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Independent recruitment of *FRUITFULL*-like transcription factors in the convergent origins of vernalization-responsive grass flowering

Martin Paliocha [a], Marian Schubert [a], Jill Christine Preston [b], Siri Fjellheim [a,*]

[a] Department of Plant Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, N-1432 Ås, Norway
[b] Department of Plant Biology, College of Agriculture and Life Sciences, The University of Vermont, Burlington, VT 05405, USA

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ABSTRACT

Flowering in response to low temperatures (vernalization) has evolved multiple times independently across angiosperms as an adaptation to match reproductive development with the short growing season of temperate habitats. Despite the context of a generally conserved flowering time network, evidence suggests that the genes underlying vernalization responsiveness are distinct across major plant clades. Whether different or similar mechanisms underlie vernalization-induced flowering at narrower (e.g., family-level) phylogenetic scales is not well understood. To test the hypothesis that vernalization responsiveness has evolved convergently in temperate species of the grass family (Poaceae), we carried out flowering time experiments with and without vernalization in several representative species from different subfamilies. We then determined the likelihood that vernalization responsiveness evolved through parallel mechanisms by quantifying the response of Pooideae vernalization pathway *FRUITFULL* (*FUL*)-like genes to extended periods of cold. Our results demonstrate that vernalization-induced flowering has evolved multiple times independently in at least five grass subfamilies, and that different combinations of *FUL*-like genes have been recruited to this pathway on several occasions.

1. Introduction

Most plant species couple endogenous and exogenous cues to regulate growth and development (Bernier, 1988; Poethig, 1990), resulting in flower, fruit, and/or seed production when conditions are favorable, thus increasing reproductive output and fitness (Bäurle and Dean, 2006; Murfet, 1977). In temperate species, the ability to respond to inductive flowering cues (i.e., attain floral competency) can often be hastened by an extended period of non-freezing cold known as vernalization (Chouard, 1960; Gaβner, 1918). Once floral competency is achieved, long days trigger the subsequent transition to reproductive growth at the shoot apical meristem (SAM). This two-step induction of flowering, prompted by the interplay of vernalization and photoperiodic cues, is found in many species across angiosperms (Andrés and Coupland, 2012; Bouché et al., 2017; Preston and Fjellheim, 2020; Preston and Sandve, 2013; Ream et al., 2012; Xu and Chong, 2018).

The vernalization-mediated flowering response is particularly well-studied in agriculturally important temperate grasses. Many grass species are identified as vernalization responsive based on their flowering behavior, but almost all are members of the temperate subfamily Pooideae (Heide, 1994). According to the current model from vernalization responsive 'winter' wheat (*Triticum* spp.) and barley (*Hordeum vulgare*) (Pooideae), cold-induced floral competency is controlled by a genetic circuit involving the mutual regulation of three central genes: *VERNALIZATION 1–3* (*VRN1–3*) (Bouché et al., 2017; Dennis and Peacock, 2009; Greenup et al., 2009; Trevaskis et al., 2007). During autumnal growth of winter wheat and barley, transcription of the flowering pathway integrator gene *VRN3/FLOWERING LOCUS T (FT)*-like is repressed by the action of the long day induced CONSTANS-like protein VRN2, resulting in a block on flowering before the onset of winter (Ream et al., 2014; Szűcs et al., 2007; Yan et al., 2004). As plants start to experience cold, expression of the *FRUITFULL* (*FUL*)-like MADS-box gene *VRN1* (*FUL1*-clade in Preston and Kellogg, 2006) gradually increases, causing the eventual repression and de-repression of *VRN2* and *VRN3*, respectively (Danyluk et al., 2003; Gu et al., 1998; Hemming et al., 2008; Higgins et al., 2010; Oliver et al., 2009; Shimada et al., 2009; Trevaskis, 2010; Trevaskis et al., 2003; Woods et al., 2016; Yan et al., 2004, 2003). Production of VRN1 is elicited by cold-induced histone modifications at the *VRN1* locus, which links the perception of winter with the acquisition of flowering competency (Deng et al., 2015;

* Corresponding author at: Department of Plant Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. BOX 5003, N-1432 Ås, Norway.
E-mail addresses: martin.paliocha@nmbu.no (M. Paliocha), marian.schubert@nmbu.no (M. Schubert), jill.preston@uvm.edu (J.C. Preston), siri.fjellheim@nmbu.no (S. Fjellheim).

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Distelfeld et al., 2009; Oliver et al., 2013, 2009). Furthermore, it is hypothesized that the florigen signal is enhanced by mutual positive feedback between the expression of *VRN1* and *VRN3*, whereby VRN1 carries out a secondary, deeply conserved function to promote flower development at the SAM (Ferrándiz et al., 2000; Gu et al., 1998; Preston and Kellogg, 2008; Tanaka et al., 2018).

