Short title:

Natural variation in *B. distachyon* flowering time

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Title:

Natural variation in Brachypodium links vernalization and flowering time loci as major flowering determinants

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One sentence summary:

Standing genetic variation for flowering time in a non-domesticated grass encompasses known and novel regulators.

Footnotes:

Author contributions

JB, FC, MO, JD, and MM conceived the study, and participated in its design and coordination. PG and IHP participated in the experiments. JB, FC, JD, and MM wrote the manuscript. All authors read and approved the final manuscript.

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Abstract

The domestication of plants is underscored by the selection of agriculturally favorable developmental traits, including flowering time, which resulted in the creation of varieties with altered growth habits. Research into the pathways underlying these growth habits in cereals has highlighted the role of three main flowering regulators: *VRN1*, *VRN2*, and *FT*. Previous reverse genetic studies suggested that the roles of *VRN1* and *FT* are conserved in *Brachypodium distachyon*, yet identified considerable ambiguity surrounding the role of *VRN2*. To investigate the natural diversity governing flowering time pathways in a non-domesticated grass, the reference *B. distachyon* accession Bd21 was crossed with the vernalization-dependent accession ABR6. Resequencing of ABR6 allowed the creation of a SNP-based genetic map at the F_4 stage of the mapping population. Flowering time was evaluated in $F_{4:5}$ families in five environmental conditions and three major loci were found to govern flowering time. Interestingly, two of these loci colocalize with the *B. distachyon* homologs of the major flowering pathway genes *VRN2* and *FT*, whereas no linkage was observed at *VRN1*. Characterization of these candidates identified sequence and expression variation between the two parental genotypes, which may explain the contrasting growth habits. However, the identification of additional QTLs suggests that greater complexity underlies flowering time in this non-domesticated system. Studying the interaction of these regulators in *B. distachyon* provides insights into the evolutionary context of flowering time regulation in the Poaceae, as well as elucidates the way humans have utilized the natural variation present in grasses to create modern temperate cereals.

Introduction

Coordination of flowering time with geographic location and seasonal weather patterns has a profound effect on flowering and reproductive success (Amasino, 2010). The mechanisms underpinning this coordination are of great interest for understanding plant behavior and distribution within natural ecosystems (Wilczek et al., 2010). Plants that fail to flower at the appropriate time are unlikely to be maximally fertile and therefore will be less competitive in the longer term. Likewise, optimal flowering time in crops is important for yield and quality: seed and fruit crops need to flower early enough to allow ripening or to utilize seasonal rains, while delayed flowering may be advantageous for leaf and forage crops (Distelfeld et al., 2009; Jung and Müller, 2009).

Although developmental progression towards flowering can be modulated in several ways, many plants have evolved means to detect seasonal episodes of cold weather and adjust their flowering time accordingly, a process known as vernalization (Ream et al., 2012). Despite the importance of flowering time, the molecular and genetic mechanisms underlying this dependency have been studied in only a few systems, notably the Brassicaceae, Poaceae, and Amaranthaceae (Andrés and Coupland, 2012; Ream et al., 2012). Three major *VERNALIZATION* (*VRN*) genes appear to act in a regulatory loop in temperate grasses. The wheat *VRN1* gene is a MADS-box transcription factor, which is induced in the cold (Yan et al., 2003; Andrés and Coupland, 2012). This gene is related to the *Arabidopsis thaliana* genes *APETALA1* and *FRUITFUL* (Yan et al., 2003; Andrés and Coupland, 2012). *VRN2* encodes a small CCT-domain protein (Yan et al., 2004) that is repressed by *VRN1* and in turn represses *FLOWERING LOCUS T* (*FT*), a strong universal promoter of flowering (Kardailsky et al., 1999; Yan et al., 2006; Andrés and Coupland, 2012; Ream et al., 2012). In cereals, active *VRN2* alleles are necessary for a vernalization requirement. Spring barley and spring wheat varieties, which do not require vernalization to flower, either lack *VRN2* (Dubcovsky et al., 2005; Karsai et al., 2005; von Zitzewitz et al., 2005), have point mutations in the conserved CCT domain (Yan et al., 2004), or possess dominant constitutively active alleles of *VRN1* (repressor of *VRN2*) (Yan et al., 2003; Fu et al., 2005) or *FT* (repressed by *VRN2*) (Yan et al., 2006).

Investigations on the regulation of flowering in the Poaceae have focused on rice (*Oryza sativa*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*), all domesticated species that have been heavily subjected to human selection over the past 10,000 years. Little

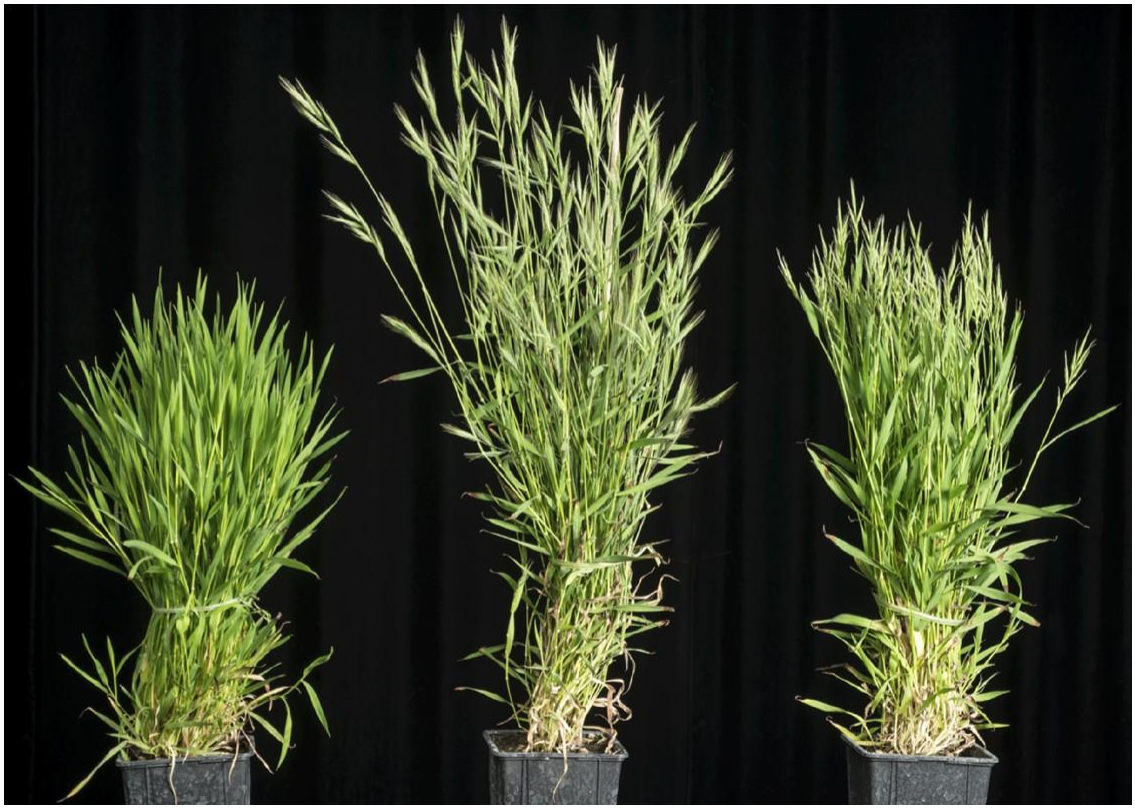
information is available on wild species within this family that have not been subjected to human selection. Such a study could provide additional insights into the standing variation present within wild systems and its likely pre-domestication adaptive significance in the Poaceae (Schwartz et al., 2010). A favorable species for such a study is *Brachypodium distachyon*, a small, wild grass, with a sequenced and annotated genome. *B. distachyon* was originally developed as a model system for the agronomically important temperate cereals (Draper et al., 2001; Opanowicz et al., 2008; The International Brachypodium Initiative, 2010; Catalán et al., 2014). With the recent availability of geographically dispersed diversity collections, we can ask how wild grasses have adapted to different climatic zones.

Previous studies have begun to explore the molecular basis of vernalization in this system. Higgins *et al*. (2010) identified homologs of the various flowering pathway genes in *B. distachyon*, and several mainly reverse genetic studies have focused on characterizing these genes further (Schwartz et al., 2010; Lv et al., 2014; Ream et al., 2014; Woods et al., 2014; Woods et al., 2016). Schwartz *et al*. (2010) did not find complete correlation between expression of *VRN1* and flowering and hypothesized that *VRN1* could therefore have different activity or roles that are dependent on the genetic background. Yet, Ream *et al.* (2014) found low *VRN1* and *FT* levels in *B. distachyon* accessions with delayed flowering, suggesting a conserved role of these homologs. Further support for a conserved role of *VRN1* and *FT* comes from the observation that overexpression of these genes leads to extremely early flowering (Lv et al., 2014; Ream et al., 2014) and RNAi-based silencing of *FT* and amiRNA-based silencing of *VRN1* prevent flowering (Lv et al., 2014; Woods et al., 2016). The role of *VRN2* in *B. distachyon* is less clear. Higgins *et al.* (2010) failed to identify a homolog of *VRN2* in *B. distachyon*; however, other studies identified Bradi3g10010 as the best candidate for the *B. distachyon VRN2* homolog (Schwartz et al., 2010; Ream et al., 2012). Recent research supports the functional conservation of *VRN2* in the role as a flowering repressor, but suggests that the regulatory interaction between *VRN1* and *VRN2* evolved after the diversification of the Brachypodieae and the core Pooideae (e.g. wheat and barley) (Woods et al., 2016).

