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Molekylærgenetisk karakterisering av vernaliseringsrespons i PACMAD-gras

Molecular genetic characterisation of vernalisation response in PACMAD grasses

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Summary

Many plants rely on a mechanism called vernalisation response to match reproductive output with favourable environmental conditions. This trait is an important adaptation to seasonal climates, like for example at high latitudes. In grasses (Poaceae), evolution of seasonally-cued flowering was one of the traits enabling certain lineages to move out of their tropical origins and diversify in temperate zones. Due to its ecological and agricultural importance, the genetic basis of vernalisation response is particularly well-studied in Pooideae grasses that predominates temperate habitats. However, little is known about vernalisation systems in other grass lineages that are adapted to cool and seasonal climates. In this study, I ask whether vernalisation response is found in a grass lineage called the PACMAD clade. To disentangle the molecular machinery governing this trait, I quantified the expression of two paralogous *FRUITFULL*-like (*FUL*-like) vernalisation genes. My results demonstrate that vernalisation response is widespread in PACMAD grasses, and found in at least four subfamilies with temperate representatives. Moreover, I found evidence for the co-option of a novel vernalisation gene (*FUL2*) into the vernalisation gene network Arundinoideae species *Molinia caerulea*.

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Background

Adequate timing of flowering is fundamental for the evolutionary success of plants. Many plant species have evolved an elaborate arsenal of physiological mechanisms that couple endo- and exogenous cues with the regulation of development and growth (Bernier 1988; Poethig 1990). Monitoring environmental changes enables plants to align reproduction with favourable conditions; for instance to avoid flowering during periods of severe stress, thus circumventing potential damage (Murfet 1977; Bäurle & Dean 2006). Environmental control of development and growth of reproductive structures is an beneficial trait, and considered an evolutionary advantage in areas with pronounced seasonal variation, for example temperate zones at high latitudes (Murfet 1977; King & Heide 2009; Amasino 2010). To prevent flowering during transient warm periods in late autumn, numerous temperate plant species evolved the ability to sense prolonged periods of cold and coordinate flowering competency accordingly. Flowering triggered or hastened by extended periods of cold is termed vernalisation response (Gaßner 1918; Chouard 1960). In vernalisation-responsive plants, long-lasting cold induces the transition of the shoot apical meristem (SAM) from vegetative to reproductive state, thus initiating the development of inflorescences. Following the establishment of flower primordia at the shoot apex, long days trigger the subsequent maturation and elongation of reproductive shoots in many temperate flowering plants. This two-step induction of flowering prompted by the interplay of vernalisation and photoperiodic cues is found in many species across the plant kingdom (Andrés & Coupland 2012; Ream *et al.* 2012; Preston & Sandve 2013; Bouché *et al.* 2017).

Grasses (Poaceae) are the third largest angiosperm family. Various molecular phylogenetic studies recognise two major radiations in the Poaceae, the so-called BOP (Bambusoideae, Oryzoideae, and Pooideae) and PACMAD clade (Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, and Danthonioideae; GPWG 2001; Duvall *et al.* 2007; GPWG II 2012; Soreng *et al.* 2015). These clades cover the majority of grass diversity, encompassing 5753 and 5726 species, respectively (Soreng *et al.* 2017). Despite the evident evolutionary success of grasses, only a few BOP and PACMAD lineages have transitioned from their ancestral tropical origins into temperate habitats (Kellogg 2001; Bouchenak-Khelladi *et al.* 2010; Visser *et al.* 2012). Diversity of some of these grass clades is positively correlated with latitude (Hartley 1973; Cross 1980; Edwards & Smith 2010; Visser *et al.* 2014). This pattern is particularly prominent in the global distribution of grass species belonging to the subfamily Pooideae. This largely temperate grass lineage is scarce in the tropics, but dominates northern latitudes. There, members of the Pooideae constitute more than 90 % of the total grass flora (Hartley 1973; Cross 1980). It is posited that the capacity to match reproduct-

ive output with the occurrence of seasons has contributed significantly to the diversification of Pooideae grasses in temperate zones characterised by pronounced seasonality (Preston & Sandve 2013; Fjellheim *et al.* 2014). Paired with the ability to acclimate to cold and tolerate frost (Sandve & Fjellheim 2010; Sandve *et al.* 2011; Preston & Sandve 2013; Vigeland *et al.* 2013), evolution of seasonally-driven flowering is regarded an important adaptation that enabled Pooideae to inhabit and diversify into temperate zones (Preston & Kellogg 2008; Ream *et al.* 2012; McKeown *et al.* 2016; Zhong *et al.* 2018). In fact, the findings of McKeown *et al.* 2016 suggest that the most recent common ancestor of Pooideae grasses possessed a vernalisation-cued network of flowering genes, a trait enabling the subsequent evolution of vernalisation response *per se*. This is regarded a key event in the evolution of temperate grasses, which is posited to have contributed markedly to the poleward expansion of the Pooideae lineage (Fjellheim *et al.* 2014).

Vernalisation regulon in grasses. Owing to the agricultural and ecological importance of many Pooideae grasses like wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), vernalisation response is particularly well-studied in this lineage. Many Pooideae species are identified as vernalisation responsive based on their flowering behaviour (Heide 1994). Moreover, the vernalisation pathway is characterised on a molecular level in several temperate cereals (Trevaskis *et al.* 2007), forage grasses (e.g. *Lolium perenne*; Andersen *et al.* 2006) and the model grass *Brachypodium distachyon* (Schwartz *et al.* 2010; Ream *et al.* 2014). According to the current model for vernalisation response in Pooideae, flowering is controlled by a genetic circuit involving the mutual regulation of three central genes called *VERNALIZATION1–3* (*VRN1–3*; Trevaskis *et al.* 2007; Dennis & Peacock 2009; Greenup *et al.* 2009; Bouché *et al.* 2017). After exposure to prolonged periods of cold, flowering is promoted by up-regulation of the *Arabidopsis thaliana FLOWERING LOCUS T* (*FT*) orthologue *VRN3* in leaves, followed by the long-distance transport of VRN3 to the shoot apex (Yan *et al.* 2006; Turck *et al.* 2008; Zeevaart 2008; Lv *et al.* 2014). During autumnal growth of vernalisation-responsive species, transcription of *VRN3* is repressed by the action of the *CONSTANS*-like gene *VRN2*, which is induced by long days and impedes flowering before winter (Yan *et al.* 2004; Szűcs *et al.* 2007; Ream *et al.* 2014). During long-lasting cold, expression of *VRN1* increases drastically (Danyluk *et al.* 2003; Trevaskis *et al.* 2003; Yan *et al.* 2003; Hemming *et al.* 2008; Oliver *et al.* 2009; Shimada *et al.* 2009). *VRN1* encodes a *FRUITFULL*-like (*FUL*-like) MADS-box transcription factor that represses *VRN2* (Gu *et al.* 1998; Yan *et al.* 2004; Shimada *et al.* 2009; Woods *et al.* 2016). Production of VRN1 is, in turn, elicited by cold-induced histone modifications at the *VRN1* locus, which links the perception of winter with the acquisition of flowering competency (Distelfeld *et al.* 2009; Oliver *et al.* 2009, 2013; Deng *et al.* 2015). The sig-

