Protein interactions of MADS box transcription factors involved in flowering in *Lolium perenne*

Stefano Ciannamea¹, Kerstin Kaufmann¹, Marta Frau¹, Isabella A. Nougalli Tonaco¹, Klaus Petersen², Klaus K. Nielsen³, Gerco C. Angenent¹ and Richard G. H. Immink¹,*

¹ Plant Research International, Business unit Bioscience, Bornsesteeg 65, 6708 PD, Wageningen, The Netherlands
² Risoe National Laboratory, Biosystems Department 330, PO Box 49, 4000 Roskilde, Denmark
³ DLF-TRIFOLIUM A/S, 31, Hoejerupvej, PO Box 19, DK-4660 Store Heddinge, Denmark

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**Abstract**

Regulation of flowering time is best understood in the dicot model species *Arabidopsis thaliana*. Molecular analyses revealed that genes belonging to the MADS box transcription factor family play pivotal regulatory roles in both the vernalization- and photoperiod-regulated flowering pathways. Here the analysis of three APETALA1 (AP1)-like MADS box proteins (LpMADS1–3) and a SHORT VEGETATIVE PHASE (SVP)-like MADS box protein (LpMADS10) from the monocot perennial grass species *Lolium perenne* is reported. Features of these MADS box proteins were studied by yeast two-hybrid assays. Protein–protein interactions among the *Lolium* proteins and with members of the *Arabidopsis* MADS box family have been studied. The expression pattern for *LpMADS1* and the protein properties suggest that not the *Arabidopsis AP1* gene, but the *SUPPRESSOR OF CONSTANS1* (*SOC1*) gene, is the functional equivalent of *LpMADS1*. To obtain insight into the molecular mechanism underlying the regulation of *LpMADS1* gene expression in vernalization-sensitive and -insensitive *Lolium* accessions, the upstream sequences of this gene from a winter and spring growth habit variety were compared with respect to MADS box protein binding. In both promoter elements, a putative MADS box transcription factor-binding site (CArG-box) is present; however, the putative spring promoter has a short deletion adjacent to this DNA motif. Experiments using yeast one-hybrid and gel retardation assays demonstrated that the promoter element is bound by an LpMADS1–LpMADS10 higher order protein complex and, furthermore, that this complex binds efficiently to the promoter element from the winter variety only. This strongly supports the model that LpMADS1 together with LpMADS10 controls the vernalization-dependent regulation of the *LpMADS1* gene, which is part of the vernalization-induced flowering process in *Lolium*.

Key words: *Lolium*, MADS box transcription factor, protein–protein interaction, vernalization.

**Introduction**

Timing of the transition from vegetative to reproductive development is determined by various environmental and endogenous factors. The synchronization of flowering time is a complex process that is best studied and understood in the model system *Arabidopsis thaliana*. In this species, flowering is determined by four major promoting pathways: long-day photoperiod, gibberellin, the autonomous pathway, and vernalization (reviewed in Jack, 2004). Although, these pathways can act independently, the balance of their signals is integrated by a common set of genes that determine the appropriate time of flowering (Putterill et al., 2004).

The vernalization requirement of *Arabidopsis* is mainly controlled by two loci: *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*). The natural allelic variation at the *FRI* and *FLC* loci, among different accessions of *Arabidopsis*, account for most of the difference in flowering time between early (summer) and late (winter) flowering ecotypes (Napp-Zinn, 1985; Burn et al., 1993; Clarke and Dean, 1994; Koornneef et al., 1994; Johanson et al., 2000;
Molecular analyses in various cereals revealed that the VRN2 locus encodes a CONSTANS (CO)-like gene and the VRN1 locus encodes an AP1-like MADS box gene. Examples of the latter are TmAP1, also named WAP1, BM5, and LpMADS1 from wheat, barley, and Lolium, respectively (Petersen et al., 2004). The expression of these three genes is strongly induced by vernalization in winter varieties (Danyluk, 2003; Murai et al., 2003; Trevaskis et al., 2003; Ciannaméa et al., 2006; Petersen et al., 2006). Furthermore, overexpression of AP1-like genes from grass species, and ectopic expression of some of these genes in model species, such as Arabidopsis and tobacco, resulted in early flowering phenotypes (Kang et al., 1997; Jeon et al., 2000; Gocal et al., 2001; Fornara et al., 2004). This all pointed to an important role for the cereal AP1-like genes in floral induction. Remarkably, no FLC-like MADS box gene has been isolated from cereals so far.