To date most studies on the regulation of flowering time of *B. distachyon* have used reverse genetic approaches to implicate the role of previously characterized genes from other species (Higgins et al., 2010; Lv et al., 2014; Ream et al., 2014; Woods et al., 2016), while only few studies have used the natural variation present among *B. distachyon* accessions to identify

flowering loci (Tyler et al., 2016; Wilson et al., 2016). Currently lacking is the characterization of loci that control variation in flowering time in a biparental *B. distachyon* mapping population. The Iraqi reference accession Bd21 does not require vernalization (Vogel et al., 2006; Garvin et al., 2008) and in addition, vernalization does not greatly reduce time to flowering in a 16 h or 20 h photoperiod (Schwartz et al., 2010; Ream et al., 2014). In contrast, the Spanish accession ABR6 can be induced to flower following a six-week vernalization period (Draper et al., 2001; Routledge et al., 2004).

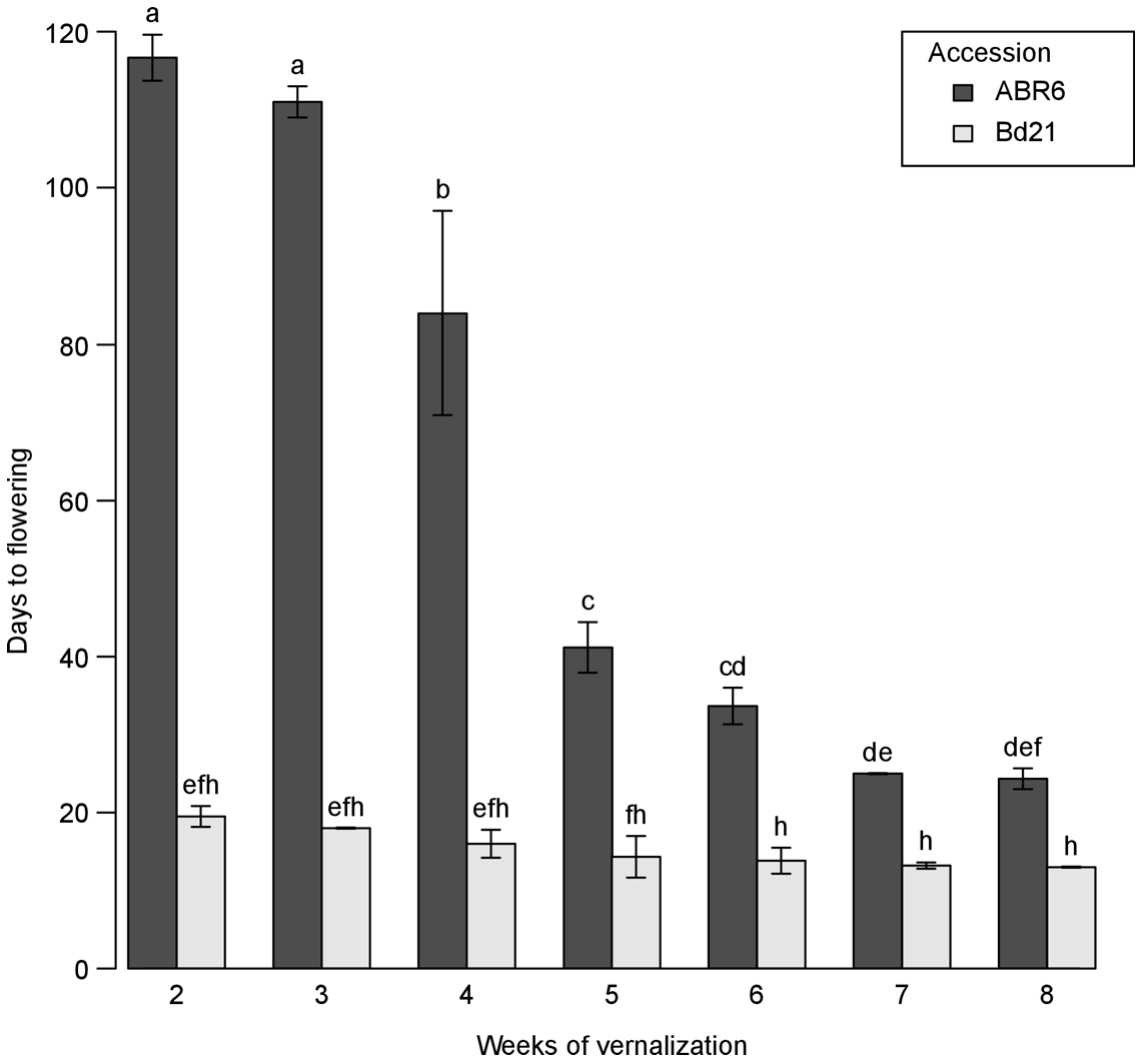
In this paper, we report on the genetic architecture underlying flowering time in a mapping population developed from ABR6 and Bd21. We observed the segregation of vernalization dependency during population advancement (Figure 1) and characterized the genetic basis of this dependency in detail at the $F_{4:5}$ stage in multiple environments. The ability to flower without vernalization was linked to three major loci, two of which colocalize with the *B. distachyon* homologs of *VRN2* and *FT*. Notably, our results further support the role of the *VRN2* locus as a conserved flowering time regulator in *B. distachyon*.



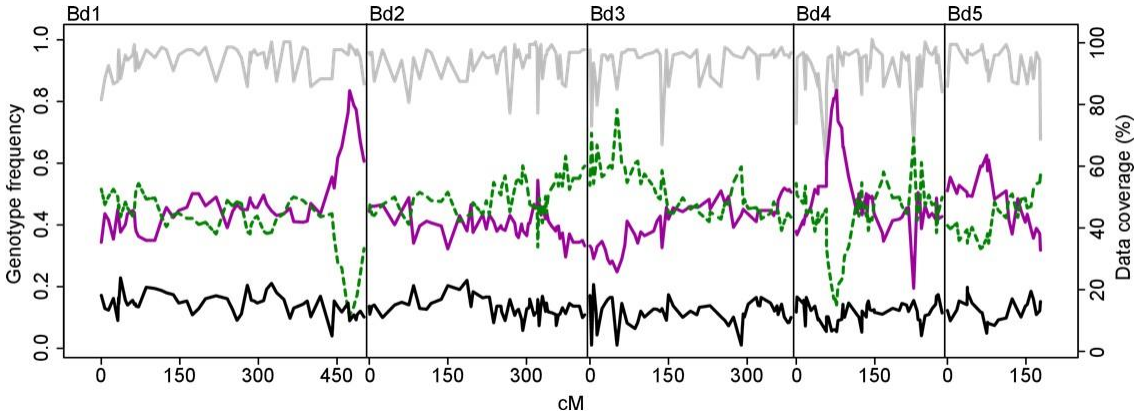
Results

Development of a B. distachyon *mapping population between geographically and phenotypically distinct accessions*

Initial investigations into the flowering time of ABR6 and Bd21 in response to different vernalization periods showed contrasting effects on the two accessions (Figure 1 and Figure 2). ABR6 responded strongly to increasing vernalization times with a reduction in flowering by 93 days, ranging from 117 days for a two-week vernalization period to 24 days for an eight-week vernalization period. This reduction in flowering time for ABR6 was not linear and the greatest drop of 43 days occurred between four and five weeks of vernalization (Figure 2). In contrast, no statistically significant difference was found with respect to the vernalization response of Bd21, although a consistent trend towards a reduced flowering time was observed. A cross was generated from these phenotypically diverse accessions for the creation of a recombinant inbred line population. To develop a SNP-based genetic map, ABR6 was resequenced and reads were aligned to the reference genome. A total of 1.36 million putative SNPs were identified between ABR6 and Bd21, of which 711,052 constituted non-ambiguous polymorphisms based on a minimum coverage of 15x and a strict threshold for SNP calling (i.e. 100% of reads with an ABR6 allele, 0% of reads with a Bd21

