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Differential expression of *VRN1* and other MADS-box genes in *Festuca pratensis* selections with different vernalization requirements

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Abstract

Most perennial and winter annual temperate grasses have a vernalization requirement (VR) for flowering, that is, they require a cold period before they can flower in response to long days. From a F1 mapping population of the outbreeding perennial forage grass *Festuca pratensis* Huds. (meadow fescue) previously used to map several quantitative trait loci (QTLs) for VR, we produced two F_2 populations divergently selected for high or low VR. The two populations were characterised for flowering behaviour and gene expression of *VRN1* as well as other MADS-box genes with a putative function in the induction of flowering. Expression of *FpVRN1* and the *VRN1*-like genes *FpMADS2* and *FpMADS3* was associated with flowering but the response of gene expression to vernalization differed between genes and populations. The expression of the *SVP*-like genes *FpMADS10* and *FpMADS16* was not affected by vernalization and did not differ between the two F_2 populations.

Additional key words: *FUL*, induction of flowering, *MADS2, MADS3, MADS10, MADS16,* meadow fescue, *SVP,* transition to reproductive development.

Introduction

The transition from vegetative to reproductive development (induction of flowering) is a result of intricate regulatory systems that respond to a variety of internal and external factors to ensure that the timing of flowering maximises fitness or yield (reviewed by King and Heide 2009). Many perennials and winter annuals have a vernalization requirement (VR), that is, they require a prolonged cold period before they can flower in response to long days. True annuals do not have a vernalization requirement. *Festuca pratensis* (meadow fescue) and *Lolium perenne* (perennial ryegrass) generally have a requirement for vernalization (Cooper and Calder 1964, Bean 1970, Heide 1988, 1994) but some accessions and cultivars can flower to a lesser or greater degree without vernalization (Bean 1970, Aamlid *et al*. 2000, Ergon *et al*. 2006).

At the molecular level, two main regulatory pathways leading to the transition to reproductive development have been characterised in cereals – the vernalization pathway and the photoperiod pathway (see Distelfeld *et al*. 2009, Greenup *et al*. 2009, Higgins *et al*. 2010, Trevaskis 2010, Hemming and Trevaskis 2011 for recent reviews). One of the major genes controlling vernalization-induced flowering in cereals is *VERNALIZATION 1 (VRN1). VRN1* is located on *Triticeae* group 5 chromosomes and is similar to *Arabidopsis thaliana APETALA1 (AP1), CAULIFLOWER (CAL)* and *FRUITFULL (FUL)* which are MADS-box transcription factors and meristem identity genes (Yan *et al*. 2003). *VRN1* is a major determinant of winter/spring growth habit in cereals. In *Triticum monococcum* (einkorn wheat) *VRN1* may be essential for flowering – a mutant with a deletion of the *VRN1* promoter and coding region remains in the vegetative phase indefinitely (Shitsukawa *et al*. 2007). *VRN1* expression is generally upregulated at the time of transition to reproductive development of the shoot apex regardless of whether that transition occurs in response to

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Abbreviations: DTH - days to heading; *FUL - FRUITFULL*; *GAPDH - GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE*; PCR - polymerase chain reaction; PHS - proportion of heading shoots; QTL - quantitative trait loci; RT - reverse transcription; SNP - single nucleotide polymorphism; *SVP - SHORT VEGETATIVE PHASE*; VR - vernalization requirement.
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vernalization or long days. Neither the mechanism by which *VRN1* leads to transition to reproductive development nor the mechanism behind repression of *VRN1* prior to vernalization in vernalization-requiring plants is fully understood. Alternative models of how *VRN1* interact with other genes to regulate vernalization-induced flowering in cereals have been proposed (*e.g*. Distelfeld *et al*. 2009, Greenup *et al*. 2009, Higgins *et al*. 2010, Trevaskis 2010).

Among forage grasses QTL-mapping of vernalization requirement (VR) has been conducted in *F. pratensis* (Ergon *et al*. 2006) and *L. perenne* (Jensen *et al.* 2005). Both these studies identified a QTL on chromosome 4 that co-located with *VRN1*. In *F. pratensis*, *VRN1* was located in a region which according to Alm *et al*. (2003) is syntenic to the *VRN1*-containing region on *Triticeae* chromosome 5. Gene expression patterns have indicated that *VRN1* is involved in the vernalization response in *L. perenne* (Andersen *et al*. 2006, Petersen *et al*. 2006) and also in the transition to reproduction induced by long days in *L. temulentum* (darnel), a species that does not require vernalization (Gocal *et al*. 2001).

There are several *VRN1*-like genes found in the grass family. These genes have been divided into the three subclades *FUL1*, *FUL2*, and *FUL3*, where *VRN1* homologues are members of the *FUL1* subclade (Preston and Kellogg 2006). Expression studies have indicated that members of *FUL1* and *FUL2* may have redundant functions in vernalization-induced transition to flowering in *Avena sativa* but not in *T. monococcum,* the latter result being consistent with the fact that *VRN1* is essential for flowering in *T. monococcum* (Preston and Kellogg 2007, 2008). Members of *FUL2* and *FUL3* have been characterised in *L. temulentum* and *L. perenne*, namely the *FUL1*-members *LtMADS1* and *LpMADS1* (=*LpVRN1*) and the *FUL2*-members *LtMADS2* and *LpMADS2* (Gocal *et al*. 2001, Petersen *et al*. 2004). These genes are expressed weakly in vegetative apices and upregulated after vernalization and/or long-day treatment depending on VR. In *L. temulentum*, some variation in the spatial expression pattern within the apex was observed suggesting some differences in the function of these two genes. In *L. perenne*, the proteins encoded by *LpMADS1* and *LpMADS2* are able to form homo- and hetero-dimers that can bind to the *LpMADS1* promoter and thus possibly take part in the regulation of *LpMADS1* (Ciannamea *et al*. 2006).

