Characterization of the Vernalization Response in *Lolium perenne* by a cDNA Microarray Approach

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Many plant species including temperate grasses require vernalization in order to flower. Vernalization is the process of promotion of flowering after exposure to prolonged periods of cold. To investigate the vernalization response in monocots, the expression patterns of about 1,500 unique genes of *Lolium perenne* were analyzed by a cDNA microarray approach, at different time points after transfer of plants to low temperatures. Vernalization of *L. perenne* takes around 80 d and, therefore, the plants were incubated at low temperatures for at least 12 weeks. A total of 70 cold-responsive genes were identified that are either up- or down-regulated with a minimal 2-fold difference compared with the common reference. The majority of these genes show a very rapid response to the cold treatment, indicating that their expression is affected by the cold stress and, therefore, these genes are not likely to be involved in the flowering process. Based on hierarchical clustering, one gene could be identified that is down-regulated towards the end of the cold period and, in addition, a few genes have been found that are up-regulated in the last weeks of the cold treatment and, hence, are putative candidates for genes involved in the vernalization response. Three of the up-regulated genes are homologous to members of the MADS box, CONSTANS-like and JUMONJI families of transcription factors, respectively. The latter two are novel genes not connected previously to vernalization-induced flowering. Furthermore, members of the JUMONJI family of transcription factors have been shown to be involved in chromatin remodeling, suggesting that this molecular mechanism, as in Arabidopsis, plays a role in the regulation of the vernalization response in monocots.

Keywords: Arabidopsis — Flowering — Lolium — Microarray — Transcription factors — Vernalization.

Abbreviations: ANOVA, analysis of variance; ChiP, chromatin immunoprecipitation; COR, COLD REGULATED; LD, long day; REML, residual maximum likelihood; SD, short day.

The nucleotide sequences for *LpOX1*, *LpCOL1* and *LpWRKY* cDNA reported in this paper have been submitted to EMBL/DBJ/GenBank under the accession numbers DQ145927, DQ145928 and DQ145929, respectively.

Introduction

Vernalization is the process of promotion of flowering after exposure to prolonged periods of cold, usually during a winter period. This process is quantitative, and depends on the extent of the cold treatment, the stage of the plant and the temperature range. The vernalization response in plants occurs only after a sufficient level of cold tolerance is achieved. In contrast, the process of cold acclimation is fast and is designed to respond to low temperatures as rapidly as possible, because plants need to be protected from freezing during fall and winter (Thomashow 1999). Besides temperature, daylength is an important environmental signal regulating the main switch from vegetative to reproductive phase. Thus photoperiod and vernalization synchronize flowering to the changing season (Reeves and Coupland 2000).

Arabidopsis remains the favourite model species to obtain insight into the complexity of flowering time regulation. During the last decade, the flowering network has been studied in detail in this species of which the complete genome sequence is known (Arabidopsis Genome Initiative 2000), and for which many necessary genetic and molecular tools are available (Mouradov et al. 2002). A large number of flowering time genes have been identified by the phenotypic analyses of several Arabidopsis mutants (Martinez-Zapater and Sommerville 1990, Koornneef 1991) and from the analysis of natural variation among various different Arabidopsis ecotypes (Johanson et al. 2000). Quantitative trait locus (QTL) mapping has been used to localize the loci linked to *FRIGIDA* (*FRI*) and FLOWERING LOCUS (*FLC*), which are to a large extent responsible for the natural variation in the vernalization response of different ecotypes. *FLC* encodes a MADS box transcription factor, which is induced by the presence of a dominant allele of *FRI* and its function as floral repressor is antagonized by vernalization in the winter annual types of Arabidopsis (Michaels and Amasino 1999, Sheldon et al. 1999). Similarly, a group of six recessive late flowering mutants (*fca, fld, ld, fve, fpa* and *fy*), under both a long day (LD) and a short day (SD) photoperiod, can be completely restored by an extensive cold period (Koornneef et al. 1991). All these genes belong to the autonomous pathway and act by limiting *FLC* expression. These results clearly show that vernalization operates in a parallel
pathway that is integrated with the light and autonomous pathway at the FLC locus.

To obtain more insight into the molecular basis of the vernalization process, flowering mutants that are no longer responsive to low temperatures were characterized. The identification and the molecular analysis of VERNALIZATION1 (VRN1) and VERNALIZATION2 (VRN2) confirmed that epigenetic changes are part of the mechanism of cellular memory of vernalization (Bastow et al. 2004). VRN1 encodes a nuclear DNA-binding protein and is involved in the regulation of the floral pathway integrator FT. The vrn1 mutant shows a reduced FT expression, while overexpression of VRN1 results in early flowering, and is associated with an increase in FT expression (Levy et al. 2002). The VRN2 protein shows homology to the Drosophila Polycomb group (PcG) protein Suppressor of zeste 12 [Su(z)12] (Gendall et al. 2001). VRN1 and VRN2 are not required for the direct down-regulation of FLC during vernalization, but are needed for the maintenance of FLC repression. It has been demonstrated that specific DNA regions of the FLC promoter and of the first FLC intron are subject to methylation and deacetylation induced by vernalization. Recently, the VTN3 (VERNALIZATION-INSENSITIVE3) gene was isolated, which encodes a protein with a plant homeodomain (PHD) and fibronectine type III repeats and is a member of the deacetylase complex that acts as a direct repressor of FLC expression at the end of the vernalization period. VTN3 is active upstream of VRN1 and VRN2, and its expression is strongly up-regulated only after a long period of exposure to cold. The transcription of VTN3 is silenced soon after the plant is exposed to higher temperatures (Sung and Amasino 2004).

