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Vernalization Downregulates Flowering Locus C in Cichorium intybus

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Declaration

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Locascio Antonella January 31st 2008

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Index

Index *6*

Riassunto *9*

Summary *11*

Chapter I **13**

The switch to flowering **13**

1.1 Flowering and determinancy: from vegetative to floral meristem 15

1.2 Termination of the floral meristem 19

1.3 Flower primordia formation at the SAM: geometrical aspects 20

1.4 Flower developmental stages 22

Chapter II **23**

Multiple pathways regulating the floral transition **23**

2.1 The photoperiod promotion pathway 23

2.1.1 A model of photoperiod measurement in plants 24

2.1.2 Control of flowering by circadian clock 27

2.2 The Autonomous Pathway 30

2.3 The Gibberellin Pathway 31

2.4 The Vernalization Pathway 32

2.4.1 Epigenetic modifications induced by vernalization 34

2.4.2 The “memory component” of the vernalization response 34

Chapter III **49**

3.1 The case of Beet and Monocots 49

3.2 Flowering in *Cichorium intybus* (chicory) 53

Chapter IV **57**

Materials and Methods **57**

4.1 Plant Material and treatment 57

4.2 RNA extraction and reverse transcription 57

4.3 Cloning of *Ci*FLC transcript and sequence analysis 58

4.4 DNA extraction and Polymerase Chain Reaction (PCR) 58
4.5 Semi-quantitative RT-PCR 58
4.6 Analyses of the circadian regulation of *Ci*FLC 59
4.7 Genome walking 59
4.8 PCR products purification 60
4.9 Preparations of DNA plasmid 60
4.10 Engineering of transgene to perform protoplast transformation 60
4.11 Protoplast isolation from chicory 61
4.12 Construction of transgene to perform *Arabidopsis* transformation 62
4.13 Construction of transgene to perform chicory transformation 62
4.14 Leaf discs transformation of chicory 63
4.15 Flowering time analyses 64
4.16 Southern blotting analyses 64
4.17 *In-situ* Hybridization 64
4.18 Cytological analyses of chicory apices 65
Chapter V 66
Results 66
5.1 Identification of sequences *At*FLC-like in chicory 66
5.1.1 Molecular cloning of chicory FLC transcripts 66
5.1.2 Sequence analysis of *Ci*FLCs 68
5.2 Southern Blotting analysis 76
5.3 Analysis of *Ci*FLC expression 77
5.3.1 Analysis of *Ci*FLC expression through the plant 77
5.3.2 Transient expression of *Ci*FLC2 and *Ci*FLC3 in protoplast 79
5.4.2 Analysis of DNA methylation 86
5.5 Genome walking 88
5.6 Functional analyses of *Ci*FLC2 91
5.6.1 Complementation of *At FRI flc3* by *Ci*FLC2 91
5.6.2 Over-expression of *Ci*FLC2 in chicory 95
5.6.3 Silencing of *Ci*FLC2 in chicory 95
Chapter VI *97*

Discussion ***97***
Literature cited ***106***
Vernalization and cell division at the shoot apex interact to affect flowering time in Raphanus sativus ***123***
Abstract ***123***
Introduction ***123***
Materials and methods ***127***
Vernalization treatment and flowering time analysis 127
Cloning of *Rs*VIN3 and *Rs*FLC 127
Semi-quantitative RT-PCR 128
Over-expression construct for functional analyses of *Rs*VIN3 128
Vernalization analyses by cell number counting 129
Results ***131***
Response to photoperiod and vernalization of *Raphanus sativus* 131
Cloning of full-coding cDNA of *Raphanus sativus* VIN3 and FLC 132
Analyses of *Rs*VIN3s and *Rs*FLC expression upon vernalization treatment 135
Complementation of *Arabidopsis vin3-4* by radish VIN3-1 136
Kinetics of vernalization in radish and arabidopsis 137
Cell division at the shoot apex of radish and arabidopsis 139
Discussion ***142***
Literature Cited ***146***
Appendix 1 .... ***150***
Appendix 2 .... ***161***
Posters produced and presented at congresses ***161***
Acknowledgements ***165***

Riassunto

La determinazione dell'epoca di fioritura nelle Angiosperme rappresenta un fattore critico nel ciclo vitale di ogni pianta. Negli ecotipi autunno-vernini di Arabidopsis, FLOWERING LOCUS C, fattore di trascrizione di tipo MADS-Box, è espresso ad un livello tale da inibire l'evento di fioritura attraverso la repressione di un gruppo di geni noti come Floral Pathways Integrators (FPIs). La regolazione positiva di FLC è dovuta al gene FRIGIDA (FRI), il quale determina un aumento nell' espressione del repressore, tale da inibire la transizione di fase.

La vernalizzazione è il principale processo di induzione della fioritura legato alla repressione di FLC. La durata del periodo di esposizione al freddo agisce in maniera quantitativa sull'entità di inibizione del repressore. Tale controllo è legato a modificazioni epigenetiche che includono la trimetilazione della lys27 nell'istone H3 (K27H3) e della Lys9 (K9H3). Il silenziamento è stabilmente mantenuto anche dopo l'innalzamento della temperatura a valori non vernalizzanti, tuttavia, viene perso in una fase non ancora determinata, ma localizzata durante o dopo l'evento meiotico.

La genetica comparativa ha dimostrato che i geni coinvolti nella determinazione dell'epoca di fioritura appartenenti alle pathways autonoma, fotoperiodica e legata alle Gibberelline, sono conservati tra Arabidopsis e un gran numero di specie coltivate, compresi legumi e cereali. Al contrario, il meccanismo di regolazione della fioritura legato alla vernalizzazione sembra solo parzialmente mantenuto, dal momento che FLC e FRI sono stati finora identificati solo nelle Brassicaceae e, recentemente, in barbabietola da zucchero, vite e pomodoro.

Il radicchio (Cichorium intybus) è una specie biennale che richiede un periodo di vernalizzazione perché l'evento di fioritura venga evocato. La forte spinta selettiva operata dall'uomo nell'ultimo cinquantennio, ha portato alla definizione di numerosi tipi di radicchio, tra cui i radicchi rossi e variegati veneti, che oltre a mostrare una notevole varietà fenotipica, differiscono nei riguardi della classe di precocità in relazione all'epoca di fioritura e conseguentemente in relazione al periodo di raccolta del prodotto commerciale. La produttività del radicchio è legata alla produzione di foglie, pertanto l'induzione della fioritura è un evento indesiderato. Data la notevole eterogeneità nei riguardi della transizione di fase, mostrata anche tra individui appartenenti ad una stessa varietà, le attuali tecniche agronomiche incontrano notevoli difficoltà nel "controllo" di tale evento. Lo studio molecolare dell'evento di fioritura in radicchio, potrebbe essere pertanto la soluzione per definire le variabili che determinano la transizione di fase e che quindi, se opportunamente modulate, possono garantire un prolungamento della fase vegetativa della pianta.

Il programma di ricerca ha avuto lo scopo di comprendere quale fosse in radicchio il meccanismo alla base dell'induzione della fioritura mediato dalla vernalizzazione, per verificare se tale meccanismo fosse comparabile con quello identificato in arabidopsis ed eventualmente, associare la diversa classe di precocità di fioritura presente tra le varietà di radicchio ad uno dei casi noti per la pianta modello.

Sono state isolate sequenze proteiche omologhe ad FLC di arabidopsis e ne è stata saggiata l'espressione nei diversi tessuti della pianta. E' stato studiato il pattern di

metilazione a livello del DNA genomico in risposta alla vernalizzazione ed in parallelo è stata saggiata la regolazione del trascritto mediante RT-PCR. Il calo nell'espressione del trascritto di FLC legato all'evento di vernalizzazione è stato relazionato ad un cambiamento nella morfologia del SAM.
L'identificazione di tratti di sequenza genomica è stata ottenuta tramite chromosome walking, ma la sequenza genica completa non è stata ancora isolata.
I dati sinora ottenuti indicano che tra radicchio e arabidopsis esistono omologie sul meccanismo di regolazione dell'espressione di FLC in seguito al processo di vernalizzazione. Tuttavia, la trasformazione del mutante nullo di arabidopsis, At FRI flc-3, con la sequenza codificante per FLC di radicchio, non ha portato al risultato atteso di recupero del fenotipo normale. Questo risultato è in disaccordo con un'ipotesi di conservazione funzionale tra FLC di radicchio e di arabidopsis. La costruzione di una linea mutante di silenziamento genico e una di sovra-espressione di FLC, permetterà di meglio definire il ruolo biologico di FLC in radicchio. Lo studio della sequenza genica, comprensiva delle regioni regolatrici promotoriali, consentirà inoltre, di chiarire il meccanismo di regolazione del trascritto di FLC. Data la sequenza genica, sarà poi possibile effettuare esperimenti di ibridazione in-situ sui cromosomi di radicchio (FISH) in modo da definire il numero di copie del gene ed eventualmente localizzarne la posizione sul cromosoma. Questo studio sarà utile per fornire ipotesi sull'evoluzione del gene.

Summary

Since proper timing of flowering is critical for the survival of plant species, plants have evolved a complex genetic network to regulate their transition to flowering in response to endogenous signals and environmental cues. In winter annuals ecotypes of Arabidopsis, a flowering repressor, FLOWERING LOCUS C (FLC), a MADS box transcription factor, is expressed at such level as to inhibit flowering in the first growing season. FLC expression is enhanced by FRIGIDA (FRI) to levels that inhibit the transition to flowering by repressing the expression of the genes often referred to as Floral Pathways Integrators.
The main process promoting flowering by the repression of FLC is the vernalization and the duration of cold has been shown to be proportional to the degree of down-regulation of FLC; such repression is maintained for the rest of the plant life even after cold exposure ends, but is restored after meiosis. The repression involves epigenetically stable modifications in FLC chromatin that include a H3 Lys27 trimethylation (H3K27me3) and a H3 Lys9 trimethylation, (Sung et al, 2006).
Interestingly, for the light-dependent, autonomous and GA integration and meristematic pathways, comparative genetic approaches show that flowering time genes are conserved between Arabidopsis and a large range of crop species, including legumes and cereals. By contrast, the vernalization pathway seems to be only partially conserved, since FLC and FRI were not characterized in dicots other than Brassicaceae, and recently in sugar beet, vitis and tomato.
Wild chicory (Cichorium intybus L.) is a biennial species which requires vernalization to flower. In Italy different types of chicory (the so called Italian red and variegate types) have been selected by farmers as leafy vegetable. These types show quite different classes of precocity in relation to flowering. Given the high heterogeneity, in regard to flowering, manifested by plants belonging to the same variety, the "control" of the switch by agronomical procedures results difficult. The knowledge about the genetic control of flowering time in chicory could be useful to enhance the vegetative phase and then, increase the productivity of the crop.
In our study, we are investigating the molecular basis that regulate the switch to flower in chicory by vernalization, to verify whether such mechanism is the same that controls flowering in Arabidopsis, and, finally, to address the diversity of the classes of precocity to one of the cases known for this model plant. We isolated FLC homologues from chicory and characterized their expression patterns in plant tissues and in response to vernalization. We also studied the pattern of cytosine methylation in chicory genomic DNA in response to vernalization. In addition, the vernalization-mediated decrease of FLC transcript was related with changes in SAM morphology. Biological function of CiFLC has been studied by AtFRIflc3 complementation. Up to now our result indicate that arabidopsis and chicory share homologies in regulating FLC expression in the vernalization response, but the absence of complementation of the mutant suggest a disagree in biological function of CiFLC or a loss of function of the transgene in Arabidopsis genetic background. Further analysis will be conducted to define if the machinery in FLC regulation and its biological function is shared between the two species. For this purpose, chicory mutants will be generated. Other aim of this work has been the identification of FLC genomic sequences in chicory. For this purpose, genome walking technique was used. Knowledge of the genomic

sequence of CiFLC will allow comparing the regulative regions with those of AtFLC and performing experiments of chromosome hybridization (i.e. FISH). The goal is identify the number of copies of the gene and characterize its position within the chromosomes. With these results we will be able to formulate hypothesis about the evolution of FLC in Cichorium intybus.

Chapter I

The switch to flowering

The timing of the transition to flowering is determined by the interaction of the endogenous developmental competence of a plant with environmental cues that signal the onset of conditions favourable for reproductive success.

Recent progress in the dissection of these diverse influences and the molecular events that regulate them has been achieved through a molecular genetic approach in the model plant *Arabidopsis thaliana* and more recently in a few other species.

To achieve reproductive success, plants must select the most favourable season to initiate reproductive development. This selection requires the existence of molecular mechanisms to continuously monitor environmental factors and to properly adapt the response to different conditions.

The environmental signals include photoperiod, light quality, and temperature; all of them regulate the correct timing to flowering (Mouradov et al. 2002).

These cues vary geographically, within local microenvironments, and seasonally in a predictable fashion along the year. *Arabidopsis* flowering is accelerated as the daylength increases, in fact this condition signals the beginning of spring and summer. This process is known as *Photoperiodic response*. *Arabidopsis* flowering is also accelerated by an extensive period of cold treatment, an environmental condition that signals the passage of winter. This process is known as *Vernalization response*. In addition, flowering is also promoted in response to stress such as overcrowding (perceived as changed light quality input), nutrient deficiency, heat and drought. Besides, endogenous signals regulate the floral transition (i.e. age, hormones, acquisition of the competence) (Simpson et al. 1999), fig.1.

Plants are able to perceive all these environmental variations and modulate their growth and development with responses that can be in the short terms (such as growth response to

ambient temperature) or in long terms (i.e. flowering response to vernalization), (Gordon et al 2002).

The complexity of responses can change in relation to the physiology of the plant and, in this case, we can distinguish between plants that complete development within a year, called *annual* plants and plant that complete its life cycle in more than a year, known as *biennals* or *perennials*. The annuals usually flower only once in their life cycle and flower initiation is immediately followed by flowers development. *Arabidopsis* is an annual species.

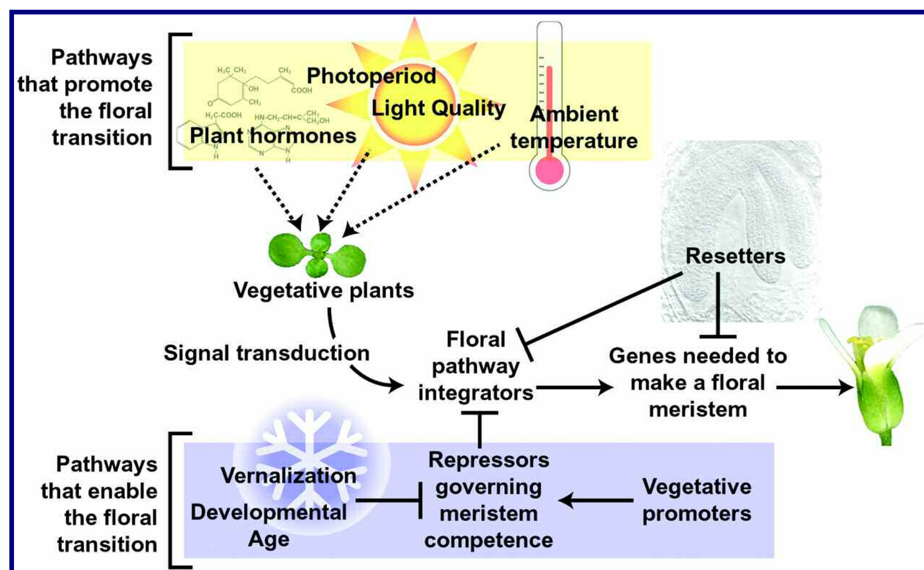


Fig.1 Schematic representation of the pathways interfering with determining of proper timing to flower in *Arabidopsis*. The pathways can be dissected in *enabling* the floral transition, laid at endogenous stimuli and *promoting* pathways, mostly connected with environmental cues. Each pathway determines genetic modifications at determinated locus, resulting in activation/repression of genes, with the final goal to obtain the reproductive transition. All these epigenetic modifications are resetted in a phase not well characterized but probably placed between meiosis and embryogenesis.

1.1 Flowering and determinancy: from vegetative to floral meristem

After its initiation during embryogenesis, the SAM begins a maturation pathway that starts in the juvenile vegetative phase. This is characterized by the production of leaf primordia with a pattern of differentiation distinct from those produced in the adult phase (Simpson et al. 1999). Juvenile leaves produce epidermal hairs (trichomes) in the adaxial surfaces, whereas adult leaves produces trichomes on both their upper and lower surface. The conversion from juvenile to adult phase is important because only the adult vegetative meristem is competent to respond to floral induction (Weigel & Nilsson 1995) and thus, regulating this transition affects the following reproductive transition.

The decision to flower triggers a new developmental program that ends with the formation of reproductive structures. In plants, most development occurs postembryonically through the continuous production of stem cells at the shoot and root apical meristems. Leaf and flower primordia emerge from the flank of the shoot apical meristems (SAM), and the transition to floral response involves a series of changes in the physiology of the plant.

In *Arabidopsis*, such as in many other plants, these changes include an acceleration of cell division at the apical meristem, elongation of the stem and at the end, formation of the flowers at the flanks of the SAM. Thus, the acquisition of floral identity by a meristem is only a subprogram of reproductive development (Blazquez et al. 2006).

The SAM functions as the main sources of new cells to sustain plant growth. The regular recruitment of meristem cells to form new organs and tissues is balanced by cell proliferation within the meristem to maintain its size relatively stable. In many cases the meristem is genetically programmed to stop producing new cells, in this case the meristem is called *determinate*. The result of a determinate meristem is an organ, such as a flower, with predictable size and form. In contrast, an *indeterminate* meristems produce part of the plant whose size and shape depend on the local environment, such as branches and roots that grow to variable lengths. The positioning of determinate and indeterminate meristems varies between species and is a major determinant of plant architecture (Sablowski 2007).

The indeterminate growth of the vegetative meristem is sustained by a small group of self renewing cells. These cells are located in the central zone (CZ) of the meristem, while some of the descendent are positioned to the peripheral zone (PZ), where they are recruited to

constitute new organ primordial. Below the CZ, the rib meristem (RM) sustains the stem growth.

The typical tunica/corpus structure found in *Angiosperms* can also be distinguished in two external layers, L1 and L2, in which the cell divisions are oriented tangentially to the meristem surface, while under the L2 layer the cell divisions not have a clearly oriented direction, this area is named L3, fig.2.

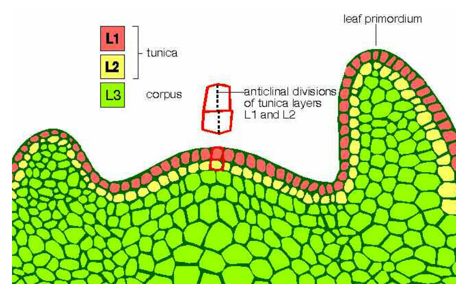


Fig.2 Representation of the apex profile. Three cell layers are distinguishable on the basis of the cell division orientation. From each layer a particular organ will develop. The epidermal tissues are generated from L1 layer, the reproductive organs are generated from the L2 layer; last, vases as xylem and phloem, are generated from the L3 layer.

SHOOT MERISTEMLESS (STM) and WUSCHEL (WUS) are two regulatory genes with central role in shoot meristem development. Both genes act sinergically in maintaining the vegetative phase of the SAM, even if WUS has a more prominent role in developmental control of meristem size and stability. In particular STM is required to delay differentiation of cells to bulk up before the recruitment into organogenesis; while WUS is required to specify the stem cells in the CZ. The domain of expression of these genes is well defined: WUS is expressed only in few cells of the L3 layer, STM is expressed throughout the meristem. In order to maintain the dominion of WUS expression, occurs a fine mechanisms which involves the CLAVATA signalling pathway (Carls & Fletcher, 2003), together with ULTRAPETALA, CORONA, PHABULOSA and PHAVOLUTA genes (Prigge et al 2005), fig.3.

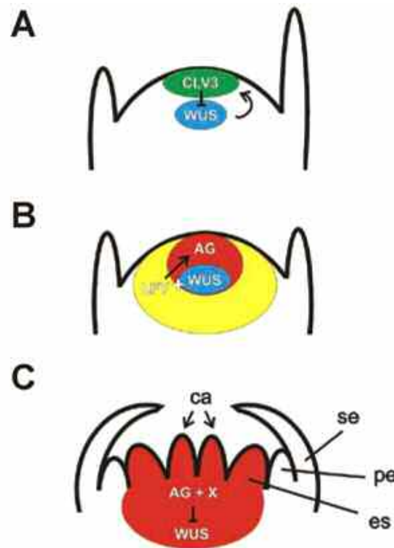


Fig.3 Molecular model of the feedback loop regulating stem cell determination in *Arabidopsis*. **A:** an indeterminate vegetative apical meristem, showing the interaction between CLV3 and WUS in their respective domain. LFY is absent in the vegetative meristem and AG expression is not induced. **B**: an early floral meristem, LFY begins to be expressed and together with WUS induces AG transcription. **C**: Carpel initiation. AG and an additional unidentified factor (X) repress WUS expression to terminate cell activity and make the meristem determinate.

The suppression of indeterminate growth of the SAM depends on floral-specific regulatory genes. During the transition, the vegetative meristem is initially converted into inflorescence meristem, which will produce floral meristems on its flanks.

The multiple environmental and endogenous signal that regulate the floral transition, converge into key regulators of floral meristem identity: APETALA1 (AP1)/CAULIFLOWER (CAL) and LEAFY (LFY) (Komeda 2004). These genes antagonize the activity of the shoot identity gene TERMINAL FLOWER 1 (TFL1), to establish floral meristems. TFL1 functions to indirectly delay the upregulation of LFY and AP1 (Parcy 2002).

AP1 and CAL encode MADS-domain transcription factors that are necessary and sufficient for the transition from inflorescence to floral meristem. AP1 is specifically expressed in young floral meristem, marking the start of flower development (Mandel et al. 1992). Overexpression of AP1 is sufficient to convert inflorescence meristem into a terminal flower (Mandel & Yanofsky 1995).

AP1 is activated by LFY and FLOWERING LOCUS T (FT) in complex with FLOWERING LOCUS D (FD) (Ruiz-Garcia et al. 1997). It plays a dual role: promotes the transcription of genes to activate the floral meristem development; suppresses the genes

required for the control of flowering time (SVP, AGL24 and SOC1), to ensure the specification of the meristem. AP1 is strongly expressed in emerging floral meristems and perianth organ primordia of floral meristem. Recently has been discovered its role as repressor of AGL4 and SOC1 in early stage of emerging floral meristems, whereas SVP in repressed later, at the stage 3 of floral meristem development. AP1 binds directly the CArGbox of SVP, AGL24 and SOC1 promoter; the different threshold levels of AP1 would discriminate for the target gene to repress. The repression of these genes ensures that reversion of floral meristems into shoot meristems will be avoided (Liu et al. 2007).

LFY encodes a transcription factor that is required for the first division of the embryo and in Gymnosperms and Angiosperms, is associated with the development of reproductive structures and is a strong regulator that specifies floral identity and thus, promotes determinancy (Tanahashi et al. 2005; Weigel et al 1992).

LEAFY has been shown to bind specific sequences present in the regulatory regions of the homeotic gene AP1 (Parcy et al. 1998), AP3 (Lamb et al 2002) and AGAMOUS (AG) (Busch et al 1999).

LEAFY protein is expressed uniformly in early floral bud, and this uniform expression results form the uniform expression of LEAFY promoter (Blazquez et al 1997), or probably from the ability of the protein to move between the cells.