nal is further enhanced by mutual positive feedback between the expression of *VRN1* and *VRN3* (Shimada *et al.* 2009). Presence of VRN3 at the shoot apex after phloem translocation results in the achievement of flowering competency by triggering floral transition of the SAM from vegetative to reproductive state (Preston & Kellogg 2008; Sasani *et al.* 2009; Trevaskis 2010). Following up-regulation of *VRN1* and vernalisation-induced release of *VRN3*, maturation and emergence of reproductive shoots is initiated by critical day lengths. Long days increase the expression of *VRN2* in leaves and *VRN1* the shoot (Dubcovsky *et al.* 2006; Trevaskis *et al.* 2006; Distelfeld *et al.* 2009; Higgins *et al.* 2010). Thus, repression of *VRN3* is reinforced in leaves, while *VRN1* continues to promote flowering in proximity to the SAM (Yan *et al.* 2003; Dubcovsky *et al.* 2006; Higgins *et al.* 2010; Ream *et al.* 2014).

MOTIVATION. Although some PACMAD species are found at high latitudes, little is known about the impact of vernalisation on flowering time in temperate grasses outside the subfamily Pooideae. Nonetheless, there is some experimental evidence for the presence of vernalisation response in a few grass species from the PACMAD clade. Evans & Knox (1969) report that in some temperate, long day-responsive ecotypes of *Themeda triandra* (Panicoideae), flowering is hastened after vernalisation treatment. Results from a growth experiment carried out on several populations of *Rytidosperma caespitosa* suggest that also some Danthonioideae may be able to accelerate flowering following exposure to long-term cold (Hodgkinson & Quinn 1978). However, no attempts have hitherto been made to detect vernalisation responsiveness across a wider spectre of temperate PACMAD lineages by increasing taxonomical resolution and screening a greater number of phylogenetically disparate species.

Assuming that seasonally controlled flowering is common in temperate PACMAD lineages, it is of interest to test if these species harness the same molecular machinery to mediate vernalisation-induced meristem transition as cold-adapted species belonging to the BOP lineage (i.e., Pooideae). Recurrent co-option of paralogous genes is a common evolutionary avenue for the evolution of complex physiological traits, such as for example C_4 photosynthesis (Christin *et al.* 2009), and cold tolerance (Sandve & Fjellheim 2010). Regarding the evolution of reproductive development in plants, transcription factors from the MADS-box family of genes are believed to play a key role by serving as a genetic toolkit for the evolution of novel regulatory pathways, including seasonally-cued flowering (Theißen 2001; Trevaskis *et al.* 2003; Schilling *et al.* 2018). As outlined above, the flowering response caused by exposure to long-term cold is conferred by a *FUL*-like MADS-box transcription factor in Pooideae grasses (Danyluk *et al.* 2003; Yan *et al.* 2003). Diversity of *FUL*-like genes is the result of several duplication events during the evolutionary history of monocots

and grasses. The first duplication gave rise to the *FUL3* lineage which is sister to the *FUL1* and *FUL2* clades that resulted from a whole-genome duplication event at the base of the grass family (Litt & Irish 2003; Paterson *et al.* 2004; Grahan *et al.* 2006; Preston & Kellogg 2006; Shan *et al.* 2007). All *FUL* genes are expressed in the shoot apical meristem during floral transition (Gu *et al.* 1998; Ferrándiz *et al.* 2000; Preston & Kellogg 2007; Danilevskaya *et al.* 2008; Kinjo *et al.* 2012), indicating that the specification of meristem and whorl identity may be the ancestral function of the *FUL* gene family. Therefore, it is likely that cold-induced up-regulation of the vernalisation perceptor *VRN1* in leaves is a derived trait. Nevertheless, also increases in *FUL2* transcript abundance during prolonged periods of cold are observed in shoots and meristems of certain Pooideae species like *L. perenne* (Petersen *et al.* 2004, 2006), *Avena sativa* (Preston & Kellogg 2007), *T. aestivum* (Chen & Dubcovsky 2012), and *B. distachyon* (Li *et al.* 2016). Given that this expression pattern resulted from to the propensity of the ancestral *FUL* to evolve a cold-response, it remains to be tested whether *VRN1* was co-opted into the vernalisation pathway in both of the major grass radiations, or if different vernalisation systems have evolved in PACMAD and BOP grasses.

In this study, I aim to test whether temperate PACMAD grasses respond to vernalisation. Many PACMAD species are found at high latitudes in climates experiencing cold winters and marked seasonal variation (Cross 1980). Presence in temperate zones implies some degree of adaptation to the climatic peculiarities characterising high latitude habitats, such as cold tolerance (Humphreys & Linder 2013), or vernalisation-mediated flowering. To determine the presence, degree, or absence of vernalisation response in PACMAD grasses, I conducted a growth experiment under controlled climatic conditions. In total 13 temperate species from the PACMAD clade were subjected to two temperature treatments simulating vernalising and control conditions to screen whether flowering is induced or significantly accelerated after the exposure to prolonged cold. Moreover, I characterise the vernalisation response of one exemplar temperate PACMAD representative on a molecular genetic level. To test which *FUL* homologues could be involved in the putative PACMAD vernalisation pathway, I quantified relative gene expression of the Pooideae vernalisation gene *VRN1* and its closest paralogue *FUL2* in leaf samples collected during the greenhouse experiment in a vernalisation responsive accession of *Molinia caerulea* (Arundinoideae).

Materials & methods

Species selection, growth conditions, and phenotyping

Study species were selected to reflect the phylogenetic diversity and geographical distribution of temperate, perennial PACMAD grasses based on a previous study involving a greenhouse experiment (Atkinson *et al.* 2016). Due to practical limitations, species with expected culm heights >180 cm (e.g. *Phragmites australis*) or species adapted to extreme environments, like salt marches (e.g. *Spartina pectinata*), were not included in the experiment despite their temperate distribution and availability. Seeds for 10 species from in total of 14 accessions were acquired from the USDA Germplasm Resources Information Network (USDA GRIN; Table 1). In addition, two native Norwegian species, *Danthonia decumbens* (Danthonioideae; three populations) and *Molinia caerulea* (two populations), were collected in nature as full-grown plants at four different locations (Table 1; Supplement 1). Moreover, seeds from one *D. decumbens* and two *Pennisetum alupecuroides* (Panicoideae) populations were collected at three locations in Europe (Table 1; Supplement 1).