All the above-discussed MADS box proteins involved in the flowering process, in both dicots and monocots, belong to the type II class of plant MADS box proteins. This class of proteins contains a conserved modular structure consisting of four different functional domains: from the N- to the C-terminus, the MADS-box (M), the intervening region (I-region), the coiled-coil keratin-like (K-box), and the C-terminal region (Riechmann and Meyerowitz, 1997). Biochemical and molecular studies have proven that specific dimerization and higher order complex formation are important for the functioning of these proteins and the interaction patterns appeared to be conserved for MADS box proteins from various plant species (Egea-Cortines et al., 1999; Immink and Angenent, 2002; Favaro et al., 2003). Furthermore, it has been shown that the CARG box is the consensus binding site for MADS domain transcription factors. This DNA motif consists of 10 bases with the consensus sequence CC(A/T)6GG (Treisman, 1986; Hayes et al., 1988). Several reports revealed that the expression of members of the MADS box family is controlled by the MADS box proteins themselves, establishing an autoregulatory loop mechanism (Schwarz-Sommer et al., 1992; Gomez-Mena et al., 2005; Zhu and Perry, 2005).

In this study, the characterization of three Lolium AP1-like genes, LpMADS1, LpMADS2, and LpMADS3, and one Lolium SVP-like gene, LpMADS10, is presented. Previous studies have shown that the expression of LpMADS1, LpMADS2, and LpMADS3 is induced during vernalization in both leaves and the shoot apex (Petersen et al., 2004), whereas, in contrast, the expression of LpMADS10 is strongly down-regulated by vernalization in the shoot apex (Petersen et al., 2006). Here the further
characterization and comparison of the \textit{LpMADS1}, \textit{LpMADS2}, and \textit{LpMADS3} genes with close relatives from \textit{Arabidopsis} is reported together with the conservation of the protein interaction patterns of the encoded proteins. Furthermore, part of the putative promoter of the \textit{LpMADS1} gene was studied by yeast one-hybrid analyses and by \textit{in vitro} binding assays. These analyses revealed a specific binding of the \textit{LpMADS1–LpMADS10} heterodimer to the CArG box present in the vernalization-responsive \textit{LpMADS1} promoter.

Materials and methods

Sequence analysis

Amino acid sequence alignments were performed using the ClustalW program (Thompson \textit{et al.}, 2004). A Neighbor-Joining tree was calculated with MEGA3.1, with the model Poisson correction (184 sites, 10 000 bootstrap replicates).

Yeast two-hybrid analyses

The \textit{LpMADS1}, \textit{LpMADS2}, \textit{LpMADS3}, and \textit{LpMADS10} open reading frames were generated by polymerase chain reaction (PCR) and subsequently cloned into \textit{pENTR}^\textregistered/D-TOPO (Invitrogen). In the next step, each obtained \textit{pENTR}^TM Directional TOPO vector was recombined with the GATEWAY destination vectors pDEST\textregistered/32 (pBDGAL4, bait) and pDEST\textregistered/22 (pADGAL4, prey) from Invitrogen. Due to technical problems, it was not possible to obtain the pBDGAL4-\textit{LpMADS10} construct. All the generated bait vectors were transformed into yeast strain PJ69-4a (MATa; James \textit{et al.}, 1996) and all prey vectors into strain PJ69-4a (James \textit{et al.}, 1996), and the transformants were selected on SD plates lacking Leu and Trp, respectively. Subsequently, the obtained bait plasmids containing the full-length sequences of \textit{LpMADS1}, \textit{LpMADS2}, and \textit{LpMADS3}, respectively, were tested for autoactivation of the yeast reporter genes. Addition of 5 mM 3-amino-1,2,4-triazole (3-AT) appeared to be sufficient to abolish the basal HIS3 expression caused by the transcriptional activity of the C-terminal domains of these three proteins. All \textit{Lolium} proteins were screened for heterodimerization capacity and in a heterologous yeast two-hybrid screening against the \textit{pBDGAL4-Arabidopsis MADS} collection (de Folter \textit{et al.}, 2005). The mating-based screening and selection for positives has been performed as described by de Folter \textit{et al.} (2005).

