Mol Genet Genomics (2011) 286:433–447
DOI 10.1007/s00438-011-0654-8

ORIGINAL PAPER

Comparative sequence analysis of *VRN1* alleles of *Lolium perenne* with the co-linear regions in barley, wheat, and rice

Torben Asp · Stephen Byrne · Heidrun Gundlach · Rémy Bruggmann · Klaus F. X. Mayer · Jeppe R. Andersen · Mingliang Xu · Morten Greve · Ingo Lenk · Thomas Lübberstedt

Received: 5 April 2011 / Accepted: 18 October 2011 / Published online: 12 November 2011

Abstract Vernalization, a period of low temperature to induce transition from vegetative to reproductive state, is an important environmental stimulus for many cool season grasses. A key gene in the vernalization pathway in grasses is the *VRN1* gene. The objective of this study was to identify causative polymorphism(s) at the *VRN1* locus in perennial ryegrass (*Lolium perenne*) for variation in vernalization requirement. Two allelic Bacterial Artificial Chromosome clones of the *VRN1* locus from the two genotypes Veyo and Falster with contrasting vernalization requirements were identified, sequenced, and characterized. Analysis of the allelic sequences identified an 8.6-kb deletion in the first intron of the *VRN1* gene in the Veyo genotype which has low vernalization requirement. This deletion was in a divergent recurrent selection experiment confirmed to be associated with genotypes with low vernalization requirement. The region surrounding the *VRN1* locus in perennial ryegrass showed microcolinearity to the corresponding region on chromosome 3 in *Oryza sativa* with conserved gene order and orientation, while the micro-colinearity to the corresponding region in *Triticum monococcum* was less conserved. Our study indicates that the first intron of the *VRN1* gene, and in particular the identified 8.6 kb region, is an important regulatory region for vernalization response in perennial ryegrass.

Keywords *Lolium perenne* · Perennial ryegrass · *VRN1* · Comparative genomics · Vernalization

Communicated by Y. Van de Peer.

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers JN969602 and JN969603 for the Veyo-GP and Falster-GP *VRN1* alleles, respectively.

Electronic supplementary material The online version of this article (doi:10.1007/s00438-011-0654-8) contains supplementary material, which is available to authorized users.

T. Asp (✉) · S. Byrne · J. R. Andersen
Department of Molecular Biology and Genetics,
Research Centre Flakkebjerg, Aarhus University, Forsøgsvej 1,
4200 Slagelse, Denmark
e-mail: Torben.Asp@agrsci.dk

H. Gundlach · K. F. X. Mayer
Institute for Bioinformatics and Systems Biology,
Helmholtz Zentrum München, 85764 Neuherberg, Germany

R. Bruggmann
Department of Biology, University of Bern,
Baltzerstrasse 6, 3012 Bern, Switzerland

M. Xu
National Maize Improvement Center of China,
China Agricultural University, 2 West Yuanmingyuan Road,
Beijing 100094, People's Republic of China

M. Greve · I. Lenk
Research Division, DLF-Trifolium Ltd, Højerupvej 31,
4660 Store Heddinge, Denmark

T. Lübberstedt
Department of Agronomy, Iowa State University,
1204 Agronomy Hall, Ames, IA 50011, USA

Introduction

To investigate the role of the *VRN1* gene in vernalization response in *Lolium perenne* (*L. perenne*) we sequenced and characterized *VRN1* alleles from two genotypes with

contrasting vernalization requirements, with the aim of identifying putative causative polymorphisms, and regulatory regions affecting vernalization response. Transition to flowering is a major shift in the developmental programme of a plant (Sung and Amasino 2004a), and in many plant species this transition is determined by seasonal changes in temperature and photoperiod (Heide 1994).

Many temperate perennial grasses have a dual induction requirement for flowering. In *L. perenne* the primary induction is achieved by low temperature and/or short days, while secondary induction requires transition to long days and is enhanced by moderately higher temperatures (Heide 1994; Aamlid et al. 2000). The requirement for vernalization varies greatly within *L. perenne* and increases generally with increasing latitude of the germplasm origin (Aamlid et al. 2000; Cooper 1960; Jensen et al. 2005). Short days have no effect at low temperatures and very little effect at high temperatures in inducing flowering, indicating that vernalization is the more important factor (Cooper 1960; Jensen et al. 2005).

Vernalization response has been extensively studied in the dicotyledonous species *Arabidopsis thaliana* (*A. thaliana*). In *A. thaliana* two loci, *Flowering Locus C* (*FLC*) and *FRIGIDA* (*FRI*), synergistically delay flowering in winter-annual accessions (Napp-Zinn 1987; Burn et al. 1993; Lee et al. 1993, 1994; Clarke and Dean 1994; Koornneef et al. 1994), and a loss-of-function mutation in either gene results in the loss of the late-flowering phenotype (Michaels and Amasino 1999; Johanson et al. 2000). The presence of a dominant allele of *FRI* elevates *FLC* expression to a level that inhibits flowering (Michaels and Amasino 1999; Sheldon et al. 1999). Vernalization overcomes the effect of *FRI* by repressing the expression of *FLC*, and this repression is stably maintained after plants are transferred to warm growth conditions (Michaels and Amasino 1999; Sheldon et al. 1999). This epigenetic repression of *FLC* provides a molecular explanation of the vernalization mediated 'memory of winter' in *A. thaliana* (Sung and Amasino 2005), which involves covalent modifications of chromatin at the *FLC* locus (Sung and Amasino 2004b; Bastow et al. 2004). In *A. thaliana*, the vernalization-mediated repression of *FLC* requires three genes: *VERNALIZATION 1* encoding a plant-specific DNA-binding protein (Levy et al. 2002), *VERNALIZATION 2* encoding a homologue of the Enhancer of Zeste that is part of a chromatin-modifying complex in *Drosophila* (Gendall et al. 2001), and *VERNALIZATION INSENSITIVE 3* encoding a protein with a PHD motif found in protein complexes involved in chromatin remodelling (Sung and Amasino 2004b). The involvement of *FLC* and *FRI* in establishing the vernalization requirement seems to be conserved in other crucifers (Osborn et al. 1997; Schranz et al. 2002), whereas other plant species such as grasses have different genes involved in establishing the vernalization requirement (Higgins et al. 2010).

The control of flowering time in cereals and grasses has been intensively studied and three key genes have been identified in the vernalization response pathway. The *VRN1* gene encodes a MADS-box transcription factor similar to the *A. thaliana* floral meristem identity gene *APETALA1* (Danyluk et al. 2003; Trevaskis et al. 2003; Yan et al. 2003), while the *VRN2* gene encodes a zinc finger-CCT domain transcription factor with no clear orthologues in *A. thaliana* or *Oryza sativa* (*O. sativa*) (Yan et al. 2004a, b), and the *VRN3* gene encodes a RAF kinase inhibitor like protein with homology to the *FLOWERING LOCUS T* (*FT*) gene from Arabidopsis (Yan et al. 2006).

The *VRN1* and *VRN2* genes were initially identified in *Triticum monococcum* (*T. monococcum*), where it was shown that the *TmVRN1* gene is dominant for the spring growth habit, and that the transcript is up-regulated by vernalization in winter genotypes, whereas transcription of the *TmVRN1* gene is independent of vernalization in spring genotypes (Yan et al. 2003). The *TmVRN2* gene is dominant for the winter growth habit and is down-regulated by vernalization and short days (Yan et al. 2004a, b; Dubcovsky et al. 2006). In *Hordeum vulgare* (*H. vulgare*), the transcript of the *VRN2* gene is also down-regulated by vernalization and under short day conditions (Trevaskis et al. 2006).

In *T. monococcum*, deletions in the *VRN1* promoter region were found to be associated with spring growth habit (Yan et al. 2003), and sequence analysis of dominant and recessive *VRN1* alleles in *H. vulgare* and polyploid wheat led to the identification of a 2.8-kb region within the first intron containing regulatory elements important for the vernalization requirement, and large deletions within this region were associated with spring growth (von Zitzewitz et al. 2005; Fu et al. 2005).

In cereals, proteins encoded by *VRN1* promote the transition from the vegetative to reproductive state of the growing shoot apex, and induction of the *VRN1* gene is accompanied by the repression of the *VRN2* gene, which, when active, prevents transcriptional activity of the *VRN3* gene. In *Triticum aestivum* (*T. aestivum*), the *VRN3* gene has been shown to act as a long-distance flowering signal (florigen) that moves from leaves to apices and promotes flowering by inducing meristem identity genes (Li and Dubcovsky 2008). This is similar to the situation known from other plant species, where it has been shown that *FT* acts as an integrator of the vernalization and photoperiod pathways (Corbesier et al. 2007; Tamaki et al. 2007; Turck et al. 2008).

