Characterization of a *MADS FLOWERING LOCUS C-LIKE* (*MFL*) sequence in *Cichorium intybus*: a comparative study of *CiMFL* and *AtFLC* reveals homologies and divergences in gene function

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Summary

• In *Arabidopsis thaliana*, the ability to flower is mainly related to a floral repressor, FLOWERING LOCUS C (FLC), which is regulated through the vernalization pathway. The genes controlling the vernalization pathway seem to be only partially conserved in dicots other than the Brassicaceae. *Cichorium intybus* (chicory) is a biennial species belonging to the Asteraceae family, and it shows an obligate vernalization requirement for flowering.

• *Cichorium intybus* MADS (MCM1, Agamous, Deficiens, SRF) FLC-like (*CiMFL*) sequences were isolated in *C. intybus* by RT-PCR and their expression patterns characterized during plant development and in response to vernalization. The biological function of CiMFL was analysed by complementation of *A. thaliana FRIGIDA* (*AtFRI);flc3*. Resetting of *MFL* expression after vernalization was analysed during microsporogenesis.

• Before vernalization, *CiMFL* is mainly expressed in the axils of young leaves. Vernalization induced *CiMFL* down-regulation under a long-day photoperiod but not under a short-day photoperiod. Furthermore, together with a decrease in *CiMFL* transcripts, cold conditions induced changes in the morphology of the shoot apical meristem and in the transition to flowering. The biological function of CiMFL was found not to be conserved.

• Our results show that the regulation of *CiMFL* expression in time and space and in relation to environmental conditions is only partially conserved with respect to *FLC* isolated from *A. thaliana*. A model for flowering repression by *CiMFL* is proposed.

Introduction

Plants undergo several developmental transitions during their life cycle, the most important of which is the transition from the juvenile phase to the adult vegetative phase, in which they are able to respond to floral inductive signals.

The modulation of flowering time results from an interaction between endogenous developmental competence and environmental cues that signal the onset of favourable conditions for reproductive success. The acquisition of floral identity by the shoot apical meristem (SAM) implies its 'determination' (Blazquez *et al.*, 2006). By means of molecular genetics approaches, the genes involved in the switch to flowering in *Arabidopsis thaliana* have been identified and assigned to inductive pathways (Salisbury, 1985; Martinez-Zapater & Somerville, 1990; Wilson *et al.*, 1992; Bernier *et al.*, 1993; Sanda & Amasino, 1996; Blazquez *et al.*, 1998; Sheldon *et al.*, 1999; Harmer *et al.*, 2001; Mouradov *et al.*, 2002; Moon *et al.*, 2003; Imaizumi & Kay, 2006; Sablowski, 2007). The environmental signals implicated in the induction of the reproductive transition include photoperiod, light quality and temperature; together these factors regulate the correct timing of flowering (Mouradov *et al.*, 2002). Moreover, the transition is often promoted by the perception of an extensive period of cold, an environmental condition that signals the end of winter; this process is known as the vernalization response (Chouard, 1960; Wellensiek, 1964; Lang, 1965; Michaels & Amasino, 2001; Henderson *et al.*, 2003).

The winter annual behaviour of some ecotypes of *A. thaliana* is conferred by the expression of the MADS (MCM1, Agamous, Deficiens, SRF)-box transcription factor gene *FLOWERING LOCUS C* (*FLC*) during the first growing season (Michaels & Amasino, 1999).

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FLC acts as the main flowering repressor. It down-regulates the expression of systemic flowering signals in the leaf (e.g. Flowering Locus T (FT)) and in response to these signals represses other genes at the meristem (e.g. Suppressor of Constans1 (SOC1) and Flowering Locus D (FD)). Vernalization represses *FLC*, making the meristem responsive to the flowering signals and allowing the leaves to produce these signals (Lee *et al.*, 2000; Sheldon *et al.*, 2000; Searle *et al.*, 2006). Following a cold treatment, the repression of *FLC* is maintained by an epigenetic mechanism that involves histone modification in *FLC* chromatin (Sung & Amasino, 2004; Dennis & Peacock, 2007; Schmitz *et al.*, 2008). In contrast, its expression is enhanced by the gene *FRIGIDA* (*FRI*) (Clarke & Dean, 1994).

FLC-like sequences have not been extensively characterized in taxa other than the Brassicaceae family. There are a few examples of the identification of *FLC*-like genes in species such as *Beta vulgaris*, *Vitis vinifera*, *Solanum tuberosum* and *Lycopersicon esculentum*, but their functions have not yet been fully characterized (Reeves *et al.*, 2007).

Cichorium intybus (chicory) is an important leaf vegetable belonging to the Asteraceae family. In the wild, it is a perennial species, while the cultivated types behave as strict biennials. In this species, the requirement of a long-day photoperiod and a period of cold seems to be absolute in order to enable the plant to flower. Sensitivity to low temperature increases with plant age, so that chicory plants gain the competence to respond to vernalization treatment only after the third true leaf has unfolded (Pimpini & Gianquinto, 1988).

The genetic control of flowering induction and differentiation in this specie is unknown. Several studies reported the effects of environmental conditions on bolting and flowering, but this information mainly came from *in vitro* cultivation of root tissues (Badila *et al.*, 1985; Demeulemeester *et al.*, 1995).

In this study we used a molecular approach to isolate *MADS FLC-LIKE* (*CiMFL*) sequences in *C. intybus* to obtain a better understanding of the genetic mechanism governing flowering induction. Given that *AtFLC* is the main repressor of flowering in *A. thaliana* and is regulated by vernalization, we were interested in characterizing its homologue in chicory. Our analyses were therefore mainly focused on the effects of vernalization and photoperiod on the regulation of *CiMFL* expression.

In comparing *AtFLC* and *CiMFL* regulation following vernalization treatments, this work provides new insights into the molecular programme controlling flowering in chicory and indicates that *CiMFL* is a homologue of *FLC* with a diverged function.

Materials and Methods

Plant growth conditions, vernalization treatment and flowering induction determination

Plants of wild chicory (*Cichorium intybus* L. (FB)) and cv. Treviso (TVT) were grown in 16-h long days (16h-LDs), under cool white fluorescence light, at 22°C. Vernalization, at 4°C and under a photoperiod of 16h-LDs or 8-h short days (8h-SDs), started when plants had developed the third true leaf. Vernalization treatments were conducted for a minimum of 7 d and a maximum of 40 d, respectively, in LD and SD conditions. After vernalization, in order to stabilize the cold treatment, the plants were transferred to a growth chamber maintained at 16°C with a 16h-LD photoperiod for 15 d. Subsequently they were moved to a growth chamber maintained at 22°C (also with a 16h-LD photoperiod) until flowering occurred.

To determine flowering induction, embedded chicory apexes were cut as described in the section '*In situ* hybridization and cytological observations' below, and SAM sections were observed under the microscope.

RNA extraction, cDNA synthesis and sequence analysis

Total RNA was extracted from leaves, apical meristems and flowers of chicory FB and TVT, using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. DNA-free RNA was obtained by DNase treatment (RNAse free DNase; Qiagen). First-strand cDNA was synthesized using SuperScript® II Reverse Transcriptase (Invitrogen) and oligo$(dT)_{12–18}$.

CiMFL cDNA was amplified by reverse transcription–PCR (RT-PCR) using degenerated primers designed based on the conserved regions obtained by aligning *FLC* sequences from *Arabidopsis thaliana* (National Center for Biotechnology Information (NCBI) GenBank accession no. NM_001085094) and *Brassica napus* (NCBI GenBank accession no. AY036888); the forward primer was 5′-RNG GTCTCGTTGAGAAAGCTCG-3′ and the reverse primer was 5′-TATTATCAGCTT CGGCT-CCCGYA-3′. PCR amplifications of the cDNA were performed with Bio-X-Act Short DNA polymerase (Bioline, London, UK) with the following cycles: 94°C for 2 min, and 30 repetitions of 94°C for 10 s, 68°C for 1 min and 70°C for 2 min. Amplicons were cloned into the pGEM-T*easy* vector (Promega) and sequenced (GenBank accession numbers FJ347969, FJ347970, FJ347971, FJ347972, FJ347973 and FJ347974). The full-length sequences were obtained by Rapid Amplification of cDNA end (RACE) reaction (Roche) using the *CiMFL*-specific primer 5′-CTCCGTGACTAGAGCCAAGAAGACCGAA-3′ to amplify the 3′-end. Sequences were edited and aligned using the software CLC Sequence Viewer 4 (CLC, Katrinebjerg, Denmark) and Lasergene DNAStar (Madison, WI, USA). Amino acid sequences were deduced using the software available online at http://www.expasy.org/tools/dna.html. A search for homology was performed using the BLASTn algorithm, with which the GenBank/European Molecular Biology Laboratory databases were queried. Phylogenetic relationships were deduced using the CLC sequence viewer workbench (http://www.clcbio.com), with application of the neighbour-joining algorithm. Values were estimated from 500 bootstrap replicates.