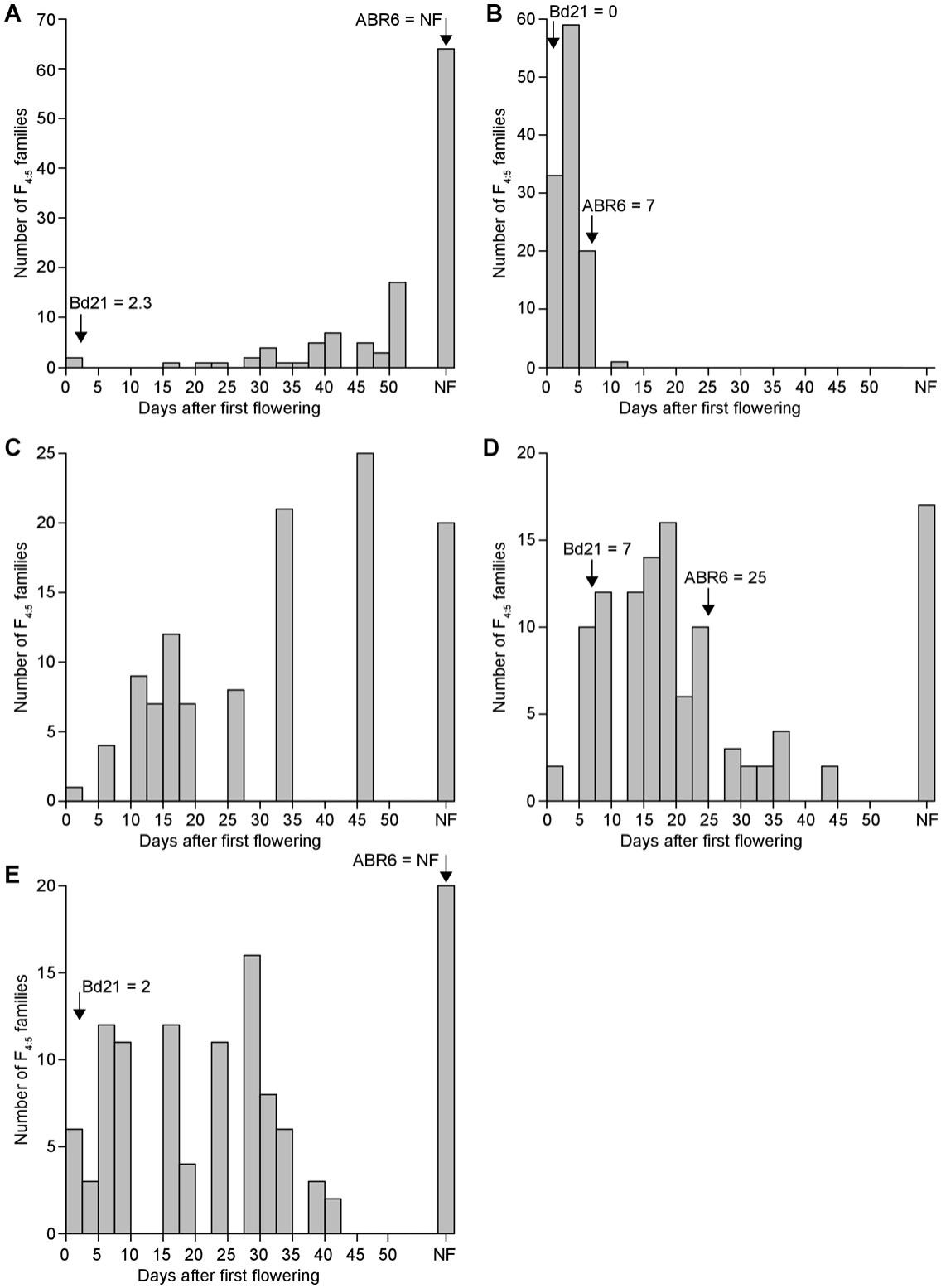


allele). Following iterative cycles of marker selection, the final genetic map consists of 252 non-redundant markers and has a cumulative size of 1,753 cM (Supplemental Figure S1). This size is comparable to the previously characterized Bd3-1 x Bd21 mapping population (Huo et al., 2011) and confirms that *B. distachyon* has a high rate of recombination compared to other grass species. The quality of the genetic map was verified by assessing the two-way recombination fractions for all 252 markers (Supplemental Figure S2). All five chromosomes were scanned for segregation distortion by comparing observed and expected genotype frequencies for each marker. The expected heterozygosity at the F_4 stage is 12.50% and the expected parental allele frequencies are 43.75% for ABR6 and Bd21 alleles, respectively. Although all five chromosomes contained regions of potential segregation distortion (Figure 3), only two loci on chromosomes Bd1 (peak at 474.1 cM) and Bd4 (peak at 77.0 cM) deviated significantly from these expected frequencies.

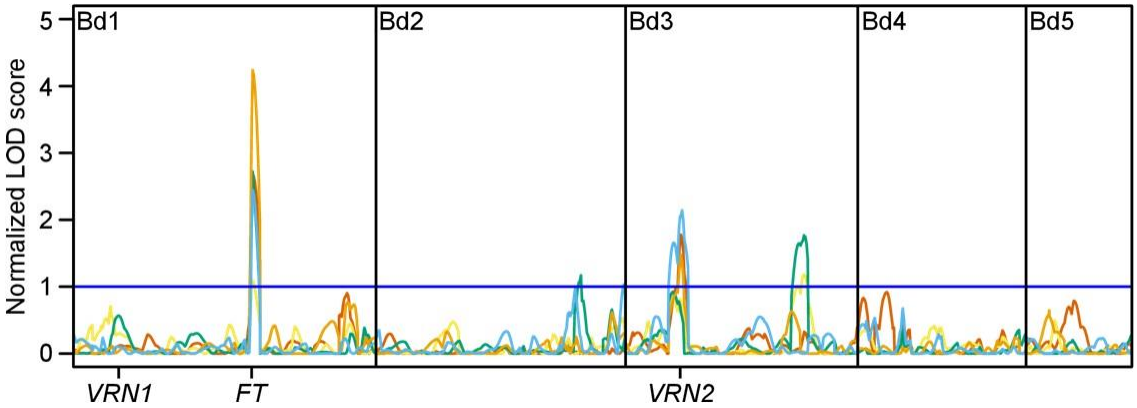


Multiple QTLs control flowering in the ABR6 x Bd21 mapping population

We evaluated the ABR6 x Bd21 $F_{4:5}$ population in a number of environments to identify the genetic architecture underlying flowering time (Supplemental Table S1 and Supplemental Data S1). Four sets of the population were grown without vernalization, whereas in one additional set flowering was scored in response to six weeks of vernalization. In all experiments, the population was exposed to natural light, although in three experiments supplemental light was used to ensure a minimum 16 h or 20 h growth period. In addition, two experiments did not have any temperature control (i.e. plants were exposed to the natural temperature in the greenhouse), three experiments had the temperature controlled at 22°C/20°C during light/dark cycles, and one experiment had the temperature maintained at a minimum of 18°C/11.5°C during light/dark cycles. Analysis of the non-vernalized environments revealed a bimodal distribution between families that flowered and families that did not flower (Figure 4). However, considerable residual variation in flowering time existed among the flowering families. For example, in Environment 5 flowering occurred over a 42-day period from 63 days to 105 days after germination (Figure 4E). Flowering in the other non-vernalized environments occurred over a similar time period (Figure 4). Interestingly, transgressive segregation for early and late flowering phenotypes was observed in Environment 4 (Figure 4D). Phenotypes in the vernalized environment were heavily skewed towards early flowering (Figure 4B). Only limited residual variation existed among the vernalized $F_{4:5}$ families and all plants flowered within 11 days from the first observation of flowering in the population. The variation in flowering time for all five environments was found to be not normally distributed. Among these diverse environments, QTL analyses using binary and non-parametric models were conservative in detecting QTLs controlling flowering time (*qFLT*) (Supplemental Table S2 and Supplemental Table S3), whereas transformation of flowering time consistently identified QTLs between environments (Supplemental Table S4 and Supplemental Table S5; Table 1 and Table 2). Three major

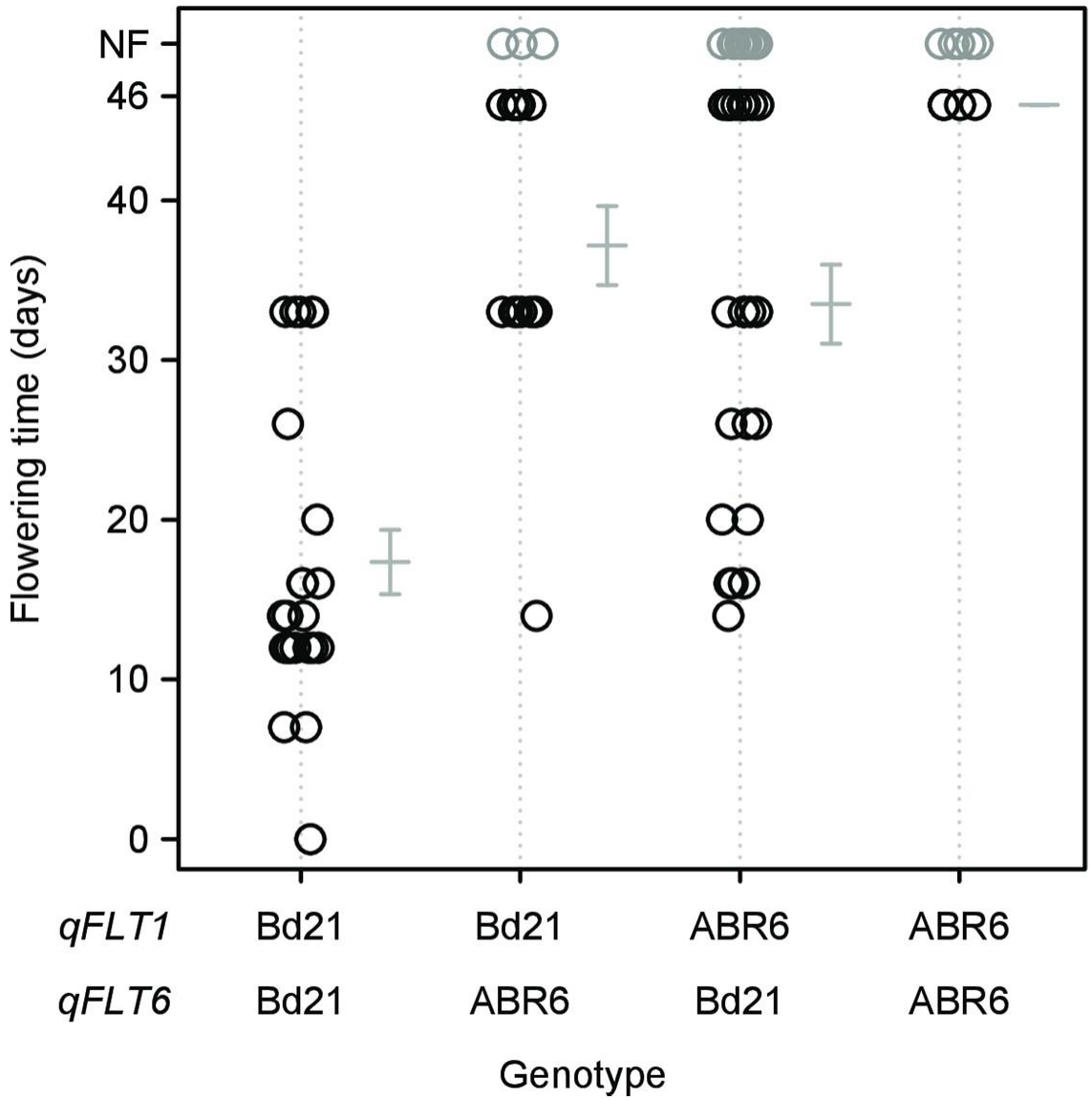


QTLs were identified on chromosomes Bd1 and Bd3 that were robustly observed using parametric and non-parametric mapping approaches (Table 1 and Table 2; Figure 5). The QTL on Bd1 (*qFLT1*, peak marker Bd1_47808182) appeared to be the major locus governing flowering time in this population, as it was the major QTL in all five environments,