Despite the importance of all these findings, it is necessary to validate the Arabidopsis model of flowering time regulation in other plant species. Rice, of which the genome sequence has been elucidated (Goff et al. 2002), is generally accepted as the model species for monocots. However, in contrast to Arabidopsis and many other cereals, rice is a subtropical species and does not require vernalization in order to flower. Therefore, the vernalization response has been studied directly in cereals that are sensitive to vernalization (Wang et al. 1995). Cereal species can be characterized as winter or spring accessions depending on their competence and ability to flower after exposure to long periods of low temperature. The spring varieties flower earlier and do not require vernalization, whereas winter varieties depend on extensive cold periods to flower. Genetic studies performed in the past revealed that the temperature-dependent timing of flowering is determined mainly by the epistatic interactions of two loci, VRN1 and VRN2 (distinct from the Arabidopsis genes with the same nomenclature). The dominance of VRN1 and the presence of a VRN2 recessive allele generate a spring growth habit. Conversely, plants with a recessive VRN1 and a dominant VRN2 allele generate a winter growth habit with a strict requirement for vernalization. Recently, the VRN1 locus of cereals was linked to a gene similar to the Arabidopsis meristem identity gene APETALA1 (API) (Murai et al. 2003, Trevaskis et al. 2003, Yan et al. 2003), and the VRN2 locus to a new gene, encoding a protein with strong similarity to CONSTANS (CO) and CONSTANS-like proteins of Arabidopsis (Yan et al. 2004). Detailed analyses have shown an epistatic interaction between VRN1 and VRN2; VRN1 expression is strongly induced in winter accessions upon an extensive period of cold treatment and, in parallel to this, VRN2 levels are progressively reduced. In the spring habit of cereals, the API-like mRNA is induced without any vernalization treatment and its expression is not repressed by VRN2.

To compare the vernalization-dependent pathway in grasses and dicot species such as Arabidopsis, a more complete set of vernalization-responsive genes in cereals needs to be elucidated. Unfortunately, the genomic complexity and the large genome size of many grasses limit a genetic approach. Therefore, we performed a microarray experiment to identify vernalization-responsive genes from Lolium perenne. We scored for changes in gene expression during a cold treatment of 12 weeks, which revealed a number of novel putative regulators that respond to vernalization.

**Results**

**Microarray preparation**

To investigate the vernalization response in L. perenne, a cDNA microarray was constructed. Two different cDNA libraries were prepared from vegetative tissues of young plants, which were grown under normal and vernalization conditions, respectively, and in addition two existing cDNA libraries were used (Petersen et al. 2004). To select against highly abundant housekeeping genes, such as genes involved in general metabolism and photosynthesis, a pre-hybridization of the libraries was performed with a mixture of six ‘housekeeping clones’ (not shown). By means of this pre-screening, approximately 25% of clones could be eliminated. The remaining clones were subjected to colony PCR and the products obtained were checked for size and yield. In total, 3,650 cDNAs that complied with our quality criteria (see Materials and Methods) were sequenced and the output file was blasted against the TAIR Arabidopsis sequence database (http://www.arabidopsis.org/). Based on this sequence information, the clones were categorized into 15 functional groups according to the GO (Gene Ontology) of biological processes (Garcia-Hernandez et al. 2002, Rhee et al. 2003; Supplementary Fig. 1). In total, about 1,500 different ‘contigs’ were obtained that subsequently were spotted as unigenes onto the microarray. In addition, the array contained another 350 L. perenne clones from which no sequence information was available and a set of controls, which were necessary for the analysis of the microarrays. All clones were spotted in duplicate onto the arrays.
Gene expression analysis