In addition to *VRN1*, grasses have at least three other *FUL*-like genes (*FUL2, FUL3,* and *FUL4*, collectively referred to as *FUL*-like genes) derived from three duplication events (Wu et al., 2017; Zhang et al., 2022). One coinciding with the τ whole-genome duplication event in commelinids giving rise to the *FUL3/FUL4 and FUL1/FUL2* lineages (Jiao et al., 2014; Zhang et al., 2022), and the σ and ρ polyploidizations generating *FUL3* and *FUL4* in Poales and *VRN1* and *FUL2* in most of Poaceae, respectively (D'Hont et al., 2012; Graham et al., 2006; Litt and Irish, 2003; McKain et al., 2016; Paterson et al., 2004; Preston and Kellogg, 2006; Preston et al., 2009; Zhang et al., 2022). All three genes are expressed in the SAM during the floral transition (Danilevskaya et al., 2008; Kinjo et al., 2012; Preston and Kellogg, 2007), consistent with their likely ancestral function in floral meristem and organ identity specification (Litt, 2007). In wheat, *FUL*-like genes are redundantly involved in spikelet and inflorescence development, as well as flowering time and plant height (Li et al. 2019). While several angiosperm *FUL*-like genes are also expressed in leaves or bracts (Gu et al., 1998; Yang et al., 2021), a role for these genes in vernalization through their upregulation in leaves has only been described in Pooideae grasses (McKeown et al., 2016; Zhong et al. 2018). This is consistent with the inferred origin of vernalization responsiveness at the base of the subfamily (McKeown et al., 2016). However, the fact that *FUL2* transcripts also increase in response to cold in the Pooideae species *Lolium perenne* (Petersen et al., 2006, 2004), *Avena sativa* (Preston and Kellogg, 2007) *Triticum aestivum* (Chen and Dubcovsky, 2012), *Schedonorus pratensis* (Ergon et al., 2016, 2013), and *Brachypodium distachyon* (Li et al., 2016) either suggests the evolution of a common upstream regulator for these genes or a propensity of grass *FUL*-like genes to be independently co-opted into vernalization pathways.

Grasses are one of the largest plant families with 11,783 species organized into 12 subfamilies (Soreng et al., 2022). Most species are found in two large clades: the largely temperate/subtropicalBambusoideae–Oryzoideae–Pooideae (BOP) clade or the mainly tropical Panicoideae–Aristidoideae–Chlorioideae–Micrairoideae–Arundinoideae –Danthonioideae (PACMAD) clade. There are also three early-diverging subfamilies with a small number of species (Anomochlooideae, Pueloideae and Pharoideae; Saarela et al., 2015; Soreng et al., 2022; Hodkinson, 2018). Although most grasses have tropical to sub-tropical distributions (Schubert et al., 2019b; Visser et al., 2014), temperate grasses have evolved multiple times in both the major BOP and PACMAD clades (Grass Phylogeny Working Group II, 2012). Pooideae dominate the grass flora in temperate, continental, and Arctic regions (Hartley, 1973), and Danthonioideae constitute a southern temperate clade (Pirie et al., 2012; Peter Linder et al., 2013; Visser et al., 2014). Furthermore, several lineages from other subfamilies, most notably Chloridoideae and Arundinoideae have also diversified into cold climate environments (Schubert et al., 2020; Atkinson et al., 2016). Despite this, little is known about the impact of vernalization on flowering in temperate grass species outside Pooideae. Evans and Knox (1969) report that in some temperate, long day-responsive ecotypes of *Themeda triandra* (Panicoideae), flowering is hastened after vernalization treatment. Furthermore, evidence from a growth experiment carried out on several populations of *Rytidosperma caespitosa*, suggests that some Danthonioideae may also be able to accelerate flowering following exposure to long-term cold (Hodgkinson and Quinn, 1978).

To determine how widespread vernalization responsive flowering is across grasses, we carry out growth experiments on a phylogenetically diverse set of temperate PACMAD species and use these data to reconstruct the minimum number of origins of vernalization responsive flowering in grasses. We then investigate the genetic basis of these origins by examining the behavior of the paralogs *VRN1* and *FUL2* during prolonged cold. We find evidence for multiple origins of vernalization responsiveness across grasses and present data supporting evolution of this trait through the parallel recruitment of different *FUL* homologs.

2. Materials and methods

2.1. Plant material

Study species were selected to reflect the phylogenetic diversity and geographical distribution of temperate, perennial PACMAD grasses based on a previous study (Atkinson et al., 2016). Seeds for five species from a total of seven accessions (Table S1) were acquired from the United States Department of Agriculture (USDA) Germplasm Resources Information Network (GRIN). Imbibed seeds for four accessions were sown out in humid soil containing equal amounts of compost and peat with a small amount of river sand. To break seed dormancy and synchronize germination, seeds were stratified in the dark at 4 ℃ for 5 days, followed by 24 h at 25 ℃. Seedlings were pricked out and transferred to individual pots. For three *Danthonia decumbens* and two *Molinia caerulea* populations, wild full-grown plants were collected at four different locations in south-eastern and western Norway (Table S2).

To synchronize plants grown from seed and collected in nature, all individuals were pre-grown at 17 ℃ under long days (16 h light, 8 h darkness) for at least four weeks in a greenhouse at the Norwegian University of Life Sciences (NMBU). At least 30 plants per population/accession were grown per treatment. Artificial light was supplied in addition to natural light during the light period using Master HPI-T Plus 400 W/645 E40 1SL light bulbs (Philips). For every population, the SAM of the largest plant was dissected prior to vernalization treatment to ensure that meristems were in the vegetative state. At least 15 plants from every population were assigned to a vernalization (8 ℃) or control treatment (20 ℃), respectively and transferred to walk-in growth chambers for 56 days (8 weeks). A relatively high vernalization temperature within the temperature range for optimal vernalization (Preston and Fjellheim, 2022) was chosen based on preliminary experiments at lower (4–6 ℃) temperatures that resulted in high *T. triandra* mortality. Two chambers per condition were used to reduce chamber effects. In each chamber photoperiod was set to short days (8 h light, 16 h darkness) and the average light irradiance was 65 μmol m^{-2} s^{-1}. Subsequently, plants were transferred back to the greenhouse, wherein emergence of the first inflorescence (bolting or 'heading') was scored as days from germination to heading (*DTH*). During the entire experiment, plants were randomized and rotated every fourth day to minimize room effects.

