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ORIGINAL PAPER

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QTL mapping of vernalization response in perennial ryegrass (*Lolium perenne* L.) reveals co-location with an orthologue of wheat *VRN1*

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Abstract The objective of this study was to map quantitative trait loci (QTL) for the vernalization response in perennial ryegrass (*Lolium perenne* L.). The mapping population consisted of 184 F_2 genotypes produced from a cross between one genotype of a synthetic perennial ryegrass variety "Veyo" and one genotype from the perennial ryegrass ecotype "Falster". Veyo and Falster were chosen among four different populations because of their contrasting vernalization requirements. In total, five QTL for the vernalization response, measured as days to heading, were identified and mapped to linkage groups (LG) LG2, LG4, LG6 and LG7. Individually, these QTL explained between 5.4 and 28.0% of the total phenotypic variation. The overall contribution of these five QTL was 80% of the total phenotypic variation. A putative orthologue of *Triticum monococcum VRN1* was amplified from genomic DNA from perennial ryegrass. PCR fragments covering the proximal part of the promoter and the 5′ end of the orthologue were subsequently PCR-amplified from both parents of the mapping population and shown to possess 95% DNA sequence identity to *VRN1*. Several polymorphisms were identified between Veyo and Falster in this fragment of the putative *VRN1* orthologue. A CAPS marker, *vrn-1*, was developed and found to co-segregate with a major QTL on LG4 for the vernalization response. This indicates that the CAPS marker *vrn-1* could be located in an orthologous gene of the wheat *VRN1*.

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Introduction

Perennial ryegrass (*Lolium perenne* L., *2x = 14*) is the most important turf and forage grass species in temperate regions (Holmes 1980). In most production systems, forage grasses should be upright growing, dense, persistent, and have a high nutritional value. Thus, for forage grasses, delay or even suppression of stem elongation and subsequently heading is an important breeding goal (Jensen et al. 2004). However, this is in conflict with the breeding goal of grass seed producers for maximal seed yield. Consequently, control of flowering, i.e., vernalization, is of pivotal interest.

Most temperate perennial grasses, including perennial ryegrass, have a dual induction requirement for flowering. Primary induction (PI) is achieved by low temperature (vernalization) and/or short days (SD), while secondary induction (SI) requires transition to long days (LD) and is enhanced by moderately higher temperatures (Heide 1994). The requirement for PI varies greatly within perennial ryegrass. In general, the PI requirement increases with increasing latitude of origin of the germplasm; some plants of Mediterranean origin require only LD in order to flower (Aamlid et al. 2000; Cooper 1960). Furthermore, SD has no effect at low temperatures and very little effect at high temperatures in inducing flowering, rendering vernalization the more important factor of these two (Aamlid et al. 2000; Cooper 1960).

The control of the vernalization response has been studied intensively in several plant species. Recently, *VRN1* was cloned by a map-based approach from diploid wheat (*Triticum monococcum*) (Yan et al. 2003). *VRN1* proved to be similar to the *Arabidopsis* meristem identity gene *AP1*. Furthermore, deletions in the promoter region adjacent to a putative MADS box protein binding site discriminated winter from spring types of diploid wheat (Yan et al. 2003). Based on this, a model for the vernalization response in diploid wheat was

proposed in which *VRN1* transcription induces flowering, *VRN2* action blocks *VRN1* transcription by binding to the promoter, and *VRN2* expression is repressed by vernalization (Yan et al. 2003). Expression-level studies of *WAP1* (Murai et al. 2002) and *BM5* (Schmitz et al. 2000) have proposed these genes as orthologues of *VRN1* in hexaploid bread wheat and barley, respectively (Trevaskis et al. 2003). In *T. aestivum*, *TaVRT-1*, a putative transcription factor associated with the vegetative to reproductive transition in cereals, was found to map to a 0.03 cM interval that was completely linked to the *VRN1* locus in *T. monococcum* (Danyluk et al. 2003). Furthermore, *OsMADS14*, a *WAP1* homologue from rice (*Oryza sativa*), shows an early flowering phenotype when overexpressed in rice (Jeon et al. 2000). Studies in *L. temulentum* have shown that an *AP1*-like MADS box gene, *LtMADS1*, is one of the first genes to be transcribed after exposure to long day conditions (Gocal et al. 2001). *LtMADS1* is closely related in sequence to *BM5* and *WAP1*, which raises the possibility that *AP1*-like genes in grasses are not only involved in the vernalization response but also induce flowering in response to photoperiod (Trevaskis et al. 2003).

To our knowledge, *LpTFL1*, a perennial ryegrass homologue of *Terminal Flower1* (*TFL1*) from *Arabidopsis*, is the only flowering time gene that has been cloned and characterised in perennial ryegrass (Jensen et al. 2001, 2004). While EST generation and SSR marker development projects are ongoing (Sawbridge et al. 2003), no sequence information has yet become publicly available. Thus, in order to shed further light on the genetics underlying the control of flowering time in perennial ryegrass, a project was initiated to map QTL for vernalization requirement. The objectives of this study are to: (1) identify QTL involved in the vernalization response in an F_2 population of perennial ryegrass derived from genotypes with divergent vernalization requirements, and (2) compare the chromosomal location of the putative perennial ryegrass orthologue of *VRN1* with QTL positions.