Another group of MADS-box genes that have a putative function in the transition to flowering in the grass family belong to the *SHORT VEGETATIVE PHASE (SVP)*-like (or *StMADS11*-like) MADS-box genes. *SVP*-like genes in *A. thaliana*, *T. aestivum*, and *Hordeum vulgare* act as negative regulators of flowering (Hartmann *et al*. 2000, De Folter *et al.* 2005, Kane *et al.* 2005, Liu *et al.* 2007, Trevaskis *et al.* 2007). The *T. aestivum* *SVP*-like gene *TaVRT2* is down-regulated by vernalization (approx. 6 weeks) and its product interacts with the VRN1 and VRN2 proteins and can bind the CArG-box in the *VRN1* promoter and repress its activity *in vivo* (Kane *et al.* 2005, 2007). Similar results have been reported from *L. perenne* - the *SVP*-like gene *LpMADS10* is repressed during cold exposure (12 weeks) and LpMADS1 (VRN1) and LpMADS10 can form heterodimers of a higher order that bind to the *LpMADS1* promoter (Ciannamea *et al.* 2006, Petersen *et al.* 2006). Based on these findings, it has been proposed that *SVP*-like genes may be involved in regulation of *VRN1* during vernalization. However, it has been shown that the CArG-box element of *TmVRN1* is not necessary for the VR in an accession of *T. monococcum* (Pidal *et al.* 2009). Moreover, Trevaskis *et al.* (2007) found that three *SVP*-like genes in *H. vulgare* were up-regulated by cold (days - 4 weeks) rather than down-regulated and that there were no difference in expression of these genes between control plants and plants that had been vernalized for 9 weeks as imbibed seeds in spite of induction of *VRN1* and flowering in these plants. Overexpression of one of the genes (*BM1*) in transgenic plants caused delayed flowering without affecting *VRN1* expression. It was concluded that in barley, it is unlikely that the *SVP*-like genes mediate derepression of *HvVRN1* during vernalization.

Several species of *Festuca* and *Lolium* are important forage and turf grass species. The two genera are closely related and their genomes have a high level of synteny (Alm *et al.* 2003, Harper *et al.* 2011). *Festulolium* hybrids have been created with the aim of combining the superior forage quality of *Lolium* with the higher level of stress tolerance in *Festuca*. The role of *VRN1* in induction of flowering may be less dominant in outcrossing grass species than in inbred cereal lines that have been under a strong selection pressure for either winter or spring growth habit. Populations of outcrossing species in genera like *Festuca* and *Lolium* may harbour more ancestral *Pooideae* genetic regulation of the vernalization pathway and studies of these species may therefore provide valuable genetic information. In perennial forage grasses, seasonal yield distribution and forage quality are highly dependent on heading/flowering time and the proportion of reproductive tillers. Also the success of commercial seed production depends on proper flower induction. The aim of the present study was to investigate the potential role of different MADS-box genes in the induction of flowering by studying their gene expression patterns in F_2 populations selected for contrasting VR phenotypes. In addition, we wanted to characterise the genetic control of VR in the original BF14/16 × HF2/7 F_1 mapping population by re-examining genotype-phenotype relationships observed in our previous study (Ergon *et al.* 2006).

Materials and methods

Divergent selections for VR, phenotypic characterisation, and tissue sampling: Individuals from the *Festuca pratensis* Huds. BF14/16 × HF2/7 F_1 mapping population (Alm *et al.* 2003) with extreme phenotypic values for VR (Ergon *et al.* 2006) were selected. Fifteen individuals that were able to flower without vernalization and fifteen individuals that required 12 weeks of vernalization were allowed to intercross in isolation to create two F_2 populations, VRmax and VRmin. Seeds from VRmin and VRmax were seeded in sand in the greenhouse in May 2004. Twenty-two plantlets of each F_2 population were transferred to peat soil, allowed to grow, and kept as clonally propagated "mother plants" from which ramets for the experiments were produced. In Exp. 1, ramets of all genotypes of each F_2 population were grown for 3 weeks and then vernalized for 6, 9, or 12 weeks before being transferred back to long day conditions for secondary induction and flowering. Non-vernalized controls were grown under long days for 5 rather than 3 weeks in order to compensate for growth occurring during vernalization. There were two replicate ramets for each genotype and treatment. One additional ramet per genotype in the 12-week vernalization treatment and in non-vernalized treatment was used for tissue sampling and gene expression analysis (described below). During vernalization, plants were exposed to 6 °C and 8-h photoperiod with irradiance of 70 µmol m^{-2} s^{-1}. Pregrowth and secondary induction was conducted in a greenhouse at 18-h photoperiod. The growth conditions were otherwise as described previously (Ergon *et al.* 2006). The secondary induction took place from the beginning of January 2005. The number of days to heading (DTH) and the proportion of heading shoots (PHS) was recorded as described earlier (Ergon *et al.* 2006). Exp. 2 was done in the same way as Exp. 1 except that the 6 and 9 weeks vernalization treatments were omitted, non-vernalized controls were produced at two different time points in order to account for different growing conditions in the greenhouse during the season, the photoperiod during vernalization was 16 rather than 8 h (due to a technical error), and the secondary induction took place from the end of May 2005. PHS was not recorded in experiment 2. Five genotypes of each F_2 population were selected for bulk analysis of MADS-box gene expression. In order to maximize the difference in VR, the earliest heading and the most abundantly flowering genotypes were selected from the VRmin population. The genotypes from the VRmax population were picked among genotypes that had a 12 weeks vernalization requirement (genotypes that headed after 9 weeks vernalization and genotypes that did not head even after 12 weeks vernalization were excluded). Plant tissue from vernalized plants was sampled at the end of the vernalization period in both experiments. Tissue from non-vernalized plants was sampled in January (Exp. 1), March and May (Exp. 2). Before sampling, the soil was rinsed off in water, the outer leaves were peeled off the individual tillers, and the 3 - 5 mm lower section of the stem bases were excised, frozen in liquid nitrogen, and kept at -80 °C.

