

# Interaction of Photoperiod and Vernalization Determines Flowering Time of *Brachypodium distachyon*<sup>1[C][W][OPEN]</sup>

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Timing of flowering is key to the reproductive success of many plants. In temperate climates, flowering is often coordinated with seasonal environmental cues such as temperature and photoperiod. Vernalization is an example of temperature influencing the timing of flowering and is defined as the process by which a prolonged exposure to the cold of winter results in competence to flower during the following spring. In cereals, three genes (*VERNALIZATION1* [*VRN1*], *VRN2*, and *FLOWERING LOCUS T* [*FT*]) have been identified that influence the vernalization requirement and are thought to form a regulatory loop to control the timing of flowering. Here, we characterize natural variation in the vernalization and photoperiod responses in *Brachypodium distachyon*, a small temperate grass related to wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). *Brachypodium* spp. accessions display a wide range of flowering responses to different photoperiods and lengths of vernalization. In addition, we characterize the expression patterns of the closest homologs of *VRN1*, *VRN2* (*VRN2-like* [*BdVRN2L*]), and *FT* before, during, and after cold exposure as well as in different photoperiods. *FT* messenger RNA levels generally correlate with flowering time among accessions grown in different photoperiods, and *FT* is more highly expressed in vernalized plants after cold. *VRN1* is induced by cold in leaves and remains high following vernalization. Plants overexpressing *VRN1* or *FT* flower rapidly in the absence of vernalization, and plants overexpressing *VRN1* exhibit lower *BdVRN2L* levels. Interestingly, *BdVRN2L* is induced during cold, which is a difference in the behavior of *BdVRN2L* compared with wheat *VRN2* during cold.

Flowering when conditions are most favorable is an important adaptive trait for reproductive success. Many plants adapted to temperate climates synchronize their flowering to coincide with seasons by monitoring cues such as temperature and photoperiod. One adaptation

to a temperature cue is vernalization, the process by which plants become competent to flower after prolonged exposure to the cold of winter. This adaptation ensures that flowering occurs only under favorable conditions in spring (Amasino, 2004; Kim et al., 2009; Amasino and Michaels, 2010).

The genetic network underlying the vernalization response has been studied intensively in the crucifer *Arabidopsis* (*Arabidopsis thaliana*; Kim et al., 2009; Amasino and Michaels, 2010). In *Arabidopsis*, the MADS box transcription factor *FLOWERING LOCUS C* (*FLC*) is a major floral repressor that is active in the fall season prior to exposure of the cold of winter (Michaels and Amasino, 1999; Sheldon et al., 1999). The FRIGIDA (*FRI*) complex promotes high *FLC* expression in the fall (Michaels and Amasino, 1999; Johanson et al., 2000; Choi et al., 2011), resulting in repression of key floral promoting genes and thus preventing flowering (Helliwell et al., 2006; Searle et al., 2006). Allelic variation at *FRI* and *FLC* account for much of the natural variation in flowering time in natural accessions of *Arabidopsis* (Shindo et al., 2005). During winter, cold induces expression of *VERNALIZATION INSENSITIVE3* (*VIN3*; Sung and Amasino, 2004) and noncoding RNAs, referred to as *COLD AIR* and *COOL AIR*, that initiate in the first intron and 3' end of *FLC*, respectively (Swiezewski et al., 2009; Heo and Sung, 2011). *VIN3* interacts with a Polycomb Repressive Complex 2 (*PRC2*) complex

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presumably to create a cold-specific PRC2 that is able to silence *FLC* (Wood et al., 2006; De Lucia et al., 2008). *COLDAIR* is thought to guide PRC2 to the *FLC* locus (Heo and Sung, 2011). PRC2 represses *FLC* by depositing H3K27 methylation at the *FLC* locus (De Lucia et al., 2008). Stable *FLC* repression after cold requires H3K9 methylation and the activities of VERNALIZATION1 (*VRN1*) and LIKE-HETEROCHROMATIN PROTEIN1 (Levy et al., 2002; Mylne et al., 2006; Sung et al., 2006). Stable repression of *FLC* permits induction of genes that promote flowering in the spring, such as *FLOWERING LOCUS T (FT)*, which encodes the mobile florigen signal that moves from leaves to the meristem (Searle et al., 2006; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007). *FT* interacts with the basic leucine zipper domain (bZIP) transcription factor *FD*, and the resulting complex activates floral homeotic genes, such as *APETALA1 (AP1)*, to promote floral meristem identity and flower development (Abe et al., 2005).

In addition to the Brassicaceae family, there are two other families of plants, Poaceae and Amaranthaceae, for which something is known about the vernalization system at the molecular level. Natural variation in the vernalization response in sugar beets (*Beta vulgaris*; Amaranthaceae) results from allelic variation at *Beta vulgaris* *BOLTING TIME CONTROL1 (BvBTC1)* (formerly the *B* locus). Dominant alleles of *BvBTC1* result in an annual habit, whereas recessive alleles (*Bvbtc1*) confer a vernalization requirement and biennial growth habit. *BvBTC1* encodes a pseudoresponse regulator with homology to the Arabidopsis *PSEUDORESPONSE REGULATOR3 (PRR3)* and *PRR7* and barley (*Hordeum vulgare*) *PHOTOPERIOD1-H1* genes (Pin et al., 2012). Dominant *BvBTC1* alleles in annual sugar beets are associated with low mRNA levels of *BvFT1*, a repressor within the *FT* subfamily, and high mRNA levels of the Arabidopsis *FT* ortholog, *BvFT2*, and this is associated with rapid flowering without vernalization. In non-vernalized plants, which are delayed in flowering, recessive *Bvbtc1* alleles are associated with high mRNA levels of *BvFT1* and low mRNA levels of *BvFT2* (Pin et al., 2012). *BvFT1* is hypothesized to act upstream of *BvFT2* because overexpression of *BvFT1* results in lower level of *BvFT2* mRNA (Pin et al., 2010). During cold in biennial beet, *Bvbtc1* expression increases and *BvFT1* expression decreases, permitting high *BvFT2* expression in subsequent long days of spring to promote flowering (Pin et al., 2010, 2012).

In wheat (*Triticum aestivum*) and barley (Poaceae), three genes are thought to participate in a regulatory loop to control the timing of flowering, namely *VRN1*, *VRN2*, and *FT* (Dennis and Peacock, 2009; Distelfeld et al., 2009a; Greenup et al., 2009). Prior to cold exposure, high levels of *VRN2* repress *FT* to prevent flowering (Yan et al., 2004b). *VRN2* is in the *CONSTANS-like* gene family and contains a putative zinc finger and a *CONSTANS*, *CONSTANS-LIKE*, and *TIMING OF CAB1 (CCT)* domain (Yan et al., 2004b; Higgins et al., 2010). Although *VRN2* acts similar to *FLC* and *BvFT1* in

that it is a floral repressor, it is unrelated to either gene, and thus far, no *BvFT1* orthologs have been found in grasses, and it remains to be determined if recently discovered *FLC-like* genes play a functional role in the vernalization process in grasses (Greenup et al., 2010; Ruelens et al., 2013). That different genes are involved in establishing the vernalization requirement in Arabidopsis, beets, and cereals (as well as consideration of paleobotanic and paleoclimatic data) indicates that vernalization systems are likely to have evolved after these groups of plants diverged (Amasino, 2010).

During cold exposure in wheat and barley, *VRN2* mRNA levels decrease and *VRN1* mRNA levels increase; after cold exposure, *VRN1* mRNA levels remain high and *VRN2* mRNA levels remain low (Yan et al., 2003, 2004b; Dubcovsky et al., 2006; Trevaskis et al., 2006; Preston and Kellogg, 2008; Sasani et al., 2009). *VRN1* is related to the *AP1*/*CAULIFLOWER (CAL)*/*FRUITFUL (FUL)* family of MADS box transcription factors that control floral meristem identity in Arabidopsis (Yan et al., 2003; Preston and Kellogg, 2008). Whereas *AP1/CAL/FUL* are expressed primarily in meristems in Arabidopsis (Liljegren et al., 1999), in grasses, *VRN1* is expressed in both meristems and leaves (Trevaskis et al., 2003; Yan et al., 2003; Preston and Kellogg, 2008; Sasani et al., 2009; Alonso-Peral et al., 2011). Cold activation of *VRN1* is accompanied by changes in chromatin in a presumed regulatory region of its first intron (Oliver et al., 2009). Opposite to that of the cold-repressed *FLC* but expected for that of a cold-induced gene, *VRN1* chromatin modifications shift from repressive (H3K27 methylation) to active (H3K4 methylation) during vernalization (Oliver et al., 2009).

In support of a model in which *VRN1* is a repressor of *VRN2*, high levels of *VRN1* early in development in lines harboring dominant *VRN1* alleles are associated with low levels of *VRN2* (Loukoianov et al., 2005; Hemming et al., 2008; Distelfeld et al., 2009a). Furthermore, *VRN2* is not repressed after cold in *vrn1* mutants, suggesting *VRN1* plays a role in maintaining repression of *VRN2* after vernalization (Chen and Dubcovsky, 2012). *VRN2* levels do however decrease during cold exposure in *vrn1* mutants, indicating there are additional factors that repress *VRN2* during cold in wheat (Chen and Dubcovsky, 2012).

In spring after vernalization, low *VRN2* levels allow for long-day photoperiod activation of *FT* in leaves and a switch from vegetative to floral meristem identity and flowering (Sasani et al., 2009). High levels of *FT* are associated with high levels of *VRN1* expression in leaves, suggesting the possibility of a positive feedback loop between *VRN1* and *FT* to “lock in” flowering (Yan et al., 2006; Shimada et al., 2009; Distelfeld and Dubcovsky, 2010). In vitro, *FT* physically interacts with the *FD* ortholog *FD-like2*, and this complex binds to the *VRN1* promoter, lending support to this part of the feedback loop model (Li and Dubcovsky, 2008).

Epistatic relationships between *VRN1*, *VRN2*, and *FT* in leaves have been revealed through the study of

existing allelic variants (Trevaskis et al., 2003; Yan et al., 2003, 2004b, 2006; Dubcovsky et al., 2005; Karsai et al., 2005; Hemming et al., 2008; Shimada et al., 2009). Particular varieties of spring barley and spring wheat, which do not require vernalization, carry either deletions of the *VRN2* locus or point mutations in the CCT domain of *VRN2* (Yan et al., 2004b; Dubcovsky et al., 2005; Karsai et al., 2005; von Zitzewitz et al., 2005; Distelfeld et al., 2009b). Therefore, an active *VRN2* is necessary for a vernalization requirement. Other spring varieties have dominant alleles of *VRN1* or *FT* that are constitutively activated (Yan et al., 2003, 2004a, 2006; Fu et al., 2005; Loukoianov et al., 2005; von Zitzewitz et al., 2005). Dominant *VRN1* alleles bypass the requirement for vernalization, presumably by down-regulating *VRN2* early in development (Loukoianov et al., 2005; Hemming et al., 2008). Plants overexpressing *FT* result in rapidly flowering plants with low levels of *VRN2* and high levels of *VRN1*, indicating that *FT* can promote *VRN1* expression in leaves (Shimada et al., 2009), whereas overexpression of *VRN2* delays flowering (Hemming et al., 2008).

Although there has been great progress in understanding the vernalization pathway in wheat and barley, the extent to which vernalization pathways are conserved in grasses remains to be determined as well as identification of additional genes involved in flowering. *Brachypodium distachyon* is a small, wild relative of temperate grasses within the Pooideae, which includes important cereal crops such as wheat, barley, oats (*Avena sativa*), and rye (*Secale cereale*). *Brachypodium* spp. is a useful model grass because of its small, completely sequenced diploid genome (International Brachypodium Initiative, 2010), simple growth requirements, large collection of accessions, inbreeding nature, and high rate of recombination (Draper et al., 2001; Vogel et al., 2006, 2009; Opanowicz et al., 2008; Filiz et al., 2009; Brkljacic et al., 2011; Huo et al., 2011; Mur et al., 2011). All of these characteristics make *Brachypodium* spp. an attractive model for studying flowering, and the vernalization response in particular, in a temperate grass.

Several studies have characterized flowering behavior in a limited number of *Brachypodium* spp. accessions (Draper et al., 2001; Vogel et al., 2009; Schwartz et al., 2010). To advance *Brachypodium* spp. as a model for studying flowering, we have characterized flowering behavior in a range of accessions in response to different photoperiods and lengths of cold treatment. In addition, we have analyzed expression of *VRN1*, *FT*, and *VRN2* genes in response to cold treatments and photoperiod to better understand what parts of the cereal regulatory loop might be conserved in *Brachypodium* spp.. Our results indicate there is rich natural diversity in photoperiod and vernalization requirements to exploit for understanding gene networks controlling flowering. In addition, we provide evidence for conserved roles of *VRN1* and *FT* in promoting flowering, whereas the role of *VRN2* is less clear.

## RESULTS

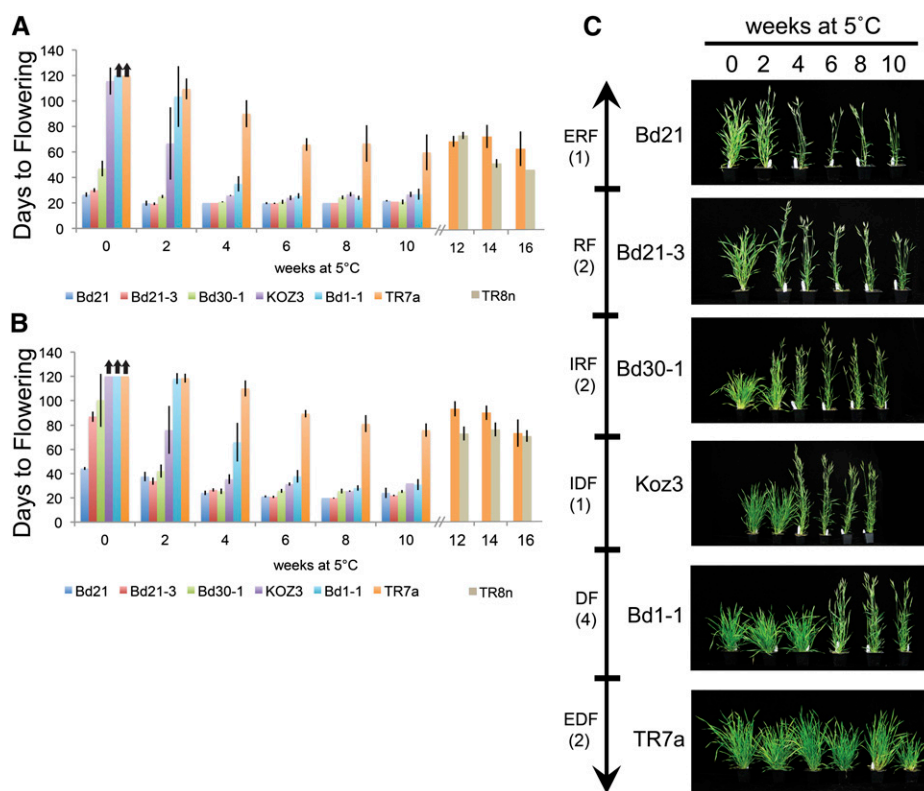
### Natural Variation of Vernalization and Photoperiod Requirement

Hundreds of diploid *Brachypodium* spp. accessions have been collected from its native range in Mediterranean Europe and the Middle East (Vogel et al., 2009; Mur et al., 2011). Preliminary studies identified a wide range of flowering behaviors in many accessions. Using this phenotypic diversity, we chose 13 accessions to evaluate in detail for flowering time under different photoperiods and the length of cold required to “saturate” the vernalization response. These 13 accessions were also chosen because they span the native range of *Brachypodium* spp. and represent the genotypic diversity within *Brachypodium* spp. based upon Simple Sequence Repeat (SSR) marker analysis (Supplemental Table S1; Vogel et al., 2009). Plants from each accession were subjected to cold treatment (5°C) in short days (8-h light, 16-h dark) as imbibed seeds in soil for varying lengths of time, followed by outgrowth in three different photoperiods: 20-h light/4-h dark, 16-h light/8-h dark, and 8-h light/16-h dark. Although a 20-h photoperiod is longer than the native photoperiod range experienced by *Brachypodium* spp. accessions, many *Brachypodium* spp. researchers use this photoperiod to accelerate growth and development, and thus including results from a 20-h photoperiod has practical value.

During cold treatments of 1 to 4 weeks, seedlings rarely emerged from the soil, but with longer cold treatments, the coleoptile typically emerged, as commonly observed in other cereals such as barley (Sasani et al., 2009; Greenup et al., 2010, 2011). During the longest cold treatments, seedlings typically reached the first leaf stage, with the second leaf visible but not fully expanded.