Gene expression was monitored at different time points during a period of 12 weeks under cold exposure. For this purpose Lolium plants were exposed to cold, and total RNA was extracted from aerial plant tissues after 1, 3, 5, 7, 19, 26, 35, 43, 57, 71 and 79 d of cold treatment. Besides this, RNA was isolated from Lolium plants before the treatment (day 0). Each sample (target) was hybridized to the array and compared with a common reference. This reference was prepared by pooling equal amounts of RNA from all samples used. To assess the
reproducibility of the microarray analysis, we performed technical (using swapped dyes) and biological replica hybridizations. A preliminary analysis of the data generated from the hybridized arrays revealed that most of the changes in gene expression were detected during the first few days after exposure to cold. It is known that immediately after exposure to cold, plants give a relatively strong response to adapt to the changed environment (Thomashow 1998, Wanner and Junttila 1999). In total, 70 genes were identified that showed a minimal 2-fold difference in mRNA accumulation compared with the level of expression of the common reference. Hierarchical clustering of these 70 genes (Fig. 1) revealed the presence of six expression clusters (Fig. 2). The genes in the first cluster (S1) exhibited high expression after cold induction and the expression is kept at this high level constantly. The second cluster (S2) comprises genes that are characterized by a rapid increase in expression in the first days of cold treatment followed by a slow down-regulation until the end of the vernalization period. The third cluster (S3) contains genes that are down-regulated during the first days of cold, and almost restore their normal expression level at later time points. Cluster S4 consists of genes that have a steady expression level during the first 6 weeks, but switch to a strong up-regulation in the last weeks of cold treatment. Cluster S5 represents five genes that show a decrease of their expression until day 19, rapidly followed by an increase during the later time points. Finally, cluster S6 represents the only gene that was down-regulated during the whole period of cold treatment.

Fig. 2 Classification of differentially expressed genes into six clusters. Hierarchical clustering has been performed on a subset of 70 selected genes of the microarray. Fold changes were 2log transformed (ratio values for at least two replications of the microarray) with respect to some selected time points (days) during vernalization. Cluster S1 contains genes that exhibit high expression after 3 d and a constant expression until the end of the cold treatment. Cluster S2 contains genes with high expression in the first days of cold treatment followed by a slow down-regulation until the end of the vernalization period. Cluster S3 contains genes that are down-regulated during the first days of cold, and almost restore their normal expression level at later time points. Cluster S4 consists of genes that have a steady expression level during the first 6 weeks, but switch to a strong up-regulation in the last weeks of cold treatment. Cluster S5 represents five genes that show a decrease of their expression until day 19, rapidly followed by an increase during the later time points. Finally, cluster S6 represents the only gene that was down-regulated during the whole period of cold treatment.
Fig. 3  Confirmation of the microarray data by Northern blot analyses. (a) cDNA microarray expression data of five selected cold-induced genes belonging to different expression clusters. In each graph, 2log-transformed fold changes (y-axis) are shown, with respect to some selected time points (days) during vernalization (x-axis). (b) Graph that displays the results from the Northern blot analysis for the same set of selected genes. All blots were hybridized with gene-specific probes, and the relative hybridization signals were normalized with the signals for the constitutive expressed actin.
ment. The remaining clones present on the microarray did not show significant differences in expression levels.

The array data were confirmed by Northern blot analyses for a small group of five selected genes. From almost every cluster, one representative gene was chosen and its expression pattern was analyzed (Fig. 3). In general, the expression patterns and levels deduced from either Northern blots or cDNA microarray appeared to be comparable.

Clusters of cold-induced and vernalization-responsive genes

To obtain more insight into the individual genes that showed cold- and vernalization-responsive expression, their sequence and homology with known genes were analyzed in more detail. Putative functions were predicted for the 70 differentially expressed genes based on annotation (NCBI/genebank, http://www.ncbi.nlm.nih.gov/) and sequence homology to known genes (Supplementary Table 1). Genes such as LEA/dehydrin (late embryogenesis abundant family group II) and COR (Cold Regulated) genes appeared to be grouped in cluster S1. LEA D11 proteins accumulate in plants in response to low temperature and have been shown to be involved in protecting macromolecules such as enzymes and lipids (Wise and Tunacliffe 2004). COR genes comprise four gene families and are induced by CBF1 (CRT/DRE-binding factor 1). Expression of these genes results in an increased freezing tolerance for non-acclimated Arabidopsis plants (Jaglo-Ottosen et al. 1998). In addition, cluster S1 includes genes that are involved in signal transduction (e.g. myo-inositol phosphate synthase, receptor protein kinase-like) and genes that participate in carbohydrate metabolism (e.g. putative Na+-dependent inorganic phosphate co-transporter). Cluster S2 includes genes encoding proteins that are involved in amino acid and protein metabolism (e.g. S-adenosylmethionine decarboxylase, carboxypeptidase I precursor). Protein denaturation is often observed when plants are grown at low temperature, as a consequence of cellular damage. Cluster S3 contains genes involved in photosynthesis (PSII) and cluster S5 comprises genes that might be related to stress (e.g. catalase) or defense (e.g. WRKY transcription factor).

In contrast to the expression profiles of the clusters that have been described above, the expression profile of the genes in cluster S4 is characterized by a late cold response. This group of seven clones includes one pathogenesis-related protein, a putative wall-associated kinase 1 protein and three cDNAs encoding transcription factors; a MADS box gene (LpMADS1, Petersen et al. 2004), a novel CONSTANS-like gene, which we named LpCONSTANS-like1 (LpCOL1) and a JUMONJI like gene (LpJMJC) (Clissold and Ponting 2001), designated LpJMJC. Based on their expression patterns, the genes of cluster S4 and the down-regulated LpOX1 gene from cluster S6 may play an important role in the vernalization response and, therefore, are characterized in more detail.