Amplification of MADS-box genes from *F. pratensis*: *FpVRN1* had previously been amplified from *F. pratensis* (Ergon *et al.* 2006). *FpMADS2* and *FpMADS3* were amplified from both genomic DNA and cDNA with the primers LpMADS2-fwd, LpMADS2-rev, LpMADS3-fwd and LpMADS3-rev (Petersen *et al.* 2004) and *FpMADS10* was amplified from cDNA with primers LpMADS10-fwd and LpMADS10-rev (Petersen *et al.* 2006). Primers LpMADS16_F1 (5'-TCTAAA TCTACTCATTTGCTCGTG-3') and LpMADS16_R1 (5'-GCGCCAATAAGCTTCTCATAAAT-3') were designed on the basis of the *L. perenne MADS16* sequence (Genbank: DQ110011) and used to amplify *FpMADS16* from cDNA. *Fp*GAPDH was amplified from cDNA using primers LpGAPDH-fwd and LpGAPDH-rev (Petersen *et al.* 2004). The amplified genes were sequenced and the similarity with their *L. perenne* counterparts as well as other known genes was determined through *BLAST* searches at http://www. ncbi.nlm.nih.gov/BLAST/.

RNA extraction, cDNA synthesis, and semi-quantitative PCR: Total RNA was extracted from the individual tissue samples using the *TRIzol* reagent (*Life Technologies, UK*). The RNA concentration was measured in a *NanoDrop 300 N.A.* spectrophotometer (*Thermo Scientific, USA*) and equal amounts of RNA from each of the five selected genotypes were combined for each of the two populations and five sampling dates creating 10 pooled samples. All DNA was removed from the pooled RNA-samples using the *TURBO* DNase treatment and removal system (*Life Technologies, UK*). cDNA was synthesized with the *SuperScript* first-strand synthesis system for RT-PCR (*Life Technologies*) using 1.6 µg of total RNA as template and random hexamers as primers in a 20 mm^3 reaction volume. Control reactions without reverse transcriptase were included. Pooling of RNA samples and cDNA synthesis was performed three times creating three sets of cDNA termed A, B, and C.

Semi-quantitative PCR and visual assessment of ethidium bromide-stained agarose gels were employed to analyse the amount of *FpVRN1, FpMADS2, FpMADS3, FpMADS10*, and *FpMADS16* transcripts in the cDNA samples. Each gene was analysed using one of the cDNA sets and the amount of *FpGAPDH* transcripts served as a control of the amount of cDNA in each sample. A *GAPDH* gene from *L. perenne* with 99 % sequence identity to the sequenced part of *FpGAPDH* has been shown to be a suitable control gene in developmental studies (Martin *et al.* 2008). cDNA (1 mm^3) was used as template in PCR reactions with 200 µM of each dNTP, 0.4 µM forward primer, 0.4 µM reverse primer, 0.05 U mm^{-3} *DyNAzyme Taq* polymerase (*Finnzymes*, Finnland), and 1× PCR buffer in 20 mm^3 reaction

volumes. *FpVRN1* was amplified from cDNA using primers lpVRN1_19 and lpVRN1_344rev (Jensen *et al.* 2005), *FpMADS2, FpMADS3, FpMADS10,* and *FpGAPDH* were amplified with the primers referred to in the previous section. For semi-quantitative PCR of *FpMADS16*, primers FpMADS16_F3 (5'-GTGCGC GTTGAGGTTTCC-3') and FpMADS16_R3 (5'-ACG TAGTTCGCACGAATGAT-3') designed on the basis of the *F. pratensis* sequence, were used to amplify a 165 bp segment of *FpMADS16*. PCR amplifications were performed in a *Mastercycler ep* (*Eppendorf AG*, Hamburg, Germany). For the amplification of *FpGAPDH*, the following cycling parameters were used: denaturation at 94 °C for 4 or 5 min followed by a variable number of amplification cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min. The other genes were amplified using the same cycling parameters except that the annealing times for *FpMADS2* and *FpMADS10* were only 20 s, the extension times for *FpMADS3* and *FpMADS16* were 1 min and 45 s, respectively, and the annealing temperatures for *FpVRN1* and *FpMADS3* were 50 and 61 °C, respectively. The number of cycles was varied in order to ensure that amplification was in the linear phase.

Mapping of *FpMADS3*: A single strand conformation polymorphism (SSCP) marker that detected different alleles of *FpMADS3* in the parents of the *F. pratensis* BF14/16 × HF2/7 F_1 mapping population (Alm *et al.* 2003) was developed. *FpMADS3* was amplified from 25 ng of genomic DNA in 10 mm^3 reaction volumes using 30 amplification cycles and otherwise as described above. PCR products were denatured and electrophoresed on mutation detection enhancement (MDE) gels prepared as described by (Bertin *et al.* 2005) by applying a constant power of 8 W for 5 h. Gels were silver-stained and individuals were recognised as either heterozygous or homozygous. Mapping was performed using *Joinmap 3.0* (Van Ooijen and Voorrips 2001) and previously collected marker data (Alm *et al.* 2003). Since the marker was heterozygous in the male parent HF2/7 only, *FpMADS3* was mapped onto the paternal map. The sequenced segments of *FpMADS10* and *FpMADS16* contained no polymorphisms and the sequenced segments of *FpMADS2* contained only single nucleotide polymorphisms (SNPs) for which all offspring were heterozygous. Hence, these three genes could not be mapped.