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Southern blot hybridization

Genomic DNA was extracted from young leaves of chicory according to the Hexadecyltrimethyl-ammonium bromide (CTAB) protocols (Doyle & Doyle, 1987). During the DNA extraction, RNase (10 ng μl^{-1}; Sigma-Aldrich) was added. Up to 10 μg of DNA extracted from leaves of different chicory cultivars (Treviso, Castelfranco, Chioggia, Lusia Adige and Verona) was digested with *Eco*RI overnight at 37°C, separated on a 0.8% (w/v) agarose gel and blotted onto a positively charged nylon membrane (Hy-bond N^+; Amersham). In order to specifically identify the MADS-box transcription factor gene *CiMFL*, a *CiMFL* probe was synthesized without the MADS-domain conserved region and labelled with dUTP-DIG (Roche). The MADS domain was removed from the *CiMFL* Coding Sequence (CDS) through amplification with the primer combination 5′-ACAGCTTCTCCTCCGGCGATAA-3′ and 5′-ATCTGGCTAGCCAAAACCTGGTTC-3′. The amplicon (423 bp) was used as a probe for hybridization. Blotted DNAs were hybridized overnight at 60°C. Filter washing and detection of anti-DIG antibody linked to alkaline phosphatase (Roche) with CDP Star (Roche) were performed according to the manufacturer's instructions.

A second Southern blotting experiments was planned to detect changes in *CiMFL* DNA methylation after vernalization. Each DNA sample of TVT chicory was digested overnight with one of two enzymes manifesting different degrees of sensitivity to cytosine methylation, in any of the cases in which methylation falls in N-CG-N (were N can be any nucleotide) and CNG sequences, Sau3AI and NdeII (Promega). DNAs were hybridized as previously described above using a DNA genomic probe of 438 bp, spanning the region from nucleotide 117 to 261 in the MADS domain, taking *CiMFL2* sequence as a reference for the nucleotide positions, plus 293 bp of intronic sequence.

In situ hybridization and cytological observations

Plant material (shoot apexes and pollinated and unpollinated flowers) was collected and fixed in 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer, pH 7.2, and incubated for 16 h at 4°C. Tissues were dehydrated by washing and incubation in a mixture of increasing ethanol-xylene concentration, according to Varotto *et al*. (2003). Dehydrated tissues were embedded in paraffin (Paraplast Plus; Sigma) and cut into 6–10-μm-thick sections using a rotary microtome (RM 2135 Leica). Sections were collected on SuperFrost Plus Slides (Menzel-Glazer, Braunschweig, Germany) and were de-waxed and treated with 10 μg ml^{-1} proteinase K (Sigma). Sense and antisense riboprobes were obtained by *in vitro* transcription using T7 and SP6 RNA polymerases (Roche) and labelled with Digoxygenin RNA labelling mix (Roche). The *CiMFL* probes were synthesized from a cDNA sequence lacking the MADS domain, as described for the Southern blot hybridization. Hybridization was conducted overnight in 50% formamide at 48°C. DIG detection and signal visualization were carried out with NBT-BCiP (Roche) following the manufacturer's instructions. After staining, slides were dried and mounted with DPX Mountant for histology (Sigma-Aldrich).

For cytological observations, shoot apexes were collected, fixed in 4% paraformaldehyde (Sigma-Aldrich) and embedded in Paraplast (Sigma-Aldrich) as described for the *in situ* hybridization procedure. All vernalized samples were collected 4 wk after moving the plants to 24°C under LDs. Sections of 6 μm for each sample were produced using a rotary microtome and collected on SuperFrost slides. After de-waxing and drying, slides were mounted with VectaShield® Mounting Medium containing 4-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA).

Slides were observed under epifluorescent light or bright field with a Leica DM4000B microscope. Images were captured with a Leica DC300F camera and processed with Adobe Photoshop 6.0.

Chicory *MFL::GFP* expression construct

CiMFL2 and *CiMFL3* coding sequences (CDS) were amplified by RT-PCR using the forward primer 5′-**AGATCT**ATGAT-TGTAATACGACTCACT-3′ and the reverse primer 5′-**GGTACC**TCTAGCTATGCATCCAACGCGT-3′. Amplicons were then separated in 1.5% (w/v) agarose and gel-purified. *Bgl*II and *Kpn*I restriction sites (in bold characters in the primer sequences) were incorporated at their 5′-ends and the stop codon was removed from the CDS through point mutations inserted in the reverse primer. PCR products were digested with *Bgl*II and *Kpn*I, and cloned into the corresponding sites of the vector pTZ-19U (Stratagene, LaJolla, CA, USA), in place of the β pre-sequence originally present in the vector. In the construct, the green fluorescent protein (*GFP*) cDNA was framed by a strong promoter (EN50PMA4) and a *nos* terminator.

Chicory protoplast transformation

Protoplasts were isolated as in Varotto *et al*. (2001). Plantlets of chicory cv. Treviso TVT were maintained on B5 medium under controlled environmental conditions, in a growth chamber at 22°C with a 12-h day:night photoperiod. Young leaves (4–5 cm in length) were cut into very small strips and placed in a filtered sterilized enzyme solution containing 0.1% (w/v) Cellulase Onozuka R10, 0.05% (w/v) Driselase and 0.02% (w/v) Macerozyme (all from Duchefa, Haarlem, The Netherlands) in WS9M medium (27 mg l^{-1} KH_2PO_4, 1.48 g l^{-1} $CaCl_2{\cdot}2H_2O$, 100 mg l^{-1} KNO_3, 250 mg l^{-1} $MgSO_4$ and 90 g l^{-1} mannitol, pH 5.6). Strips were incubated overnight in this enzyme solution, in the dark at 28°C. Gentle shaking was maintained.

The protoplast suspension was then filtered with 200-μm nylon mesh (Sigma-Aldrich) and centrifuged at 130 *g* for 10 min. The pellet was re-suspended in FS13S buffer (27 mg l^{-1} KH_2PO_4, 1.48 g l^{-1} $CaCl_2{\cdot}2H_2O$, 100 mg l^{-1} KNO_3,

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250 mg l^{-1} $MgSO_4$ and 130 g l^{-1} saccharose) and centrifuged again (100 *g* for 10 min). The pellet was then washed in WS9M medium as described above and re-suspended in calcium-mannitol buffer ($MaCa_3$) (0.5 M mannitol, 20 mM $CaCl_2$ and 0.1% MES, pH 5.8, with KOH). After centrifugation at 100 *g* for 5–10 min the protoplasts were re-suspended in $MaCa_3$ buffer, and 10–20 μg of the pTZ-19U vector carrying the *CiMFL2::GFP* or *CiMFL3::GFP* cassette was added. After 5 min of incubation at room temperature, a fresh solution of Polyethylene Glicol (PEG) 40% was added. Transformed protoplasts were centrifuged at 100 *g* for 5 min and the pellet was re-suspended in WS9M medium. At this point, protoplasts were cultured in the dark, overnight (18–20 h) at room temperature for the expression of the fusion protein.

Semiquantitative RT-PCR for *CiMFL* mRNA quantification

For quantification of *CiMFL* transcripts, cDNA was prepared from young leaves. Leaves were collected from 10 plants for each treatment (LD or SD) in the growth chamber at either 4 or 24°C. Serial dilutions of the concentrated first-strand cDNA reaction were prepared.

A gene-specific primer combination (5′-CAGTCACGACGTTGTAAAACGACGGC-3′ with 5′-CGCCAAGCTATTTAGGTGACACT-3′) was used to amplify four *CiMFL* sequences at the same time, while the primer combination 5′-CAGTCACGACGTTGTAAAACGACGGC-3′ with 5′-GAGCGACGGATGCGTCACAGAGAACAGAA-3′ was used for *CiMFL2*-specific amplification.

PCR conditions were controlled by *18S* amplification with the *18S* primers forward 5′-GGAGCCATCCCTCCGTAGTTAGCTTCTT-3′ and reverse 5′-CCTGTCGGCCAAGGCTATATACTCGTTG-3′.

All the PCRs were performed with Bio-X-Act Short DNA polymerase (Bioline), with the following cycles: 94°C for 2 min, followed by either 26 cycles (for *18S*) or 30 cycles (for *FLC*) of 94°C for 10 s, 58°C for 12 s and 72°C for 45 s, in a PerkinElmer 9600 thermocycler (PerkinElmer, Waltham, MA, USA). Amplified fragments were separated on a 1.5% (w/v) agarose gel.

For the analysis of *CiMFL2* expression during the day, leaf samples were collected throughout the day every 4 h, starting at 07:00 h (time 0). The cDNA was diluted and each dilution was amplified with selective primers for *CiMFL2* using the above cycling conditions. Images were captured using Kodak Molecular Imaging Software.