To account for differential growth in the vernalized and control plants, corrected *DTH* (DTH_C) was calculated using temperature-adjusted days, rather than subtracting the entire duration of the temperature treatment from *DTH*. Assuming a linear relationship between growth and temperature (Baskerville and Emin, 1969), it was presumed that plants in the control treatment (17 ℃) accumulated 2.125 times more heat units than vernalized plants, given a growth baseline below 8 ℃. DTH_C for vernalized plants was thus calculated as:

$$DTH_C = DTH - \left[L \cdot \frac{(T_C - T_V)}{T_C}\right]$$

where L the length of the vernalization period (56 days), T_C the temperature for the control group (17 ℃), and T_V the vernalization temperature (8 ℃) (Baloch et al., 2003; Kirby et al., 1989; McKeown et al., 2016; Preston and Fjellheim 2022).

2.2. Sampling, RNA extraction, and cDNA synthesis

To test if *VRN1* and *FUL2* are induced by cold in species from the

PACMAD clade, we selected three species (*M. caerulea, D. decumbens*, and *B. gracilis*) for analysis of gene expression in leaves under vernalization. During the growth chamber experiments, leaf tissue from the longest leaf was collected for RNA extraction at zeitgeber time 3 (ZT3; i.e., 3 h after lights on) for three different time points: before the plants were moved to the growth chambers (day 0), and after six weeks (day 42) and eight weeks (day 56) of vernalization. A TissueLyser II bead mill and 3 mm tungsten carbide beads (QIAGEN) were used to disrupt deep frozen leaf tissue. Total RNA was isolated with RNeasy Plant Mini Kit (QIAGEN), following the manufacturer's instructions, including the additional centrifugation and elution step. Complementary DNA (cDNA) was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) following the protocol provided by the manufacturer.

2.3. Target gene isolation

The target genes *M. caerulea VRN1* (*McVRN1*), *D. decumbens VRN1* (*DdVRN1*), *M. caerulea FUL2* (*McFUL2*), and *D. decumbens FUL2* (*DdFUL2*) were PCR-amplified from cDNA using primers designed by Preston and Kellogg (2006) and McKeown et al. (2016) as well as RT-qPCR primers created in this study (Table S4). Amplicons were purified with ExoSAP-IT (Affymetrix), sub-cloned using the pGEM-T Easy cloning vector system (Promega) and transformed into chemically competent *Escherichia coli* JM109 cells (Promega). All steps were performed following the manufacturer's protocol but using half the reaction volume for the ligation reaction with 1.5 μL PCR product. After plating and 24 h of incubation, successfully transformed colonies were picked from the growth medium. Sub-cloned PCR products were then amplified from the plasmid vector using M13 forward and reverse primers. Partial coding sequences were obtained by Sanger dideoxy sequencing performed at the University of Vermont (UVM) Integrative Genomics Resource using SP6 sequencing primers. Residual plasmid vector contamination was removed from putative *VRN1* and *FUL2* sequences using NCBI's UniVec database (NCBI Resource Coordinators, 2017) and blastn v2.7.1 (Altschul et al., 1990; Camacho et al., 2009; Zhang et al., 2000) with default search parameters prior to further analysis.

Target gene sequences for *T. triandra* were obtained by genome assembly of raw reads of seven genome-skimmed individuals (Dunning et al., 2017; Olofsson et al., 2016). Sequence data were downloaded from NCBI's Sequence Read Archive (Leinonen et al., 2011) and assembled using MaSuRCA v3.2.6 (Zimin et al., 2013) with *k*-mer length $k = 106$ estimated with KmerGenie v1.7051 (Chikhi and Medvedev, 2013), and SOAPdenovo2 r240 for scaffolding (Luo et al., 2012). Target genes were identified using megablast v2.7.1 (Camacho et al., 2009) with default search strategy and introns removed manually to obtain coding sequences. For *Bouteloua gracilis*, genes were identified by PCR amplification with primers designed for *T. triandra* and *D. decumbens* and confirmed by Sanger dideoxy sequencing and subsequent phylogenetic analysis.

2.4. Phylogenetic analysis

Target gene sequences were added to a representative selection of 54 *FUL* homologs from 32 monocot taxa (McKeown et al., 2016; Preston and Kellogg, 2006) and realigned using the R package DECIPHER v2.17.1 (Wright, 2016, 2015). *FUL3* sequences were retrieved from GenBank (Benson et al., 2012) and added to the multiple sequence alignment with MAFFT v7.505 L-INS-I using the –keeplength and –add options (Katoh and Standley, 2013). After manual inspection and adjustment of the alignment, the best nucleotide substitution model was determined based on AICc calculations by the modelTest function from the R package phangorn v2.5.5 (Darriba et al., 2012; Schliep, 2011). Gene trees were inferred using BEAST v1.10.4 (Suchard et al., 2018) and BEAGLE v3.1.2 (Ayres et al., 2012), assuming an uncorrelated, log-normal relaxed clock (Drummond et al., 2006), a general time-reversible substitution model including gamma distributed rate variations with four discrete categories, and invariable sites (GTR + Γ + I; Hasegawa et al., 1985; Tavaré, 1986; Yang, 1994), and a Yule two-parameter prior (Gernhard, 2008; Yule, 1925). Two independent BEAST analyses were run for 1.0×10^8 generations and sampled every 1,000th generation. Convergence of both runs combined was assessed using Tracer v1.7.1 (Rambaut et al., 2018) with 25 % of the trees discarded as burn-in. The maximum clade credibility tree was rescaled to reflect posterior node heights and visualized with ggtree v3.4.0 (Yu et al., 2017).