In *L. perenne*, the major quantitative trait locus (QTL) explaining 28% of the phenotypic variation for vernalization response in the mapping population VrnA was identified on linkage group (LG) 4 and found to co-localize with the *LpVRN1* gene, a putative orthologue of the *VRN1*

gene from *T. monococcum* (Jensen et al. 2005). In the VrnA F_2 mapping population, the *VRN1* allele from the Veyo-Grand Parent (GP) genotype co-segregated with F_2 genotypes with a low vernalization requirement, while the *VRN1* allele from the Falster-GP genotype co-segregated with F_2 genotypes with a high vernalization requirement (Jensen et al. 2005).

The objectives of this study were to (1) identify, sequence, and characterize allelic Bacterial Artificial Chromosome (BAC) clones covering the *VRN1* locus from *L. perenne* genotypes with contrasting vernalization requirements, (2) annotate the *VRN1* locus with the aim of identifying putative causative polymorphisms, regulatory regions, and repetitive elements, (3) annotate the genes in the proximity of the *VRN1* locus in *L. perenne*, and to (4) perform comparative sequence analysis of the *L. perenne VRN1* locus with the corresponding regions of barley, wheat, and rice.

Materials and methods

Identification of BAC clones containing the *VRN1* gene

A BAC library with an estimated five-time genome coverage consisting of 101,376 clones with an average insert size of 99 kb has been constructed (Farrar et al. 2007) from one of the heterozygous parents, NV#20F1-30, of a F_2 mapping population designed to study vernalization response in *L. perenne* (Jensen et al. 2005). DNA pools of the BAC library were screened by PCR as described in Farrar et al. (2007) to identify BAC clones containing the *VRN1* gene. The PCR reactions (10 µl) contained 1 µL of a 1:10 dilution of BAC DNA, 0.5 U of Hotmaster *Taq* polymerase (Eppendorf, Hamburg, Germany), 1× PCR buffer, 10 pmol of primer *LpVRN1*_19for (5′-TCTCCTCTTCTTCCCCACTG), 10 pmol of primer *LpVRN1*_344rev (5′-AGTCGGTTGCGAACTCGTAG), and 6 nmol dNTPs. PCR was performed in a MJ-Research PTC-225 Peltier Thermal Cycler (MJ Research. Inc., Waltham, MA, USA): 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 (Jensen et al. 2005). Genomic DNA from the *L. perenne* genotype NV#20F1-30, that initially was used for constructing the BAC library, was used as a positive control. The insert size of the selected BAC clones was determined as described in Farrar et al. (2007).

VRN1 CAPS marker analysis of BAC clones

A *VRN1* Cleaved Amplified Polymorphic Site (CAPS) marker analysis of the BAC clones was performed using the same primer pair and PCR conditions as described above. The primer pair amplifies a 337-bp fragment of the Veyo-GP allele and a 339-bp fragment of the Falster-GP allele. The PCR fragments were digested with 5 U *Pvu*II (Fermentas, UAB, Vilnius, Lithuania), separated on a 1.5% agarose gel and stained with ethidium bromide as described in Jensen et al. (2005).

BAC sequencing and assembly

Purified BAC DNA was obtained using the Montage BAC_{96} Miniprep Kit (Millipore, Ballerica, MA, USA) and sheared by nebulization for 60 s at 10 pounds per square inch. The sheared DNA was blunt ended, dephosphorylated, and ligated into the pCR®4 Blunt-Topo® vector as described in the Topo® Shotgun Subcloning Kit (Invitrogen, Carlsbad, CA, USA). Individual clones were picked and plasmid DNA was isolated using the Montage Plasmid $Miniprep_{96}$ Kit (Millipore, Ballerica, MA, USA). The clones were sequenced in the forward and reverse directions using DYEnamic™ ET Dye Terminator chemistry and analysed on a MegaBACE 1000 (GE Healthcare Life Sciences, Piscataway, NJ, USA). Base calling and quality assessment were done using PHRED (Ewing and Green 1998). Reads were assembled with PHRAP and edited with CONSED (Gordon et al. 1998). Gaps were closed by a combination of direct BAC sequencing, sequencing of subclones using nested primers or sequencing of PCR fragments spanning the region between contig ends. The finished assemblies of BAC sequences were found to agree with their restriction maps.

Sequence analysis and annotation

Sequence analysis and annotation was essentially performed as described by Bruggmann et al. (2006). The BAC sequences were analysed for Long Terminal Repeat (LTR) retrotransposons using LTR_STRUC (McCarthy and McDonald 2003), and using RepeatMasker with a customised repeat library. The analysis for coding potential was performed by applying extrinsic (homology based) and intrinsic (ab initio gene prediction) methods. Spliced alignments of cDNA and EST sequences were obtained using the GenomeThreader program (Gremme et al. 2005). The EST collection included assemblies of all available monocot EST sequences, and the cDNAs included sequences from monocot plants. For ab initio detection, the two gene-finding programmes FGeneSH++ (Salamov and Solovyev 2000) and GeneMarkHMM (Lukashin and Borodovsky 1998) using monocot and *Zea Mays*/*O. sativa* matrix, respectively, were applied.

Comparative sequence analysis

The PipMaker alignment and visualisation tool (Schwartz et al. 2000) was used for cross-species comparison of the *VRN1* locus. Sequences covering the *VRN1* locus of

H. vulgare (GenBank: AY750993, AY750994, AY750995, and AY750996), *T. monococcum* (GenBank: AY188331), *T. aestivum* (GenBank: AY747597, AY747599, AY747600, AY747601, AY747603, AY747604, and AY747606), *Triticum turgidum* (*T. turgidum*) (GenBank: AY747598 and AY747602), *Aegilops tauschii* (*Ae. tauschii*) (GenBank: AY747605), and *AP1* from *O. sativa* (GenBank: AF377947) were identified and retrieved from GenBank. These sequences, together with the two *L. perenne* BAC sequences (GenBank: JN969602 and JN969603), were submitted to the MultiPipMaker server (http://pipmaker.bx.psu.edu/pipmaker/).

Plant materials for expression analysis

Expression analysis was performed to quantify transcript levels of the *VRN1* transcript in Falster-GP and Veyo-GP. Plant materials were harvested at 0, 2, 4, and 6 weeks of vernalization as previously described (Andersen et al. 2006). RNA was extracted from leaves (100 mg) of Falster-GP and Veyo-GP by the Tri-reagent method (Sigma, St. Louis, USA), according to the manufacturer's instructions, and resuspended in 30 µl DEPC-treated water. The concentration of RNA was measured on a GeneQuant II spectrophotometer (Pharmacia Biotech, Piscataway, NJ, USA). First-strand cDNA synthesis was performed on a total of 5 µg pooled RNA. The RNA solutions were adjusted to a volume of 10 µl with water and 500 ng of B26 primer (5′-GACTCGAGTCGACATCGATTTTTTT TTTTTTTTTT-3′) (500 ng/µl) (Frohman et al. 1998) was added. The primer/RNA/water mixture was heated to 70°C for 10 min and then cooled on ice. Four µl of 5× first-strand buffer (Invitrogen GmbH, Karlsruhe, Germany), 1 µl of RNAguard™ porcine RNase inhibitor (30 U/µl), 2 µl DTT (0.1 M), and 1 µl dNTPs (10 mM) were added and the mixture was incubated at 37°C for 2 min. Finally, 1 µl (200 U) of the Superscript II reverse transcriptase (Invitrogen GmbH, Karlsruhe, Germany) was added and the mixture, 20 µl total, was incubated at 42°C for 50 min. Following incubation, the mixture was diluted with water to 200 µl of which 1 µl was used per quantitative real-time PCR reaction.

Quantitative real-time PCR expression analysis

Quantitative real-time PCR (qPCR) reactions were performed as described in Andersen et al. (2006) according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (Bustin et al. 2009). The SYBR Green PCR Master Mix was used for qPCR, which is a premix containing all the components, except primers, template, and water necessary to perform qPCR using SYBR® Green I Dye (Applied Biosystems, Foster, CA, USA). The reactions were carried out in a total of 10 µl: 5 µl SYBR Green PCR Master Mix, 600 nM forward primer, 600 nM reverse primer (Table 1), 1.0 µl cDNA template, and water up to 10 µl. Reactions were loaded onto 384-well plates, and the qPCR reactions were carried out on an ABI7900HT Sequence Detection System (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions. Expression level units were calculated by the $2^{-\Delta\Delta C_T}$ method, which quantifies the amount of target sequence as fold change relative to a calibrator sample (in this case Falster-GP at 0 weeks of vernalization) and normalized to *Actin* which was used as a reference gene (Livak and Schmittgen 2001).