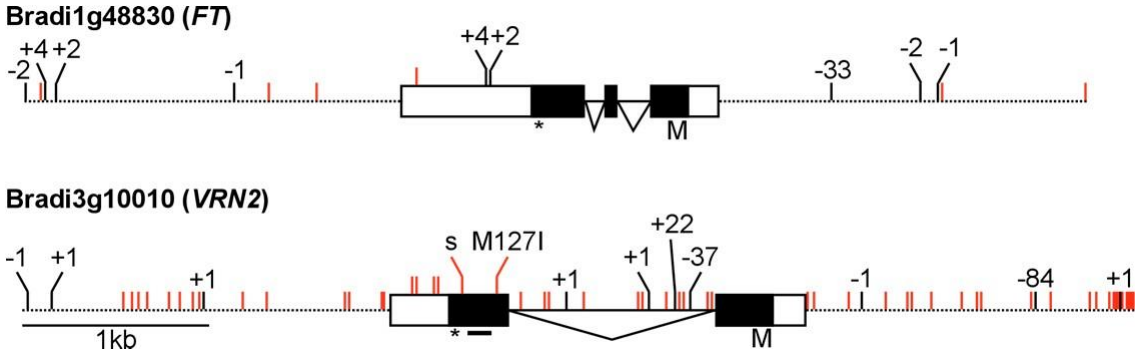
explaining the most phenotypic variation (phenotypic variance explained; PVE) compared to any other QTL (Table 2). PVE values for this locus ranged from 15.9% to 37.5%. Another QTL on Bd3 (*qFLT6*, peak marker Bd3_8029207) was also detected in all five studies, though its contribution was only significant in three environments. PVE values for the statistically significant QTLs ranged from 11.8% to 18.7%. Bd21 alleles at these two loci promoted early flowering, whereas individuals with ABR6 alleles at both loci had maximal flowering time or did not flower within the timescale of the experiment (Figure 6). Interestingly, in the two environments where this former locus did not have a significant contribution, two other QTLs were identified. A QTL on Bd3 (*qFLT7*, peak marker Bd3_44806296) explained 13.6% and 14.0% of the variation observed in these studies and a QTL on Bd2 (*qFLT3*, peak marker Bd2_53097824) was identified through a combination of non-parametric and parametric analyses of Environments 4 and 5. Additional QTLs on Bd1 (*qFLT2*), Bd2 (*qFLT4*), Bd3 (*qFLT5*), and Bd4 (*qFLT8*) were not significant in more than one of the environments tested (Table 1).

Previous studies identified the *B. distachyon* homologs of flowering regulators from *Arabidopsis*, wheat, barley, and rice (Higgins et al., 2010; Ream et al., 2014). The one-LOD support intervals of all statistically significant QTLs were combined to identify the maximal one-LOD support interval for each QTL. Several of the previously identified *B. distachyon* homologs of flowering regulators are candidate genes underlying these QTLs (Table 3). Although several homologs fall within the one-LOD support intervals of *qFLT1* on Bd1 (292.1 - 305.6 cM) and *qFLT6* on the short arm of Bd3 (72.9 - 97.0 cM), these loci also harbor the *B. distachyon* homologs of *FT* (Bradi1g48830) and *VRN2* (Bradi3g10010), which have been previously implicated in flowering time regulation in *B. distachyon* through a series of mainly reverse genetic studies (Lv et al., 2014; Ream et al., 2014; Woods et al., 2014; Woods et al., 2016).



Natural variation in FT *and* VRN2

Analysis of the resequencing and RNAseq data allowed an initial evaluation of candidate genes underlying these QTLs. A *de novo* assembly was created from the ABR6 resequencing reads and the resulting contigs were probed with the Bd21 sequences of *FT* (Bradi1g48830) and *VRN2* (Bradi3g10010), enabling the identification of structural variation between ABR6 and Bd21 (Figure 7; Supplemental Table S6). Spliced alignment of RNAseq reads permits further characterization of candidate genes underlying an identified QTL through the confirmation of polymorphisms between two parental genotypes, verification of annotated candidate gene models, qualitative assessment of expression of candidate genes in the sampled tissue, and discovery of potential splice variants.

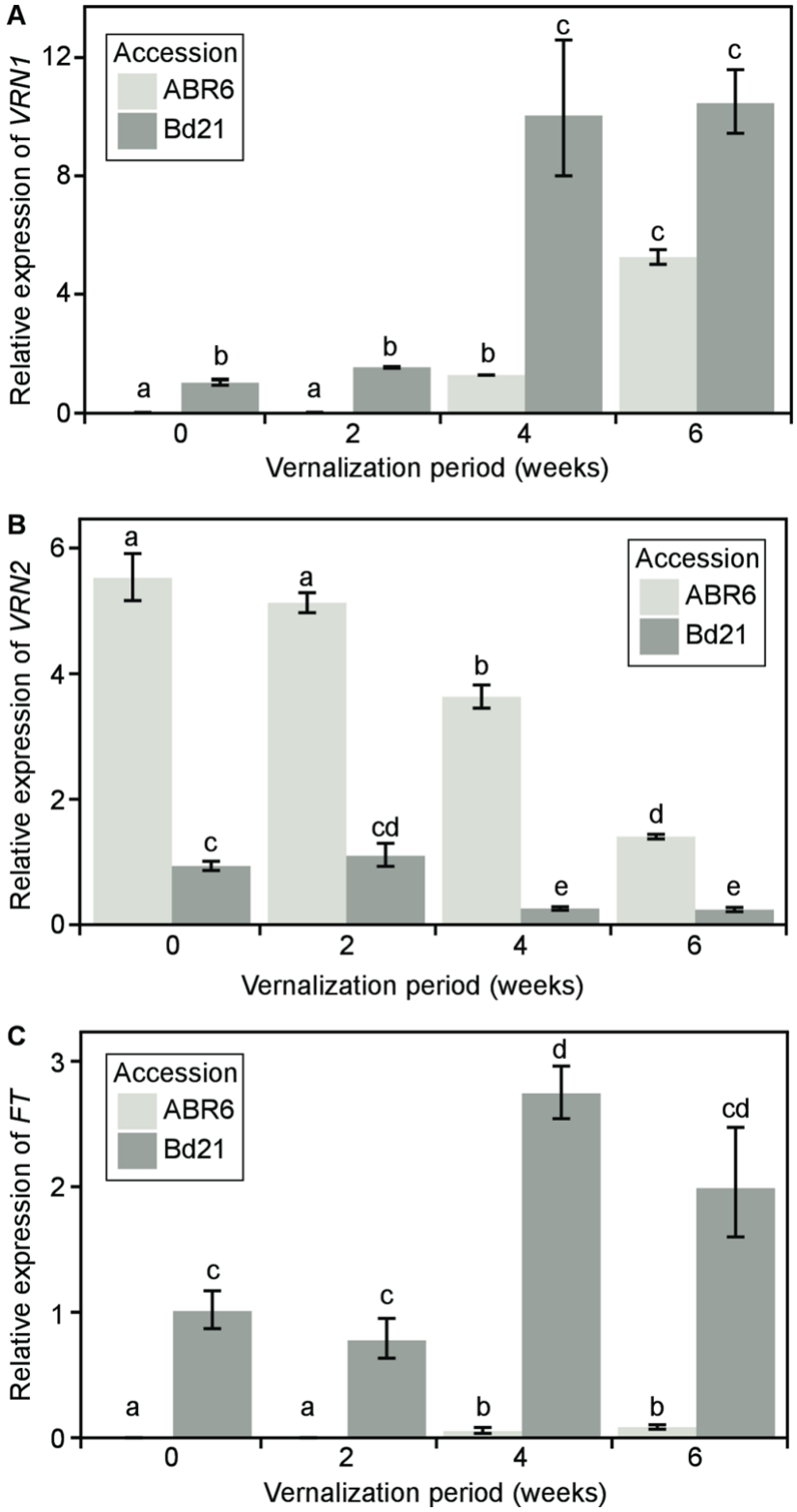


No polymorphisms were found in the coding sequence of Bradi1g48830, the *B. distachyon* homolog of *FT*. However, two indels (two and four bp, respectively) and a SNP mapped to the 3'-UTR. Additionally, two SNPs and three indels (including a 33 bp indel 590 bps upstream of Bradi1g48830) were found in the promoter region (2 kb upstream). The terminator region (2 kb downstream) contained three SNPs and four indels. Bradi1g48830 was not expressed in ABR6 and barely detectable in Bd21 (only two reads mapped to the gene). Owing to the low expression, it was not possible to confirm the published gene model with our RNAseq data.