Production, selection and analysis of transgenic *A. thaliana* plants

The *CiMFL2* sequence, including its 3′ untranslated region (UTR) (703 bp), was cloned into the pENTR™/D-TOPO vector (Invitrogen) to enable use of the Gateway™ recombination system (Invitrogen) for insertion of *CiMFL2* into the expression vector pMDC32, which carried the 2X35S promoter and *nos* terminator (ABRC; The Ohio State University). The construct was introduced into *Escherichia coli* strain TOP10 (Invitrogen) for sequencing. *Agrobacterium tumefaciens* strain C58i_pMD990 (kindly provided by R. Amasino's laboratory) was subsequently transformed with the over-expression construct through electroporation. An *A. thaliana FLC* null mutant in the *FRI* background, *flc-3* (Redei, 1962; Lee *et al.*, 1993; Michaels & Amasino, 1999), was transformed with the selected *A. tumefaciens* using the floral dip method (Clough & Bent, 1998). Transformants were selected by supplying 25 mg l^{-1} hygromycin on Murashige and Skoog (MS; Duchefa) medium. Plants were grown in a growth chamber under long-day conditions at 24°C. Hygromycin-resistant plants were transferred to soil and grown for analysis of flowering time and phenotype. The presence of the transgene in the *A. thaliana* genome was verified by PCR, using primers annealing to the *35S* promoter and the *nos* terminator sequences flanking the *CiMFL2* CDS in the cassette (forward primer 5′-CTATCCTTCGCAAGACCCTTCCTCT-3′ and reverse primer 5′-AATCAT CGCAAGACCGGCAACAGGATTC-3′).

Time to flowering was measured in T1 plants as the total leaf number in the rosette at flowering, when the inflorescence stem reached approximately 1–3 cm in length.

Results

Identification of *CiMFL* cDNAs

Chicory *FLC*-like sequences were identified by PCR-based cloning using primers designed on the basis of the *FLC* sequences of *A. thaliana* and *B. napus* available in GenBank, and named *CiMFL*s. Specifically, degenerated primers were designed based on the MADS-box conserved region and on the CDS 3′-end. *CiMFL* transcripts were amplified by RT-PCR in a homozygous wild accession of chicory (FB line) which showed perennial behaviour. Subsequently, we isolated *CiMFL* sequences in the self-incompatible heterozygous cultivar Treviso (TVT), which showed biennial behaviour and required vernalization to flower.

Three partial cDNA sequences homologous to *AtFLC* were identified in the FB wild chicory line and were designated *CiMFLa*, *CiMFLb* and *CiMFLc*; the lengths of the cDNA fragments were as follows: *CiMFLa*, 503 bp; *CiMFLb*, 236 bp; and *CiMFLc*, 182 bp (GenBank accession numbers FJ347972, FJ347973 and FJ347974). Sequence analysis led to the identification of a putative open reading frame (ORF) for *CiMFLa*, while premature stop codons were retrieved inside the CDS of *CiMFLb* at positions 212 and 217, and in *CiMFLc* at positions 109 and 124 (data not shown).

Four *CiMFL* sequences were isolated in TVT chicory. Because the 5′-CDS was already covered, gene-specific primers were designed to obtain the missing 3′-end of the four amplicons by 3′ RACE reactions. Full-length cDNA sequences were gel-purified and named *CiMFL1*, *CiMFL2*, *CiMFL3* and *CiMFL4*

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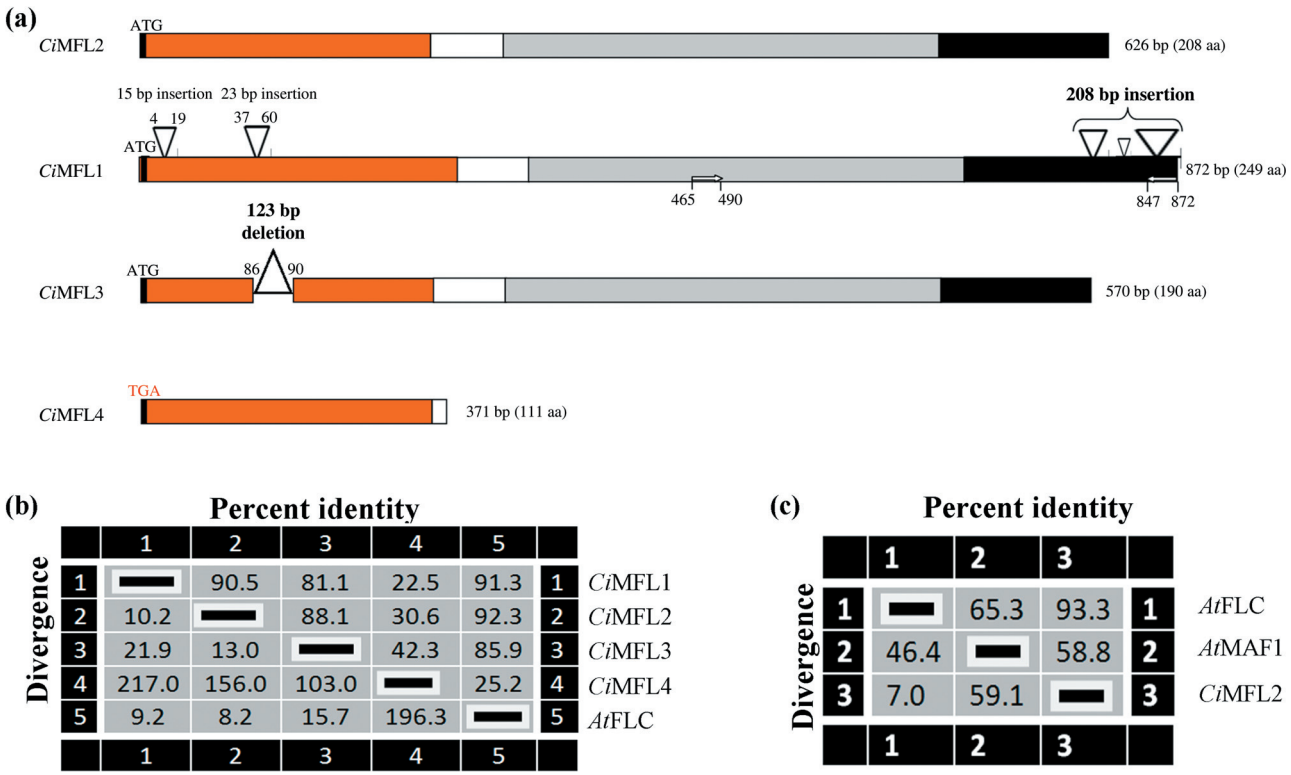

Fig. 1 Structure of *Cichorium intybus* transcripts and sequence analyses. (a) Approximate boundaries of the MADS-box, I-region, K-box and C-terminal domain are indicated. Referring to the *C. intybus MADS FLOWERING LOCUS C-LIKE 2* (*CiMFL2*) sequence, some insertions are identifiable in *CiMFL1* from the nucleotides 4–19, from 37–60 and other shorter insertions are present at the 3′-end, resulting in 208 extra bases and 24 substitutions in the overall coding sequence. Two inverted repeats of 26 nucleotides in *CiMFL1* are indicated by white arrows. Using the software MFOLD (http://mfold.bioinfo.rpi.edu) we determined that the folding of the sequence of *CiMFL1* could result in a hairpin structure (data not shown). With respect to *CiMFL2*, *CiMFL3* shows a deletion of 123 nucleotides inside the MADS domain, corresponding to the stretch of *CiMFL2* sequence from position 86 to position 209; *CiMFL4* lacks a putative open reading frame, having the methionine start codon replaced by a stop codon (TGA), and its sequence is truncated after the I-domain. (b) The percentage identity of amino acid sequences of *C. intybus MFL*s and *Arabidopsis thaliana FLC* (AAD21249) was calculated with the formula: (matches × 100)/length of aligned region (with gaps), while the divergence was calculated as follow: divergence (*i*,*j*) = 100 [distance (*i*,*j*)]/total distance, where distance (*i*,*j*) = sum (residue distances) + (gaps × gap penalty) + (gap residues × gap length penalty). (c) Percentage identity of amino acid sequences of CiMFL2, AtFLC and AtMAF (MADS Affecting Flowering) 1 (AAK37527).

(GenBank accession numbers FJ347968, FJ347969, FJ347970 and FJ347971). Details of these sequences are given in Fig. 1.

A nucleotide comparison between the partial *CiMFL* sequences cloned from wild chicory and the full-length sequences isolated from the cultivated accession TVT showed that two of the transcripts were shared between the FB line and TVT. Specifically, the deduced amino acid sequence of MFLa matched that of MFL2 (91% identity), while the sequence of MFLb matched that of MFL4 (100% identity). *MFL1* and *MFL3* seem to be unique to TVT, while *CiMFLc* appears to be characteristic of the wild accession. Interestingly, CiMFL2 possesses a distinctive insertion of 14 amino acids into the MADS domain when compared with FLC proteins from other species available in the GenBank database (Fig. 2a).