Yeast one-hybrid experiments

The MATCHMAKER One-Hybrid System (catalogue no. K1603-1) was used to study protein–DNA interactions. For this purpose, the putative promoter fragments of \textit{LpMADS1} from \textit{Lolium perenne} (clone F6, DLF-TRIFOLIUM) and from \textit{Lolium multiflorum} (variety, Westerwoldcim, WW) were amplified by \textit{Pfu} polymerase and subsequently cloned into the pGEM-T Easy vector (Promega). For the F6 fragment, the primers PRO596 (GAGCCTAAATCATGCG-CACGAGCGACCC) and PRO597 (TCTGAAAGGTTCTCCG-TTCCGGCAAGGG) were used, giving a fragment of 516 bp, and for the WW fragment primers PRO598 (GACGCTATCGGGACACGGA- GACGACGATGCT) and PRO599 (TCTGAGAATCTGGGGGCA- GATGCGCG), yielding a fragment of 568 bp. After enzymatic digestion by \textit{SacI} and \textit{XhoI}, the F6 and WW promoter fragments were gel purified and cloned into the \textit{SacI-} and \textit{XhoI-} linearized pHISi vector (Clontech), resulting in the plasmids pHIS-F6 and pHISi-WW, respectively. Initially, pHISi-F6 and pHISi-WW were integrated into the yeast genome of strain PJ69-4a (James \textit{et al.}, 1996) and, subsequently, the obtained yeast clones were selected on a concentration range of 3-AT, to identify clones with a low level of background (<20 mM 3-AT) expression for the HIS3 reporter gene. For the screening of a promoter fragment against a heterodimer and the heterologous yeast one-hybrid screening, the conventional yeast one-hybrid system was slightly modified, to enable expression of two putative DNA-binding proteins. All identified \textit{Arabidopsis} MADS box transcription factor dimers (De Folter \textit{et al.}, 2005) and the \textit{LpMADS1–LpMADS10} heterodimer were reconstituted in yeast strain PJ69-4a. For this purpose, the yeast expression vector pTFT1 (Egea-Cortines \textit{et al.}, 1999) was made Gateway compatible by cloning the Gateway RiB cassette (Invitrogen) into the blunted EcoRI restriction site, giving plasmid pARC352. Subsequently, the complete \textit{Arabidopsis} MADS box transcription factor collection was recombined into the pARC352 destination vector. In the next step, the various ‘pTFT1-MADS’ constructs were transformed to yeast clones that already contain a specific ‘pADGAL4-Arabidopsis MADS’ construct (de Folter \textit{et al.}, 2005). The \textit{LpMADS1–LpMADS10} heterodimer was reconstituted in yeast as pADGAL4-LpMADS1 and pTFT1-LpMADS10.

Electromobility shift assay (EMSA)

As templates for the probes, the plasmids pHISi-F6 and pHISi-WW, described above, were used. The DNA probes for the shifts were produced by PCR with biotinylated primers PRO716 (FW, pHISi vector, GTAATACGACTCACTATAAGG) and PRO717 (RV, pHISi vector, ATCGATTGCACGACTTGG) using \textit{Pfu} polymerase, and gel purified using the QIAEXII-kit. Either 30 or 40 fmol of probes were used per lane (see figure legends for details). The full \textit{LpMADS1} and \textit{LpMADS10} coding sequences as well as C-terminally deleted versions were amplified using modified primers introducing \textit{Neol} and \textit{Bam}HI sites, and then cloned into the pSPUTK vector. \textit{LpMADS1-MIK1} encodes a protein of 158 amino acid residues, and \textit{LpMADS1-MIK2} encodes a protein of 174 amino acid residues. The resulting constructs were checked for PCR errors by sequencing. \textit{In vitro} translation and EMSA assays were performed as described in Kaufmann \textit{et al.} (2005).

Results

\textbf{Lolium MADS box genes potentially involved in flowering}

Previous studies in \textit{L. perenne} have identified three \textit{AP1}-like genes named \textit{LpMADS1}, \textit{LpMADS2}, and \textit{LpMADS3}, and one gene that belongs to the SVP/AGL24 clade, named \textit{LpMADS10} (Petersen \textit{et al.}, 2004, 2006). Based on their expression patterns and similarities with close relatives from \textit{Arabidopsis}, these genes may potentially be involved in controlling the transition from vegetative to generative growth. To obtain a better insight into the sequence similarities between the proteins encoded by these \textit{Lolium} genes and to compare these proteins with the MADS box regulators of flowering in \textit{Arabidopsis}, a phylogenetic tree has been generated (Fig. 1A). Subsequently, an alignment (Fig. 1B) has been made for the predicted amino acid sequences of \textit{LpMADS1}, \textit{LpMADS2}, and \textit{LpMADS3}, and the \textit{Arabidopsis} proteins \textit{AP1} and \textit{FUL}, which are the most closely related based on the phylogenetic tree. The same has been done for \textit{LpMADS10} and the \textit{Arabidopsis} proteins \textit{SVP} and \textit{AGL24} (Fig. 1C). The comparison
Fig. 1. Phylogenetic tree and alignments for the Lolium MADS box transcription factor proteins. (A) Phylogenetic tree. Comparison was made using the amino acid sequence of MADS box proteins from Arabidopsis (AGL24, SVP, SOC1, FLC, MAF1–5, AGL6, AGL13, SEP1–4, AP1, CAL, and FUL) and Lolium perenne (LpMADS1, LpMADS2, LpMADS3, and LpMADS10). Bootstrap values are given in percentages and are indicated above each branch. (B) Alignment of LpMADS1, LpMADS2, and LpMADS3 of Lolium with the AP1 and FUL proteins from Arabidopsis. (C) Alignment of LpMADS10 from Lolium with SVP and AGL24 from Arabidopsis and TaVRT-2 from Triticum aestivum.
between LpMADS1 and LpMADS2 revealed a high degree of identity (71%, similarity 79%), while, in contrast, LpMADS3 and LpMADS1 share only 48% (similarity 64%) of their amino acid sequences. The Arabidopsis AP1 and FUL proteins have 51% (similarity 68%) and 49% (similarity 68%) of their sequence in common with LpMADS1, respectively, and both Arabidopsis proteins share 45% (similarity 62% and 59%, respectively) of their sequence with LpMADS3. The C-terminal domain of the LpMADS1, LpMADS2, and LpMADS3 proteins showed a less conserved amino acid sequence, though a conserved FUL-like motif (L/MPPWML) can be identified at the very C-terminal region of all three Lolium proteins. Although, there is clear overlap in the protein sequences, based on this comparison it cannot be concluded which of these LpMADS genes is the candidate gene that has a function similar to AP1 from Arabidopsis.