2.5. RT-qPCR

To quantify the relative abundance of *VRN1* and *FUL2* mRNA from the exemplar taxa *D. decumbens* 'SY', *M. caerulea* 'HV', *T. triandra* 'NSW', and *B. gracilis*, gene-specific RT-qPCR primers were designed using Primer3 v4.1.0 with default settings (Untergasser et al., 2012). Two housekeeping genes, *ELONGATION FACTOR 1α* (*EF1α*) and *UBIQUITIN 5* (*UBQ5*), served as references for the relative quantification and were amplified using primers designed by McKeown et al. (2016). Amplicon identity of target and reference genes was confirmed by Sanger dideoxy sequencing (Eurofins GATC and Azenta GENEWIZ). Primer efficiencies were determined using a 2-fold dilution series (Schmittgen and Livak, 2008), starting with a 1:10 cDNA dilution. Amplification efficiencies were between 0.90 and 1.10 for all primer pairs (Bustin et al., 2009; Pfaffl, 2001).

Transcript abundance was quantified with an Applied Biosystems 7500 Fast instrument (ThermoFisher Scientific; *M. caerulea*) or a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories; *B. gracilis*, *D. decumbens*, and *T. triandra*), using Applied Biosystems SYBR Select Master Mix (ThermoFisher Scientific) with a total reaction volume of 10 μL per well. Quantification was carried out on five biological replicates (except *M. caerulea* week 8, vernalized, where $n = 4$ and *B. gracilis* week 6, where $n = 3$) and three technical replicates. Fluorescence data for each gene were pre-processed using the CPP function from the R package chipPCR v0.0.8–10 (Rödiger et al., 2015). Amplification curves were normalized between 0 and 1 and smoothed using a 3-point Savitzky–Golay filter (Savitzky and Golay, 1964). The slope of the overall background trend (baseline) was estimated by linear regression and subtracted from the fluorescence signals (Rödiger et al., 2015). Quantification cycles were determined by calculating the second derivative centre (geometric mean of the second derivative minimum and maximum) of the normalized, smoothed and baseline-corrected amplification curves (Tellinghuisen and Spiess, 2014). Mean expression of every gene at the first sampling point (week 0) was used as internal reference to calculate ΔC_q. Target gene expression was then normalized relative to the geometric mean of *EF1α* and *UBQ5* expression ($\Delta\Delta C_q$) (Vandesompele et al., 2002). All C_q values were corrected by the amplification efficiency of their corresponding RT-qPCR primers.

2.6. Statistical analyses

Computations and statistical analyses were carried out in R v4.0.2 (R Core Team, 2020). Flowering data was analyzed using Mann–Whitney *u*-tests (Mann and Whitney, 1947). Effects of temperature treatment, time and interaction between time and treatment on gene expression were analyzed using two-way ANOVAs using the lm function from R's stats package (R Core Team, 2020) omitting data from the reference time point (week 0). Post-hoc tests were carried out with multcomp v1.4–14 (Hothorn et al., 2008) using Tukey-type contrast matrices to construct appropriate general linear hypotheses between vernalized and non-vernalized material after 6 and 8 weeks, respectively.

3. Results

3.1. Identification of vernalization-responsive species

In total, 12 accessions from seven PACMAD species occurring in the temperate zone were surveyed in this study (Table S1-S2). Statistical analysis of differential flowering time was performed for populations that produced at least five flowering individuals per treatment until the termination of the experiment after 300 days (12 populations, see Fig. 1). In 11 of these 12 accessions, vernalized plants flowered significantly earlier ($P < 0.05$; Mann–Whitney *u*-test) than non-vernalized plants (Fig. 1). One population of *T. triandra* (Panicoideae) originating from Eastern Cape, South Africa (PI 206348; Table S2; 'ZA1′ in Fig. 1) was the only flowering accession that did not significantly respond to vernalization. The strongest response to vernalization was observed in *B. gracilis* (Chloridoideae) and one population of *M. caerulea* (Arundinoideae) collected as full-grown plants in Hvaler, south-eastern Norway ('HV' in Fig. 1).

3.2. Candidate gene identification

For two of the study species (*D. decumbens* and *M. caerulea*), partial coding sequences for *VRN1* and its paralog *FUL2* were obtained by bacterial plasmid sub-cloning. Subsequent Sanger sequencing from the vector yielded one 400 bp nucleotide sequence for *McVRN1* and *DdFUL2*. Primers designed for the RT-qPCR assay based on these sequences were used to amplify and isolate *M. caerulea FUL2* (*McFUL2*, using *DdFUL2* qPCR primers) and *D. decumbens VRN1* (*DdVRN1*, using *McVRN1* qPCR primers). This approach resulted in the amplification of shorter *McFUL2* and *DdVRN1* regions relative to *DdFUL2* and *McVRN1*. Thus, a 334 bp sequence of *DdVRN1* was isolated, in addition to a 115 bp amplicon of *McFUL2*. Partial coding sequences of *Themeda triandra VRN1* (*TtVRN1*) and *FUL2* (*TtFUL2*) recovered from genomic DNA were 785 bp and 714 bp long, respectively. Sequences from *Bouteloua gracilis* material generated with RT-qPCR primers from *D. decumbens* had lengths of 104 bp (*BgVRN1*) and 159 bp (*BgFUL2*), respectively.

