN1* is BdRVR1. This protein represses *BdVRN1* prior to vernalization by increasing repressive chromatin modifications at the *BdVRN1* locus (Woods et al. 2017). In line with this, *BdRVR1* expression was significantly higher in *bdvrt2* mutants during devernalization at 24 °C (Fig. 6). These results point toward *BdVRT2* being involved in an epigenetic mechanism to regulate vernalization and flowering.

To investigate the overlap of DEGs in our *bdvrt2-T1* mutant to the *bdrvr1* mutant reported by Woods et al. (2017), we conducted
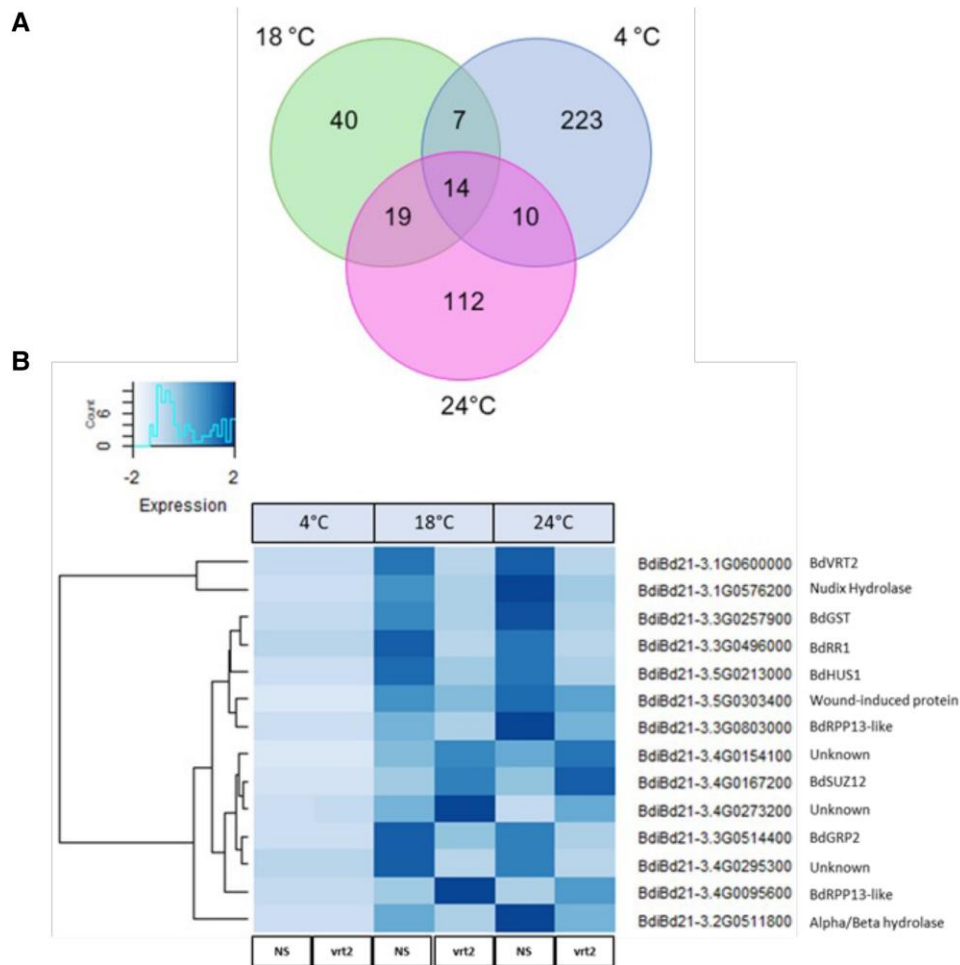