Primer design for divergent selection and germplasm evaluation

Primers were designed to distinguish between the Falster-GP allele containing the 8.6-kb insertion/deletion (indel) in the first intron of *VRN1*, and the Veyo-GP allele lacking this indel. VRN1_Veyo (F: CTTCGCCTAAGCCAAAC AGT, R: TTAGGTGGCCTCGTGTCTTC) was designed to amplify a 418-bp region in the first intron, amplifying across either side of the insertion. VRN1_Falster (F: as above, R: GAAAAATATCAACATTCAAATACCAAA) was designed to amplify a 407-bp region in the first intron, to include the 5′ end of the insertion.

Establishment of divergent selection populations for vernalization response

The divergent selection populations for vernalization response were established according to Lübberstedt et al. (2003). The populations were initiated by performing pair crosses in all combinations between five *L. perenne* genotypes; LTS03 (Falster-GP), LTS04 (Veyo-GP), LTS11 (Lp 34–551), LTS15 (INRA4), and LTS16 (INRA5) contrasting for vernalization response (Xing et al. 2007). The same number of seedlings from each of the combinations of pair crosses was taken at random and combined to form the Syn0 population. The Syn0 plants were allowed to pollinate

Table 1 Primers used for quantitative real-time PCR experiments

Region	Forward	Reverse
VRN1–exon 1	5′ GCAAGCGGATCGAGAACAAGAT	5′ TCTCGTGCGCCTTCTTGAG
Actin	5′ CTGGAATTGCTGATCGCATG	5′ TGGTAGGAGCAAGGGCAGTG

each other under controlled conditions, and the seed was collected in bulk to constitute the Syn1 (=C0) population. Divergent selection for vernalization requirement was performed by subjecting the C0 population (500 plants) to phenotypic selection for vernalization requirement over two generations to produce (1) a C2+ population with high vernalization requirement, (2) a C2− population with no vernalization requirement, and (3) a population without any conscious selection for vernalization requirement. Plants selected for the C1+ and the C2+ populations required at least 8 weeks of vernalization to initiate flowering, while plants selected for the C1− and C2− populations were selected if they flowered without vernalization.

Plant materials for germplasm evaluation of Veyo-GP and Falster-GP *VRN1* alleles

A total of 380 individual plants from 13 commercial varieties of *L. perenne* and nine genotypes of DLF-Trifolium breeding material were selected for evaluation of presence or absence of Veyo-GP and Falster-GP VRN1 alleles. The commercial varieties used were Aberavon, Aberdart (IBERS, UK), Cancan, Foxtrot, Indiana, Stefani (DLF-Trifolium, Denmark), Lipresso (Eurograss, Germany), Matrix (Cropmark, New Zealand), Pastour, Ohio, Hamilton (Limagrain, France), Respect, and Romark (Cebeco, The Netherlands) (Table 2). Genomic DNA was isolated from all 380 plants and used for genotyping.

Results

BAC clone identification

A total of 101,376 BACs were screened for individual clones containing the *VRN1* gene. Three BAC clones were identified and analysed using a *VRN1* cleaved amplified polymorphic sequence (CAPS) marker (Jensen et al. 2005) to determine the grandparental origin of BAC clones. Previous studies using the *VRN1* CAPS marker have shown that both the Veyo-GP and the Falster-GP genotypes are homozygous for their respective alleles (Jensen et al. 2005). One BAC clone was derived from the Falster-GP genotype, and two BAC clones were derived from the Veyo-GP genotype of the F_2 mapping population (Fig. 1). Based on CAPS marker analysis and insert size, two BAC clones, 7D23 (Falster-GP allele) and 74D14 (Veyo-GP allele), were selected for sequencing.

Table 2 Heading classification of the commercial varieties

Variety	Heading date class
Aberavon	Intermediate to late
Aberdart	Intermediate
Cancan	Late
Foxtrot	Intermediate to late
Hamilton	Early
Indiana	Early to intermediate
Lipresso	Early
Matrix	Early
Ohio	Late
Pastour	Late
Respect	Intermediate
Romark	Late
Stefani	Late

BAC sequencing and assembly

The initial assembly of the region surrounding the *VRN1* locus was accomplished by Sanger sequencing of random sheared sub-clones with an average insert size of 1.0–1.5 kb. A total of 1,559 and 1,485 PHRED $\geq$ 20 reads were used for the assembly of the Falster-GP and Veyo-GP contiguous sequences, respectively, producing 15 non-vector contigs for the Veyo-GP BAC clone and five non-vector contigs for the Falster-GP BAC clone. Gaps were closed by a combination of direct sequencing of BAC clones, complete sequencing of sub-clones using nested primers, and sequencing of PCR fragments spanning the region between contig ends. The final sequence for the Veyo-GP BAC clone was 77,368 bp with an average coverage of 9.1, while the final sequence for the Falster-GP BAC clone was 55,912 bp with an average coverage of 13.3.

Gene content, structure, and distribution

Two genes were identified on the 77-kb contiguous Veyo-GP sequence (Table 3). These genes were annotated *Lp_74D14_1* and *Lp_74D14_2*. The *Lp_74D14_1* gene is

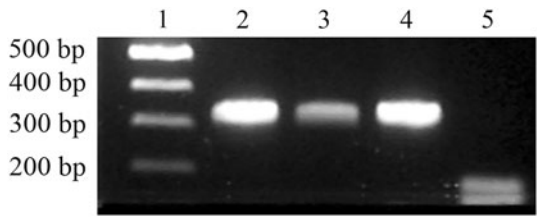

Fig. 1 *VRN1* CAPS marker analysis of the selected *Lolium perenne* BAC clones. *Lane 1* 100 bp DNA Ladder marker; *Lane 2* undigested PCR fragment of the *VRN1* gene from the Falster-GP genotype; *Lane 3* digested PCR fragment of the *VRN1* gene from the Falster-GP genotype; *Lane 4* undigested PCR fragment of the *VRN1* gene from the Veyo-GP genotype; *Lane 5* digested PCR fragment of the *VRN1* gene from the Veyo-GP genotype. A fragment of the *VRN1* gene was amplified from the BAC clones by PCR, digested with *Pvu*II, and separated on an agarose gel as described in "Materials and methods"

Table 3 Characteristics of the predicted genes identified at the *VRN1* locus in *Lolium perenne*

Genotype	Gene name	Size (bp)	Structure[a]	Protein[b]	Predicted function	*Oryza sativa* locus id[c]
Falster-GP	*Lp_7D23_1* (*VRN1*)	16,292	8E, 7I	244	Vernalization response	Os03g54160
Veyo-GP	*Lp_74D14_1* (*VRN1*)	7,726	8E, 7I	244	Vernalization response	Os03g54160
Veyo-GP	*Lp_74D14_2*	3,359	4E, 3I	606	Unknown	Os03g54150

[a] The number of exons (*E*) and the number of introns (*I*) deduced either from comparison with cDNAs or from prediction programmes

[b] The number of amino acid residues

[c] For each *Lolium perenne* gene, homologues found on *O. sativa* chromosome 3 are indicated with their locus id

located at nucleotide position 19,916 to 27,641, while the *Lp_74D14_2* gene is located on the complementary strand at nucleotide position 31,778 to 28,421. The *Lp_74D14_1* gene was identified as the *VRN1* gene, while the *Lp_74D14_2* gene has homology to an expressed gene in *O. sativa* with unknown function (GenBank: NP_00105 1299) (Fig. 2). The gene structure model of the *Lp_74D1 4_1* gene (*VRN1*) was determined by a spliced alignment against a full-length cDNA (GenBank: AY198326). The *Lp_74D14_1* gene has eight exons and seven introns, which is the same genomic organization as the *VRN1* genes from *T. monococcum*, *H. vulgare*, and *AP1* from *O. sativa*. The deduced amino acid sequence of the *Lp_74D14_1* gene is 87, 86, and 83% identical to those of the *VRN1* gene from *T. monococcum*, *H. vulgare*, and *AP1* from *O. sativa*, respectively.