Materials and methods

Selection of parents for the mapping population

Two diploid varieties (Veyo and Borvi) and two diploid ecotypes (Falster and Kleppe) were selected for their contrasting behaviour with regard to vernalization requirements (Aamlid et al. 2000). The varieties Veyo and Borvi originate from Italy and England and the ecotypes Falster and Kleppe from Denmark and Norway, respectively. Individual plants were selected as parents for the mapping population based on their contrasting primary and secondary induction requirements, as determined in the following two preliminary experiments.

Plants were sown at 3-week intervals in 7 cm pots and raised under non-inductive conditions in the greenhouse for 8 weeks. Subsequently, plants were transferred to PI in a growth chamber and six vernalization treatments were applied: vernalization at 0, 3, 6, 9, 12 or 15 weeks at 6°C with an 8 h daylength (200 $\mu mol.m^{-2}.s^{-1}$ PAR). The first sowing comprised 18 pots of each of the four populations, nine of which were control plants that were never transferred to PI. Later sowings comprised nine pots of each population. After PI, the plants were transplanted into 12 cm pots and transferred to SI in the greenhouse under daylight conditions starting in April 2002. Temperatures ranged from 14 to 36°C with a daily mean of 20°C, and daylengths between 14 and 18 h. Heading date was recorded when the top spikelet emerged through the leaf sheath of the flag leaf. Flowering date was recorded when more than 50% of the spikelets on the first inflorescence had visible anthers.

Development of the mapping population

Perennial ryegrass variety Veyo and perennial ryegrass ecotype Falster were chosen from the populations tested because of their contrasting vernalization requirements (Fig. 1). The mapping population generated consisted of 184 F_2 genotypes derived from a cross between a single genotype from the synthetic perennial ryegrass variety Veyo and a single genotype from the perennial ryegrass ecotype Falster (in the following, the two individual genotypes selected as grandparents (GP) of the cross will be denoted Veyo-GP and Falster-GP). Specifically, initial crosses were produced between a single genotype from Veyo and a single genotype from Falster by open pollination in isolation. After seed harvest 50 F_1 seeds were sown and five clones of each plant was produced.

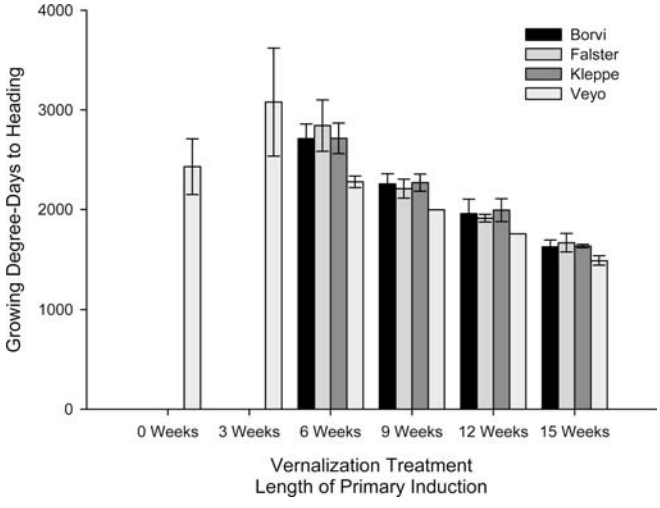

Fig. 1 Mean and standard deviations for growing degree days to heading under different vernalization treatments. No error bars are added for Veyo at 9 and 12 weeks of PI since all the plants headed at the same date. Plants were subjected to 0–15 weeks of PI (6°C and 8 h daylength) followed by greenhouse conditions

F_1 plants were pair-wise matched on the basis of flowering time and five clones of each were subsequently intercrossed by open pollination in isolation to produce two-way pseudo-testcross F_2 populations. Selection of the mapping population from these crosses were carried out based on the amount of seed obtained. The cross selected had the F_1 genotypes F1-30 and F1-39 as parents. In the rest of this paper this is referred to as the F_2 population VrnA. Plants were grown in fully-fertilised peat and watered every day with a complete nutrient solution.

Phenotyping of the mapping population for the vernalization response

One hundred and eighty-four F_2 genotypes of VrnA were sown in September 2002 together with parent (F1-30 and F1-39) and grandparent (Veyo-GP and Falster-GP) plants, re-potted into 1 l pots containing fully-fertilised peat, and grown in the greenhouse at 18°C day temperature and 16°C night temperature with a day-length of 16 h. In December 2002, all 184 F_2 genotypes were cloned to produce four clones and left in the greenhouse for regrowth. Plants were transferred to vernalization chambers in March 2003 and vernalized for 6 weeks at 6°C and an 8 h daylength (approximately 200 $\mu mol.m^{-2}.s^{-1}$ PAR). Since the largest differences between Veyo and Falster were seen at 6 weeks of PI (Fig. 1), this treatment was chosen to screen VrnA. After vernalization, plants were transferred to the greenhouse until the end of the experiment, in August 2003. Heading date was recorded when the top spikelet emerged through the leaf sheath of the flag leaf. From 3 to 9% of the individual plants in the four replicates were not flowering by termination of the experiment; these were treated as missing.