**Differential expression of Lolium transcription factors during vernalization**

Among the genes of cluster S4, the transcription factor LpMADS1 appeared to have a very low basal expression level during the first 43 d of cold treatment, after which its transcript starts to accumulate to higher levels. Its expression profile was confirmed by Northern blot analysis (not shown) and is in line with the published data and proposed regulatory role of this gene in vernalization (Petersen et al. 2004). Close homologs of LpMADS1 from other monocot species, such as WAP1 from Triticum aestivum (Murai et al. 2003), TmAPI from Triticum monococcum (Yan et al. 2003) and BMS from barley (Trevaskis et al. 2003), have also been shown to be involved in the vernalization response.

Another cDNA clone (DQ145928) of cluster S4, with a similar expression pattern to LpMADS1, encodes a CONSTANS-like protein (named LpCOL1). In Arabidopsis, the flowering time gene CONSTANS (CO) belongs to a family of 17 putative transcription factors defined by two conserved domains, the zinc finger region and the CCT domain (CO, CO-like, TOC1) at the N- and the C-terminus of the protein, respectively (Putterill et al. 1995, Strayer et al. 2000, Robson et al. 2001). The LpCOL1 clone isolated in this study contains the 3'-untranslated region (UTR) and the 3' end of a predicted open reading frame (ORF) that is homologous to the group III type of CO-like proteins (Griffiths et al. 2003). Based on this, a phylogenetic tree was generated and an alignment was made with the predicted amino acid sequences of the Arabidopsis and rice group III CO-like proteins (Fig. 4). This analysis revealed that LpCOL1 shows the highest degree of identity at the amino acid level with the zinc finger protein C60910 from Oryza sativa (70%), but does not have a clear homolog in Arabidopsis. Recently, the expression of the clone C60910 (renamed OsP) was monitored during the vegetative and reproductive phase of rice, and appeared to remain stable. However, the expression of this gene was found to be under circadian control under both SD and LD photoperiods, following a similar oscillation pattern to O. sativa HEADING DATE 1 (OsHd1) (Shin et al. 2004).

A third transcription factor in cluster S4 appeared to encode for a JUMONJI-like transcription factor (LpJMJC). This clone has 75% identity at the amino acid level with a JUMONJI domain-containing protein of rice (AAR06534).

Fig. 4  Phylogenetic tree and alignment of the selected C-terminal peptide sequences of CONSTANS-like group III proteins. (a) Phylogenetic tree. Comparisons were made using the amino acid sequence of CONSTANS-like proteins from the plant species Arabidopsis (AtCOL 9, AtCOL 10, AtCOL 11, AtCOL 12, AtCOL 13, AtCOL 14 and AtCOL 15), Lolium perenne (LpCOL1), Oryza sativa (OsN and C60910) and Triticum monococcum (VRN2). Arabidopsis (CO) and L. perenne (LpCO) were included as outgroups. Bootstrap values are indicated above each branch found in 1,000 bootstrap replicates. (b) Alignment of the same proteins as in (a). The conserved amino acids of the CCT domain are shown in a box.
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Fig. 4
Recently, Clissold and Ponting (2001) identified several eukaryotic proteins containing JmjC domains and classified them into seven groups based on sequence similarity.

In addition to these three transcription factors from cluster S4, a putative WRKY transcription factor, LpWRKY1 (DQ145929), was selected for further analyses based on its identity and expression pattern. The microarray analysis revealed a decrease in mRNA levels after the first days of cold exposure but its expression was clearly up-regulated towards the end of the vernalization period (Fig. 3). In Arabidopsis, it has been shown that many WRKY proteins are involved in a multitude of responses to biotic and abiotic stresses (Eulgem et al. 2000). The deduced amino acid sequence of LpWRKY1 shows the highest percentage of sequence identity with the AtWRKY50 protein of Arabidopsis (54% identity). AtWRKY50 belongs to one (IIC) of the five distinct subgroups of the group II WRKY members of transcription factors as characterized by Eulgem et al. (2000). A phylogenetic tree was generated using LpWRKY1 from Lolium and the amino acid sequences of all Arabidopsis group II WRKY proteins (Supplementary Fig. 2). Analysis of LpWRKY1 revealed that this clone contains, like other members from Arabidopsis (AtWRKY50, AtWRKY51 and AtWRKY59), the hallmark heptapetide, WRKYGGK with a single amino acid substitution compared with the consensus sequence. Remarkably, the loss of binding to the TGTACC DNA element, known as the W box, is correlated with this mismatch in the heptapeptide motif (Dong et al. 2003).