Figure 5. DEGs shared across temperature treatments between control (NS) and *bdvrt2* mutant plants. **A)** Venn diagram showing the number and overlap of DEGs after 3 d of 4 °C vernalization, an 18 °C warm treatment after 2-wk of non-saturating vernalization, and a 24 °C devernalization treatment after 2-wk of non-saturating vernalization. Pairwise comparisons are NS control plants versus *bdvrt2* mutants. **B)** Heatmap showing the differential expression and identity of the 14 overlapping genes from the center of the Venn diagram in **A)**. The scale is based on scaled Fragments Per Kilobase of exon model per Million mapped values.
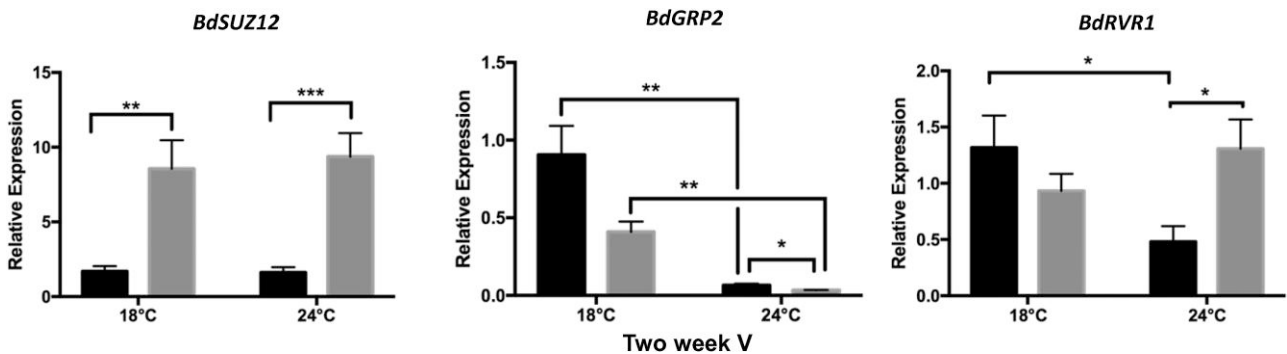