Greater sequence variation was observed at Bradi3g10010, the *B. distachyon* homolog of *VRN2*, and its flanking regions. Only 1.9 kb of the promoter region is present on the Bradi3g10010 contig, but this region contains 29 SNPs and three indels (including an 84 bp indel 1.4 kb upstream of Bradi3g10010). The 2 kb terminator region contains 14 SNPs and three 1 bp indels. Additionally, 11 SNPs and four indels (including a 37 bp and a 22 bp indel) were localized in the intron, two SNPs in the coding sequence, and four SNPs in the 3'-UTR. Bradi3g10010 was expressed in leaves from both Bd21 and ABR6 and spliced alignment of RNAseq reads confirmed the published annotation of Bradi3g10010 for both ABR6 and Bd21. Moreover, the six SNPs predicted in the exons were supported by the RNAseq data and these may contribute to the observed effect on flowering time in this mapping population. Two SNPs map to the annotated coding sequence and four SNPs map to the 3'-UTR. One of the two SNPs in the annotated coding sequence is predicted to cause a non-synonymous mutation (Figure 7).

Expression of VRN1, VRN2, and FT in response to vernalization

To understand the transcriptional dynamics of *VRN1*, *VRN2*, and *FT* in response to vernalization, we assessed steady state levels of mRNA expression in plants at the fourth leaf stage after exposure to two, four, and six weeks of vernalization at 5°C or to no vernalization



(Figure 8). *VRN1* and *FT* had a similar pattern in steady state levels of gene expression in response to vernalization (Figure 8A and 8C). For both genes, very low levels of expression were observed in ABR6, whereas Bd21 had fairly high levels of transcript abundance. After experiencing four weeks of vernalization, ABR6 had similar levels of *VRN1* transcript as

Bd21 without vernalization treatment. In contrast, *FT* expression had a marginal increase after four and six weeks of vernalization in ABR6 relative to no vernalization or two weeks of vernalization. *FT* expression levels were significantly lower than Bd21 across all periods of vernalization. Both *VRN1* and *FT* expression increased significantly between Bd21 examples vernalized for two or four weeks. *VRN2* expression in ABR6 was inversely correlated with the length of vernalization, with similar levels of expression after no vernalization and two weeks vernalization and increasingly lower levels of expression after four and six weeks of vernalization (Figure 8B). Bd21 exhibited a similar reduction in *VRN2* expression, although lower levels of expression were observed without vernalization compared to ABR6 with six weeks vernalization. The trends of all three genes highlighted the importance of four weeks of vernalization as the inflection point in transcriptional abundance, which coincides with a significant reduction in days to flowering in ABR6 (Figure 2).

Discussion

In our advancement of the ABR6 x Bd21 RIL population, we observed substantial variation in flowering time. To define the genetic architecture of flowering time, we developed a comprehensive genetic map and assessed $F_{4:5}$ families in multiple environments. We uncovered three major QTLs, with two QTLs coincident with the *B. distachyon* homologs of *VRN2* and *FT*. Interestingly, *VRN1* was not associated with flowering time and was found to have no mutations within the transcribed sequence (Supplemental Table S6). Further minor effect QTLs were identified, suggesting that additional regulators play a role in controlling flowering time in *B. distachyon*.

Segregation distortion in the ABR6 x Bd21 population

Segregation distortion is a common observation in the development of mapping populations in plants, including grasses such as rice, *Aegilops*, maize, or barley (Xu et al., 1997; Faris et al., 1998; Lu et al., 2002; Muñoz-Amatriaín et al., 2011). In the ABR6 x Bd21 population, significant deviation from expected genotype frequencies was observed at two loci on chromosomes Bd1 and Bd4 (Figure 3). Interestingly, heterozygosity was not affected at these loci, but the ABR6 allele was overrepresented. It is likely that these loci are linked to traits that were inadvertently selected during population advancement based on genetic and/or environmental factors. Several genetic mechanisms can contribute to segregation distortion in intraspecific crosses, including hybrid necrosis (Bomblies and Weigel, 2007), genes involved in vernalization requirement and flowering time (such as the *vrn2* locus in the Haruna Nijo x OHU602 doubled-haploid barley population (Muñoz-Amatriaín et al., 2011)), or preferential transmission of a specific parental genotype. While segregation distortion at these loci was not associated with the identified flowering time QTLs, canonical resistance genes encoding nucleotide-binding, leucine-rich repeat proteins are present at the Bd4 locus (Bomblies et al., 2007; Tan and Wu, 2012).

The genetic architecture of flowering time in B. distachyon

In *Arabidopsis*, natural variation has been used as a complementary forward genetics-based approach for investigating flowering time (Koornneef et al., 2004). In our work, we identified two major QTLs controlling flowering time (*qFLT1* and *qFLT6*; Figure 6) in both vernalized and non-vernalized environments that colocalized with the *B. distachyon* homologs of *FT* (Bradi1g48830) and *VRN2* (Bradi3g10010). These observations are consistent with previous

reverse genetic studies on the role of *FT* and *VRN2* in controlling flowering time (Lv et al., 2014; Ream et al., 2014; Woods et al., 2014; Woods et al., 2016). Two additional QTLs on chromosomes Bd2 (*qFLT3*) and Bd3 (*qFLT7*) were detected in two environments, whereas three minor effect QTLs (*qFLT2*, *qFLT4*, *qFLT5*, and *qFLT8*) were found in individual environments only. Two recent genome-wide association studies (GWAS) used the natural variation found within *B. distachyon* germplasm to identify SNPs associated with flowering time (Tyler et al., 2016; Wilson et al., 2016). Tyler *et al.* (2016) identified nine significant marker-trait associations, none of which overlap with the QTLs identified in our study. In contrast, Wilson *et al.* (2016) identified a much simpler genetic architecture consisting of three significant marker-trait associations, one of which could be linked to *FT*. These additional QTLs and marker-trait associations identified in our study and the GWAS studies could either correspond to one of the identified homologs of flowering genes in *B. distachyon* (Table 3; compare Higgins *et al.* 2010) or constitute novel loci as hypothesized by Schwartz *et al.* (2010). With the exception of the proximal QTL on Bd2 (*qFLT3*), all QTLs in our study were contributed by ABR6 (Table 1). Bd21 has previously been classified as a "spring annual" (Schwartz et al., 2010) or "extremely rapid flowering" (Ream et al., 2014). However, increased vernalization times still led to a modest reduction in flowering time (Figure 2), which is explained by the detection of a QTL contributed by Bd21.

We hypothesized that structural variation between ABR6 and Bd21 would underlie the observed variation in flowering time. No structural variation in *FT* was observed between ABR6 and Bd21 in the coding sequence, however, several indels map to the promoter region (Figure 7). These polymorphisms may explain expression differences between these two accessions. As expected, no *FT* expression was found in ABR6 seedlings, and only two Bd21 RNAseq reads mapped to this gene. Steady-state expression levels of *FT* in the fourth leaf were significantly lower in ABR6 relative to Bd21 without vernalization (Figure 8C). After four weeks vernalization, *FT* expression levels increased in ABR6, although they were significantly lower than Bd21 steady-state levels after any level of vernalization. It was previously shown that in barley, wheat, and *B. distachyon*, *FT* expression is upregulated after vernalization (Sasani et al., 2009; Chen and Dubcovsky, 2012; Ream et al., 2014). Our observations indicate that *FT* is expressed in Bd21 and increases less than *VRN1* in response to vernalization. In contrast, *FT* in ABR6 only increases marginally after four weeks of vernalization and remains significantly below the levels observed in Bd21 after no vernalization.

Interestingly, an intact copy of the flowering repressor *VRN2* is also present in Bd21 (Ream et al., 2012), which does not have a strong vernalization response (Vogel et al., 2006; Garvin et al., 2008). The lack of vernalization requirement in some *B. distachyon* accessions cannot, therefore, be explained by an absence of *VRN2* (Ream et al., 2014). Intriguingly, early-flowering mutants identified in genetic screens have thus far not mapped in the *VRN2* region (Ream et al., 2014). Moreover, expression levels for *VRN2* also did not vary among early and late flowering accessions and *VRN2* mRNA levels are likely not rate limiting (Ream et al., 2014). An earlier study by Schwartz *et al.* (2010) described potential correlation between different *VRN2* alleles and flowering time. The authors did not rule out the effects of population structure and proposed that elucidating the role of *VRN2* in *B. distachyon* will require more in-depth genetic studies. A recent comprehensive analysis of population structure in *B. distachyon* collections revealed that flowering time, and not geographic origin, is indeed the major distinguishing factor between genotypically distinct clusters (Tyler et al., 2016). Our results confirm *VRN2* as an important flowering regulator in the ABR6 x Bd21 mapping population and highlight structural and expression variation between parental accessions. However, none of the SNPs identified in the coding sequence map to the CCT domain. A point mutation in this domain results in a spring growth habit in cultivated *Triticum monococcum* accessions (Yan et al., 2004). It is unclear whether the structural variation surrounding *VRN2* corresponds to the allelic variation observed by Schwartz *et al.* (2010). Woods and Amasino (2016) hypothesize that even though *VRN2* may not be involved in vernalization control in *B. distachyon*, it may still possess an ancestral role in flowering regulation. This is further supported by the observation that *VRN2* expression is not controlled by *VRN1* in *B. distachyon*, yet *VRN2* was found to be a functional repressor of flowering in this species (Woods et al., 2016). We observed a negative correlation between *VRN2* transcript accumulation and vernalization period in ABR6 and Bd21 (Figure 8B). Similar decreases were observed for ABR6 and Bd21, although transcript abundance in Bd21 were significantly lower than ABR6 under any vernalization period. Therefore, our identification of natural variation in *VRN2* among geographically diverse *B. distachyon* accessions further supports *VRN2* as a core flowering regulator in this non-domesticated grass.