LEAFY is activated by FT/FD besides being activated by gibberellin, which also functions as a signal to promote the shift to reproductive development (Blazquez et al 1998).

To maintain the indeterminate inflorescence meristem, AP1/CAL and LFY must be activated only in the floral primordia. The expression of LFY and AP1/CAL in the inflorescence meristem is prevented by TERMINAL FLOWER (TFL) which encodes a homologue of FT, but has the opposite function (Kobayashi et al 1999).

The interactions between FT, AP1/CAL, LFY and TFL ensure a sharp and stable transition to floral identity. After the FT/FD activation, LFY and AP1/CAL reinforce the expression of each other and together then activate the floral development programme.

1.2 Termination of the floral meristem

Termination of the meristem is part of the flower development programme set in motion by AP1 and LFY.

After being inducted at the flanks of the inflorescence meristem, the floral meristem differentiates four whorls of organs: sepals, petals, stamens and carpels. The identity of each type of organ is determinated by a specific combination of MADS-domain proteins according to the recent model A-B-C-D-E (fig.4) that has already replaced the old ABC model.

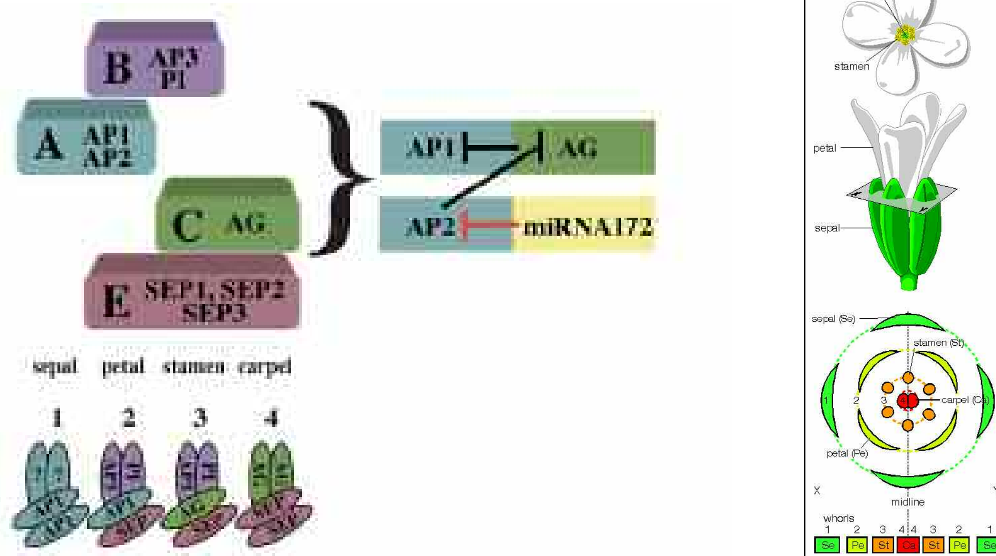


Fig. 4. Revised ABC model of flower development. A, B, C and E are four activities present in adjacent whorls of the flower. These activities function combinatorially to specify the identity of the four organs in the flower: sepals, petals, stamens and carpels. The majority of ABC genes code for MADS proteins. Recent evidences suggest that all the MADS proteins function together in complexes. Tetramers of MADS proteins specify floral organ identity (colored ovals). Interactions demonstrated to be direct are indicated in red (Jack 2004).

One of these MADS-box protein is AP1, which after its earlier role in specifying floral identity, participates in the development of the perianth organs (sepals and petals). LFY after the floral transition activates genes encoding MADS-box proteins required for stamen and carpel development (i.e. APETALA3, PISTILLATA, AGAMOUS).

AGAMOUS has the prominent role in terminate the meristem, doing so antagonize the function of meristem maintenance of genes such as STM or WUS. In fact, it has been proved that WUS expression is decreased after AG expression is activated and it disappears by the time carpel primordial are initiated (Mayer et al. 1998). Interestingly, WUS participates at the AG activation together with LFY, thus AG functions in a negative feedback loop that terminates WUS expression and meristem activity in the floral bud. Among the genes activated by AG, *GA4* whose protein catalyzes the final step in the biosynthesis of bioactive gibberellin. Because the gibberellin would antagonize meristem activity, a localized increase in gibberellins levels might mediate the meristem termination (Shani et al. 2006).

Other genes are known to promote floral determinancy. One of them is SUPERMAN, which limits stamen number and controls cell proliferation in the centre of the floral meristem (Sakai et al. 1995) and URL that limits the number of organs as well.

1.3 Flower primordia formation at the SAM: geometrical aspects

A fundamental process in shoot morphogenesis is the formation of lateral organs, such as leaves and flowers, which takes place at the shoot apical meristem (SAM).

Shape and size changes in the course of leaf or flower formation are directional and growth of these organs is determinate (Lyndon & Battey 1985). Thus, early in lateral organ formation, the SAM surface must be divided into two portions of different fate and growth (determinate vs. indeterminate), which is the process named *SAM surface partitioning* (Lyndon 1998). In the course of partitioning, a portion of SAM periphery becomes the lateral organ primordial, while the remaining portion maintains the SAM character. In plant with an apical inflorescence the switch from the vegetative to reproductive phase of development is often accompanied by a change in lateral organ identity from leaves to bracts or flowers. In *Arabidopsis,* it is known that the first periclinal divisions are in different tunica layers accordingly to the primordium identity (L1 if leaf, L2 if flower) (Vaughan 1955).

With the aid of curvature computation and electron microscopy, it is possible to study the changes in geometry of the SAM in the course of early flower primordium development.

For *Arabidopsis* has been observed that the flower primordium is preceded by the formation of a shallow crease, followed by a bulge to end in the floral organs specification. During the stage from the shallow crease to the bulge, the large adaxial portion of the shallow crease changes into a bulge, the remaining smaller abaxial portion retains the shallow crease shape. This abaxial crease however does not persist and is soon incorporated into the bulge. When bulging occurs in the shallow crease, adjacent SAM cells form one more crease. Such a developmental sequence could be interpreted in the way shown in fig.5.

The bulge is the floral primordium proper, formed at the bottom of the putative bract axil. The second axil would be the axil of the flower primordium, formed at the boundary between the flower primordium and the SAM (Dorota 2006).

A similar sequence of events has been proposed by Long and Barton, who based their interpretation on gene expression pattern. The stage 0 corresponds to the appearance of STM expression on the meristem flanks.

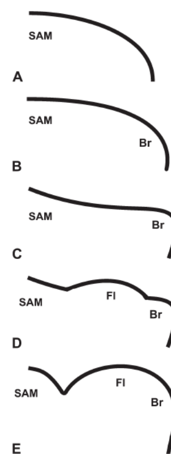


Fig. 5. Proposed interpretation of consecutive stages in early flower primordium development in *Arabidopsis*. Outlines of shoot apex periphery are shown as they would appear in median longitudinal sections, with the apex centre on the left. The curvature of the outlines represents the median curvature of the apex surface. The stage of initial bulging leading to the shallow crease and putative bract (Br) formation (A–C) is followed by bulging in the shallow crease when the flower primordium proper (Fl) develops (D). Two creases are shown in (D), the remnants of the first formed axil are visible on the right side of the floral primordium while the second formed axil is on its left. The abaxial crease flattens out during consecutive stage (bulge stage shown in E.

1.4 Flower developmental stages

Flower development has been divided into 12 stages using a series of landmark events (fig. 6). Stage 1 begins with the initiation of a floral buttress on the flank of the apical meristem. Stage 2 commences when the flower primordium becomes separate from the meristem. At the stage 3 sepal primordial arise and grow to overlie the primordium (stage 4). Petal and stamen primordial appear next at the stage 5, and are soon enclosed by the sepals (stage6). During stage 6, petals primordial grow slowly, whereas stamen primordial enlarges more rapidly. Stage 7 begins when the medial stamens become stalked. These soon develop locules, this characterizes the stage 8. A long stage 9 begins with the petal primordial becoming stalked and during this stage all organ lengthen rapidly. This includes the gynoecium, which commences growth as an open-ended tube during stage 6. When the petals reach the length of the lateral stamens, stage 10 commences. Stigmatic papillae appear soon after (stage11), and the petals rapidly reach the height of the medial stamens (stage 12). This final stage ends when the 1-mm long bud opens (Smyth et al. 1990).

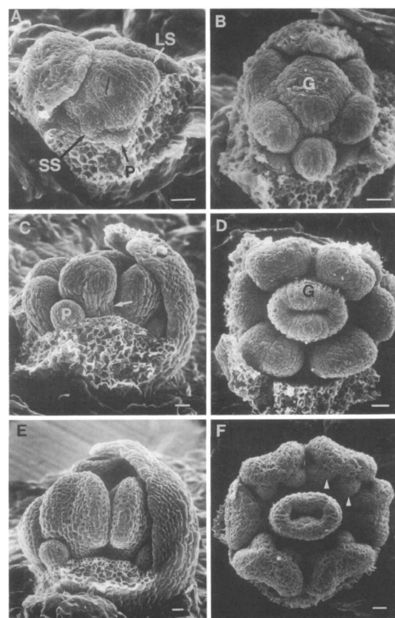


Fig. 6 A-F Individual Buds at Stages 5 to 8. **(A)** A bud at stage 5. One of the small petal primordia (P) is indicated. The primordia of the long stamens (LS) are larger than that of the short stamen *(SS).* **(B)** Lateral view of a bud at stage ***6***. The stamen primordia are now dome shaped, whereas the petal primordia are still relatively small. The gynoecium (G) will arise from the central dome of cells. **(C)** Media1 view of a bud at stage 7 showing that the long stamen primordia are now constricted toward their base (arrow). The petal primordia (P) have become dome shaped. **(D)** Vertical view **of** a stage-7 bud showing that the stamens do not yet show locule ridges on their adaxial surface. The gynoecium (G) is growing vertically as a slotted tube. **(E) A** bud at stage 8 in which the stamen primordia have increased markedly in size, especially in relation to the petal primordia. **(F)** Vertical view of a stage-8 bud in which locules (arrows) are now clearly visible in the stamens. Bars = 10 µ*m*.

Chapter II

Multiple pathways regulating the floral transition

Genes affecting flowering time in *Arabidopsis* have been identified through two complementary strategies. The first analyzed the variation present in the natural ecotypes of *Arabidopsis* that vary in flowering time. The second approach used a diverse spectrum of induced mutations that result in early or late flowering behaviour. The results of these studies are consistent with a model in which several pathways regulate the expression of a few key genes known as flowering signal integrators whose main function is to regulate the expression of genes specifying flower meristem identity (Kardailsky et al 1999).

Five pathways partially independent have been described: the photoperiodic pathway, the autonomous pathway, the vernalization pathway, the light quality pathway and the gibberellin pathway.

2.1 The photoperiod promotion pathway

The timing to flower has an important impact on reproductive success. One of the most important environmental cues affecting the transition is the change in day length (photoperiod).

On the basis of the photoperiodism (response or capacity to respond to photoperiod) is possible identify these categories of plants: long-day (LD) plants, in which flowering occurs when the day becomes longer than a crucial length; short-day (SD) plants, in which flowering occurs when days become short and day-neutral (DN) in which flowering is not regulated by photoperiod (Salisbury 1985).

Environmental cues are sensed by the leaves while the response occurs mostly at the apex, requiring a long-range communication within the plant.

In the 1930s, based on grafting experiments, Mikhail Chailakhyan postulated the existence for a "florigen" signal that should be produced in the leaf and than relocated to the apex (Chailakhyan 1968).

Much effort has been made toward the characterization of the "florigen", and to date only few evidences are gravitating around FT (Jaeger & Wigge 2007; Corbesier, et al 2007; Mathieu et al.2007).

Within the past few years, crucial findings have brought new insight into the molecular mechanism of photoperiodic flowering, and there have been several recent breakthroughs in the study of photoperiodism.

2.1.1 A model of photoperiod measurement in plants

Several models have been proposed to explain how the photoperiodic information is perceived by the plant and the integrated into a developmental process. The most famous of these models is the "external coincidence model", proposed by Erwin Bunning in 1936 (fig. 7). In this model light plays two crucial roles. One is resetting the circadian clock, which is important for generating the daily oscillation of a key regulator. The second is regulating the activity of this component.

Photoperiodic responses will be triggered only when regulator levels overcome a threshold in coincidence with daylight, which constitute the external signal (Imazumi & Kay 2006).

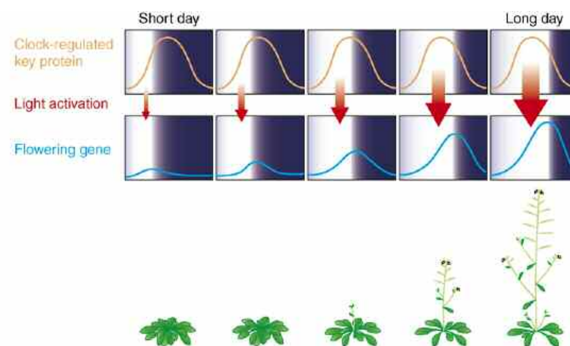


Fig. 7 The external coincidence model: an example of the photoperiodic flowering response in long-day (LD) plants. The function of the clock-regulated key regulator, which induces the expression of the flowering gene, is regulated by light, therefore, flowering will be accelerated when the late-afternoon expression of the key regulator and the presence of daylight coincide.

Circadian rhythms, as leaf movement and floral opening, are governed by an internal timekeeper, referred to as the circadian clock.
The clock includes a network of transcription factors arranged in interlocking negative-feedback loops (Lakin-Thomas 2000). The clock maintains an internal estimate of the passage of the time and schedules physiological processes to occur at appropriate time of the day. In order to remain synchronized with the environment, the circadian clock is reset or entrained by specific cues the relay information about the external time. These cues comprise: light-dark cycle, temperature and nutrient availability (Roenneberg & Merrow 1998).
A model to describe the principles of clock architecture, considers an input pathway entraining a core oscillator which generates rhythmic outputs. Many components of the input pathway are themselves outputs of the clock and the rhythmic output from the clock may feedback to affect the functioning of the core oscillator (Harmer et al. 2001).
In addition evidences suggest that each cell of the plant has a core circadian oscillator located in (Webb 1998).
Resetting of the clock involves a change in phase that does not alter the internal sequence of processes, but re-aligns that sequence with the daily environmental progression (Millar 2004). This mechanism allows the organism to adjust toward changing daylength and time of dawn during seasonal transitions (Devlin 2002).
Light signals are perceived by phytochrome (PHYA to PHYE) red and far-red light photoreceptors and cryptochrome (CRY1, CRY2) blue-light photoreceptors. PHYs and CRYs exhibit circadian oscillations at mRNA level, and this may contribute to rhythmic sensitivity of the clock to light in concert with other factors, such as EARLY FLOWERING 3 (ELF3) (Somers et al. 1998).
ELF3 acts to antagonize light input into the clock during the night and also contribute to resetting of the oscillator (McWatters et al. 2000). ZEITLUPE (ZTL) and LOV/KELCH PROTEIN 2 (LKP2) could be additional mediator of light input. In particular ZTL would be a blue-light receptor (Salomè & McClung 2005).
The circadian clock can also be entrained by temperature cycles in which the day and night temperature differ by 2-4°C (Balasubramanian et al. 2006). The molecular basis of the temperature entrainment of the clock is not well understood (Devlin 2002). Nevertheless, it

has been demonstrated that growth temperature can also affects the flowering induction by influencing the expression of a strong flowering-promoter FLOWERING LOCUS T (Blazquez et al 2003).

The central oscillator in *Arabidopsis* is proposed to consist of elements arranged in interlocking transcriptional loops (Hayama & Coupland 2003).
A mathematical analysis based on a previous existing model, has indicated a simplified mechanism by which the core oscillator would work (Locke et al .2005).
The core oscillators consist of morning factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and evening factors, TIMING OF CAB EXPRESSION1 (TOC1), EARLY FLOWERING 4 (ELF4) and LUX ARRHYTHMO (LUX) (Alabadì et al 2001; Wang & Tobin 1998; Strayer et al 2000).
LHY and CCA1 are enhanced by TOC1, they show peak of expression rhythmic with the maximum raised at the dawn.
In the mathematical model, the light activates a hypothetical factor Y, which induces the expression of TOC1. TOC1 acts via another hypothetical component X to induce LHY expression and LHY and TOC1 both act to repress the expression of Y. The identity of Y has not been determinated, but experimental data suggest that its function might be fulfilled by GIGANTEA (GI), (Mizoguchi et al 2005).
Other proteins that could be considerate part of the core oscillator are the PRRs. The members of this family are expressed from the dawn to the dusk in sequence PRR9-PRR7-PRR5-PRR3-TOC1, (Mizuno & Nakamichi 2005). CCA1 regulates the expression of PRR7and PRR9, by directly binding their promoter. The PRRs seems to feedback to regulate CCA1 and LHY expression, (Farrè et al 2005). ELF4 and LUX are proposed as component of the core oscillator only recently.
LUX is a MYB-factor co-ordinately expressed with TOC1 and is repressed by CCA1 and LHY. ELF4 is required to maintain the rhythmic outputs and for light induced activation of CCA1 and LHY. ELF4 is inhibited by CCA1 and LHY, indicating another loop in the core oscillator (Hazen et al 2005; Doyle et al. 2002). All the factors citated are represented in fig.8, (Gardner et al 2006).

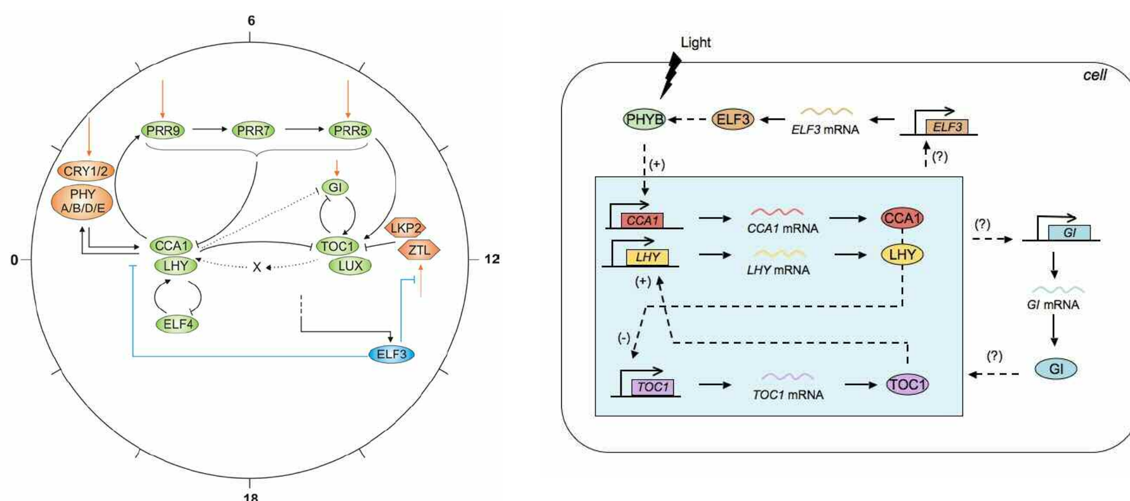


Fig 8. The model is based on recent analyses which indicate that multiple feedback loops exist within the *Arabidopsis* clock. PHYs, CRYs and ELF3 are under transcriptional control by the circadian clock. The circle around the model indicates time of day, with 0 representing dawn. Components are positioned within the circle according to their approximate maximal transcript abundance in continuous light, with the exception of LKP2 and ZTL which are not transcribed in a circadian-dependent manner and thus are represented by hexagons. Positive interactions are shown with arrows, and negative interactions are indicated by perpendicular lines. Dotted lines indicate interactions assumed from a mathematical model but not conclusively demonstrated experimentally. Components and interactions associated with light perception are shown in orange, and components and interactions involved in gating are shown in blue. The circadian regulator of ELF3 is unknown and therefore control of ELF3 by the clock is indicated by a dotted line (Gardner et al 2006)

2.1.2 Control of flowering by circadian clock

The outputs of the circadian clock are pathways that lead to physiological and biochemical rhythms such as photosynthesis, leaf movement, hypocotyls elongation, stomatal movement and circumnutation. One well defined output pathway is the photoperiodic response pathway that controls the transition from vegetative growth to flowering.

Arabidopsis is sensitive to day length and flowers in LDs. This response is partially regulated by the circadian clock and GI is the component of the clock that regulates the induction. GI interacts with SPYNDLY (SPY) and together induce CONSTANS expression. Under SD, CO expression peaks during the night phase, but in LD the peak occurs in the light period. This leads to the production of active CO which is capable of inducing FT. CO is regulated at transcriptional level, being repressed during the light part of the day, by several DOF factors (i.e. CDF1, CDF2, CDF3 and COG1). Recently, through the analysis of T-DNA tagged mutants and tissue specific RNAi transgenic plants, has been

demostred that these factors act upstream of CO in the control of flowering time (Fornara oral communication 2007).

To regulate CO protein stability, phyA and cry signals function in an antagonistic manner to phyB signals: phyA and cry protect CO protein from degradation, whereas phyB promotes its degradation. Several experiments have demonstrated that CO activates FT in the phloem. The mechanism by which CO triggers flowering from the phloem involves the cell-autonomous activation of FT expression (An et al. 2004; Valverde et al 2004; Suarez-Lopez et al. 2001). Although CO induces FT expression, CO protein does not contain a typical DNA binding domain and, hence, it was postulated that CO protein interacts with transcription factors that directly bind to the FT promoter. Recently one such possible mechanism has been reported. CO physically interacts with HAP5 which together with HAP2 and HAP3b constitutes the heterotrimeric CCAAT-binding factor complex, fig.9 (Cai et al. 2007).

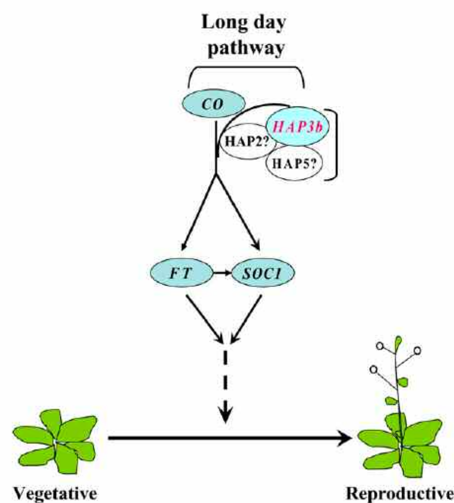


Fig.9. Model of CO interactions in promoting FT expression. HAP2, HAP3b and likely, HAP5 would form a heteroprotein complex that together with CO, participate at the activation of FT (Cai et al. 2007).

It has been shown that light stabilizes the CO protein in the evening under long days when CO mRNA abundance reaches its peak. Regulated RNA metabolism is an important molecular scheme functioning in flowering-time control in plants. It is known that a group of genes belonging to the autonomous pathway encodes RNA binding proteins, and regulates flowering initiation through its activity. Recently, it has been demonstrated that

miR172, a microRNA, is regulated by daylength and that the daylength effect on miR172 is disrupted in *co* and *ft* mutants (Schmid et al 2003), suggesting a role for miR172 in photoperiodic regulation of flowering. Later in another work has been showed that miR172 regulates flowering by regulating FT and GI seems regulates this microRNA abundance at the miRNA processing step rather than at the transcriptional level. Therefore, it appear that GI-mediated photoperiodic flowering is governed by the coordinated interaction of two distinct genetic pathways: one mediated via CO and the other mediated via miR172 and its target, fig. 10 (Jumg et al. 2007).