Imbibed seeds were sown out in humid soil containing equal amounts of compost and peat with a small amount of river sand. To break seed dormancy, trigger and synchronise germination, seeds were stratified at 4 °C for 5 days, followed by 24 h at 25 °C. Stratification took place in dark growth chambers and seeds were covered with plastic foil to keep the soil sufficiently humid. After germination, seedlings were pricked out and transferred to individual pots (diameter 12 cm, volume 3 L). At least 30 plants per population were grown per treatment. In order to synchronise plants grown from seeds and collected in nature, all individuals were pre-grown at 17 °C under long days (16 h light, 8 h darkness) for at least 4 weeks in the greenhouse at Vollebekk forsøksgård, NMBU. Artificial light was supplied in addition to natural light during the light period using MASTER HPI-T PLUS 400W/645 E40 ISL light bulbs (Philips Lighting, Eindhoven, The Netherlands). Shoot apical meristems of the largest plant from every population were dissected prior to vernalisation treatment to ensure that meristem transition from vegetative to reproductive state had not already taken place. None of the plants sacrificed for meristem dissection had initiated floret development, thus allowing me to continue the growth experiment.

Individual plants from each population were divided into two equal groups, consisting of at least 15 individuals each. Plants assigned to vernalisation and control treatment were transferred to two walk-in growth chambers. Two chambers were used per condition. Temperatures were set to 8 °C and 17 °C, respectively. Photoperiod was set to short day with 8 h light and 16 h darkness. In the growth chambers, average irradiance was approximately 65 μmol m^{-2} s^{-1} at plant level. Temper-

Table 1: Overview of the 22 accessions included in the growth experiment, their systematic placement, and approximate geographical origin. Bioclimatic variables BIO4 (temperature seasonality; standard deviation) and BIO11 (mean temperature of the coldest quarter; °C) estimated from the approximate geographic origin using the WorldClim v1.4 data set (Hijmans *et al.* 2005) with 5' resolution. S: grown from seeds, F: collected as full-grown plants in nature.

Species	Subfamily	GRIN ID	Geographical Origin	BIO4	BIO11	S/F
Arundinella hirta	Panicoideae	PI 246756	Japan, Miyagi, Sendai	8.1	2.1	S
Pennisetum alopecuroides	Panicoideae	–	Germany, Markgröningen	6.4	1.2	S
Pennisetum alopecuroides	Panicoideae	–	Spain, Gran Canaria	2.5	16.4	S
Themeda triandra	Panicoideae	PI 206348	South Africa, Eastern Cape	4.9	6.1	S
Themeda triandra	Panicoideae	PI 281968	Australia, New South Wales	5.7	10.5	S
Themeda triandra	Panicoideae	PI 365061	South Africa, Limpopo	3.1	13.0	S
Themeda triandra	Panicoideae	PI 208024	South Africa, Transvaal	2.9	16.9	S
Bouteloua curtipendula	Chloridoideae	PI 476980	USA, South Dakota	11.2	−6.2	S
Bouteloua gracilis	Chloridoideae	PI 591814	USA, South Dakota	10.7	−5.3	S
Bouteloua gracilis	Chloridoideae	W6 50713	USA, Nebraska	10.0	−4.4	S
Bouteloua hirsuta	Chloridoideae	PI 674899	USA, South Dakota	11.3	−6.8	S
Calamovilfa longifolia	Chloridoideae	W6 50718	USA, Nebraska	10.0	−4.4	S
Muhlenbergia montana	Chloridoideae	PI 674945	USA, Colorado	7.6	−10.8	S
Muhlenbergia wrightii	Chloridoideae	PI 674964	USA, Colorado	7.6	−10.8	S
Molinia caerulea	Arundinoideae	–	Norway, Østfold	6.8	−1.4	F
Molinia caerulea	Arundinoideae	–	Norway, Hedmark	7.6	−5.2	F
Danthonia californica	Danthonioideae	W6 40424	USA, Oregon	6.7	−1.8	S
Danthonia decumbens	Danthonioideae	–	Portugal, Cascais	3.4	11.1	S
Danthonia decumbens	Danthonioideae	–	Norway, Østfold	7.6	−5.2	F
Danthonia decumbens	Danthonioideae	–	Norway, Buskerud	7.5	−4.4	F
Danthonia decumbens	Danthonioideae	–	Norway, Sogn og Fjordane	4.3	0.9	F
Danthonia intermedia	Danthonioideae	W6 35433	USA, Utah	8.7	−5.3	S

ature treatment lasted 56 days (8 weeks). Subsequently, all plants were transferred to greenhouse and grown at 17 °C, long days (natural light, supplemented with 16 h artificial light) until the emergence of the first inflorescence ('heading'). During the entire experiment, plants were randomised in the greenhouse and growth chambers to minimise room effects. Heading date was recorded approximately every second day. Days to heading (DTH) were calculated from the date plants were transferred into the growth chambers/beginning of the vernalisation treatment.

In order to account for differential growth in the vernalised and control plants, heading date 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 & Emin 1969), it was presumed that plants in control treatment (17 °C) accumulated 2.125 times more heat units than vernalised plants, given a growth baseline below 8 °C. DTH for vernalised plants were thus corrected in the following manner

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

where DTH_C are corrected days to heading, L the length of the vernalisation period (56 days), T_C the temperature for the control group (17 °C), and T_V the vernalisation temperature (8 °C) (Kirby *et al.* 1989; Baloch *et al.* 2003; McKeown *et al.* 2016). In that way, vernalisation days were heat unit-corrected by subtracting 29.68 days from DTH.

Sampling, RNA extraction, and cDNA synthesis

Leaves for RNA extraction were collected at three different time points from two of the focal species, *D. decumbens* and *M. caerulea*. Samples were taken the day vernalisation treatment began prior to moving the plants into the growth chambers (day 0), after six weeks of vernalisation (day 44), and the day plants were transferred back to the greenhouse (day 56). Sampling was conducted at zeitgeber time 3 (ZT3; i.e., 3 hours after lights on). Leaf tissue was retrieved from the longest leaf, flash-frozen in liquid nitrogen, and stored at -80 °C in 2.0 mL microcentrifuge tubes until RNA extraction. Prior to RNA isolation, leaf tissue was disrupted and homogenised using a TissueLyser II bead mill and 3 mm tungsten carbide beads (both QIAGEN, Hilden, Germany). Total RNA was purified using a RNeasy Plant Mini Kit (QIAGEN Sample & Assay Technologies, Sweden), following the manufacturer's instructions, including the additional centrifugation and elution step. From these extracts, complementary DNA (cDNA) was synthesised using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) following the protocol provided by the manufacturer. Isolated RNA was stored at -80 °C and cDNA at -20 °C in 1.5 mL nuclease-free microcentrifuge vials.