There is a great range of flowering behaviors among the 13 accessions. Although any categorization of this wide spectrum of flowering behavior is somewhat arbitrary, we find it useful to categorize the 13 accessions into six classes based upon the number of days it takes to flower without prior vernalization in both 16- and 20-h daylength and the length of cold exposure required to saturate the vernalization response. These classes are extremely rapid flowering, rapid flowering, intermediate rapid flowering, intermediate delayed flowering, delayed flowering and extremely delayed flowering. Flowering time was measured as days to flowering for all accessions (Fig. 1; Supplemental Fig. S1, A, B, E, and F) as well as primary leaf number on the main (or parent) shoot at flowering (Supplemental Fig. S1, C, D, G, and H). In all accessions tested, days to flowering was highly correlated with leaf number ( $r^2 = 0.93$ ; Supplemental Fig. S2).

Accession Bd21 is the sole member of the extremely rapid flowering class; it is the only accession tested that flowers rapidly (less than 45 d) in both 16-h and 20-h photoperiods without prior cold exposure, although there is a slight vernalization response when grown in a 16-h photoperiod (Fig. 1, A and B; Supplemental Fig. S1).



**Figure 1.** Vernalization and photoperiod interact to determine flowering time in a range of *Brachypodium* spp. accessions. A and B, Imbibed seeds of accessions were cold treated at 5°C in soil in an 8-h photoperiod for the indicated length of time (weeks), followed by outgrowth in a growth chamber set to 20-h light/4-h dark (A) or 16-h light/8-h dark (B). Flowering time is measured as the number of days to spike emergence (Zadoks scale = 50) from the end of cold treatment. Arrows indicate treatments where plants did not flower within the 120 d of the experiment. For TR8n in A and B, only flowering data for 12, 14, and 16 weeks of cold is presented (see Supplemental Fig. S1 for all flowering data for this accession.) C, Photographs of representative plants from each of the six flowering classes (described in the text) after 60 d of outgrowth in a 16-h photoperiod after vernalization. Number in parentheses below class name represents the number of accession(s) evaluated in this study that fall within that particular flowering class. ERF, Extremely rapid flowering; RF, rapid flowering; IRF, intermediate rapid flowering; IDF, intermediate delayed flowering; DF, delayed flowering; EDF, extremely delayed flowering. Bar = 5 cm.

Bd21-3 and Bd3-1 are in the rapid flowering class because they have a conditional vernalization requirement; these accessions do not require cold to flower rapidly in a 20-h photoperiod (20–30 d to flower [DTF]), but 2 to 3 weeks of cold significantly accelerates flowering in a 16-h photoperiod (Fig. 1, A and B). This is evident when considering both days to heading after vernalization and the total number of leaves produced by the main shoot at flowering (Supplemental Fig. S1, C and G). Bd21-3 and Bd3-1 therefore have a facultative vernalization requirement, dependent upon the photoperiod in which they are grown.

All of the other accessions tested have a strong vernalization response in a 20-h photoperiod as well as a 16-h photoperiod. We place accessions Bd30-1 and Bd2-3 in an intermediate rapid flowering class, as they are delayed in flowering compared with accessions in the rapid-flowering class, flowering within an average of 50 to 60 d with significant variability in flowering time without vernalization in a 20-h photoperiod (Fig. 1A). Bd30-1 and Bd2-3 exhibit delayed flowering when grown

in 16 h of light (approximately 100 d), yet a short cold treatment of 2 to 3 weeks is sufficient to allow rapid flowering (Fig. 1B).

Without vernalization, none of the accessions within the three remaining delayed flowering classes flowered in a 20-h photoperiod upon termination of the experiment after 120 d of growth (Fig. 1A; Supplemental Fig. S1A). Plants that failed to flower continued to produce leaves and also produced a number of secondary tillers from the main shoot, resulting in a bushier plant (Fig. 1C; Supplemental Figs. S1C and S3). Given that none of these remaining classes flowered without vernalization, the length of cold required to saturate the vernalization response was the sole criterion used to categorize these accessions. Accessions BdTR12c and Koz-3 are placed in an intermediate delayed flowering class, given that as little as 2 to 4 weeks of cold exposure accelerated their flowering by over 50 d (Fig. 1, A and B; Supplemental Fig. S1). Accessions Bd1-1, RON-2, Tek-11, and Bd29-1 are placed in a delayed flowering class, as they require 6 to 8 weeks of cold to saturate their vernalization

response (Fig. 1, A and B; Supplemental Fig. S1). In the case of BdTR8n and BdTR7a, flowering occurred after 60 d in a 20-h photoperiod even after 10 weeks of cold, in contrast to 20 to 30 d for the other accessions tested, and thus BdTR8n and BdTR7a are placed in an extremely delayed flowering class (Fig. 1A; Supplemental Fig. S1). To determine if longer cold treatments might further accelerate flowering of BdTR8n and BdTR7a, imbibed seeds were exposed to 12, 14, and 16 weeks of cold. Sixteen weeks of cold accelerated BdTR8n flowering by about 20 d in a 20-h photoperiod, whereas there was no acceleration of flowering of BdTR7a, which still flowered around 60 d after cold treatment (Fig. 1A). Photographs of representative plants from each of the six classes are presented in Figure 1C. Additional photographs of representative plants from all accessions  $\times$  treatments at 60 d after cold treatment are shown in Supplemental Fig. S3.

None of the *Brachypodium* spp. accessions flowered in an 8-h photoperiod within 120 d even after 10 weeks of cold treatment (Supplemental Figs. S4 and S5). There are, however, varieties of wheat and barley in which inflorescence development is initiated in short days, but inflorescence emergence is severely delayed or does not occur in short days (Evans, 1987; Dubcovsky et al., 2006). Dissection of *Brachypodium* spp. meristems on the main shoot of at least three plants from each treatment after 120 d of outgrowth in short days revealed all of the meristems were vegetative with no morphological sign of floral transition (data not shown). However, spike emergence is observed in some Bd21 and Bd21-3 plants grown in short days after over 200 d of growth, and meristems show signs of morphological transition to flowering by 180 d of growth (data not shown). Thus, most of the accessions tested behave as obligate long-day plants, but the most rapid flowering accessions, Bd21 and Bd21-3, have a facultative long-day response flowering under short 8-h photoperiods when grown for long periods.

We also note that there is a range of heights among accessions (see also Draper et al., 2001; Filiz et al., 2009), and cold treatment reduces the height of many accessions (Supplemental Fig. S6). Clearly, some of the variation in height is attributed to differences in flowering time. However, some accessions that received a saturating cold treatment and that flowered at the same time showed substantial variation in height. For example, Bd21-3 reached 23 cm and Bd29-1 reached 45 cm after saturating cold treatments, and both averaged six leaves at flowering (Supplemental Figs. S1C and S6C). To evaluate height differences without the complication of differences in flowering time, we measured height among accessions grown in a noninductive 8-h photoperiod, where all plants remained in a vegetative state. Again, there is a range of heights among accessions, and Bd3-1 and Bd2-3 are among the tallest accessions (Supplemental Figs. S5 and S6). However, cold treatment did not have a large effect on height within an accession in a noninductive 8-h photoperiod. Accounting for the minimum height that many of the accessions reach, even after long cold treatments, a minimum

headspace of 70 cm is typically needed when growing many different accessions in a controlled environment chamber.

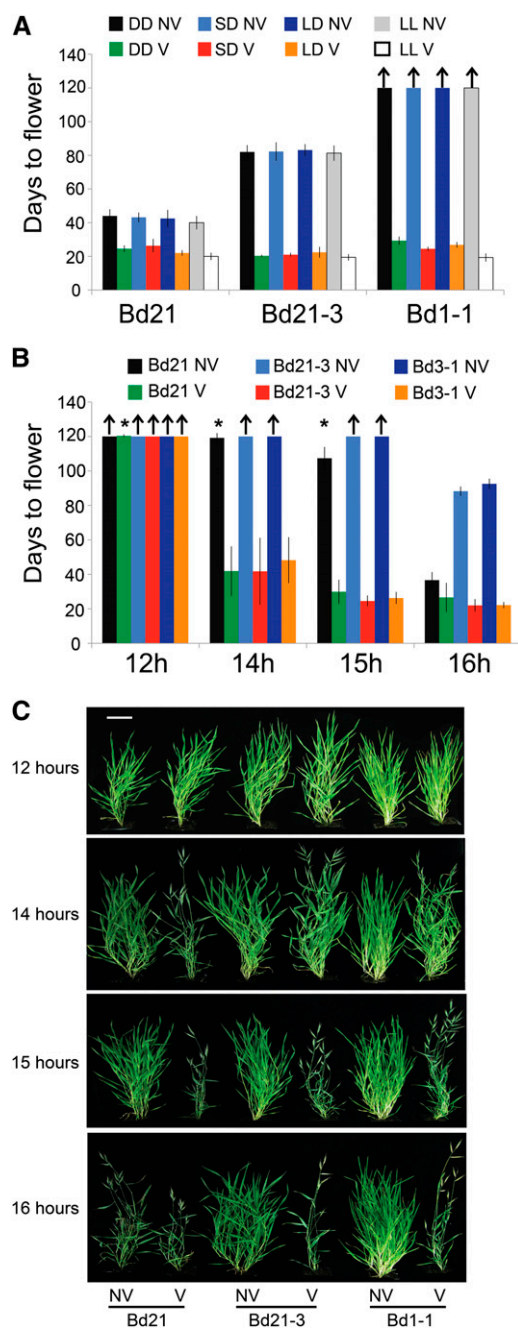
### Interaction of Vernalization and Photoperiod

To test if there is any effect of photoperiod on the process of vernalization, imbibed seeds from Bd21, Bd21-3, and Bd1-1 were exposed to one of four light regimes during cold treatment: no light, short days (8-h light/16-h dark), long days (16-h light/8-h dark), and continuous light. All plants flowered rapidly after a saturating cold treatment ended, regardless of the photoperiod regime experienced during cold exposure, when compared with their respective nonvernalized control plants, which were exposed to the same photoperiod treatment for an equivalent developmental time (Fig. 2A; Supplemental Fig. S7). However, as discussed above, only a minimal amount of development occurs when vernalizing imbibed seeds; thus, although there is no effect of photoperiod on the effectiveness of vernalization on imbibed seeds, it is possible that the photoperiod during cold exposure of mature plants could affect flowering.

Some *Brachypodium* spp. accessions flower more rapidly with increasing photoperiod, such as Bd21, Bd21-3, and Bd3-1 (Fig. 1, A and B). To explore the minimum photoperiod that promotes rapid flowering following vernalization, imbibed seeds from Bd21, Bd21-3, and Bd3-1 were sown in soil and exposed to 3 weeks of cold treatment (5°C, 16-h photoperiod) followed by growth in one of four photoperiods: 12-h light/12-h dark, 14-h light/10-h dark, 15-h light/9-h dark, or 16-h light/8-h dark. Fourteen hour or longer photoperiods significantly accelerate flowering following vernalization in Bd21, Bd21-3, and Bd3-1 (Fig. 2, B and C; Supplemental Fig. S8). By contrast, unvernallized plants and plants that experienced a 12-h photoperiod following vernalization exhibited delayed flowering (Fig. 2, B and C; Supplemental Fig. S8). Generally, longer photoperiods resulted in less variation and more rapid flowering among individuals after cold treatment.

### Temperature Range of the Vernalization Response

Several studies have defined a range of temperatures that satisfy the vernalization requirement in different plant species or accessions within a species (Ahrens and Loomis, 1963; Chujo, 1966; Trione and Metzger, 1970; Rawson et al., 1998; Wollenberg and Amasino, 2012). To define the temperature range effective for *Brachypodium* spp. vernalization, we exposed seedlings (Zadoks stage 10–11; Zadoks et al., 1974) from Bd21-3 and Bd1-1 to a range of temperatures (vernalization treatment under a long-day 16-h photoperiod) and assessed flowering compared with nonvernalized controls. Because time and temperature result in different growth rates among the different vernalization treatments, flowering time is presented as the number of primary leaves on the main



**Figure 2.** Effect of different photoperiods during and after vernalization on flowering time. A, Imbibed seeds sown on plates were exposed to 5°C for 3 weeks (Bd21, Bd21-3) or 7 weeks (Bd1-1) in either no light (DD), 8-h light/16-h dark (SD), 16-h light/8-h dark (LD), or constant light (LL). Nonvernalized controls were exposed to a similar light regime to an equivalent stage of development that the vernalized seedlings reached by the end of the cold treatment. Plants were grown in a 16-h photoperiod following vernalization after transplanting to soil. B, Imbibed seeds sown in soil were exposed to 5°C for 3 weeks (under a 16-h photoperiod) before shifting to one of four photoperiods: 12-h light/12-h dark, 14-h light/10-h dark, 15-h light/9-h dark, or 16-h light/8-h dark. For A and B, flowering time reflects the number of days to spike emergence (Zadoks scale = 50) from the end of cold treatment. Arrows indicate treatments where plants did not flower during the 120 d of the experiment. Asterisk indicates some plants within a

shoot at flowering (see Supplemental Fig. S9 for flowering time presented as days to flowering).

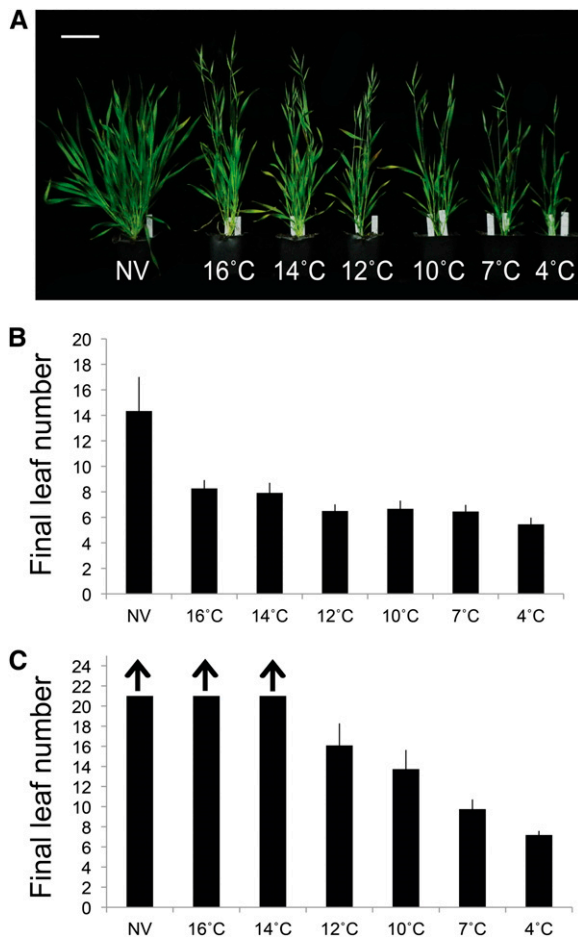
Interestingly, temperatures averaging as high as 16°C had some accelerating effect on flowering in Bd21-3, but there was little, if any, effect in Bd1-1 (Fig. 3). In Bd1-1, acceleration of flowering was evident after a 12°C cold treatment (Fig. 3C). One caveat to this interpretation is that Bd21-3 and Bd1-1 vary in the time it takes to satisfy their vernalization requirement (3 versus 7 weeks, respectively), and hence additional growth that occurred during cold treatment of Bd1-1 plants may have influenced the ability to vernalize at higher temperatures. Regardless of this, it is clear that temperatures as high as 12°C accelerate flowering in Bd1-1.

### *VRN1* and *FT* Promote Flowering in *Brachypodium* spp.

*Brachypodium* spp. has *VRN1/AP1*- and *FT*-containing gene families, in which clearly identifiable orthologs to wheat *VRN1* and wheat *FT* are present (Higgins et al., 2010). To determine the relationship between flowering time and mRNA levels of *VRN1* or *FT* in *Brachypodium* spp., we measured mRNA levels of these genes in various accessions grown without cold treatment in a 20-h photoperiod. These accessions were selected because they vary in flowering time and vernalization requirement (Fig. 1A; Supplemental Fig. S1). There was a clear correlation between elevated levels of *VRN1* and *FT* and rapid flowering among the accessions. *VRN1* and *FT* levels were highest in Bd21 (DTF = 27), followed by Bd3-1 (DTF = 34) and Bd21-3 (DTF = 30; Fig. 4, A and C). Accessions that showed delayed flowering (50 d or longer) had low levels of *VRN1* and *FT* (Fig. 4, A and C). These data agree with the correlation between flowering time and *VRN1* and *FT* levels previously published in a sample of *Brachypodium* spp. accessions (Schwartz et al., 2010).