The LpOX1 gene is down-regulated during vernalization

The microarray analysis showed that the expression of only one gene was significantly decreased during the time course of cold exposure (Fig. 3). This LpOX1 cDNA (DQ145927) encodes a protein which shares 44% identity with a 2OG-Fe(II) oxygenase enzyme (NP_910590) from rice. The same amino acid region, named 2OG-Fe (II) oxygenase, is present in the sequence of an ACC oxidase protein (BAA76387). In plants, 2-oxoglutarate (2OG)- and Fe(II)-dependent dioxygenases catalyze the formation of ethylene and also catalyze the hydroxylation and desaturation reactions in the synthesis pathways of other plant hormones, pigments and compounds such as gibberellins, anthocyanidins and flavones (Aravind and Koonin 2001).

Expression profiling of the vernalization-responsive genes under different photoperiods

Changes in photoperiod are often a prerequisite for the developmental switch from the vegetative to the reproductive phase of a plant. Previous studies in Arabidopsis revealed that the photoperiod response is inter-related to the temperature pathway and that they together influence developmental growth (Fowler et al. 2001). Furthermore, it is known that perennial ryegrass is insensitive to day length for flowering induction during the vernalization period (Aamlid et al. 2000).

However, after vernalization, flowering is induced by LD conditions. To assess the role of photoperiod in regulating the genes of cluster S4 and S6, the vernalization treatment of Lolium was performed under both SD and LD light regimes, and the expression patterns of the LpMADS1 (S4), LpCOL1 (S4) and LpOX1 (S6) genes were monitored under these distinct photoperiodic conditions. For this purpose, aerial plant material was collected from the Lolium plants grown under SD and LD conditions at different time points (after 1, 6, 19, 35, 43, 57 and 71 d of cold treatment), and subsequently, total RNA was extracted. Northern blot analysis of the obtained expression patterns revealed that differences in photoperiod during vernalization did not have a substantial effect on the expression levels of these genes (data not shown).

Discussion

In the present study, we performed a comprehensive analysis of the vernalization process in L. perenne by a cDNA microarray approach. Our results showed transcriptional changes upon cold treatment for at least 70 genes. The obtained expression patterns led to the clustering of the affected genes into six classes, to which putative functions could be attributed based on the expression profiles and the predicted identity of genes present in each class. For the induction of flowering by vernalization, a long period of low temperatures is essential and, therefore, genes involved in the vernalization response are expected to have an expression pattern in accordance with this behaviour. Although not many genes appeared to be up- or down-regulated after a long exposure to cold, we were able to identify a few genes with the expected expression pattern and, furthermore, their identity and homology to members of families of important regulators point to possible roles in the regulatory mechanism underlying the vernalization process.

The first days of cold treatment were included in the analysis because many biochemical and physiological changes in a plant occur rapidly after exposure to low temperature conditions and analysis of the complete period allows separation of cold acclimation and the vernalization response. Many genes that are directly responsive to the cold treatment or changing environmental conditions are dramatically up-regulated during the first few days. Among them are expected genes such as COR/LEA (Cold-Responsive, Late Embryogenesis Abundant) genes, which play a role in cryo-protection by stabilizing the cellular membranes (Thomashow 1999).

Among the genes differentially expressed towards the end of the vernalization period, LpMADS1 was identified, which is of particular interest because many members of the MADS box transcription factor family appear to be involved in flower induction. Recent reports revealed that the genes at the VRN1 locus of various cereals belong to the APETALA1/AP1/AGL9 (SEP3) clade of MADS box transcription factors (Litt and Irish 2003). Examples are WAP1 from T. aestivum (Murraits et al. 2003), TmAP1 from T. monococcum (Yan et al. 2003) and BM5.
from barley (Trevaskis et al. 2003). Because \textit{LpMADS1} is close in sequence to these MADS box genes, it might be the candidate for the VRN1 locus in Lolium. Up-regulation of this gene at the end of the vernalization period has been reported before (Petersen et al. 2004) and this clearly shows that the microarray analysis performed in this study is done in a proper manner and enables identification of putative regulators of the vernalization response.