Reexamination of genotype-phenotype relationships in F_1: In our previous work (Ergon *et al.* 2006), we found that VR in the BF14/16 × HF2/7 F_1 mapping population was mainly controlled by several loci on chromosome 4F. There were strong maternal effects and no paternal effects of 4F markers on VR. The five markers with the strongest effect on VR (*Xpsr39* at 34.0 cM, *Xpsr115* at 40.6 cM, *Xibf543a* at 52.4 cM, *Xwg644* at 54.3 cM, and *Xwg114* at 57.2 cM) as well as *FpVRN1* at 54.3 cM were selected as descriptors of two maternal haplotypes and the phenotype-genotype relationship in the F_1 population was examined by studying the distribution of haplotypes among the two main phenotypic classes (VR = 12 weeks and VR = 0 weeks). Individuals with crossovers or with one or more unknown allele(s) were excluded from the analysis. In addition, the F_2-individuals selected for gene expression analysis were genotyped for *FpVRN1* using the assay described earlier (Ergon *et al.* 2006).

Results

Two divergently selected F_2 populations, VRmax and VRmin, were characterised for VR and vernalization response. In the VRmin population, there was some flowering in non-vernalized plants and in plants vernalized for 6 weeks. Around 1 % of shoots headed at these two treatments whereas 11.9 and 14.2 % of the shoots headed after 9 and 12 weeks of vernalization, respectively (Table 1). The VRmax population was not able to flower without vernalization and after 9 and 12 weeks of vernalization 1.3 and 8.5 % of the shoots

Table 1. The effect of vernalization on proportion of heading shoots (PHS) in vernalized and non-vernalized plants of two F_2 populations (VRmin and VRmax) selected for contrasting vernalization requirement from the *Festuca pratensis* F_1 mapping family B14/16 × HF2/7 (Exp. 1). Data for the subpopulations selected for gene expression analyses are also shown. Clonal plants were vernalized at 6 °C and 8-h photoperiod for 0, 6, 9, or 12 weeks. The values given are the means of the genotypes ± SE, the genotype ranges are given in brackets. Statistical difference between F_2 populations according to a Students *t*-test is indicated with * ($P \leq 0.05$) or ** ($P \leq 0.001$).

Population	Vernalization 0 weeks	6 weeks*	9 weeks**	12 weeks
F_2 population VRmin (17 genotypes)	1.0 ± 0.6 (0 - 8.0)	1.2 ± 0.5 (0 - 7.9)	11.9 ± 2.0 (0 - 29.6)	14.2 ± 3.0 (0 - 39)
F_2 population VRmax (18 genotypes)	0	0	1.3 ± 0.7 (0 - 12.4)	8.5 ± 2.1 (0 - 25)
VRmin selected 5 genotypes	2.6 ± 1.7 (0-8.0)	2.8 ± 1.5 (0 - 7.9)	17.8 ± 3.8 (6.9 - 29.6)	24.0 ± 4.4 (11 - 39)
VRmax selected 5 genotypes	0	0	0	13.3 ± 4.5 (1 - 25)

Table 2. The effect of vernalization on days to heading (DTH) in vernalized and non-vernalized plants of two F_2 populations (VRmin and VRmax) selected for contrasting vernalization requirement from the *Festuca pratensis* F1 mapping family B14/16 × HF2/7. Data for the subpopulations selected for gene expression analyses are also shown. Clonal plants were vernalized at 6 °C and 8-h photoperiod for 0, 6, 9, or 12 weeks and transferred to long day conditions in the greenhouse on the same date. Days to heading were counted after the transfer. Plants that headed before this time point were given a value of 0. Means of those genotypes that flowered ± SE. The genotype ranges are given in the first bracket. The number of genotypes that flowered and the total number of genotypes in each treatment is given in the second bracket. NF - no flowering. Statistical difference between F_2-populations according to a Students *t*-test is indicated with * ($P \leq 0.05$) (analyzed for the 9 and 12 weeks vernalization treatments only).

Population	Exp. 1 0 weeks	6 weeks	9 weeks	12 weeks*	Exp. 2 0 weeks	12 weeks
F_2 population VRmin (17 genotypes)	35 ± 10 (16-52) (3/17)	58 ± 2 (51-64) (5/17)	47 ± 5 (0-72) (16/17)	29 ± 7 (0-82) (12/17)	8 ± 3 (0-22) (9/15)	16 ± 8 (0-34) (5/6)
F_2 population VRmax (18 genotypes)	NF	NF	59 ± 5 (51-73) (4/18)	46 ± 2 (38-64) (14/18)	NF	30 ± 1 (24-38) (15/18)
VRmin selected 5 genotypes	44 ± 8 (36-52) (2/5)	56 ± 3 (51-59) (3/5)	31 ± 9 (0-51) (5/5)	15 ± 8 (0-42) (5/5)	4 ± 2 (0-10) (5/5)	16 ± 8 (0-34) (5/5)
VRmax selected 5 genotypes	NF	NF	NF	45 ± 5 (39-64) (5/5)	NF	30 ± 3 (24-38) (5/5)