As shown in Figures 1 and 2, accessions that are rapid flowering when grown in 20-h photoperiods exhibit delayed flowering under shorter photoperiods. For example, Bd21 and Bd21-3 flower later in a 16-h photoperiod (DTF = 44 and 87, respectively) compared with a 20-h photoperiod (DTF = 27 and 30, respectively) in the absence of cold treatment. Consistent with this, *VRN1* and *FT* levels are significantly lower in Bd21 and Bd21-3 plants grown in a 16-h photoperiod compared with plants grown in a 20-h photoperiod (Fig. 4, B and D). *FT* and *VRN1* are not detected in Bd21 or Bd21-3 plants grown in an 8-h photoperiod (Fig. 4, B and D), consistent with the observation that neither of these accessions flowered within 120 d when grown in this photoperiod (Supplemental Fig. S4). In addition, *FT* and *VRN1* mRNA levels were undetectable in Bd1-1 in all photoperiods, correlating with the delayed flowering of this line in the absence of cold treatment (>120 d; Fig. 4, B and D).

treatment did not flower after 120 d. C, Photos of 60-d-old plants from B. NV, Nonvernalized; V, vernalized. Bar = 5 cm.



**Figure 3.** Temperature range that satisfies the vernalization requirement. Imbibed Bd21-3 or Bd1-1 seeds were sown in soil and allowed to germinate to Zadoks stages 07 to 10. Seedlings were then transferred to different vernalization temperatures under a 16-h photoperiod for 3 weeks (Bd21-3) or 7 weeks (Bd1-1) before shifting to outgrowth in a 16-h photoperiod. Outgrowth conditions averaged 22°C during the light period and 18°C during the dark period under a 16-h photoperiod. During vernalization, temperatures fluctuated diurnally in a 16-h photoperiod (data not shown). A, Representative images of Bd21-3 plants after different temperatures during cold treatment (all plants are the same age). Flowering time of Bd21-3 (B) or Bd1-1 (C) plants measured as the number of primary leaves produced by the main shoot at the time of flowering. Arrows indicate plants did not flower during the 100 d of the experiment. NV, Nonvernalized. [See online article for color version of this figure.]

To evaluate the role of *VRN1* and *FT* in *Brachypodium* spp. flowering, we generated transgenic Bd21-3 plants that express the *VRN1* or *FT* complementary DNA (cDNA) under control of a *Panicum virgatum* ubiquitin promoter (Mann et al., 2012). We generated 10 independent transgenic lines that segregate for the *VRN1* transgene *UBI:VRN1* and three independent transgenic lines that segregate for the *FT* transgene *UBI:FT*. In a 16-h photoperiod, in which wild-type Bd21-3 is delayed in flowering without undergoing vernalization, transgenic plants harboring *UBI:VRN1* flowered within an average of 21 to 29 d and produced an average of five to six leaves (Fig. 5, A and B; Supplemental Fig. S10A). Strikingly, *UBI:FT*

transgenic plants flowered after about 12 d and produced four leaves (Fig. 5, A and C; Supplemental Fig. S10B).

*VRN1* expression levels in the leaves of 10 independent *UBI:VRN1* transgenic lines is significantly higher ( $P < 0.05$ ) compared with wild-type Bd21-3 plants or sibling plants that did not harbor the transgene (Fig. 5D). *FT* expression is also significantly elevated in the leaves of the *UBI:VRN1* transgenic plants compared with control plants ( $P < 0.05$ ), consistent with the rapid flowering phenotype of these plants and indicating that *VRN1* plays a role in promoting *FT* expression in leaves (Fig. 5E). *FT* expression levels in the leaves of three independent *UBI:FT* transgenic lines is significantly higher ( $P < 0.01$ ) compared with wild-type Bd21-3 plants that did not harbor the transgene, correlating with the early flowering phenotype of these lines (Fig. 5H). Additionally, *VRN1* expression is significantly up-regulated ( $P < 0.01$ ) in the *UBI:FT* transgenic lines compared with wild-type Bd21-3 (Fig. 5G).

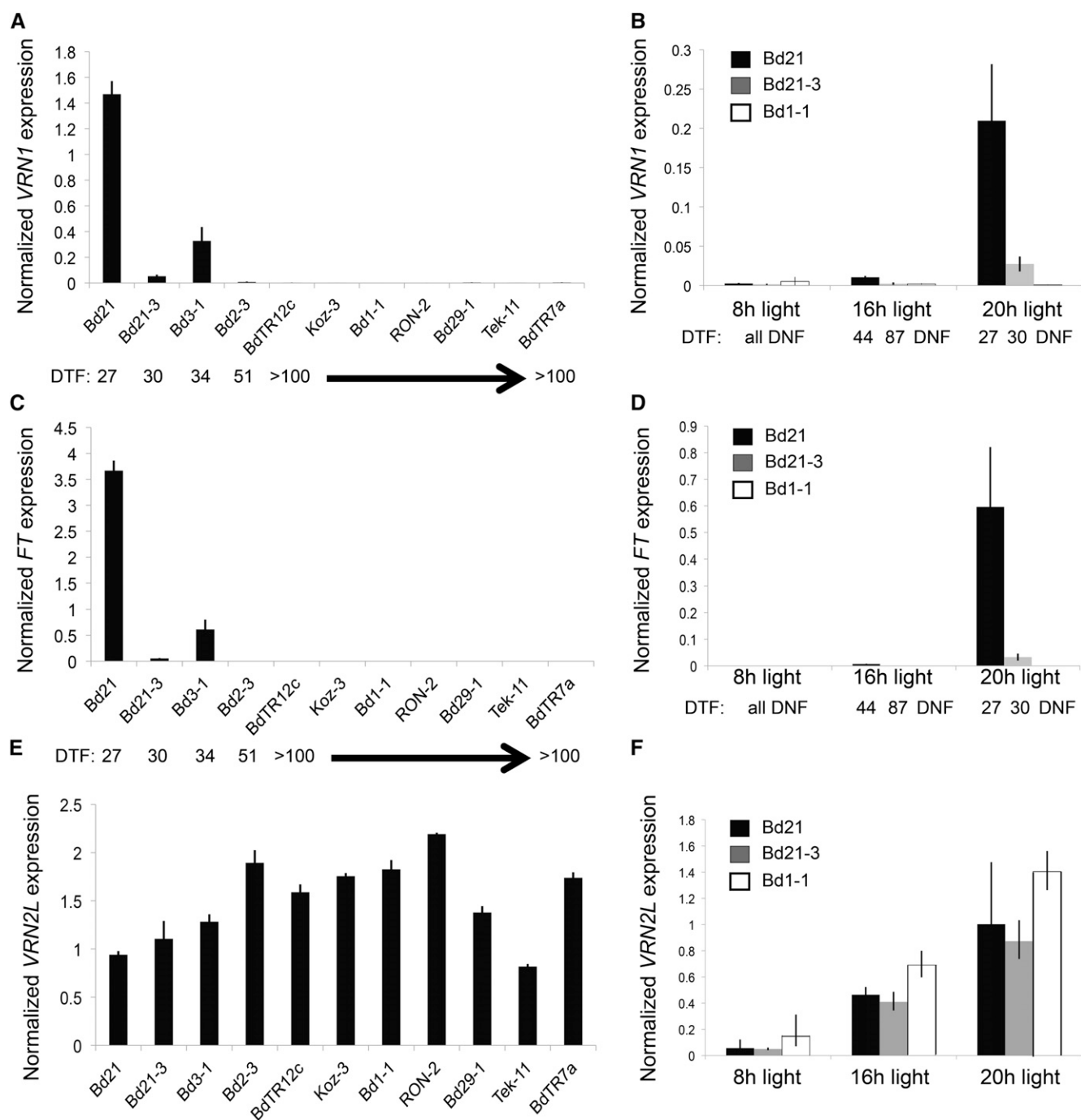
#### Effect of Vernalization (Cold) on *VRN1* and *FT* in *Brachypodium* spp.

In wheat and barley, *VRN1* levels increase during cold and remain elevated following cold (Trevaskis et al., 2003; Yan et al., 2003; Preston and Kellogg, 2008; Sasani et al., 2009), whereas *FT* levels are higher in vernalized plants following cold treatment compared with nonvernalized plants (Yan et al., 2006; Hemming et al., 2008; Sasani et al., 2009; Shimada et al., 2009). We tested the influence of cold treatment (performed under a 16-h long-day photoperiod) on expression of the *VRN1* and *FT* orthologs in three *Brachypodium* spp. accessions.

*VRN1* mRNA levels increase quantitatively during increasing length of cold treatment in Bd1-1 and were significantly higher in all accessions tested by the end of the saturating cold treatment (Fig. 6A). *FT* levels are undetectable in all samples at the end of the cold treatment (data not shown). After a saturating cold treatment of imbibed seeds, *VRN1* and *FT* expression was significantly higher in the leaves of vernalized samples of all accessions compared with the respective nonvernalized samples (Fig. 6, B and C), correlating with rapid flowering. However, *VRN1* levels were significantly lower in leaves of all accessions after a subsaturating cold treatment compared with saturating cold treatments (Fig. 6B). The lower *VRN1* levels measured after a subsaturating cold treatment correlates with delayed flowering in these accessions after this treatment (Fig. 6B; Supplemental Fig. S11). *VRN1* levels remained high in leaves of Bd21, Bd21-3, and Bd1-1 plants that were fully vernalized, and plants flowered rapidly after this cold treatment (Fig. 6B; Supplemental Fig. S11; data not shown).

#### A Gene with Homology to Wheat *VRN2* Is Up-Regulated by Cold

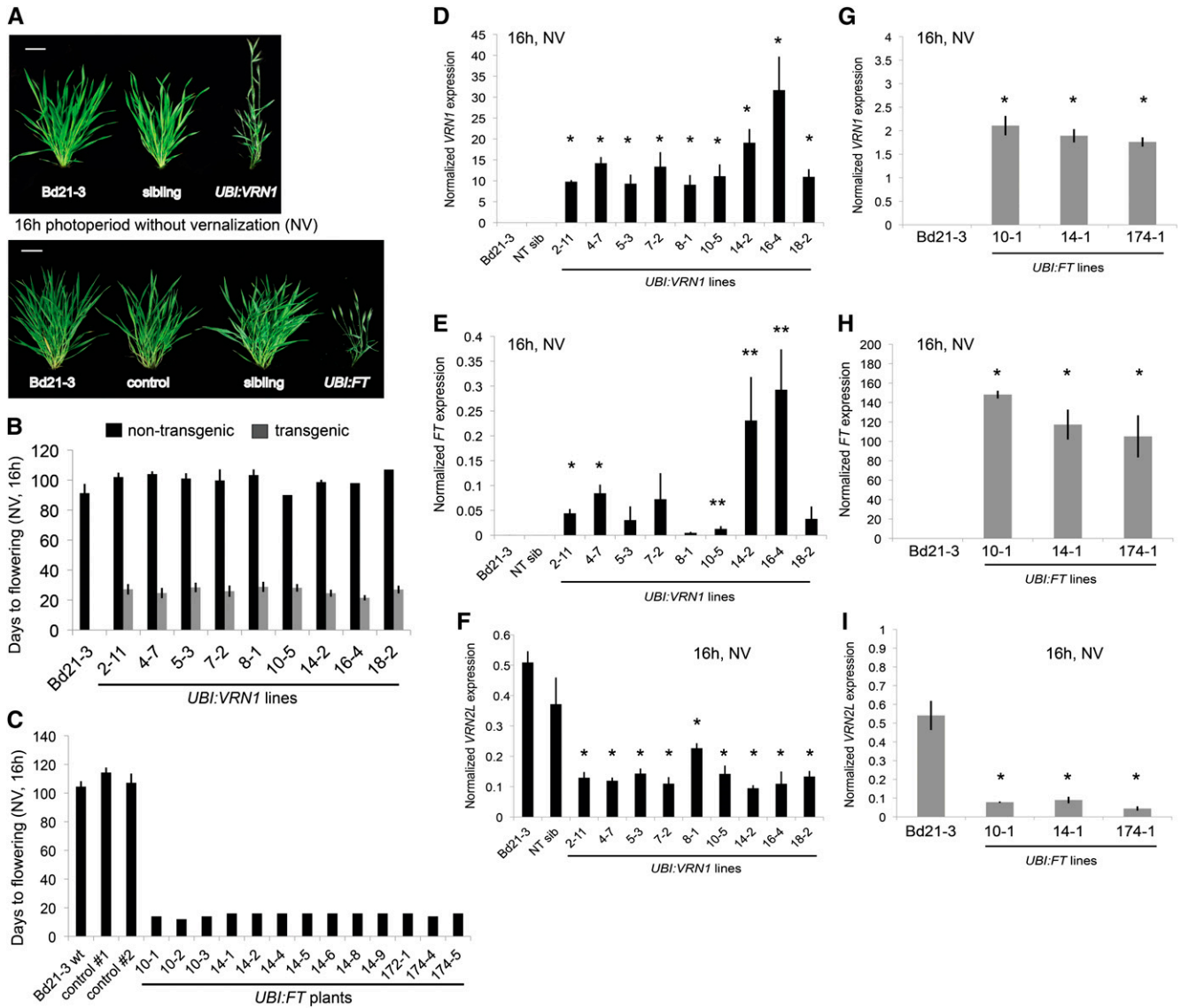
The closest homolog of wheat *VRN2* is *Bradi3g10010* (Higgins et al., 2010; Ream et al., 2012). We refer to



**Figure 4.** *VRN1*, *VRN2L*, and *FT* expression in different accessions and photoperiods without cold treatment. A, C, and E, RT-qPCR of *VRN1* (A), *FT* (C), and *VRN2L* (E) expression in the second leaf of *Brachypodium* spp. accessions. Plants were grown nonvernalized in a 20-h photoperiod and harvested at leaf stage 2 (Zadoks stages = 12–13). B, D, and F, RT-qPCR of *VRN1* (B), *FT* (D), and *VRN2L* (F) expression in the second leaf of *Brachypodium* spp. accessions (Bd21, Bd21-3, and Bd1-1) grown nonvernalized in 8-, 16-, or 20-h photoperiods and harvested at leaf stage 2 during the middle of the photoperiod. Bars represent the average of three biological replicates  $\pm$  1 sd. DNF, Did not flower during 120 d of growth.

this gene as *VRN2-like* (*VRN2L*) because Bayesian phylogenetic studies using the CCT domain place *Bradi3g10010* within the *VRN2* clade; however, the *VRN2* and *Ghd7* (a photoperiod response regulator) clades are unresolved but may in fact be orthologous

(for additional discussion, see Ream et al., 2012). Additionally, BLAST searches using *VRN2L* in the most recently released versions of the wheat (Brenchley et al., 2012) and barley genomes (Mayer et al., 2012) do not reveal a closer homolog to *Bradi3g10010* than

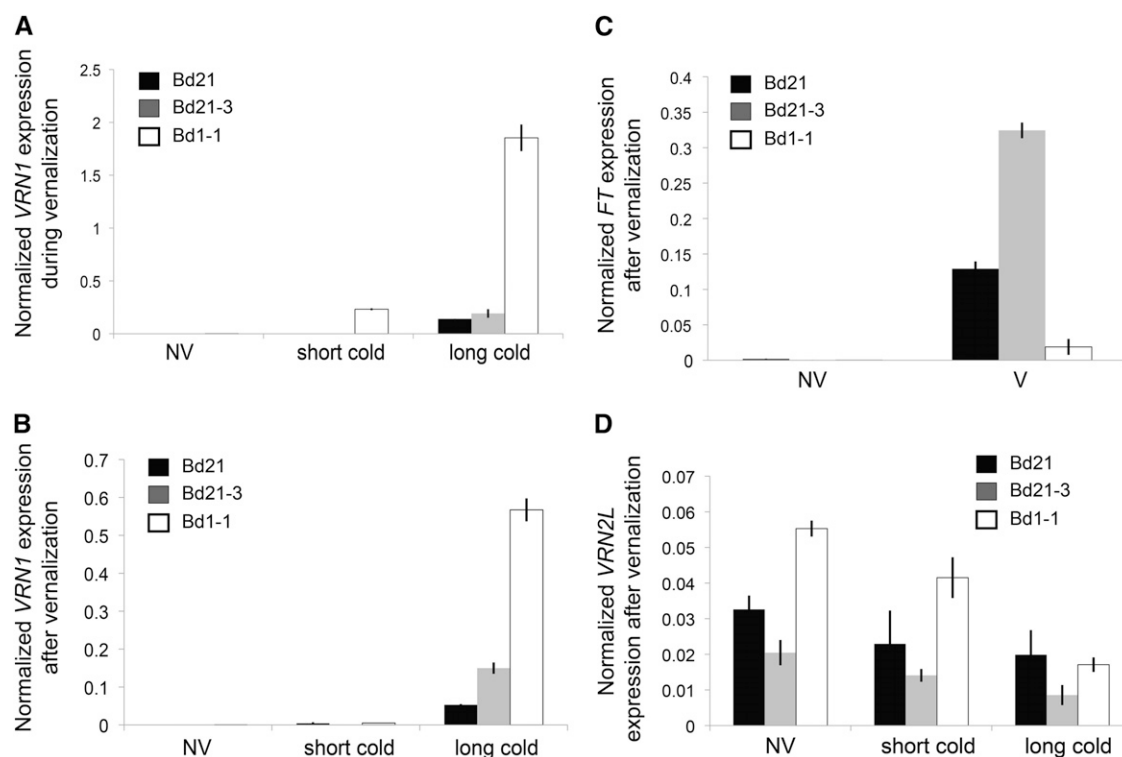


**Figure 5.** *VRN1* and *FT* overexpression cause rapid flowering. A, Representative photos of Bd21-3 wild-type, nontransgenic sibling plants (sibling), empty pANIC10A vector transformed plants (control), and *UBI:VRN1* and *UBI:FT* plants grown nonvernalized in a 16-h photoperiod. Bar = 4 cm. B, Flowering time of Bd21-3 wild-type compared with independent *UBI:VRN1* transgenic lines. Dark gray indicates plants carrying *UBI:VRN1*, and light gray indicates sibling plants segregating from the same primary transformant mother plant that do not carry the transgene. C, Flowering time averages of Bd21-3 wild-type and empty vector control plants (control) compared with flowering time of individual *UBI:FT* transgenic plants from different transgenic lines. For B and C, plants were grown nonvernalized in a 16-h photoperiod, and bars represent the average  $\pm$  1 sd. D to I, RT-qPCR data of leaf 2 from two-leaf stage *UBI:VRN1*, *UBI:FT*, or Bd21-3 control plants grown nonvernalized in a 16-h photoperiod. *VRN1* expression (D and G), *FT* expression (E and H), and *VRN2L* expression (F and I). For D to I, bars represent the average of three biological replicates  $\pm$  1 sd. Single asterisk indicates *P* value < 0.01, and two asterisks indicate *P* value < 0.05. NT sib, Nontransgenic sibling plant. [See online article for color version of this figure.]