Phylogenetic analysis of *FLC* and *CiMFL2* sequences was carried out using the aligned deduced amino acid sequences, both considering the whole coding sequence and considering only the MADS domain (Fig. 2b,c). *MADS Affecting Flowering* (*MAF*) sequences were inserted into this alignment as an out-group. *MAF* genes constitute a group of transcriptional factors that are very similar to *FLC* genes, but are classified as a separate group supported by a high bootstrap value (Tadege *et al.*, 2001). Phylogeny construction using the whole *FLC* coding sequence showed that *CiMFL2* is grouped together with *A. thaliana FLC*, supported by a high bootstrap value of 491 out of 500, and is separated from the *MAF* genes (Fig. 2b). However, the neighbour-joining tree created by aligning only the MADS-box domain revealed that *CiMFL2* is neither in the cluster of the *MAF* family nor in the group including *AtFLC* (Fig. 2c). These results support the view that the MADS-domain sequence in *CiMFL2* with the additional amino acids in its first trait of sequence (Fig. 2a) represents a peculiar characteristic of the gene that could explain the evolution of a diverged function in spite of its high sequence homology with *AtFLC*.

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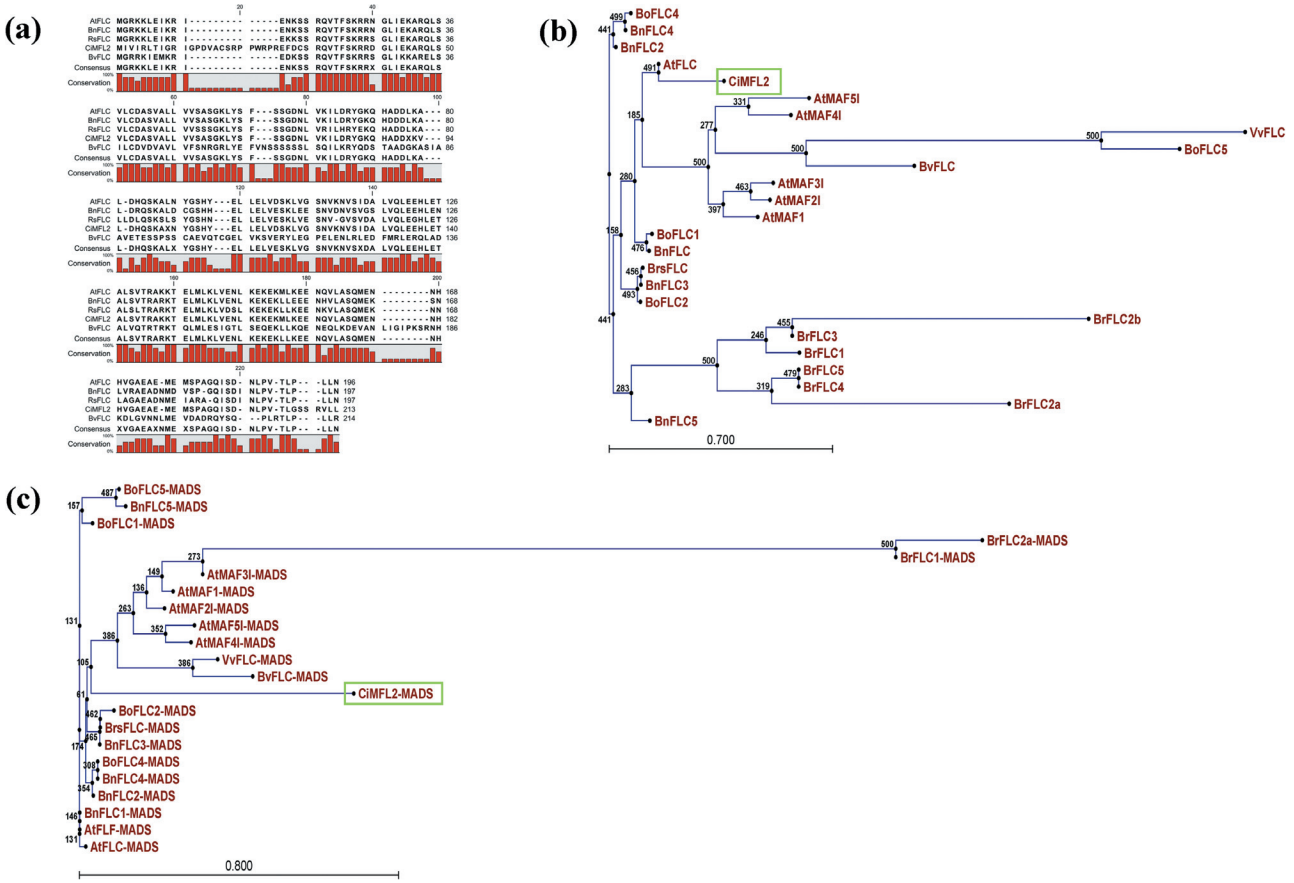

Fig. 2 Phylogenetic analysis of *Cichorium intybus MADS FLOWERING LOCUS C-LIKE 2* (*CiMFL2*). (a) Partial amino acid alignment of *FLOWERING LOCUS C* (*FLC*) sequences isolated from *Arabidopsis thaliana* var. Columbia (*AtFLC*, accession number NM_001085094), *Brassica napus* (*BnFLC*, accession number AY036888), *Raphanus sativus* (*RsFLC*, accession number AAP31676), *Cichorium intybus* var. Treviso (*CiMFL2*, accession number FJ347969) and *Beta vulgaris* (*BvFLC*, accession number EF036526). Gaps are indicated in pink, and a high degree of residual conservation by the red bar plot. The alignment was produced using the ClustalW algorithm in the CLC Sequence Viewer 4 software. A region of 14 amino acids inside the MADS domain of *CiMFL2* characterizes the *Cichorium* species. A high identity emerges between *AtFLC* and *CiMFL2*. (b) Phylogenetic analysis of the deduced amino acid sequences of *C. intybus MFL2*, *A. thaliana MADS Affecting Flowering* (*MAF*) (*AtMAF1*: AAK37527; *AtMAF2I*: AAO65307; *AtMAF3I*: AAO65310; *AtMAF4I*: AAO65315; *AtMAF5I*: AAO65320), *A. thaliana FLC* (*AtFLC*: AAD21249) and 14 *Brassica FLC* sequences (*BoFLC1*: CAJ77613; *BoFLC3*: CAJ77614; *BoFLC4*: AAQ76275; *BoFLC5*: CAJ77618; *BrFLC1*: AAO13159; *BrFLC2*: AAO86066 + AAO86067; *BrFLC3*: AAO13158; *BrFLC5*: AAO13157; *BrsFLC*: AAP31678; *BnFLC1*: AAK70215; *BnFLC2*: AAK70216; *BnFLC3*: AAK70217; *BnFLC4*: AAK70218; *BnFLC5*: AAK70219). (c) Phylogenetic analysis of the MADS-box domain of *C. intybus MFL2*, *A. thaliana MAF* (*AtMAF1*: AAK37527; *AtMAF2I*: AAO65307; *AtMAF3I*: AAO65310; *AtMAF4I*: AAO65315; *AtMAF5I*: AAO65320), *A. thaliana FLC* (*AtFLC*: AAD21249) and nine *Brassica FLC* sequences (*BoFLC1*: CAJ77613; *BoFLC4*: AAQ76275; *BoFLC5*: CAJ77618; *BrsFLC*: AAP31678; *BnFLC1*: AAK70215; *BnFLC2*: AAK70216; *BnFLC3*: AAK70217; *BnFLC4*: AAK70218; *BnFLC5*: AAK70219). *Brassica rapa FLC* sequences were not included in this alignment because the sequences in GenBank were incomplete in the MADS region. Trees were produced using a neighbour-joining algorithm; numbers at the nodes denote bootstrap support out of 500 replicates.

The presence of *MFL* in the genome of chicory was confirmed by Southern blot analysis. Genomic DNA from different cultivars of chicory and from *A. thaliana* was digested and hybridized with a *CiMFL2* cDNA probe lacking the MADS domain. Three major hybridizing fragments were detected (Supporting Information Fig. S1).

A second Southern blot hybridization was performed in which the TVT chicory genome was hybridized with a 439-bp DNA probe corresponding to a fragment of a genomic sequence comprising an intron and isolated by genome walking. At least four hybridization signals were observed.

Taken together, our results (data not shown) support the conclusion that more copies of *CiMFL* are present in the genome of *C. intybus*.