The temperature in the greenhouse from January until the transfer to PI in March ranged from 12.9 to 24.3°C, while the greenhouse temperature post-PI until the completion of heading (18 June) ranged from 11.6 to 39.5°C. The experiment was divided into two independent experiments; each experiment was performed as an alpha-lattice with two replications and one pot as the experimental unit.

DNA extraction and molecular marker analysis

Genomic DNA was extracted from the 184 F_2 genotypes, parents (F1-30 and F1-39) and grandparents (Veyo-GP and Falster-GP), according to Guidet et al. (1991). DNA was spooled out, washed once in 2 ml 70% ethanol at room temperature, air-dried and re-suspended in 0.5 ml R40 (40 mg/ml RNaseA in TE pH 8.0). DNA was diluted to a concentration of 25 ng/µl for use in PCR reactions.

SSR and AFLP analysis was performed as described by Jensen et al. (manuscript in preparation). In total 33, SSRs (41% of the tested SSRs were found to be polymorphic in this population), 59 AFLPs and 1 CAPS marker were genotyped on all 184 F_2 genotypes with an average of 20% missing data points (minimum 0.5%, maximum 35%). All SSR primer sequences are published [http://www.grasp-euv.dk; Jensen et al. (in preparation); Jones et al. 2001; Kubik et al.1999, 2001].

Identification of a putative *VRN1* orthologue and development of a CAPS marker

Primers were designed based on the alignment of DNA sequences *VRN1* (Yan et al. 2003), *BM5* (Schmitz et al. 2000) and *OsMADS14* (Jeon et al. 2000) from diploid wheat, barley and rice, respectively. The primers VRN1ex13for (5′-AAGGTGCAGCTGAAGCGG) and VRN1ex176rev (5′-GGTGGAGAACTCGTAGAGC) (MWG Biotech, Ebersberg, Germany) amplified a 165-bp fragment by PCR, using a mix of genomic DNA from four genotypes of VrnA as the DNA template. Each PCR reaction (10 µl) contained 0.5 U of *Taq* polymerase, 1× PCR buffer, 20 pmol of each primer, 6 nmol dNTPs and 25 ng of total genomic DNA. PCR was performed in a MJ Research PTC-225 Peltier Thermal Cycler as follows: 5 min at 94°C, followed by 30 cycles of 20 s at 94°C, 20 s at 50°C and 60 s at 65°C, with a final extension of 5 min at 65°C. PCR fragments were excised from a 1.5% TAE agarose gel, purified using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), and cloned into pCR2.1-TOPO (Invitrogen, Groeningen, The Netherlands). Sequencing reactions were prepared using the DYEnamic ET dye terminator kit and sequenced on the MegaBACE 1000 96 capillary electrophoresis system (Amersham Biosciences, Piscataway, N.J., USA).

Based on this sequence, similarity searches were performed using BLAST algorithms (Altschul et al. 1990) against GenBank accessions (http://www.ncbi.nlm.nih.gov/Genbank/index.html). A cDNA sequence of perennial ryegrass (GenBank accession no. AY198326), very similar to *VRN1* of diploid wheat was found.

Primers lpVRN1_19for (5′-TCTCCTCTTCTTCCCCACTG) and lpVRN1_344rev (5′-AGTCGGTTGCGAACTCGTAG) were designed based on this cDNA sequence. A fragment of 337 bp and a fragment of 339 bp were amplified from Veyo-GP and Falster-GP, respectively. PCR, cloning and sequencing were performed as described above. The fragments from Veyo-GP and Falster-GP were aligned, and several polymorphisms were identified. Two of these polymorphisms resided in a recognition site of the restriction enzyme *Pvu*II. This was utilised as a gene-targeted CAPS marker (*vrn-1*) to distinguish between the Veyo-GP and Falster-GP allele of the putative *VRN1* orthologue. PCR reactions applying primers lpVRN1_19for and lpVRN1_344rev were set up for all individuals in our mapping population. Five microlitres of PCR amplification products were digested with 5.0 U of *Pvu*II

(Fermentas, Vilnius, Lithuania). Digestion products were separated by electrophoresis in 1.5% TAE agarose gels and bands were revealed with ethidium bromide.

Linkage map construction

Map construction was carried out using the Haldane mapping function within the software JoinMap 3.0 (Van Ooijen and Voorrips 2001). All linkage groups (LG) were grouped with a minimum of LOD 6.0 except for LG5, which grouped at LOD 4.0. The map consists of seven linkage groups, named according to the chromosome assignment found in the ILGI perennial ryegrass population (http://ukcrop.net/perl/ace/search/FoggDB).

Data analysis and QTL mapping

Data were transformed from days to heading to growing degree-days to heading, in order to account for the effect of varying temperature regimes before, during, and after vernalization on the time to heading. Growing degree-days (GDD) were calculated using the formula $GDD = (T_{max} + T_{min}/2)\text{-}T_{base}$, where T_{max} is the daily maximum temperature, T_{min} is the daily minimum temperature and T_{base} is the basal temperature set at 3°C, and defined as the temperature below which development of perennial ryegrass ceases (Niels Roulund, DLF Trifolium, personal communication). Subsequently, GDD to heading were summed for individual plants.