In our study of the natural variation between two morphologically and geographically diverse *B. distachyon* accessions we failed to implicate *VRN1* as a flowering regulator. However,

VRN1 expression during and after cold treatment and the failure of *VRN1* silenced lines to flower suggests a conserved role of *VRN1* as a promoter of flowering (Woods and Amasino, 2016; Woods et al., 2016). Interestingly, a QTL in the Bd21 x Bd1-1 *B. distachyon* mapping population colocalized with *VRN1* and the light receptor *PHYTOCHROME C* (*PHYC*) (Woods et al., 2016). Between ABR6 and Bd21, sequence variation was found in the promoter and terminator regions of *VRN1* and a strong positive correlation was observed with extended periods of vernalization (Figure 8A), particularly at four weeks vernalization, which was a critical inflection point for flowering time in ABR6. Despite this sequence and expression variation, *VRN1* was not found to contribute to flowering time in the ABR6 x Bd21 mapping population. Interestingly, an assessment of allelic variation in 53 *B. distachyon* accessions currently available in Phytozome (Version 11.0.2, https://phytozome.jgi.doe.gov) found that none of these accessions possess structural variation in the *VRN1* annotated coding sequence. These findings suggest that *VRN1* is a crucial regulator of flowering in *B. distachyon* and under strong selection pressure.

Conclusions

Thanks to their economic and evolutionary importance, flowering time pathways are of particular interest in the cereals and related grasses. Our report adds to this body of research by using natural variation to map vernalization dependency in a *B. distachyon* mapping population. Since *B. distachyon* is partly sympatric with the wild relatives of wheat and barley, it seems likely that the species would have been subject to similar selective pressure and therefore is a useful model for understanding pre-domestication or standing variation. We investigated this standing variation by assessing segregation of flowering regulators in a mapping population derived from two geographically diverse accessions of *B. distachyon*. Notably, we found additional support for the roles of *FT* and *VRN2* in controlling flowering in wild temperate grasses. Additionally, allelic variation may explain the ambiguity around the role of the *VRN2* homolog observed in *B. distachyon*. Further fine-mapping will be required to confirm the roles of these genes in *B. distachyon* flowering time. However, we also detected novel components in the form of additional QTLs, which reflects the power of studying natural variation in mapping populations derived from phenotypically diverse parents. During population advancement, we have observed a variety of additional morphological and pathological characteristics segregating in this population and it will serve

as a useful resource for other researchers investigating standing variation in non-domesticated grasses.

Materials and Methods

Plant growth for assessing ABR6 and Bd21 vernalization response

Six seeds for ABR6 and Bd21 were germinated on paper (in darkness at room temperature) and transferred to an equal mixture of the John Innes Cereal Mix and a peat and sand mix (Vain et al., 2008) four days after germination. Vernalization was initiated 14 days after germination for either two, three, four, five, six, seven, or eight weeks (8 h day length; 1.2 klux light intensity; 5°C). The different sets were staggered to ensure that all sets left vernalization on the same date. After vernalization plants were grown in a Sanyo Versatile Environmental Test Chamber (Model MLR-351; 16 h photoperiod; 8.0 klux light intensity; 22°C/20°C day/night temperatures) for 35 days and then transferred to a greenhouse without light and temperature control (late April to mid July 2013; Norwich, UK). Days to flowering was measured from the end of vernalization until the emergence of the first spike and was averaged across all six biological replicates (only five replicates were available for Bd21 after 7 weeks of vernalization). Statistical significances were assessed by pairwise comparisons using *t*-tests with pooled standard deviations and Bonferroni correction for multiple comparisons.

Resequencing of ABR6

Seedlings were grown in a Sanyo Versatile Environmental Test Chamber (16h photoperiod; 8.0 klux light intensity; 22°C) in an equal mixture of the John Innes Cereal Mix and a peat and sand mix. Seven-week-old plants were placed in darkness for three days prior to collecting tissue. Genomic DNA was extracted using a standard CTAB protocol and a library of 800 bp inserts was constructed and sequenced with 100 bp paired-end reads and an estimated coverage of 25.8x on an Illumina HiSeq 2500. Library preparation and sequencing was performed at The Genome Analysis Centre (Norwich, UK). The resulting reads were mapped to the Bd21 reference sequence (Version 1) (The International Brachypodium Initiative, 2010) with the Galaxy wrapper, which used the BWA (Version 0.5.9) *aln* and *sampe* options (Li and Durbin, 2009). Polymorphisms between ABR6 and Bd21 were identified with the *mpileup2snp* and *mpileup2indel* tools of *VarScan*

(Version 2.3.6) using default settings (Koboldt et al., 2009). A *de novo* assembly was created from the raw ABR6 reads using default settings of the CLC Assembly Cell (Version 4.2.0) and default parameters. Potential structural variation between ABR6 and Bd21 was investigated by performing a BLAST search with the Bd21 regions of interest against the ABR6 *de novo* assembly and mapping contigs for hits with at least 95% identity and an E-value under $1e^{-20}$ to the Bd21 reference sequence (Version 3).

Development of the ABR6 x Bd21 F_4 population and genetic map

The *B. distachyon* accessions ABR6 and Bd21 were crossed and three ABR6 x Bd21 F_1 individuals, confirmed as hybrid by SSR marker analysis (data not shown), were allowed to self-pollinate to generate a founder F_2 population comprised of 155 individuals. After single seed descent, DNA was extracted from leaf tissue of 114 independent F_4 lines using a CTAB gDNA extraction protocol modified for plate-based extraction (Dawson et al., 2016). SNPs for genetic map construction were selected based on a previously characterized Bd21 x Bd3-1 F_2 genetic map to ensure an even distribution of markers relative to physical and genetic distances (Huo et al., 2011). SNPs without additional sequence variation in a 120 bp window were selected every 10 cM. The Agena Bioscience MassARRAY design suite was used to develop 17 assays that genotyped 449 putative SNPs using the iPLEX Gold assay at the Iowa State University Genomic Technologies Facility (Supplemental Data S2). Markers were excluded for being monomorphic (106), dominant (34), or for missing data for the parental controls (33). Heterozygous genotype calls for some markers were difficult to distinguish and classified as missing data. Additional SNPs between ABR6 and Bd21 in six markers developed for the Bd21 x Bd3-1 F_2 genetic map (Barbieri et al., 2012) were converted into CAPS markers (Konieczny and Ausubel, 1993) (Supplemental Table S7). The integrity of these 282 markers was evaluated using R/qtl (Version 1.33-7) recombination fraction plots (Broman et al., 2003). Two markers were removed for not showing linkage and one marker was moved to its correct position based on linkage. Genetic distances were calculated using the Kosambi function in MapManager QTX (Version b20) (Manly et al., 2014). Removal of unlinked and redundant markers produced a final ABR6 x Bd21 F_4 genetic map consisting of 252 SNP-based markers (Supplemental Data S3). Segregation distortion was assessed using a chi-square test with Bonferroni correction for multiple comparisons.

Plant growth and phenotyping of flowering time in the ABR6 x Bd21 $F_{4:5}$ families

Three to five plants for each of the 114 ABR6 x Bd21 $F_{4:5}$ families were grown under five different environmental conditions as detailed in Supplemental Table S1. For the phenotyping performed in Aberystwyth, individual seeds were sown in 6 cm pots with a mixture of 20% grit sand and 80% Levington F2 peat-based compost. Seeds were grown for 2 weeks in greenhouse conditions (22°C/20°C, natural light supplemented with 20 h lighting) and then either maintained in the greenhouse or transferred to a vernalization room for six weeks (16 h day length, 5°C). Plants were returned to the greenhouse following vernalization and grown to maturity. Flowering time was defined as the emergence of the first inflorescence and was measured from the first day that flowering was observed in the entire mapping population. Flowering time was averaged across the individuals of an $F_{4:5}$ family. For the phenotyping performed in Norwich, plants were first subjected to growth conditions and pathogen assays as described in Dawson *et al.* 2015. Plants were germinated in a peat-based compost in 1 L pots and grown for six weeks in a controlled environment room (18°C/11°C, 16 h light period). Six weeks post germination, the fourth or fifth leaf of each plant was cut off for pathological assays. The plants were transplanted into 9 cm pots with an equal mixture of the John Innes Cereal Mix and a peat and sand mix (Vain et al., 2008) and transferred to the respective growth environments for flowering assessment (Supplemental Table S1). Flowering time was defined as the emergence of the first inflorescence within an $F_{4:5}$ family and was measured from the first day that flowering was observed in the entire mapping population. Families that did not flower 60 days after emergence of the first inflorescence in the mapping population were scored as not flowering.

Quantitative trait locus analysis for flowering time

Flowering phenotypes were assessed for normality using the Shapiro-Wilk test (Royston, 1982). In an initial analysis, phenotypic values were converted into a binary classification based on whether families flowered (F) or did not flower (NF). Interval mapping was performed with the *scanone* function in R/qtl under a *binary* model with conditional genotype probabilities computed with default parameters and the Kosambi map function (Xu and Atchley, 1996; Broman et al., 2006). Simulation of genotypes was performed with a fixed step distance of 2 cM, 128 simulation replicates, and a genotyping error rate of 0.001. Statistical significance for QTLs was determined by performing 1,000 permutations and controlled at $\alpha = 0.05$ (Doerge and Churchill, 1996). Non-parametric interval mapping was performed with similar parameters in R/qtl under an *np* model (Kruglyak and Lander, 1995).