*HvVRN2* and *TmVRN2* (Zinc Finger-CCT1 [ZCCT1]/ZCCT2).

Given that there are no closer homologs to wheat *VRN2* than *BdVRN2L*, we hypothesized that *BdVRN2L* might play a *VRN2*-like role in *Brachypodium* spp. As stated earlier, *VRN1*, *VRN2*, and *FT* are thought to form a regulatory loop in wheat and barley. If the same regulatory loop exists in *Brachypodium* spp.,

plants that overexpress *VRN1* should have high levels of *FT* and low levels of *VRN2L*. *VRN2L* expression was significantly lower in all of the *UBI:VRN1* lines (*P* < 0.05) and *UBI:FT* lines (*P* < 0.01) compared with wild-type Bd21-3 plants or nontransgenic sibling plants (Fig. 5, F and I). These data support the hypothesis that *VRN2L* plays a role analogous to wheat *VRN2* in a feedback loop model with *VRN1* and *FT*



**Figure 6.** *VRN1*, *FT*, and *VRN2L* gene expression with and without cold treatment of imbibed seeds. Imbibed seeds were given a subsaturating (short cold) vernalization treatment (1 week at 5°C for Bd21 and Bd21-3; 3 weeks at 5°C for Bd1-1) or a saturating (long cold) vernalization treatment (3 weeks at 5°C for Bd21 and Bd21-3; 7 weeks at 5°C for Bd1-1). Leaf 2 was harvested 11 d after removing from cold (B–D) or from emerging shoot tissue at the end of the cold treatment (A). Tissue was harvested at a similar stage of development from nonvernalized controls. Plants were grown in a 16-h photoperiod during and after vernalization. For C, Bd1-1 was treated for 6 weeks of cold. A, RT-qPCR of *VRN1* in shoot tissue of Bd21, Bd21-3, and Bd1-1 during cold treatment. B, RT-qPCR of *VRN1* in leaf 2 of Bd21, Bd21-3, and Bd1-1 after cold treatment. C, RT-qPCR of *FT* in leaf 2 of Bd21, Bd21-3, and Bd1-1 after cold treatment. D, RT-qPCR of *VRN2L* in leaf 2 of Bd21, Bd21-3, and Bd1-1 after cold treatment. Bars represent mean of three biological replicates  $\pm$  SE. Black indicates Bd21, gray indicates Bd21-3, and white indicates Bd1-1. NV, Nonvernalized; V, vernalized.

(Yan et al., 2006; Shimada et al., 2009; Distelfeld and Dubcovsky, 2010).

In wheat and barley, *VRN2* expression is lower in short days versus long days (Dubcovsky et al., 2006; Trevaskis et al., 2006; Sasani et al., 2009). In the tropical cereals rice (*Oryza sativa*) and *Zea mays*, *GHD7* represses flowering in long days and transcripts of *GHD7* are correspondingly higher in long days versus short days (Xue et al., 2008; Hung et al., 2012). We measured *VRN2L* expression in *Brachypodium* spp. accessions Bd21, Bd21-3, and Bd1-1 under different photoperiods (Fig. 4F). *VRN2L* is more highly expressed with increasing photoperiod in all three accessions (Fig. 4F), consistent with behavior of wheat *VRN2* and rice *Ghd7*.

We analyzed *VRN2L* levels in different accessions to determine if there was a correlation with flowering time in a 20-h photoperiod, as is the case with *VRN1* and *FT*. *VRN2L* expression varied by less than 3-fold among the accessions tested (Fig. 4E). Furthermore, there was lack of a strong correlation between flowering time and *VRN2L* expression among different *Brachypodium* spp. accessions. For instance, Bd2-3 flowers at around

50 d without cold in a 20-h photoperiod but had higher levels of *VRN2L* than Tek-11 (Fig. 4E), a line that did not flower in a 20-h photoperiod unless exposed to at least 8 weeks of cold (Fig. 1). Thus, if *VRN2L* plays a repressive role in *Brachypodium* spp. flowering, *VRN2L* mRNA levels are not likely to be rate limiting.

In wheat and barley, *VRN2* is down-regulated in response to cold and is stably repressed after cold (Yan et al., 2004b; Trevaskis et al., 2006). *VRN2L* expression was lower in leaves derived from vernalized imbibed seeds (measured after vernalization was completed) compared with nonvernalized plants (Fig. 6D). Furthermore, *VRN2L* mRNA levels measured in plants after the end of the cold treatment progressively decreased after exposure to increasing durations of cold in Bd1-1 and slightly decreased in Bd21 and Bd21-3 (Fig. 6D). *VRN2L* levels were very low or undetectable in shoot tissues emerging from a germinating seed during cold. Consistent with this, *VRN2* levels in wheat and barley are very low or undetectable in similar tissues during cold (Sasani et al., 2009).

During cold, wheat and barley *VRN2* levels decrease significantly in leaves (Yan et al., 2004b; Trevaskis et al.,

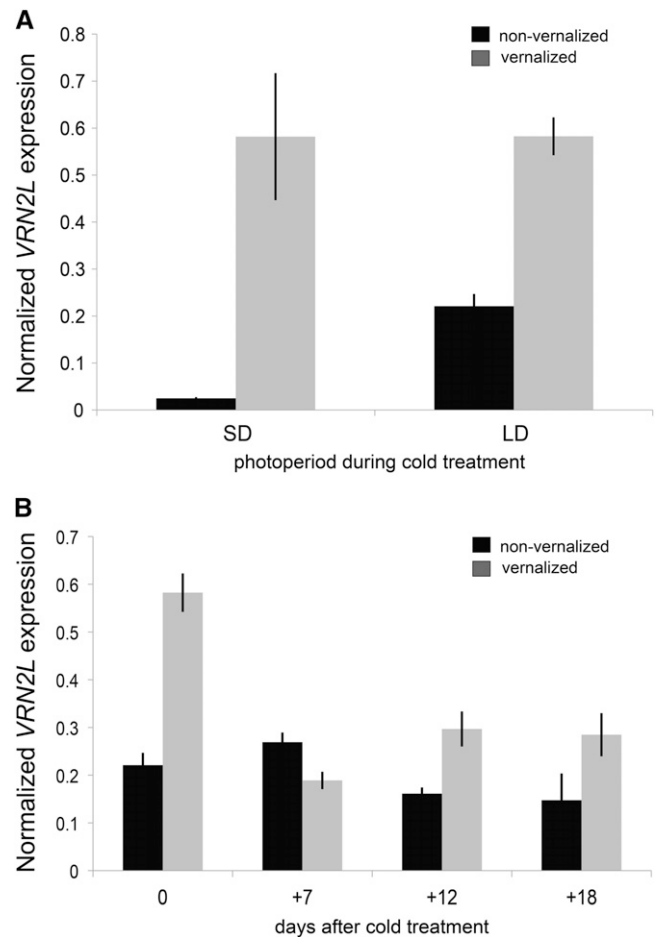
2006), consistent with *VRN2*'s role as a floral repressor and similar to the decrease in *FLC* levels during vernalization in *Arabidopsis* (Michaels and Amasino, 1999; Sheldon et al., 1999). Surprisingly, *VRN2L* mRNA levels increase in leaves during cold exposure of Bd21-3 seedlings in both short and long days (Fig. 7A; Supplemental Fig. S12D). This pattern of expression is in stark contrast to that observed for *VRN2* in wheat (cv G3116) or barley (cv Sonja), where *VRN2* is repressed in leaves during cold exposure in long days (Supplemental Fig. S12, E and F; Yan et al., 2004b; Trevaskis et al., 2006; Sasani et al., 2009) and where *VRN2* mRNA levels are very low or undetectable in short days, regardless of cold treatment (Dubcovsky et al., 2006; Trevaskis et al., 2006; Sasani et al., 2009). As a control for the cold treatment, we also measured *VRN1* mRNA levels. Similar to wheat and barley *VRN1*, *Brachypodium* spp. *VRN1* mRNA levels increased in the leaves by the end of cold (Supplemental Fig. S12, A–C).

Seven days after cold exposure of seedlings, *VRN2L* mRNA levels were slightly lower in vernalized versus nonvernalized plants (Fig. 7B), similar to the results presented in Figure 6 in leaves derived from vernalized imbibed seeds. However, this difference was abolished after 12 days following vernalization (Fig. 7B). Similar to observations with imbibed seed vernalization (Fig. 6, A and B), *VRN1* mRNA levels were high in leaves of vernalized seedlings at the end of the cold treatment and remained high in vernalized plants following cold (Supplemental Fig. S13, A and B). *FT* mRNA levels were undetectable in leaves of seedlings at the end of cold treatment (data not shown) but were significantly higher after cold treatment in vernalized plants (Supplemental Fig. S13C).

To more precisely determine when *VRN2L* levels increase in leaves during cold, Bd21-3 seedlings were grown to the two-leaf stage and shifted to one of several lengths of cold in a 16-h photoperiod ranging from hours to weeks. *VRN2L* levels in leaf 2 peaked after 25 h of cold and were still higher in plants treated for 1 to 4 weeks of cold compared with nontreated plants (Fig. 8), similar to the patterns observed in Figure 7A and Supplemental Figure S12D. In addition, we analyzed *VRN2L* levels from a recently published microarray data set (Li et al., 2012), where Bd21 was treated with different periods of cold ranging from 0 to 24 hours. In this data set, *VRN2L* levels also increased during short cold, peaking between 10 and 24 h of cold treatment. In summary, *VRN2L* mRNA levels peak during short-term cold exposure, but levels remain elevated even after several weeks of cold compared with noncold-treated plants. We also note that the *VRN2L* cold induction pattern is different than the cold induction pattern of *VRN1*, where expression increases gradually over several weeks of cold (Supplemental Fig. S14).

## DISCUSSION

We have characterized flowering time variation in a variety of *Brachypodium* spp. accessions in relation to

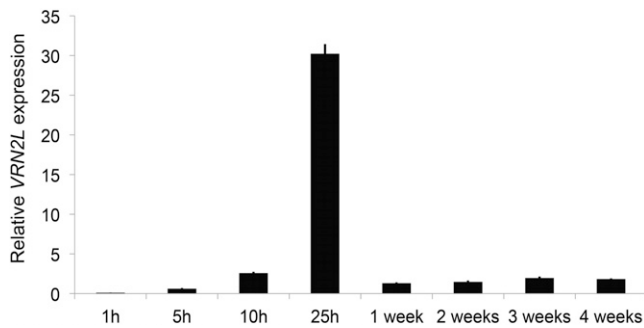


**Figure 7.** *VRN2L* gene expression in leaves of vernalized plants during and after cold treatment. Seedlings were grown to the two-leaf stage (Zadoks stage = 12) before transferring to 5°C for 3 weeks. After cold treatment, plants were grown in a 16-h photoperiod. A, RT-qPCR of *VRN2L* in leaf 2 of nonvernalized (black) versus vernalized (gray) Bd21-3 at the end of a saturating vernalization treatment conducted in short days (8-h photoperiod) or long days (16-h photoperiod). B, RT-qPCR of *VRN2L* in leaf 2 of nonvernalized (black) versus vernalized (gray) Bd21-3 after cold treatment performed in 16-h photoperiod. Bars represent the average of three biological replicates  $\pm$  1 sd.

photoperiod and vernalization. There is a wide range of variation for flowering time behaviors, and flowering time can be manipulated quite readily by varying either cold exposure or photoperiod (Fig. 1). Our study provides a foundation for characterizing the genetic determinants of natural variation in flowering behavior at a molecular level.

## Photoperiod and Temperature Influence on Flowering

For the majority of *Brachypodium* spp. accessions, 6 weeks of vernalization followed by a 20-h photoperiod will generally result in plants that flower at the same time and at a similar developmental stage (Fig. 1). Exceptions to this observation are accessions in the extremely delayed flowering class such as BdTR7a and



**Figure 8.** *VRN2L* expression in response to cold. A, RT-qPCR of *VRN2L* in leaf 2 in plants that received the indicated lengths of cold treatments (5°C) in a 16-h photoperiod. Expression values of each treatment are shown relative to noncold-treated plants harvested at the same time of day. Bars represent an average of three biological replicates  $\pm$  1 sd.

BdTR8n, which require longer cold exposure to flower rapidly after a shift to warm conditions (Fig. 1, A and B; Supplemental Fig. S1). Although a 20-h photoperiod is not a native photoperiod where these accessions are found, we chose this photoperiod because it is a common daylength used by many laboratories to grow *Brachypodium* spp., and this photoperiod provides an additional parameter to classify flowering behavior among accessions. Conversely, all accessions tested will remain vegetative for at least 120 d if grown in an 8-h photoperiod (Supplemental Fig. S4). Vernalization is effective in imbibed seeds for all accessions as initially reported for Bd1-1 (Schwartz et al., 2010).

*Brachypodium* spp. accessions have previously been grouped into spring and winter classes based on flowering behavior in a 20-h photoperiod without vernalization (Schwartz et al., 2010). We have expanded this analysis and categorized *Brachypodium* spp. accessions into six different flowering time classes (Fig. 1C). Although the boundaries distinguishing these classes is arbitrary, this more discriminating classification system will inform genome-wide association studies and provide researchers with a framework for growing different accessions in controlled laboratory environments. Future studies using a larger set of accessions will explore in greater depth whether any associations exist between geography and flowering behavior. In our study, accessions from Iraq typically flowered most rapidly with little or no vernalization requirement. Turkish accessions showed a range of flowering behaviors (2 weeks of cold to greater than 10 weeks of cold to accelerate flowering), and two Spanish accessions also differed substantially in vernalization requirement.

Given the photoperiods within the native growth habitats of *Brachypodium* spp., most lines might actually behave as winter annuals. This is clearly supported by evidence of delayed flowering of Bd21, Bd21-3, and Bd3-1 in a 14-h photoperiod (compared with 16-h or 20-h photoperiods) and acceleration of flowering of these accessions in a 14-h photoperiod after vernalization

(Fig. 2). In the absence of cold, some winter cereals transition to flowering more rapidly after a short-day treatment followed by growth in long days compared with growth in long days alone (Evans, 1987; Dubcovsky et al., 2006; Turner et al., 2013). Although a previous study did not provide evidence of this response when using long days of 20-h for accessions Bd21-3, Bd3-1, and Bd30-1 (formerly DNSCA-6; Schwartz et al., 2010), testing these accessions in shorter, native long-day photoperiods should be informative. We have observed natural variation in the ability of short days to replace cold in accelerating flowering in *Brachypodium* spp. under more native photoperiods (D.P. Woods, T.S. Ream, and R.M. Amasino, unpublished data).