Analysis of variance was performed to partition the different sources of variation for the traits measured using SAS Proc GLM and SAS Proc Mixed (SAS Institute, Cary, N.C.). Significant differences between means were calculated using a *t*-test. Heritability for the trait GDD to heading was estimated from the pooled data from the experiments using the formula $h^2 = \sigma^2_g / [(\sigma^2_g + \sigma^2_{ge}/u)] + (\sigma^2_e/ru)$ where σ^2_g, σ^2_e and σ^2_{ge} were the estimates of genetic, environment and G×E variances derived from the mean square expectations of the ANOVA, $u = 2$ is the number of environments, and $r = 2$ is the number of replicates. Least square means (Lsmeans) for all traits were calculated using the Proc Mixed analysis in SAS.

The Lsmean values for GDD to heading were used to map QTL associated with the vernalization response using interval mapping with the software MAPQTL 4.0 (Van Ooijen et al. 2002). This program allows for the detection of QTL in cross-pollinating species such as perennial ryegrass. A sequential procedure was used for QTL detection. Firstly, the LOD threshold for the significance test was estimated from 1,000 permutations of the quantitative trait data. Both the chromosome-wide significance thresholds (αc) and the genome-wide significance threshold (αg) were calculated. Both thresholds were established at a significance level of 5%. Secondly, forward selection with interval mapping followed by automatic cofactor selection was performed. The procedure commenced with the selection of markers at significant LOD peaks as co-factors and was extended via the inclusion of a subset of flanking markers as co-factors on a single linkage group. This process was repeated for all linkage groups, while keeping significant loci ($P > 0.02$) as co-factors until LOD peaks stabilised. Subsequently, multiple QTL mapping (MQM) was performed (Van Ooijen et al. 2002). LOD scores on all linkage groups were inspected to check if they were below the significance threshold. If this was the case their cofactor loci were removed as being false positives, and MQM mapping was repeated in case any were found. QTL positions were assigned to local LOD score maxima. Confidence intervals of the map position were indicated by a two-LOD support interval.

The effect of individual QTL was calculated using the nearest marker in two different ways. For loci with two segregating alleles, effects were calculated using the formulae: $(AA - aa)/2 =$ additive effect and $Aa - (AA + aa)/2 =$ dominance effect, where AA, aa and Aa are the means for GDD to heading for the different genotype classes ($A =$ Veyo-GP allele). In the case of three segregating alleles, parental allele effects and an interaction effect were calculated (Table 1; Knott et al. 1997). The maternal and paternal effects do not refer to the cytoplasmic effects but merely follow the definition by Knott et al. (1997). No loci mapping close to a QTL had four segregating alleles.

Results

Selection of parents for the mapping population

Since GDD to heading and flowering were completely correlated (data not shown) only results from GDD to heading are presented. Veyo had significantly fewer GDD to heading for all PI treatments compared to the other three populations, which were not significantly different from each other. At 0 and 3 weeks of PI only Veyo headed (Fig. 1). In contrast, at 6 weeks of PI all four populations headed. All populations required fewer GDD to heading with increased length of PI. Furthermore, the differences observed for GDD to heading between Borvi, Falster, Kleppe and Veyo decreased from 6 to 15 weeks of PI, although Veyo continued to head earlier. The largest differences in GDD to heading were seen between Veyo and Falster at 6 weeks of PI

Table 1 Model used to test the effect of QTL alleles for heterozygous parents with three alleles segregating (modified from Knott et al. 1997)

Parental cross	$Q_1Q_2 \times Q_2Q_3 \rightarrow Q_1Q_2, Q_1Q_3, Q_2Q_2, Q_2Q_3$
Maternal effect	$(Q_1Q_2 + Q_1Q_3) - (Q_2Q_2 + Q_2Q_3)$
Paternal effect	$(Q_1Q_2 + Q_2Q_2) - (Q_1Q_3 + Q_2Q_3)$
Interaction effect	$(Q_1Q_2 + Q_2Q_3) - (Q_1Q_3 + Q_2Q_2)$

(Fig. 1). Consequently, perennial ryegrass variety Veyo and ecotype Falster were selected as parental material for the generation of an F_2 mapping population for the analysis of vernalization requirement.

Identification of a putative *VRN1* orthologue in perennial ryegrass

PCR amplification revealed two segregating alleles for *vrn-1* in VrnA. Fragments amplified from both grandparents of the mapping population VrnA revealed a 95% DNA sequence identity to *VRN1* (Fig. 2). Genotyping with the CAPS marker *vrn-1* showed that the parents of the mapping population were heterozygous. Furthermore, the 184 F_2 genotypes of VrnA showed a 1:2:1 segregation of the *vrn-1* marker alleles.

Statistical parameters of the mapping population VrnA

Analysis of variance results indicated that the variation among genotypes for GDD to heading was highly significant. The phenotypic values of the grandparents (Veyo-GP and Falster-GP), the parents (F1-30 and F1-39) and the VrnA F_2 population are presented in Table 2. The two grandparents were significantly different at $P < 0.001$ with Veyo-GP heading earlier than Falster-GP at 6 weeks of PI. Both parents were intermediate compared to the grandparents with regard to GDD to heading, and no significant differences were found between the means. This was also the case between the means of F1-30 and the VrnA F_2 population where no significant differences were found. However, significant differences at 0.05 level were found between the mean of F1-39 and the VrnA F_2 population. Heritability was high at 0.87. The average frequency of GDD to heading for the F_2 genotypes (Fig. 3) was monomodal and normally distributed. Transgressive segregation was observed with some F_2 genotypes heading earlier than Veyo-GP.