LpMADS10 shares 50% (similarity 70%) amino acid sequence with SVP and only 41% (similarity 65%) with AGL24. Furthermore, LpMADS10 appeared to be 76% identical (similarity 90%) to the putative floral repressor TaVRT-2, recently isolated from Triticum aestivum (Kane et al., 2005). Based on this sequence comparison and the expression analyses performed by Kane and colleagues (2005) and Petersen et al. (2006), LpMADS10 might be the most similar in function to TaVRT-2 and SVP, from wheat and Arabidopsis, respectively.

### Protein–protein interactions of the Lolium MADS box proteins

Because comparison of amino acid sequences between Arabidopsis and Lolium AP1-like and SVP/AGL24-like proteins does not provide sufficient clues to predict the functions of the Lolium genes, the properties of the encoded proteins were investigated. The high similarity between LpMADS1 and LpMADS2 protein sequences suggests that they may have similar functions and, hence, overlapping interaction patterns, as has been shown for other redundant proteins such as SEPALLATA1 (SEP1) and SEP3 (de Folter et al., 2005). To obtain insight into the dimerization specificity of the four Lolium proteins, a binary yeast two-hybrid screen has been performed (Table 1). Both LpMADS1 and LpMADS2 were able to form homodimers and heterodimers with each other; while, in contrast, LpMADS3 behaves differently in these interactions. Remarkably, all three AP1-like Lolium proteins were able to heterodimerize with the LpMADS10 protein.

To investigate the interaction capacity of the four Lolium proteins in more detail, a heterologous yeast two-hybrid screening was performed by screening against the entire collection of Arabidopsis MADS box proteins fused to the GAL4-binding domain (BD) (de Folter et al., 2005). This heterologous interaction screen may provide information about the conservation of protein features between Lolium and Arabidopsis. Not all combinations could be tested, because some of the Arabidopsis proteins have been shown to give rise to autoactivation in this yeast system (de Folter et al., 2005). The results of the screening for LpMADS1 and LpMADS2 are shown in Table 2. As expected, both proteins have an identical interaction pattern. Notably, many protein interactions were observed between these two Lolium proteins and Arabidopsis MADS box proteins involved in flowering time, such as SVP, AGL24, and SOC1, but also with proteins playing a role in floral organ formation, such as FUL, AGAMOUS (AG), and SEP2. LpMADS10 has a number of interactions in common, but also revealed distinct interactors (Table 2). Remarkably,

### Table 1. Yeast two-hybrid analysis for the LpMADS proteins

Yeast was spotted onto medium lacking adenine and medium lacking histidine, supplemented with 5 mM 3-AT, and all combinations that grew on both selection media were scored as an interaction (+). No interaction is indicated by –.

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<th>Interactors of:</th>
<th>Full protein name</th>
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<td>LpMADS1</td>
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<td>AG</td>
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<tr>
<td>SHP1</td>
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<td>SEP2/I/II</td>
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<td>SVP</td>
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### Table 2. Heterologous yeast two-hybrid screen

Interactions between LpMADS1, LpMADS2, LpMADS3, and LpMADS10, and the Arabidopsis MADS box protein collection. LpMADS3 did not interact with any of the Arabidopsis proteins. All interaction events resulted in yeast growth on selective medium lacking adenine and selective medium lacking histidine, supplemented with 5 mM 3-AT. No interaction is indicated by –.
LpMADS3 failed to interact with any protein of the Arabidopsis MADS box collection. Resequencing the AD-LpMADS3 construct did not reveal mutations, and the same construct was used in the screen for interactions with the other Lolium proteins (Table 1), which exclude a trivial reason for the lack of interactors.