The gene model of the *Lp_74D14_2* gene was predicted to include four exons and three introns, supported by an EST from a *L. perenne* in-house database and a full-length *O. sativa* cDNA (GenBank: NP_001051299). The deduced amino acid sequence is 45% identical to the corresponding protein in *O. sativa*, and the C-terminal end of the protein has weak homology (*E*-value 0.005) to the DnaJ domain of proteins associated with the hsp70 heat-shock chaperone system.

In the 56-kb contiguous sequence from Falster-GP a partial *VRN1* gene, annotated as *LP_7D23_1*, was identified at nucleotide position 45,506–48,256, corresponding to the first exon and 2.6 kb of the first intron of the gene. The remaining 7.7 kb of the BAC clone downstream the partial *VRN1* gene showed no homology to the corresponding region of the Veyo-GP sequence containing the remaining 2.8 kb of the first intron and the remaining six introns and seven exons. This suggests that the *VRN1* gene in the Falster-GP genotype is either truncated or, alternatively, that a large indel is present in the first intron of the gene. A PCR-based strategy was adopted using genomic DNA from the Falster-GP genotype as template, to reveal if the *VRN1* gene is truncated. Additional 6 kb of contiguous sequence was subsequently obtained from the Falster-GP genotype containing the remaining part of the *VRN1* gene. Comparative sequence analysis of the *VRN1* gene from the two genotypes identified an 8.6-kb region which represents either an insertion in Falster-GP *VRN1* allele, or a deletion in the Veyo-GP *VRN1* allele in the first intron of the gene (Fig. 2).

Repeat elements

The two contiguous BAC sequences were analysed for repeat elements using LTR_STRUC (McCarthy and McDonald 2003) and RepeatMasker with a customised repeat library consisting of a non-redundant set of 5,707 repeat element sequences and classified by a hierarchical repeat classification scheme (Haberer et al. 2005). The repeat library was used to mask and classify the repeat element in the BAC sequences. The repeat content of the Veyo-GP and the Falster-GP BAC sequence was 42 and 58%, respectively (Table 4). In the Veyo-GP BAC sequence 84% of the repeat elements were Class I LTR retrotransposons, while the Class I LTR retrotransposons constituted only 68% of the repeat elements in the Falster-GP BAC sequence (Table 4). A total of 6.4 and 9.1% of the repeat elements were classified as Class I non-LTR retrotransposons in the Veyo-GP and Falster-GP BAC sequences, respectively, while Class II DNA transposons represented 3.2 and 18.2% of the Veyo-GP and Falster-GP BAC sequence repeat elements, respectively (Table 4). The position and orientation of the different transposable elements are depicted in Fig. 2.

LTR_STRUC (McCarthy and McDonald 2003) was used to identify novel full-length LTR retrotransposons by searching for structural features characteristic of such elements. Two novel full-length LTR retrotransposons were identified and annotated as *Lotte_7D23_1* and *Camilla_7D23_1* in the Falster-GP BAC sequence at nucleotide position 6,781–15,261 and on the complement strand at nucleotide position 37,548–19,839, respectively (Table 4; Fig. 2). Both repeat elements showed homology to the *Athila*-like repeat element *Sabrina* from *H. vulgare* (Choudhuri et al. 2004). For the allelic Veyo-GP sequence a fragment of the *Camilla* repeat element was identified and annotated as *Camilla_74D14_1*. It is located at the very 5′ end of the Veyo-GP BAC sequence on the complementary strand at nucleotide position 11,617–1 (Fig. 2). The *Lotte* repeat element was not present in the Veyo-GP BAC sequence as the location of this repeat element in the

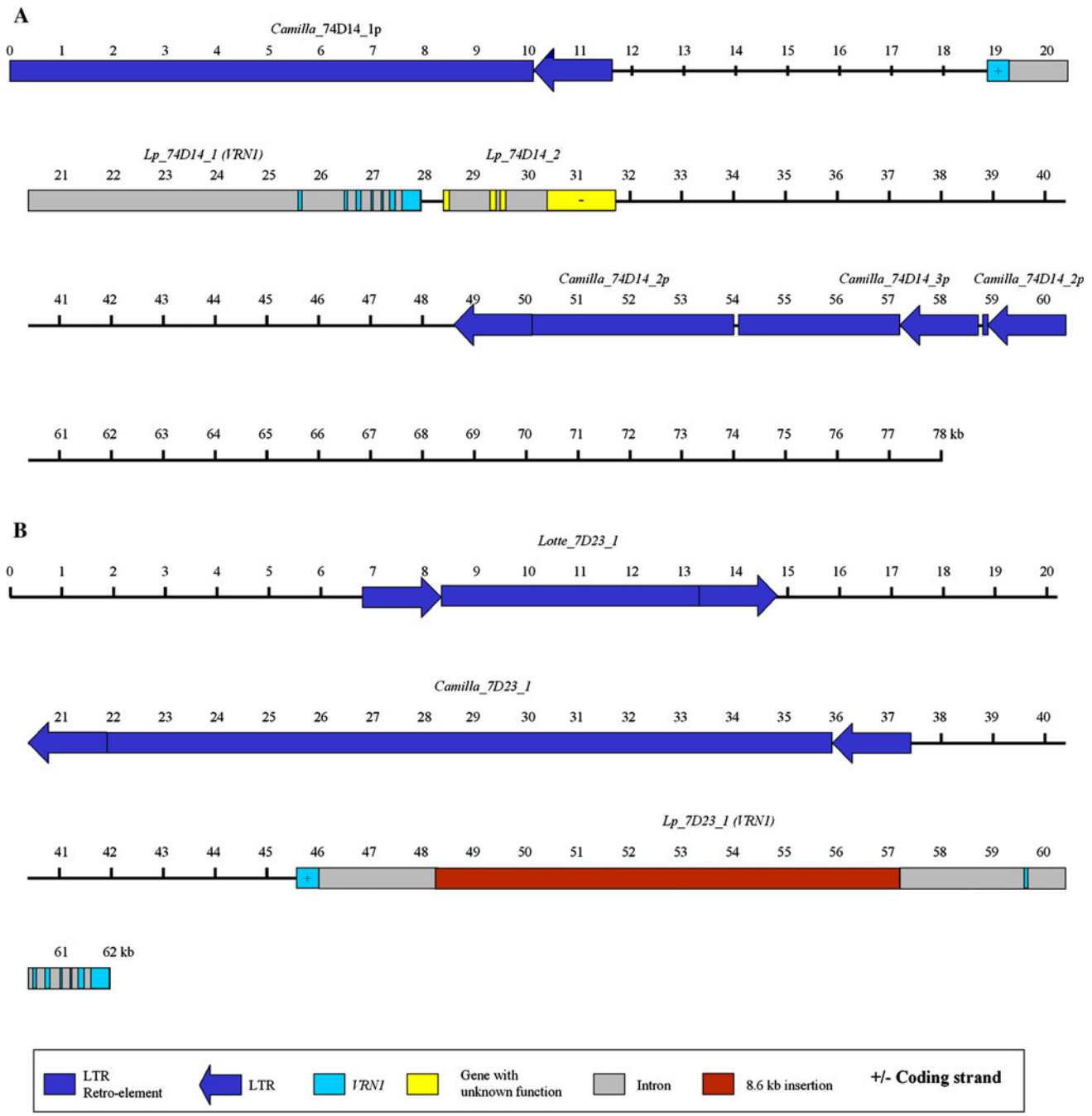

Fig. 2 Organization of the *Lolium perenne VRN1* locus. The 77-kb region from the genotype Veyo-GP (**a**) and the 62-kb region from the genotype Falster-GP (**b**) are arrayed linearly and the major features are highlighted. The annotation of transposable elements was performed as described by SanMiguel et al. (2002). In the names of repeat elements, p at the end means a partial repeat element with deletions of portions of LTR and/or coding region. Regions given the same names suggest that a repeat element sequence was interrupted, likely by the insertion of another repeat element. Genes and repeat elements were identified as described in "Materials and methods"

Falster-GP BAC sequence is 5′ to where the allelic Veyo-GP sequence starts.

A *Camilla* repeat element, annotated as *Camilla_74D14_2*, with an internal deletion was identified in the Veyo-GP BAC sequence on the complementary strand at position 60,593–48,522. The repeat element has intact LTR and Target Site Duplication (TSD) in both ends and the internal deletion was most likely caused by the insertion of another nested and truncated *Camilla* repeat element, annotated as *Camilla_74D14_3*, on the complementary strand at position 58,787–53,910. The 5′ TSD and LTR were not identified in the nested and truncated *Camilla* repeat element indicating that this element is truncated in the 5′ end (Fig. 2).