None of the accessions tested flower under short 8-h daylength after 120 d of outgrowth, even after receiving long vernalization treatments. Additionally, meristems of all tested accessions remained vegetative throughout the 120-d growth period with no signs of floral transition. Together, this suggests that it is unlikely that the *Brachypodium* spp. accessions tested contain photoperiod-insensitive alleles. Photoperiod-insensitive alleles within specific genotypes of wheat and barley lead to rapid flowering in both long and short daylength and exhibit normal spike development (Dubcovsky et al., 2006; Beales et al., 2007; Turner et al., 2013). This contrasts with studies in wheat in which genotypes have been discovered that carry CARG-box mutations within the promoter region of *VRN1*, resulting in early initiation of spike development in short days but subsequent slower growth of the spikelet in short days compared with long days (Dubcovsky et al., 2006).

A range of temperatures will satisfy the vernalization requirement (Fig. 3), similar to that found in studies of wheat (Ahrens and Loomis, 1963; Chujo, 1966; Trione and Metzger, 1970; Rawson et al., 1998). Temperatures as high as 16°C have been reported to accelerate flowering via the vernalization pathway in *Arabidopsis* (Wollenberg and Amasino, 2012). In wheat, temperatures between 0°C and 12°C have been reported to be effective, although higher temperatures generally result in less effective vernalization (Chujo, 1966). This is not unexpected given that in nature, plants or seeds will experience a range of cold temperatures over the course of winter. Nonetheless, temperatures from 2°C to 6°C are optimal to ensure a complete vernalization response and thus more uniform rapid flowering in response to long days across a range of accessions.

### *VRN1* and *FT* Promote Flowering

The *VRN1* ortholog in *Brachypodium* spp. has a conserved role similar to that in wheat and barley in promoting flowering. The *Brachypodium* spp. *FT* ortholog also has a floral promoting effect, as is the case across a range of flowering plant families. *VRN1* and *FT* levels correlate with flowering behavior in nonvernalized and vernalized plants, and overexpression of these genes results in rapid-flowering plants (Figs. 4–6). Long-term

cold induces *VRN1* expression, and this correlates with higher *FT* expression after vernalization (Fig. 6; Supplemental Figs. S13 and S14). Overexpression of *VRN1* and *FT* both lead to rapid flowering in a 16-h photoperiod. Consistent with studies from wheat and barley, which suggest a positive feedback loop exists between *FT* and *VRN1*, we observed high mRNA levels of *FT* in *UBI:VRN1* lines and high mRNA levels of *VRN1* in *UBI:FT* lines (Yan et al., 2006; Shimada et al., 2009; Distelfeld and Dubcovsky, 2010), suggesting this part of the model is conserved in *Brachypodium* spp. and throughout the Pooideae.

In wheat and barley, rapid-flowering lines often harbor natural allelic variants of *VRN1* or *FT* (Yan et al., 2003, 2004a, 2006; Fu et al., 2005; Loukoianov et al., 2005; von Zitzewitz et al., 2005). Dominant alleles of *VRN1* and *FT* in wheat and barley are able to bypass the vernalization requirement, even in the presence of functional *VRN2* alleles. Future studies will examine whether there is an association between certain alleles of *VRN1*, *FT*, and *VRN2L* with flowering. Sequences of many accessions are currently being generated and will enable such a study (<http://genome.jgi.doe.gov/genome-projects/>).

#### *Brachypodium* spp. *VRN2L* Is Distinct from Wheat/Barley *VRN2*

In contrast to *VRN1* and *FT*, the role of *VRN2L* in *Brachypodium* spp. flowering is less clear. *VRN2L*, the closest homolog to wheat/barley *VRN2* identified to date in *Brachypodium* spp., shares some features in common with wheat/barley *VRN2* (Ream et al., 2012). Specifically, *VRN2L* is expressed more highly as days get longer (Fig. 4F; Ream et al., 2012), and after vernalization of imbibed seeds, *VRN2L* mRNA levels are lower in leaves (Fig. 6). Searches of assemblies of other *Brachypodium* spp. accessions with a range of flowering behaviors has so far not revealed a gene with closer homology to wheat *VRN2*, nor have these searches revealed a *VRN2* homolog in the region of synteny that is deleted in Bd21 (for discussion, see Ream et al., 2012).

On the other hand, several features of *Brachypodium* spp. *VRN2L* are distinct from *VRN2* in wheat and barley. First, *VRN2* mRNA levels decrease during cold in wheat and barley and remain low at least 14 days after vernalization in wheat (Yan et al., 2004b); by contrast, *Brachypodium* spp. *VRN2L* mRNA levels increase during cold exposure (Fig. 7; Supplemental Fig. S12D). The cold-mediated increase occurs quite rapidly (within a day) relative to the time it takes to satisfy the vernalization requirement (several weeks; Fig. 8). The cold-induced expression of *VRN2L* in leaves is reminiscent, at least to some degree, to that of the *C-REPEAT BINDING FACTOR* genes that have roles in cold acclimation (Thomashow, 2010). Further tests are necessary to determine whether *VRN2L* in *Brachypodium* spp. has any role in cold acclimation.

Second, *Brachypodium* spp. *VRN2L* levels are only slightly lower after seedling vernalization, and this

repression is not observed past 12 days following the end of cold exposure (Fig. 7). A confounding variable regarding seedlings that have undergone vernalization (but not in the case of imbibed seeds where *VRN2L* mRNA is essentially not detected at this developmental stage) is the several-fold higher mRNA levels of *VRN2L* following cold treatment relative to nonvernalized plants. After moving to warm temperatures, *VRN2L* levels do drop relative to the higher levels present immediately at the end of cold treatment in vernalized plants, but 7 d after cold exposure, they are only marginally lower than the mRNA levels in nonvernalized plants. By 12 d following cold exposure, as discussed above, *VRN2L* levels are not lower than the nonvernalized controls. This pattern of expression is not indicative of a gene acting as a floral repressor that is targeted by a vernalization response pathway.

*Brachypodium* spp. *VRN2L* mRNA levels in leaves do not always correlate with *VRN1* or *FT* expression in the absence of cold, highlighting another feature distinguishing *Brachypodium* spp. *VRN2L* from wheat or barley *VRN2*, where high mRNA levels of *FT* and *VRN1* consistently correlate with low mRNA levels of *VRN2* (Trevaskis et al., 2006; Yan et al., 2006; Hemming et al., 2008; Shimada et al., 2009). The inconsistent correlation of *VRN2L* with *VRN1* and *FT* is highlighted by two examples. In the first case, rapid-flowering accessions Bd21, Bd21-3, and Bd3-1 have high mRNA levels of *VRN1* and *FT* but not correspondingly lower levels of *VRN2* when compared with other accessions (Fig. 4E). However, in the second case, high mRNA levels of *VRN1* and *FT* (i.e. generated via a *UBI:VRN1* or *UBI:FT* transgene) do correlate with lower *VRN2L* mRNA levels (Fig. 5F).

In summary, *Brachypodium* spp. is a useful model for understanding the genetic networks conferring a vernalization response in a temperate grass. It is likely that the roles of *VRN1* and *FT* are conserved in vernalization in *Brachypodium* spp. compared with cereals. Future studies will reveal the extent of conservation in the vernalization network between *Brachypodium* spp. and economically important cereals such as wheat, oats, and barley.

## MATERIALS AND METHODS

### Plant Growth

Accession information is located in Supplemental Table S1. Seeds were imbibed overnight in distilled water at 5°C prior to sowing. Individual plants were grown in MetroMix 360 (Sungrow) in plastic pots and fertilized weekly with Peters Excel 15-5-15 Cal-Mag and Peters 10-30-20 Blossom Booster (RJ Peters). Growth chamber temperatures averaged 22°C during the light period and 18°C during the dark period. Plants were grown under T5 fluorescent bulbs (5,000 K, Lithonia), and light intensities averaged around approximately 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at plant level. To minimize light intensity differences within each chamber, plants were rotated several times per week. For experiments in Figures 2 and 3, plants were grown under T8 fluorescent bulbs, and light intensities averaged around 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at plant level. Developmental stage was recorded using the Zadoks scale (Zadoks et al., 1974). Nonvernalized control plants were synchronized to the developmental stage plants had reached when they were removed from vernalization. In Figure 3, plants were

at the same developmental stage when entering the vernalization treatment. Additionally, for the rest of the experiments all plants entered the cold at the same developmental stage.

## Plant Measurements

Flowering time of vernalized plants was measured as the number of days from the end of vernalization (or emergence of the coleoptile in nonvernalized plants) to the first day upon which emergence of the spike was detected (Zadoks scale = 50; Zadoks et al., 1974). The number of primary leaves derived from the main shoot was recorded at the time of heading to control for development. For plants that flowered, height was measured from the base of the plant to the tallest point of the plant. For nonflowering plants, height was measured from the soil level to the highest point of the tallest leaf.

## RT-qPCR assays

RNA was isolated from leaf tissue or whole shoot tissue using Plant Concert RNA Reagent (Life Technologies) following the manufacturer's recommendations. RNA samples were treated with Turbo DNase (Life Technologies) according to the manufacturer's protocol prior to first-strand cDNA synthesis. RNA integrity was assessed by a combination of A260/A280 and A260/A230 measurements and running samples on a denaturing formaldehyde-agarose gel as recommended in the Qiagen RNAeasy Mini Handbook. Only those samples with 260/280 and 260/230 ratios greater than 1.7 and showing no degradation by gel electrophoresis were used. For each sample, no real-time (RT) controls were performed. cDNA was made using 1  $\mu$ g of total RNA per sample and M-MLV Reverse Transcriptase (Promega) and poly-T primers (IDT). For quantitative PCR (qPCR), 3  $\mu$ L cDNA (diluted 5-fold following first-strand synthesis) was amplified using Takara ExTaq II in a 20- $\mu$ L reaction containing 6  $\mu$ L water, 10  $\mu$ L SybrGreen Takara ExTaqII Master Mix, and 1  $\mu$ L 10  $\mu$ M forward and reverse primers. qPCR reactions were amplified on an ABI 7500 Fast system with the following parameters: 95°C for 30 s and 40 cycles of 95°C for 5 s, 60°C for 5 s, and 72°C for 34 s, followed by a melting curve program. Fifty-five degrees Celsius was used for annealing in all samples for Supplemental Figure S12. Fluorescence data were collected during the 72°C extension steps and during the melting curve program. Gene expression was quantified using the  $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001), and all primer efficiencies ranged from 95% to 110%. Expression data for each gene is shown normalized to *UBIQUITIN-CONJUGATING ENZYME18* (*Brachypodium distachyon*; Hong et al., 2008; Schwartz et al., 2010), *Actin* (barley [*Hordeum vulgare*]; Trevaskis et al., 2006), or *TRANSLATION ELONGATION FACTOR1* (wheat [*Triticum aestivum*]; Distelfeld and Dubcovsky, 2010). Primer pairs used to amplify each gene are listed in Supplemental Table S2.

## Generation of *UBI:VRN1* and *UBI:FT* Transgenics

*VRN1* and *FT* cDNAs were amplified from Bd21-3 cDNA pooled from vernalized and nonvernalized leaf tissue. cDNAs were gel extracted (Qiagen) and were cloned into pENTR-D-TOPO (Life Technologies) using the manufacturer's protocol. Clones were verified by sequencing. pENTR-cDNAs were recombined into pANIC10A (Mann et al., 2012) using Life Technologies LR Clonase II following the manufacturer's protocol. Clones were verified by sequencing in pANIC10A and then transformed into chemically competent *Agrobacterium tumefaciens* strain Agl-1. Plant callus transformation was performed as described (Vogel and Hill, 2008). Independent transgenic lines were genotyped for the transgene using a cDNA-specific forward and pANIC vector AcV5 tag reverse primer (Supplemental Table S1). Primer pairs used to clone each cDNA are listed in Supplemental Table S1.

## Supplemental Data

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

**Supplemental Figure S1.** Vernalization timecourses of *Brachypodium* spp. accessions recorded as days to flowering and leaf count on the main shoot grown in 20-h and 16-h photoperiods.

**Supplemental Figure S2.** Correlation of days to flowering and final leaf number.

**Supplemental Figure S3.** Photographs of *Brachypodium* spp. accessions after 60 d of outgrowth post-vernalization.

**Supplemental Figure S4.** Vernalization timecourse of *Brachypodium* spp. accessions grown in an 8-h photoperiod.

**Supplemental Figure S5.** Photographs of *Brachypodium* spp. accessions grown in an 8-h photoperiod after 120 d of outgrowth.

**Supplemental Figure S6.** Height of *Brachypodium* spp. accessions in different photoperiods following cold treatment.

**Supplemental Figure S7.** Effect of different photoperiods during vernalization on flowering time.

**Supplemental Figure S8.** Effect of different photoperiods after vernalization on flowering time.

**Supplemental Figure S9.** Effect of different temperatures during vernalization on flowering time.

**Supplemental Figure S10.** Primary leaf number on the main shoot at flowering of *UBI:VRN1* and *UBI:FT*.

**Supplemental Figure S11.** Vernalization timecourse for Bd21-3 and Bd1-1.

**Supplemental Figure S12.** *VRN1* and *VRN2L* gene expression in leaves of wheat, barley, and *Brachypodium* spp. during cold.

**Supplemental Figure S13.** *VRN1* and *FT* gene expression in leaves of vernalized plants during and after cold treatment.

**Supplemental Figure S14.** *VRN1* expression in response to varying lengths of cold.

**Supplemental Table S1.** Origin of *Brachypodium* spp. accessions used in this study and assignment to flowering class.