To allow a good comparison between the heterologous interaction patterns of the Lolium MADS box proteins and the interaction patterns of the Arabidopsis MADS box proteins involved in flowering, Venn diagrams were created (Fig. 2). Comparison of the LpMADS1 sequence with the sequences of Arabidopsis MADS box genes revealed that AP1 and FUL are the closest relatives, suggesting that they may play a similar developmental role (Fig. 1A, B). However, AP1 expression is not detectable in vegetative organs, but starts to be expressed in the floral meristem at an early stage of floral development and specifies the identity of sepals and petals (Mandel et al., 1992; Mandel and Yanofsky, 1995). In contrast to the function of AP1, there are indications suggesting that LpMADS1 is an early regulator of the flowering process in Lolium (Petersen et al., 2004, 2006). Therefore, it was taken into consideration whether LpMADS1 and SOC1, despite their low sequence identity (38%), may share the same interacting proteins, since both proteins function as flowering regulators in Lolium and Arabidopsis, respectively. Remarkably, almost all proteins with which SOC1 interacts appear to form dimers with LpMADS1 as well (Fig. 2A). The interaction map of AP1 completely overlaps with the interaction map of LpMADS1, and the same is the case

**Fig. 2.** Comparison of interaction maps for Lolium and Arabidopsis MADS box proteins. (A) Venn diagrams showing the yeast two-hybrid interactions of LpMADS1 identified in this study and the interaction maps of AP1, SOC1, and FUL according to de Folter et al. (2005). The Venn diagram of AP1 is presented in a green eclipse, FUL in brown, LpMADS1 in orange, and SOC1 in light blue. (B) Venn diagrams showing the yeast two-hybrid interactions of LpMADS10 identified in this study and the interaction maps of SVP and AGL24 according to de Folter et al. (2005). The Venn diagram of LpMADS10 is presented in an orange eclipse, SVP in green, and AGL24 in light blue. Not all combinations could be tested because some of the Arabidopsis proteins have been shown to give rise to autoactivation in this yeast system (de Folter et al., 2005).
for FUL. However, AP1 and FUL lack many interactions observed for LpMADS1 and SOC1, indicating that they are clearly distinct with respect to interaction selectivity.

In Fig. 2B the Venn diagram is given for LpMADS10 and the Arabidopsis proteins SVP and AGL24. LpMADS10 has a broader set of interactors than the two Arabidopsis proteins, but all MADS proteins forming dimers with SVP and AGL24 also interact with LpMADS10.

**Characterization of the putative LpMADS1 promoter sequence**

To obtain more insight into the role of LpMADS1 in the flowering process and how the expression of this gene is regulated, the upstream sequences of a spring and winter growth habit variety were analysed. Recently, Petersen and colleagues (2006) isolated and analysed the putative LpMADS1 promoter from the winter variety L. perenne F6, and from two spring growth habit varieties, L. temulentum and L. multiflorum Westerwoldicum (WW). The F6-1 winter variety is vernalization dependent for flowering and its LpMADS1 gene is up-regulated towards the end of the vernalization process. In contrast, the two spring varieties flower independently from vernalization and have expression of LpMADS1 throughout the vegetative phase. Here, the LpMADS1 F6 (winter) and WW (spring) promoter sequences were compared. In Fig. 3, an alignment is given for these two promoter sequences. Remarkably, among the InDel (internal deletions) and SNPs (single nucleotide polymorphisms) found, a deletion of 9 bp was identified in the spring variety WW, directly adjacent to a conserved putative MADS box transcription factor-binding site, the CArG box [CC(AT)6AG]. Previously, similar work in wheat showed a deletion adjacent to a putative CArG box in the promoter sequences of many spring accessions (Yan et al., 2003, 2004). The presence of a putative MADS box transcription factor-binding site (CArG box) suggests either that LpMADS1 is regulated by another MADS box transcription factor or, alternatively, that it is able to regulate its own expression via an autoregulatory feedback loop. Both negative and positive autoregulatory feedback mechanisms have been hypothesized for plant MADS box transcription factors (Schwarz-Sommer et al., 1992; Trobner et al., 1992; Goto and Meyerowitz, 1994; de Folter et al., 2005; Gomez-Mena et al., 2005).

**Yeast one-hybrid experiments with LpMADS1 winter and spring promoter fragments**

For MADS box transcription factors it is known that they bind DNA either as homodimers or as heterodimers in vitro (Riechmann et al., 1996; West et al., 1998). Based on the observations that the LpMADS1 promoter contains a putative CArG box and that LpMADS1 forms a heterodimer with LpMADS10, it has been hypothesized that the LpMADS1–LpMADS10 heterodimer could act as a negative autoregulator complex for the LpMADS1 promoter (Petersen et al., 2006). Furthermore, Petersen and colleagues suggested that the deletion in close proximity to the CArG box in the putative LpMADS1 promoter of the spring accessions may affect the binding by the MADS box protein(s). To test these hypotheses, LpMADS1 winter and spring promoter elements were tested in the yeast one-hybrid system against various Lolium MADS box heterodimers. The results of this screening are presented in Table 3 and revealed that the LpMADS1–LpMADS10 heterodimer was able to bind strongly to the F6 winter promoter fragment, while it barely interacted with the WW spring promoter fragment. Similarly, although weaker than the LpMADS1–LpMADS10 heterodimer, binding to the LpMADS1 F6 promoter fragment was scored for

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**Fig. 3.** Comparison of the LpMADS1 upstream sequence of the F6 (winter) variety and the WW (spring) variety. The putative CArG box is marked in grey and a box is placed around the stretch of DNA used for the gel-shift experiments.