Figure 6. Expression validation for genes implicated in epigenetic modifications during vernalization–devernalization. Material used for RNA-seq was also used for RT-qPCR. Error bars are SEM ($n = 5$). The asterisks indicate the *P*-value of ANOVA with a post hoc Tukey HSD test: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. V, vernalized.

a Fisher's exact test, which revealed that 29 genes were shared across datasets with 17 genes showing opposite expression patterns within the 2 mutants ($P < 0.0001$). This suggests that *BdVRT2* and *BdRVR1* are involved in the same genetic pathway and may act antagonistically to regulate *BdVRN1* or other floral promoters under devernalizing conditions. To identify

other genes within this genetic pathway that were not identified by RNA-seq, we conducted qPCR on genes known to play roles in vernalization-dependent flowering time regulation (Fig. 7, Supplementary Fig. S3). In 2-leaf stage seedlings at 18 °C following incomplete vernalization (Fig. 7), known floral promoters such as *BdVRN1* and *BdFT1* were significantly downregulated in
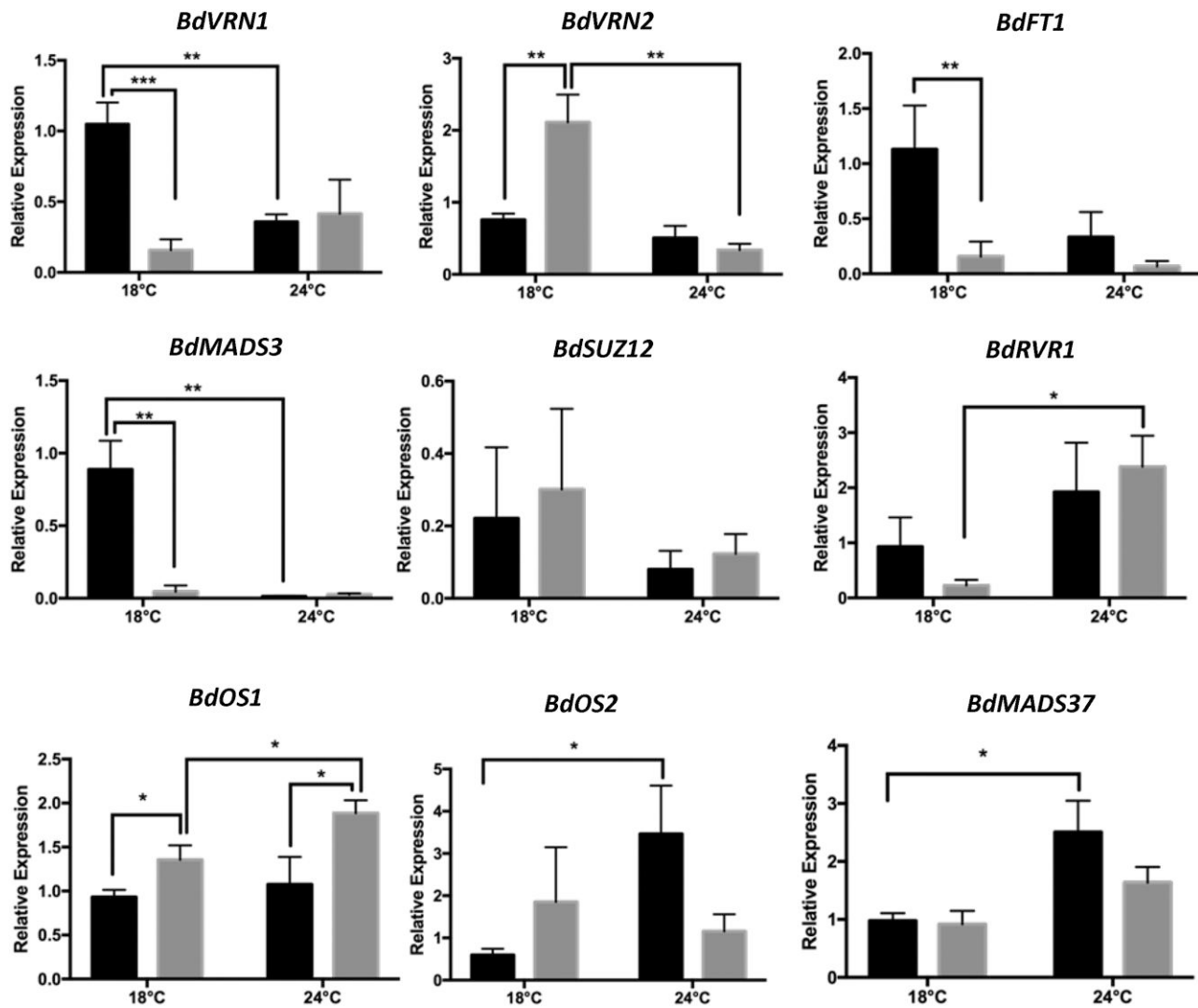
Figure 7. Key regulators of flowering time are differentially expressed in *bdvrt2* mutants at the 2-leaf stage. Black bars, segregating null siblings; gray bars, *bdvrt2* T-DNA mutants. Whole shoots were used. The asterisks indicate the *P*-value from Student's *t* tests of normalized data: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Error bars represent SEMs ($n = 5$).

the mutant compared with the NS, consistent with their upregulation in *TaVRT2* overexpression wheat lines (Xie et al. 2021). By contrast, repressors of the floral transition like *BdVRN2* and the *FLC* homolog *BdOS1* were upregulated in the mutant, also potentially explaining the delayed flowering phenotype. Interestingly, *BdOS2* and *BdMADS37* were upregulated in the NS at 24 °C compared with 18 °C, which implicates them in the devernalization process.