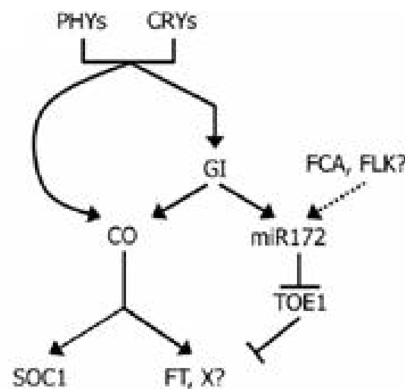


Fig.10. A schematic working model for miR172 functioning in photoperiodic flowering. miR172 mediates light signals from GI and promotes flowering by inducing FT independent of CO.

Recent advances show new regulator acting in the photoperiodic pathway, LONG VEGETATIVE PHASE 1-1D (LOV1-1D). The major role of LOV1-1D is to control CO expression; in particular LOV1-1D controls flowering time by negatively regulating CO expression. It acts only in LD conditions, but not in SD, so that LOV1-1D has been introduced into the photoperiodic control of flowering. However it has been displayed a further role in the response toward the cold temperature. In fact, loss of LOV1 function results in hypersensitivity to cold. Thus, LOV1 seems responsible for the freezing

tolerance, due to an upregulation of response genes, such as COLD-REGULATED 15A (COR15/A) and COLD INDUCED 1 (KIN1), (Yoo et al. 2007).
It is noteworthy to mention a new class of genes, also involved in the control of flowering time through the photoperiodic pathway and the circadian clock, the TEMPRANILLO genes. It has been recently stated, that TEM1 and TEM2 act in parallel with CO in FT regulation, but their function is to repress the gene. Thus, a balance between CO and TEM activities would contribute to a sharply control of flowering time (Pelaz oral communication 2007).

2.2 The Autonomous Pathway

The autonomous pathway includes genes whose mutants show a photoperiod-independent late flowering phenotype that can be reverted by vernalization (Zapater & Somerville. 1990).
Genes included in this pathway are FCA, FY, FPA, LUMINIDEPENDENS (LD), FLC, FVE and FLK (Sanda & Amasino 1996).
FLD, LD and FVE, exert its role in transcriptional regulation, whereas FCA, FY, FPA and FLK, are post-transcriptional regulators.
Molecular analyses have revealed that the role of these genes converges into the down regulation of a gene, which is a strong flowering repressor and that belongs to the vernalization pathway, FLOWERING LOCUS C (FLC) (Michaels & Amasino 1999; Sheldon et al. 2000; Rouse et al. 2002). In fact, autonomous pathway mutants exhibit late flowering phenotype and high level of FLC expression.
FCA and FY interact between them. FY plays a role in RNA 3'-end processing and is required for growth and development in plants (Henderson et al. 2005). FCA feedbacks its expression by promoting premature cleavage and polyadenilation within intron 3, to generate non-functional FCA transcript. It has been demonstrated that auto regulation of the transcript, requires the interaction with FY (Quesada et al 2003; Simpson et al. 2003; Amasino 2003).

The mechanism by which FCA and FY regulate FLC is unknown, but might involve 3'-end processing. It is, instead, known that FLC is a target of FVE. The analysis of the chromatin in *fve* mutant shows an increased acetylation of the H3 or H4 in immunoprecipitation assay, which probably cause an increased level of FLC transcription, resulting in a late-flowering mutant (Ausin et al. 2004). Even in *fld* mutant the chromatin of FLC resulted modified. In particular a greater acetylation of H4 was recognized (He et al.2003). These two evidences suggest cooperation between FLD and FVE in negatively regulate FLC by participating in the deacetylation of chromatin as part of a HDAC complex (Amasino 2004).

2.3 The Gibberellin Pathway

Gibberellins are a class of plant hormones involved in the regulation of flower development in *Arabidopsis*, but even in many other aspects of plant growth and development, including seed germination, stem elongation, leaf expansion and flower induction (Langridge 1957; Ross et al 1997). The gibberellic effect is particularly apparent under non-inductive photoperiod (short-days), (Wilson et al. 1992). The analysis of mutant deficient in GA biosynthesis shows a retarded growth of all floral organs, especially abortive stamen development that result in complete male sterility.
Increased responsiveness of phyB mutants to exogenous GAs also suggests an interaction between phytochrome and GA signalling (Reed et al. 1996).
The role of GAs in LFY and SOC1 activation has recently been analyzed (Blazquez et al. 1998; Blazquez & Weigel 1997; Moon et al. 2003). GAs are also involved in FT activation, in SD GAs are required for flowering and FT displays a peak of expression, suggesting GAs might be responsible for FT induction in these growth conditions (Gomez-Mena et al. 2001; Pineiro et al 2003).
Recent advances have shown that GA regulates the various plant developmental programs by suppressing a group of DELLA protein nuclear repressors. There are 5 DELLA proteins: GAI, RGA, RGL1, RGL2 and RGL3; all containing an N-terminal DELLA domain which is involved in the inactivation by GA signals (Dill et al. 2001).

GA regulates flower development by opposing the function of DELLA repressors and by partly promoting the expression of floral homeotic genes: APETALA 3, PISTILLATA and AG, other than promoting LFY expression (Cheng et al. 2004).

2.4 The Vernalization Pathway

Vernalization is the process whereby the floral transition is promoted through exposure of plants to long periods of cold temperature or winter. The requirement of cold aligns the flowering with the season to ensure that the reproductive phase occurs under favourable conditions (Henderson et al 2003).

The vernalization response has been analysed in numerous plant species and studied at the physiological level (Michaels & Amasino 2001). Relative to other temperature responses, vernalization displays unique characteristics. First, the response requires exposure to a long period, usually weeks, of low temperatures, to be effective (Lang 1965). Second, it appeared that mitotic activity of somatic tissue was an important factor for this response to occur (Wellensiek 1964).

A peculiar characteristic of the vernalization response is its pronounced quantitative nature. So that, increasing the period of low temperature, flowering time is progressively accelerated. However, the response is saturable, reaching a point at which further exposure to cold does not lead to additional acceleration of flowering (Lang 1965). The gradual nature of the vernalization response suggests the existence of a progressive accumulation of a floral promoter coupled with the removal or repression of a floral inhibitor.

Cold treatment is believed to induce a developmental state that is mitotically inherited; at the end of the treatment flowering is promoted and will occurs after some weeks from the return at the higher temperature. It is possible to assume that vernalization serves to provide competency to flower (Chouard 1960).

The physiological characteristics of vernalization suggest that the response involves a form of epigenetic “memory”, which is stable through the mitotic generation of cells and can be considered to be an epigenetic switch.

There is requirement for vernalization both in monocarpic than polycarpic species. Among the monocarpic those that flower in the second growing season are called biennals o winter annuals. *Arabidopsis* show both summer annual than winter annual accession and this make easier the understanding about vernalization requirement.

Napp-Zinn was one of the pioneering in the studying of vernalization response. He found that in certain crosses between natural accessions of *Arabidopsis*, a single dominant locus, which he named *FRIGIDA (FRI),* was responsible for the cold requirement (Napp-Zinn 1987). *FRI* is a single copy gene, encoding a protein with two potential coiled-coil domains (Johanson et al. 2000). The biochemical function of FRI remains to be determined, although it is know its nuclear localization and strong expression in meristematic regions. The allelic variation at *FRI* is an important determinant of flowering time. The accessions *Columbia* and *Landsberg erecta* of *Arabidopsis,* result in an early flowering phenotype because they carry non functional *FRI* alleles. In particular *Columbia* has a 16 bp deletion inside the first exon which causes a premature STOP into the second exon, whereas *Landsberg* has an acquired insertion-deletion event at the beginning of the *FRI* open-reading frame, which removes the putative START-codon (Johanson et al 2000). Moreover *Ler* show a 1.2-kb insertion of a Mutator-line transposable element (TE) in the first intron of FLC. This TE is responsible for the inability of FRI to upregulate FLC (Gazzani et al. 2003) and renders FLC subject to repressive chromatin modifications mediated by short interfering RNAs generated from homologous transposable elements in the genome (Liu et al. 2004).

The major role of FRI, however, has been recognized in the up-regulation of a gene, which is a key repressor of flowering, referred as *FLOWERING LOCUS C.* The presence of dominant alleles of *FLC* is necessary for FRI to confer a vernalization requirement (Koornneef et at. 1994). Recently *SUPPRESSOR OF FRIGIDA4 (SUF4)* has been shown to be necessary for the FRI-associated late-flowering phenotype, which results from a high level of FLC activity (Kim. et al 2006). FRIGIDA ESSENTIAL 2 (FES1) and FRIGIDA-LIKE1 (FRL1), are two other proteins that likely act in a complex with FRI in the FLC positive regulation (Schmitz et al. 2006; Michaels et al. 2004).

In general winter annual ecotypes have dominant alleles of FRI and FLC, whereas rapid-cycling types have either a no-functional *fri* allele (Michaels et al 2003) or a weak *flc* allele

(Gazzani et al 2003). Vernalization represses the FRI-mediated increase in FLC expression (Michaels 1999).

2.4.1 Epigenetic modifications induced by vernalization

The cloning and characterization of FLC revealed some key aspects of the molecular basis of vernalization (Michaels & Amasino 1999; Sheldon et al 1999). Vernalization represses FLC by mitotically stable epigenetic modifications that are maintained even after the exposure to cold has ended.

2.4.2 The "memory component" of the vernalization response

The establishment and maintenance of silenced chromatin states during epigenetic regulation has been associated with several modifications at both the DNA and histone level (Bird 2001; He et al. 2004).

Vernalization treatment was reported to induce a global reduction in DNA methylation levels (Burn et al. 1991; Finnegan et al. 1998). In order to address FLC repression to a reduction in DNA methylation through the vernalization, the chromatin of FLC has been analysed before and after cold treatment and no change in the status of methylation was found (Finnegan et al. 2005).

The recent characterization of FLC repressors and activators has shown that some of the regulatory modification are at histone level; in particular regard acetylation and methylation of specific residues of specific histones, referred as the "histone code"(Turner 2002).

In the fall season, *FLC* chromatin is in an active state, that is the chromatin is enriched in acetylation of lysine 9 and 14 and trimethylation of Lys 4 of the histone 3, which are hallmarks of active genes (Bastow et al. 2004; He et al.2004). After the passage through the cold of the winter, the *FLC* chromatin becomes enriched in methylation of Lys9 and Lys 27 of the histone 3 (hallmarks of repressed genes), whereas the level of modification responsible for the active chromatin are extremely reduced.

Vernalization-induced repression of *FLC* activity is dependent upon temperature induction of VERNALIZATION INSENSITIVE 3 (VIN3), which is recruited to a protein complex, the VRN2 Polycomb Repressive Complex 2 (PRC2) associated with the FLC gene (Dennis & Peacock 2007).

The constituent of the VRN2 complex are VRN2, CURLY LEAF (CLF1), SWINGER (SWN1), and FERTILIZATION INDIPENDENT ENDOSPERM 1 (FIE1) (Wood et al. 2006).

It has been shown that there is a positive correlation between length of exposure at the cold, VIN3 induction, promotion of flowering and modifications to particular residues in the histones associated with *FLC* chromatin. It was suggested that the amount of VIN3 recruited by the VRN2 complex is proportional with the level of histone-modification, thus with the level of transcriptional activity at the *FLC* locus.

VIN3 encodes a PHD-finger protein. Usually, this kind of proteins is found to be part of chromatin remodelling complexes (Sung S., Amasino R. 2004). VRN2 encodes a homolog of the Polycomb group protein *Suppressor of Zeste 12 (Su(z)12)* (Gendall et al. 2001); VRN1 is a protein with two plant-specific B3 domains and binds dsDNA non-sequence specifically in vitro; the general chromosome association of VRN1 supports the idea that it is not an FLC-specific regulator, which also appears to be the case of VRN2 (Levy et al. 2002).

Recently, a genetic approach has led to the identification of two other VIN3-interacting proteins, referred to as VIN3-LIKE1 (VIL1) and VERNALIZATION 5 (VRN5). VIN3 and VIL1/VRN5 interact through a C-terminal domain and mutations at these loci cause a vernalization-insensitive phenotype that prevent the accumulation of the repressive modifications at the FLC histones (Sung et al. 2006; Greb et al.2006).

In *Drosophila*, maintenance of the repressed state requires a PRC1 complex. In *Arabidopsis* such a complex has been revealed by reverse genetics approach and identified with LIKE HETEROCHROMATIN PROTEIN 1 (LHP1). LHP1 is required to maintain repressed state of FLC after cold exposure, but not for the initiation of H3K9 methylation (Mylne et al. 2006; Sung et al 2006). Because VRN1 is required for the H3K9me2 mark at *FLC*, its

activity may be required for LHP1 association with FLC; however no interaction between LHP1 and VRN1 has been demonstrated (Mylne et al. 2006).
Resetting of *FLC* is essential for maintaining a vernalization requirement. The resetting is necessary to perpetuate a vernalization requirement in successive generations. The specific stage at which resetting occurs is unknown, but the evidences speculate that would occurs during the meiosis, because it is detectable in the early embryo but FLC activity is lacking in the developing endosperm. Moreover, it has been reported that in contrast to chromosomes in mitotic divisions, there is no VRN1 associated with the chromosomes in the male meiotic divisions. VRN1 is the only protein known to be associated with *FLC* regulation and the vernalization response that has been shown to have a difference in presence between mitosis and meiosis. This consideration suggests that the absence of VRN1 may predispose *FLC* to reset to the normal level of activity in the new embryo. The S phase of the second pollen mitosis occurs in the pollen tube and this may be the time when a new complement of histones associate with *FLC* locus re-establishing the state of *FLC* activity (Mylne et al. 2006).
In the sequence of events, VIN3 initiates histone deacetylation at FLC, this promotes and enables the H3K27 dimethylation by a VRN1-containing complex. The proteins involved in methylation of the H3, belong to the SET domain protein family. KYP and SUVH2 are among them. LHP1 binds specifically H3K9 dimethylated, thus contributing to the epigenetic silencing of FLC. It is thought that VRN1 first would methylates H3K9, then LHP1 can bind the region and maintain the repressive state (Jiang et al 2007).

2.5 The Integrators Pathway

A large number of genes acting within the pathways has been cloned and currently analysed to understand how they are linked to each other and how the corresponding proteins function (Amasino 2004; Bastow & Dean 2003; Simpson & Dean 2002; Sung & Amasino 2004; Jack 2004).
Two genes play a prominent role at the “end” of these promotion cascades. CO is probably the most downstream actor, specific for the photoperiod pathway (Valverde et al. 2004).

FLC is the point of convergence of the autonomous and vernalization pathway. Through the regulation of CO and FLC, the flowering signals lead to the induction of a set of genes called Floral Meristem Identity (FMI), responsible for the fate change of the meristem emerging from the flanks of a shoot apex (Long & Barton 2000). This group of gene comprises LFY, AP1 and CAULIFLOWER (CAL) (Kieffer & Davies 2001; Lohmann & Weigel 2002).

The regulators that integrate the inputs from the different flowering cascades of signals and that convey the resulting outcome to FMI genes at the shoot apex are named Integrators. Among the integrators, the gene LEAFY, SOC1/AGL20 and FT. These three genes constitute a novel pathway known as the Floral Pathway Integrators.

LFY plays a prominent role during flower development. Its expression precedes the floral transition, for that can be used as marker of flowering induction. The transcript appears in young leaf primordia to increase until reach the maximum in young floral meristems (Blazquez et al 1997; Blazquez et al. 1998). LFY encodes a transcription factor, capable to travel from cell-to-cell via plasmodesmata (Parcy et al 1998, Session et al 2000, Wu et al 2003). The protein binds cis-elements present in AP1 and AG regulatory sequences (Bush et al 1999; Lohmann et al. 2001) and seems to be regulated by day-length , even if the mechanisms to date, is still unknown (Nillson et al 1998; Weigel & Nillson 1995). The photoperiod promotion effects on LFY may be mediated by SOC1 or by a second MADS gene, AGAMOUS-LIKE24 (AGL24), (Jack 2004).

FT belongs to the photoperiod pathway (Koorneef et al. 1991). FT mRNA is transcript at leaf level, but FT protein acts at the shoot apical meristem. It has been shown that FT moves from the companion cells to the SAM, through the vasculature direct toward the emerging primordia, which later will differentiate into flower (Jaeger & Wigge 2007). This transport occurs through symplastic unloading from the phloem into the apical meristem region. It has been demonstrated that the amount of FT mRNA in the leaves, produced after three days in LD, is sufficient to enable the flowering at the shoot apical meristem (Corbesier et al. 2007).

In the shoot apex FT interacts with FD, forming part of a transcriptional complex to induce the activation of other genes involved in the flowering transition (i.e. SOC1, LFY, AP1) (Valverde et al. 2004; Abe et al. 2005). FT acts redundantly with TWIN SISTER OF FT (TSF), which antagonize completely FT function (Mathieu et al. 2007).

SOC1/AGL20 encodes for a MADS box transcription factor. The transcript is expressed mostly in leaves and in the shoot apex, during floral transition its expression raise the apex. SOC1 expression is absent from stage 1 flower meristem and reappear in the centre of older flower meristem (Borner et al. 2000; Lee et al 2000; Samach et al. 2000).
FLC functions to repress the floral activator SOC1 (Lee et al., 2000; Hepworth et al. 2002). SOC1 is activated by the long-day promotion pathway via CO (Samach et al. 2000) as well as by the GA pathway (Borner et al. 2000; Moon et al. 2003). Integration of the FLC and CO signals is mediated by discrete elements in the SOC1 promoter (Hepworth et al. 2002). A consensus MADS binding sequence in the SOC1 promoter can be bound by FLC in vitro. Mutation of this binding sequence abolishes repression of SOC1 by FLC. Although a CO-responsive region of the SOC1 promoter also was defined, binding of CO to this sequence could not be demonstrated,
Although the GA-responsive element in the SOC1 promoter has not been defined, it is clear that removal of the FLC repression of SOC1 is not sufficient to result in high SOC1 transcript levels; upregulation of SOC1 also requires positive activation by either the GA or the long-day promotion pathway. In short days, the GA pathway is the only pathway that can activate SOC1 (Moon et al. 2003).

2.5.1 Interactions between the Integrators

The integrators are linked to each other by forming an intricate gene regulatory network fig. 11, (Parcy 2005). For instance, recent evidence suggests that FT might be able to up-regulate progressively LFY (Schmid et al. 2003). FT compete with TFL1 (TERMINAL FLOWER 1) about the regulation of LFY expression in the apex. In fact, in wild-type plant (Ractcliffe et al. 1998), LFY expression normally does not enter the SAM, because of the

TFL1 expression, which has an opposite function of FT, until the excess of FT expression overcome TFL1 inhibition.

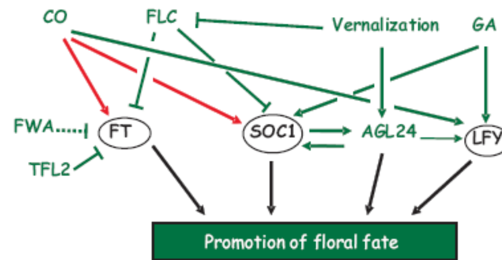


Fig.11 Gene regulatory network around Floral Pathway Integrators. Red arrows represent direct interactions, dashed arrows represent post-transcriptional regulation and plain arrows represent transcriptional regulation. Floral Pathway Integrators are circled.

In addition FT is involved in the control of SOC1 expression, even if this relationship needs to be further investigated (Schmid et al. 2003).

SOC1 has been proposed to induce LFY (Jack 2004, Mouradov et al. 2002) with the contribution of AGLS24 (Yu et al 2002). AGL24 is induced by vernalization and might participate in the FLC-independent vernalization effect even inducing SOC1 expression (Michaels et al. 2003a).

Recently, it has been proposed the existence of a loop between LFY and AGL24. In effect, AGL24 seems an early target of transcriptional repression by LFY and AP1. Without such repression, continued AGL24 expression in floral meristems is sufficient to cause floral reversion. This indicate that LFY and AP1 promote floral development not only by positively regulating genes activated in flower development, but also by repressing AGL24, a promoter of inflorescence fate (Yu et al 2004).

2.5.2 Regulation of FMI by the Integrators

AP1 and CAL are expressed after LFY in the stage 1 of floral meristem. The activation of AP1 by LFY is postulated to be direct (Wagner et al. 1999). The AP1 promoter contains a sequence that can be bound in vitro by the LFY protein (Parcy et al. 1998), but this

sequence has not yet been demonstrated to be necessary in planta for LFY activation of AP1.

FT is capable to induce AP1 independently of LFY (Ruiz-Garcia et al. 1997), but the mechanism is not yet understood. The fact that CAL cannot rescue for the loss of AP1 in *lfy ap1* double mutant, suggests that FT is not able to induce CAL independently of LFY, or that CAL and AP1 meristem identity functions are not exactly equivalent (Parcy 2005).

Genetic data shows that AP1 induction is delayed in both *lfy* and *ft* single mutants, indicating that LFY might act synergistically with FT.

2.6 The key repressor of flowering time: FLOWERING LOCUS C, FLC

FLC is a key component of the response to vernalization. It belongs to the MADS-box transcription factor and is expressed predominantly in the shoot and root apices and vasculature. FLC plays its role in quantitatively delay flowering by repressing the floral pathway integrators (Michaels & Amasino 1999; Sheldon et al. 1999).

The function of FLC when full expressed is to repress both the expression of systemic flowering signals in the leaf (i.e. FT), and the response to these signals at the meristem (i.e. SOC1 action, FD upregulation). Vernalization represses FLC in both tissues rendering the meristem responsive to the flowering signals and allowing the leaves to produce these signals (Searle et al 2006), fig 12.

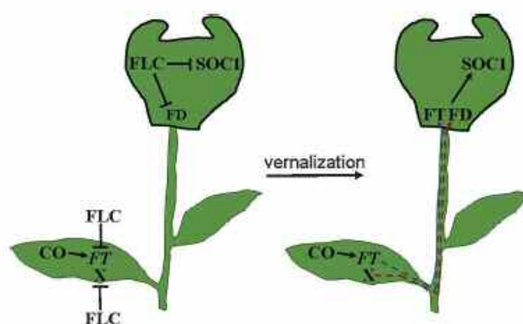


Fig.12 A schematic diagram illustrating the interactions between FLC and the photoperiod pathway in the leaf and meristem and the effect of vernalization on these interactions. Prior to vernalization, FLC acts in the leaf to repress transcription of FT and of other genes that are illustrated as X. In the meristem, FLC represses transcription of FD and of SOC1. Vernalization reduces FLC expression both in the leaf and the meristem. FD is also required at the meristem for SOC1 expression, and because FT and FD interact, this heterodyne might activate SOC1 expression directly or indirectly. The increase in FD expression does is blocked by expression of FLC in the leaves. The reduction of FLC expression in the

meristem during vernalization allows FD and SOC1 expression in the meristem to rise in response to the X signal and FT/FD, respectively.

Allelic variations at the FLC locus contribute to the natural variations in vernalization requirement (Michaels et al. 2003; Gazzani et al. 2003). The early flowering phenotype results mostly, by changes in the regulation of expression rather that alteration of protein function. However, during a study regard to the natural variations in flowering behaviour, three natural FLC alleles were discovered. In all the cases, protein function was severely affected (Lempe et al. 2005), fig.13. One of the allele was null, because it lacks exons 2 to 6 and does not rescue the null mutation *flc-3* in *Columbia-FRI* background; the second, because of an alternative splice acceptor site, has a deletion in the last exon. The last one shows an alternative splice acceptor site in the last intron, which adds additional sequences and causes frame shift of the sequences of the last exon (Lempe et al. 2005).