Target gene isolation

The target genes *VRN1* and *FUL2* were PCR-amplified from *D. decumbens* and *M. caerulea* cDNA using primers designed by Preston & Kellogg (2006) and McKeown *et al.* (2016) as well as RT-qPCR primers created in this study (Supplement 1, 2 & 4, see below). The resulting amplicons were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA), sub-cloned using the pGEM-T Easy cloning vector system (Promega, Madison, WI, USA), and transformed into chemically competent *Escherichia coli* JM109 cells (Promega, Madison, WI, USA). All steps were performed following the protocol provided by the manufacturer, however using half the reaction volume for the ligation reaction with 1.5 mL 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 in the University of Vermont

Cancer Centre Advanced Genome Technologies Core (Burlington, VT, USA) using SP6 sequencing primers. 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 with default search parameters (Altschul *et al.* 1990; Zhang *et al.* 2000; Camacho *et al.* 2009) prior to further analysis.

Phylogenetic analysis

The identity of target genes isolated from *M. caerulea* and *D. decumbens* was evaluated by Bayesian phylogenetic analysis. Target gene sequences were added to an existing alignment of *FUL1–3* nucleotide sequences from 31 monocot taxa (Preston & Kellogg 2006; McKeown *et al.* 2016). Next, a multiple sequence alignment was generated using the R package `DECIPHER` v2.6.0 (Wright 2015, 2016). 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` v2.3.1 (Schliep 2011; Darriba *et al.* 2012). Gene trees were inferred by Bayesian evolutionary analysis carried out using BEAST v1.7.5 (Drummond & Rambaut 2007; Drummond *et al.* 2012), assuming an uncorrelated, log-normal relaxed clock (Drummond *et al.* 2006). Nucleotide substitution rates were approximated using the GTR + Γ + I model (Hasegawa *et al.* 1985; Tavaré 1986; Yang 1994) according to the results from `modelTest`, and a Yule two-parameter prior (Yule 1925; Gernhard 2008). Heterogeneity of the substitution rate was approximated with four discrete Γ rate categories (Yang 1994). Two chains were run independently on the ORION computer cluster (Centre for Integrative Genetics, NMBU) for 2.0×10^7 generations and sampled every 10 000. generation. Convergence of both chains combined was assessed using `RWTY` v1.0.1 (Warren *et al.* 2017), with 25 % of the trees discarded as burn-in. The tree with maximum clade credibility was visualised with `ggtree` v3.6 (Yu *et al.* 2017).

RT-qPCR

Gene-specific primers were designed to amplify the candidate genes *VRN1* (*FUL1*) and *FUL2* using PRIMER3 v4.1.0 with default settings (Untergasser *et al.* 2012; Supplement 2). Additionally, to assess the acquisition of flowering competency, *VRN3* was quantified using primers from McKeown *et al.* (2016) (Supplement 4). Two housekeeping genes, *EF1α* and *UBQ5*, served as references for the relative quantification and were amplified using primers designed by McKeown *et al.* (2016) (Supplement 4). Amplicon identity of target and reference genes was confirmed by sequencing. Primer efficiencies were determined using a 2-fold dilution series (Schmittgen & Livak 2008), start-

ing with a 1:10 cDNA dilution. Amplification efficiencies were between 0.90 and 1.10 for all primer pairs (cf. Pfaffl 2001; Bustin *et al.* 2009). Efficiencies of primers designed to quantify *DdVRN1* did, however, not fall within the desired range, likely due to the presence of multiple *DdVRN1* alleles and/or splice variants in the *D. decumbens* material. Therefore, *D. decumbens* was excluded from further RT-qPCR analysis.

Gene expression in was quantified on an Applied Biosystems 7500 Fast instrument (ThermoFisher Scientific, Waltham, MA, USA) using the Applied Biosystems SYBR Select Master Mix (ThermoFisher Scientific, Carlsbad, CA, USA) with a total reaction volume of 20 µL per well (Supplement 5). Quantification was carried out on five biological replicates (four replicates for week 6, vernalised and week 8, control), with three technical replicates per biological replicate. 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 normalised between 0 and 1, and smoothed using a 3-point Savitzky–Golay filter (Savitzky & 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 (C_q *sensu* Bustin *et al.* 2009) were determined by calculating the second derivative centre (geometric mean of the second derivative minimum and maximum) of the normalised, smoothed and baseline-corrected amplification curves (Tellinghuisen & Spiess 2014; Rödiger *et al.* 2015). Average 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 normalised 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 efficiency of their corresponding primers.

Statistical testing

Computations and statistical analyses were carried out in R v3.4.3 (R Core Team 2017). All statistical tests were carried out on an $\alpha = 0.05$ level of significance. Non-normal distributed data was contrasted performing Mann–Whitney *u*-tests, a two-sample variant of the Wilcoxon rank-sum test (Mann & Whitney 1947; Blair & Higgins 1980). In cases where the normality criterion was not violated, statistical testing was carried out using Student's *t*-test or Welch's two-sample *t*-test (Student 1908; Welch 1947). Variance ratios in flowering data were tested using the χ^2 statistic developed by Bartlett (1937) rather than F, since the F-test of equality of variances is sensitive to non-normality (Snedecor & Cochran 1989), like for instance in the highly aggregated heading data.

Results

Identification of vernalisation-responsive species

In total 22 accessions from 13 PACMAD species occurring in the temperate zone were surveyed (Table 1). Statistical analysis on 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 Figure 1). In 11 of these 12 accessions, vernalised plants flowered significantly earlier ($P < 0.05$, Mann–Whitney u-test) than non-vernalised plants (Figure 1). One population of *Themeda triandra* (Panicoidae) originating from Eastern Cape, South Africa (PI 206348; Table 1) was the only flowering accession that did not respond significantly to vernalisation (*'ZA1'* in Figure 1). The strongest response to vernalisation in means of DTH_C was observed in *Bouteloua gracilis* (Chloridoideae) and one population of *M. caerulea* collected as full-grown plants in Hvaler, south-eastern Norway (*'HV'* in Figure 1). In three of the flowering species/populations (*B. gracilis*, *M.caerulea 'HV'*, *T. triandra 'NSW'*; Figure 1), variation in heading date was significantly different between non-vernalised and vernalised plants ($P < 0.05$, Bartlett's χ^2-test).