In contrast to 18 °C, no differences in gene expression between the NS and mutants at the 2-leaf stage were observed at 24 °C, except for *BdOS1* (Fig. 7). We speculated that devernalization of the NS at 24 °C resulted in differential regulation of these genes relative to the 18 °C treatment, concealing differences between the NS and the mutant, and that it was during this stage that transcriptional reprogramming in response to the environment was occurring. Subsequent analysis of gene expression in the 3-leaf stage seedlings revealed that this may have been the case (Supplementary Fig. S3). At the 3-leaf stage, although there was no significant difference in *BdVRN1* expression between the NS and *bdvrt2* mutants, the expression of *BdVRN1* in the mutants at 24 °C was still on average lower than the NS (Supplementary

Fig. S3). The modified expression of key vernalization genes suggests that *bdvrt2* mutants are not in a comparable state of vernalization to the NS, and the low/high levels of promoters/repressors may explain the delay in flowering time of the *bdvrt2* mutants.

Discussion

We provide evidence that *BdVRT2* contributes to the length and stability of the vernalization requirement for timely flowering in Brachypodium, a role likely conserved in cereal grasses (Purvis and Gregory 1945). While there are clearly different observations of gene expression that suggest that not all is necessarily conserved between this model species and the related domesticated crops, we think it is unlikely that these signify major functional differences. Rather, the observed differences seem to occur between different varieties or accessions within the same species and because of specific conditions in which gene expression is measured. This is the case for *TaVRT2* in wheat, with seemingly opposite results between varieties (Kane et al. 2005, 2007; Trevaskis et al. 2007; Dubcovsky et al. 2008). Also, the gene function of *TaVRT2* has thus far only been studied in a spring accession

and through ectopic expression or gain of function alleles (Xie et al. 2021; Adamski et al. 2021). The latter results do not necessarily reflect gene function accurately as the expression is established at unnatural levels in unusual times and tissue locations. Hence, we feel that the loss-of-function characterization of *BdVRT2* significantly contributes to our understanding of its biological role, both in this model species and related crop species.

The interpretation of our results as evidence of the devernalization occurring in Brachypodium may come as a surprise because the temperatures we used are both in the moderate (18 to 24 °C) and the hot range (e.g. 32 °C). Devernalization is most often considered as unusual and occurring from unlikely hot temperatures that reverse vernalization. We think, however, that there is a continuum between reversibility of vernalization at ambient temperatures and the more extreme process of devernalization, as extensively studied in Arabidopsis with a focus on *FLOWERING LOCUS C* (Antoniou-Kourounioti et al. 2018). In both processes, partial vernalization is reversed and while devernalization is mostly known as a phenomenon in crop breeding to be avoided, it is probably just a more extreme version of reversing partial vernalization. The more the vernalization progresses, the more extreme the temperatures that are required to reverse it. This range of temperatures that result in reversibility of vernalization or devernalization appears to be more moderate in the *bdvrt2* mutants. We therefore consider the role of *BdVRT2* to be in reinitiating the process of vernalization after it has been interrupted, possibly directly through an epigenetic process. High levels of epigenetic regulators like *BdSUZ12* and *BdRVR1* in *bdvrt2* mutants suggest that, in wild-type plants, negative regulation of these epigenetic factors by BdVRT2 stabilizes the vernalization state.

An outstanding question resulting from our data is at what level or levels *BdVRT2* is regulated. We only monitored regulation at the transcript level and while the data suggest fluctuating temperatures as the driving factor for *BdVRT2* gene expression, it seems likely to us that additional regulation at the protein level contributes to its function. This is the case for Arabidopsis SVP and would follow from more general trends of protein biochemical functions being relatively conserved. We intend to study this further, as this could potentially explain the variable effects of ectopic expression on flowering time observed in wheat (Adamski et al. 2021; Li et al. 2021; Xie et al. 2021).