Identity of newly generated *FUL*-like nucleotide sequences was confirmed by generating a gene tree using Bayesian inference. Putative *VRN1* and *FUL2* sequences were placed in two clades together with *VRN1* and *FUL2* orthologs from other PACMAD taxa, respectively (Fig. 2). The topology of the inferred gene tree is congruent with the results of Preston and Kellogg (2006), whose multiple sequence alignment served as the basis for the phylogenetic analysis. Consistent with previous findings (McKeown et al., 2016; Preston and Kellogg, 2006; Zhang et al., 2022), strong support for a gene duplication event at the base of the Poaceae giving rise to the paralogs *VRN1* and *FUL2* was found in the inferred gene tree. Within the *FUL2* clade, the division of the grass family into early-diverging and 'crown Poaceae' (BOP and PACMAD) is evident and well supported ($PP \geq 0.95$; Fig. 2). The division into lineages above subfamily-level received less support in the *VRN1* lineage ($PP = 0.73$; Fig. 2). Nevertheless, PACMAD taxa formed a distinct clade. Despite their relatively short length, the putative *VRN1* and *FUL2* sequences isolated from *D. decumbens*, *B. gracilis*, *M. caerulea*, and *T. triandra* were placed with other PACMAD taxa within the predicted clade (Fig. 2).

3.3. Gene expression in response to vernalization

Based on the hypothesis that *FUL*-like genes have been independently recruited for vernalization responsiveness in PACMAD grasses, we predicted that *VRN1* and/or *FUL2* transcription would increase significantly over time only in our cold-treated plants, manifesting in a significant time point by treatment interaction. Significant effects of temperature treatment on gene expression were detected for *DdVRN1* and *DdFUL2* ($P < 0.000$, ANOVA, Table S3), whereas sampling time had a significant effect on *McFUL2* ($P < 0.05$, ANOVA, Table S3). Post-hoc tests revealed significant differences in gene expression between vernalized and non-vernalized individuals for *DdVRN1* ($P < 0.005$), *DdFUL2* ($P < 0.000$) after six and eight weeks, respectively, and *McFUL2* after eight weeks ($P < 0.05$, Tukey's HSD test) with consistently higher expression levels in vernalized material. No treatment effects were found for *BgVRN1, BgFUL2, McVRN1, TtVRN1* or *TtFUL2* (Fig. 3, Table S3).

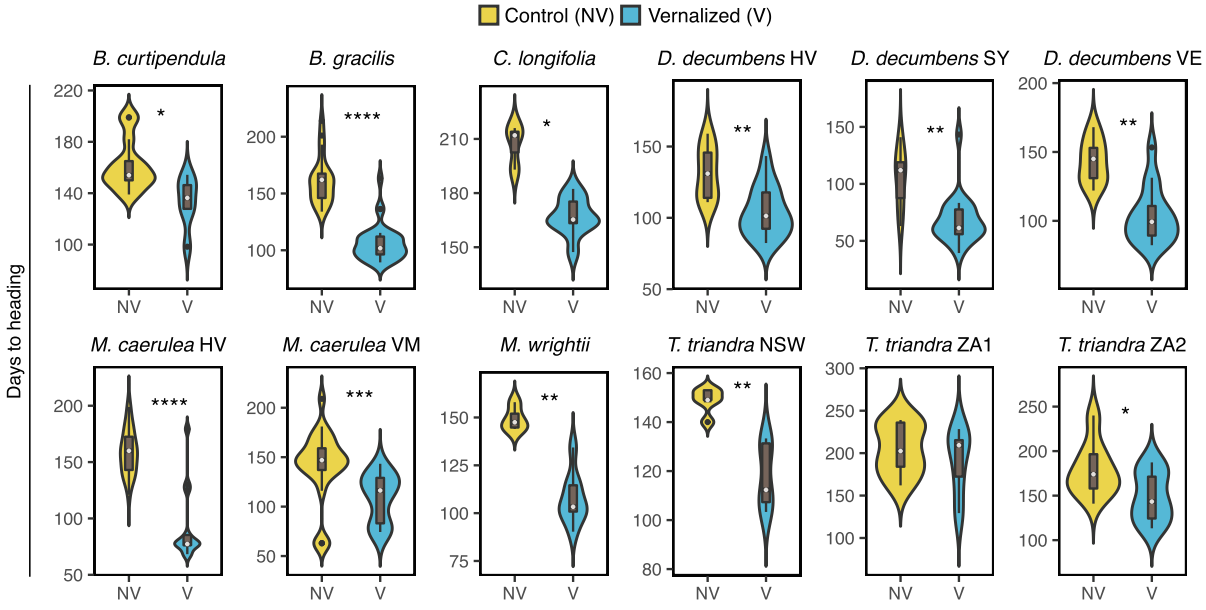

Fig. 1. Flowering behavior of 12 PACMAD accessions subjected to two different temperature treatments, measured in heat unit-adjusted days to heading (DTH_C). Colored areas represent density of the data and are scaled to resemble sample size, i.e., percentage of flowering plants. Grey rectangles indicate the interquartile range, lines 95 % confidence intervals, light dots the median, and dark dots outliers. The experiment was terminated after 300 days, and non-flowering individuals were omitted from the analysis. Significance codes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (Mann–Whitney *u*-test).