For parametric mapping, flowering time data was transformed (T) using the following approaches: (T1) the removal of all $F_{4:5}$ families that did not flower within the timescale of the experiment, (T2) transforming all non-flowering phenotypic scores to one day above the maximum observed, and (T3) transforming by ranking families according to their flowering time. For the third transformation approach (T3), the earliest flowering family was given a rank score of 1 and subsequent ordered families given incremental scores based on rank (2, 3, 4, etc.). When two or more families had shared flowering time, they were given the same rank and the next ranked family was given an incremental rank score based on the number of preceding shared rank families. Non-flowering families were given the next incremental rank after the last flowering rank. For all three transformations, composite interval mapping was performed under an additive model (H_0:H_1) using QTL Cartographer (Version 1.17j) with the selection of five background markers, a walking speed of 2 cM, and a window size of 10 cM (Zeng, 1993, 1994; Basten et al., 2004). Statistical significance for QTLs was determined by performing 1,000 permutations with reselection of background markers and controlled at α = 0.05 (Doerge and Churchill, 1996; Lauter et al., 2008). One-LOD support intervals were estimated based on interval mapping (Lander and Botstein, 1989).

RNAseq of ABR6 and Bd21

Plants were grown in a controlled environment room with 16 h light at 22°C and fourth and fifth leaves were harvested as soon as the fifth leaf was fully expanded (roughly 28 days after germination). RNA was extracted using the TRI Reagent (Sigma-Aldrich®) according to the manufacturer's specifications. TruSeq libraries were generated from total RNA and mean insert sizes were 251 bp and 254 bp for ABR6 and Bd21, respectively. Library preparation and sequencing was performed at The Genome Analysis Centre (Norwich, UK). Sequencing was carried out using 150 bp paired-end reads on an Illumina HiSeq 2500 and ABR6 and Bd21 yielded 38,867,987 and 37,566,711 raw reads, respectively. RNAseq data quality was assessed with FastQC and reads were removed using Trimmomatic (Version 0.32) (Bolger et al., 2014) with parameters set at ILLUMINACLIP:TruSeq 3-PE.fa:2:30:10, LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, and MINLEN:100. These parameters will remove all reads with adapter sequence, ambiguous bases, or a substantial reduction in read quality. The sequenced reads were mapped to the Bd21 reference genome using the TopHat (Version 2.0.9) spliced alignment pipeline (Trapnell et al., 2009).

RT-qPCR analyses

ABR6 and Bd21 seeds were surface sterilized (70% ethanol for 30 seconds, washed in autoclaved dH_2O, 1.3% sodium hypochlorite for 4 minutes, washed in autoclaved H_2O three times), transferred to moistened Whatman filter paper, left at room temperature in darkness overnight, and vernalized for either two, four, or six weeks (in darkness at 5°C). A control set was surface sterilized and transferred to filter paper overnight, but not vernalized. Following vernalization, plants were transferred to soil and grown in a Sanyo Versatile Environmental Test Chamber in conditions similar to Environment 2 (20h photoperiod; 4.0 klux light intensity; 22°C/20°C). Once fully expanded, fourth leaves were collected in the middle of the photoperiod and flash frozen in liquid nitrogen.

Total RNA was extracted using TRI reagent according to manufacturer's instructions (Sigma-Aldrich®). RNA samples were treated with DNase I (Roche) prior to cDNA synthesis. Quality and quantity of RNA samples were assessed using a NanoDrop spectrophotometer followed by agarose electrophoresis. First-strand cDNA was synthesized according to manufacturer's instructions (Invitrogen). Briefly, 1 μg of total RNA, 1 μL of 0.5 μM poly-T primers, and 1 μL of 10 mM dNTP were incubated at 65°C for 5 min and 4°C for 2 min, with subsequent reverse transcription reactions performed using 2 μL of 10x reverse transcription buffer, 4 μL of 25 mM $MgCl_2$, 2 μL of 0.1 M DTT, 1 μL of RNaseOUT (40 U/μL), and 1 μL of SuperScript III reverse transcriptase (200 U/μL) at 50°C for 50 min. Reverse transcription was inactivated by incubating at 85°C for 5 min and residual RNA was removed with the addition of 1 μL Rnase H (2 U/μL) and incubation at 37°C for 20 min.

Quantitative real time PCR was performed in 20 μL reaction volumes using 10 μL of SYBR-Green mix (Sigma-Aldrich), 1 μL of 10 μM forward and reverse primers, 4 μL water, and 4 μL of cDNA diluted 10-fold. The program for PCR amplification involved an initial denaturation at 95°C for 3 min and then 40 cycles of 94°C for 10 sec, 60°C for 15 sec, and 72°C for 15 sec. Fluorescence data was collected at 72°C at the extension step and during the melting curve program on a CFX96 Real-Time system (Bio-Rad).

Relative gene expression was determined using the $2^{-\otimes\otimes CT}$ method described by Livak and Schmittgen (2001) using *UBIQUITIN-CONJUGATING ENZYME18* (*Brachypodium distachyon*; (Hong et al., 2008); Schwartz *et al.* 2010) for normalization. All primers were

previously used by Ream *et al.* (2014) and had PCR efficiency ranging from 95 to 110%. Statistical analysis of gene expression was performed using R (Version 3.2.3). Comparisons between all genotype by treatment combinations were made with pairwise *t*-tests using log transformed relative expression levels, with *p*-values corrected for multiple hypothesis testing based on the Benjamini-Hochberg approach.

Accession numbers for data in public repositories

Raw resequencing reads of ABR6 have been submitted to the NCBI Short Read Archive under the BioProject ID PRJNA319372 and SRA accession SRX1720894. The ABR6 *de novo* assembly has been deposited at DDBJ/ENA/GenBank under the accession LXJM00000000. The version described in this paper is version LXJM01000000. Raw RNAseq reads have been submitted to the NCBI Short Read Archive under the BioProject ID PRJNA319373 and SRA accessions SRX1721358 (ABR6) and SRX1721359 (Bd21).

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Tables

Table 1. Significant flowering time QTLs (*qFLT*) in the different environments identified using several binary, non-parametric, and parametric approaches.

Locus	Chr[a]	cM	Allele[b]	E1[c]	E2	E3	E4	E5
qFLT1	Bd1	297.6	Bd21	B, T2, T3, NP[d]	T1, T3, NP	T2, T3, NP	T2, T3	T1, T2, T3, NP
qFLT2	Bd1	465.2	Bd21	T2	-	-	-	-
qFLT3	Bd2	338.3	ABR6	-	-	-	NP	T2, T3
qFLT4	Bd2	409.0	Bd21	-	T1, T3	-	-	-
qFLT5	Bd3	60.8	Bd21	-	-	-	T1	-
qFLT6	Bd3	91.2	Bd21	T2, T3	T1, T3	T2, T3	-	-
qFLT7	Bd3	294.6	Bd21	-	-	-	T2, T3, NP	B, T2, T3, NP
qFLT8	Bd4	90.1	Bd21	-	-	-	NP	-

[a]Chromosome

[b]Allele that reduces flowering time

[c]Environment (see Supplemental Table S1)

[d]QTL analyses were performed with interval mapping using binary classification (B) and non-parametric analysis (NP), and composite interval mapping using transformed data (T1, T2, and T3).

Table 2. Significant QTLs from composite interval mapping of transformed flowering time phenotypes (T3) in the ABR6 x Bd21 $F_{4:5}$ families.

ENV[a]	Locus	Chr[b]	cM	EWT[c]	LOD	AEE[d]	PVE[e]	1-LOD SI[f]
1	*qFLT1*	Bd1	297.6	3.06	12.96	2.87	36.3%	296.1 - 305.6
1	*qFLT6*	Bd3	91.2	3.06	4.51	1.64	11.8%	ND
2	*qFLT1*	Bd1	297.6	3.09	7.59	0.82	20.0%	296.1 - 305.6
2	*qFLT4*	Bd2	409.0	3.09	3.20	0.47	6.7%	403.2 - 411.0
2	*qFLT6*	Bd3	93.2	3.09	6.64	0.79	18.2%	72.9 - 97.0
3	*qFLT1*	Bd1	297.6	3.20	8.61	1.50	31.1%	292.1 - 303.6
3	*qFLT6*	Bd3	91.2	3.20	5.69	1.20	18.7%	74.9 - 97.0
4	*qFLT1*	Bd1	297.6	3.19	3.49	1.77	15.9%	292.1 - 305.6
4	*qFLT7*	Bd3	294.6	3.19	3.79	1.59	14.0%	273.9 - 300.7
5	*qFLT1*	Bd1	297.6	3.17	8.62	3.43	37.5%	294.1 - 301.6
5	*qFLT3*	Bd2	338.3	3.17	3.70	-1.75	9.9%	323.7 - 348.0
5	*qFLT7*	Bd3	294.6	3.17	5.61	2.02	13.6%	275.9 - 302.0

[a]Environment (see Supplemental Table S1)

[b]Chromosome

[c]Experiment-wide permutation threshold

[d]Additive effect estimate for transformed phenotypes

[e]Percent of phenotypic variance explained

[f]One-LOD support interval (cM); ND denotes QTLs not detected using standard interval mapping.

Table 3. Previously identified *B. distachyon* homologs of flowering regulators in *Arabidopsis* (*At*), hexaploid and diploid wheat (*Ta* and *Tm*), barley (*Hv*), and rice (*Os*) within the one-LOD support intervals of the statistically significant QTLs under transformation T3.