One of these putative novel regulators is the identified \textit{LpCOL1} gene, which has been selected because of its up-regulation towards the end of the vernalization period, like \textit{LpMADS1}. Furthermore, it is homologous to the \textit{CO}-like family of plant transcription factors, members of which have been shown to be involved in the flowering process. Studies in Arabidopsis revealed that the \textit{CO} gene (Puterill et al. 1995, An et al. 2004) plays an important role in the photoperiod flowering pathway. Recently, studies in perennial ryegrass (\textit{L. perenne}) have provided insight into the function of the \textit{LpCO} gene, demonstrating conservation of function between monocot and dicot species (Martin et al. 2004). \textit{LpCOL1} appeared to be different from \textit{LpCO} and belongs to group III of the \textit{CO-like} gene family (Griffiths et al. 2003). A function is not known for any of the members of this group, but the strong up-regulation towards the end of the cold period for \textit{LpCOL1} suggests a role in vernalization-induced flowering. The \textit{VRN2} gene isolated from wheat encodes another protein with similarity to \textit{CO} and \textit{CO-like} proteins of Arabidopsis, but, in contrast to \textit{LpCOL1}, this gene is down-regulated during the vernalization period. Interestingly, neighbor joining cluster analysis of the \textit{CO-like} proteins showed that the \textit{VRN2} proteins form a separate group closely related to group IV (Yan et al. 2004). The \textit{CO-like} proteins belonging to group IV represent a novel class of this family and are found in cereals such as barley and rice only, and not in Arabidopsis. In addition, the absence of any \textit{VRN2} orthologs in rice suggests that the group IV of \textit{CO-like} proteins may be specific to the temperate cereal species adapted to cold (Yan et al. 2004). Despite the fact that \textit{VRN2} and \textit{LpCOL1} are members of the same family, they belong to two different groups. Notably, group III, to which \textit{LpCOL1} belongs, includes members of various species, such as Arabidopsis, rice and Lolium, and this may suggest that they represent a class of genes that are more generally involved in the flowering induction process.

A similar expression pattern of induction by a prolonged period of cold was observed for the \textit{JUMONJI}-like gene \textit{LpJMJC}. This family of transcription factors has been characterized in different organisms, such as in animals, fungi and plants, and might be involved in chromatin remodeling (Balciunas and Ronne 2000, Clissold and Ponting 2001). At present, limited information is available about the function of members of this family in plants. However, Noh and colleagues recently found two novel floral regulatory genes, \textit{ELF6} (\textit{EARLY FLOWERING 6}) and \textit{REF6} (\textit{RELATIVE OF EARLY FLOWERING 6}), both encoding \textit{JUMONJI} proteins. Chromatin immunoprecipitation (ChIP) analysis, using material from the \textit{ref6} mutant, showed hyperacetylation of the promoter region and first intron of the flower repressor \textit{FLC} (Noh et al. 2004). In addition, previous ChIP experiments, using antisense against VRN1 and VRN3, showed that the same domains of \textit{FLC} were enriched for histone deacetylation and methylation (Sung and Amasino 2004). Thus, these modifications probably lead to silencing of the floral repressor \textit{FLC} by deacetylation and methylation of regulatory sequences of the \textit{FLC} gene. In line with this and the results from the ChIP experiments in the \textit{ref6} mutant, Noh and colleagues have suggested that the \textit{REF6} \textit{JUMONJI} protein might be a component of a histone deacetylase 1,2 (HDAC1/2) co-repressor complex involved in specific down-regulation of \textit{FLC}. Although, \textit{LpJMJC} is not a close homolog of \textit{REF6}, its up-regulation towards the end of the vernalization process as has been identified in this study and the fact that it is a member of the same family of transcription factors suggest an important role for this gene in the regulation of the vernalization response in Lolium.

Based on the study of \textit{ELF6} and \textit{REF6} in Arabidopsis (Noh et al. 2004), \textit{LpJMJC} may act at the molecular level as well through chromatin remodeling. It would be of great interest to determine whether the expression of the known cereal vernalization-responsive genes \textit{VRN1} (\textit{LpMADS1}) and \textit{VRN2} might be regulated by chromatin modifications by proteins such as \textit{LpJMJC}, in a similar manner to that which has been shown for the Arabidopsis \textit{FLC} gene (He et al. 2004).

\section*{Materials and Methods}

\textbf{Plant material}

\textit{Lolium perenne} plants were initially grown in soil under normal greenhouse conditions (21°C, 16 h light/8 h dark, LD). For the primary induction (vernalization), 3-week-old plants were transferred to a growth chamber with a temperature below 5°C, for 12 weeks (10 h light/14 h dark, SD). Following vernalization, plants were transferred to the greenhouse (21°C, 16 h light/8 h dark, LD) for secondary induction.

Lolium plants that were not subjected to the vernalization treatment failed to flower once that they were transferred to the greenhouse (21°C, 16 h light/8 h dark, LD).

For photoperiod studies, 2-week-old Lolium plants were transferred to a temperature below 5°C for 12 weeks under either an LD (16 h light/8 h dark) or an SD (8 h light/16 h dark) photoperiod.

\textbf{RNA extraction and purification}

Total RNA was isolated from aerial parts of Lolium plants using the LiCl method described by Verwoerd et al. (1989). For the preparation of microarray targets, total RNA was digested with RNase-free DNase I (Roche Diagnostics Nederland B.V., Almere, The Netherlands) and the RNA was further cleaned with the RNasy Mini kit (Qiagen Benelux B.V., Venlo, The Netherlands). The concentration and the purity of RNA were determined by measuring the absorbance at 260 nm (\textit{A}_{260}) and 280 nm (\textit{A}_{280}) in a spectrophotometer.