**Supplemental Table S2.** Primer Sequences.

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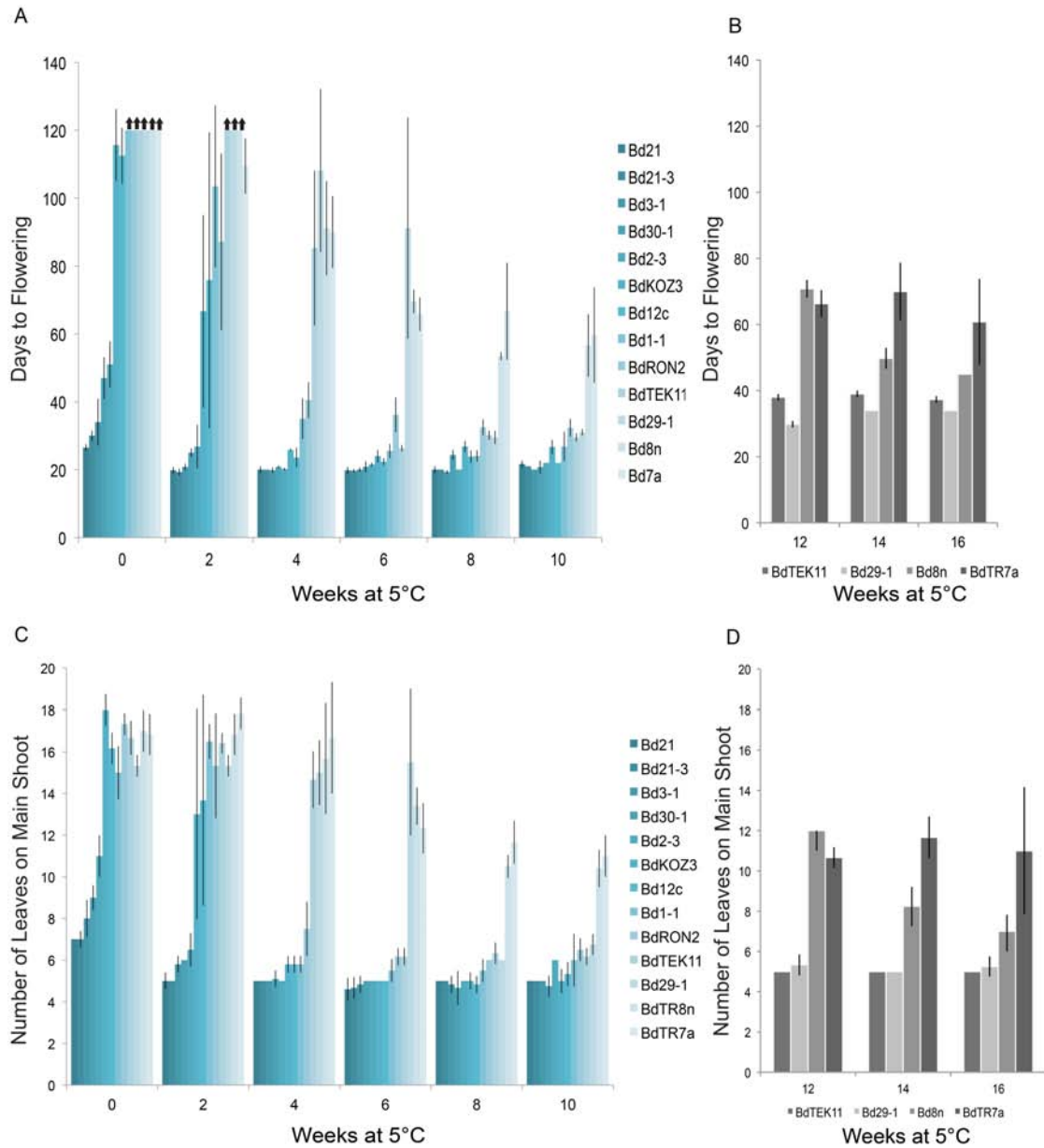
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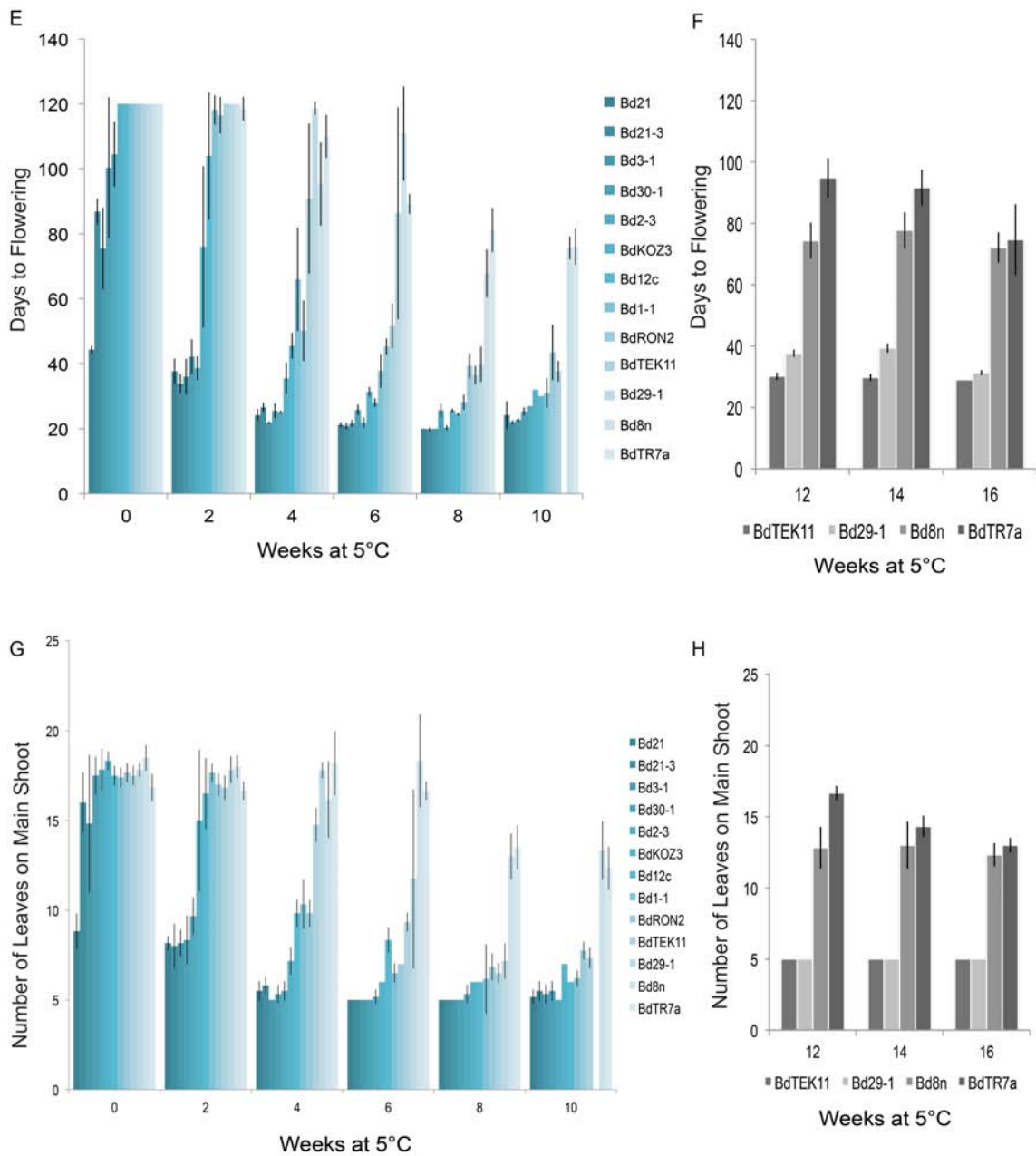
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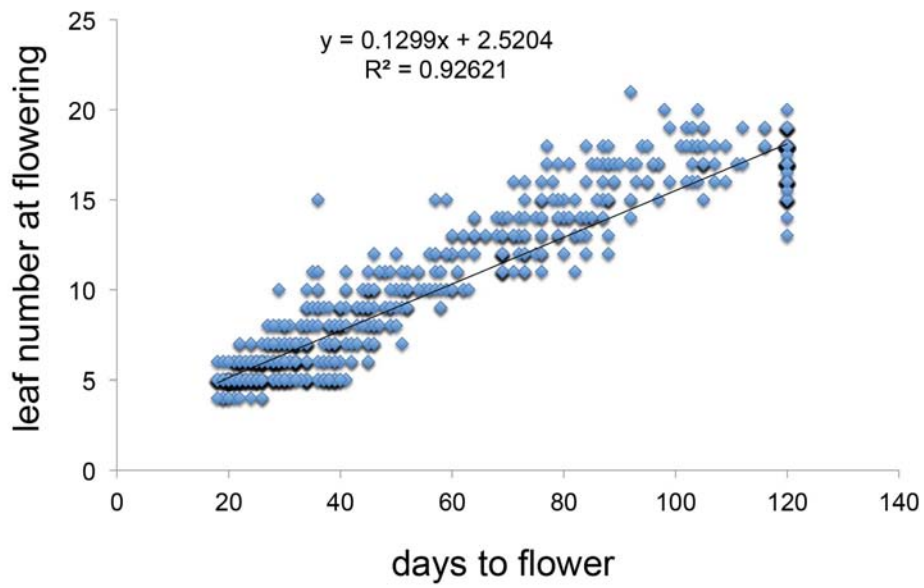
## Supplemental Figures



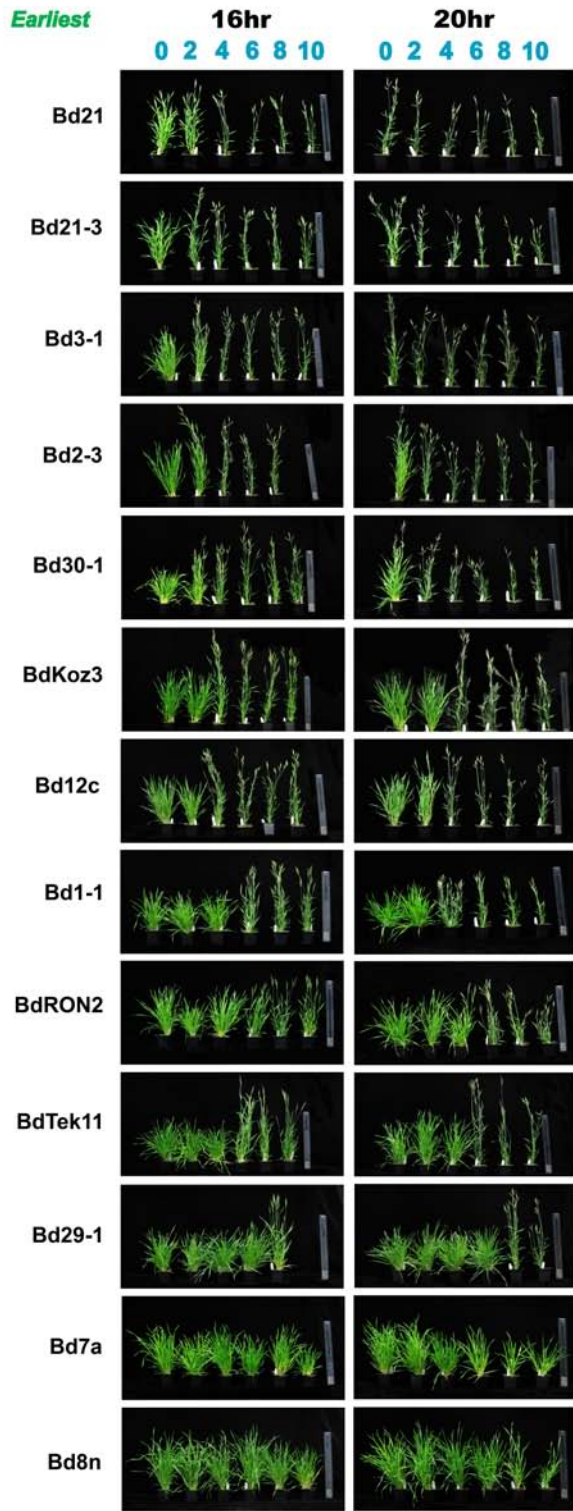
**Figure S1. Vernalization timecourses of *Brachypodium* accessions grown in a 20-hour photoperiod.** Days to flowering of *Brachypodium* accessions grown in a 20-hour photoperiod after imbibed seeds were cold treated for the indicated number of weeks at 5°C. Final leaf number of *Brachypodium* accessions grown in a 20-hour (B) or 16-hour (D) photoperiod after the indicated number of weeks of cold treatment. Bars represent the average  $\pm$  one standard deviation. Flowering time reflects the number of days to spike emergence (Zadoks' scale=50) from the end of cold treatment. Primary leaves were counted on the main shoot until emergence of the spikelet, or, in the case of plants that had not flowered, 120 days after the end of the cold treatment.



**Figure S1. Vernalization timecourses of *Brachypodium* accessions grown in a 16-hour photoperiod.** (E-F) Days to flowering of *Brachypodium* accessions grown in a 16-hour photoperiod after imbibed seeds were cold treated for the indicated number of weeks at 5°C. (G-H) Final leaf number of *Brachypodium* accessions grown in a 16-hour photoperiod after the indicated number of weeks of cold treatment. Bars represent the average  $\pm$  one standard deviation. Flowering time reflects the number of days to spike emergence (Zadoks' scale=50) from the end of cold treatment. Primary leaves were counted on the main shoot until emergence of the spikelet, or, in the case of plants that had not flowered, 120 days after the end of the cold treatment.

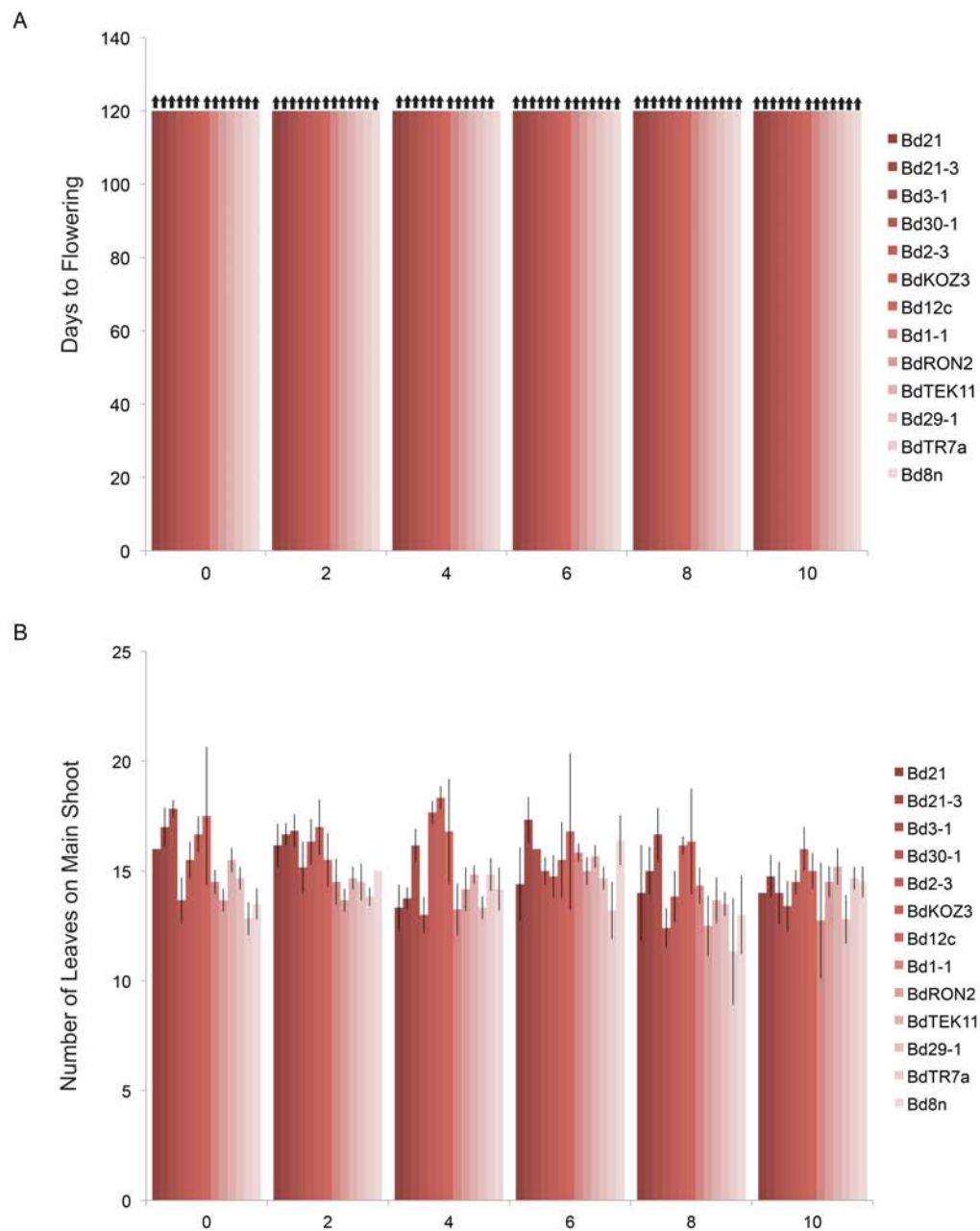


**Figure S2. Correlation of days to flowering and final leaf number.** Scatterplot of days to flowering (x-axis) vs. leaf number at flowering (y-axis) for plants used in the 16-hour and 20-hour vernalization timecourses in Fig. 1. Plants that had not flowered by 120 days were assigned a value of 120 days.