Despite its occurrence in several disparate plant species, the process of devernalization remains relatively under-studied in grasses and crops and its ecological implications have not been fully explored. We have gathered evidence showing that devernalization depends on several factors including the length of time at vernalizing temperatures prior to devernalizing ones and the temperature being experienced. We also confirm that the vernalized state becomes more stable with increasing exposure to cold temperatures, similar to what has been observed for other species (Purvis and Gregory 1945). In our observations, a 4-d 18 °C disruption in vernalization can lead to a delay in flowering despite exposure to enough days typically required to saturate the vernalization response. With autumn/winter temperatures projected to increase and become more variable, reversibility of vernalization or "devernalization" should be taken into consideration when modeling for impacts on flowering time due to warming climates for our important grass crops.

Materials and methods
Plant material
Wild-type *Brachypodium distachyon* accession Bd21-3 was originally obtained from the US Department of Energy (DOE) Joint Genome

Institute (JGI) and bulked-up at the University of Leuven under standard greenhouse conditions. T-DNA insertional mutant seed of *BdVRT2* (Bradi1g45812) (line JJ7712) in the Bd21-3 background was also ordered from the DOE-JGI, with plants being genotyped and propagated for at least 3 generations before use. The segregating lines that no longer contained the T-DNA insertion were used as null sibling controls. Genotyping was conducted as described on the JGI website (https://jgi.doe.gov/our-science/science-programs/plant-genomics/brachypodium/brachypodium-t-dna-collection/). For CRISPR editing of *BdVRT2*, a single guide RNA was designed targeting the first exon of *BdVRT2* and cloned into vector pZMUBI-BdCas9-BdU6Pro-gRNA-NOS under the care of John Vogel. Embryogenic callus was cultured and transformed as described in Alves et al. (2009). T0 and T1 generation plants were genotyped by sequencing the sgRNA target region of *BdVRT2*: 5′-GAACAGCCCGCG CCGCCGCT-3′. The genotyping primers are listed in Supplementary Table S1.

Experimental conditions and phenotyping
Wild-type Bd21-3 seeds were sown into a pot containing a 3:1 mix of soil and vermiculite. A total of 25 seeds were used per condition with 5 seeds sown per pot. For vernalization, the pots were placed into a Conviron growth chamber at 4 °C under short days (8 h light/16 h dark, 50 μmol m^{-2} s^{-1}) before being moved to a Lovibond growth chamber at various temperatures under long days (16 h light/8 h dark, 100 μmol m^{-2} s^{-1}). Non-vernalized controls were sown and moved directly to a Lovibond growth chamber at 22 °C under long days. To determine how they behaved under more natural conditions, wild-type Bd21-3 plants were also grown in an unheated, naturally lit greenhouse experiment starting in October or December 2021 in Leuven, Belgium: 50.8823° N, 4.7138° E, with a time-series of aboveground tissues collected for qPCR.

For the moderate temperature experiments comparing Bd21-3 VRT2 mutants to their respective controls, 40 plants per treatment per genotype were sown as described previously and vernalized in darkness at 4 °C for 2 wk. The plants were placed in a Lovibond growth chamber (16 h light/8 h dark, 100 μmol m^{-2} s^{-1}) at either 18 or 24 °C for the duration of the experiment. The experiments were repeated twice. To analyze the effect of the duration of devernalizing temperatures during vernalization, 20 seeds were sown per condition and placed into a Conviron growth chamber at 4 °C under short days (8 h light/16 h dark, 50 μmol m^{-2} s^{-1}). The pots were covered with aluminum foil to prevent exposure to light and growth of germinated seedlings. A total of 4 conditions were tested: the seeds were vernalized fully for 4 wk (control); after 1 wk, the pots were moved to a Lovibond at 18 °C under long days (16 h light/8 h dark, 100 μmol m^{-2} s^{-1}) for devernalization for either 1 d (V-1), 2 d (V-2), or 4 d (V-4). After the devernalization treatment, the pots were returned to 4 °C to complete vernalization. After vernalization, the pots were moved to a Lovibond growth chamber at 22 °C under long-day conditions (16 h light/8 h dark, 100 μmol m^{-2} s^{-1}) until flowering. Non-vernalized controls were sown and moved directly to 22 °C for growth. The experiments were ended after 120 d. Any non-flowering plants were given a flowering time of 120 d as a standard to assist with data analysis.