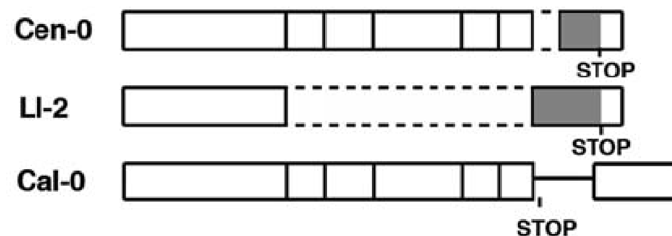


Fig.13 Changes in FLC transcripts in three accessions. Dotted lines indicate exons that are missing in part or completely. These deletions cause frame shifts and thus altered amino acid sequences (grey boxes). Premature stop codons are indicated. The reasons for the aberrant transcript processing in Ll-2 are not known. In Cen-0, an alternative splice acceptor site in the last exon is used, leading to a deletion of exon sequences and a frame shift, whereas in Cal-0 an alternative splice acceptor site in the last intron is used, which adds additional sequences and also causes a frame shift of sequences of the last exon.

Five homologues of FLC have been characterized in the *Arabidopsis* genome, they are referred to as MADS AFFECTING FLOWERING 1(MAF1) to MAF5 (Ratcliffe et al. 2003). MAF1 is also known as FLM (Scortecci et al 2001) or AGL27 (Alvarez-Buylla et al

2000). FLM is a floral repressor but does not seem to be involved in the vernalization response; instead it would act in the photoperiod pathway (Scortecci et al 2003). MAF2 is another floral repressor which shows a pronounced vernalization response when subjected to short periods of cold (Ratcliffe et al 2003). MAF3 and MAF4 could be floral repressor too, whereas MAF5 increases its expression during vernalization, so may play a role opposite to FLC (Ratcliffe 2003).

As above mentioned, *FLC* is repressed by vernalization and the degree of repression is proportional to length of the cold treatment and correlates with the extent of the promotion of flowering (Sheldon et al. 2000b).

Which regions of the *FLC* gene are important for non-vernalized expression and for the stable repression by vernalization, have been recently determined fig. 14 (Sheldon 2002). A 75-bp region in the *FLC* promoter, containing three basic domain/Leu zipper (b-ZIP) binding motifs, is necessary for non-vernalized expression. To characterize the sequences required for the vernalization response, a progressive deletion of regions into the FLC gene were made. A region of 6kb including 2kb of the promoter sequence, the first two exons and intron I, was sufficient for the initial vernalization-induced repression and for the maintenance of the downregulation (Sheldon et al 2002). In particular, it has been verified that the intron I is the most important for the maintenance of the vernalization-induced repression of *FLC*, but for the stable downregulation of the expression, the promoter sequences in conjunction with intragenic regions are required. It has been determined that at least 272 bp upstream of the ATG is sufficient in combination with intragenic sequence for the initial downregulation (Sheldon et al 2002).

The 75-bp sequence in the promoter has been characterized and seems to be involved in gene regulation by ABA or light (Iwasaki et al 1995; Terzaghi et al. 1995).

Moreover, the intragenic regions are important for the repression of FLC by FCA, even without the promoter sequences.

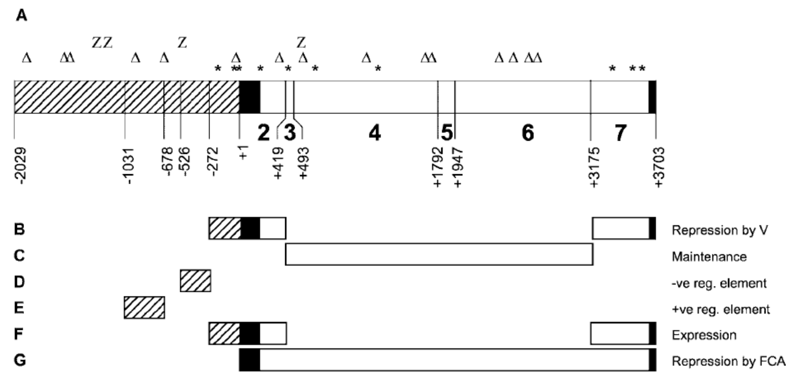


Fig 14. (A) Representation of the *FLC* sequence contained within the 6-kb construct. GAGAG sites and potential PHO and Zeste recognition motifs are indicated (asterisk, triangle, and Z, respectively). (B) Regions sufficient for vernalization (V)-induced repression of FLC expression. (C) Region required for the maintenance of vernalization-induced repression. (D) Region containing potential negative regulatory elements. (E) Region containing potential positive regulatory elements. (F) Regions sufficient for expression in non vernalized plants. (G) The repression of FLC expression by FCA requires the 4-kb intragenic region.

It is known that all the MADS-box protein act in complex with other proteins to regulate a specific plant developmental process. However, the molecular mechanism by which different MADS-domain proteins select their specific target gene is elusive so far. FLC, being a MADS-box protein has to be part of a protein complex too. To date, nothing is reported in bibliography, but it seems that FLC should be component of a complex together with AP1 and SVP (Ilha Lee, personal communication, October 2007). In addition, it has been displayed that FLC can bind a CArG box region in the first intron of the SEP3 gene. This demonstrates that FLC can even exert a role in negative regulation of gene involved in flower structures development, other than toward genes involved in flower time determination (Kaufmann & Angenent, oral communication 2007).

2.6.1 Activation of FLC

As mentioned above, a key activator of FLC is FRI. FRI upregulates FLC at RNA levels (Michaels & Amasino 1999; Sheldon et al. 2000). Genetic analysis of natural variation in flowering time has also identified AERIAL ROSETTE 1 (ART1) from the extremely late-flowering accession Sy-0; ART1 acts synergistically with FRI to upregulates FLC (Poduska et al 2003).

Genetic analyses have revealed many of the components involved in the regulatory network governing FLC expression: VERNALIZATION INDIPENDENT 4 (VIP4), VIP3, PHOTOPERIOD INDEPENDET EARLY FLOWERING (PIE1), EARLY IN SHORT DAYS 4 (ESD4) (Zhang & Van Nocker 2002; Zhang et al. 2003; Noh & Amasino 2003; Reeves et al. 2002; Murtas et al 2003). The molecular mechanism by which these activators work in FLC expression is unknown.

A complex named PAF1 and comprising the proteins VIPs 1-7, EARLY FLOWERING 8 (ELF8) and ELF7, is required for the transcriptional activation of FLC (He et al. 2006).

Recent evidences show that SUF11 could enhance the activity of VIP5, affecting FLC expression, by an unknown mechanism (Juhyun K. oral communication 2007)

In yeast, this complex also interacts with the histone methyl transferase SET1 and SET2, which are involved respectively in methylation of histone H3K4 and H3K36 residues, marks of transcriptional active chromatin. In *Arabidopsis* the homologues of SET1 and SET2 seem to be ELF7/8 and EARLY FLOWERING IN SHORT DAYS (EFS) (Zhao et al. 2005; Kim SY et al. 2005). The PAF1 complex is also required for the expression of MAF2 and FLM.

HUA2 is a gene that plays a role in the control of flowering acting on FLC, FLM and MAF2. It has a role in RNA processing, and together with the PAF1 Complex, enhances the expression of several genes that delay flowering, including FLC (Doyle et al. 2005).

ABH1 is another protein involved in FLC regulation at RNA level. It forms a complex with CBP80 constituting the large subunit of the nuclear cap-binding complex. Mutation *abh1*, displays an early flowering phenotype both under short- and long-days. The cap-binding complex has been implied in regulating different level of mRNA metabolism in yeast. In *Arabidopsis* it has been shown an influence of *abh1* on intron 1-dependent pre-mRNA maturation processes of FLC; in particular the absence of ABH1 disturb the correct splicing of FLC pre-mRNA, leading to the accumulation of a non-functional transcript with an unspliced intron 1 (Kuhn et al. 2007).

Members of the FLC clade, have a conserved nucleotide sequence in the 5’-UTR that is within the region of the highest level of H3-K4 trimethylation. This region comprises a 26-bp region immediately downstream of the transcription starting point and 7-bp region immediately upstream of the start codon. A deletion of this conserved region prevents *FLC*

up-regulation by *FRI* or autonomous-pathway mutations. Trimethylation H3K4 is thought to promote gene expression by the recruiting of an ATP-hydrolyzing chromatin-remodelling protein, which in *Arabidopsis* is indicated as PIE1. It is proposed that PIE1 binds the 5' region of *FLC*, FLM and MAF2 chromatin when these regions are enriched for H3K4 trimethylation resulting from ELF7/8-dependent recruiting of the EFS methyltransferase. The autonomous pathway would act preventing the ability of ELF7 and ELF8 to activate *FLC*. In presence of a dominant active allele of *FRI*, this ability of the autonomous pathway is blocked (Hu et al. 2006).

Besides, epigenetic modifications at the histones level, activation of *FLC* requires another further condition to occur. The histone variant H2A.Z is necessary for transcriptional activity of *FLC* and serves as marker for the "on" state (Deal et al. 2007). The genes ESD1 (EARLY IN SHORT DAYS 1), SUF3, ARP6 (Actin Related Protein 6) (Deal et al. 2005) and PIE1 are involved in H2A.Z variant replacement (Kobor et al. 2004). Recently, it has been found another protein, SERRATED LEAVES AND EARLY FLOWERING (SEF), interacting with ARP6 and PIE1, and classified as an homolog of Swc6, a component of the yeast SWR1 complex, that might play a role in the control of cell cycle progression or developmental processes, such as leaf and flower morphology, contributing also to the FLC regulation (March-Diaz et al. 2007). H2A.Z-HTA8, -HTA9 and -HTA11 are three histones homologs to H2A.Z variant in *Arabidopsis*; they act redundantly; the resulting FLC expression rate, is regulated by the overall level of the three H2A.Zs, placed around the FLC promoter and the 3' region of the gene (Choi et al. 2007).

2.6.2 Repression of FLC

FLC is negatively regulated mainly by two via: the vernalization pathway and the autonomous pathway. The repression operated by the vernalization treatment has been elucidated before (par. 2.4.2).

The autonomous pathway functions to limit the accumulation of FLC mRNA and acts in parallel with the vernalization pathway. In the absence of *FRI*, this pathway is the major regulator of FLC (Koorneef et al 1991; Simpson & Dean 2002). Although all members in this pathway act to limit FLC activity, is possible to address the different proteins into

distinct functions. FCA, FY, FPA, FLK and LD are RNA-binding proteins, whereas FLD, FVE and FELATIVE OF EARLY FLOWERING 6 (REF6) are comprised in the chromatin remodelling factors. FLD shares sequence identities with an animal Lysine Demethylase (LSD1). LSD1 demethylates H3K4, FVE is part of a HDAC complex; REF6 is predicted to demethylate H3K36 (Klose et al. 2006).

In plant there are 4 homologs of LSD1 which are named FLD, LDL1 (LSD-1 LIKE), LDL2 and LDL3. LDLs function is unknown, but they seem redundant with FLD in repressing FLC expression. LDL1 and LDL2 act specifically on FLC and not on the other members of the clade; in addition, LDL1 and 2 are involved in FWA repression. It is noteworthy to say that FLD and FVE, together with the vernalization treatment, repress FLC, but not participate at the silencing of the gene (Jiang et al 2007).

Recent advances indicate the involvement of a histone acetyltransferase, HAC1, in the regulation of flowering via FLC. Very few information are available about this protein, but evidences suggest that HAC1 affects flowering time by epigenetic modification of factor upstream of *FLC*. *hac1* mutant responds normally to day length, vernalization and gibberellin treatment, displays an increased level of FLC, MAF4 and MAF5, thus suggesting that HAC1 acts in the autonomous pathway. However, HAC1 does not seem to regulate *FLC* directly, because no changes in the epigenetic modification of *FLC* chromatin were observed in the mutant. One possible explanation for the phenotype is that HAC1 acetylates the histones of an unknown factor that represses *FLC* and/or MAF4 and 5. This unknown factor could be a component of the autonomous pathway (Deng et al. 2007).

From the analysis of mutants with an altereted timing of flowering, emerged the mutation *bri1*. BRI1 is a receptor for the brassinosteroid. It has been shown that the mutant has an increased level of FLC and that the combinations of double-triple mutant with gene in the autonomous pathway display an enhanced late-flowering phenotype. Because *bri1* still responds to vernalization, GAs and photoperiod, and not direct relation emerged from the screening of mutants, it has been suggested a role for BRI1 as assistant of the repression of FLC mediated by the autonomous pathway (Domagalska et al. 2007).

It has been shown that all the epigenetic modifications imposed in the *FLC* chromatin by the vernalization, are transmitted to the genes flanking the *FLC* locus. The gene *UPSTREAM OF FLC (UFC)*, which has unknown function, is located 4.7 kb upstream of

FLC. Like *FLC, UFC* is downregulated by vernalization, even if the repression not stable after the returning to warmer temperature condition. The fact that relocating *FLC* in other regions of the genome, the genes flanking are modified as *FLC* after vernalization, suggest that *FLC* is the spreader of modifications. The hypothesis is that *FLC* can bind a vernalization responsive factor, which facilitates the spread of repressive chromatin from *FLC* to genes on either side, including introduced genes (Finneganet al. 2004).

2.6.3 A role for small RNAs in the regulation of FLC

DCLs are ribonucleases that generate small RNA species from double stranded RNA. Each of the DCL generates a particular class of small RNA. For example DCL1 generates miRNA, whereas DCL3 heterochromatic siRNA. DCL1 and DCL3 despite the different RNAs generated, share a functionally redundant role in repression of *FLC* repression or some *FLC* activator.

The small RNAs generated from DLC1 and DCL3 have not been isolated, so far, but it is supposed that they could act targeting the 3' end of *FLC*, in a region downstream to the mature FLC transcript (Schmitz et al. 2007).

Small RNAs corresponding to the reverse strand of *FLC* in the region corresponding to the 3' to the major poly(A) site were recently detected. An antisense RNA that covered this region was found, and this RNA contained an intron not present in the sense strand, suggesting the production from an antisense transcription mechanism. The small RNAs derived from the 3' region of *FLC* through the activity of NRPD1a, generate an antisense transcript that is a target for DICER-LIKE 3(DLC3). The small RNAs are 30- and 24- bp long, and would be involved in recruitment of chromatin complexes to specific *FLC* sequences that methylate histones at specific nucleosomes located downstream from the major poly(A) site, leading to reduced expression of *FLC*. Two mechanisms are plausible to account for how the small RNAs determine the reduction in *FLC* expression. The first proposes a reduction of FLC mRNA because of a reduced level of transcription. The second involves an inefficient polyadenilation of FLC transcripts, so that aberrant transcripts could be produced and processed by a DCL3, reinforcing silencing at the FLC 3' end. In this last hypothesis the genes FCA/FY from the autonomous pathway can be

involved. In fact FCA/FY could regulate the poly(A) site choice by recruiting specific transcript or stabilizing weak poly (A) site interactions. However, not evidences support this model (Swiezewski et al. 2007).

Chapter III

Flowering in other specie

3.1 The case of Beet and Monocots

It is likely that FLC represses long day induction of FT in *Brassica napus* as probably in other Brassicaceae species. In fact, the FLC orthologues from *Brassica* delay flowering when introduced in *Arabidopsis* background (Tadege et al. 2001). Vernalization treatment downregulates the FLC orthologues and promotes flowering, thus it is likely that the same interactions between FT, FD and SOC hold in this species.

FLC-like has been identified even in sugarbeet, tomato, potato and grape.

In sugarbeet the downregulation of *Bv*FLI is not stable and its activity is upregulated on return to normal temperatures, but when introduced into *Arabidopsis* it still repress flowering. Four RNA variants were identified in immature leaf and shoot apex cDNA libraries, fig 15, (Schmitz et al. 2007). The variants differ by two K-domain indels arranged in all four possible pairwaise combinations; all variants share the same translation stop codon. The four variants presumably are the result of differential processing of a single genomic locus. Vernalized sugar beets however are prone to inflorescence reversion, dependent on the environmental conditions immediately following vernalization (such as SD and rapid increase in temperature).

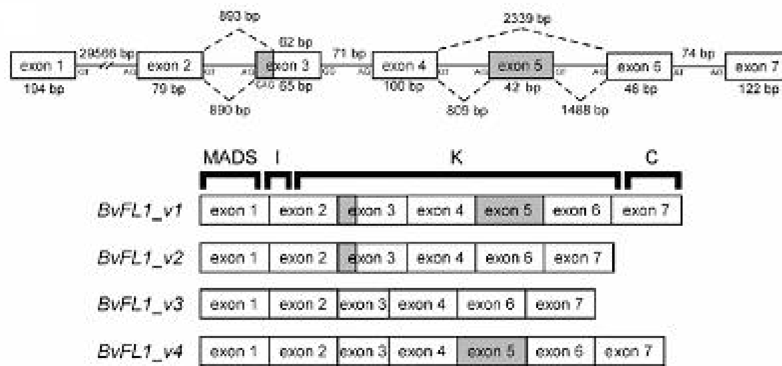


Fig. 15 Genomic structures of the sugar beet FLC homolog BvFL1. Dashed lines indicate the four putative alternative splicing possibilities required to generate mRNA variants BvFL1_v1–v4. Coding regions affected by splice site variation are shaded. Approximate boundaries of the MADS, I, K, and C domains are indicated for BvFL1_v1.

The level of *Bv*FLC transcript has been examined and it returned at the same level prior to the vernalization, after returning at warming temperatures; this data is consistent with the propension toward the reversion. In addition, other line of evidences allows the authors to suggest that the "bolting gene" is not *Bv*FLC, but it may lie outside the vernalization pathway; more likely it could be a member acting in the photoperiodic pathway. However, this specie shows an obligate vernalization requirement maybe because of a failure to recognize a long-day flowering cue such that vernalization became the only effective pathway to flowering (Reeves et al. 2007).

Detailed analysis of genes involved in vernalization and long day responses in plants other than *Arabidopsis*, come from the monocot cereals, wheat and barley.
Genetic analyses have identified three genes controlling the vernalization requirement in wheat and barley: VRN1, VRN2 and FT (VRN3), fig 16. These genes have been isolated and shown to regulate also the promotion of flowering by long days. VRN1 encodes an APETALA1-Like MADS box transcription factor and regulates meristem identity. It is induced by vernalization and accelerates the transition to reproductive development at the SAM. VRN3 is induced by long day, translocated from the leaf to the shoot apex to trigger flowering (Yan et al. 2006; Tamaki et al. 2007). VRN2, acts as floral repressor, can be considered the orthologue of *At*FLC. It integrates vernalization and day-length responses by

repressing VRN3 until plants are vernalized. VRN2 in fact prevents the expression of VRN3.

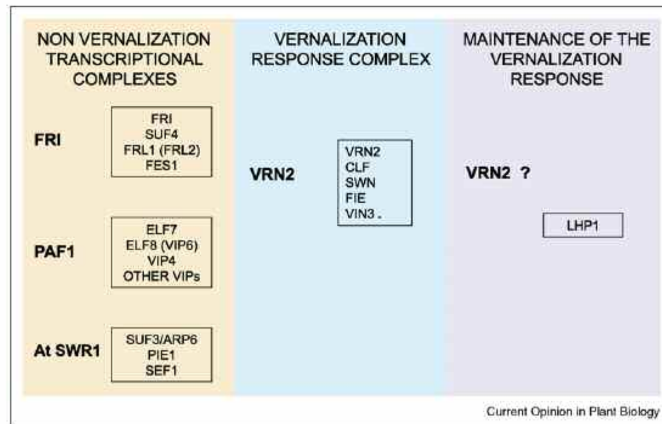


Fig. 16 Relationship between environmental cues and gene action in flowering in *Arabidopsis* and barley and wheat. In the long days of autumn FT is repressed (by FLC in *Arabidopsis* and VRN2 in wheat and barley). In winter low temperatures repress FLC in *Arabidopsis* or in wheat and barley VRN1 is induced. VRN2 is repressed by short days. In the long days of spring, FT is induced and causes flowering in both systems (Dennis & Peacock 2007).

VRN2 is expressed in LD, but is not expressed in SD. These considerations suggest the hypothesis that VRN2 blocks VRN3 in LD, by direct interaction with VRN3. VRN2 decreases when plant is vernalized under LD, whereas VRN1 expression increases. VRN1 has been proposed to repress expression of VRN2, so that the decrease seen during the vernalization could be attributed to the VRN1 induction (Yan et al. 2004). When plants are vernalized under SD, VRN2 expression is low and is not affected by vernalization, fig. 17 (Trevaskis et al. 2006), thus is unlikely that VRN2 plays a role in the vernalization response.

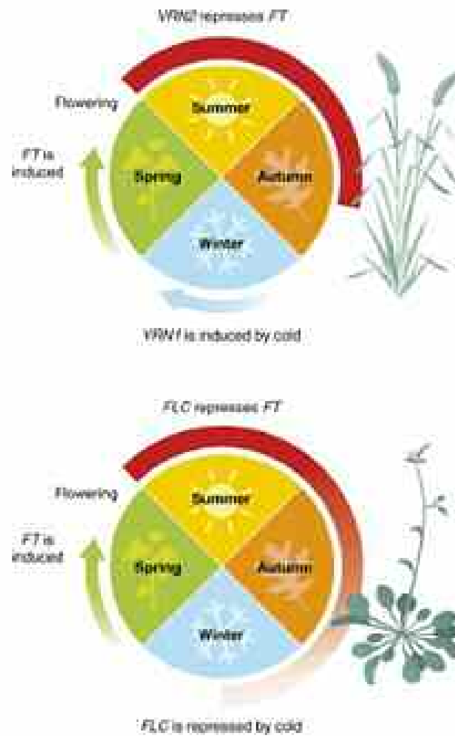


Fig. 17 Molecular basis of vernalization-induced flowering in cereals versus *Arabidopsis*. In the temperate cereals (top), VRN2 represses FT and blocks long-day promotion of flowering before winter. VRN2 is not expressed in the short days of winter, when VRN1 is induced by prolonged exposure to cold. After winter, VRN1 expression remains high. This promotes inflorescence initiation and represses VRN2, to allow long-day induction of FT to accelerate reproductive development. When flowering occurs, VRN1 expression is reset to establish the vernalization requirement in the next generation. In *Arabidopsis* (bottom), FLC is expressed before winter and represses FT. Vernalization represses FLC, and this allows long-day induction of FT (and SOC1) to promote flowering in spring. FLC expression is reset during meiosis to establish the vernalization requirement in the next generation (Trevaskis et al 2006).

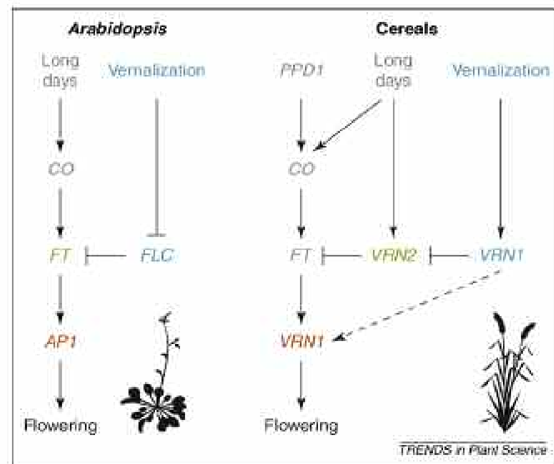


Fig 18 A comparison of the molecular pathways regulating flowering time in *Arabidopsis* and the temperate cereals. Vernalization and long days promote flowering in *Arabidopsis* (left) and in the temperate cereals (right). The day-length response is conserved (grey). CONSTANS (CO) activates FLOWERING LOCUS T (FT) expression. This requires PHOTOPERIOD1 (PPD1) in cereals. In *Arabidopsis*, FLOWERING LOCUS C (FLC) blocks long-day induction of FT but is repressed by vernalization. In the temperate cereals, VRN2 blocks long-day induction of FT before winter, but VRN1 is induced by vernalization to repress VRN2 and allow long-day induction of FT. In *Arabidopsis*, the vernalization and day-length response pathways intersect at FT, which can be described as a floral integrator gene (green). In cereals, VRN2 is a floral integrator gene. In both *Arabidopsis* and in the temperate cereals, activation of flowering causes expression of genes that promote inflorescence

meristem identity (red), such as APETALA1 (AP1) in Arabidopsis. VRN1 acts as both a flowering time gene in the vernalization response pathway (blue) and as a meristem identity gene during reproductive development (red), (Trevaskis et al 2006).