\textbf{RNA gel blot analysis}

Total RNA (5–15 µg) was denatured using the glyoxal method (Sambrook et al. 1989) and separated on a 1.4% (w/v) agarose gel.
After separation, the gel was capillary blotted onto Hybond-N’ membranes (Amersham Pharmacia, Roosendaal, The Netherlands). Gene-specific fragments from all of the tested genes were used as probes for hybridization. The probes were labeled by random oligonucleotide priming (Feinberg and Vogelstein 1984), and blots were hybridized under stringent conditions as described by Angenent et al. (1992). Subsequently, blots were stripped by washing at 100°C in 0.5× SSC, and re-hybridized with a 1,048 bp actin fragment to determine the loading. Based on these data, the obtained gene-specific signals were normalized (Quantity One-1D, BIO-RAD, Veenendaal, The Netherlands).

**Preparation of cDNA libraries**

Four cDNA libraries were prepared from *L. perenne*. Two libraries (viralized and non-viralized) were constructed in the Stratagene UNI-ZAP XR vector (Petersen et al. 2004) and two in the Stratagene HybriZAP-2.1 Two-Hybrid vector. The ‘vegetative’ libraries were prepared from RNA isolated from aerial parts of 3-week-old vegetative Loli num plants, which were never exposed to cold. The ‘viralized’ libraries were prepared from the aerial parts of plants subjected to 90 d of vernalization. The titers of the libraries varied between 8×10^6 and 3×10^7 p.f.u. ml⁻¹ (these were amplified libraries) and the average insert size was 1.1 kb. In vivo mass excision and isolation of library plasmid DNA was done according to the manufacturer’s description.

**Microarray preparation and isolation of unigenes**

To select against highly expressed housekeeping genes, a pre-screening of the cDNA libraries was performed. Each independent colony-forming unit of *Escherichia coli* re-assembled in LB freez ing medium (36 mM K₂HPO₄, 13.2 mM KH₂PO₄, 1.7 mM trisodium citrate, 0.4 mM MgSO₄·7H₂O, 6.8 mM (NH₄)₂SO₄, 4.4% glycerol (v/v), 10 g of trigo n, 5 g of yeast extract, 5 g of NaCl) supplemented with 100 mg liter⁻¹ ampicillin, and aliquoted in 96-well microtiter plates. Following growth at 37°C, each individual clone was deposited with a 96-pin replicator (Westburg, Leusden, The Netherlands) on an agar selection plate (100 mg liter⁻¹ ampicillin) and grown. The 96-well microtiter plates were stored at −80°C, as glycerol stock. The obtained colonies were transferred to Hybond-N’ membranes (Amersham Pharmacia). Subsequently, the membranes were placed on Whatmann paper saturated with denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 5–10 min and then placed on Whatmann paper saturated with neutralization buffer (1.5 M NaCl, 0.5 M Tris–HCl, pH 7.2) for 5–10 min. The membranes were hybridized with a mixture of clones involved in general metabolism and photosynthesis (Cbl a/b-binding protein (CAA44777), ribulose-biphosphate carboxylase (AF097361), PSI (M61146), malate dehydrogenase (AF346003), coat protein (AAQ82724) and putative RNA-dependent polymerase (AAK97522)]. The majority of these ‘housekeeping’ genes could be eliminated by this pre-hybridization of the libraries. The remaining 80% of clones were subjected to PCR to amplify the inserts. The PCR was prepared in 100 µl, containing specific forward and reverse primers designed on the vector sequences directly flanking the insert, 1× PCR buffer, 0.4 µM of each primer, 0.1 mM dNTPs and 1.25 U of Taq polymerase. PCR was performed as follows; 94°C for 2 min; five cycles of 94°C for 30 s, 56°C for 1 min and 1.5 min at 72°C; 40 cycles of 94°C for 30 s, 54°C for 1 min and 5 min at 72°C. Afterw ards, the PCR products were purified with the QiaQuick 96 PCR purification kit (Qiagen). A small aliquot of each PCR purified product was loaded on a 1.5% (w/v) agarose gel to assess quality and quantity. All fragments smaller than 200 bp or with a yield lower than 2 µg were eliminated. In total, 3,650 amplified clones have been selected and sequenced (BigDye kit v1.1 RR-100, Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands), yielding 1,500 unique contigs based on blasting against the NCBI sequence databases. DNASTAR software (DNASTAR, Madison, WI, USA) was used for DNA analysis.

Besides the approximately 1,500 amplified unigenes, another 350 clones obtained from the PCRs, but from which no sequence information was available, and a set of control clones were spotted. All fragments were arrayed from 384 microtiter plates onto GAPS aminosilane-coated microslide glass (Corning, Acton, MA, USA) using a PixSys 7500 arrayer (Cartesian Technologies, Irvine, CA, USA) equipped with Chip-maker 3-quin pins (Telechem, Sunnyvale, CA, USA). Slides were post-processed as described before (van Doorn et al. 2003).