headed, respectively. Thus VRmin responded strongly to 9 weeks of vernalization whereas VRmax required at least 12 weeks of vernalization to have a similar response. After 9 and 12 weeks of vernalization, the VRmin population headed on average 12 - 17 d earlier than the VRmax population, respectively (Table 2). Heading in both VRmin and VRmax was generally more rapid in Exp. 2 than in Exp. 1. In non-vernalized plants of VRmin, this is likely to be due to a season-dependent induction of flowering. In vernalized plants of both populations, it may also be due to the long days during vernalization in Exp. 2. The phenotypic difference between the two groups of genotypes selected for gene expression analyses was maximised by selecting five early and abundantly flowering genotypes of VRmin. Average HS in selected VRmin genotypes were 2.6, 2.8, 17.8, and 24.0 % after 0, 6, 9, and 12 weeks of vernalization, respectively (Table 1). VRmax genotypes were selected among those that required no less and no more than 12 weeks of vernalization, in average 13.3 % of the shoots in the selected VRmax genotypes headed after 12 weeks of vernalization. Selected VRmin genotypes vernalized for 12 weeks headed on average 30 and 14 d earlier than the selected VRmax genotypes in Exp. 1 and 2, respectively (Table 2).

A 280 bp cDNA fragment of *FpMADS2* [GenBank: GU477495] containing the transition between the coding region and the 3'UTR was amplified using *L. perenne MADS2* primers. At the cDNA level, the amplified region of *FpMADS2* had 98 % identity with *LpMADS2* whereas at the protein level it had 100, 97, 97, and 87 % identity with LpMADS2 (Petersen *et al.* 2004), LtMADS2 (Gocal *et al.* 2001), AsFUL2 (Preston and Kellogg 2006), and TaAGL29 (Zhao *et al.* 2006), respectively.

A 201 bp cDNA fragment of *FpMADS3* [GenBank:GU574697] was amplified using *LpMADS3* primers. The amplified *FpMADS3* fragment lies in the C-terminal region of the encoded protein and has 86, 61, and 58 % identity at the protein level with LpMADS3 (Petersen *et al.* 2004), *H. vulgare* BM3 (Schmitz *et al.* 2000) and TaAGL10 (Zhao *et al.* 2006), respectively. FpMADS3 has a 6 or 8 amino acid deletion relative to LpMADS3, BM3, and TaAGL10.

A 216 bp cDNA fragment [GenBank:GU574699] of *FpMADS10* containing the transition between the coding region and the 3'UTR was amplified using *LpMADS10* primers. The whole sequence had 97 % identity with *LpMADS10* (Petersen *et al.* 2004) at the cDNA level and 100, 90, and 87 % identity at the protein level with LpMADS10, TaAGL11 (Zhao *et al.* 2006), and *H. vulgare* BM10 (Trevaskis *et al.* 2007), respectively.

Using *LpMADS16* primers, a fragment approximately 1 kb in size was amplified from *F. pratensis* cDNA of which 999 bp were sequenced [GenBank:GU574700]. This sequence covers the whole coding region as well as 58 bp of the 5'UTR and 281 bp of the 3'UTR. At the protein level, FpMADS16 has 97, 86, 84, and 77 % identity with LpMADS16, *H. vulgare* VRT-2 (Szücs *et al.* 2006), *T. aestivum* VRT-2/AGL36 (Kane *et al.* 2005, Zhao et al 2006), and *H. vulgare* BM10 (Trevaskis *et al.* 2007), respectively.

A weak expression of *FpVRN1* was observed in non-vernalized plants at all three sampling dates but only in plants that were able to flower without vernalization (VRmin) (Fig. 1). *FpVRN1* was strongly upregulated by the vernalization treatment in both experiments, both in VRmin and in plants that required vernalization in order to flower (VRmax). The *FpVRN1* expression in vernalized plants appeared to be similar in the two populations and in the two experiments in spite of the fact that both the timing and the abundancy of flowering differed between populations and between experiments.

FpMADS2 was expressed in non-vernalized plants, but the expression varied between the two populations. There was a strong expression of *FpMADS2* in non-vernalized VRmin plants and a very weak expression in

non-vernalized VRmax plants at all three time points. The effect of vernalization on *FpMADS2* expression also differed between populations. *FpMADS2* was down-regulated by vernalization in VRmin but upregulated in VRmax.

FpMADS3 was expressed in non-vernalized VRmin plants at all three time points but the signal strength was weaker than that of *FpMADS2* and no expression could be observed in non-vernalized VRmax plants. Expression was upregulated in vernalized VRmax plants as compared to non-vernalized plants whereas it appeared to be unaffected by vernalization in VRmin plants.

The expression patterns of *FpVRN1, FpMADS2*, and *FpMADS3* had some similarities and some disimilarities. Expression was stronger in non-vernalized VRmin plants than in non-vernalized VRmax plants. *FpVRN1* was strongly upregulated by vernalization in both populations whereas *FpMADS2* and *FpMADS3* were only upregulated in VRmax plants and down-regulated or not affected in VRmin plants. *FpMADS10* and *FpMADS16* expression appeared not to be affected by vernalization or by genotype but was constitutively expressed at high levels. When accounting for total amount of RNA in the samples as revealed by the GAPDH control reactions, the difference in photoperiod during vernalization between the two experiments did not have any evident effects on expression of the various genes which indicates that effects of the vernalization treatment are due to low temperature.