3.2 Flowering in *Cichorium intybus* (chicory)

Chicory is native to Europe, Asia and North-Africa, and very extensively cultivated in North-Eastern of Italy, as leafy vegetable and as an industrial raw material to obtain inulin from roots.

Different types of chicory (the so called Italian red and variegate types) have been selected by farmers as leafy vegetable. These types show quite different classes of precocity in relation to flowering time. Currently, the main types cultivated in the Veneto region of Italy are: “Rosso di Chioggia”(CHT), early “Rosso di Treviso” (TVP), late “Rosso di Treviso” (TVT), fig. 19-A, “Variegato di Castelfranco”(CTF) and “Rosso di Verona” (VR).

Wild chicory (*Cichorium intybus* L.), fig. 19-D, is a biennial or perennial plant, while the cultivated types behave as strict biennials and during the first year they show a vegetative growth with a rosette comprising more than 80 leaves, depending on the variety and the climatic conditions. In the second year, at the beginning of the spring, the plants enter a new period of growth and produce new leaves until later, the rising temperature and long photoperiod promote the bolting. It results in the development of a flower stalk, approximatively 60-100 cm (fig.19-B), which branches and develops racemes of capitula (fig. 19-F). Flowering begins in May to June. The blue flower (fig. 19-C) open early in the morning and under optimal light and temperature conditions, anthesis is completed before 10 a.m. The seed (fig. 19-E) is the only plant material used for variety commercial propagation; despite this, little research has been done on chicory seed production (Lucchin et al. 2008).

The requirement of a long-day photoperiod (LD) seems absolute in order to flower.

Margara (1977) divided the floral induction process into two sequential phases, *pre-induction*, at which the plant acquires sensitivity to LD (‘photo inducibility’), and *photo-induction*, characterized by a LD requirement. The critical daylength was estimated to be 13 h.

Low temperatures (vernalization) cause earlier and more intensive bolting and flowering in chicory, stimulating plant photo inducibility (Pimpini & Gianquinto 1988; Gianquinto & Pimpini1989 and 1995).
Most available information on the photoperiodic response of chicory comes from *in vitro* cultivation of root tissues (Paulet 1985; Demeulemeester et al. 1995).
According to the genotype, the requirement of a period of low temperature may be absolute or facultative. Vernalization occurs at a temperature below 8°C and sensitivity increases with plant age. It has been demostred that plants are competent to respond to the vernalization treatment only after the third true leaf has been unfolded (Pimpini et al. 1988). Exposure to relatively high temperature (20–25°C) soon after vernalization has a devernalizing effect (Gianquinto 1997). The crops could not tolerate high ambient temperatures and promote stalk elongation when the temperature exceeded 32°C (Krausenbaum 1996).
The genetic control of flowering induction and differentiation is still unknown. Seed stalk emission may depend on the number of leaves which form the rosette, or day length, or both. Many studies reported about the effect of environmental conditions on bolting and flowering of chicory, but this information comes from *in vitro* cultivation of root tissue (Badila et al. 1985; Demeulemeester et al. 1995) and from studies conducted on specific cultivated varieties, so that the results cannot be extended among the different varieties of chicory. In fact, while *Arabidopsis* was not selected by the farmers and thus, conserve its wild genotype, the varieties of chicory derive from a strong human selection. As consequence, the data collected from one particular genotype are not representative for the specie.
In this crop, flower initiation is undesirable, since the rapid growth of the flower-stalk greatly reduces the marketable yield. Extensive bolting mostly occurs in the spring crop, when transplanting takes place in late winter (February-March), due to low temperatures and extending day length. An anticipated or early bolting has to be accurately avoided also because, other that obtains a shedding of unwanted seeds, resulting in weed chicory in the field of the following year, it causes a net loss of commercial production. This situation poses two problems: first, the need of non-bolting varieties able to render the crop at least partially independent of the temperature during the first stage of development; second the need of technical procedures able to permit that selection and seed production of the

selected plants may take place in the same growing season, early enough to proceed according to an annual cadence (Lucchin et al. 2008).
Superior genotypes can be maintained and propagated by *in vitro* culture: chicory explants regenerate easily via organogenesis (Varotto et al. 1997), while so far, embryogenesis has been achieved in hybrids *Cichorium intybus x Cichorium endivia* (Helleboid et al. 1998). The breeding program could also help in developing new varieties late flowering, but unfortunately the genetic control of flowering and development in chicory are far from being understood. Moreover, the breeding may encounter difficulties related to: a) the biennial life-cycle; b) the sporophytic incompatibility system which generally prevents both self-fertilization and intermating among plants with identical incompatibility phenotype, thus, strongly reducing the possibility of intermating (Varotto et al. 1995); c) the difficulty to obtain the synchrony of bolting of the different genotypes through an adjustment of flowering by means of a controlled exposure to long days.
The aim of this work was to obtain a better knowledge of the genetic governing flower induction and vernalization requirements in *Cichorium intybus.* Since *At*FLC is the main flowering repressor characterized until now, its homologous were isolated and characterized in chicory and the expression pattern in response to vernalization determined. The present work introduces new progresses in understanding the molecular mechanism controlling flowering in chicory and establishes the bases for further development of tools for providing varieties with known flowering behaviour.

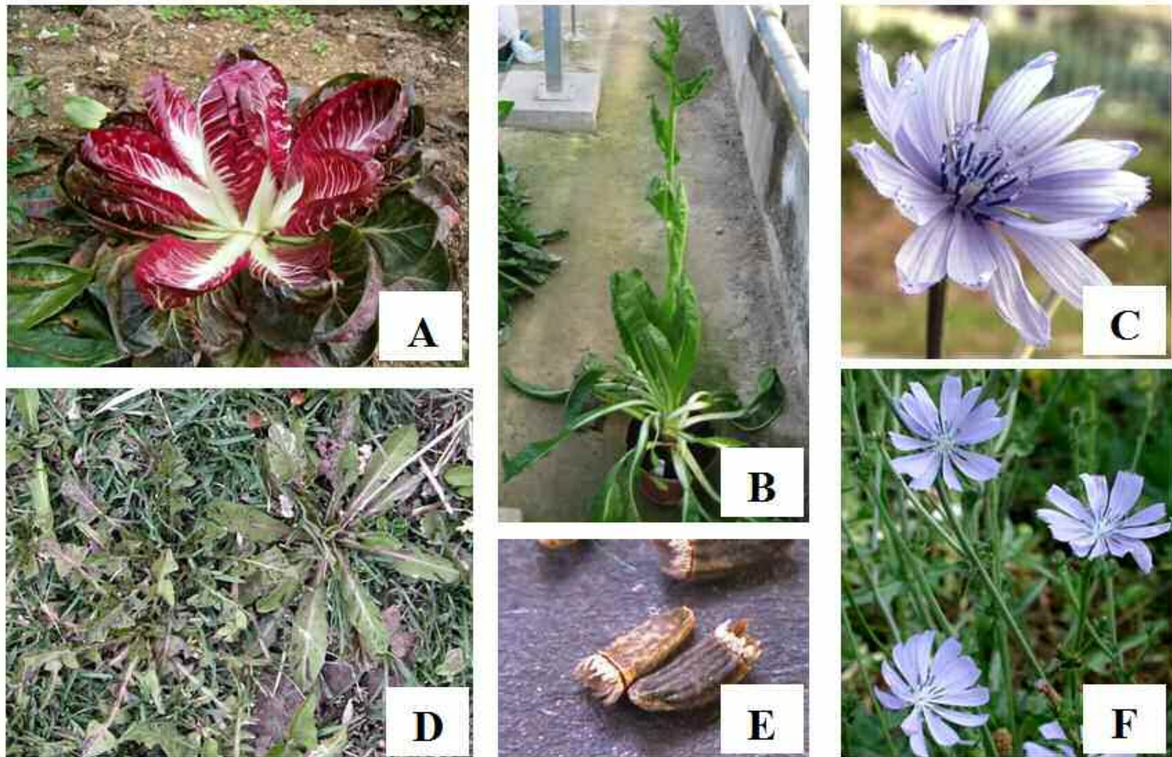


Fig 19 Chicory-TVT plant as appeared during the cultivation in soil (**A**); **B** an inducted TVT chicory with flower stalk; **C** blue flower of TVT chicory; **D** wild chicory in nature field; **E** seeds; **F** racemes of capitula inflorescences of wild chicory.

Chapter IV

Materials and Methods

4.1 Plant Material and treatment

Chicory (*Cichorium intybus* cv *late Rosso di Treviso* L.) was grown in growth chambers with a long day photoperiod, with cool fluorescence light and at 22°C. Plant vernalization was conducted when plants had the third true leaf (stage believed to be the beginning of the competence for the plant to respond at the treatment) at 4°C and with a cycle of LD or SD condition for a minimum of 7 days to a maximum of 60 days. After vernalization, plants were transferred in a growth chamber at 16°C-LD for 15 days to fix vernalization before to move the plants at 22°C-LD until flowering occurred.

4.2 RNA extraction and reverse transcription

2μg of total RNA were extracted from various tissues of chicory according to the manufactures instruction from the "*RNeasy Plant Mini Kit*", Qiagen. In order to remove any trace of genomic DNA, DNase treatment was performed during RNA extraction by using *RNAse free DNase* from Qiagen.

cDNA of chicory was obtained through reverse transcription of total RNA, performed with *SuperScript II Reverse Transcriptase* (Invitrogen), using 0.5μg of Oligo(dT)$_{12\text{-}18}$, supplied by the kit.

4.3 Cloning of *Ci*FLC transcript and sequence analysis

The cDNA of *Ci*FLC was isolated by reverse transcription-PCR (RT-PCR) using primers designed from *At*FLC conserved sequence (forward primer 5'-GGA GCA GCA GAC CCG TCG GCT AC-3' and reverse primer 5'- CTT GGC GTA ACT GCC CCA CTACT- 3'), by using Bioline Short Taq DNA polymerase (Fermentas) and cycle 94°C 2', 94°C 10'' 68°C 1' 72°C 2', for 30 repetitions. The amplicons were cloned into *pGEM-Teasy* vector (Promega) and sequenced by the sequencers ABI 3730 XL, ABU 3700 and ABI 3100, from the CRIBI service of the University of Padova (for details see http://bmr-genomic.it). Sequences were analysed by using blastn, blastx and tblastx algorithms to determine proteinic and nucleic similarities. Then, sequences were edited and aligned using the software Lasergene DNAStar version 7.

4.4 DNA extraction and Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from young leaves of chicory according to CTAB protocols. RNase treatment was performed during extraction by using RNase 10ng/µl concentrated, from Sigma-Aldrich. DNA integrity and quality was checked by visualizing the sample on 0.8% agarose gel. The amount of DNA was determined by spectrophotometer analysis. PCR amplifications of genomic traits of *Ci*FLC gene were performed in GeneAmp PCR System 9700 machine, using Advantage Taq DNA Polymerase from BD-Clontech.

4.5 Semi-quantitative RT-PCR

In order to analyse the response of chicory to cold treatment, and determine the number of days required for a status of full vernalization, *Ci*FLC cDNA amount was checked. The transcript was amplified from cDNA obtained from plants vernalized and not, at different time-points and under different photoperiodic conditions. Amplifications were conducted by using the gene specific primer combination 5'- CGG CGG CTG ATA TAA TCA CAG

GAA TC-3’ and 5’-CAG TCA CGA CGT TGT AAC GAC GC-3’, to amplify the four *Ci*FLCs sequences previously identified, and the combination 5’-ATG TCG GGC GAG TTA AGC CGG-3’ with 5’- AGT AGT ATC AGA GTT TCT GGT CG- 3’ for selective amplification of *Ci*FLC2.

The amount of cDNA dilutions was determined by using the 18S transcript as internal control. The primers used for the 18S amplification were: forward 5’-GGA GCC ATC CCT CCG TAG TTA GCT TCTT-3’ and reverse 5’-CCT GTC GGC CAA GGC TAT ATA CTC GTTG-3’. All the PCR reactions were conducted using *Phusion High Fidelity Taq*, Finnzymes, with cycling instruction as suggested by the guideline; 29 and 26 cycles were respectively used for 18S and *Ci*FLC amplification in all the experiments.

4.6 Analyses of the circadian regulation of *Ci*FLC

A semi-quantitative RT-PCR has been performed to check if *Ci*FLC2 mRNA level changed according to a 24-h rhythm imposed by the circadian clock. Sample collection started at the dawn (time 0) through the day every 4 hours (4 am- 8am- 12 am -16pm -20 pm- 24pm- 4am). Leaf materials were collected and total RNAs were isolated and treated with *DNAse I* (Qiagen). *Ci*FLC2 abundance was analysed by RT-PCR using dilution 1:20 - 1:30 of the original RT reactions. *Ci*FLC2 was amplified by using the selective primers forward 5’- TTA GGC TTA GAG TCT AGC TGT-3’ and reverse 5’- AGT CTG ATC AGA TGG ACC TAC AG- 3’. Amplicons were separated in 1.2% agarose gel and images were captured by Kodak Molecular Imaging software.

4.7 Genome walking

The isolation of *Ci*FLC gene sequences has been carried out by PCR amplification and genome walking through traits of genomic sequences in libraries obtained using the *GENOME-WALKER Kit*- Clontech. All the details for library construction and PCR amplification are available in the Genome-Walker protocol. The primers used for PCR amplification were: **1**- 5’-CAG TCA CGA CGT TGT AAA ACG ACG GC -3’; **2**- 5’-GTC

GGA TTC AGT TCC AGC TCC TGA CAT- 3’; **3**- 5’- GTA GCC GAC CCT TCA AAG TCT TTG AAC GTC T- 3’; each of then was used in combination with the Adaptor Primers supplied by the kit.

4.8 PCR products purification

PCR reactions were visualized on 1.2% agarose gel, thus PCR amplicons were isolated for successive cloning and sequencing by using *Montage-PCR kit,* Millipore and *QIAquick Gel Extraction kit* – Qiagen.

4.9 Preparations of DNA plasmid

Plasmid DNAs were isolated from clones previously identified as positive in the screening through PCR-colonies, using *Plasmid Mini Kit*- Qiagen, according to the manufacturer instructions.

4.10 Engineering of transgene to perform protoplast transformation

The coding sequence of *Ci*FLC2 was cloned upstream of the GFP reporter gene in the modified vector pTZ-19U (fig.1). Stop codon was removed from *Ci*FLC2 sequence, and a KpnI restriction site was inserted at the 3’ end of the reverse primer 5’- ACG CGT GAA TGA AGC GTT TAG AGG TACC-3’. The forward primer had the adjunctive sequence for the enzyme BglII resulting in 5’- AGT GAG GAT CGC TAC GGC CAT AGA TCT-3’. The vector was subsequently cloned into *E. coli* JM-109 strain.

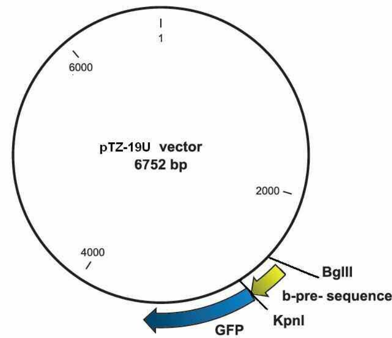


Fig.1. Circular map for the pTZ-19U vector modified. ***Ci***FLC2 was inserted in the vector at the place of the beta pre-sequence.

4.11 Protoplast isolation from chicory

Plantlets of Chicory cv. Treviso were maintained on solidified (0.8% w/v agar) B5 medium (Gamborg et al., 1968) under controlled environmental conditions, in growth chamber at 22°C with 12h day/night photoperiod. Protoplasts were isolated from young leaves (4–5 cm in length) , which were cut into very small strips and placed in a filtered sterilised enzyme solution containing 0.1% (w/v) Cellulase Onozuka R10, 0.05% (w/v) Driselase, 0.02% (w/v) Macerozyme in WS9M solution (27 mg/1 KH_2PO_4, 1.48 g/1 $CaCl_2$ $2H_2O$, 100 mg/1 KNO_3, 250 mg/l $MgSO_4$ and 90 g/1 mannitol at pH 5,6). Strips from leaves were incubated in 20 ml of this enzyme solution and digestion carried out at 28°C for 14–16 h, with gentle agitation and in the dark.

After digestion, the protoplast suspension was filtered through a stainless steel sieve and centrifuged at 1200 rpm for 10 min. Surnatant was removed while the pelleted protoplasts were resuspended in FS13S buffer (KH_2PO_4 0.027g/l, $CaCl_2H_2O$ 1.48 g/l, KNO_3 0.10 g/l, $MgSO_4$ 0.25 g/l, saccharose 130 g/l) and centrifuged again (1000 rpm for 10 min). A disc floating of protoplasts was removed, transferred into a COREX glass centrifuge tube and washed in medium WS9M by centrifuge as above. Protoplast pellet was resuspended in 2 ml of Mannitol-Calcium buffer, $MaCa_3$ (0.5M mannitol, 20mM$CaCl_2$, 0.1%MES, pH to 5.8 with KOH, filter) and protoplasts were counted on the grid of a Burker slide. After counting, protoplasts were centrifuged at 1000 rpm for 5-10 min. Pellet was resuspended in 300 μl of $MaCa_3$ and 10-20 μg of plasmid carrying *Ci*FLC::GFP were added. After a short incubation of 5 min at room temperature, 300μl of fresh and filtered solution of PEG 40%,

were added drop-by-drop. After a centrifuge at 1000 rpm for 5 min the pellet was resuspended with 2 ml of WS9M and cultured at 20°C C in the dark over-night. Transformed protoplasts were observed with a DM4000B microscope- Leica, equipped with a Leica DC300F Camera and Leica Image Manager 50 software (Leica Microsystems-England). Pictures were processed by using Adobe Photoshop 6.0 (Adobe System).

4.12 Construction of transgene to perform *Arabidopsis* transformation

The coding sequence of *Ci*FLC2 was cloned into the vector pENTR^{TM}/D-TOPO-Invitrogen, to exploit the Gateway^{TM} recombination system and finally, insert *Ci*FLC2 into the expression vector pMDC32 (kindly supplied by the *Arabidopsis* Biological Resource Center-ABRC, The Ohio State University). Subsequently, the construct was cloned into *E. coli* strain TOP10 (Invitrogen) for sequencing, to rule out any possible mistakes during cloning.

Agrobacterium tumefaciens C58i_pMD90 conceded by R. Amasino lab, University of Wisconsin was subsequently transformed through electroporation.

Arabidopsis flc-3 mutant (Michaels & Amasino 1999) transformation was carried out by using the floral dip method (Clough & Bent 1998). Transformed plants were selected under 20mg/ml hygromicin and 50mg/ml kanamycin supplied in the medium.

4.13 Construction of transgene to perform chicory transformation

A 306 bp sequence from *Ci*FLC2 cDNA (nt 313-619) was amplified by RT-PCR using forward primer 5' G**GG TAC** ATT GGA GTG AGC TGA GCT A -3' and reverse primer 5'- G**GG TAC** CGA TCT GGA CCG TGA GATT-3'. Both primers included a KpnI site (in bold and underlined) at their 5'-ends. The PCR product was cloned into pGEM-Teasy and excised from the vector by digesting with KpnI. The fragment was cloned into the vector pHANNIBAL at the KpnI site. The vector contained the CaMV 35S promoter and the *Nos* terminator in sense orientation. The resulting plasmid was verified by sequencing and named pHANNIBAL-*Ci*FLCS. Subsequently, the anti-sense fragment containing the

306bp *Ci*FLC2 was amplified from the pHANNIBAL-*Ci*FLCS plasmid using forward primer 5'- CG**G GAT CC**G GTT CAC ACT ATG AGC TA-3' and reverse primer 5'-CG**G GAT CC**GA GAG TTA CCG GAA GAT T- 3'. Both primers contained a BamHI site in their 5'-ends (in bold and underlined). PCR product was cloned into the pGEM-Teasy vector and after verifying the sequence, was digested by BamHI and cloned at the BamHI site in the pHANNIBAL-*Ci*FLCS vector in anti-sense orientation. The resulting RNAi construct was named pHANNIBAL-*Ci*FLCS+*Ci*FLCA. The presence of right and left border flanking the RNAi cassette allowed the Gateway™ recombination to insert the cassette into the expression vector pMDC32 (figure2). The construct obtained was named pMDC32-35S::*Ci*FLCS+*Ci*FLCA and has been used to transform discs leaf of chicory.

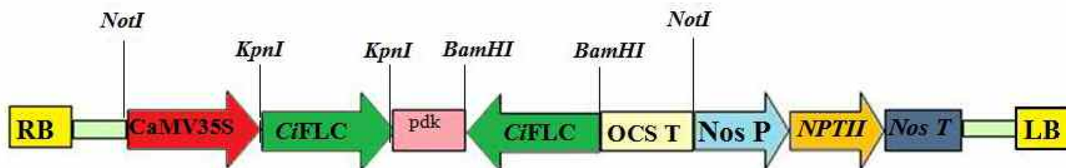


Fig.2 Schematic diagram of *Ci*FLC RNAi construct. The construct driven by CaMV 35S promoter consisted of an inverted repeat of 306 bp fragment corresponding to nt 313-619 of *Ci*FLC2 cDNA. *Ci*FLC repeats were separated by an intron from pdk. The neomycin phosphotransferase selectable marker gene (NPTII) was driven by the Nos promoter. The T-DNA left border and the right border and the position of enzyme sites are indicated.

4.14 Leaf discs transformation of chicory

Leaf discs were excised from young leaves of chicory cv late "rosso di Treviso". After a short incubation (5 min) in a suspension of agrobacterium carrying the pMDC32 35S::*Ci*FLC2 construct or the pMDC32-35S::*Ci*FLCS+*Ci*FLCA, discs were drained in a sterile filter paper, placed on Petri dish containing BFR (MS salt, saccharose, MES, micro agar, NAA, BAP; pH 5.8 with KOH) medium and incubated in the dark at 28°C. After two days, discs were transferred in a new Petri containing the same BFR medium, but supplemented either with kanamycine and cefotaxime. The transformants regenerated from the discs, could be selected for the hygromicin resistance and checked by PCR or Southern Blot for the presence of the transgene in the genome.

4.15 Flowering time analyses

Flowering time was determined by the number of rosette leaves formed when the inflorescence stem reached approximately 1 to 3 cm long. The flowering time of arabidopsis transformants carrying the *Ci*FLC ORF and not, respectively, were assessed. Flowering time of chicory was determinated by observation of SAM profile at the microscope.