**EMSAs**

*In vitro* EMSAs were performed to confirm the differences in binding of MADS domain proteins to the two varieties of the *LpMADS1* promoter. The probes were derived from DNA fragments surrounding and including the putative CArG box (Fig. 3), cloned into the pHISi vector. Binding of LpMADS1 and LpMADS10 homodimers to the winter (F6) and spring (WW) type CArG boxes was identified; however, binding was stronger to the winter type (Fig. 4A). A similar difference in binding efficiency between the F6 and WW promoter elements was observed with the LpMADS1 and LpMADS10 heterodimer (Fig. 4A). Surprisingly, with this combination of proteins, a supershift was obtained, which suggests that a higher order complex is formed by these two proteins. To gain a better understanding of the composition of the LpMADS1- and LpMADS10-containing complex, deletion constructs have been generated for LpMADS1. Subsequently, these C-terminally deleted versions of LpMADS1 were used together with the full-length LpMADS10 protein, in order to distinguish homo- from heterodimers based on height differences of the retarded bands in the native gel. The results demonstrate that LpMADS10 is able to form DNA-binding heterodimers with both C-terminally deleted versions of LpMADS1. This is indicated by the intermediate bands in the gel shifts (Fig. 4B). No supershifted band was observed in the shifts with C-terminally deleted versions of LpMADS1 together with LpMADS10, underlining the importance of the C-terminus in higher order complex formation of MADS domain proteins.

**Heterologous targeted yeast one-hybrid screening**

To determine whether other MADS box transcription factors are able to bind to one of the two *LpMADS1* promoter elements, a yeast one-hybrid screening against a collection of MADS box proteins and MADS box protein dimers was performed. Unfortunately, such a collection is currently not available for *Lolium*. However, as mentioned before, comparison of protein–protein interaction maps from MADS box proteins derived from different species, such as *Antirrhinum*, *Petunia*, and *Arabidopsis*, revealed a high degree of conservation (Immink and Angenent, 2002). Therefore, it was decided to screen the *Lolium LpMADS1* winter and spring promoter fragments against the collection of *Arabidopsis* MADS box transcription factors and their identified dimers (De Folter et al., 2005; see Materials and methods). The various yeast clones expressing a single *Arabidopsis* MADS box protein or a dimer were mated with either the winter or summer promoter element in front of the HIS3 reporter gene and spotted onto selective plates containing 30 mM 3-AT to select for putative binding factors. The results of this experiment were validated by an independent replicate experiment and revealed that a number of single MADS box proteins from *Arabidopsis* bind to the F6 winter type promoter element, but fail to bind to the WW spring type promoter fragment (Table 4). Possibly, the presence of the deletion flanking the CArG box in the putative spring promoter (WW) affects the DNA binding affinity. In line with this, only the putative winter promoter (F6) revealed interactions with a selective subset of the *Arabidopsis* MADS box dimers (Table 4). Remarkably, six of these dimers consist of at least one protein that is involved in the flower transition process, such as the dimers AGL24–AGL6, AGL97–MAF1, and SEP3–SVP.

### Table 3. Yeast one-hybrid analysis with LpMADS1 upstream sequences

The yeast clones with the integrated WW (spring) and F6 (winter) reporter constructs both gave background growth on selective medium lacking histidine and supplemented with 3-AT, up to a concentration of 15 mM. Strong growth on selective medium is indicated by +, slow growth by +/-, and no growth, and hence the lack of a protein–DNA interaction, by –.

<table>
<thead>
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<th>Promoter element</th>
<th>pADGAL4 insert</th>
<th>pTFT1 insert</th>
<th>Selection medium lacking HIS with the indicated concentration 3-AT (mM)</th>
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<tbody>
<tr>
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<td>–</td>
<td>–</td>
<td>5 15 20 25 30 40 50 60</td>
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Role of MADS box proteins in vernalization-induced flowering of Loliun

Three genes have been isolated from L. perenne that belong to this AP1/AGL9 clade, named LpMADS1, LpMADS2, and LpMADS3 (Petersen et al., 2004) from which LpMADS1 co-localizes with the VRN1 locus (Jensen et al., 2005). The expression of the LpMADS1 gene is strongly up-regulated upon prolonged periods of cold (Petersen et al., 2004; Ciannamea et al., 2006), and is further enhanced by long-day photoperiods (Petersen et al., 2004). The expression of LpMADS2 and LpMADS3 is also induced by vernalization and enhanced by long-day conditions, however, to a lesser extent. These expression patterns and the strong homology with other cereal VRN1 genes suggest a role for LpMADS1, LpMADS2, and LpMADS3 in the vernalization-mediated induction of flowering.