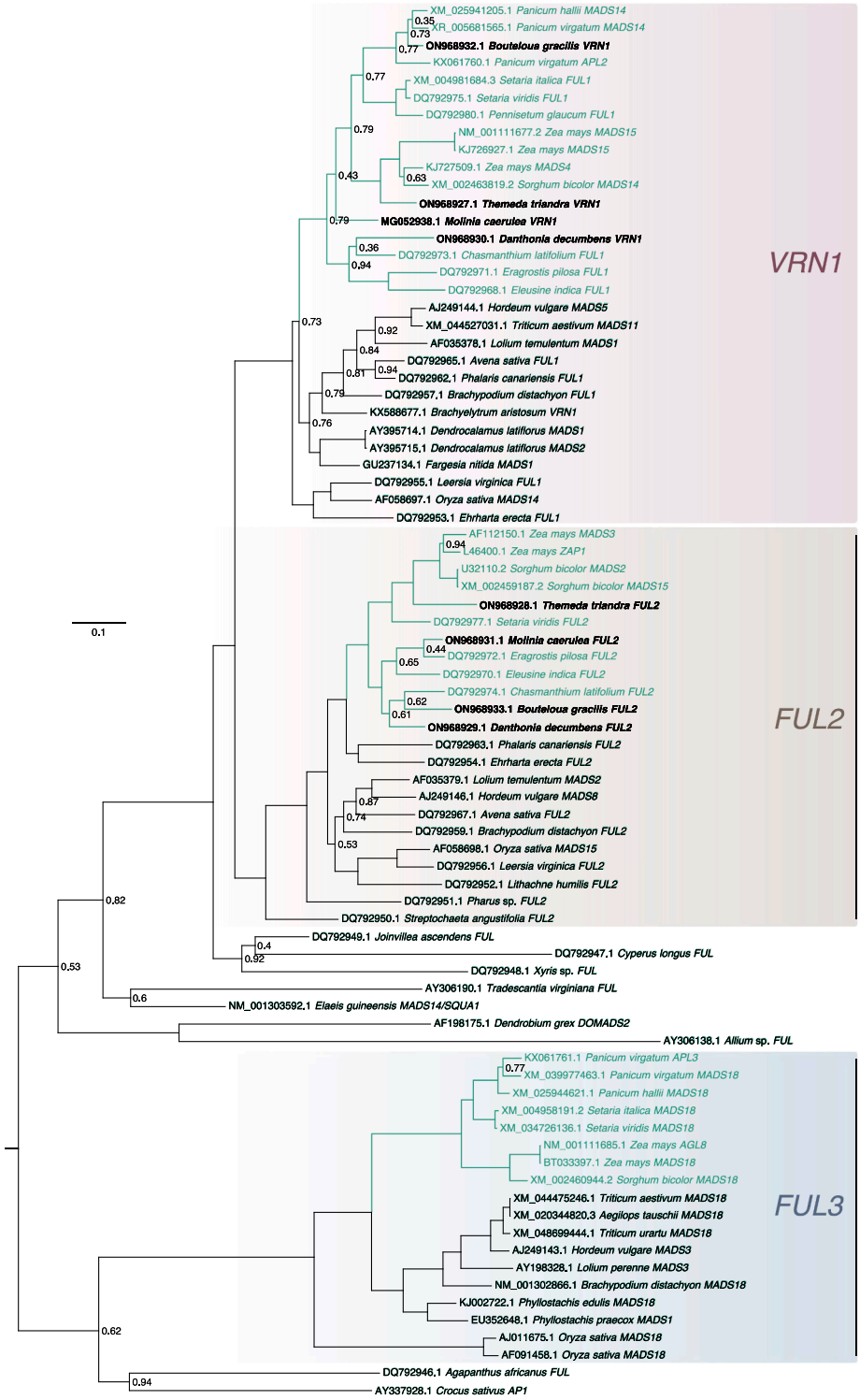

Fig. 2. Maximum clade credibility tree of *FUL*-like genes from 38 different monocot taxa inferred with BEAST. Branch lengths are scaled to represent relative nucleotide substitution rates. Sequences generated in this are highlighted in bold and other PACMAD taxa are highlighted in green. Numbers at nodes denote $PP < 0.95$ and identifiers are GenBank accession numbers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Vernalization responsiveness in PACMAD grasses

Significantly hastened flowering was observed in vernalized individuals of seven species from four different subfamilies (Panicoidae, Chloridoideae, Arundinoideae, and Danthonioideae), suggesting that vernalization-cued flowering may be a widespread phenomenon in temperate PACMAD grasses. Our results corroborate earlier findings on a few species (Evans and Knox, 1969; Hodgkinson and Quinn, 1978). Given that the majority of PACMAD taxa occur in tropical and subtropical climates, a vernalization response likely evolved independently in different temperate PACMAD lineages, concomitant with their transition to habitats that experience seasonal cold. Furthermore, recent estimates place the split between BOP and PACMAD grasses at ~ 81.42–80.2 million years ago (Ma) (Huang et al., 2022; Schubert et al., 2019b), pre-dating the seasonality increase in high latitudes during the Eocene–Oligocene boundary (Eldrett et al., 2009) that likely triggered