4.16 Southern blotting analyses

10µg of DNA extracted from young leaves of chicory plants vernalized and not, were probed with a *Ci*FLC cDNA probe dUTP-DIG (Roche) labelled, lacking the MADS-domain region or through a genomic sequence obtained by genome walking (see par. 5.4 Chapter 5 Results). To remove the MADS-box, *Ci*FLC was previously amplified using primers combination 5'-TTA TCG CCT TAG GAG AAG CTGT-3' and 5'-GTT CCG GTA ACT CTC CCA CTA CT-3'.

In order to determine eventual changes in the pattern of DNA methylation of chicory after the treatment, each DNA combination was digested with the restriction enzymes Sau3AI and NdeII. These enzymes displayed a different sensibility regards the methylation at the same restriction site (GATC). After digestion, DNA fragments were purified and concentrated by using *High Pure Purification kit* from Roche.

Southern blotting experiments and signal detections were performed according to "*The DIG System User's Guide for Filter Hybridization*"-Roche, Boeringer Mannheim.

4.17 *In-situ* Hybridization

Plant materials were collected and fixed in 4% paraformaldheyde (SIGMA), in 0.1M phosphate Buffer pH 7.2, (NaH_2PO_4 5M and Na_2HPO_4 0.5M are mixed to reach the final concentration 0.1M) and 0.1% gluteraldheyde (SIGMA). Samples were then, incubated for 16 hours at 4°C. After fixation, tissues were dehydrated by washing and incubation in

solution at increasing ethanol- xylene concentration, until the samples were only in xylene (protocol described by Varotto et al. 2003). From this point drops of Paraplast Plus (Sigma-Aldrich) were added to the samples in order to progressively substitute the xylene and finally embed the tissues.

The embedded samples were cut in 6- 10 µm sections using a microtome (RM 2135 Leica) and collected in SuperFrost Plus Slides (Menzel-Glazer) to be subsequently deparaffinized and treated with 10µg/ml Proteinase K (Sigma). Sense and antisense probes, were obtained by *in vitro transcription*, using the Digoxigenin RNA labelling mix (Roche) and T7 and SP6 polymerases (Roche), according to the manufacturer instructions. The sequence of the probe corresponded to *Ci*FLC without the MADS-domain, previously described. Hybridization was conducted in 50% formamide at 48° O/N. DIG detection and signal visualization were carried out using NBT and BCiP (both Roche). Thus, the slides were incubated for 16-24 h in the dark at room temperature.

Colorimetric reaction was blocked with ddH_2O for 5 min. The coloration is then developed as follows: 60 sec in EtOH 70%, 30 sec in EtOH 100%, 60 sec in EtOH:Xyl 1:1, 30 sec in Xyl 100%.

Slides were dried and mounted with DPX Mountant for histology (Fluka Biochemika). Hybridized samples were observed with a Leica digital microscope.

4.18 Cytological analyses of chicory apices

Apices from plant vernalized and not, were collected, fixed and embedded as for in-situ hybridization. 6µm sections were produced by using a microtome, and collected in SuperFrost slides to be later deparaffinized as for *in-situ* protocol. Sections were finally dried and mounted with VectaShield® Mounting Medium with DAPI (Vector Laboratories USA). Slides were observed with a fluorescence microscope (Leica DM 4000B).

Chapter V

Results

5.1 Identification of sequences *At*FLC-like in chicory

5.1.1 Molecular cloning of chicory FLC transcripts

Chicory and *Arabidopsis* both require vernalization to flower. Since *FLC* has been identified as the major repressor to flowering in *Arabidopsis*, we decided to assess the presence of *FLC* sequences in chicory. According to the sequence of *Arabidopsis and Brassica spp.*, degenerated primers were designed on the MADS-domain, in the exon downstream of the MADS box and at the level of the C-terminal end (par.4.3). At first, *Ci*FLC was amplified by RT-PCR in a wild accession of chicory that we named F2 line. This population shows a strong self- incompatibility. Our propose was the identification of FLC in cultivated accessions of chicory, finally, we also isolated the gene in a cultivar manifesting a biennial phenotype and requiring vernalization to flower: late Rosso di Treviso (TVT). Three *Ci*FLC homologs were identified in the F2 population of wild chicory and they count 503 bp, 236 bp and 182 bp respectively (fig. 1a). Their nucleotidic sequence is showed in fig. 1-Appendix1. Four *Ci*FLCs were identified in TVT chicory (fig. 1b). Two of these sequences were highly homologous between F2 population and TVT (100% of amino acidic identity). In particular, we found that FLC2 corresponds to FLCa, while FLC4 corresponds to FLCb. FLC1 and FLC3 seem peculiar to TVT, while FLCc seems characteristic of the wild accession. The amino acidic alignment of the transcripts is shown in fig.2.

Fig.1. Agarose gel image showing FLC amplification by RT-PCR, in wild chicory F2 and in cultivated chicory TVT. ***a)*** RT-PCR was conducted on F2 chicory (in the picture beside). The result showed only two amplicons but after cloning and sequencing of the PCR products, three sequences were identified. These sequences correspond to three variants of *Ci*FLC-like, that we named FLCa, FLCb and FLCc; the ladder used is *1kb plus* from Invitrogen. ***b)*** RT-PCR was conducted on leaf samples of TVT chicory (in the picture beside). 4 amplification products corresponding to the four *Ci*FLCs like were identified, cloned and sequenced. The ladder is *1kb plus* from Invitrogen.

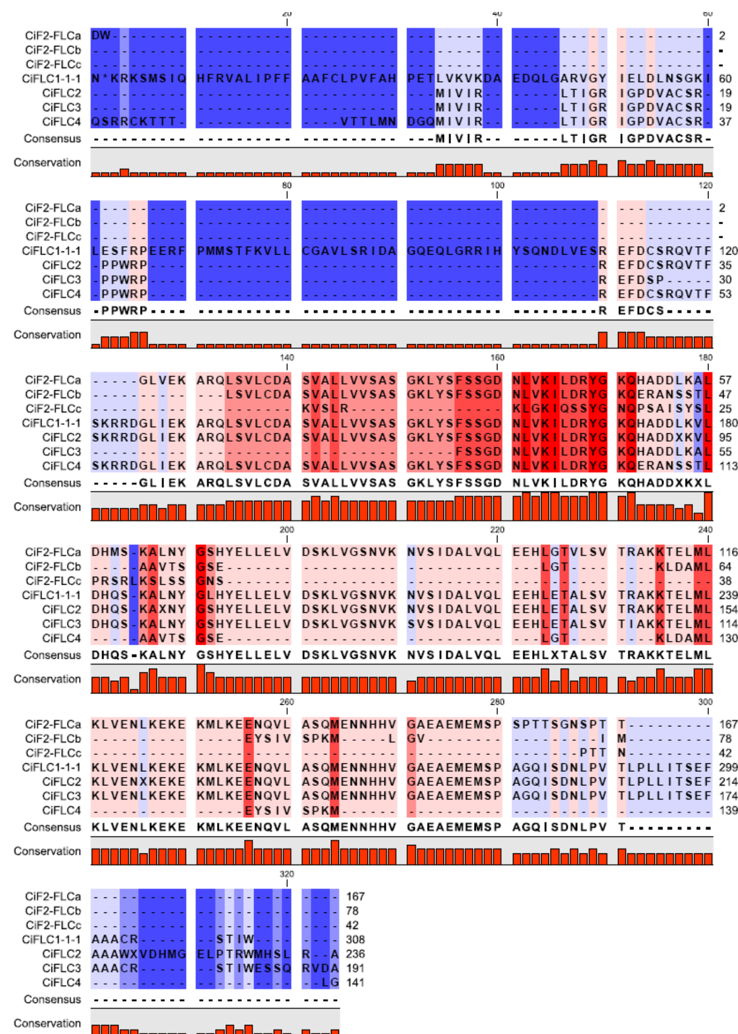


Fig.2. Amino acidic alignment between sequences of *Ci*FLCs isolated from wild chicory-F2 and cv Treviso. cDNAs from the wild chicory are indicated as *a, b, c;* while the transcripts isolated in cv Treviso are indicated by *1, 2, 3* and *4*. Note the high conservation between the sequences *a* and *2*; and between *b* and *4*. The sequence *Ci*FLC1 is conserved in its central part in respect to the other *Ci*FLCs. *Ci*FLCc is a variant totally different from the others. The identical amino acid residues are indicated

by red background, the different amino acid residues are indicated by graduation of blue background. The degree of conservation is indicated by the graph below the consensus. Alignment was created by using clustalW algorithm.

5.1.2 Sequence analysis of *Ci*FLCs

The cDNAs corresponding to *Ci*FLC homologs were cloned into the *pGEM-Teasy vector* (Promega) and the nucleotidic sequences were translated *in silico* into amino acidic sequences using the software provided on-line on the Expasy database (http://www.expasy.org). Alignment of these sequences was carried out by Lasergene DNAStar 7 Package and CLC Combined Workbench 3 software.

*Ci*FLC1 is 925 bp long, corresponding to 308 amino acids. Compared to *At*FLC, it shows a percentage of amino acidic identity of 69.8%; a 60 aa insertion at the 3' end and a 14 aa insertion into the MADS box domain were observed.

*Ci*FLC2 is 703 bp long, corresponding to 236 amino acids. This sequence is the more similar to that of *At*FLC (72% of amino acidic identity).

*Ci*FLC3 is 507 bp long and is identical to *Ci*FLC2, but for a missing lack 40 aa sequence inside the MADS domain (52% of amino acidic identity with *At*FLC).

*Ci*FLC4 is only 425 bp long; translation of this sequence reveals an amino acidic sequence that partially resembles the 3'-end of FLC and contains 6 amino acids from the MADS-box (GLVEKA) (36% of amino acidic identity with *At*FLC).

Nucleotide sequences and protein sequences are shown in figure 3 and fig. 4 respectively.

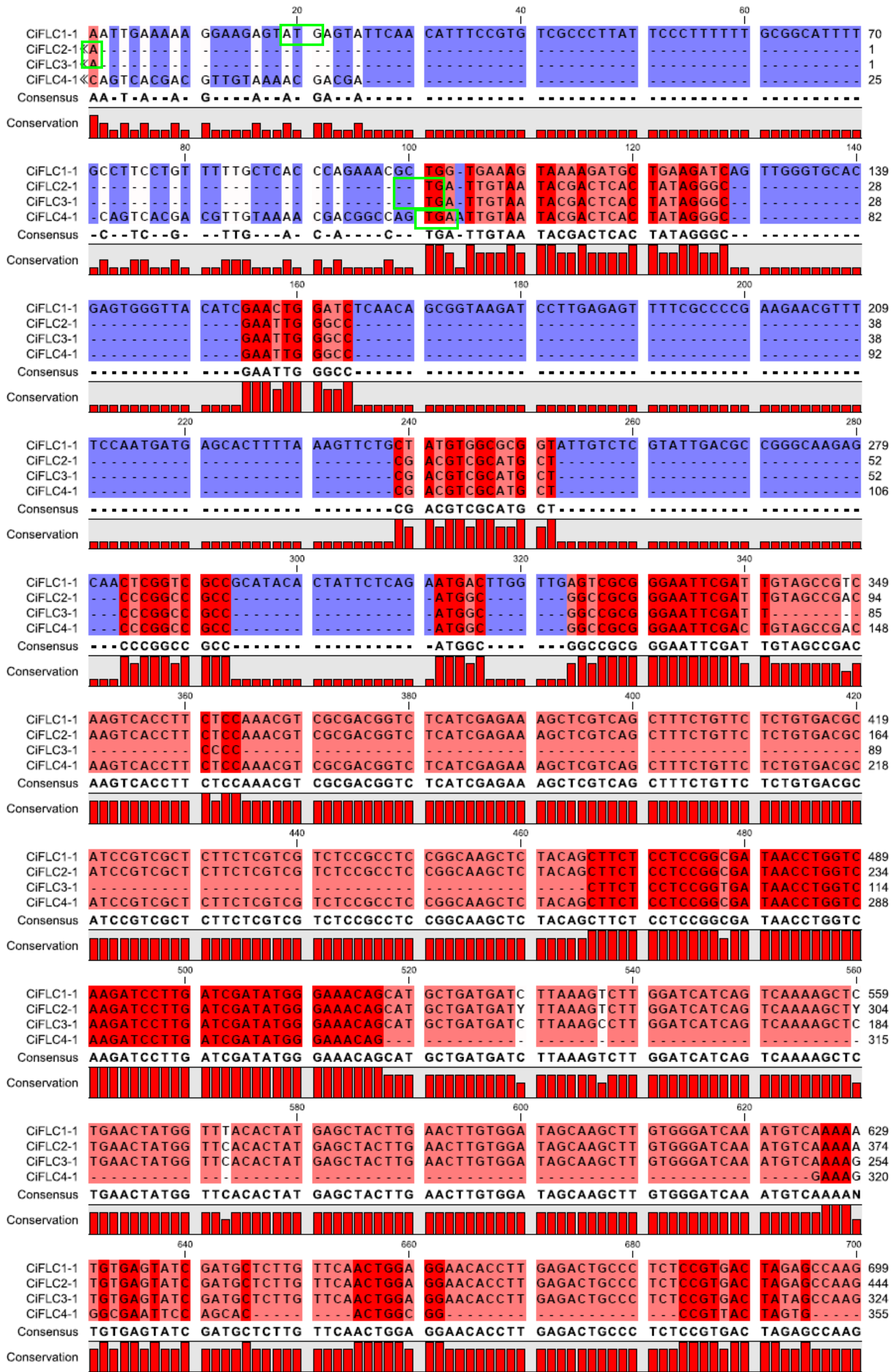
It is important to note that in all the sequences described above, the START codon, was deduced on the bases of the *Arabidopsis* sequence, but still not verified because the full-coding sequences were not been yet isolated (fig. 3).

We focused our study on the functional characterization of *Ci*FLC2, which is the candidate with the highest protein similarity to the *At*FLC structure and presents a sequence conserved in wild chicory F2 (Fig.4, 5 and 6). For nucleotidic sequence alignment see Fig 2- Appendix 1.

The alignment of the amino acidic sequences among the FLC-like isolated so far, shows that *Ci*FLC2 possesses its own characteristic insertion into the MADS domain that, being

also present in the wild F2 population, can be proposed as peculiar for the *Cichorium* species (fig. 7).

The blasting report against the NCBI database for this 14 aa additional sequence does not reveal any information about the putative function of the sequence.


CiFLC1-1
CiFLC2-1
CiFLC3-1
CiFLC4-1
Consensus
Conservation

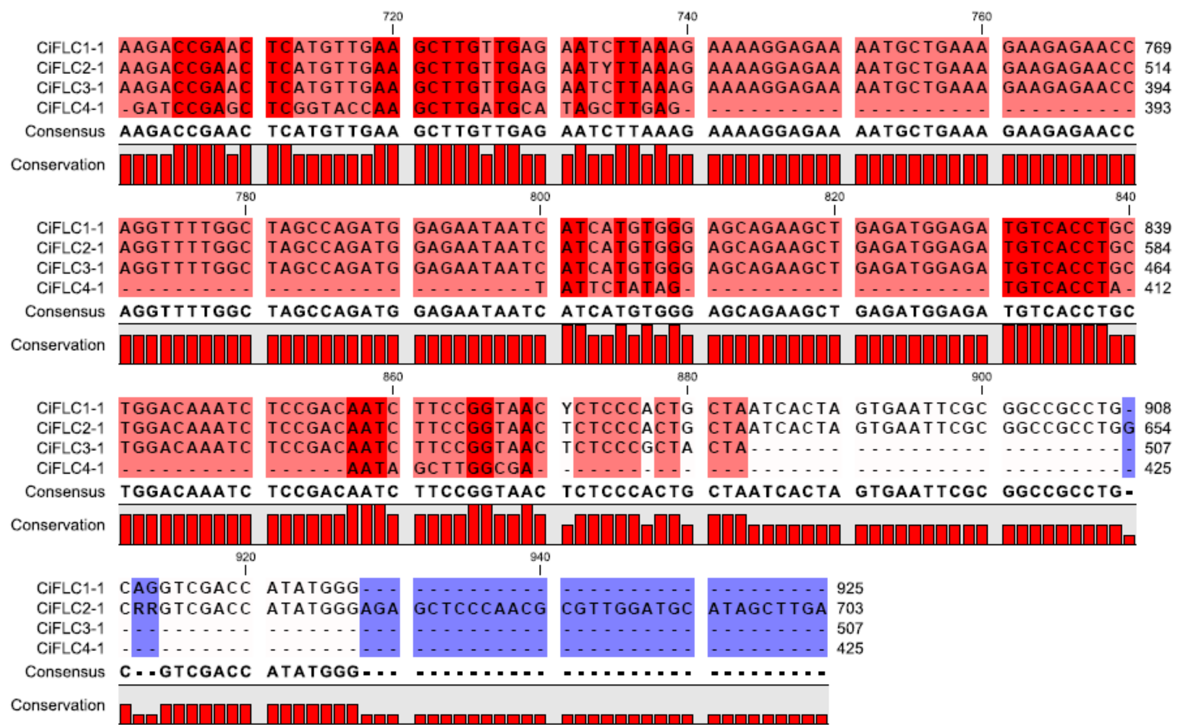


Fig.3. Nucleotidic alignment of partial cDNA sequences corresponding to the hypothesized full-coding cDNAs of the chicory FLC variants. The supposed START codon is underlined by a green box, the STOP codon is not identified. Interesting to note that in FLC4 the START codon is not an ATG but TGA, which usually is read as STOP code. Alignment has been produced by ClustalV V algorithm.

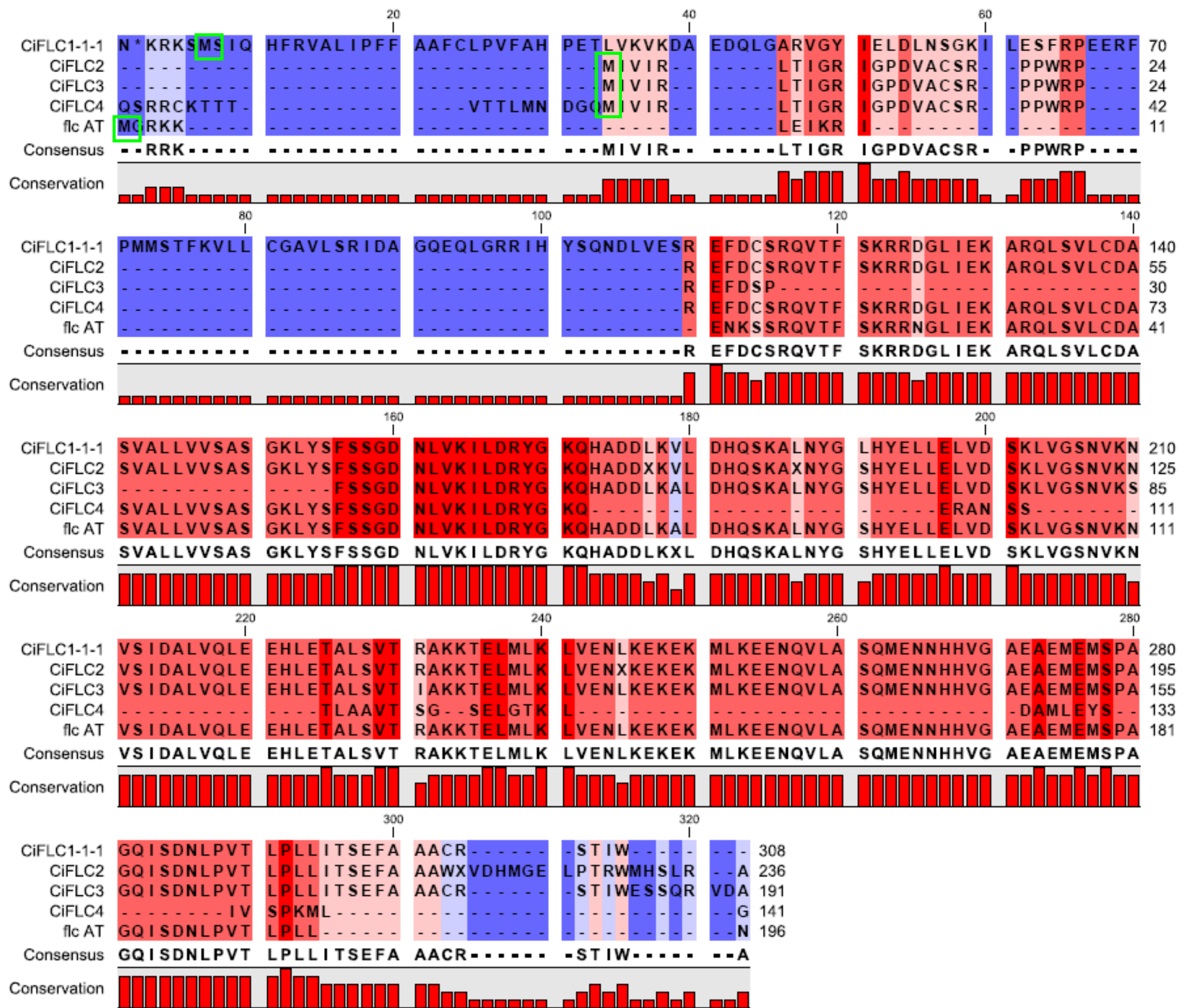


Fig.4. Amino acidic alignment between *Ci*FLCs variants and FLC isolated in *Arabidopsis*. A high conservation among the sequences is evidenced from the red background and from the graph below the consensus. The domains conserved comprise most of the MADS domain until the end of the FLC sequence, with exception of *Ci*FLC4 that lack in many pieces of sequence and *Ci*FLC3 that lack 40 amino acids inside the MADS box. It is noteworthy to note the high conservation between *Ci*FLC2 and *At*FLC. START codon is evidenced by a green box. Alignment has been produced by ClustalW.