The mapping population contained three SNPs in *FpMADS3* that allowed the mapping of this gene between *p65m78_730* and *Xablpgp106* at 49.7 cM on the paternal

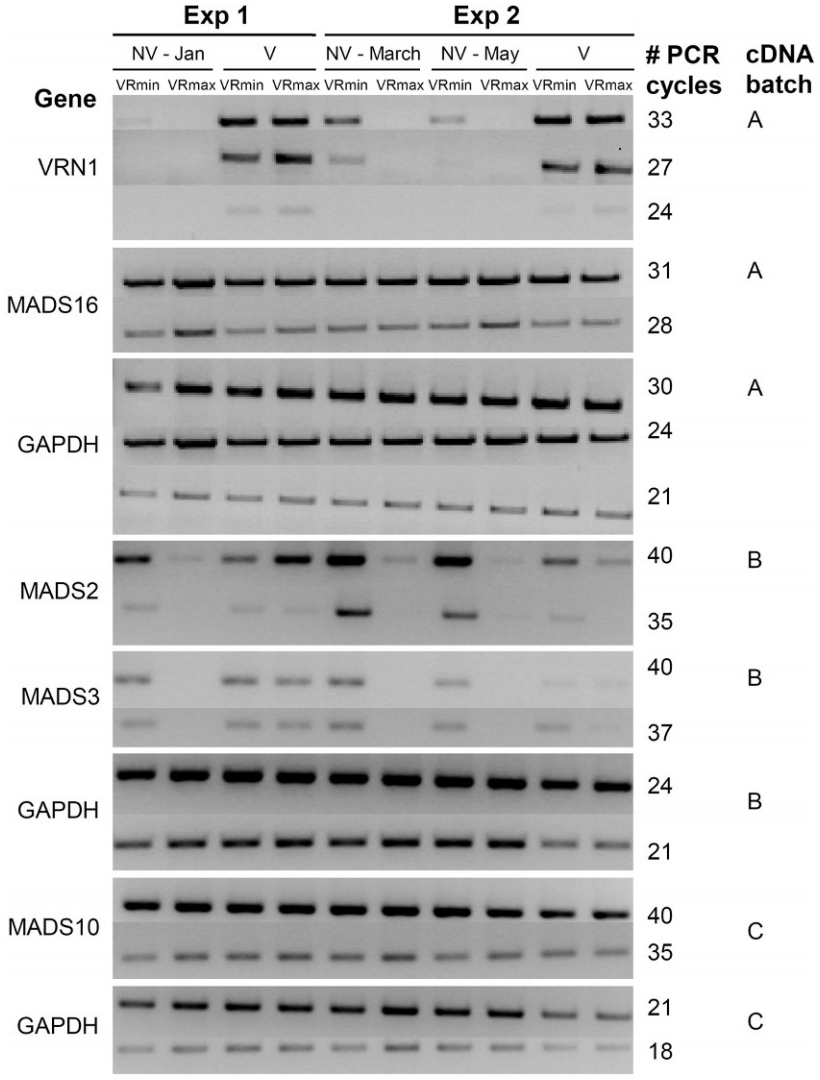

Fig. 1. MADS-box gene expression in shoot apex tissues of vernalized (V) and non-vernalized (NV) plants of two F_2 populations (VRmin and VRmax) with contrasting vernalization requirement. V plants were vernalized at 6 ° C for 12 weeks, whereas NV plants were grown in the greenhouse and sampled in January, March, and May. Total RNA from five genotypes selected from each population was pooled prior to cDNA synthesis. Three sets of cDNA were used as templates for PCR (A, B, and C). The analysis of GAPDH serves as a control for the amount of cDNA in the sample.

map of chromosome 2F (Fig. 2).

The relationship between the VR phenotype on one hand and the maternal haplotype defined by six VR-associated markers on 4F (*Xpsr39*, *Xpsr115*, *Xibf543a*, *Xwg644, FpVRN1*, and *Xwg114*) on the other hand was examined in the F_1 family. Variation in VR in the F_1 family was determined by maternal alleles in this region on 4F and the analysis performed here revealed that the *abaaba* haplotype is associated with VR and that the *babbab* haplotype is associated with the ability to flower without vernalization. Almost all F_1 individuals that contained the *babbab* haplotype were able to flower without vernalization (39 of 42 individuals), and almost all F_1 individuals with a VR containing the *abaaba* haplotype (32 of 35 individuals). However, among all the F_1 individuals that had the *abaaba* haplotype many of them were able to flower without vernalization (15 of 47 individuals). Similarly, among the F_1 individuals that were able to flower without vernalization many of them did not contain the *babbab* haplotype (15 of 54 individuals). These results indicate that *babbab* is quite but not completely dominant for tha ability to flower without vernalization, but that there are also other genes in the population conferring this trait.

The F_2 plants analysed for gene expression were also genotyped for *FpVRN1*-alleles. Only two of the five VRmin individuals and one of the five VRmax individuals had inherited the *FpVRN1* b-allele (the allele present in the *babbab* haplotype associated with the ability to flower without vernalization). For comparison, the frequency of individuals carrying the b-allele among the 15 F_1 parents of VRmin and VRmax were 11/15 and 5/15, respectively.

Discussion

The two divergently selected F_2 populations differed in VR, PHS, and DTH. Some of the VRmin genotypes were able to flower to a limited extent without vernalization whereas none of the VRmax genotypes had this ability. Although genotypes that were able to flower without vernalization in a way resembled spring cereals, they differ from spring cereals in that they flower sparsely without vernalization and respond strongly to vernalization in terms of a higher PHS. The VRmin population responded strongly to 9 weeks of vernalization whereas the VRmax population required at least 12 weeks to have a similar response. After vernalization, the VRmin population flowered approximately 2 weeks earlier than the VRmax population.