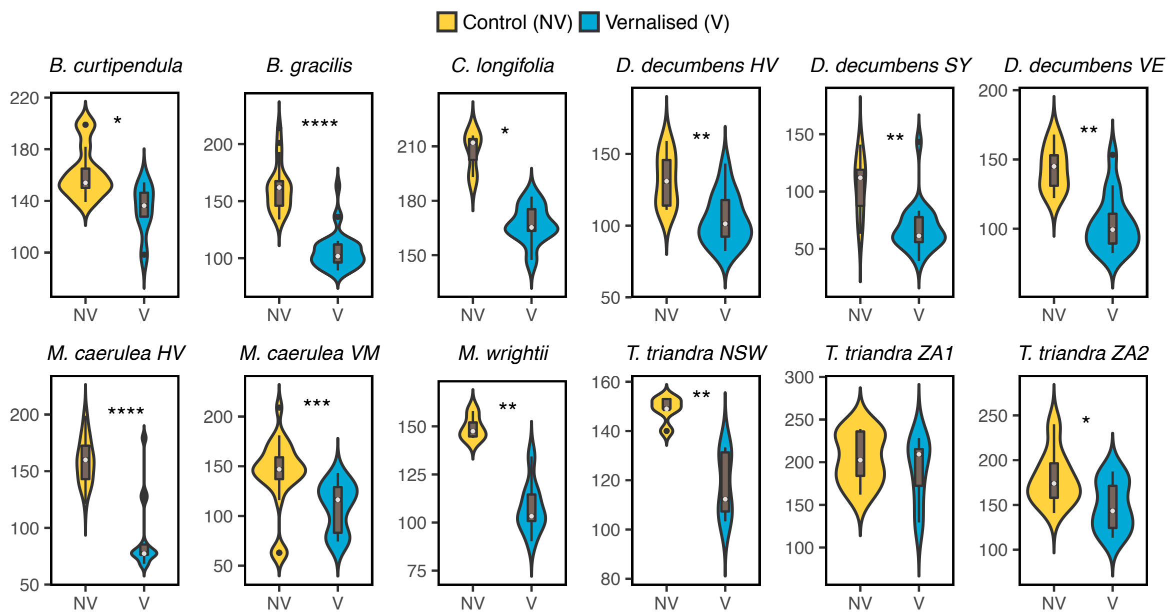

FIGURE 1: Flowering behaviour of 12 PACMAD accessions subjected to two different temperature treatments, measured in heat unit-adjusted days to heading (DTH_C). Coloured areas represent the density of the data and are scaled to resemble sample size, i.e. percentage of flowering plants. Grey rectangles 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 further analysis. *Significance levels*: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (Mann–Whitney u-test).

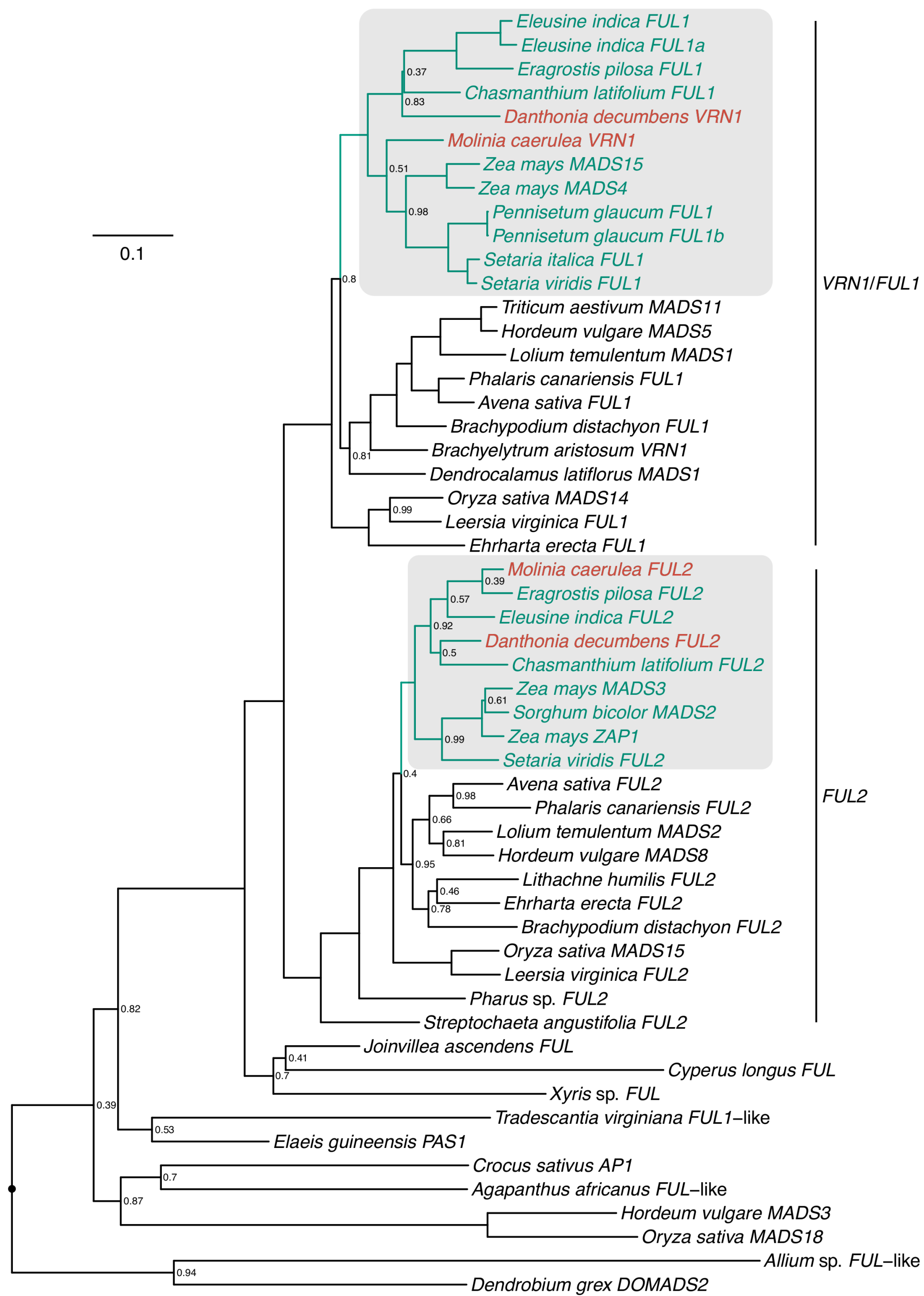

FIGURE 2: Maximum clade credibility tree of *FUL*-like genes from 33 different monocot taxa inferred with BEAST. Branch length scaled to resemble nucleotide substitution rate; node heights rescaled to reflect posterior mean node heights for the clades. Taxa belonging to the PACMAD clade highlighted in green, newly generated sequences highlighted in red. Numbers at the nodes denote $PP < 0.99$.

Candidate gene identity

Partial coding sequences for *VRN1* and its paralogue *FUL2* were obtained for two of the study species (*D. decumbens* and *M. caerulea*). Sequences of *D. decumbens FUL2* (*DdFUL2*) and *M. caerulea VRN1* (*McVRN1*) transcripts were isolated by bacterial plasmid sub-cloning and subsequently sequenced from the vector. This yielded one 400 bp nucleotide sequence per gene. 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) (Supplement 4). This approach resulted in the amplification of smaller *McFUL2* and *DdVRN1* regions relative to *DdFUL2* and *McVRN1*. Thus, a 334 bp sequence of *DdFUL2* was isolated, in addition to a short, 115 bp *McVRN1* amplicon.