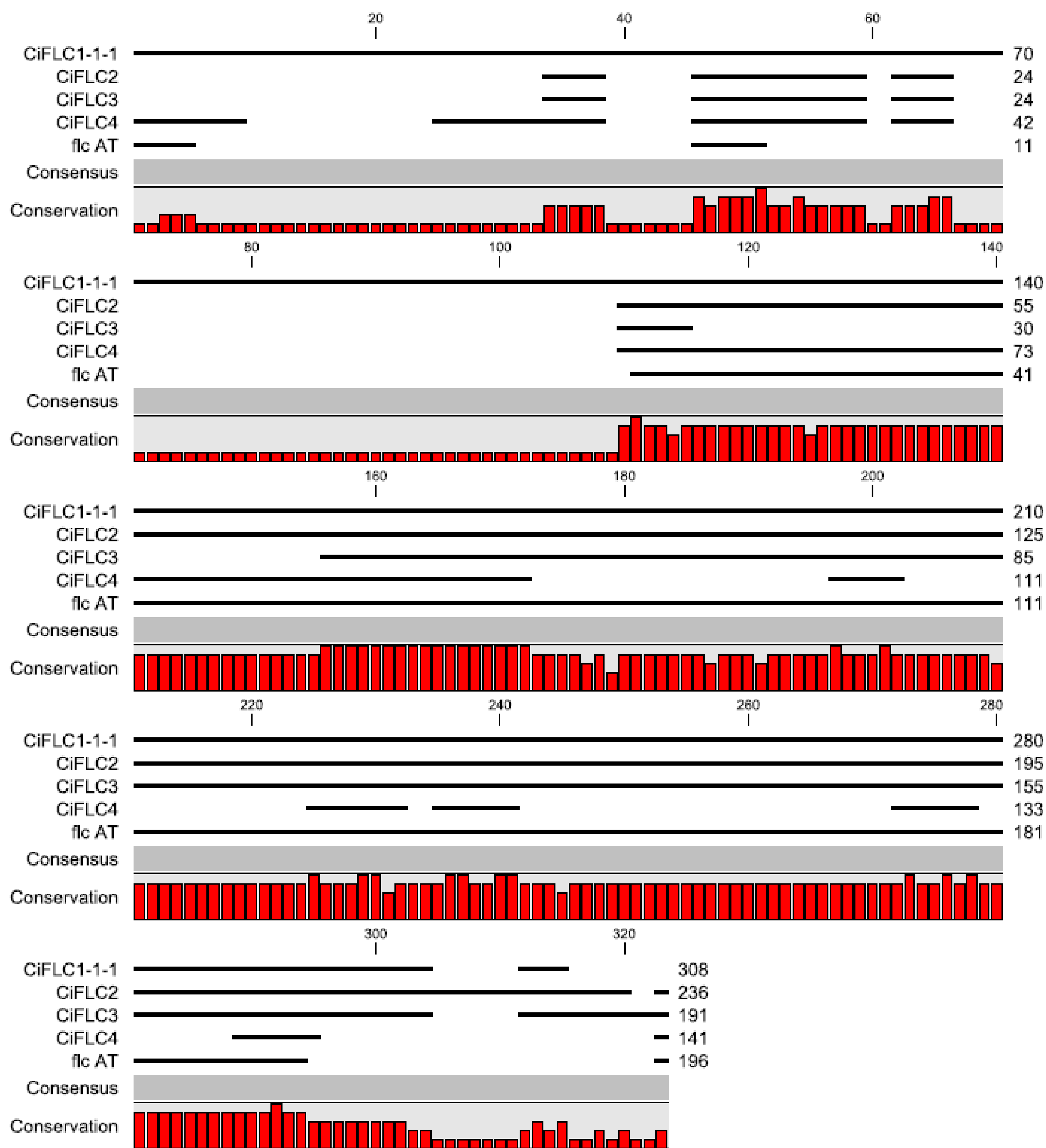


Fig.5. Schematic view for the amino acidic alignment showed in fig 4.

```
FLC At ecotype Col-0-1  MGRKKLEIKR I--------- -----ENKSS RQVTFSKRRN GLIEKARQLS VLCDASVALL VVSASGKLYS  56
                CiFLC2  MIVIRLTIGR IGPDVACSRP PWRPREFDCS RQVTFSKRRD GLIEKARQLS VLCDASVALL VVSASGKLYS  70
             Consensus  MXXXXLXIXR IGPDVACSRP PWRPREXXXS RQVTFSKRRX GLIEKARQLS VLCDASVALL VVSASGKLYS
          Conservation

FLC At ecotype Col-0-1  FSSGDNLVKI LDRYGKQHAD DLKALDHQSK ALNYGSHYEL LELVDSKLVG SNVKNVSIDA LVQLEEHLET  126
                CiFLC2  FSSGDNLVKI LDRYGKQHAD DXKVLDHQSK AXNYGSHYEL LELVDSKLVG SNVKNVSIDA LVQLEEHLET  140
             Consensus  FSSGDNLVKI LDRYGKQHAD DLKXLDHQSK ALNYGSHYEL LELVDSKLVG SNVKNVSIDA LVQLEEHLET
          Conservation

FLC At ecotype Col-0-1  ALSVTRAKKT ELMLKLVENL KEKEKMLKEE NQVLASQMEN NHHVGAEAEM EMSPAGQISD NLPVTLPLL-  195
                CiFLC2  ALSVTRAKKT ELMLKLVENX KEKEKMLKEE NQVLASQMEN NHHVGAEAEM EMSPAGQISD NLPVTLPLLI  210
             Consensus  ALSVTRAKKT ELMLKLVENL KEKEKMLKEE NQVLASQMEN NHHVGAEAEM EMSPAGQISD NLPVTLPLLI
          Conservation

FLC At ecotype Col-0-1  ---------- ---------- -----N  196
                CiFLC2  TSEFAAAWXV DHMGELPTRW MHSLRA  236
             Consensus  TSEFAAAWXV DHMGELPTRW MHSLRX
          Conservation
```

Fig. 6. Amino acidic alignment between *Ci*FLC2 and *At*FLC ORF. In this view, high conservation among the amino acidic residues is indicated by red background and amino acid number is indicated on the right. Alignment has been produced by ClustalW algorithm

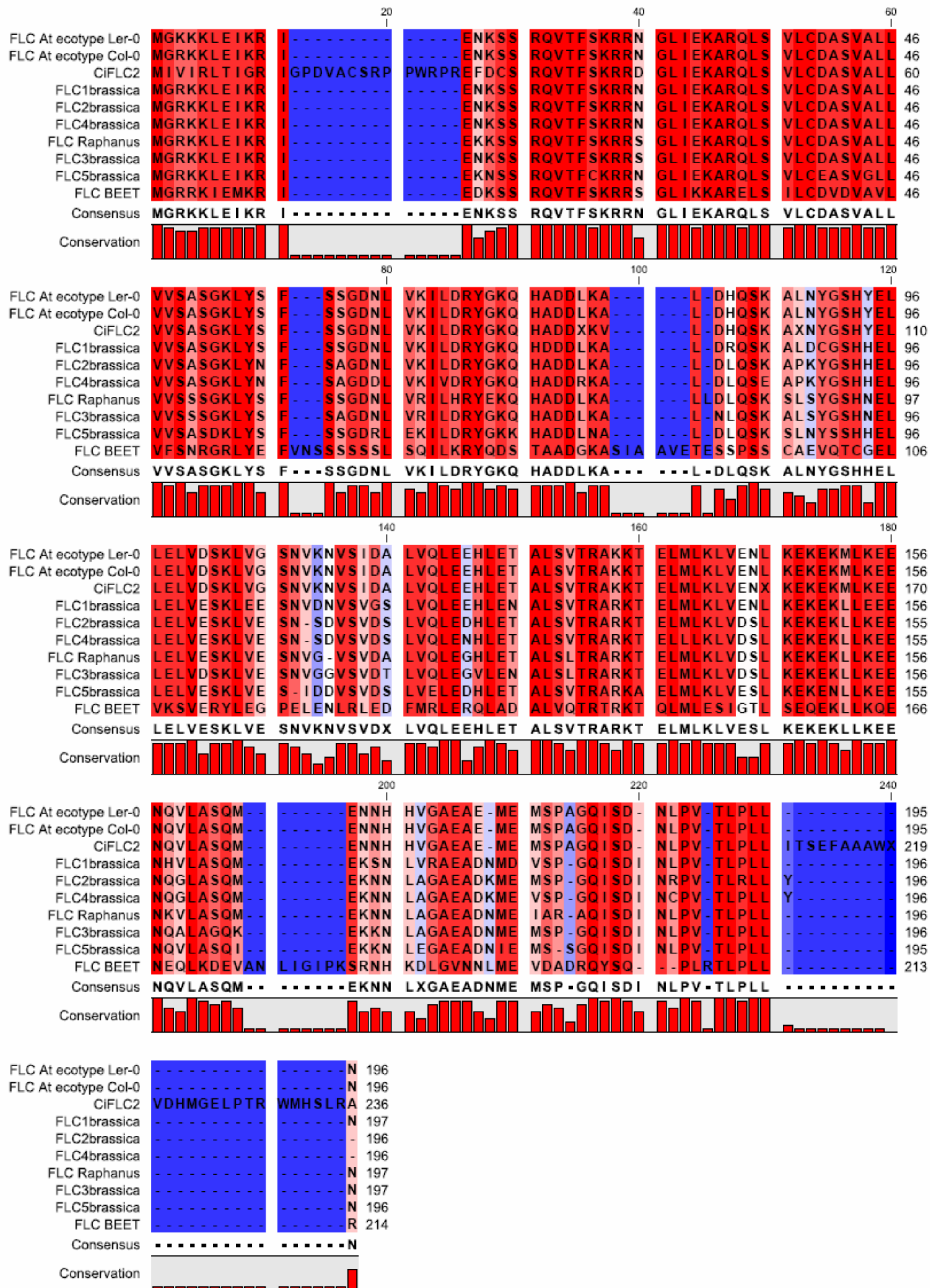


Fig 7. Amino acidic alignment of FLC sequences isolated in *Brassica, Raphanus, Arabidopsis, Beet* and *Cichorium* species. A short region (14 amino acids) is additional only in *Ci*FLC2, so that we could suppose that this region could be characteristic of the *Cichory* specie. A high similarity emerges between the sequence of FLC isolated in chicory and the one isolated in *Arabidopsis*; only 14 amino acidic substitutions are valuables.

5.2 Southern Blotting analysis

RT-PCR showed the presence of 4 *Ci*FLC-like transcripts. In order to define if these 4 variants could be addressed to 4 different gene copies or splice variants, we set up a southern blotting experiment.

Leaf materials were collected from seven different chicory cultivars: Lusia Adige, LA; red of Verona, VR; wild type chicory, F2; Treviso late flowering, TVT; red of Chioggia late flowering, CHT; red of Chioggia early flowering, CHP; variegate of Castelfranco, CTF and *Arabidopsis* var. *Columbia* (At). DNA was extracted, digested with EcoRI and probed with a cDNA *Ci*FLC2 sequence lacking the MADS domain and labelled with Digoxigenin.

The result in figure 8 shows the presence of more than one hybridization band, with 3 of them present in all the cultivars.

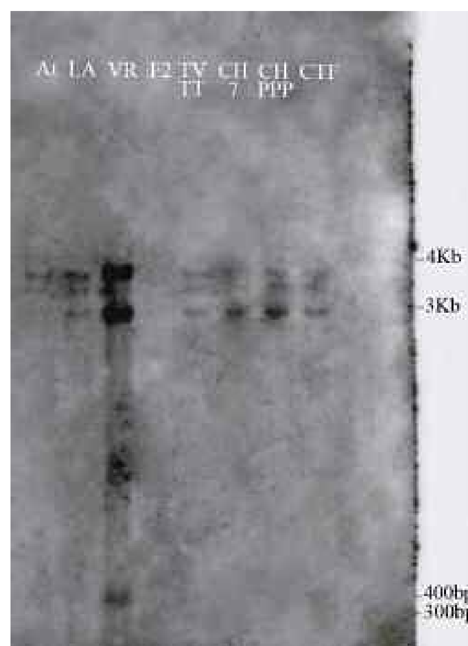


Fig.8 Southern blotting showing the pattern of hybridization of a cDNA *Ci*FLC probe, lacking the MADS domain, against genomic DNAs. Seven different cultivar of chicory have been analysed and compared to arabidopsis. DNA was extracted from leaves and digested with a six-cutter enzyme, EcoRI. In F2 is not observed a pattern of hybridization because the sample was lost during the loading in the agarose gel. Three signals are instead well evident in all the cultivars of chicory, in a range comprise between 3 to 4 kb of length. In this range only two bands of arabidopsis are represented while a higher signal over 4 kb is weakly detected. The cv VR shows a peculiar pattern of hybridization, being the only one with an additional signal of approximatively 380bp. This pattern could be however addressed to the higher amount of DNA loaded for VR, respect to the other cultivars.

5.3 Analysis of *Ci*FLC expression

5.3.1 Analysis of *Ci*FLC expression through the plant

FLC is a floral repressor that in *Arabidopsis* has been shown to be expressed in young leaf primordia, shoot apical meristem (SAM) and root apical meristem (RAM). Localization of FLC has been related with its function. In fact, FLC directly represses SOC1 and FT in the phloem and meristem delaying flowering. We have tested mRNA expression by RT-PCR in chicory and found that FLC was detectable in leaves, apex and in floral tissues, from the stage of bud to mature flower (Fig 9 A and B).

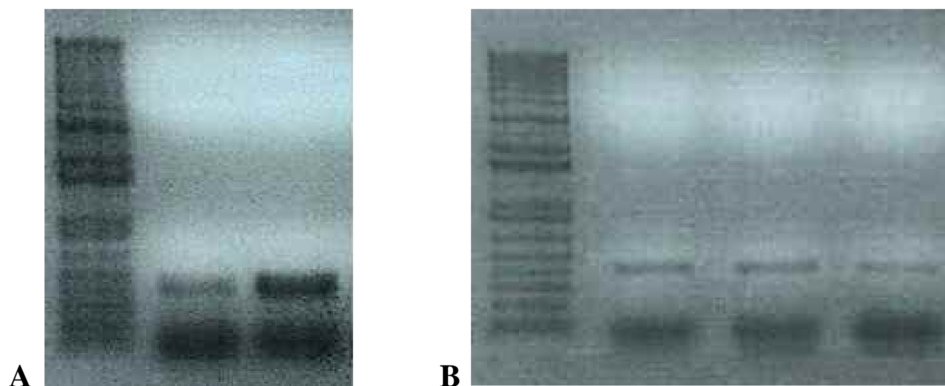
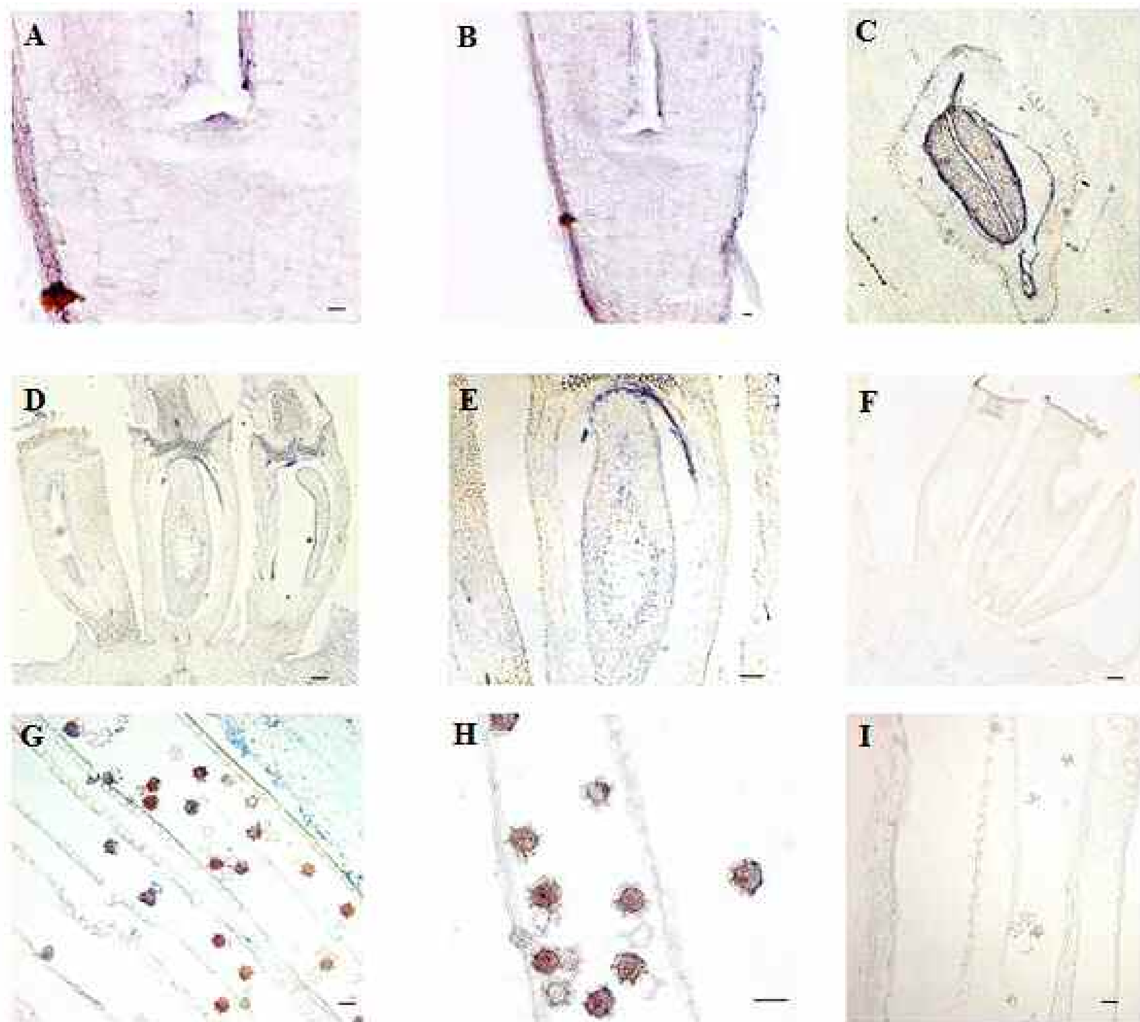


Fig. 9. RT-PCR showing FLC expression in different tissues of wild chicory. Signal is just qualitative, PCR is not normalized. **A)** *Ci*FLC expression in SAM (first lane) and leaves, (second lane). **B)** *Ci*FLC cDNA expression in bud, mature flower closed into sepals and fertilized flower.

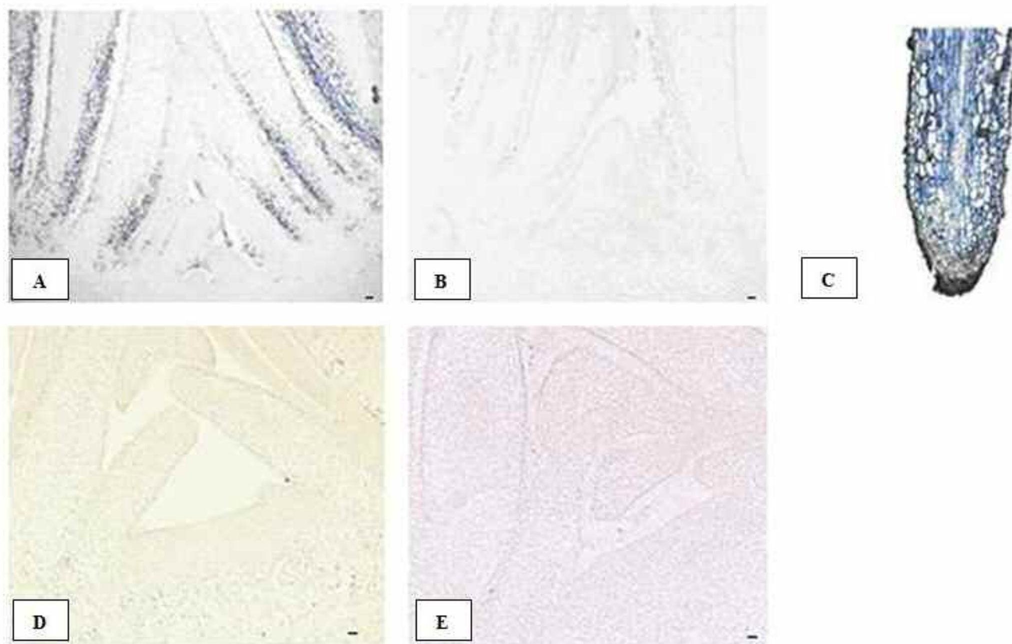
In order to improve our understanding of *Ci*FLC expression and localization, we performed an *in-situ* hybridization on flower tissues and meristematic apices.

*Ci*FLC was detectable in the early embryonic stage at the SAM level and in the cotyledon margins. The signal was moreover detectable in floral tissues, in particular was weakly visible inside the ovules and in the mature pollen. The signal was not detectable in the negative control sections (Tav.1). Finally, *Ci*FLC resulted expressed in the RAM (Tav.2-C), but was not detectable at the level of SAM in the adult plant, whereas was strongly expressed in the adaxial side of the leaf primordia, (Tav.2-A, B).

Our experiment revealed that *Ci*FLC was quickly downregulated by vernalization and that the status of full vernalization coincided with the change of the SAM morphology. In order to prove our hypothesis, we carried out an *in-situ* hybridization in the SAM of vernalized plants and, as expected no signal was detected in the apex neither at the adaxial face of the leaf primordia (Tav.2-D).



Tav1. In situ hybridization showing FLC expression in different tissues. In **A** longitudinal section of embryo. The signal is significant in the SAM and around the cotyledons. In **C** transversal section of embryo. The signal is detected in the margins of the cotyledons. In **D** longitudinal section of flower. The ovules are well defined and a weak expression of FLC is appreciable. In **E** particular of ovule showed in picture D. In **F** negative control. None expression is noticeable. In **G** longitudinal section of anthers. The pollen grains shown an expression which is insignificant in the control, picture **I**, but worthy in **G** and **H**. Bars= 100µm.



Tav.2. *In situ* hybridization on SAM and RAM of chicory. In **A** longitudinal section of apex of chicory not vernalized. FLC expression is detectable in leaf primordia, in particular in the adaxial surface of the leaf but is not detectable in the apex. In **B** negative control. In **C** longitudinal section of root. FLC results expressed through the RAM. In **D** longitudinal section of SAM of chicory in a plant vernalized 30 days. None signal is detectable through the apex and the leaves as in the negative control (**E**). Bars= 100µm.

5.3.2 Transient expression of *Ci*FLC2 and *Ci*FLC3 in protoplast

In order to verify the functionality of the transcript chosen in this work, we tested whether the sequence was actually *translated* into protein and, eventually, *where* the *Ci*FLC protein localized. For this purpose we generated a construct carrying the β-GFP sequence present in the vector pTZ-U19. The STOP codon of *Ci*FLC2 first, and *Ci*FLC3 later, were deleted and immediately fused with the start codon of the GFP reporter into the vector.
Protoplast preparation has been carried out from leaves of chicory cultivar TVT and two different experiments of transformations were performed.
In figure 10 we show the sub-cellular localization of *Ci*FLC2. Analogous expression was detected for *Ci*FLC3 (not shown). Nuclear localization of the FLC protein is revealed by the GFP expression. This result is consistent with the function of FLC as a transcription factor. The coincidence in the expression pattern of *Ci*FLC2 and *Ci*FLC3, lead us to

exclude a role for the 14 amino acids insertion in the MADS-box as responsible for the correct localization of *Ci*FLC2.

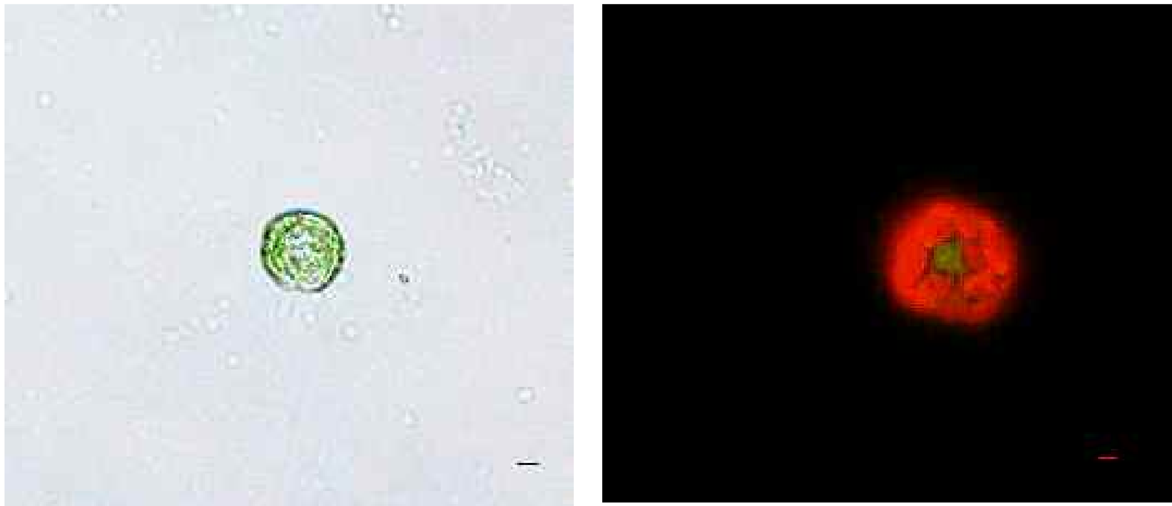


Fig. 10 GFP expressions in chicory protoplast harbouring the *Ci*FLC2::GFP construct. Expression was visualized into the nucleus. Images were observed at the fluorescence microscope. Under white light, on the left, and under fluorescence light, on the right. Bars=100µm

5.4 Determination of vernalization requirement and mechanism of *Ci*FLC regulation

5.4.1 Repression of *Ci*FLC expression by vernalization

To determine whether chicory FLCs, as *At*FLC, are involved in controlling flowering and are downregulated by the vernalization pathway, semi-quantitative RT-PCR was carried out and the pattern of FLC expression in response to vernalization was analysed. Seedlings of TVT chicory were vernalized at 4°C both under LD and SD photoperiod for 0, 7, 15 and 30 days.