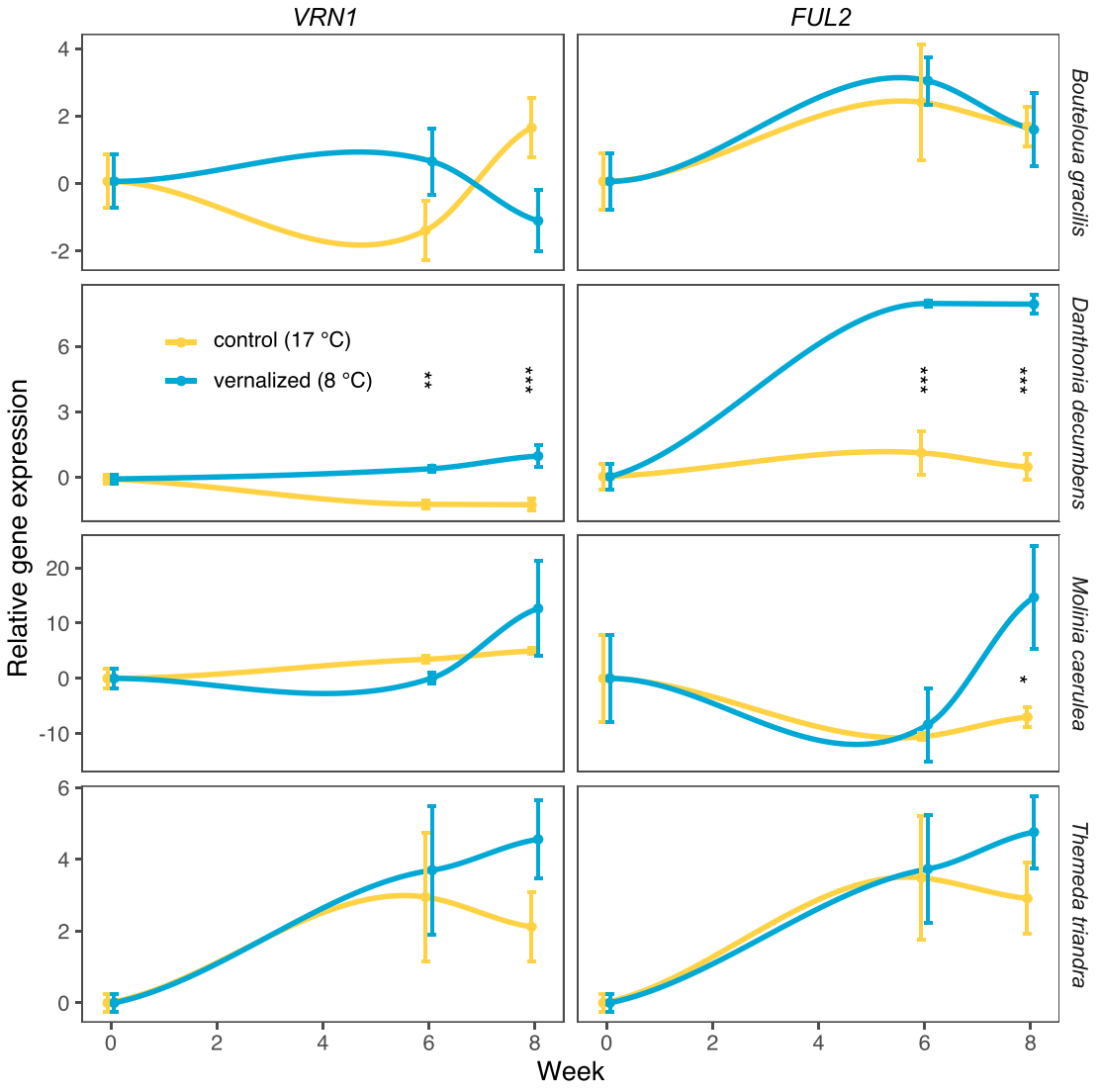

Fig. 3. Relative expression levels of *VRN1* and *FUL2* in the temperate PACMAD species *Bouteloua gracilis*, *Danthonia decumbens*, *Molinia caerulea*, and *Themeda triandra* with (blue lines) and without (yellow lines) vernalization. Significance codes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ according to a Tukey's HSD test comparing treatments at specific timepoints. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the evolution of vernalization response in Pooideae (Fjellheim et al., 2014; McKeown et al., 2016; Preston and Sandve, 2013; Zhong et al., 2018). Rather than coinciding with a period of cooling, the BOP–PACMAD partition coincides with a period of global warming following the Cretaceous thermal maximum (Clarke and Jenkyns, 1999; Huber et al., 2002; Wilson et al., 2002), which makes the early evolution of vernalization response at the base of the PACMAD clade unlikely.

In addition to large variation in responses between species, we also found variation in vernalization responses between populations for *D. decumbens, M. caerula* and *T. triandra*, in line with previous findings for *T. triandra,* as well as several Pooideae species (Evans and Knox, 1969; Heide, 1994) (Fig. 1). Vernalization sensitivity has been shown to follow environmental clines in, for example, *Arabidopsis thaliana* (Lewandowska-Sabat et al., 2012; Mitchell-Olds and Schmitt, 2006; Riihimäki and Savolainen, 2004; Wollenberg and Amasino, 2012), *B. distachyon* (Schwartz et al., 2010; Tyler et al., 2016), and *Phleum pratense* (Fiil et al., 2011). In each case, populations originating from coastal environments, distinguished by milder winters, had stronger responses to vernalization than continental populations, suggesting that the need to time flowering adequately may be greater in environments with indistinct transitions between seasons. This is congruent with the flowering behavior observed in *M. caerulea*, where the coastal Hvaler population ('HV', Fig. 1) turned out to have a stronger vernalization response than cold-treated individuals originating from a slightly more continental habitat (Vestmarka/'VM', Fig. 1).