Identity of newly generated *M. caerulea FUL*-like nucleotide sequences was confirmed by generating a gene tree using Bayesian evolutionary analysis. Putative *VRN1* and *FUL2* sequences were nested within a monophyletic clade together with orthologues from other PACMAD taxa (Figure 2). The topology of the inferred gene tree is largely congruent with the results of Preston & Kellogg (2006), whose multiple sequence alignment served as the basis for the phylogenetic analysis. Consistent with previous findings (Preston & Kellogg 2006; McKeown *et al.* 2016), strong support for a gene duplication event at the base of the Poaceae giving rise to the paralogues *VRN1* and *FUL2* is found in the inferred gene tree. Within the *VRN1* clade, the division of the grass family into early-diverging and 'crown Poaceae' (BOP & PACMAD) is evident and well supported (*PP* = 0.8; Figure 2). The division into lineages above subfamily-level received less support in the *FUL2* lineage (*PP* = 0.4; Figure 2). Nevertheless, PACMAD taxa formed a distinct, monophyletic clade. Despite their relatively short length, alleged *VRN1* and *FUL2* sequences isolated from *M. caerulea* and *D. decumbens* were placed with other PACMAD taxa within the predicted clade (Figure 2). Successful identification of these candidate genes permitted to carry on with the design of the mRNA quantification assay.

Gene expression in response to vernalisation

In order to test candidate gene expression during temperature treatment, transcription profiles of the paralogues *VRN1* and *FUL2* in addition to the *FT*-homologue *VRN3* were determined by RT-qPCR in one population of *Molinia caerulea*, a temperate PACMAD representative from the grass subfamily Arundinoideae (Soreng *et al.* 2015, 2017). This population with pronounced vernalisation response ('*VM*'; Figure 1) yielded RNA extracts of particularly good quality, rendering it a suitable substrate for gene expression analysis. Since I was not able to design suitable primers

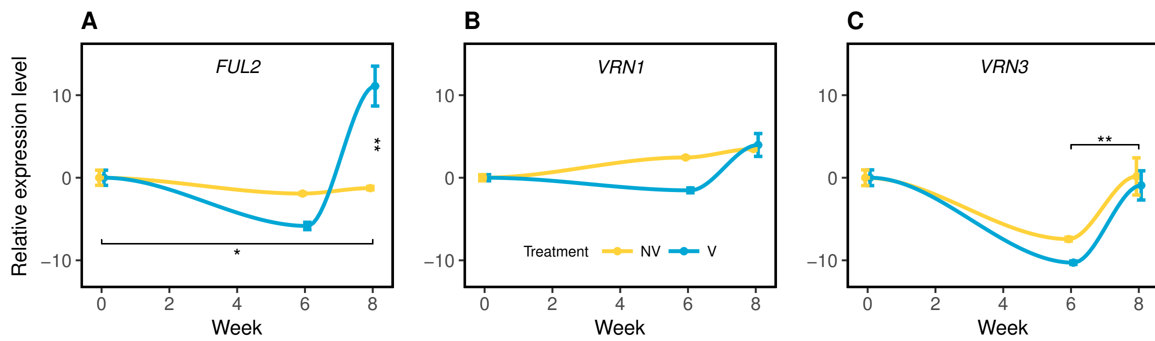

Figure 3: Relative expression levels of [A] *FUL2*, [B] *VRN1*, and [C] *VRN3* in the temperate grass *Molinia caerulea* (Arundinoidae) under cold (V) and control (NV) conditions. *Significance levels*: * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test). Error bars are standard errors of the mean.

for the quantification of *DdVRN1* transcripts, no RT-qPCR was carried out for *D. decumbens*.

In vernalised plants, relative transcript abundance of *FUL2* was significantly higher at the end of the vernalisation period compared to the first time point ($P = 0.024$, $t = 2.54$, $df = 19.3$; Figure 3A). Between week 0 and 6, *FUL2* transcription declined. This decrease was, however, not statistically significant relative to the first time point and non-vernalised plants. Relative *FUL2* mRNA abundance was significantly higher in vernalised relative to non-vernalised plants at the end of the the temperature treatment ($P = 1.762 \times 10^{-3}$, $t = 3.69$, $df = 11.1$; Figure 3A). Relative expression of *VRN1* was largely constant during the growth chamber experiment in both, vernalised and non-vernalised individuals (Figure 3B). There was no significant difference of *VRN1* expression between week 0 and week 8 and between treatments at all time points (Student's *t*-test). *VRN1* expression equalised between both treatments towards week 8. Transcript abundance of *VRN3* decreased significantly between week 0 and 6, following the same pattern as *FUL2*, albeit more pronounced ($P < 0.01$ in both treatments). *VRN3* expression increased significantly from week 6–8 in both treatments ($P < 0.01$), approaching the approximate same level as before the start of the temperature treatment (i.e., week 0). For all gene targets, variation in mRNA concentration was greater towards the end of the temperature treatment (Figure 3).

Discussion

Vernalisation responsiveness in PACMAD grasses. Flowering data generated during the growth experiment suggest that temperate grasses outside the Pooideae are responsive to vernalisation, corroborating earlier findings (e.g. Evans & Knox 1969; Hodgkinson & Quinn 1978). Significantly hastened flowering was observed in vernalised individuals of seven species from four different subfamilies (Panicoidae, Chloridoideae, Arundinoideae, and Danthonioideae), suggesting

that vernalisation-cued flowering may be a rather widespread phenomenon in temperate PACMAD grasses. However, considerable differences were evident between different species and populations. In line with previous findings (e.g. Evans & Knox 1969), different ecotypes of the same species had different vernalisation requirements, as observed in *T. triandra* and *M. caerulea*. Whereas vernalised plants of two of the three focal *T. triandra* populations flowered significantly earlier than non-vernalised individuals, no significant difference in flowering time was detected in the *T. triandra* population originating from Eastern Cape, South Africa (Table 1; Figure 1). Given the relatively cold origin of the latter population (BIO11 = 6.1 °C; Table 1), it is possible that the vernalisation temperature (8 °C) was too high to trigger a detectable vernalisation response. However, since many *T. triandra* ecotypes are characterised as short-day plants (Evans & Knox 1969; Evans 1975), it is also plausible that this population has lost its vernalisation response, relying solely on photoperiod to synchronise reproductive output with favourable environmental conditions. Future studies examining the response of various PACMAD species to different combinations of day length and vernalisation treatments will thus be important to disentangle crosstalk between the photoperiodic and vernalisation pathway, as has been done in numerous temperate Pooideae (e.g. Trevaskis *et al.* 2006; Schwartz *et al.* 2010; Ream *et al.* 2014; Bettgenhaeuser *et al.* 2017).