Table 4 Occurrence and distribution of repetitive DNA at the *VRN1* locus in *Lolium perenne*

Repeat class	BAC clone 74D14					BAC clone 7D23				
	No. of hits	% of repeat elements	No. of bases (bp)	% of repeat bp's	% of sequence	No. of hits	% of repeat elements	No. of bases (bp)	% of repeat bp's	% of sequence
Class I: retroelement	28	90.3	31,587	96.8	40.8	17	77.3	31,786	97.4	56.9
LTR retrotransposon	26	83.9	31,117	95.4	40.2	15	68.2	31,485	96.5	56.3
Ty1/copia	9	29.0	1,940	6.0	2.5	1	4.6	77	0.2	0.1
Ty3/gypsy	0	0	0	0	0	2	9.1	340	1.0	0.6
TRIM	0	0	0	0	0	0	0	0	0	0
Other LTR	17	54.8	29,177	89.4	37.7	12	54.6	31,068	95.2	55.6
Non-LTR retrotransposon	2	6.5	470	1.4	0.6	2	9.1	301	0.9	0.5
LINE	1	3.2	393	1.2	0.5	0	0	0	0	0
SINE	1	3.2	77	0.2	0.1	2	9.1	301	0.9	0.5
Unclassified retrotroelement	0	0	0	0	0	0	0	0	0	0
Class II: DNA Transposon	1	3.2	813	2.5	1.1	4	18.2	780	2.4	1.4
DNA transposon superfamily	1	3.2	813	2.5	1.1	2	9.1	341	1.0	0.6
CACTA superfamily	1	3.2	813	2.5	1.1	1	4.6	177	0.5	0.3
hAT superfamily	0	0	0	0	0	1	4.6	164	0.5	0.3
Mutator superfamily	0	0	0	0	0	0	0	0	0	0
Tc1/Mariner superfamily	0	0	0	0	0	0	0	0	0	0
PIF/Harbinger	0	0	0	0	0	0	0	0	0	0
Other transposon family	0	0	0	0	0	0	0	0	0	0
MITE	0	0	0	0	0	2	9.1	439	1.4	0.8
Stowaway	0	0	0	0	0	1	4.6	163	0.5	0.3
Tourist	0	0	0	0	0	1	4.6	276	0.9	0.5
Other MITE	0	0	0	0	0	0	0	0	0	0
Unclassified DNA transposon	0	0	0	0	0	0	0	0	0	0
Class III: helitron	0	0	0	0	0	1	4.6	66	0.2	0.1
Simple sequence	0	0	0	0	0	0	0	0	0	0
High copy number gene	1	3.2	75	0.2	0.1	0	0	0	0	0
Other repeats	1	3.2	147	0.5	0.2	0	0	0	0	0
Total repeats	31	100	32,622	100	42.2	22	100	32,632	100	58.4

Allelic- and comparative sequence analysis of the *VRN1* locus

Allelic sequence analysis using the GenAlyzer software (Choudhuri et al. 2004) revealed that the two contiguous sequences share approximately 36.3 kb of allelic sequence. Within this region covering the 5′ flanking region and the entire *VRN1* gene, a total number of 38 single-nucleotide polymorphisms and 15 indels were identified, ranging in size from 1 to 8,565 bp.

The 8,565-bp insertion located in the first intron of the *VRN1* gene in the Falster-GP genotype was analysed for LTR retrotransposons using LTR_STRUC (McCarthy and McDonald 2003). However, no structural features characteristic of such elements could be identified, suggesting that the insertion is not a LTR retrotransposon. A putative transposon-like protein was identified in the insertion at position 7,787–8,134 which has homology to Class II CACTA DNA transposons of the En/Spm sub-class, suggesting that the insertion could be a repeat element of this class. However, no terminal inverted repeats could be identified suggesting that the element is either truncated or nonautonomous. Furthermore, comparative sequence analysis of the 8.6-kb fragment with the *VRN1* gene from *T. monococcum*, *H. vulgare,* and polyploid wheat (von Zitzewitz et al. 2005; Yan et al. 2003; Fu et al. 2005) showed that the insertion has no homology to the *VRN1* gene from these species.

Comparative analysis of DNA sequences from different species is a powerful approach for identifying coding and functional non-coding sequences, because functional sequences tend to evolve at a slower rate than non-functional sequences. A Percent Identity Plot (PIP) of *VRN1* spring and winter alleles of *L. perenne*, *T. aestivum*, *T. turgidum*, *T. monococcum*, *H. vulgare*, *Ae. tauschii*, and *AP1* from *O. sativa* was created using MultiPIPMaker (Schwartz et al. 2000) and used for comparative sequence analysis of coding sequences and to identify conserved non-coding sequences between spring and winter alleles.

The first exon appears to be the most conserved part of the coding sequence with an identity of 93%, while the remaining exons are less conserved with an identity between 75 and 90% (Supplementary Fig. 1). The 5′ region of the first intron of the *VRN1* gene in *L. perenne* shows homology to the corresponding regions from the other species included in this study, while the 3′ region of the first intron shows no significant homology. A 2.8-kb region within the 5′ region of the first intron of the *VRN1* gene in cereals has previously been defined as the "critical region" containing putative regulatory elements for the vernalization response, and deletions within this region have been associated with spring growth habit (Fu et al. 2005); however, the 8.6-kb indel region in *L. perenne* does not overlap with the "critical region" discriminating winter and spring types in the cereals identified by Fu et al. (2005). Comparative sequence analysis of *L. perenne*, *O. sativa* (GenBank: NP_001051299), and *T. monococcum* (GenBank: AY188331) sequences revealed furthermore that the *Lp_74D14_2* gene located on the complementary strand close to the 3′ end of the *VRN1* gene in *L. perenne* and *O. sativa* is absent in *T. monococcum*. In *H. vulgare*, sequence information for this genomic region is not available so far. The gene is expressed in both *O. sativa* and *L. perenne* as supported by an EST from *L. perenne* (in-house EST database) and *O. sativa* (GenBank: NP_001051299). No additional sequences with significant homology to this gene were identified in GenBank.

Expression analysis of the *VRN1* gene during vernalization

In a previous study it has been shown that the Veyo-GP and the Falster-GP genotypes are homozygous for their respective *VRN1* alleles (Jensen et al. 2005), which makes it possible to perform allele-specific expression analysis of the *VRN1* gene in the two genotypes. Transcript levels of the *VRN1* gene during vernalization were studied by qPCR in the Veyo-GP and Falster-GP genotypes before and during vernalization using primers designed to the first exon.

To check for potential contamination, mis-priming, and primer-dimer artefacts a quality control was performed by melting curve analysis to validate that all samples had a similar melting temperature (data not shown). The *Actin* gene was used as a reference gene for the qPCR analysis as it previously has been shown to be a suitable reference

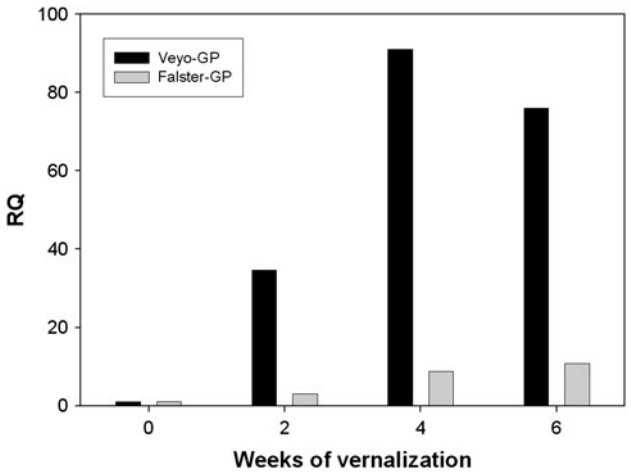

Fig. 3 *VRN1* transcript levels in Veyo-GP and Falster-GP plants before and during vernalization. The expression levels are the averages of three biological replicates. Relative quantification is expressed as fold-difference relative to *VRN1* transcript level of Falster-GP plants at 0 weeks of vernalization

Table 5 Divergent recurrent selection for vernalization response and allelic composition at the *VRN1* locus in *L. perenne*