**Preparation of fluorescent targets**

Total RNA, isolated from Loli num plants, prior to vernalization and at different time points during vernalization (after 1, 3, 5, 7, 19, 26, 35, 43, 57 and 79 days), was used for first-strand cDNA synthesis. The reverse transcription reaction was performed in a 50 µl volume containing 40 µg of RNA spiked with 1.0 ng of in vitro synthesized luciferase mRNA (Promega, Leiden, The Netherlands) with 2 µg of oliog(dT) 21-mer, 500 µM each of dATP, dGTP and dCTP, 300 µM dUTP, 200 µM aminonialyl-dUTP; 5× first strand buffer (Invitrogen Life Technologies, Breda, The Netherlands), 10× dithiothreitol (DDT; 0.1 M), 40 U of RNaseOUT (Invitrogen Life Technologies, Breda, The Netherlands) and 300 U of SuperScript II RNase H- Reverse transcriptase (Invitrogen Life Technologies, Breda, The Netherlands). Reverse transcription was performed at 37°C for 2 h. The samples were ethanol precipitated and dissolved in 20 µl of Tris-EDTA pH 8.0, after which cDNA/mRNA hybrids were denatured at 98°C for 5 min and immediately chilled on ice. RNA was degraded by adding 5.0 µl of 1 M NaOH (10 min at 37°C). After adding 5.0 µl of 1 M HEPES pH 6.8 and 4.0 µl of 1 M HCl, the cDNA was precipitated with ethanol and resuspended in 10 µl of 0.1 M sodium carbonate buffer pH 9.3. Cy5 and Cy3 monofunctional NHS esters (Amersham Pharmacia) were dissolved in 45 µl of dimethyl sulfoxide (DMSO) and used to label the modified cDNA at room temperature for 30 min. Finally, the labeled cDNA was ethanol precipitated twice and dissolved in 8.1 µl of MQ water.

**Microarray hybridization**

Aliquots (8.1 µl) of each fluorescent target were mixed together in a hybridization solution (Ambion, 80 µl). Prior to use, the microarray slide was pre-heated at 50°C and the fluorescent targets were heated at 95°C (1 min), cooled on ice and mixed with the pre-heated hybridization buffer (68°C). A total of 65 µl was applied to the microarray under a Gene Frame (ABgene, Hamburg, Germany) and the hybridization was carried out overnight at 50°C. After hybridization, the slides were washed by agitation successively at room temperature in 1× SSC, 0.1% (v/v) SDS (5 min) followed by 0.1× SSC, 0.1% SDS (v/v) (5 min) twice. Subsequently, slides were rinsed briefly in 0.1× SSC before drying by centrifugation (5 min, 27×g).

**Data collection, normalization and cluster analysis**

Arrays were scanned with the ScanArray Express HT (Perkin Elmer, Boston, MA, USA). Image analysis and signal quantification were performed with AIS software (Imaging Research, Ontario, Canada). Background fluorescence was calculated on the basis of the fluorescence signal of the negative control genes, the yeast (Saccharo myces cerevisiae) aspartate kinase, yeast imidazole-glycerol-phosphate dehydratase, yeast phosphoribosyl-aminoimidazole carboxylase and jellyfish (Aequoria victoria) green fluorescent protein (van Doorn et al. 2003). Signals not reaching the threshold level of 0.5× background were omitted from the analyses. As a typical experiment design, we used a ‘common reference’ and consecutive time point samples. The
average variance of this design of time course experiment is estimated to be 2.00. The intensity ratios, after median normalization, were calculated for each clone, and a log-transformation was performed. All the clones that were missing ratio values for >3 time points were excluded from the analysis. The clustering analysis was done using an unsupervised data analysis approach of unweighted pair group method with arithmetic mean (UPGMA) algorithm and a Pearson coefficient of similarity measure for the expression levels. This was followed by self-organizing map clustering.

**Statistical analysis of microarray data**

Two different statistical approaches, ANOVA (analysis of variance) and REML (residual maximum likelihood), have been used to analyze the obtained microarray data, in order to perform a quality control, a location effect check and a variance component determination for all the arrays. The quality control was carried out to assess the reproducibility between plots A vs. B (the two blocks A and B are spotted in duplicate onto the array containing the same clones). In order to compare technical and biological variability, a variance components analysis was carried out using the model clone (time points/biological duplicates/arrays/replicates). Biological replicates were nested within time points, arrays within biological replicates (those are, in fact, the swaps for one of the biological replicates) and replicate spots within arrays. Strong outliers between the A and B replicates were omitted from further analyses using the Genstat regression/ANOVA criterion. After this, 1,808 of the 1,920 clones were kept for further analyses. These were analyzed with ANOVA and REML for significance of the time differences. From the REML analyses (separate analyses per clone), the means per time point and the SEMs were obtained. From the ANOVA F-probability and REML χ-probability, it can be deduced whether time points differed significantly (criteria: both <0.01). From the t-probabilities, the significance of the three time point differences can be found.

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