Genetic map construction

The total map length was 490.4 cM with an average distance between markers of 5 cM (Fig. 4). The largest distance between markers was found on LG2 with an interval of 29 cM. Sixty percent of all mapped markers (56/93) showed distorted segregation at an experiment-wise ($P < 0.05$) level which was almost exclusively due to significantly distorted segregation at the gamete level (data not shown). Of the 56 distorted markers, 33 were AFLP markers and 23 were SSR markers. The largest proportion of distorted markers was found on LG1 where all markers showed distortion with the Veyo-GP allele being underrepresented. However, markers showing distorted segregation were found on all linkage groups (Fig. 4).

Identification of QTL associated with the vernalization requirement

Five QTL for the vernalization response measured as GDD to heading were identified and mapped to LG2, LG4, LG6 and LG7 (Table 3 and Fig. 4). Individually, the QTL identified explained between 5.4 and 28.0% of the total phenotypic variation. The overall contribution of these five QTL was approximately 80% of the total phenotypic variation. Of the five QTL identified, two mapped 5 cM apart on LG2. These two QTL had LOD scores of 7.2 and 5.7 and explained 18.6 and 16% of the total phenotypic variation, respectively. The QTL of

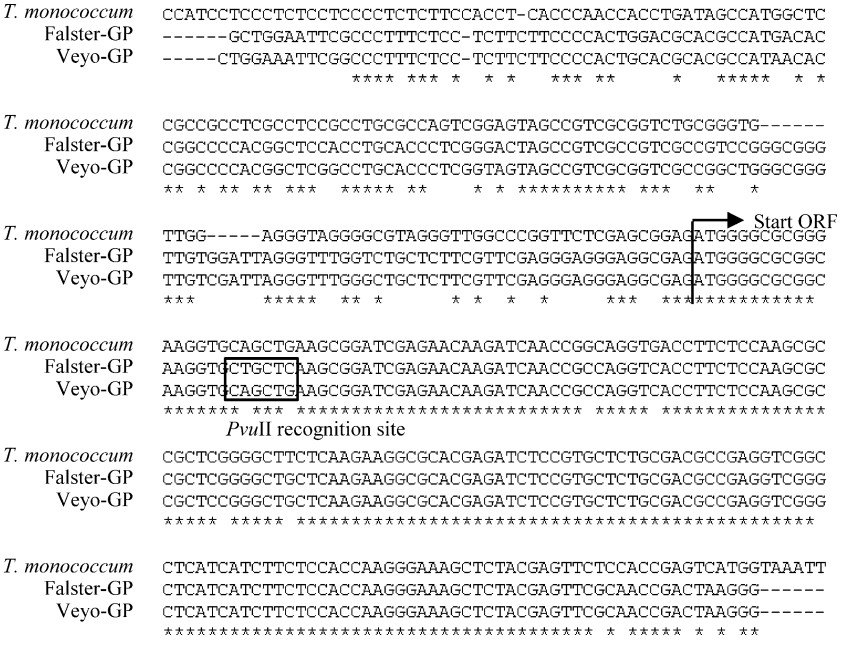

Fig. 2 Comparison between PCR fragments amplified from the grandparental genotypes (Veyo-GP and Falster-GP) of the mapping population VrnA and *VRN1*. Ninety-five percent identity in the ORF region of the fragment, as well as some conserved segments in the 5′ UTR were found. A *PvuII* recognition site was found and used to design a CAPS marker distinguishing between the two grandparental genotypes

Table 2 Mean and standard deviation (SD) for GDD to heading for the grandparents of the VrnA population (Veyo-GP and Falster-GP), the parents (F1-30 and F1-39) and the VrnA F_2 population. H^2, Heritability across experiments

	Veyo-GP (Mean ± SD)	Falster-GP (Mean ± SD)	F1-30 (Mean ± SD)	F1-39 (Mean ± SD)	VrnA		H^2
					Mean ± SD	Range	
GDD	943 ± 133	1,946 ± 214	1,187 ± 134	1,083 ± 175	1,287 ± 215	705–2,070	0.87

largest effect was found on LG4. This QTL was localised with a LOD score of 17.2 and explained 28.0% of the total phenotypic variation (Fig. 5). Significantly, this QTL was observed to co-localise with the CAPS marker *vrn-1*. The QTL of smallest effect was mapped to LG6 with a LOD score of 4.11 and explained 5.4% of the total phenotypic variation (Table 3). Finally, a QTL, which explained 12.9% of the total phenotypic variation, was mapped to LG7 with a LOD score of 10.3.

In terms of QTL effects on days to heading, averaging GDD over the total growth period (including the cold treatment period) indicates that 15.6 GDD equates to 1 day. Therefore, the QTL on LG2 (*B3-D12*) with an additive effect of 47.6 GDD corresponds to a change in flowering time of approximately 3.1 days. Similarly, the QTL mapped to LG4 (*vrn-1*) with an additive effect of 117.8 GDD corresponds to a change in flowering time of approximately 7.6 days, while the QTL mapped to LG6 (*B1-A8*) with an additive effect of 83.7 GDD corresponds to a change in flowering time of approximately 5.4 days (Table 3).