Plant name	Selection experiment	Veyo-GP allele	Falster-GP allele
LTS03	Founder	0	1
LTS04	Founder	1	0
LTS11	Founder	0	1
LTS15	Founder	0	1
LTS16	Founder	0	1
2005-10/110999-Ep. 009	GRASP:C2+	0	1
2005-10/110999-Ep. 041	GRASP:C2+	0	1
2005-10/110999-Ep. 069	GRASP:C2+	0	1
2005-10/110999-Ep. 096	GRASP:C2+	0	1
2005-10/110999-Ep. 108	GRASP:C2+	0	1
2005-10/110999-Ep. 114	GRASP:C2+	0	1
2005-10/110999-Ep. 127	GRASP:C2+	0	1
2005-10/110999-Ep. 129	GRASP:C2+	0	1
2005-10/110999-Ep. 132	GRASP:C2+	0	1
2005-10/110999-Ep. 136	GRASP:C2+	0	1
2005-10/110999-Ep. 148	GRASP:C2+	0	1
2005-10/110999-Ep. 150	GRASP:C2+	0	1
2005-10/110999-Ep. 153	GRASP:C2+	0	1
2005-10/110999-Ep. 155	GRASP:C2+	0	1
2005-10/110999-Ep. 157	GRASP:C2+	0	1
2005-10/110999-Ep. 159	GRASP:C2+	0	0
2005-10/110999-Ep. 161	GRASP:C2+	0	1
2005-10/110999-Ep. 184	GRASP:C2+	0	1
2005-10/110999-Ep. 226	GRASP:C2+	0	1
2005-10/110999-Ep. 233	GRASP:C2+	0	1
2005-10/110999-Ep. 234	GRASP:C2+	0	1
2005-10/110999-Ep. 241	GRASP:C2+	0	1
2005-10/110999-Ep. 248	GRASP:C2+	0	1
2005-10/110999-Ep. 269	GRASP:C2+	0	0
2005-10/110999-Ep. 314	GRASP:C2+	0	1
2005-10/110999-Ep. 330	GRASP:C2+	0	1
2005-10/110999-Ep. 335	GRASP:C2+	0	0
2005-10/110999-Ep. 339	GRASP:C2+	1	1
2005-10/110999-Ep. 363	GRASP:C2+	0	1
2005-10/110999-Ep. 366	GRASP:C2+	0	1
2005-10/110999-Ep. 379	GRASP:C2+	0	1
2005-10/110999-Ep. 392	GRASP:C2+	0	0
2005-10/110998-Ep. 068	GRASP:C2−	1	0
2005-10/110998-Ep. 032	GRASP:C2−	1	1
2005-10/110998-Ep.254	GRASP:C2−	1	0
2005-10/110998-Ep. 267	GRASP:C2−	0	0
2005-10/110998-Ep. 242	GRASP:C2−	1	0
2005-10/110998-Ep. 172	GRASP:C2−	1	1
2005-10/110998-Ep. 141	GRASP:C2−	1	1
2005-10/110998-Ep. 140	GRASP:C2−	0	1
2005-10/110998-Ep. 455	GRASP:C2−	0	1
2005-10/110998-Ep. 129	GRASP:C2−	1	1
2005-10/110998-Ep. 185	GRASP:C2−	1	1

Table 5 continued

Plant name	Selection experiment	Veyo-GP allele	Falster-GP allele
2005-10/110998-Ep. 207	GRASP:C2−	1	1
2005-10/110998-Ep. 240	GRASP:C2−	1	0
2005-10/110998-Ep. 451	GRASP:C2−	1	0
2005-10/110998-Ep. 137	GRASP:C2−	1	0
2005-10/110998-Ep. 264	GRASP:C2−	1	0
2005-10/110998-Ep. 079	GRASP:C2−	1	1
2005-10/110998-Ep. 076	GRASP:C2−	1	1
2005-10/110998-Ep. 453	GRASP:C2−	1	1
2005-10/110998-Ep. 166	GRASP:C2−	1	1
2005-10/110998-Ep. 450	GRASP:C2−	1	0
2005-10/110998-Ep. 011	GRASP:C2−	1	1
2005-10/110998-Ep. 110	GRASP:C2−	1	0
2005-10/110998-Ep. 147	GRASP:C2−	1	1
2005-10/110998-Ep. 085	GRASP:C2−	1	1
2005-10/110998-Ep. 186	GRASP:C2−	1	1
2005-10/110998-Ep. 248	GRASP:C2−	1	1
2005-10/110998-Ep. 423	GRASP:C2−	1	1
2005-10/110998-Ep. 098	GRASP:C2−	1	0
2005-10/110998-Ep. 205	GRASP:C2−	1	1
2005-10/110998-Ep. 160	GRASP:C2−	1	0
2005-10/110998-Ep. 247	GRASP:C2−	1	1

gene for qPCR analysis in *L. perenne* (Andersen et al. 2006).

In the Veyo-GP genotype, the *VRN1* gene is induced 35-, 91-, and 76-fold after 2, 4, and 6 weeks of vernalization, respectively. In contrast, the *VRN1* gene in the Falster-GP genotype is only induced 3-, 9-, and 11-fold after 2, 4, and 6 weeks of vernalization, respectively (Fig. 3). Thus, the transcription of the *VRN1* gene is induced to higher levels in Veyo-GP genotype plants as compared with Falster-GP genotype plants. In the non-vernalized Falster-GP controls, transcription of the *VRN1* gene is completely repressed, whereas it is induced at a low level in the Veyo-GP genotype (data not shown).

Divergent selection for vernalization response

A total of 32 genotypes from each of the C+ and C− populations alongside the founding genotypes were tested for the presence or absence of the Veyo-GP and Falster-GP *VRN1* alleles. Of the founder genotypes only LTS03 (Veyo-GP) showed a product with the Vrn1_Veyo primers, whereas the other four genotypes only showed a product with the VRN1_Falster primers. A significant difference (X^2; $p < 0.05$) in accumulation of the Veyo-GP and Falster-GP alleles was found between the C+ and C-populations. In the C2+ population, one genotype amplified a product with both primers and 28 genotypes amplified with only the VRN1_Falster primers. In the C2− population, 29 genotypes amplified a product with the VRN1_Veyo primers, and 20 genotypes amplified with the VRN1_Falster primers (Table 5). This clearly demonstrates that selection for no vernalization requirement resulted in the accumulation of plants with at least one Veyo-GP allele lacking the 8.6-kb insertion, whereas selection for high vernalization requirement has resulted in accumulation of plants homozygous for the Falster-GP allele.

Germplasm evaluation for Veyo-GP and Falster-GP *VRN1* alleles

The VRN1_Veyo and VRN1_Falster primers were used to genotype the indel in 380 genotypes representing a wide range of *L. perenne* germplasm from commercial varieties and breeding material. Seven genotypes, three homozygous and four heterozygous, carried the Veyo-GP allele lacking the insertion in the first intron of *VRN1* (Supplementary Table 1). All seven genotypes originated from the cultivar Matrix, which is a very early heading cultivar bred in New Zealand. The VRN1_Falster primers amplified the expected product in 312 out of 380 genotypes and 65 genotypes failed to amplify a product with either primer set.

Discussion

An orthologue of the *VRN1* gene has been identified and mapped to a major QTL on LG 4 for the vernalization response in *L. perenne*, explaining 28% of the phenotypic variation. The QTL was mapped in an F_2 population, 'VrnA', derived from a cross between a single genotype from the variety Veyo with a low vernalization requirement, and a single genotype from the ecotype Falster, with a high vernalization requirement. In the VrnA population, the *VRN1* allele from the Veyo-GP genotype co-segregates with a QTL allele for low vernalization requirement, while the *VRN1* allele from the Falster-GP genotype co-segregates with a QTL allele for a high vernalization requirement (Jensen et al. 2005).

To identify the polymorphisms at the *VRN1* locus responsible for the contrasting vernalization requirement, we identified and sequenced two allelic BAC clones of the *VRN1* locus from the two grandparent genotypes. We were able to identify a 8.6-kb deletion in the first intron of the *VRN1* gene in the allele from the genotype Veyo-GP which has a low vernalization requirement. This deletion was in a recurrent selection experiment associated with genotypes with a low vernalization response.