Congruent with findings from vernalisation experiments conducted on several Pooideae species (reviewed in Heide 1994), our data also indicate population-specific vernalisation requirements. Difference in flowering acceleration observed in both *M. caerulea* populations included in the experiment could be an artefact of plants being collected during different life-stages or climatic conditions, or caused by population-specific vernalisation responsiveness. Vernalisation sensitivity has been shown to follow environmental clines in for example *Arabidopsis* (Brassicaceae; Riihimäki & Savolainen 2004; Mitchell-Olds & Schmitt 2006; Lewandowska-Sabat *et al.* 2012; Wollenberg & Amasino 2012), *B. distachyon* (Schwartz *et al.* 2010; Tyler *et al.* 2016), and *Phleum pratense* (Poaceae: Pooideae; Fiil *et al.* 2011). In each case, populations originating from coastal environments, distinguished by milder winters, had stronger responses to vernalisation 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 behaviour observed in *M. caerulea*, where the coastal Hvaler population ('*HV*', Figure 3) turned out to have a stronger vernalisation response than cold-treated individuals originating from a slightly more continental habitat (Vestmarka/'*VM*', Figure 3). Allelic variation at different vernalisation loci, such as *VRN1*, are known to determine different vernalisation phenotypes ranging from non-responsive to vernalisation-responsive to obligate vernalisation-requiring cultivars/accessions in

temperate cereals and *B. distachyon* (Szűcs *et al.* 2007; Trevaskis *et al.* 2007; Rhoné *et al.* 2010; Schwartz *et al.* 2010). It would thus be of interest to test whether allelic differences in *McFUL2* could account for the distinct flowering behaviours observed in the two *M. caerulea* populations.

Genetic architecture of pacmad vernalisation response. Ancient angiosperm diversifications within temperate zones are hypothesized to have been facilitated by the repeated evolution of cold adaptations such as vernalisation responsiveness (Ream *et al.* 2012; Preston & Sandve 2013; McKeown *et al.* 2016; Woods *et al.* 2016). Experimental evidence from this study indicates that niche transition of now temperate pacmad lineages might have been facilitated by the evolution of a flowering gene network responsive to long-term cold. Despite their inferred independent origins, results from the relative gene expression analysis suggest that the vernalisation pathways of pacmad grasses involve genes homologous to Pooideae vernalisation genes.

Interestingly, vernalisation seems to affect flowering in *M. caerulea* through the up-regulation of a *FUL*-like gene that is paralogous to the temperate Pooideae vernalisation gene *VRN1* (*FUL1*). 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 vernalisation responsive in other tissues (specifically the sam), my data currently support the independent recruitment of grass-specific *FUL*-like paralogues into a vernalisation-mediated flowering pathway. Differential neofunctionalisation is a plausible evolutionary avenue that would explain such a scenario (Hughes 1994; Force *et al.* 1999; He & Zhang 2005). The reason for the propensity of *FUL*-like genes to evolve cold-responsiveness might stem from their ancestral function(s). In the case of *VRN1* and *FUL2*, the pre-duplication ancestor is inferred to have been involved in determining floral meristem identity (Theißen *et al.* 1996; Gu *et al.* 1998; Litt & Irish 2003; Preston & Kellogg 2007). Comparative RNA *in situ* hybridisation indicates that all *FUL*-like genes are strongly expressed in spikelet and floret meristems (e.g. Gocal *et al.* 2001; Preston & Kellogg 2007; Danilevskaya *et al.* 2008; Preston & Kellogg 2008; Ergon *et al.* 2013; Li *et al.* 2016), emphasising their conserved, ancestral role in determining inflorescence meristem identity (Preston & Kellogg 2007; Preston *et al.* 2009). In bop grasses, *VRN1* is expressed in all floral whorls of the inflorescence meristem and postulated to specify overall meristem identity (e.g. Moon *et al.* 1999; Gocal *et al.* 2001; Preston & Kellogg 2007; Kinjo *et al.* 2012), congruent with e-class transcription factors in the abcde model of floral development (Theißen 2001; Murai 2013; Callens *et al.* 2018). On the other hand, *FUL2* is proposed to exert a more specific function by regulating the differentiation of whorl-primordia into particular anatomical structures in numerous species (Gu *et al.* 1998; Ferrándiz *et al.* 2000; Gocal *et al.* 2001; Preston & Kellogg 2007,

2008), supporting the hypothesis that the primordial *FUL*-like gene was involved in mediating meristem transition (Preston *et al.* 2009), and that the cold-induced up-regulation and subsequent co-option of *VRN1* into the Pooideae vernalisation pathway is a derived trait (Preston & Kellogg 2007; Preston *et al.* 2009; Li *et al.* 2016; McKeown *et al.* 2016). Duplication and expansion of *FUL*-like genes at the base of the Poaceae led to sub- and neofunctionalisation among *FUL*-like paralogues, resulting in distinct expression patterns and developmental roles of *VRN1* and *FUL2* during inflorescence development (Preston & Kellogg 2007; Preston *et al.* 2009). Up-regulation of *FUL2* in *M. caerulea* leaves during vernalisation might thus be another example of independent recruitment of closely related genes as a key mechanism for the convergent evolution of complex physiological adaptations, similar to the parallel co-option of paralogous genes in convergent evolution of cold tolerance (e.g. Sandve & Fjellheim 2010), and C_4 photosynthesis (e.g. Christin *et al.* 2009). A probable reason for preferential recruitment of *FUL*-like genes into the vernalisation systems of different grasses might be that the ancestral gene was highly expressed in response to cold. It has been shown that high transcript abundance facilitates the co-option of genes into adaptive physiological pathways (Moreno-Villena *et al.* 2018). It is thus possible that *FUL2* evolved a cold response prior to its recruitment into the *M. caerulea* vernalisation regulon, mirroring the scenario inferred for *VRN1* in Pooideae (McKeown *et al.* 2016). This hypothesis could be tested by profiling the expression of *FUL2* during vernalisation in more PACMAD species and use these data to infer the ancestral state of *FUL2* expression during cold at the base of the clade.

An interesting aspect of our study warranting further investigation is the timing of *FUL2* recruitment into the PACMAD vernalisation pathway. In Pooideae, the regulon perceiving, amplifying, and transmitting the vernalisation signal is mostly conserved (although, see Woods *et al.* 2017), and is posited to have evolved early in the subfamily (McKeown *et al.* 2016). Given that the majority of PACMAD taxa are adapted to tropical and sub-tropical conditions, vernalisation response likely evolved independently in different temperate PACMAD lineages concomitant with their transition to seasonal habitats. The split between BOP and PACMAD grasses is estimated to have occurred approximately 54.9 (± 7) Ma ago (Christin *et al.* 2014; Cotton *et al.* 2015), thus predating the seasonality increase in high latitudes during the Eocene–Oligocene boundary (Eldrett *et al.* 2009) that likely triggered the evolution of vernalisation response in Pooideae (Ream *et al.* 2012; Preston & Sandve 2013; Fjellheim *et al.* 2014; McKeown *et al.* 2016). Rather, the BOP–PACMAD partition coincides with a period of marked changes in temperature during the Paleocene–Eocene thermal maximum (Kennett & Stott 1991; Zachos *et al.* 2005; Cotton *et al.* 2015), which makes the early evolution of vernalisation response at the base of the PACMAD clade unlikely. This opens up

for the possibility that *FUL2* recruitment into the *M. caerulea* vernalisation regulon happened at the tip of the lineage as an adaptation to increased seasonality, hence independent of the *VRN1* co-option in Pooideae. Absence of *FUL2* in the leaf and shoot transcriptome of *Arundo donax* (Arundinoideae; Sablok *et al.* 2014; Barrero *et al.* 2015; Evangelistella *et al.* 2017), a close relative to *M. caerulea*, either indicates that vernalisation genes have been lost or pseudogenised in other PACMAD lineages, or that vernalisation responsiveness has evolved multiple times harnessing different genetic mechanisms, thus supporting the hypothesis of multiple independent origins of vernalisation response in PACMAD grasses.