4.2. Genetic architecture of the PACMAD vernalization response

Our results suggest that the vernalization pathways of some PACMAD grasses involve genes homologous to the Pooideae vernalization genes *VRN1* and *FUL2*. In *M. caerulea*, vernalization seems to affect flowering through the up-regulation of a *FUL2*-like gene. Whereas *McFUL2* transcripts drastically increase following eight weeks of cold, leaf *McVRN1* transcription remains low regardless of temperature treatment. Although it is not possible to entirely discount that *McVRN1* is vernalization responsive in other tissues (specifically the SAM), our data support the independent recruitment of grass-specific *FUL*-like paralogs into a vernalization-mediated flowering pathway, possibly through differential neofunctionalization (Force et al., 1999; Hughes, 1994; He and Zhang, 2005). In *D. decumbens*, both *DdVRN1* and *DdFUL2*

are upregulated through vernalization, similar to the case in wheat (Chen and Dubcovsky, 2012; Li et al. 2019). Although our expression data indicate that *VRN1* and *FUL2* are involved in vernalization induced flowering in PACMAD grasses, we lack functional characterization to fully demonstrate this. Following this, our expression data preclude strong conclusions about functional redundancy in the vernalization pathway. However, VRN1 and FUL2 are known to be functionally redundant in other contexts, such as in promoting the floral transition (Yang et al., 2021). Functional redundancy among *FUL*-like genes might provide flexibility for fine-tuning flowering responses to specific environmental conditions. Although *T. triandra TtVRN1* and *TtFUL2* showed the expected trend of upregulation in response to cold, further sampling is warranted in the face of our non-significant results. In contrast, gene expression patterns of *BgVRN1* or *BgFUL2* do not support the recruitment of *VRN1/FUL2* paralogs into the vernalization pathway, begging the question as to whether other grass *FUL*-like genes (*FUL3* and *FUL4*) might be involved (cf. Li et al., 2019).

The propensity of *FUL*-like genes to be repeatedly co-opted into the vernalization pathway might stem from their ancestral function(s). In the case of *VRN1* and *FUL2*, their pre-duplication ancestor is inferred to have been involved in determining floral meristem identity (Gu et al., 1998; Litt and Irish, 2003; Preston and Kellogg, 2007; Theiβen et al., 1996). Comparative RNA *in situ* hybridization indicates that all *FUL*-like genes are strongly expressed in spikelet and floret meristems (Danilevskaya et al., 2008; Ergon et al., 2013; Gocal et al., 2001; Li et al., 2016; Preston and Kellogg, 2008, 2007; Yang et al., 2021), emphasizing their conserved, ancestral role in determining inflorescence meristem identity (Preston et al., 2009; Preston and Kellogg, 2007). In BOP grasses, *VRN1* is expressed in all floral whorls of the inflorescence meristem and postulated to specify overall meristem identity (Gocal et al., 2001; Kinjo et al., 2012; Moon et al., 1999; Preston and Kellogg, 2007), congruent with E-class transcription factors in the ABCDE model of floral development (Callens et al., 2018; Theiβen, 2001). On the other hand, *FUL2* is proposed to work with *VRN1* to exert a more specific function by regulating the differentiation of whorl-primordia into particular anatomical structures in numerous species (Ferrándiz et al., 2000; Gocal et al., 2001; Gu et al., 1998; Preston and Kellogg, 2008, 2007; Wu et al., 2017; Yang et al., 2021). These data support the hypothesis that the ancestral *FUL*-like gene was involved in mediating the transition to inflorescence development (Preston et al., 2009), and that the cold-induced up-regulation and subsequent co-option of *VRN1* into the Pooideae vernalization pathway is a derived trait (Li et al., 2016; McKeown et al., 2016; Preston et al., 2009; Preston and Kellogg, 2007).

Duplication and expansion of *FUL*-like genes at the base of the Poaceae has led to sub- and neofunctionalization among *FUL*-like paralogs, resulting in distinct expression patterns and developmental roles of *VRN1* and *FUL2* during inflorescence development (Preston et al., 2009; Preston and Kellogg, 2007). Up-regulation of *McFUL2* in *M. caerulea* and *DdFUL2/DdVRN1* in *D. decumbens* during vernalization might be another example of independent recruitment of closely related genes. In this case, we hypothesize that *FUL*-like gene recruitment has been a key mechanism underlying convergent origins of a complex physiological adaptation, like the parallel co-option of paralogous genes in convergent evolution of cold tolerance (Sandve and Fjellheim, 2010; Schubert et al., 2019a; Vigeland et al., 2013), C_4 photosynthesis (Christin et al., 2009), and floral zygomorphy (Hileman, 2014).

An interesting aspect of our study warranting further investigation is the timing of *VRN1/FUL2* recruitment into the PACMAD vernalization pathway. In Pooideae, the regulon perceiving, amplifying, and transmitting the vernalization signal is mostly conserved (although, see Woods et al., 2017), and is posited to have evolved after the origin of the subfamily (McKeown et al., 2016). This opens up the possibility that the recruitment of *VRN1/FUL2*-mediated vernalization in the different PACMAD subfamilies happened more recently in these clades as adaptations to increased temperature seasonality, and hence was temporally independent of the *VRN1/FUL2* co-option in Pooideae. Although *FUL*-like genes have often been recruited into the regulatory pathway conferring vernalization response, other genes might be involved in vernalization-responsive species where neither *VRN1* nor *FUL2* are cold responsive. This supports the hypothesis of multiple independent origins of vernalization response in PACMAD grasses harnessing different genetic mechanisms.

5. Concluding remarks

Taken together, our results provide a basis for the evolutionary and functional analysis of vernalization response and its underlying genetic machinery in PACMAD grasses. A vernalization response was detected in species from all four PACMAD subfamilies tested. We found evidence consistent with one or both *VRN1/FUL2* paralogs being involved in vernalization-mediated flowering of *M. caerulea* (Arundinoideae) and *D. decumbens* (Danthonioideae), and perhaps also *T. triandra* (Panicoideae). This suggests that *VRN1*, *FUL2*, and possibly other *FUL*-like genes like *FUL3* and *FUL4* (Chen and Dubcovsky, 2012; Li et al., 2019; Yang et al., 2021) are easily co-opted into adaptations to deal with increased temperature seasonality.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Newly generated VRN1 and FUL2 sequences are available on GenBank under accession numbers ON968927–ON968933 and MG052938.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2022.107678.

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