Discussion

The flowering requirement of six European populations of perennial ryegrass has recently been studied (Aamlid et al. 2000). The Mediterranean variety Veyo responded as quantitative long day plants with little winter requirement, whereas the Danish ecotype Falster required at least 9 weeks of vernalization for saturation of primary induction. Both populations consistently ranked at opposite extremes when comparing days to heading after different vernalization treatments; that is Veyo required significantly fewer days to heading than Falster (Aamlid et al. 2000). Preliminary experiments indicated that the largest differences in GDD to heading between the grandparents of the mapping population were achieved at 6 weeks of vernalization. Choosing this treatment ensured that the largest variation possible would be obtained in a mapping population for heading date as a result of vernalization treatment.

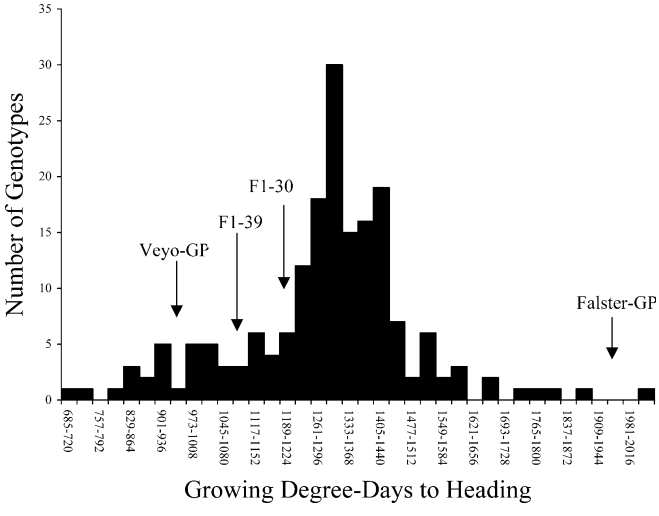

Fig. 3 Frequency of GDD to heading for the VrnA mapping population after vernalization for 6 weeks at 6°C with an 8 h daylength. *Veyo-GP*, grandparent with little vernalization requirement, *Falster-GP*, grandparent with strong vernalization requirement, *F1-30* and *F1-39* parents used for the cross

Heritability for heading date was found to be 0.87 in this population. This is comparable to what has been found in meadow fescue (0.71, Fang et al. 2004), barley (0.81, See et al. 2002) and rice (0.90, Mei et al. 2003). High heritability substantially increases the power of QTL detection and decreases the bias of QTL effect estimates (Utz and Melchinger 1994).

Significant deviations ($P < 0.05$) from expected segregation ratios for individual markers were identified on all linkage groups, with LG1 and LG3 having the largest number of distorted markers (Fig. 4). This is consistent with earlier studies in which high levels of segregation distortion have been observed, especially affecting LG3 (Jones et al. 2002a), LG5 and LG7 of perennial ryegrass (Armstead et al. 2004). The self-incompatibility loci *S* and *Z* of perennial ryegrass have been mapped to LG1 and LG2 (Thorogood et al. 2002), which could explain the distortion for markers mapping to these two chromosomes. Furthermore, markers previously mapped to LG3 also show association with an *S* locus (Thorogood et al. 2002). For LG1, the Veyo-GP alleles are under-represented, whereas on LG2 the Falster-GP alleles are under-represented. Single locus distortion on LG4, LG5, LG6 and LG7 could be due to "ghost markers" due to marker bands resulting from more than one sequence (Frisch et al. 2004). This phenomenon is likely to have a greater impact on populations with four compared to two alleles (i.e., out-crossing vs. in-breeding species).

The total map length was 490 cM using the Haldane mapping function. Previously, map lengths of 814 cM (Jones et al. 2002a), 811 cM (Jones et al. 2002b), 628 cM (Armstead et al. 2004), 372 and 179 cM (Forster et al. 2004), determined by the Kosambi mapping function, have been reported for perennial ryegrass. The use of the Haldane function compared to the Kosambi mapping function results in longer map lengths. LG1, LG5 and