For the transcriptomic experiments during vernalization, the plants were grown for 3 wk in a growth room under long days (16 h light/8 h dark, 115 μmol m^{-2} s^{-1}) at 22 ± 2 °C before being moved to 4 °C under short days (8 h light/16 h dark, 50 μmol m^{-2} s^{-1}). Subsequently, 3 d later, 6 replicates of whole shoot tissues were harvested for RNA. For devernalization transcriptomic analyses, the seeds were incompletely vernalized for 2 wk at 4 °C and

then moved to a Lovibond chamber at either 18 or 24 °C under long days (16 h light/8 h dark, 100 μmol m^{-2} s^{-1}) for germination. Whole shoot samples were harvested for RNA when plants reached the 3-leaf stage using 6 replicates per condition.

RNA sequencing

The aboveground tissue was harvested for total RNA extraction using the TRIsure method (Bioline), and DNase treatment was given using TURBO DNAase (Invitrogen). Totally, 5 biological replicates were used for both RNA-seq studies. Sequencing and bioinformatic analysis were performed by Novogene, UK. Over 15 gigabytes of transcriptomic data for each sample were obtained from the Illumina HiSeq 4000 platform. The R package DESeq2 was used to analyze differential gene expression. The genes with adjusted P-value <0.05 and logFC >1 were annotated using descriptions from Phytozome and Ensemble plants. NCBI Blast was used to identify previously annotated proteins. Data were deposited in the SRA database with the project number PRJNA1118079.

Quantitative PCR

Precisely 500 ng of whole shoot RNA was used to synthesize cDNA using the SensiFast cDNA synthesis kit (Bioline). Quantitative RT-PCR was performed with 3 technical and 5 biological replicates using the StepOne Real-Time PCR system and the relative expression levels were calculated using the $\Delta\Delta Ct$ method with *BdUBC18* (Ubiquiting Conjugating enzyme 18) as a reference gene. Transcript abundance of known flowering time genes, *BdVRN1*, *BdVRN2*, *BdFT1*, and *BdVRT2* (Distelfeld et al. 2009; Ream et al. 2014; Woods et al. 2016), was assessed, as well as *FLC* relatives, *BdODDSOC1* (*BdOS1*), *BdOS2,* and *BdMADS37*. *TaODDSOC2* was previously shown to be differentially regulated in wheat by incomplete vernalization (Dixon et al. 2019). We analyzed gene expression in 2- and 3-leaf stage seedlings, as this is around the time of the floral transition (Hong et al. 2011; Ream et al. 2014).

Statistical analyses

Significant differences between temperature treatments or genotypes within experiments were determined based on analysis of variance (ANOVA) using the aov function in R version 4.0.3, followed by post hoc tests. Student's t tests were conducted for simple pairwise comparisons, whereas Bonferroni-corrected Kruskal–Wallis or Tukey honestly significant difference (HSD) tests were conducted to take into account multiple comparisons.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession number PRJNA1118079.

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Author contributions

A.K., M.L., and K.G. conceived the experiments; A.K., M.L., A.V., M.U.H., M.V.D., and S.E. performed the experiments; A.K., J.C.P., and K.G. wrote the article; and all authors approved of the article.

Supplementary data

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

Supplementary Figure S1. T-DNA insertion model and expression of *BdVRT2* after 3 wk of growth.

Supplementary Figure S2. Leaf and tiller numbers of *bdvrt2* mutants and NS lines grown at 18 or 24 °C after incomplete 2-wk vernalization or complete 4-wk vernalization.

Supplementary Figure S3. Key regulators of flowering time are differentially expressed in *bdvrt2* mutants at the 3-leaf stage.

Supplementary Table S1. Primers for CRISPR edit genotyping and qPCR.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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