*Latest*

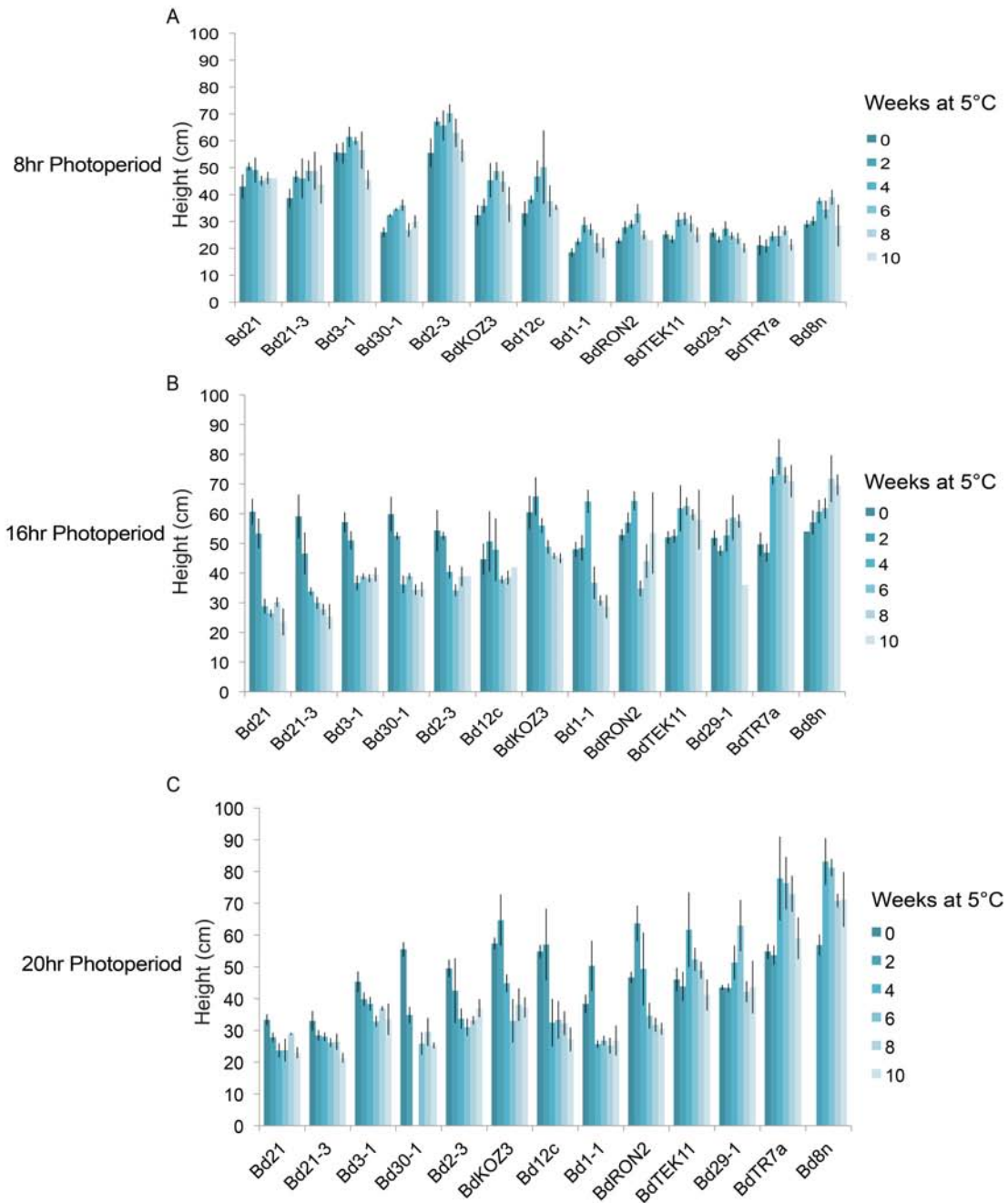
**Figure S3.** Photographs of *Brachypodium* accessions after vernalization. Photographs of representative plants from each treatment X photoperiod were taken 60 days after removing from cold treatment in the indicated photoperiod.



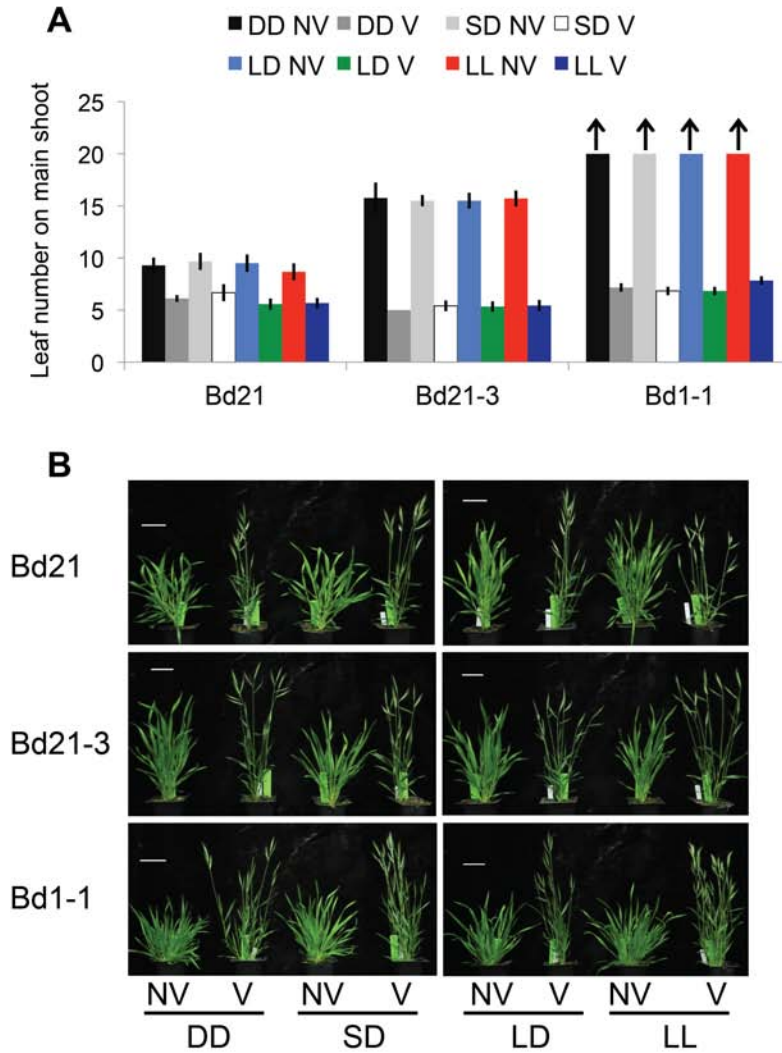
**Figure S4. Vernalization timecourse of *Brachypodium* accessions grown in an 8-hour photoperiod.** (A) Days to flowering or (B) primary leaf number on main shoot of *Brachypodium* accessions grown in an 8-hour photoperiod after imbibed seeds were cold treated for the indicated number of weeks at 5°C. Bars represent the average  $\pm$  one standard deviation. Flowering time reflects number of days to spike emergence (Zadoks' scale=50) from the end of cold treatment. Primary leaves were counted on the main shoot 120 days after the end of the cold treatment. Arrows indicate plants within a treatment had not flowered within 120 days of the experiment.



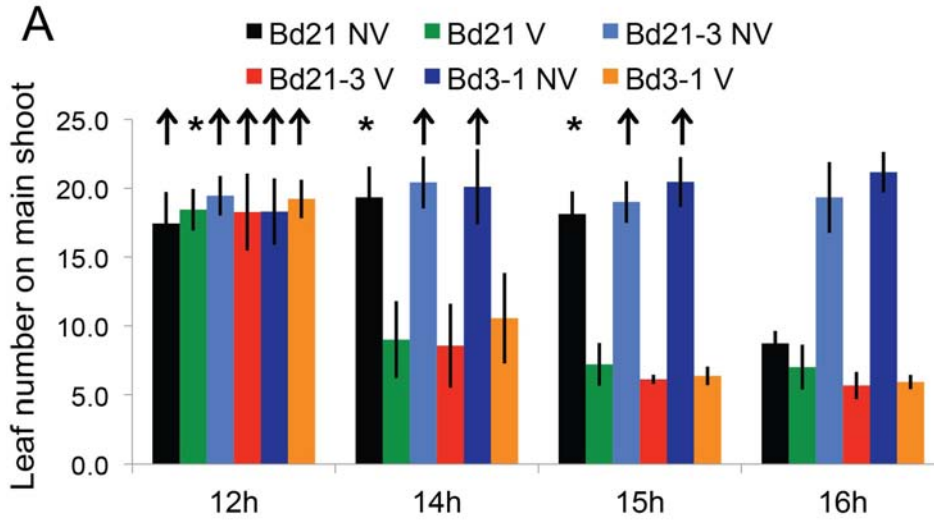
**Figure S5. Photographs of *Brachypodium* accessions grown in an 8-hour photoperiod.** Photographs of representative non-vernalized plants from each accession were taken 120 days after emergence of the coleoptile. Bar = 5 cm.



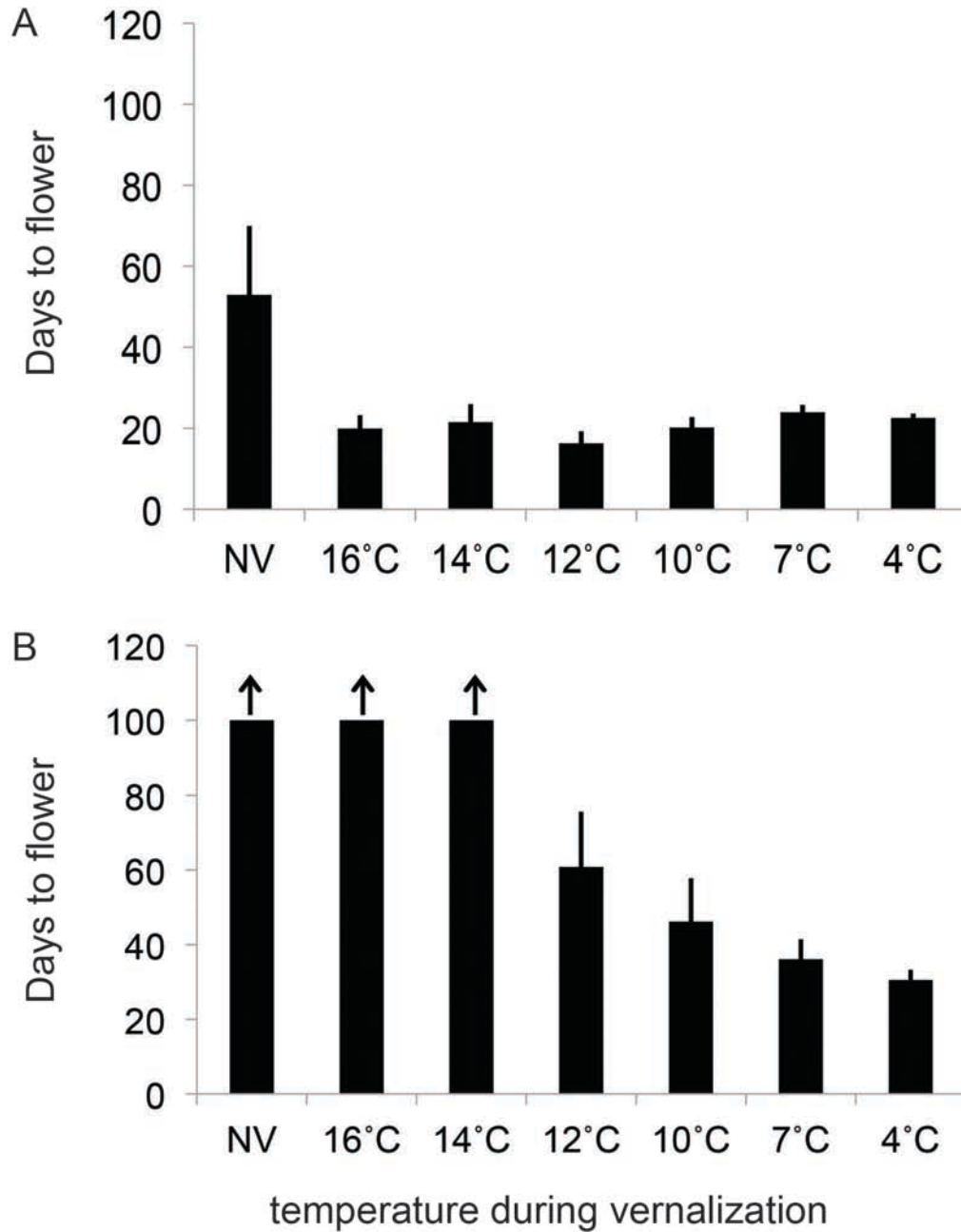
**Figure S6. Height of *Brachypodium* accessions in different photoperiods following cold treatment.** Height of *Brachypodium* accessions after cold treatment when grown in a (A) 8-hour photoperiod (B) 16-hour photoperiod or (C) 20-hour photoperiod. Height was recorded after 120 days of outgrowth from the soil level (base of plant) to the tallest point of the plant. Bars represent the average +/- one standard deviation.



**Figure S7. Effect of different photoperiods during vernalization on flowering time.** Imbibed seeds sown on plates were exposed to three weeks (Bd21, Bd21-3) or seven weeks (Bd1-1) at 5°C in either: DD: no light, SD: 8-hours light/16-hours dark, LD: 16-hours light/8-hours dark, LL: constant light. Non-vernalized (NV) controls were exposed to a similar light regime to an equivalent stage of development that the vernalized seedlings reached by the end of the cold treatment. Plants were grown in a 16-hour photoperiod following vernalization. (A) Number of primary leaves on the main shoot at flowering (NV=non-vernalized, V=vernalized). Bars represent +/- one standard deviation. (B) Representative photographs of non-vernalized and vernalized plants from each treatment. Scale bar= 5 cm.



**Figure S8. Effect of different photoperiods after vernalization on flowering time.** (A) Imbibed seeds were treated for three weeks at 5°C before shifting to one of four photoperiods: 12-hours light/12-hours dark, 14-hours light/10-hours dark, 15-hours light/9-hours dark, or 16-hours light/8-hours dark. Flowering time is presented as the number of primary leaves on the main stem at flowering. Arrows indicate treatments where plants did not flowering during the 120 days of the experiment. "\*" indicates some plants did not flower during this treatment after 120 days. NV=non-vernalized, V=vernalized.



**Figure S9. Effect of different temperatures during vernalization on flowering time.** Days to flowering of plants vernalized as seedlings (Zadok's stage=10) for (A) three weeks (Bd21-3) or (B) seven weeks (Bd1-1) in a 16-hour photoperiod and then placed into "warm" conditions in a 16-hour photoperiod, along with non-vernalized (NV) plants. Bars represent +/- one standard deviation. Arrows indicate that plants within a treatment did not flower by 100 days.