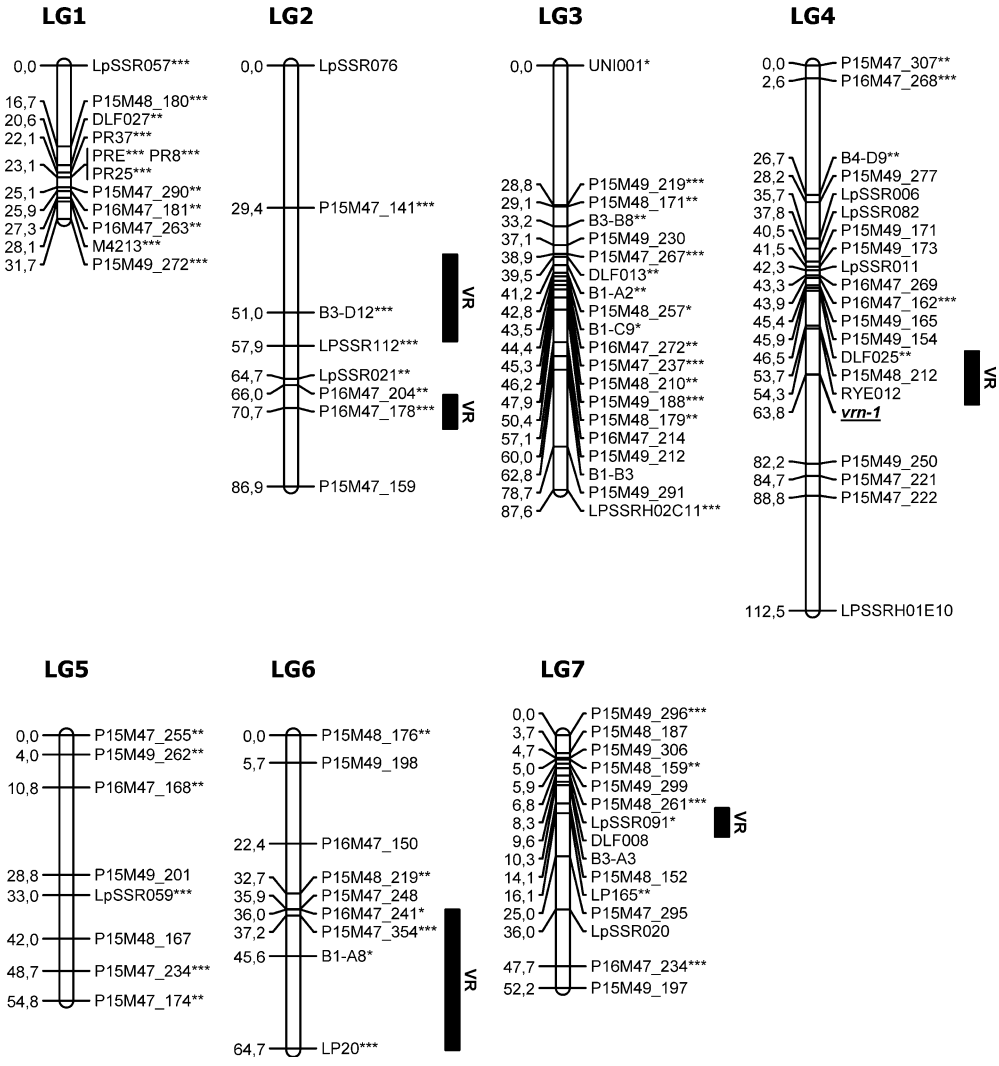

Fig. 4 Location of QTL responsible for GDD to heading at 6 weeks of vernalization in the VrnA population. *VR*, vernalization response. AFLP loci are indicated in the format PxxMxx_xxx. Remaining loci are all SSR loci (http://www.grasp-euv.dk/; Jones et al. 2001; Kubik et al.1999, 2001) except for the CAPS marker *vrn-1*. Distorted segregation ratios for individual markers: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$

LG7 were shorter in this study as compared to previous studies, which might be overcome by adding more markers. However, another explanation could be a lack of homology between homologous chromosomes from the two genetically distant grandparent genotypes Falster and Veyo due to deletions or translocations, as has been recently described at the sequence level in maize (Fu and Dooner 2002). This would lead to reduced pairing and crossing-over during meiosis and hence to contracted map lengths. This phenomenon has been observed in cereals (Benavente and Orellana 1992; Sybenga 1999).

In total, five QTL on four chromosomes were found. F_2 genotypes possessing Veyo-GP alleles generally headed earlier than F_2 genotypes possessing alleles from the late heading grandparent Falster-GP. Heterozygotes carrying one Veyo-GP and one Falster-GP allele showed intermediate GDD to heading, similar to the F_1 genotypes (Fig. 3). Interestingly, transgressive segregation was found towards genotypes heading earlier than Veyo-GP. While this does not immediately fit with the above observation, it is possible that the observed transgression might be due to undetected Falster-GP complementary alleles accelerating flowering.

Table 3 QTL for vernalization response measured as GDD to heading after vernalization at 6 weeks at 6°C in the VrnA F_2 population

LG	Loci	LOD	αc	αg	Additive	Dominance	Maternal	Paternal	Interaction	Percentage
2	*B3-D12*	7.23	2.9	3.8	47.65	−70.23				18.6
2	*P16M47_178*	5.67	2.9	3.8						16.0
4	*Vrn-1*	17.19	2.9	3.8	117.81	−20.96				28.0
6	*B1-A8*	4.11	2.6	3.8	83.70	82.72				5.4
7	*LpSSR115*	10.29	2.7	3.8			−135.26	330.31	117.56	12.9

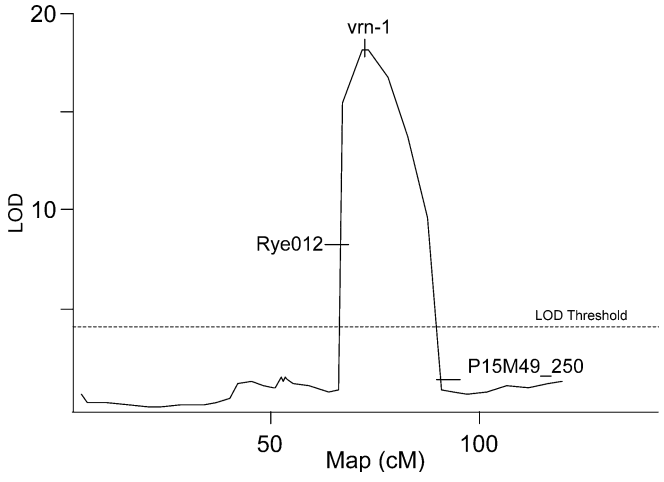

Fig. 5 LOD profile of the vernalization response on LG4. The CAPS marker *vrn-1* is located at the maximum LOD 18.19 at 72 cM. The LOD threshold of 3.8 ($P < 0.05$) was found after 1,000 permutations