Day length possibly also influences the expression of flowering genes in *M. caerulea*. During chilling and short day-treatment, the expression of the flowering promoter *VRN3* decreased in both, non-vernalised and vernalised plants before rising back to its initial level. This could be interpreted as a signature of interaction between the photoperiodic and vernalisation-mediated flowering pathway, meaning that flowering is actively repressed under sub-optimal photoperiods (e.g. short days; Dubcovsky *et al.* 2006; Trevaskis *et al.* 2006; Hemming *et al.* 2008). Increase in *VRN3* transcript abundance might thus be triggered by low levels of *FUL2* present in vernalised and non-vernalised plants. However, an important limitation to this study is the lack of expression data for vernalisation genes immediately after vernalisation treatment, under warm conditions and long days. If *FUL2* triggers flowering in a fashion similar to *VRN1* in Pooideae, it might be possible that *VRN3* mRNA levels rise to a higher level in vernalised plants than in controls after vernalisation under long days. However, this postulate is not supported by experimental evidence and should be focus of future experiments including pre- and post-vernalisation samples.

Concluding remarks

Taken together, my results provide a basis for the further evolutionary and functional analysis of vernalisation response and its underlying genetic machinery in PACMAD grasses. I found strong evidences for the presence of a genetic switch accelerating flowering following vernalisation in one PACMAD species (*M. caerulea*, Arundinoideae), indicating that representatives from this subfamily are adapted to habitats characterised by pronounced seasonality. Moreover, vernalisation response was detected in species from three other lineages (Panicoideae, Chloridoideae, Danthonioidae), suggesting that similar genetic adaptations may be found in other PACMAD species. In addition to other accommodations to cool climates (like cold tolerance in Danthonioideae; Humphreys & Linder 2013), presence of a seasonally-cued network of flowering genes may contribute explaining

the occurrence and persistence of certain PACMAD grasses in temperate habitats.

Comparative analysis of the entire vernalisation regulon across the PACMAD clade would reveal if the responses evident from my data share a common evolutionary origin, or whether different mechanisms have evolved in different lineages. It would therefore be interesting to assess the evolutionary history of *VRN2*- and *VRN3*-homologues in the PACMAD clade and quantify their expression in response to vernalisation in different tissues. Furthermore, it would be of interest to measure the vernalisation sensitivity of selected PACMAD species by treating plants with different combinations of vernalisation temperatures and periods. Many grasses are short-day plants, meaning that future studies should choose experimental approaches accounting for differences in day length responses.

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Supplementary information

Supplement 1: Sampling locations for *Molinia caerulea*, *Danthonia decumbens*, and *Pennisetum alupecuroides* (WGS 84). Life-stage: s: seeds, f: full-grown.

Species	Subfamily	Latitude	Longitude	Country	Location	Stage
Pennisetum alopecuroides	Panicoideae	48.90348	9.08654	Germany	Markgröningen	S
Pennisetum alopecuroides	Panicoideae	27.81924	−15.57880	Spain	Gran Canaria	S
Molinia caerulea	Arundinoideae	59.08860	11.03807	Norway	Hvaler	F
Molinia caerulea	Arundinoideae	59.94413	11.99221	Norway	Vestmarka	F
Danthonia decumbens	Danthonioideae	38.74711	−9.42143	Portugal	Cascais	S
Danthonia decumbens	Danthonioideae	59.93588	11.99152	Norway	Hvaler	F
Danthonia decumbens	Danthonioideae	59.90340	10.29282	Norway	Sylling	F
Danthonia decumbens	Danthonioideae	61.49050	5.39508	Norway	Vevring	F

Supplement 2: Primers used for target gene isolation from complementary DNA (cDNA) by polymerase chain reaction (PCR).

Name	Sequence (5'–3')	Direction	Gene	Reference
VRN1_34F	GAGAACAAGATCAACCGSCAGGT	F	*McVRN1*	McKeown *et al.* 2016
VRN1_478R	TTATTCTCCTCCTGCAGTGA	R	*McVRN1*	
ZAP4F	ATCTCCGTSCTCTGYGA	F	*DdFUL2*	Preston & Kellogg 2006
ZAP1oneR	GARGKKGCTCAGCATCCAT	R	*DdFUL2*	

Supplement 3: PCR program for the isolation of target gene sequences using Bullseye Red Taq DNA Polymerase Master Mix (Midwest Scientific, Valley Park, MO, USA).

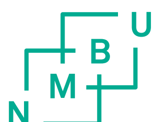
Stage	Temperature	Duration (min)	Cycles
Activation	94 °C	5:00	1
Denaturation	94 °C	0:30	40
Annealing	55 °C	0:30	
Extension	72 °C	10:00	
Completion	72 °C	5:00	1
Hold	4 °C	–	∞

Supplement 4: Primers used for target gene quantification by RT-qPCR.

Name	Sequence (5'–3')	Direction	Gene	Note/Reference
McVRN1_29F	GTCGCGCTCATCATCTTCTC	F	*VRN1*	used to isolate *DdVRN1*
McVRN1_190R	TCTATATTCGTGGCGCCAGT	R	*VRN1*	
DdFUL2_F212	ATTCGTGGCACCAATTTCCC	F	*FUL2*	used to isolate *McFUL2*
DdFUL2_R363	CGTCATCGTCTTCTCCCCAA	R	*FUL2*	
McVRN3.F	GCGGACATTCTACACACTCG	F	*VRN3*	McKeown *et al.* 2016
McVRN3.R	GGATCCCCATGGTCGGAC	R	*VRN3*	
GrassUBQ5F	CGCCGACTACAACATCCAG	F	*UBQ5*	McKeown *et al.* 2016
GrassUBQ5R	TCACCTTCTTGTGCTTGTGC	R	*UBQ5*	
EF1α_594F	GTGACAACATGATTGAGAGG	F	*EF1α*	McKeown *et al.* 2016
EF1α_1064R	AGGTGTGGCAGTCCAGCACTG	R	*EF1α*	

Supplement 5: Protocol for mRNA quantification by RT-qPCR using the Applied Biosystems (ABI) SYBR Select Master Mix and an ABI 7500 Fast instrument.

Stage	Temperature	Duration (min)	Cycles
Hold	50 °C	2:00	1
Activation	95 °C	2:00	
Denaturation	94 °C	0:03	40
Annealing	58 °C	0:05	
Detection	60 °C	0:36	
Melt Curve Detection	95 °C	0:15	–
	60 °C	1:00	
	95 °C	0:15	
	60 °C	0:15	



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