Effect of vernalization and photoperiod on *CiMFL* down-regulation

To determine whether chicory *MFLs* were down-regulated by vernalization, the pattern of *CiMFL* expression in response to vernalization was analysed using semiquantitative RT-PCR. Seedlings of TVT chicory were vernalized at 4°C under both

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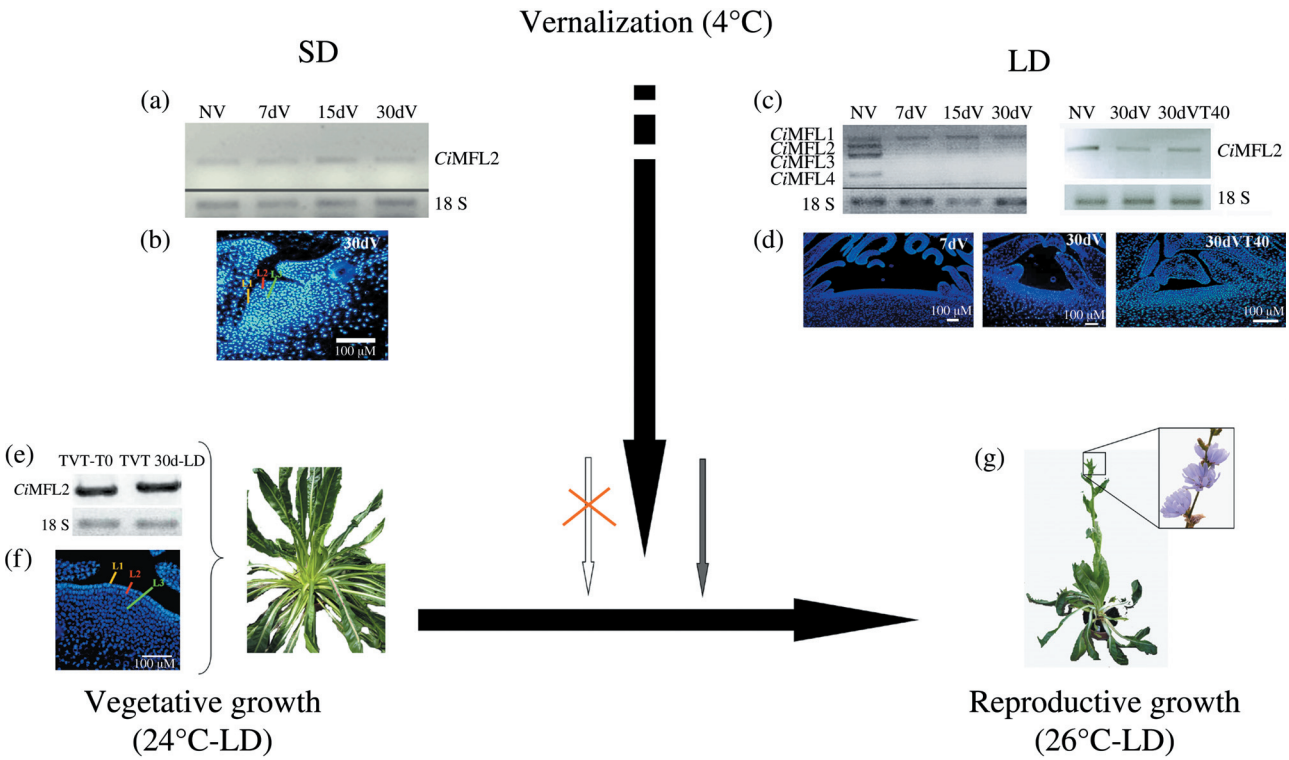

Fig. 3 Vernalization and photoperiod effects on down-regulation of *Cichorium intybus MADS FLOWERING LOCUS C-LIKE* genes (*CiMFLs*). Cold treatment was performed at 4°C under long-day (LD) and short-day (SD) photoperiods. RNA extractions were performed on leaf material after 7, 15 and 30 d of each treatment. (a, b) Seedlings of *C. intybus* cv. Treviso (TVT) were grown under an LD photoperiod and moved into a cold room at 4°C in SD conditions. RNA was isolated from leaf material after 7, 15 and 30 d of treatment. *CiMFL2* down-regulation was not appreciable; either the apical meristem maintained a vegetative appearance after shifting in LDs, demonstrating that cold treatment under an SD photoperiod is not effective in promoting flowering in chicory. (c) *CiMFL2*, *CiMFL3* and *CiMFL4* were down-regulated by low temperature in LD conditions after only 7 d. (d) Correspondingly, an effect on inflorescence meristem (IM) induction was revealed by the modification of the cell organization in the profile of the shoot apical meristem (SAM) (d-7 days of vernalization (dV)); the effect for the lost of stratification is the 'flattening' and the increase of the apex diameter. In plants vernalized for 30 d (30 dV), the stage of IM induction was advanced, as demonstrated by the change in the curvature of the meristem (d-30 dV). The effects of the vernalization treatment were maintained (T 40) after the end of the cold treatment (c-30 dV T 40) and the wrinkled surface of the apical meristem characterized the early stages of differentiation of the IM (d 30 dV T 40). *CiMFL2* was always detectable in TVT seedlings grown at 24°C under LDs until 30 d of growth (e), and the vegetative meristem showed a vegetative structure in which the L1, L2 and L3 layers were distinguishable (f).

SD and LD photoperiods for 7, 15 and 30 d. As shown in Fig. 3, under LD conditions the variants *CiMFL2*, *CiMFL3* and *CiMFL4* were down-regulated after 7 d of treatment; at the same time, *CiMFL1* maintained a constitutive level of expression. In addition, we observed that, while 7 d of treatment in LDs was enough to trigger down-regulation of *CiMFL*s, vernalization had no effects on *CiMFL* expression under the SD photoperiod and particularly did not have an effect on *CiMFL2* expression (Fig. 3, left side).

Given that vernalization under the SD photoperiod did not promote *CiMFL2* down-regulation, we tested the possibility that its regulation might be dependent on the photoperiod rather than on vernalization. To explore this hypothesis, we monitored *CiMFL2* expression by semiquantitative RT-PCR and found that, without a vernalization treatment, the LD photoperiod is not sufficient to induce *CiMFL2* down-regulation (Fig. 3, bottom).

The response of *CiMFL2* towards vernalization, strictly depends upon daylength, which stimulated our interest in the effect of this environmental factor on *CiMFL2* regulation. To investigate the possibility of a regulatory effect of photoperiod on *CiMFL2* expression, transcript levels were monitored by RT-PCR throughout the day. We found that the level of expression for this gene remained high and stable throughout the day and night (data not shown).

The data indicate that vernalization promotes down-regulation of *CiMFL*, but cold conditions exert their effect only under LD conditions. In fact, in plants vernalized under SDs, the expression level of *CiMFL* was maintained, and when the same plants were shifted to LDs they did not flower. In addition, one of the *CiMFL* transcripts analysed (*CiMFL1*) responded differently to vernalization, because it was not affected by cold induction in LD conditions either. We assume that *CiMFL1* is transcribed from a different *MFL*

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locus than *MFL2* in the *C. intybus* genome and is not regulated by vernalization.

Effect of *CiMFL* down-regulation on floral transition

To shed light on the involvement of *CiMFL* down-regulation upon cold treatment in floral transition, we investigated changes in the SAM profile during inflorescence meristem (IM) initiation. In *C. intybus* the vegetative SAM had a concave shape and its three cell layers, L1, L2 and L3, were well defined (Fig. 3f). Conversely, in a plant vernalized for 7 d, a month after the end of the cold treatment (15 d after fixation of the vernalization treatment) the SAM had a completely different profile. It was 'flattened', and its three-layer (L1, L2 and L3) structure had lost its original organization, as a thick cell layer was formed by anticlinal divisions of the meristematic cells. An increase in the meristem diameter was also observed (Fig. 3d; 7 days of vernalization (dV) and 30 dV). Similarly to *A. thaliana* and other dicots, this cytological rearrangement characterizes the early stages of IM organization. Later, differentiation of the IM was evident when the cells in the thick layer began to assume a new organization: the profile of the apex appeared superficially wrinkled, mirroring the effect of a new pattern of cell division in the inner cells of the meristem (Fig. 3d; 30 dV T 40).

SAM profiles were also analysed in plants vernalized for 40 d under the SD photoperiod, and then shifted to LDs to await flowering. DAPI staining of SAM sections demonstrated that vernalization in SDs was not sufficient to induce the formation of the IM (Fig. 3b).

To determine whether apical meristem induction was promoted by cold, we analysed the SAM profile of chicory grown for a month in LDs at 22°C. The SAM maintained its vegetative structure, indicating that an LD photoperiod cannot induce IM differentiation (data not shown).

In addition, we verified that the repression of *CiMFL2* induced by vernalization in LDs was maintained after the treatment ended and led to changes in the organization of the IM (Fig. 3c).

These results indicate that *CiMFL2* down-regulation is associated with a reorganization of SAM morphology and with IM differentiation.