Sequence analysis of the 8.6-kb region identified a putative transposon protein with homology to Class II CACTA DNA transposons of the En/Spm sub-class (Pereira et al. 1986). Class II elements move via a DNA intermediate, where the elements are excised from the genome and integrated elsewhere. Excision and reintegration requires an enzyme known as transposase. The terminal regions of all CACTA elements show a similar sequence organization. They are flanked by short terminal inverted repeats that terminate in the CACTA motif, which serves as a recognition site for the transposase protein (Lewin 1997). In most cases, sequence conservation between different families is limited to this short motif, which makes it almost impossible to identify new elements based on the TIR of known elements. In addition, CACTA elements contain sub-terminal repeats of 10- to 20-bp units that are repeated in direct and inverted orientation (Lewin 1997). Thus, CACTA transposons are difficult to identify and new elements are usually only found because of the presence of a transposase-like protein. In the 8.6-kb region a transposase-like protein was identified, but no terminal inverted repeats were identified, suggesting that the element might be truncated. Another explanation might be that the element is a nonautonomous CACTA element, with no functional transposase gene, and thus depends on trans-acting factors encoded by independent autonomous copies present in the same genome for the transposition (Gierl et al. 1989; Bennetzen 2000).

Both alleles of the *VRN1* gene were independently mapped in the VrnA mapping population and found to co-localize with the major QTL for the vernalization response on LG 4 (data not shown). Furthermore, in a small selection experiment an accumulation of plants homozygous for the Falster-GP allele was observed in plants selected for a high vernalization requirement, while the Veyo-GP allele accumulated in plants selected for a no vernalization requirement.

Although there were a number of polymorphisms present between both alleles, the greatest difference was due to the occurrence of the large indel in the first intron. Previous studies in cereals have already implicated regions of the first intron of *VRN1* in the control of vernalization. We therefore hypothesise that the indel in the first intron of *VRN1* may be responsible for the contrasting vernalization requirements of these two *L. perenne* genotypes. In polyploid wheat species and *H. vulgare*, a 2.8-kb region within the first intron of the *VRN1* gene was identified as a "critical region" for winter growth habit, and deletions within this region are associated with spring growth habit, suggesting that this region might contain intronic regulatory elements for the vernalization response (von Zitzewitz et al. 2005; Fu et al. 2005). While the results reported here support the presence of regulatory regions within the first intron across grasses, the 8.6-kb indel region in *L. perenne* does not overlap with the region discriminating winter and spring types in the cereals. Furthermore, it seems that the Veyo-GP allele (deletion allele) is rare in *L. perenne* germplasm, while in contrast the Falster-GP allele (insertion allele) is widespread. Thus, given the low frequency of the Veyo-GP allele in the *L. perenne* germplasm it is most likely that a deletion has occurred in the Veyo-GP. While this does not exclude common regulatory mechanisms, it might suggest that genetic regulation of the vernalization response has diverged between cereals and perennial grasses.

In cereals, proteins encoded by *VRN1* and *VRN3* promote flowering and the early flowering (spring type) allele is dominant for both genes, whereas the protein encoded by *VRN2* represses flowering and the late-flowering (winter type) allele is dominant. Thus, only recessive *VRN1* and *VRN3* alleles and at least one dominant *VRN2* allele should be present in an individual to confer effective winter types, while all other allelic combinations would result in spring types. The observation that the *VRN1* Veyo-GP allele conferring no vernalization requirement is the dominant allele is thus in agreement with the results from cereals. However, comprehensive allele sequencing will be required to validate this hypothesis for the *VRN2* and *VRN3* genes in *L. perenne*.

The two *L. perenne* BAC sequences presented in this work provide the first large sequenced genomic regions from *L. perenne* and, thus, give a first insight into the genomic organization and genome composition of

L. perenne. The repeat content of the Veyo-GP and the Falster-GP BAC sequences was 42 and 58%, respectively. However, the true repeat content can only be estimated more accurately when more genomic *L. perenne* sequence data become available. The predominant repeat elements at the *VRN1* locus in *L. perenne* are Class I LTR retrotransposons with similarity to the *Athila*-like LTR retrotransposon *Sabrina* from *H. vulgare* (Shirasu et al. 2000), while Class II repeat elements constitute only approximately 2.5% of the repetitive elements.

The average gene density observed in the two *L. perenne* BAC sequences is one gene per 44 kb. This value is similar to observations made for 100 random BAC sequences from *Z. mays*, where an average gene density of one gene per 43 kb has been observed (Haberer et al. 2005). However, increasing evidence shows the presence of gene-rich regions and a wide variation of gene densities in cereal genomes (Keller and Feuillet 2000). For example, for *Z. mays* an 18-fold variation in gene density ranging from 0.5 genes per 100 kb up to 10.7 genes per 100 kb has been observed (Haberer et al. 2005). More recently, for gene-rich *Z. mays* contiguous regions in the size of several megabases a gene density of one gene per 27–33 kb has been reported (Bruggmann et al. 2006).

Since the genome sizes of *Z. mays* (2,671 Mb) and *L. perenne* (2,034 Mb) are within the same range, it can be speculated that the gene density in *L. perenne* is similar to the gene density in maize (Bennett and Smith 1976; Evans et al. 1972). The observed genome density of one gene per 44 kb at the *VRN1* locus of *L. perenne* is, therefore, in agreement with the expected gene density. However, more genomic sequence of *L. perenne* is needed for a more accurate estimate of the average gene density.

The gene order and orientation at the *VRN1* locus in the *L. perenne* genotype Veyo-GP showed micro-colinearity to the corresponding region on chromosome 3 in *O. sativa*, where both genes identified in the Veyo-GP BAC sequence had the same order and orientation. In the corresponding region from *T. monococcum* chromosome 5, the microcolinearity was less conserved, because the *Lp_74D14_2* gene with unknown function, present in both *L. perenne* and *O. sativa*, was not identified.

In general, *L. perenne* exhibits a large genotypic variation in vernalization requirement (Aamlid et al. 2000; Skøt et al. 2005), where the Veyo-GP and Falster-GP genotypes are among the extremes. The fact that the Veyo-GP allele is rare in the germplasm suggests that *VRN1* polymorphisms, e.g., in the promoter or first intron, other than the indel reported here contribute to finer scale variation in the vernalization response in *L. perenne*.

Acknowledgments This work was supported by a grant from the framework "Biotechnology and applied plant genetics in plant breeding" from The Directorate for Food, Fisheries and Agricultural Business under the Danish Ministry of Food, Agriculture and Fisheries. Dorthe Strue Nielsen and Kirsten Vangsgaard are acknowledged for their excellent technical assistance.

References

Aamlid TS, Heide OM, Boelt B (2000) Primary and secondary induction requirements for flowering of contrasting European varieties of *Lolium perenne*. Ann Bot 86:1087–1095

Andersen JR, Jensen LB, Asp T, Lübberstedt T (2006) Vernalization response in perennial ryegrass (*Lolium perenne* L.) involves orthologues of diploid wheat (*Triticum monococcum*) *VRN1* and rice (*Oryza sativa*) *Hd1*. Plant Mol Biol 60:481–494

Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA, Dean C (2004) Vernalization requires epigenetic silencing of *FLC* by histone methylation. Nature 427:164–167

Bennett MD, Smith JB (1976) Nuclear DNA amounts in angiosperms. Phil Transac Royal Soc London Series B-Biol Sci 334:309–345

Bennetzen JL (2000) Transposable element contributions to plant genome evolution. Plant Mol Biol 42:251–269

Bruggmann R, Bharti AK, Gundlach H, Lai J, Young S, Pontaroli AC, Wei F, Haberer G, Fuks G, Du C et al (2006) Uneven chromosome contraction and expansion in the maize genome. Genome Res 16:1241–1251

Burn JE, Smyth DR, Peacock WJ, Dennis ES (1993) Genes conferring late flowering in *Arabidopsis thaliana*. Genetica 90:147–155

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622

Choudhuri JV, Schleiermacher C, Kurtz S, Giegerich R (2004) Genalyzer: interactive visualization of sequence similarities between entire genomes. Bioinformatics 20:1964–1965

Clarke JH, Dean C (1994) Mapping *FRI*, a locus controlling flowering time and vernalization response in *Arabidopsis thaliana*. Mol Gen Genet 242:81–89

Cooper JP (1960) Short-day and low-temperature induction in *Lolium*. Ann Bot 24:232–246

Corbesier L, Vincent C, Jang SH, Fornara F, Fan QZ, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C (2007) FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science 316:1030–1033

Danyluk J, Kane NA, Breton G, Limin AE, Fowler DB, Sarhan F (2003) *TaVRT-1*, a putative transcription factor associated with vegetative to reproductive transition in cereals. Plant Physiol 132:1849–1860