A comparison of LpMADS1 with the Arabidopsis MADS box proteins revealed a high similarity with FUL and, based on this, FUL might be the functional homologue of LpMADS1. However, the activity of the FUL gene is required for carpel and fruit development (Gu et al., 1998) and, furthermore, FUL has an early and redundant function in the specification of floral meristem identity, together with AP1 and CAL (Gu et al., 1998; Ferrandiz et al., 2000). Therefore, despite the fact that LpMADS1 and FUL are close in amino acid sequence, they seem to fulfil different functions. Functionally, LpMADS1 acts more like SOC1 from Arabidopsis, which is a dosage-dependent mediator of flowering and, like LpMADS1, this gene is strongly and positively regulated by the vernalization and autonomous pathways, and weakly by the photoperiod pathway (Moon et al., 2005; Sheldon et al., 2006). Completely in

Table 4. Heterologous yeast one-hybrid screening of the LpMADS1 promoter elements against the Arabidopsis MADS box proteins and MADS box protein dimers

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<th>Binding MADS box proteins</th>
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(T. aestivum; Danyuk et al., 2003; Murai et al., 2003), TmAP1 (Triticum monococcum; Yan et al., 2003), and BM5 (barley; Trevaskis et al., 2003). Three genes have been isolated from L. perenne that belong to this API/AGL9 clade, named LpMADS1, LpMADS2, and LpMADS3 (Petersen et al., 2004), from which LpMADS1 co-localizes with the VRN1 locus (Jensen et al., 2005). The expression of the LpMADS1 gene is strongly up-regulated upon prolonged periods of cold (Petersen et al., 2004; Ciannamea et al., 2006), and is further enhanced by long-day photoperiods (Petersen et al., 2004). The expression of LpMADS2 and LpMADS3 is also induced by vernalization and enhanced by long-day conditions, however, to a lesser extent. These expression patterns and the strong homology with other cereal VRN1 genes suggest a role for LpMADS1, LpMADS2, and LpMADS3 in the vernalization-mediated induction of flowering.

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line with this hypothesis, the heterologous yeast two-hybrid screen performed in this study revealed that LpMADS1 forms dimers with the same partners as SOC1. Furthermore, the expression pattern of LpMADS1 is very similar to that of SOC1 (Moon et al., 2005), which also supports the idea that these genes are functionally related. The question remains of how two closely related MADS box genes from Lolium and Arabidopsis have evolved different functions. Recently, Causier and colleagues (2005) showed that two orthologous genes from Arabidopsis and Antirrhinum, SHATTERPROOF (SHP) and PLENA (PLE), respectively, have clearly different functions. Surprisingly, the parologue of SHP, which is AG in Arabidopsis, is the functional homologue of PLE, while they have not been derived from the same ancestral gene. This example shows that duplicated genes may evolve independently by neo-functionalization and diverge in function from the orthologous genes that originated by speciation. In the case of AP1 and SOC1, their ancestral MADS box gene may have duplicated into an AP1 and SOC1 lineage before the separation of monocot and dicot species, and even before the origin of angiosperms. This is reminiscent of the situation for AG-SHP, one of the ancient paralogues, i.e. AP1 has diverged, while SOC1 retained its original function, which is conserved in monocots. Although evidence was not provided that the Lolium AP1-like gene LpMADS1 is the functional equivalent of the Arabidopsis SOC1 gene, the results suggest that these MADS box genes play a similar role in the floral transition process.

**Do LpMADS1, LpMADS2, and LpMADS3 have redundant functions?**

LpMADS1, LpMADS2, and LpMADS3 have been placed in the AP1/AGL9 clade of MADS box genes based on phylogenetic analysis and, furthermore, the expression of all three genes is induced by prolonged periods of cold (Petersen et al., 2004). This suggests that these three genes may play redundant roles in vernalization-induced flowering. Previous phylogenetic studies placed LpMADS1 in a subgroup together with the supposed orthologous genes LtMADS1, BM5, OsMADS14, and ZMM4, while LpMADS2 grouped together with LtMADS2, BM8, OsMADS15, and ZAP1, from L. temulentum, barley, rice, and maize, respectively (Gocal et al., 2001; Petersen et al., 2004). Remarkably, there are strong similarities in gene expression patterns between members of the first group (LpMADS1, BM5, and LtMADS1), whose transcripts are induced in leaves from vernalized plants. In contrast, the members of the second group (LpMADS2, BM8, and LtMADS2) are not or only to a small extent expressed in leaves. Nevertheless, in the shoot apical meristem (SAM), the expression of both LpMADS1 and LpMADS2 is induced by vernalization (Petersen et al., 2004). It has been shown here that LpMADS1 and LpMADS2 have an identical heterologous interaction pattern and interact specifically with many of the Arabidopsis MADS box proteins involved in the flowering process. Taken together, these findings strongly suggest that LpMADS1 and LpMADS2 are, at least partially, functionally redundant and are both involved in vernalization-induced flowering. LpMADS3 is also upregulated in the SAM by vernalization, but it is clearly distinct from LpMADS1 and LpMADS2 and is placed in a separate subgroup that contains only monocot genes (Petersen et al., 2004). Like LpMADS1, the LpMADS3 protein forms a heterodimer with LpMADS10, but it is not able to interact with any of the Arabidopsis MADS box proteins. Based on these results, it cannot be ruled out that LpMADS3 also has a function as a floral inducer; however, it has most probably diverged in function when compared with the related genes LpMADS1 and LpMADS2.