Expression analysis of *CiMFL* at the transcript level

To better characterize the *MFL* sequences isolated from chicory, we analysed expression of the gene in different plant tissues and organs by RT-PCR. *CiMFL2* expression was detected in leaves, in shoot apexes and in both unpollinated and pollinated flowers (data not shown).

To determine *CiMFL* expression at the tissue level, we performed *in situ* hybridization experiments. With our probe we were not able to selectively hybridize a specific *CiMFL* variant (i.e. *CiMFL1*, *CiMFL2* or *CiMFL3*). In fact, the main differences among the sequences of *CiMFL1*, *CiMFL2* and *CiMFL3* are located in the MADS domain, which we had to remove from the probe in order to make the hybridization *MFL*-specific. Thus, from here onwards, '*CiMFL* expression' is used to refer to the general expression of a pool of multiple sequences.

CiMFL expression was detected in the embryonic apical meristem (Fig. 4a) and in the epidermal tissue of the cotyledons (Fig. 4b). In 4-wk-old nonvernalized plants the hybridization signal was detected in the leaf axils and in the adaxial surface of the leaf (Fig. 4c). Shoot apexes and leaves of vernalized plants did not show any *MFL* expression (Fig. 4d). In flower sections the hybridization signal was localized in the pollen grains (Fig. 4e,f).

CiMFL2 and CiMFL3 protein localization

The localization of CiMFL proteins was assessed using *GFP* reporter fusions with *CiMFL2* and *CiMFL3* CDS (*CiMFL2::GFP* and *CiMFL3::GFP*). We selected these two *CiMFL* cDNAs to investigate whether the deletion of 14 amino acids inside the MADS domain of CiMFL3 could be responsible for a different cellular localization of the protein or could affect translation. In transformed chicory TVT protoplasts, the CiMFL2 protein was localized in the nucleus (Fig. S2). The same localization was observed for CiMFL3 (data not shown), thus excluding a specific role for the 14 amino acid sequence insertion in protein localization.

Resetting of *CiMFL* expression after vernalization

Semiquantitative RT-PCR and *in situ* hybridization experiments showed that *CiMFL* was down-regulated by cold treatment and that after flowering the expression of *CiMFL* was up-regulated in pollen (Figs 3,4). Moreover, in Southern blot analysis we found that methylation of *CiMFL* chromatin is not involved in its silencing (data not shown).

To determine at which stages during gametogenesis *CiMFL* expression was resumed, *in situ* hybridizations were carried out on chicory flowers at different developmental stages during microsporogenesis. This allowed us to follow the expression of *CiMFL* transcripts in the anther during meiosis. In chicory pollen, ontogeny has been divided into five developmental stages: pollen mother cells (PMCs), meiocytes, tetrads, microspores and pollen (Varotto *et al.*, 1996).

In flower buds from vernalized chicory plants, the expression of *CiMFL* was detected in the anthers starting at the pollen mother cells stage (Fig. 5a,b). In particular, during pre-meiosis expression was also detected in the meiocytes as well as in the surrounding tapetum (Fig. 5c–e). In post-meiosis, *CiMFL* expression was observed in dyads, in tetrads, in the surrounding tapetum, in young microspores and in pollen (data not shown), as in nonvernalized plants (Fig. 4). This result suggests a possible resetting of *CiMFL* expression in the male reproductive structures of vernalized plants, in the sporogenous tissues before meiosis, and in the highly specialized nourishing cells of the tapetum before their degeneration.

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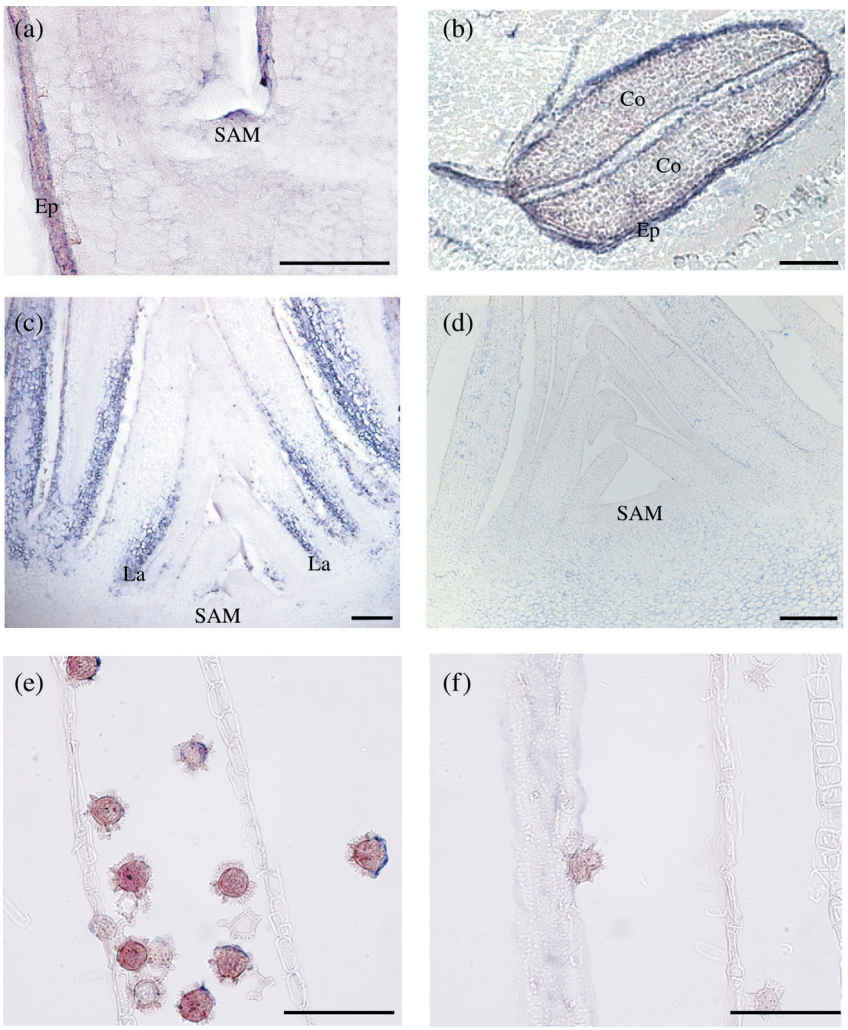

Fig. 4 *In situ* hybridization in different tissues of *Cichorium intybus* cv. Treviso (TVT) plants. The tissue sections were hybridized with an antisense RNA *C. intybus MADS FLOWERING LOCUS C-LIKE* (*CiMFL*) probe lacking the MADS domain. (a) A longitudinal section of a mature embryo. *CiMFL* was detected at significant levels in the shoot apical meristem (SAM) and on the epidermis of cotyledons (Ep). (b) A cross-section of an embryo. A strong signal was detected in the epidermal tissue (Ep) of the cotyledons (Co). (c) A longitudinal section of the SAM of a nonvernalized TVT plant. *FLOWERING LOCUS C* (*FLC*) expression was detected in leaf axils (La); no signal was detected in the SAM. (d) A longitudinal section of a shoot apex from a vernalized TVT plant. The characteristic 'flat' profile of the committed meristem is evident. No signal corresponding to *CiMFL* was detected. (e) A longitudinal section of anthers. Pollen grains showed the hybridization signal, which was absent in pollen hybridized with the sense probe (f). Bars, 100 μm.

Functional complementation of *CiMFL2* in *A. thaliana*

The *A. thaliana flc3* null mutant (Michaels & Amasino, 1999) shows an early flowering phenotype as a result of the knockdown of *FLC* expression. To assess the biological function of CiMFL2, the transcript was ectopically expressed in the *A. thaliana FLC* null mutant *flc3*. The sequence, driven by the *35S* promoter, was inserted into a binary vector containing a gene for hygromycin resistance for transgenic selection. The construct was transformed into *A. tumefaciens* strain C58i_pMD990. *Arabidopsis thaliana* plants were stably transformed using the floral dip method. Transgenic plants were selected by antibiotic resistance and analysed for the presence of the transgene by PCR, using construct-specific primers (data not shown). The *35S::CiMFL2* lines of *A. thaliana* did not show a typical late-flowering phenotype (measured by counting the total number of leaves in T1 plants; Fig. 6) although they expressed the *CiMFL2* transgene. Nonetheless, we were struck by the phenotype of the plants and particularly by the arrangement and morphology of the leaves. In fact, as shown in Fig. 6, seedlings of the transformants had unusually curled leaves, with irregular margins. Even after transplanting, transformants maintained the leaf phenotype, which was, however, lost after flower stalk emergence. This phenotype was observed in 80% of the independent transformants analysed (60 plants). The *A. thaliana* mutant transformed with the empty vector showed a regular phenotype (Fig. 6).