Dubcovsky J, Loukoianov A, Fu D, Valarik M, Sanchez A, Yan L (2006) Effect of photoperiod on the regulation of wheat vernalization genes *VRN1* and *VRN2*. Plant Mol Biol 60:469–480

Evans GM, Rees H, Snell CL, Sun S (1972) The relationship between nuclear DNA amount and the duration of the mitotic cycle. Chromosom Today 3:24–31

Ewing B, Green P (1998) Basecalling of automated sequencer traces using phred. II. Error probabilities. Genome Res 8:186–194

Farrar K, Asp T, Lübberstedt T, Xu M, Thomas AM, Christiansen C, Humphreys MO, Donnison IS (2007) Construction of two *Lolium perenne* BAC libraries and identification of BACs containing candidate genes for disease resistance and forage quality. Plant Breed 19:15–23

Frohman MA, Dush MK, Martin GR (1998) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85:8998–9002

Fu D, Szücs P, Yan L, Helguera M, Skinner JS, von Zitzewitz J, Hayes PM, Dubcovsky J (2005) Large deletions within the first intron in *VRN-1* are associated with spring growth habit in barley and wheat. Mol Gen Genomics 273:54–65

Gendall AR, Levy YY, Wilson A, Dean C (2001) The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell 107:525–535

Gierl A, Saedler H, Peterson PA (1989) Maize transposable elements. Annu Rev Genet 23:71–85

Gordon D, Abajian C, Green P (1998) Consed: a graphical tool for sequence finishing. Genome Res 8:195–202

Gremme G, Brendel V, Sparks ME, Kurtz S (2005) Engineering a software tool for gene structure prediction in higher organisms. Inf Softw Technol 47:965–978

Haberer G, Young S, Bharti AK, Gundlach H, Raymond C, Fuks G, Butler E, Wing RA, Rounsley S, Birren B, Nusbaum C, Mayer KF, Messing J (2005) Structure and architecture of the maize genome. Plant Physiol 139:1612–1624

Heide OM (1994) Control of flowering and reproduction in temperate grasses. New Phytol 128:347–362

Higgins JA, Bailey PC, Laurie DA (2010) Comparative genomics of flowering time pathways using *Brachypodium distachyon* as a model for the temperate grasses. PLoS ONE 5(4):e10065

Jensen LB, Andersen JR, Frei U, Xing Y, Taylor C, Holm PB, Lübberstedt T (2005) QTL mapping of vernalization response in perennial ryegrass (*Lolium perenne* L.) reveals co-location with an orthologue of wheat *VRN1*. Theor Appl Genet 110:527–536

Johanson U, West J, Lister C, Michaels S, Amasino R, Dean C (2000) Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. Science 290:344–347

Keller B, Feuillet C (2000) Colinearity and gene density in grass genomes. Trends Plant Sci 5:246–251

Koornneef M, Blankestijn-de Vries H, Hanhart C, Soppe W, Peeters T (1994) The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the *Lansberg erecta* wild-type. Plant J 6:911–919

Lee I, Bleecker A, Amasino R (1993) Analysis of naturally occurring late flowering in *Arabidopsis thaliana*. Mol Gen Genet 237:171–176

Lee I, Michaels SD, Masshardt AS, Amasino RM (1994) The late-flowering phenotype of *FRIGIDA* and *LUMINIDEPENDENS* is suppressed in the *Lansberg erecta* strain of Arabidopsis. Plant J 6:903–909

Levy YY, Mesnage S, Mylne JS, Gendall AR, Dean C (2002) Multiple roles of *Arabidopsis VRN1* in vernalization and flowering time control. Science 297:243–246

Lewin B (1997) Transposons. In: Lewin B (ed) Genes VI. Oxford University Press, New York, pp 563–595

Li C, Dubcovsky J (2008) Wheat FT protein regulates VRN1 transcription through interactions with FDL2. Plant J 55:543–554

Livak KJ, Schmittgen TD (2001) Analysis of relative gene Expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. Methods 25:402–408

Lübberstedt T, Andreasen BS, Holm PB et al (2003) Development of ryegrass allele-specific (GRASP) markers for sustainable grassland improvement—a new framework V project. Czech J Genet Plant Breed 39:125–128

Lukashin AV, Borodovsky M (1998) GeneMark.hmm: new solutions for gene finding. Nucleic Acids Res 26:1107–1115

McCarthy EM, McDonald JF (2003) LTR_STRUC: a novel search and identification program for LTR retrotransposons. Bioinformatics 19:362–367

Michaels SD, Amasino RM (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell 11:949–956

Napp-Zinn K (1987) Vernalization: environmental and genetic regulation. In: Atherton JG (ed) Manipulation of flowering. Butterworths, London, pp 123–132

Osborn TC, Kole C, Parkin IAP, Sharpe AG, Kuiper M, Lydiate DJ, Trick M (1997) Comparison of flowering time genes in *Brassica rapa*, *B. napus* and *Arabidopsis thaliana*. Genet Soc Am 146:1123–1129

Pereira A, Cuypers H, Gierl A, Sommer ZS, Saedler H (1986) Molecular analysis of the En/Spm transposable element system in Zea mays. EMBO J 5:835–841

Salamov A, Solovyev V (2000) Ab initio gene finding in *Drosophila* genomic DNA. Genome Res 10:516–522

SanMiguel PJ, RamaKrishna W, Bennetzen JL, Busso C, Dubovsky J (2002) Transposable elements, genes and recombination in a 215-kb contig from wheat chromosome $5A^m$. Funct Integr Genomics 2:70–80

Schranz ME, Quijada P, Sung SB, Lukens L, Amasino R, Osborn TC (2002) Characterization and effects of the replicated flowering time gene *FLC* in *Brassica rapa*. Genetics 162:1457–1468

Schwartz S, Zhang Z, Frazer KA, Smit A, Riemer C, Bouck J, Gibbs R, Hardison R, Miller W (2000) PipMaker—a web server for aligning two genomic DNA sequences. Genome Res 10:577–586

Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ, Dennis ES (1999) The *FLF* MADS box gene: a repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. Plant Cell 11:445–458

Shirasu K, Schulman AH, Lahaye T, Schulze-Lefert P (2000) A contiguous 66-kb barley DNA sequence provides evidence for reversible genome expansion. Genome Res 10:908–915

Skøt L, Humphreys MO, Armstead I, Heywood S, Skøt KP, Sanderson R, Thomas ID, Chorlton KH, Sackville Hamilton NR (2005) An association mapping approach to identify flowering time genes in natural populations of *Lolium perenne* (L.). Mol Breed 15:233–245

Sung S, Amasino RM (2004a) Vernalization and epigenetics: how plants remember winter. Cur Opin Plant Biol 7:4–10

Sung S, Amasino RM (2004b) Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature 427:159–164

Sung S, Amasino RM (2005) Remembering winter: toward a molecular understanding of vernalization. Annu Rev Plant Biol 56:491–508

Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3a protein is a mobile flowering signal in rice. Science 316:1033–1036

Trevaskis B, Bagnall DJ, Ellis MH, Peacock WJ, Dennis ES (2003) MADS box genes control vernalization-induced flowering in cereals. Proc Natl Acad Sci USA 100:13099–13104

Trevaskis B, Hemming MN, Peacock WJ, Dennis ES (2006) *HvVRN2* responds to daylength, whereas *HvVRN1* is regulated by vernalization and developmental status. Plant Physiol 140:1397–1405

Turck F, Fornara F, Coupland G (2008) Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. Annu Rev Plant Biol 59:573–594

von Zitzewitz J, Szucs P, Dubcovsky J, Yan L, Francia E, Pecchioni N, Casas A, Chen TH, Hayes PM, Skinner JS (2005) Molecular and structural characterization of barley vernalization genes. Plant Mol Biol 59:449–467

Xing Y, Frei U, Schejbel B, Asp T, Lübberstedt T (2007) Nucleotide diversity and linkage disequilibrium in 11 expressed resistance candidate genes in *Lolium perenne*. BMC Plant Biol 7:43

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene *VRN1*. Proc Natl Acad Sci USA 100:6263–6268

Yan L, Helguera M, Kato K, Fukuyama S, Sherman J, Dubcovsky J (2004a) Allelic variation at the *VRN-1* promoter region in polyploid wheat. Theor Appl Genet 109:1677–1686

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J (2004b) The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. Science 303:1640–1644

Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Dubcovsky J (2006) The wheat and barley vernalization gene *VRN3* is an orthologue of FT. Proc Natl Acad Sci USA 103:19581–19586