None of the genotypes in the F_2 population VRmax were able to flower without vernalization. Thus, the ability to flower without vernalization is inherited as a dominant trait in the F2 populations. We have previously shown that the VR variation in the F1 mapping population is mainly controlled by several genes on chromosome 4F where *FpVRN1* is located (Ergon *et al.* 2006). The fact that there were strong maternal effects and no paternal effects of 4F markers on VR in F_1 showed that the female parent (BF14/16) is heterozygous for genes on 4F controlling VR variation in the F_1 family. This, together with the analysis of the distribution of F_1 plants among genotypic and phenotypic classes done in the present study, indicates that the maternal *babbab* haplotype (defined by markers *Xpsr39*, *Xpsr115*, *Xibf543a*, *FpVRN1, Xwg644,* and *Xwg114* on 4F) is dominant for the ability to flower without vernalization. *VRN1* is dominant for a spring growth habit in cereals and may be a factor in determining the phenotype in the F_1 and F_2 populations of *F. pratensis* studied here. However, the fact that the markers *Xpsr39* and *Xpsr115*, located 20 and 14 cM distally to *FpVRN1,* have the strongest effects on VR suggests that other genes on 4F are responsible (Ergon *et al.* 2006). Fayt *et al.* (2007) located *Vrd1* and *Vrd2*, two genes in wheat that have a dominant effect in reducing the required length of vernalization, to chromosome 4A and 5D, respectively, using monosomic substitution lines. Interestingly, *Xpsr39* and *Xpsr115* are also located on *Triticeae* chromosome 4 and 5, respectively (Alm *et al.* 2003). We may therefore speculate that *Xpsr39* and *Xpsr115* are linked to *Vrd1* and *Vrd2* and that these genes are responsible for the identified QTLs distal to *VRN1*.

Considering that the *babbab* haplotype is dominant for the ability to flower without vernalization, it is puzzling that the *abaaba/babbab* heterozygous female parent (BF14/16) expresses a VR phenotype, whereas the *abaaba* homozygous male parent (HF2/7) is able to flower without vernalization. This can be explained if the phenotypes of the parents are due to epistatic action of genes located elsewhere. The distribution of F_1 plants among genotypic and phenotypic classes also shows that in addition to the QTLs identified on 4F, there must be one or more other loci controlling VR in the population. In addition, one of the five VRmax and only two of the five VRmin genotypes used for gene expression analysis had inherited the *VRN1*-allele associated with the ability to flower without vernalization. This may be due to several reasons, such as overcrossing events between *FpVRN1* and stronger distal QTLs, or a selection of genes controlling early flowering rather than VR when VRmin parents and VRmin individuals were picked out. In any case, the results show that the variation in the ability to flower without vernalization in the F_2 population is at least partly controlled by other loci than *FpVRN1*, *Xpsr39*, and *Xpsr115*. These loci could be some of the QTLs detected for traits related to induction of flowering on other chromosomes than 4F in our earlier study (Ergon *et al.* 2006) but they could also be genes whose effects are masked in the F_1 population.

FUL-like MADS-box genes in the *Poaceae* have been divided into the three subclades *FUL1, FUL2*, and *FUL3*,

where *LtMADS1, TaMADS11 (WAP1), HvMADS5 (BM5)*, and *AsFUL1* (all cereal *VRN1* homologues) are members of the *FUL1* subclade; *LtMADS2, TmFUL2a, HvMADS8 (BM8)*, and *AsFUL2* are members of the *FUL2* subclade; and *HvMADS3* is a member of the *FUL3* subclade (amongst others) (Preston and Kellogg 2006). *FpVRN1* and *LpMADS1* are likely orthologs of *VRN1* (Jensen *et al.* 2005, Ergon *et al.* 2006) and can therefore be considered as members of the *FUL1* subclade. *FpMADS2* and *LpMADS2* are very similar to *FUL2* members *LtMADS2* and *AsFUL2* and are therefore likely members of the *FUL2* subclade. *FpMADS3* had 87 and 61 % similarity with *LpMADS3* and *HvMADS3* (*BM3*) at protein level, respectively. Although the similarity is limited, the fact that *FpMADS3* was mapped to 2F, which has a high level of synteny with the *Triticeae* chromosome 2 series (Alm *et al.* 2003) where *HvMADS3* is located (Schmitz *et al.* 2000), indicates that *FpMADS3*, *LpMADS3*, and *HvMADS3* could be orthologous genes belonging to the *FUL3* subclade. The wheat and barley

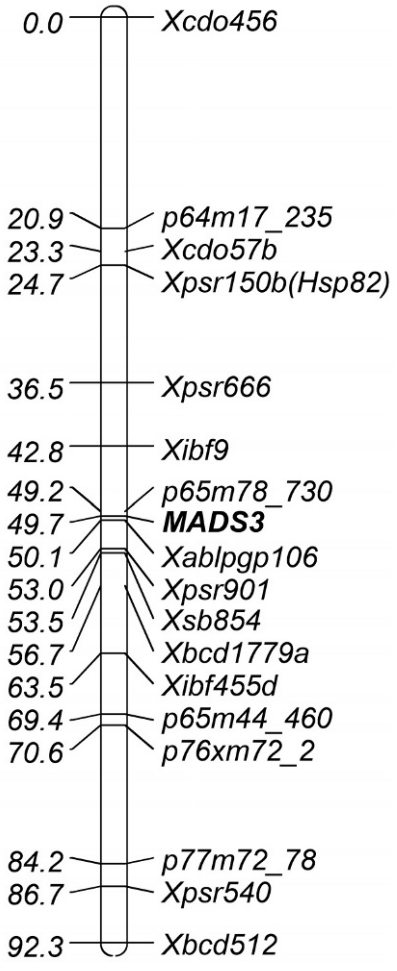

Fig. 2. Map position of *MADS3* on chromosome 2F. The *Festuca pratensis* F_1 mapping family B14/16 × HF2/7 was genotyped for a single strand conformation polymorphism in *MADS3* which was subsequently mapped onto the paternal map of chromosome 2F obtained by Alm *et al.* 2003.