Locus	Chr[a]	1-LOD SI[b]	*B. distachyon* gene	Homologous genes[c]
qFLT1	Bd1	292.1 - 305.6	Bradi1g45810	*AtAGL24*, *TaVRT2*, *OsMADS55*
			Bradi1g46060	*AtABF1*
			Bradi1g48340	*AtCLF*, *OsCLF*
			Bradi1g48830	*AtTSF*, *HvFT1*, *OsHd3a*/*OsFTL2*
qFLT3	Bd2	323.7 - 348.0	Bradi2g53060	*AtFDP*
			Bradi2g54200	*AtNF-YB10*
			Bradi2g55550	*AtbZIP67*
qFLT4	Bd2	403.2 - 411.0	Bradi2g60820	*AtFY*, *OsFY*
			Bradi2g62070	*AtLUX*, *OsLUX*
qFLT6	Bd3	72.9 - 97.0	Bradi3g08890	*OsFTL13*
			Bradi3g10010	*TaVRN2*, *TmCCT2*, *OsGhd7*
			Bradi3g12900	*AtHUA2*
qFLT7	Bd3	273.9 - 300.7	Bradi3g41300	*OsMADS37*
			Bradi3g42910	*AtSPY*, *OsSPY*
			Bradi3g44860	*OsRCN2*

[a]Chromosome

[b]Combined maximal one-LOD support interval (cM) from all significant QTLs

[c]Identified in Higgins *et al.* 2010 and Ream *et al.* 2012

Figures

Figure 1. Flowering behavior within the ABR6 x Bd21 mapping population. Three months after a six-week vernalization period, ABR6 (left) is not flowering, whereas Bd21 (center) is flowering and an individual in the ABR6 x Bd21 mapping population displays an intermediate flowering phenotype (right).

Figure 2. Effect of vernalization on flowering time in ABR6 and Bd21. Days to flowering was measured from the end of vernalization for seven different vernalization periods. After vernalization plants were grown in a growth chamber (16 h photoperiod) for 35 days and then transferred to a greenhouse without light and temperature control (late April to mid July 2013; Norwich, UK). Mean days to flowering and standard error are based on six biological replicates. Different letters represent statistically significant differences based on pairwise comparisons using *t*-tests with pooled standard deviations and Bonferroni correction for multiple comparisons.

Figure 3. Segregation distortion in the ABR6 x Bd21 F_4 population. For each marker of the genetic map the frequencies of F_4 individuals with homozygous ABR6 genotype (solid magenta), homozygous Bd21 genotype (dashed green), or heterozygous genotype (solid black) were calculated (scale on left). Data coverage (percent of F_4 individuals with genotype calls per marker) is represented by the gray line (scale on right).

Figure 4. Frequency distribution of flowering time in the ABR6 x Bd21 population. Flowering time was measured from the first day that flowering was observed in the entire population. (A) Environment 1 (April to July, natural light supplemented for 20h, 22°C/20°C, no vernalization), (B) Environment 2 (April to July, natural light supplemented for 20h, 22°C/20°C, six weeks vernalization), (C) Environment 3 (May to July, natural light and temperatures, no vernalization), (D) Environment 4 (September to November, natural light supplemented for 16h, minimum 18°C/11.5°C, no vernalization), (E) Environment 5 (March to May, natural light and temperatures, no vernalization). Flowering times for the parental lines are indicated by arrows (no data for Environment 3). NF = not flowering.

Figure 5. Linkage mapping of flowering time in the ABR6 x Bd21 population. Time to flowering for 114 $F_{4:5}$ families of the population was transformed into ordered rank values,

QTL analysis performed using composite interval mapping under an additive model hypothesis test (H_0:H_1), and plotted based on normalized permutation thresholds. The blue horizontal line represents the threshold of statistical significance based on 1,000 permutations. Orange = Environment 1(April to July, natural light supplemented for 20h, 22°C/20°C, no vernalization), blue = Environment 2 (April to July, natural light supplemented for 20h, 22°C/20°C, six weeks vernalization), red = Environment 3 (May to July, natural light and temperatures, no vernalization), yellow = Environment 4 (September to November, natural light supplemented for 16h, minimum 18°C/11.5°C, no vernalization), green = Environment 5 (March to May, natural light and temperatures, no vernalization). See Supplemental Table S1 for full environmental details. The genetic positions of the previously identified homologs of *VRN1*, *VRN2*, and *FT* are indicated (compare Higgins *et al*. 2010 and Ream *et al.* 2012).

Figure 6. Phenotype by genotype plot for the two major loci controlling flowering time in the ABR6 x Bd21 mapping population. Days to flowering in Environment 3 for the ABR6 x Bd21 $F_{4:5}$ families homozygous at *qFLT1* and *qFLT6* shows that the Bd21 alleles at these two loci promote early flowering. Error bars represent one standard error; NF = not flowering.

Figure 7. Comparison of the flowering regulators *FT* and *VRN2* between the *B. distachyon* accessions Bd21 and ABR6. Contigs of the ABR6 *de novo* assembly were aligned to the Bd21 reference sequence (Version 3) and polymorphisms were identified in the genes of interest and 2kb promoter and terminator sequence (1.9kb promoter for *VRN2*). Red ticks represent SNPs and black ticks represent indels. The length of indels (bp) is shown with + for insertion and – for deletion. The amino acid change of the non-synonymous SNP in *VRN2* is indicated. s = synonymous SNP; dashed line = promoter or terminator; white box = 5'-UTR or 3'-UTR; black box = exon; black line = intron; M = methionine/translation start; star = translation stop; black bar under *VRN2* = CCT domain.

Figure 8. *VRN1*, *VRN2*, and *FT* expression in fourth leaf of ABR6 and Bd21 after varying periods of cold treatment. Seeds were imbibed with water and not vernalized or vernalized for two, four, or six weeks, and transferred to a growth chamber with parameters similar to Environment 2. Fully expanded fourth leaves were harvested in the middle of the photoperiod. Relative gene expression of *VRN1* (A), *VRN2* (B), and *FT* (C) was determined using RT-qPCR and analyzed using the $2^{-\otimes\otimes Ct}$ method. All genes were normalized to 1 based

on Bd21 expression with no cold treatment (0 weeks) and *UBQ18* was used as internal control. Bars represent the mean of three biological replicates with error bars showing ± 1 standard error. Different letters represent statistically significant differences based on pairwise *t*-tests using a multiple hypothesis corrected *p*-value threshold of 0.05 with the Benjamini-Hochberg approach.

Supplemental Material

Supplemental Figure S1. Linkage groups of ABR6 x Bd21 genetic map.

Supplemental Figure S2. Two-way recombination fraction plot for the ABR6 x Bd21 F_4 population.

Supplemental Table S1. Summary of the environmental conditions tested.

Supplemental Table S2. Significant QTLs from interval mapping of binary classification of flowering time phenotypes in the ABR6 x Bd21 $F_{4:5}$ families.

Supplemental Table S3. Significant QTLs from interval mapping using a non-parametric model for flowering time phenotypes in the ABR6 x Bd21 $F_{4:5}$ families (NP).

Supplemental Table S4. Significant QTLs from composite interval mapping of transformed flowering time phenotypes in the ABR6 x Bd21 $F_{4:5}$ families (T1).

Supplemental Table S5. Significant QTLs from composite interval mapping of transformed flowering time phenotypes in the ABR6 x Bd21 $F_{4:5}$ families (T2).

Supplemental Table S6. Summary of the structural variation between Bd21 and ABR6 for the flowering regulators Bradi1g48830 (*FT*), Bradi3g10010 (*VRN2*), and Bradi1g08340 (*VRN1*).

Supplemental Table S7. Five cleaved amplified polymorphic sequences (CAPS) markers included in the ABR6 x Bd21 genetic map design.

Supplemental Data S1. Raw, binary, and transformed flowering time data for the ABR6 x Bd21 $F_{4:5}$ families in the five environments tested.

Supplemental Data S2. Sequence information used to develop iPLEX assays for the 247 MassARRAY markers in the ABR6 x Bd21 genetic map design.

Supplemental Data S3. ABR6 x Bd21 genetic map.

Supplemental Figure S1. Linkage groups of ABR6 x Bd21 genetic map. Cumulative cM distances and SNP marker names are shown to the left and right of each chromosome, respectively. cM distance at the F_4 stage was estimated using the Kosambi function. SNP marker names consist of the corresponding chromosome and physical position in the Bd21 reference genome (Version 3).

Supplemental Figure S2. Two-way recombination fraction plot for the ABR6 x Bd21 F_4 population.

Supplemental Table S1. Summary of the environmental conditions tested.

Supplemental Table S2. Significant QTLs from interval mapping of binary classification of flowering time phenotypes in the ABR6 x Bd21 $F_{4:5}$ families.

Supplemental Table S3. Significant QTLs from interval mapping using a non-parametric model for flowering time phenotypes in the ABR6 x Bd21 $F_{4:5}$ families (NP).

Supplemental Table S4. Significant QTLs from composite interval mapping of transformed flowering time phenotypes in the ABR6 x Bd21 $F_{4:5}$ families (T1).

Supplemental Table S5. Significant QTLs from composite interval mapping of transformed flowering time phenotypes in the ABR6 x Bd21 $F_{4:5}$ families (T2).

Supplemental Table S6. Summary of the structural variation between Bd21 and ABR6 for the flowering regulators Bradi1g48830 (*FT*), Bradi3g10010 (*VRN2*), and Bradi1g08340 (*VRN1*).

Supplemental Table S7. Five cleaved amplified polymorphic sequences (CAPS) markers included in the ABR6 x Bd21 genetic map design. Marker names consist of the corresponding chromosome and physical position in the Bd21 reference genome (Version 3). Synonymous names refer to Barbieri *et al.* 2012.

Supplemental Data S1. Raw, binary, and transformed flowering time data for the ABR6 x Bd21 $F_{4:5}$ families in the five environments tested.

Supplemental Data S2. Sequence information used to develop iPLEX assays for the 247 MassARRAY markers in the ABR6 x Bd21 genetic map design. Marker names consist of the corresponding chromosome and physical position in the Bd21 reference genome (Version 3) and SNPs are indicated in square brackets.

Supplemental Data S3. ABR6 x Bd21 genetic map. “A” genotype calls refer to ABR6, “B” genotype calls to Bd21, “H” genotype calls to heterozygous markers and “-“ to missing data points.

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