**Regulation of LpMADS1 expression**

Although there is strong evidence that LpMADS1 is involved in the induction of flowering upon a prolonged period of cold, it is still not clear how the up-regulation of its expression by vernalization is regulated at the molecular level. Based on the facts that the expression of LpMADS10 is down-regulated upon vernalization, that the upstream sequence of the LpMADS1 gene contains a CArG box, which is the consensus binding site for MADS box transcription factors, and that LpMADS1 and LpMADS10 are able to heterodimerize, it is tempting to speculate that this heterodimer is involved in the regulation of LpMADS1 expression. Recently, Petersen and colleagues (2006) hypothesized a possible scenario for the regulation of LpMADS1 expression. According to their model, LpMADS1 expression is repressed by a default status, characterized by non-inductive environmental conditions. Then, during vernalization, down-regulation of LpMADS10, the presumed SVP orthologue, permits the increase of LpMADS1 expression. Thus, the control of flowering is controlled by the delicate balance between LpMADS10 and LpMADS1 transcripts levels. A similar method of regulation has been suggested for the LpMADS1 homologue from wheat, TaVRT1 (Kane et al., 2005). The yeast one-hybrid and EMSA experiments performed in this study support this hypothesized control mechanism. Both the LpMADS1 and LpMADS10 homodimers are able to bind the putative LpMADS1 promoter and bind more efficiently to the LpMADS1 F6 (winter) promoter element than to the LpMADS10 F6 (winter) promoter sequence. Besides binding by these homodimers, the LpMADS1–LpMADS10 heterodimer appeared to bind strongly to the F6 (winter) promoter element only. Probably, the LpMADS10 homodimer is involved in the initial repression of LpMADS1 expression in winter accessions during the vegetative stage. Subsequently, LpMADS10 expression drops due to the
prolonged exposure to low temperatures, which coincides with an up-regulation of LpMADS1 expression. This may result in the preferred formation of LpMADS1–LpMADS10 heterodimers, which then further activate LpMADS1 expression. Finally, LpMADS10 expression is completely switched off, and LpMADS1 expression can be maintained via a positive autoregulatory loop by an LpMADS1-containing protein complex.

For all tested Lolium homodimers and heterodimers, a weaker binding, or even no binding, was obtained when the LpMADS1 WW (spring) promoter element was used. This lack of binding suggests that the small deletion present in the WW (spring) promoter fragment, just downstream of the CArG box, disturbs the protein–DNA binding. This is particularly interesting because the CArG box itself is identical in both promoter fragments and it provides for the flowering process in both monocots and dicots.

Surprisingly, the LpMADS1–LpMADS10 heterodimer seems to bind to the LpMADS1 F6 (winter) promoter element as a higher order complex. In line with the quartet model (Egea-Cortines et al., 1999; Theissen and Saedler, 2001), it is hypothesized that this is a complex consisting of two LpMADS1–LpMADS10 heterodimers (Fig. 5). However, the quartet model predicts that the two dimers within the complex bind to two adjacent CArG boxes, upon bending of the DNA, but the LpMADS1 promoter fragments used in this study contains only one CArG sequence motif. However, a 2.1 kb LpMADS1 upstream sequence has been isolated recently (K Petersen and KK Nielsen, unpublished results), and this DNA sequence appeared to contain two additional putative CArG boxes, which can be used as binding sites by the higher order complex. In conclusion, the results presented here provide strong evidence that LpMADS1 plays an important role in the vernalization-induced flowering process, in a way similar to SOC1 in Arabidopsis. Furthermore, LpMADS1 expression could be regulated by the LpMADS1 protein itself via an autoregulatory mechanism, possibly in a higher order complex with other MADS box proteins. This study shows once more the importance of plant MADS box transcription factors, and their dynamic behaviour, in the regulation of the flowering process in both monocots and dicots.

**Acknowledgements**

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