genes *FpMADS10* and *FpMADS16* are similar to *TaAGL11*, *BM10*, and *VRT-2/AGL36* which belong to the SVP/StMADS11 clade of MADS-box genes (Kane *et al.* 2005, Zhao *et al.* 2006, Trevaskis *et al*. 2007).

Expression analyses were performed on pooled RNA samples from five selected genotypes from each of the two F_2 populations divergently selected for high or low VR (Tables 1 and 2). Although random effects of the selection of genotypes may occur, differences in expression patterns between the two groups are likely to be related to the phenotypic differences in VR observed between the two F_2 populations.

FpVRN1 expression in the divergently selected F_2 populations was positively associated with the competence to flower and/or to the transition to flowering; this is in agreement with the expression patterns found in winter and spring genotypes of other cereals and grasses. The fact that vernalization had a strong up-regulating effect in both populations indicates that *FpVRN1* expression plays a role in the vernalization-dependent induction of flowering, occurring in both populations.

The *FUL1, FUL2,* and *FUL3* homologues *FpVRN1, FpMADS2* and *FpMADS3* were differently expressed in the two selected populations before vernalization and they are differently regulated by vernalization. Among non-vernalized plants, all three genes had a higher expression in VRmin than in VRmax. In VRmin, vernalization was associated with an increased expression of *FpVRN1* but not of *FpMADS2* and *FpMADS3.* In VRmax, vernalization was associated with an increased expression of all three genes. Expression patterns of *FpMADS2* and *FpMADS3* were in agreement with the expression patterns found in *L. perenne*, *L. temulentum*, *T. monococcum*, and *A. sativa* (Petersen *et al.* 2004, Preston and Kellogg 2008). These genes, like *VRN1*, generally appear to be expressed in apices during vernalization or around the time of transition to reproductive development. An interesting observation is that the expression of *FpMADS2* and *FpMADS3* was not enhanced by vernalization in VRmin in spite of the fact that vernalization has a strong enhancing effect on flowering. *FpMADS2* and *FpMADS3* expression was, however, associated with the difference between non-vernalized VRmin and VRmax plants as was also the case for *FpVRN1*. In addition to possible redundant and/or differential functions of these three genes, the fact that their encoded proteins have been found to form heterodimers that appear to act in the regulation of *VRN1* expression suggests that they may also interact in the induction of flowering.

The *SVP*-like genes *FpMADS10* and *FpMADS16* appeared to be constitutively expressed in both populations in our experiments. This is in contrast to results from *L. perenne* where *MADS10* expression was down-regulated by vernalization (Petersen *et al.* 2006). However, it has been found that *SVP*-like genes in barley are actually up-regulated by cold and down-regulated after the transition to flowering (Trevaskis *et al.* 2007). Although there is a possibility of differential regulation

and function of *SVP*-like genes within and between species, it is also possible that the down-regulation observed in some studies is actually due to the transition to flowering rather than to the vernalizing treatment itself and that the apices in our study had not reached the developmental stage where down-regulation occurs. Some studies indicate that SVP-like proteins act as repressors of *VRN1* and that *VRN1* expression during vernalization is due to a down-regulation of a *SVP*-like gene (Kane *et al.* 2005, 2007, Petersen *et al.* 2006). Such a mechanism is not supported by our results, since *FpVRN1* expression is enhanced during vernalization without *FpMADS10* and *FpMADS16* being down-regulated.

Conclusions

The divergently selected F_2 populations differ in the ability to flower without vernalization, vernalization response, and the length of vernalization required for a response to occur. The ability to flower without vernalization is inherited as a dominant trait in the mapping population. Although loci on chromosome 4F has a major effect on VR in the F_1 population, the effect of these loci in the parents and in the F_2 populations are affected by epistatic interactions with other loci in the genome. Expression of *FpVRN1, FpMADS2*, and *FpMADS3* is associated with flowering in the two F_2 populations. However, the effect of vernalization on gene expression differs between genes and F_2 populations indicating different regulation mechanisms. Our results do not support a role of *MADS10* and *MADS16* as repressors of *VRN1, MADS2*, or *MADS3* in our *F. pratensis* mapping population. The study of divergently selected populations developed from a single cross is a way of identifying gene effects against the heterogeneous genetic background present in populations of outbreeding species. With more detailed phenotypic and genotypic characterisation of a larger number of individuals in such populations, we would be able to more accurately identify the loci on 4F and their individual effects on different components of the "vernalization requirement" (*i.e.* the ability to flower without vernalization and the required length of vernalization) as well as on different responses to vernalization (*i.e.* proportion of heading shoots and days to heading). Candidates for the genes near markers *Xpsr39* and *Xpsr115* on 4F that have dominant effects on the ability to flower without vernalization and possibly the required length of vernalization could be identified with detailed comparative mapping of this region with sequenced genomes in the grass family. Transcriptome analyses and more detailed analyses of expression patterns in genotypically and phenotypically well characterised individuals will give a better understanding of the genetic control of vernalization and induction of flowering in *F. pratensis*.

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