Using gene-specific primers for transgene amplification, mRNA expression in transgenic *A. thaliana* was confirmed by RT-PCR (data not shown). The positive transgenic lines showed the expected *CiMFL2* transcript, but while the level of expression was high in young plants, in 10-d-old nonvernalized plants transcription was drastically reduced. *CiMFL2* was detectable, albeit at a low level, in all the transformants and its expression did not explain the earliness of the phenotype. Taken together, these results indicate that *CiMFL2* is not a functional *FLC* homologue in *C. intybus.*

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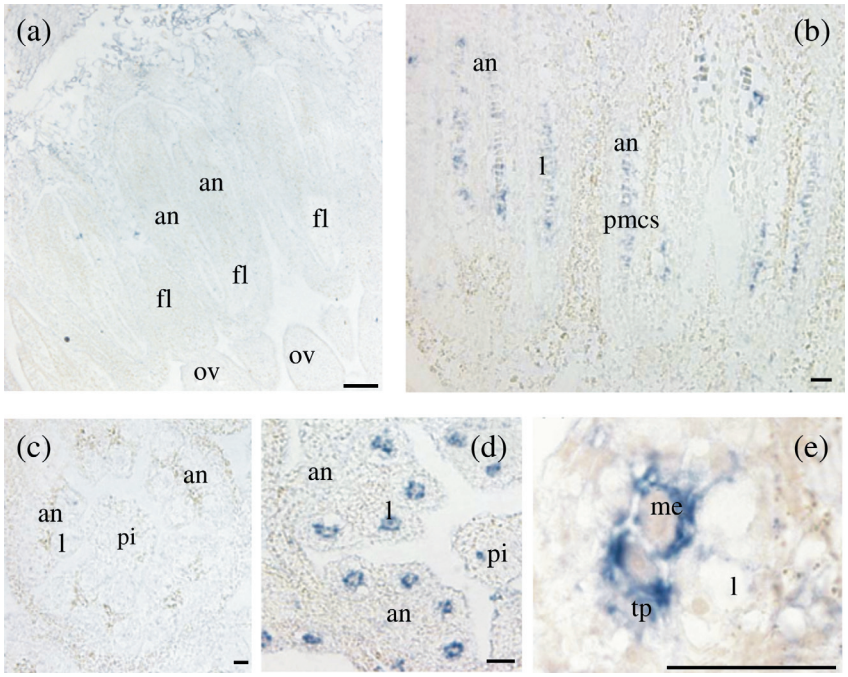

Fig. 5 *In situ* hybridization in flower tissues of *Cichorium intybus* cv. Treviso (TVT). Tissues were probed with either an antisense or a sense RNA *MADS FLOWERING LOCUS C-LIKE 2* (*CiMFL2*) probe lacking the MADS domain. (a) A longitudinal section through a floret hybridized with a sense riboprobe as a negative control, at the stage of pollen mother cells: no hybridization signal is detectable in the florets. (b) A longitudinal section through a floret hybridized with the antisense riboprobe at the stage of pollen mother cells: the signal is visible in the anther locule mainly in pollen mother cells. (c) A cross-section through a floret hybridized with a sense *CiMFL2* probe, at the meiocyte stage during microsporogenesis: no signal is detectable. (d) Cross-sections through anthers of a floret at the meiocyte stage during microsporogenesis: a hybridization signal is visible in meiocytes and in the tapetum cells surrounding the meiocytes. (e) Enlargement of the section in (d) showing evidence of a *CiMFL* signal, notably around the meiocytes and in the proximity of the tapetum cells. Bars: (a) 300 μm; (b–e) 100 μm. an, anthers; fl, floret; l, locule; me, meiocytes; ov, ovaries; pi, pistil; pmcs, pollen mother cells; tp, tapetum.

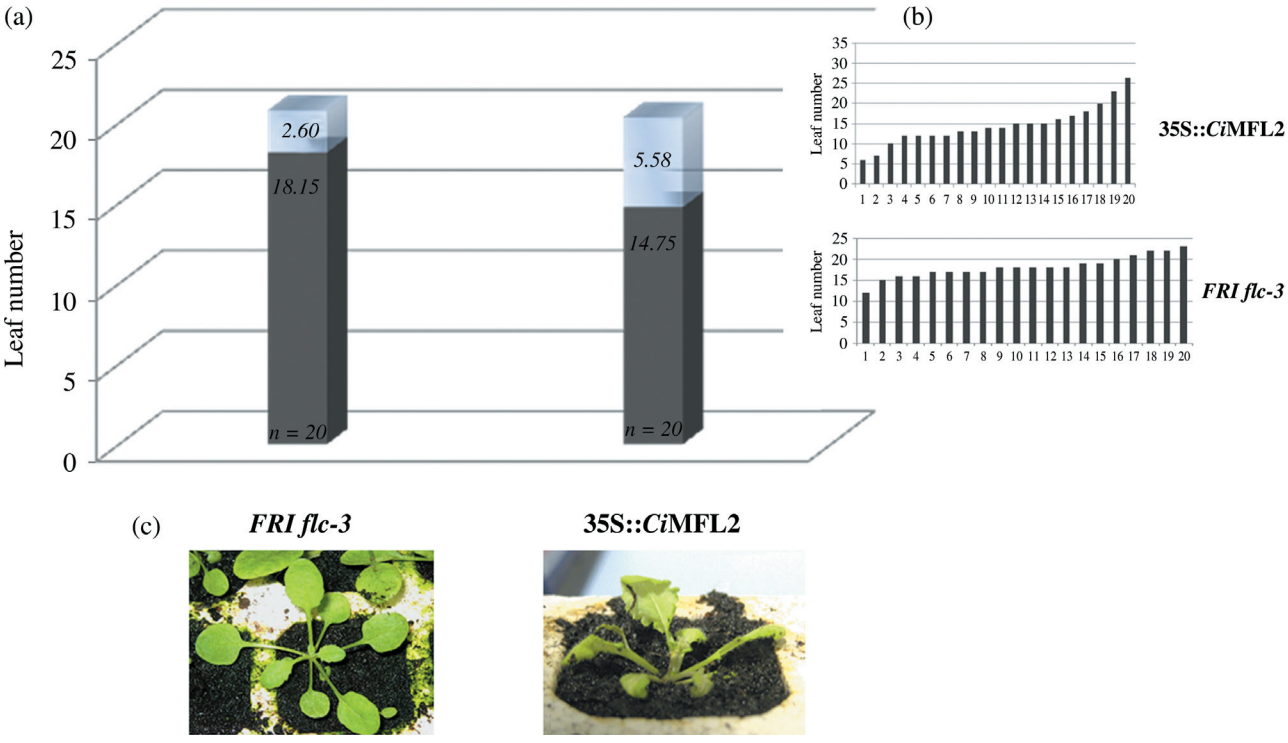

Fig. 6 Flowering time measured by leaf counting after the introduction of the *Cichorium intybus MADS FLOWERING LOCUS C-LIKE 2* (*CiMFL2*) transgene into *Arabidopsis thaliana*. (a) Expression of *CiMFL2* did not cause an increase in time to flowering with respect to the untransformed control. Light grey blocks indicate 1 standard deviation from the mean. (b) Distribution of total leaf number at flowering in individual T1 transformants. (c) Phenotype of the *35S::CiMFL2* transformant showed an altered leaf phenotype compared with the *AtFRI;flc3* null mutant.

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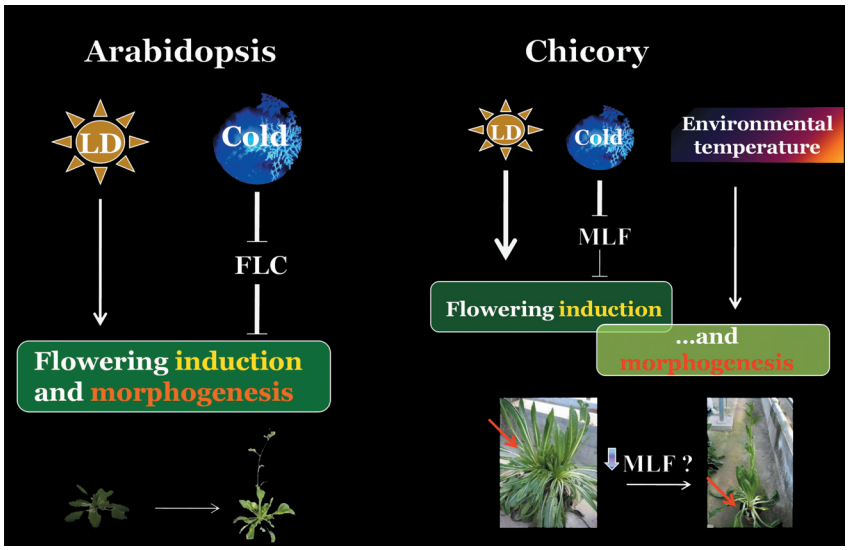

Fig. 7 Model of flowering transition in *Cichorium intybus* (chicory). In *Arabidopsis thaliana*, flowering induction and morphogenesis operate as a single step. Long days (LDs) and vernalization act synergistically to down-regulate *FLOWERING LOCUS C* (*FLC*) transcription. If *FLC* is not silenced it strongly represses the flowering transition. In chicory the processes of induction and flower morphogenesis occur in two different steps. In this model, LDs and vernalization both act on shoot apical meristem (SAM) induction and *MADS FLOWERING LOCUS C-LIKE* (*MFL*) silencing. When the environmental temperature reaches a critical value (23°C) after the induction, morphogenesis can take place. In this model, the role of *CiMFL* and its level of expression do not seem to be determinant in establishing the proper time to flowering, and thus *CiMFL* cannot be defined as the 'bolting' gene. Nevertheless, in chicory, after flowering morphogenesis a modification in the leaf habitus is observed. While in *A. thaliana* the leaves in the rosette remain unaltered throughout the vegetative phase and during flowering, in chicory a dramatic modification of the degree of opening is observed. This alteration is the same as observed in the phenotype of the *Cimfl* mutant (data not shown).