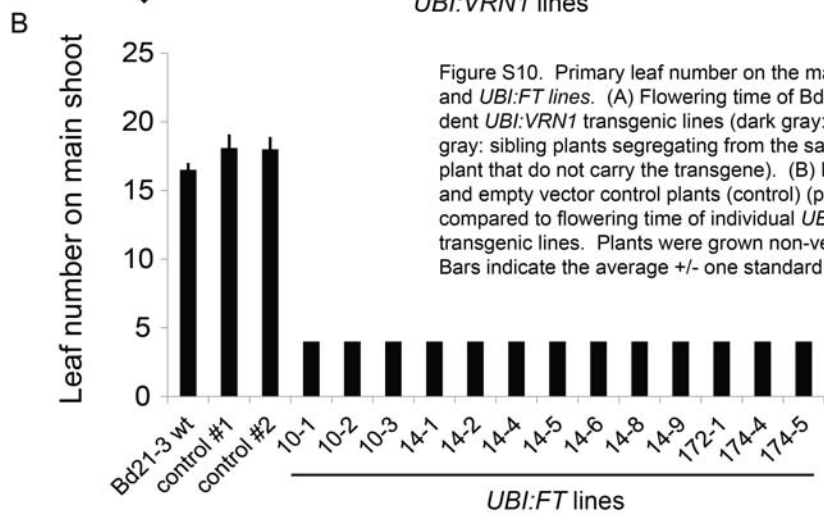
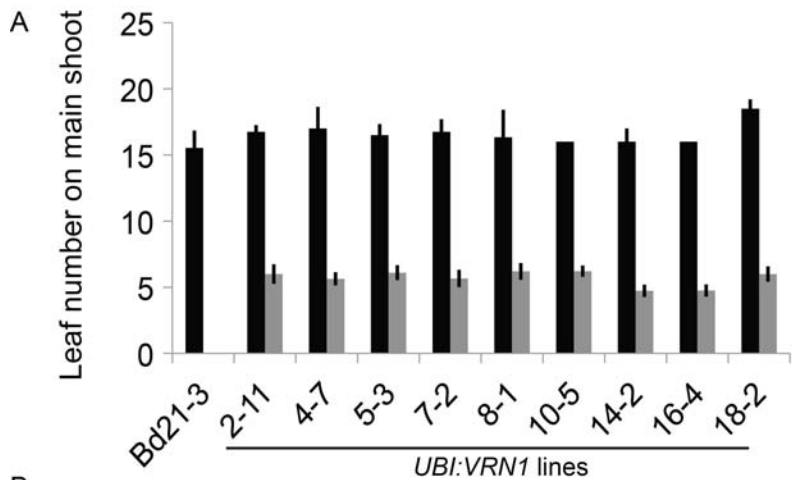
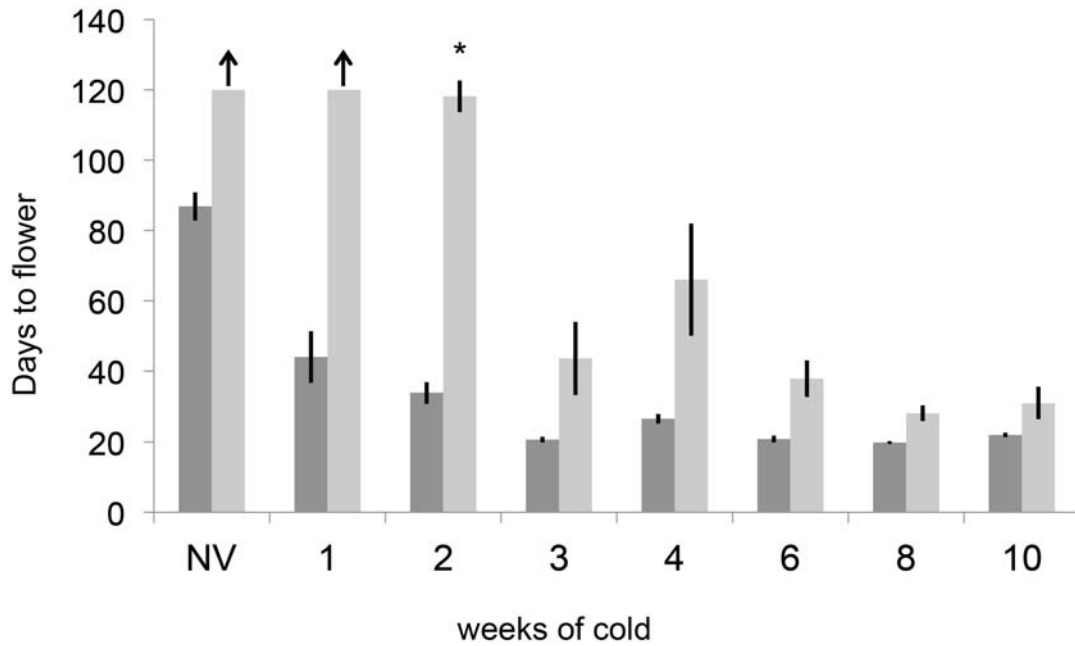
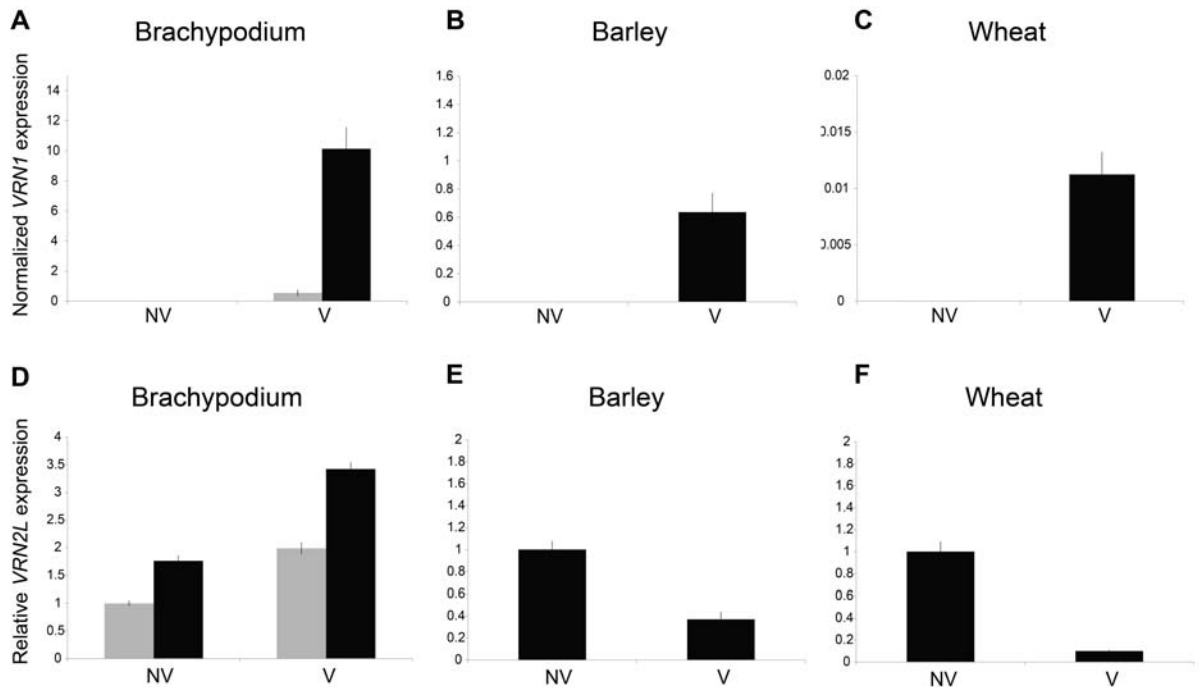


Figure S10. Primary leaf number on the main shoot at flowering of *UBI:VRN1* and *UBI:FT* lines. (A) Flowering time of Bd21-3 wildtype compared to independent *UBI:VRN1* transgenic lines (dark gray: plants carrying *UBI:VRN1*; light gray: sibling plants segregating from the same primary transformant mother plant that do not carry the transgene). (B) Flowering time of Bd21-3 wildtype and empty vector control plants (control) (presented as averages of 6-12 plants) compared to flowering time of individual *UBI:FT* transgenic plants from different transgenic lines. Plants were grown non-vernalized in a 16-hour photoperiod. Bars indicate the average +/- one standard deviation.

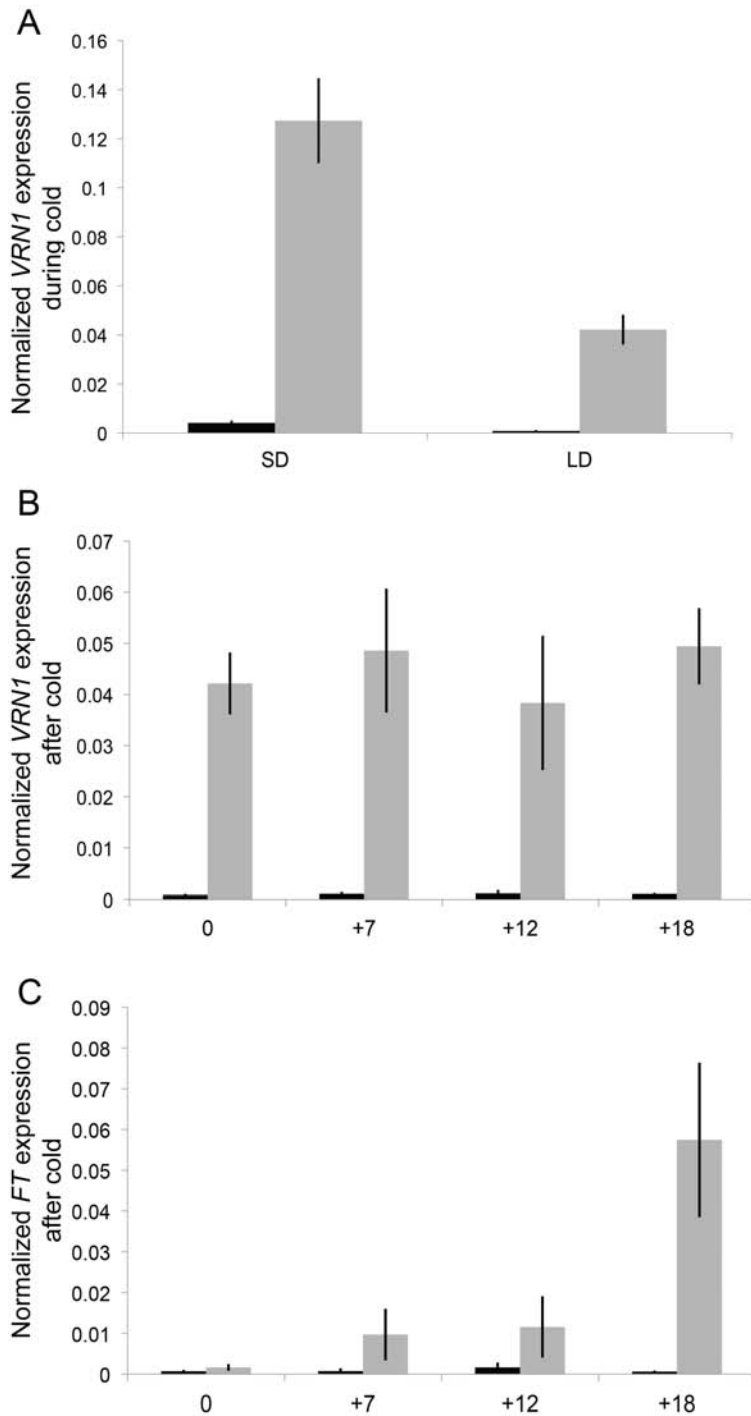


**Figure S11. Vernalization timecourse for Bd21-3 and Bd1-1.** Imbibed seeds were treated at 5°C for the indicated number of weeks before transferring to a 16-hour photoperiod. “\*” indicates that some plants within the treatment did not flower during the 120 days of the experiment. An arrow indicates that no plants flowered during the course of the experiment. Bars indicate the average +/- one standard deviation.



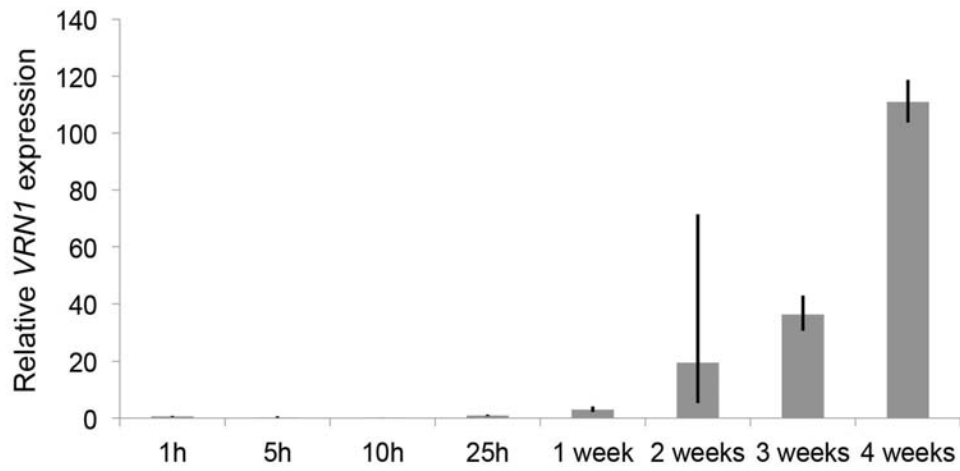
**Figure S12. *VRN1* and *VRN2L* gene expression in leaves of wheat, barley and *Brachypodium* during cold.**

*VRN1* and *VRN2L* expression in leaf 2 of *Brachypodium*, wheat and barley sampled at the end of the cold treatment in a 16-hour photoperiod. Plants were grown together in the same growth chamber during the experiment. (A, B, C) RT-qPCR of *VRN1* expression in (A) Bd21-3 (gray), Bd1-1 (black), (B) Sonja (H.v.) and (C) G3116 (T.m.). (D, E, F) RT-qPCR of *VRN2L* and *VRN2* expression shown relative to non-vernalized plants in (D) Bd21-3 (gray), Bd1-1 (black), (E) Sonja (H.v.) and (F) G3116 (T.m.). As primer sets and standard curves were different for each species in the qPCR assay, expression levels between species are not directly comparable. Bd21-3 was treated at 5°C for 4 weeks, whereas Bd1-1, Sonja, and G3116 were treated at 5°C for 8 weeks. Bars represent an average of three biological replicates +/- one standard deviation. NV=non-vernalized, V=vernalized.



**Figure S13. *VRN1* and *FT* gene expression in leaves of vernalized plants during and after cold treatment.**

Seedlings were grown to the two-leaf stage (Zadoks' stage=12) before transferring to 5°C for three weeks. After cold treatment, plants were grown in a 16-hour photoperiod. (A) RT-qPCR of *VRN1* in leaf 2 of non-vernalized (black) vs. vernalized (gray) Bd21-3 at the end of a saturating vernalization treatment conducted in short days (8-hour photoperiod) or long days (16-hour photoperiod). (B) RT-qPCR of *VRN1* and (C) *FT* in leaf 2 of non-vernalized vs. vernalized Bd21-3 at the end of cold (0), +7, +12 and +18 days after vernalization (cold treatment performed in 16-hour photoperiod). For (A) and (B), bars represent the average of three biological replicates +/- one standard deviation. For (C), bars represent +/- one standard error.



**Figure S14. *VRN1* expression in response to cold.**

(A) RT-qPCR of *VRN1* in leaf 2 in plants that received the indicated lengths of cold treatments (5°C) in a 16-hour photoperiod. Expression values of each treatment are shown relative to non-cold treated plants harvested at the same time of day. Bars represent an average of three biological replicates  $\pm$  one standard deviation.

## Supplemental Tables

Accession/ flowering class	Origin	Seed Origin
Bd21/ERF	Iraq	Garvin et al. 2008
Bd21-3/RF	Iraq	Vogel et al. 2008
Bd3-1/RF	Iraq	Garvin et al. 2008
Bd30-1/IRF	Spain	Schwartz et al. 2010
Bd2-3/IRF	Iraq	Garvin et al. 2008
KOZ3/IDF	Turkey	Vogel et al. 2009
TR12c/IDF	Turkey	Feliz et al. 2009
Bd1-1/DF	Turkey	Garvin et al. 2008
Bd29-1/DF	Ukraine	Garvin et al. 2008
RON2/DF	Spain	Schwartz et al. 2010
TEK11/DF	Turkey	Vogel et al. 2009
TR7a/EDF	Turkey	Feliz et al. 2009
TR8n/EDF	Turkey	Feliz et al. 2009

Table S1. Origin of *Brachypodium* accessions used in this study and assignment to flowering classes (Extremely Rapid Flowering, ERF; Rapid Flowering, RF; Intermediate Rapid Flowering, IRF; Intermediate Delayed Flowering, IDF; Delayed Flowering, DF; Extremely Delayed Flowering, EDF).

Primer	Primer sequence	Purpose	Reference
UBC18 qPCR	GTCACCCGCAATGACTGTAAGTTC	RT-qPCR	this paper
UBC18 qPCR	TTGTCTTGCGGACGTTGCTTTG	RT-qPCR	this paper
VRN1 qPCR	GCTCTGCAGAAGGAACCTTGTTG	RT-qPCR	this paper
VRN1 qPCR	CTAGTTTGCGGGTGTGTTTGCTC	RT-qPCR	this paper
VRN2L qPCR	ATGCATGAGAGAGAGGCGAAGG	RT-qPCR	this paper
VRN2L qPCR	TCGTAGCGGATCTGCTTCTCGTAG	RT-qPCR	this paper
FT qPCR	TTCGGGAACAGGAACGTGTCCAAC	RT-qPCR	this paper
FT qPCR	AGCATCTGGGTCTACCATCACGAG	RT-qPCR	this paper
HvActin qPCR	GCCGTGCTTTCCTCTATG	RT-qPCR	Trevaskis et al. 2006
HvActin qPCR	GCTTCTCCTTGATGTCCCTTA	RT-qPCR	Trevaskis et al. 2006
HvVRN1 qPCR	GCATAAGTTGGTTCTTCTGGCTCTG	RT-qPCR	Trevaskis et al. 2007
HvVRN1 qPCR	GCCTCATCATCTTCTCCACCAA	RT-qPCR	Trevaskis et al. 2007
HvVRN2ab qPCR	GAGCCACCATCGTGCCATTC	RT-qPCR	Trevaskis et al. 2006
HvVRN2ab qPCR	GCCGCTTCTTCTCTTCTC	RT-qPCR	Trevaskis et al. 2006
TmTEF1 qPCR	GCCCTCCTTGCTTTCACTCT	RT-qPCR	Distelfeld et al. 2010
TmTEF1 qPCR	AACGCGCCTTTGAGTACTTG	RT-qPCR	Distelfeld et al. 2010
TmVRN1 qPCR	AAGAAGGAGAGGTCACCTGCAGG	RT-qPCR	Yan et al. 2006
TmVRN1 qPCR	GGCTGCACTGCCGCA	RT-qPCR	Yan et al. 2006
TmZCCT2 qPCR	CCACCATCGTGCCATTCT	RT-qPCR	Distelfeld et al. 2009
TmZCCT2 qPCR	CCCACCATCATCTCTGTATCAA	RT-qPCR	Distelfeld et al. 2009
VRN1 cDNA F	caccatggggcgcgggaaggtgcag	cloning VRN1 cDNA	this paper
VRN1 cDNA R	gccgctgatgtggctcacc	cloning VRN1 cDNA	this paper
FT cDNA F	caccATGGCCGGGAGGGACAGGGAC	cloning FT cDNA and genotyping UBI:FT lines	this paper
FT cDNA R	GGGGTACATCCTCTGCCGCC	cloning FT cDNA	this paper
VRN1 F	CTAGCCAAGGCAGGGTAGAC	genotyping UBI:VRN1 lines	this paper
pANIC10A AcV5 R	aga cca gcc gct cgc atc ttt cca ag	genotyping UBI:VRN1 and UBI:FT lines	this paper

Table SII. Primer sequences.