The dominance effect for the two largest QTL on LG2 and LG4 is negative, resulting in earlier heading. This significant negative (early heading) heterosis has previously been noted for F_1 hybrids in rice (Mei et al. 2003).

In a recent QTL study of heading date where plants were vernalized under natural conditions in the field, genetic synteny was found between a region on perennial ryegrass LG7 explaining 44% of the phenotypic variance with a region of rice chromosome 6 known to contain the *Hd3* heading date locus (Armstead et al. 2004). The same region explained 12.9% of the phenotypic variation for heading date in this study. Armstead et al. (2004) also identified a minor QTL on LG2, whereas in this study two linked QTL were mapped to LG2 explaining 18.6 and 16% of the phenotypic variation, respectively. Due to the limited number of markers in common between these two studies, it is difficult to conclude if the QTL identified on LG7 and LG2 in both studies are identical. Additional QTL specific to each of both mapping populations have been identified.

A major QTL on LG4 was found to co-localise with the CAPS marker *vrn-1* in VrnA. This major QTL for the vernalization response is closely linked to the marker *Rye012*, as a minor heading date QTL in the study of Armstead et al. (2004), and QTL for ear emergence and inflorescence production (Hayward et al. 1994). Vernalization loci have been mapped in several cereal species (for reviews, see Laurie 1997; Snape et al. 2001). *VRN1* and *VRN2* are the most important determinants conditioning the vernalization response in diploid wheat (Dubcovsky et al. 1998; Tranquilli and Dubcovsky 2000). *VRN1* and *VRN2* map to the distal end of the long arm of chromosome $5a^{m}L$ (Dubcovsky et al. 1998). Comparative mapping within cereals has identified a number of loci and one QTL orthologous to *VRN1* (Dubcovsky et al. 1998): (1) *Vrn-A1* maps to the long arm of chromosome 5A and is the most potent vernalization requirement gene of hexaploid wheat (*T. aestivum*) (Pugsley 1971; Snape et al. 1976), (2) *Vrn-H1* (originally *Sh2*) maps to the long arm of chromosome 5H in barley (Takahashi and Yasuda 1971; Laurie et al. 1995), (3) *Vrn-R1* of rye (*Secale cereale*) maps to chromosome 5R (Plaschke et al. 1993), and (4) a QTL for the vernalization response in oat (*Avena sativa*) which maps to chromosome 6 (LG24) (Holland et al. 1997; Van Deynze et al. 1995). By comparative mapping it has been found that several regions of wheat chromosome 5 and oat chromosome 6 are syntenic to perennial ryegrass LG4 (Alm et al. 2003). This supports the hypothesis that the QTL for the vernalization response identified on perennial ryegrass LG4 and shown to co-localise with the CAPS marker *vrn-1* could be an orthologue of the wheat *VRN1*. In wheat the locus *VRN2* maps closely to *VRN1*. Since only one QTL is found in this region in perennial ryegrass, *VRN2* is either not present or it is not segregating in the VrnA population and therefore was not detected.

The vernalization and photoperiod responses are the primary factors determining the switch from vegetative to reproductive growth. The ability to control this process has many practical and scientific implications. Since grass species are mainly grown for their vegetative production, the shift from vegetative growth to flowering leads to a reduction in feed quality. The controlled inhibition of flowering would thus result in a significant increase of feed quality. In this study six genotypes had two or more clones that did not flower before the termination of the phenotyping experiment. For the *vrn-1* CAPS marker located on LG4 close to the major QTL for GDD to heading, all six F_2 genotypes were homozygous for the allele from the late heading grandparent Falster. For the remaining four markers mapping close to QTL the findings were not as consistent. The genotypes were either homozygous with regards to the Falster-GP, heterozygous, or carried alleles from the early flowering Veyo-GP. These non-flowering or very late flowering genotypes could be used for the production of varieties that would be suitable for areas with very mild winters, thereby delaying or hindering the transition from vegetative to reproductive growth and keeping a high forage quality throughout the growing season. However, seed production would necessarily have to be done in areas with cold winters to ensure proper vernalization and thereby seed production. Furthermore, with the control or inhibition of flowering, the practical exploitation of transgenic perennial ryegrass varieties would be feasible. Currently, the spread of transgenes to other varieties or wild populations of the same species would be inevitable due to the efficient outbreeding nature of perennial ryegrass.

Other potential research goals may include the production of very early flowering genotypes with shorter generation times. In the VrnA F_2 population transgressive genotypes were found that flowered before the initiation of vernalization. Thus, the exploitation of these

ryegrass genotypes might allow production of to two to three generations per year. This would be beneficial for the rapid development both of research and breeding populations.

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