Discussion

In this work we investigated the transition to flowering mediated by *MFL* down-regulation through the vernalization pathway in *C. intybus*.

Chicory cannot be considered a model plant for molecular biology studies because of its strong auto-incompatibility mechanism which makes it difficult to obtain inbred lines (Varotto *et al.*, 1995). Moreover, its long life-cycle, its unsequenced genome and the absence of established technical procedures hamper the use of this species in successful breeding programmes. For all these reasons, we encountered several problems in our efforts to isolate and characterize the *MFL* gene.

Identification of *CiMFL* sequences

Four *CiMFL* transcripts were identified and sequenced in the heterozygous cultivar Treviso (TVT) and named *CiMFL1*, *CiMFL2*, *CiMFL3* and *CiMFL4*. Of these, *CiMFL2* and *CiMFL3* were investigated in more detail, as they were initially considered putative orthologues of the *A. thaliana FLC*. In this study, the variants *CiMFL1* and *CiMFL4* were not characterized in relation to flowering induction. In fact, *CiMFL1* does not respond to vernalization treatment and shows a divergent sequence at the 3′-end of the CDS with respect to the other *MFL* sequences isolated; in contrast, *CiMFL4* has a stop codon at the beginning of its sequence and lacks many of the conserved regions of MADS-box genes (i.e. the MADS-box, K-box and I-region).

The overlapping patterns of localization and the effectiveness of the vernalization treatment in down-regulating *CiMFL2* and *CiMFL3* supported our hypothesis that *CiMFL2* and *CiMFL3* are two expressed alleles of the same putative *CiMFL* locus (95.8% amino acid identity). A comparison between the amino acid sequence of *CiMFL2* and those of *FLC*-like genes of other species showed that there is a 14 amino acid region within the MADS domain that is unique to *C. intybus*. Three other *CiMFL*-like sequences were isolated in a wild accession of chicory (FB) and were named *CiMFLa*, *CiMFLb* and *CiMFLc*. Alignment of the variants isolated in TVT with those identified in FB revealed a high degree of amino acid identity between CiMFL2 and CiMFLa (91%), suggesting that *CiMFLa* could be the functional allele of the *MFL* locus in the FB homozygous line.

The results from the Southern hybridization suggested that more sequences of *CiMFL* are present in the genome of chicory (Fig. S1). Two inverted repeats at the 3′ end of the transcript characterize *CiMFL1* and the computational analyses showed that this variant forms a hairpin structure, which may be involved in miRNA biogenesis. Moreover, *CiMFL1* is neither down-regulated by the vernalization treatments and nor involved in the induction of flowering mediated by cold treatments. These observations suggest that *CiMFL1* might be codified by a different *MFL* genomic sequence and differently regulated at the transcriptional level from the *CiMFL2* and *CiMFL3* alleles.

CiMFL down-regulation and IM differentiation

In this study, for the first time we analysed the vernalization response of *C. intybus*, both monitoring *CiMFL* expression and observing SAM morphology during and after cold treatments. We showed that the vernalization treatment was effective in down-regulating *CiMFL2* and that the repression was stably maintained. A period of 7 d of cold treatment at 4°C, under LDs, was enough to induce full down-regulation of all *CiMFLs*,

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apart from *CiMFL1*, for which constitutive expression was maintained, demonstrating that *CiMFL* is clearly an essential component of the vernalization response (Fig. 7).

CiMFL down-regulation was associated with a change in the morphology of the SAM, which was transformed from a 'concave' profile, corresponding to a three-layer (L1, L2 and L3) organization characteristic of a vegetative meristem, to a 'flat' apex in which the three-layer organization was lost and the meristem appeared to be committed to flowering.

We observed that the LD photoperiod itself was not able to induce flowering in chicory and that *CiMFL2* expression was unaffected by the rhythms of the circadian clock.

However, vernalization treatments were completely ineffective under SDs and neither *CiMFL2* down-regulation nor changes in SAM morphology were observed. These results are apparently in contrast to those previously reported for *A. thaliana* (Lee & Amasino, 1995), *Raphanus sativus* (J.-Young Yun *et al.*, unpublished data), *Brassica napus* (Fowler *et al.*, 2001) and *Sinapis alba* (D'Aloia *et al.*, 2008). In fact, in *A. thaliana* and related species, LD conditions allowed more effective vernalization, with longer periods of vernalization being needed to down-regulate *FLC* expression under SDs.

Along with a photoperiodic effect on IM differentiation in vernalized plants, we noted a determinant effect of temperature on the differentiation of floral structures. A decrease of 2–4°C had marked consequences for the flowering behaviour of vernalized plants. Even if the apex presented all the characteristics of an IM and the photoperiod was favourable, the emergence of the floral stalk was arrested and resumed only when the temperature increased again. The critical value of the temperature for flowering was over 23°C (A. Locascio *et al.*, unpublished results). Taken together, our results for the vernalization treatments confirm that *CiMFL* is a key gene for the transition to flowering in *C. intybus*, and that both photoperiodic and vernalization pathways contribute to *CiMFL* silencing, but they also indicate that *CiMFL* down-regulation itself induces differentiation of the IM. However, this last process is not sufficient to trigger flowering. We hypothesize that there is an additional mechanism controlling floral structure differentiation after IM formation in chicory.

Given the similarity in behaviour and sequence between *AtFLC* and *CiMFL2*, also confirmed by the phylogenetic analysis, we investigated whether *CiMFL2* could rescue the null mutant *AtFRI;flc3*. *CiMFL2* was not able to rescue this null mutant. Instead, we observed a peculiar 'leaf phenotype' in all the independent transformants, and this altered phenotype was maintained until the plants flowered. Although in these plants the level of transcript was higher compared with that of *AtFLC*, they still showed an early flowering phenotype. One hypothesis is that the additional sequence present in the *CiMFL2* MADS domain impairs the formation of the MADS complex. A second hypothesis is that the transgene is recognized as a foreign sequence and a mechanism of silencing would block *CiMFL2* expression. A third possibility is that CiMFL2 is not homologous in function to AtFLC and has a new function: the regulation of leaf orientation. An irregular phenotype was also observed during *BnFLC* characterization. In particular, in transgenic lines of *B. napus* inflorescence development was disturbed and fertility slightly reduced as a result of irregular anther development (Tadege *et al.*, 2001).

Our idea that *CiMFL2* might control leaf orientation is supported by several lines of evidence. First, it did not appear to be the only determinant of flowering except under certain conditions (i.e. certain photoperiod and temperature conditions). Secondly, its pattern of expression in nonvernalized chicory adult plants was limited to the leaf axils. Thirdly, chicory plants committed to flowering drastically changed their leaf organization to allow floral stalk emergence.

To test this last hypothesis, we are currently analysing chicory mutants in which *CiMFL2* is either over-expressed or silenced. Preliminary results show that over-expression of *CiMFL2* inhibits differentiation of transformed calli, while the silenced line shows a leaf phenotype. Nevertheless, better characterization of these mutants is required.

CiMFL resetting

As *FLC* in *A. thaliana*, *CiMFL2* down-regulation is maintained after the cold treatment ended. A study on the resetting of *FLC* repression produced by vernalization in *A. thaliana* has recently been published (Sheldon *et al.*, 2008). The authors reported that both paternally and maternally derived *FLC* genes were reset in the progeny. In particular, in vernalized plants the paternal *FLC* allele was transcribed in the anthers, and also in somatic tissues and in the sporogenous PMCs, but there was no transcription in mature pollen. In chicory, during microsporogenesis, the *CiMFL* transcript was localized in the meiocytes and in the tapetum, and then in the dyads and tetrads in free microspores and in pollen grains. These results are consistent with the pattern of resetting found in *A. thaliana*.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Southern blot showing the copy number of *MADS FLOWERING LOCUS C-LIKE* (*MFL*) in the *Cichorium intybus* genome.

Fig. S2 Green fluorescent protein (GFP) expression in chicory protoplasts harbouring the CiMFL2::GFP protein fusion.

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