As shown in fig. 11-A three of the four *Ci*FLC variants responded after 7 days of cold treatment under LD. Plant vernalized in LD flowered after two months after the treatment.

From this point, for all the reason mentioned before, we decided to focus our study only on *Ci*FLC2 expression. The primers used to selectively amplify this variant were: forward primer 5'-AGT AGT GGG AGA GTT ACC GGA AG-3' and reverse primer 5'-CAC ATT TTT GAC ATT TGA TCC CAC A-3'. Under SD the molecular effect of vernalization was not significant, (fig. 11-B). In fact, plants vernalized in SD never flowered before two growing seasons.

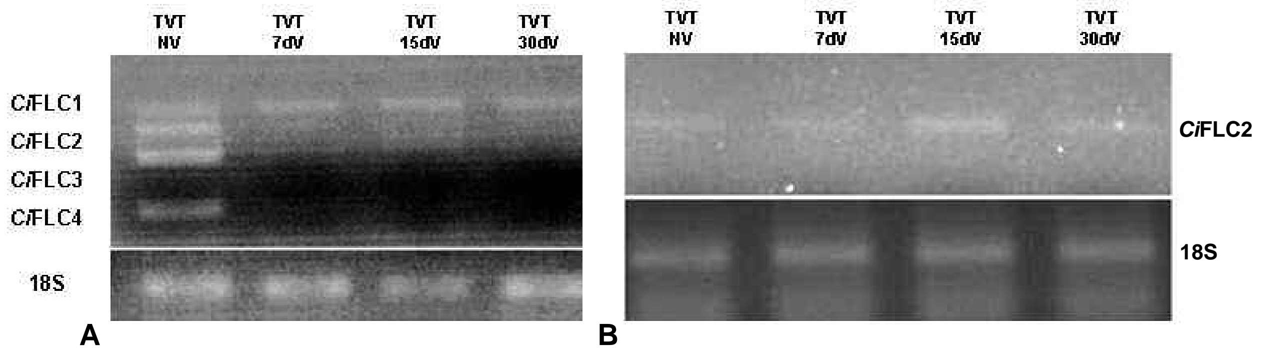


Fig. 11 Agarose gel image showing results of FLC downregulation by vernalization in chicory. **A)** Seedling of TVT chicory were grown under LD photoperiod and moved into a cold room at 4°C-LD when the third true leaf was visible. RNA extraction was prepared from leaves after 7, 15 and 30 days of treatment. The level of *Ci*FLC decreased rapidly after 7 days. **B)** Seedling of TVT chicory were grown under LD photoperiod and moved into a cold room at 4°C-SD when the third true leaf was emitted. RNA preparation was made from leaves material after 7, 15 and 30 days of treatment. FLC did not decrease even after 30 days.

Total number of leaves is the parameter generally used to compare different flowering behaviours in regards to diverse environmental conditions or genetic backgrounds. Because chicory takes a very long time before that inflorescence is visible (more than 3 months after vernalization, when plants have about 200 leaves) we looked for another parameter to use in our study. It is known that in the model plant *Arabidopsis*, the vegetative shoot apical meristem undergoes modifications of its shape after that induction has been triggered. For this reason we analysed the SAM shape in non-vernalized and vernalized chicory plants, so that we would relate a FLC decrease to morphological modifications of the SAM. We observed that until the SAM was vegetative, its shape was concave and the three layers L1,

L2 and L3 were well distinguishable. After a period of vernalization, when FLC expression was strongly decreased, SAM resulted flattened and looked as, a thick layer of cells substituted the three domains L1, L2 and L3, and the diameter of the apex was enlarged, (fig. 14-C). All these modifications agree with the geometrical arrangements which characterize the switch from vegetative to a reproductive meristem. After this stage we were able to follow the phases of flower formation reported in figure 12.

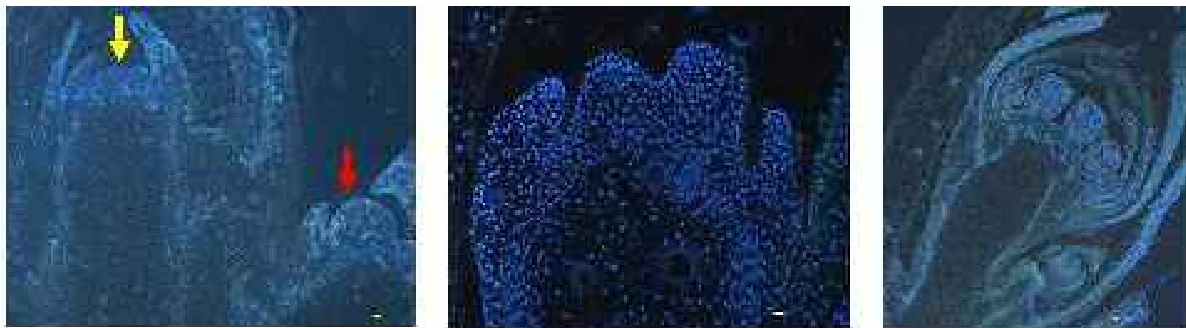


Fig.12 Fluorescent microscope images of different stages of floral development in chicory. From the left to the right, in the first picture is possible to note two different stages of flower meristem initiation indicated by the arrows. The meristem indicated by the yellow arrow is in early stage of differentiation, marked by the flat profile. The meristem below, indicated by the red arrow, is in advanced differentiation, floral primordia are in formation. Beside an advanced stage of differentiation, where rearrangement of the meristem is well appreciable. In a scale of evolution this stage can be placed between the two meristems indicated in the first picture. In the third picture the floral structures are defined. Bars=100 µm

In the case of vernalization under SD photoperiod, FLC level remained high (Fig. 11B) and SAM shape maintained the vegetative structure, fig.14-G. According to this data we can say that the SAM shape is related to FLC downregulation in chicory.

Because in SD we did not observe any change in FLC2 level of expression and any rearrangement in SAM shape, we were interested in investigating the possibility that *Ci*FLC2 regulation might be dependent only from the photoperiod rather than from

vernalization. In order to explore this hypothesis we monitored FLC2 expression in TVT seedlings grown at 22°C-LD, then we analysed FLC2 expression at two time points: when plants emitted the third true leaf (TVT 0) and after 30 days of growth (TVT 30D-LD). As results in figure 13, *Ci*FLC2 expression was not affected; neither SAM shape was changed (fig. 14-H). This result confirms the regulative role for the vernalization on FLC expression.

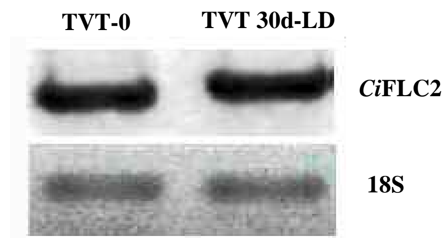


Fig. 13 Semiquantitative RT-PCR for leaf sample of plants grown at 22°C in LD. Through RT-PCR we showed that *Ci*FLC2 transcript level did not change even after 30 days of growth in LD. PCR conditions were controlled by using the 18S amplification as internal control.

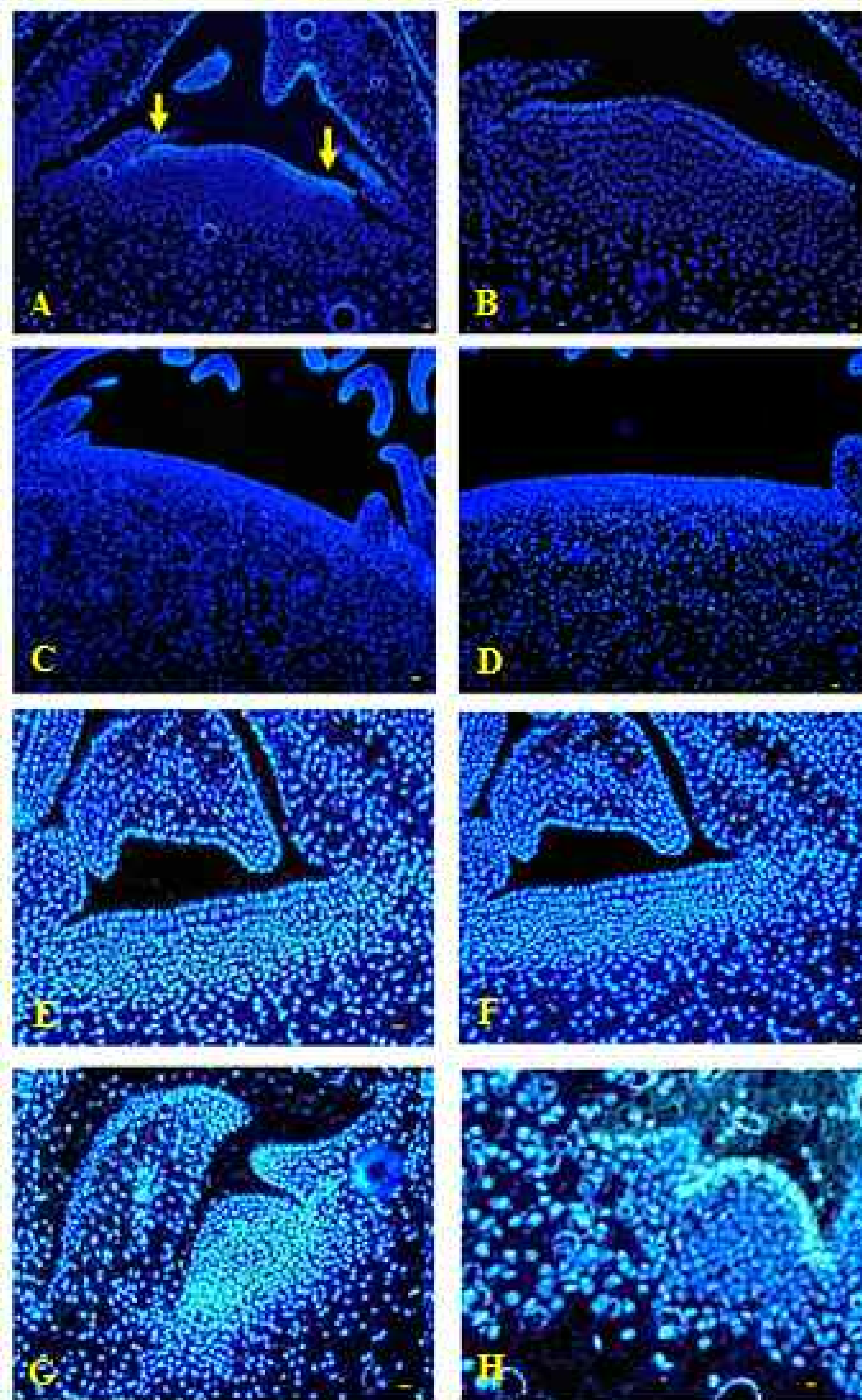


Fig.14 Longitudinal sections through a shoot apical meristem (SAM) of *Cichorium intybus* cv. Treviso stained with DAPI. **A, B**: Adult plants were not vernalized. The three layers L1, L2 and L3 are well defined. Leaf primordia are indicated by the arrows in fig.B. **C, D**: young plants were vernalized 7 days at 4°C in LD conditions. Only one thick layer of cells is distinguishable and the diameter of the SAM is drastically increased. In **E, F**: Young plants vernalized 30 days. Floral meristem begins its organization. **G,** Young **p**lants were vernalized for 30 days at 4 °C in SD conditions. SAM organization with L1, L2 and L3 layers is still maintained. **H**, Young plants were grown at 22°C for 30 days after emission of the third true leaf, in LD conditions. Bars=100µm

In chicory we observed that vernalization elicited its response under LD, but not under SD. In order to understand the reason of this molecular control, we decided to analyse FLC level of expression during the day and verify if this expression was affected by the rhythms imposed from the circadian clock. Seedlings of TVT chicory were grown under LD conditions at 23°C. Leaf material was collected to analyse by RT-PCR the *Ci*FLC2 mRNA. Samples were collected at different time points during the day. The results in fig. 15 demonstrated that FLC2 transcription was unaffected by the circadian clock. So that, the missing response of *Ci*FLC2 at the cold treatment in SD could not be addressed at a not favourable expression of *Ci*FLC2 during the day.

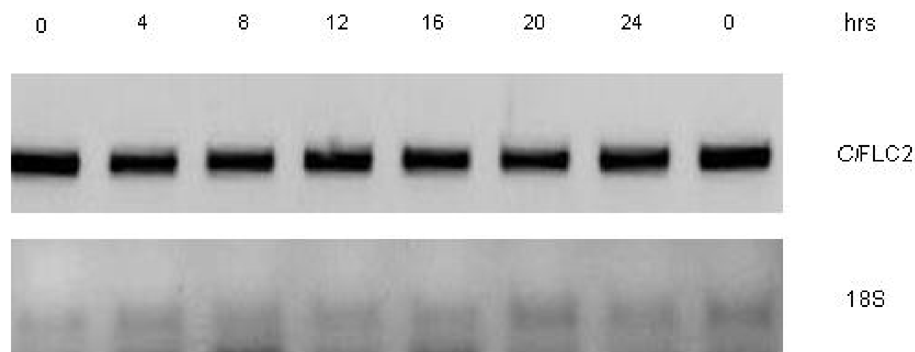


Fig15. RT-PCR showing the pattern of *Ci*FLC2 expression during the day when plants grown in LD. PCR conditions were normalized using 18S amplification as internal control. Each lane corresponds to the hour of which the sample has been collected. As you can see the level of *Ci*FLC2 expression did not change during the day.

In order to verify if FLC repression were stable after plants returned at the warm condition, the level of FLC expression was analysed 40 days from the end of the cold treatment and apex profile was examined.

As shown in fig. 16, FLC expression was lower in respect to the not vernalized samples, according to the "flat" profile of the meristem. This result confirms that the machinery operating FLC repression works in the same manner than in *Arabidopsis.*

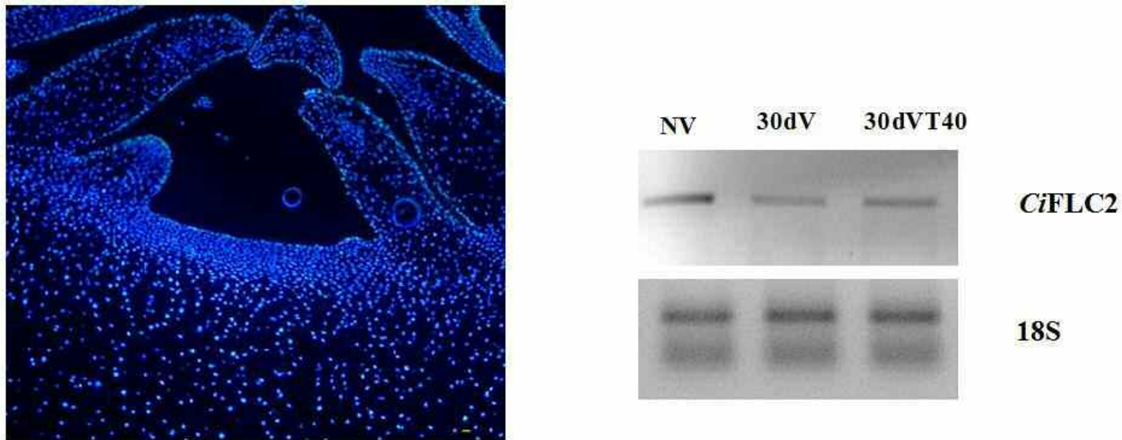


Fig.16. On the left a SAM profile of a plant vernalized 30 days and grown at warm temperature (<26°C) for 40 days. The picture shows a "flat" shape, characteristic of an inducted meristem in an early stage of the floral meristem organization; bar=100 µm. On the right FLC2 expression was examined by RT-PCR. RNA was extracted from leaf sample of TVT chicory non vernalized (NV), vernalized for 30 days (30dV), vernalized for 30 days and moved at warmer temperature for 40 days after cold treatment (30dVT40). The level of FLC remained low for the samples vernalized while a higher FLC expression is appreciable in the NV plant. 18S amplification has been used as internal control.

5.4.2 Analysis of DNA methylation

It is know that a vernalization treatment often affects the pattern of methylation of many genes. The addition of methyl groups to the nucleotides of the chromatin, results in a hindrance for the transcriptional machinery. The impossibility to access chromatin determines a different level of expression for the correspondent gene (Yuehui He, Amasino R.2004). Semi-quantitative RT-PCR in chicory revealed a relationship between *Ci*FLC expression and cold treatment. In order to define whether the downregulation of *CiFLC* was attributable to variation in the methylation pattern, we set out a Southern blotting experiment. DNA was digested with a couple of isoschizomer enzyme that exhibit different sensitivity toward methylation. In particular, we used Sau3AI, which is sensitive against the deossicytosine methylation (*dcm*), but is insensitive to the deossiadenosine methylation (*dam*) and NdeII which is insensitive to *dam* methylation.

Seedlings of TVT chicory were vernalized at 4°C in LD conditions. Leaves were collected and genomic DNA was extracted and restricted with Sau3AI and NdeII respectively. The blotted DNAs were probed with a sequence of *Ci*FLC cDNA lacking the MADS-box

domain. The results showed that FLC repression after vernalization did not involve methylation as epigenetic modification for its transcriptional regulation (fig.17).

To confirm the previous pattern of hybridization, we stripped the filter and re-hybridized it using a DNA probe, whose sequence was obtained by genome walking, and corresponding approximatively to the end of the second exon and the beginning of the second intron. This new result confirms that FLC methylation did not change after cold treatment (fig. 18). In addition, it is worthy to note that the profile of hybridization agrees with the previous southern experiments. In fact, more copies of *CiFLC* are still visible.

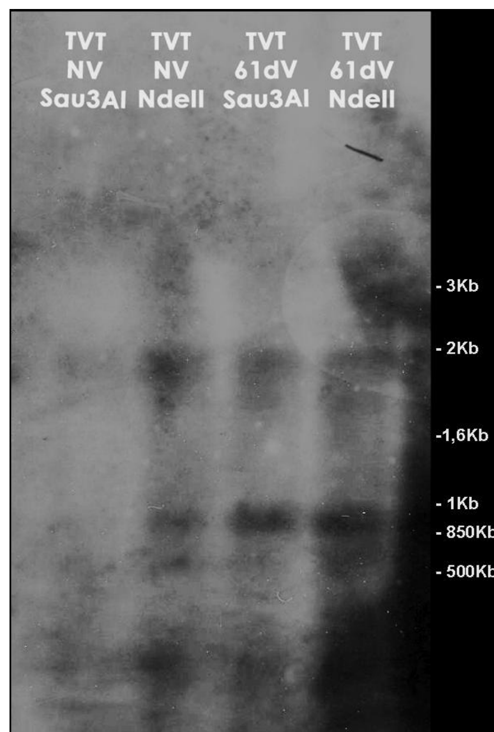


Fig.17. Southern blotting showing the pattern of *CiFLC* methylation before and after vernalization. DNAs were extracted from leaves of chicory (TVT) in plants not vernalized and vernalized for 61days. Each sample was digested alternatively with Sau3AI and its isoschizomer NdeII. DNAs were purified after restriction and loaded in a 0.8% agarose gel. Hybridization with a *Ci*FLC cDNA probe detected at least four sequences. The higher at 2kb, the second at approximatively 1.8 kb, the third at 900 bp and the latest at 700 bp. The pattern was coincident before and after vernalization.

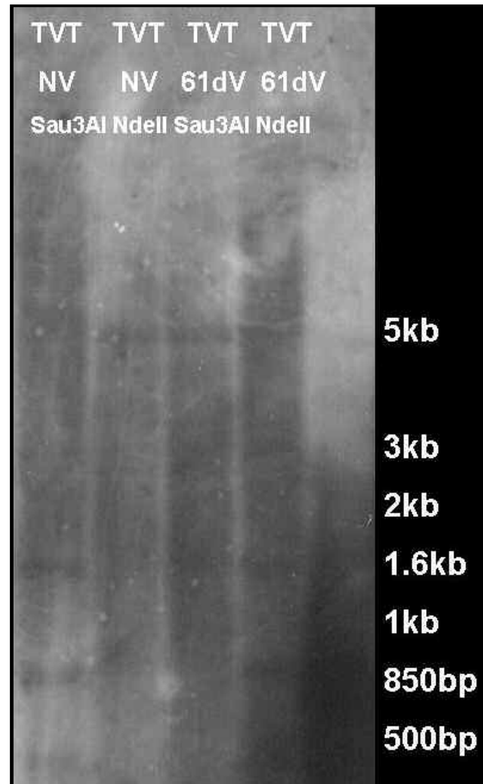


Fig 18. Southern blotting showing the pattern of *CiFLC* methylation before and after vernalization. Hybridization was conducted in the same filter in fig.15 but using a DNA probe corresponding to the region across the second exon-second intron of the gene. The pattern of hybridization showed four sequences. The bigger at 5Kb, below at 3kb, 1.6kb and last at 850bp.

5.5 Genome walking

Chicory is a crop of agronomic interest, but is one of the species less known. In fact, none cDNA neither genomic libraries are available to date.

Studying of *Ci*FLC regulation required at least the isolation of the region of the first intron, which in *Arabidopsis* is known to be the main region subject to the transcriptional control. That region is a target for histonic modifications operated by the PAF1 complex in the case of FLC activation and by the VRN2-complex and VIN3 in the case of transcriptional repression (He et al. 2004; Wood et al.2006). Several experiments have been oriented in the effort to isolate fragments of genomic sequence, including RACE and Inverse-PCR.

First of all, in order to obtain the full-length sequences from the partial cDNAs of *Ci*FLC before isolated, a Random Amplification of cDNA Ends (RACE) reaction was carried out. Many primers were used in combination with the universal primer supplied from the commercial kit (Roche Diagnostic), but in all the cases, after that several PCR conditions were tested, a consistent background, due to the presence of many amplicons, was present. Part of products was sequenced, but not any homology with FLC was revealed. For the majority of the amplicons, isolation as single sequence was impossible. Given the unfeasibility to isolate the full-length of FLC by RACE reactions, we tried to amplify regions of DNA sequence by PCR and inverse PCR, using many primers combinations. Three sequences in this case were identified. The first, 3kb long, was amplified with the primers 5'-TAA GGG AAT TTG GTC ATG AGA TTA TCA-3' and 5'-TGA ATG GCG AAT GGA CGC GCC CTG-3', fig.19A. Peculiarity of this fragment was the complexity to sequence it. In fact, many regions in the amplicon were repeated and inverted; moreover, regions of similarity with the cloning plasmid were shown. Thus, primers used to sequence the fragment, often worked both on the plasmid and in part of sequences inside the 3kb amplicon, resulting in an indecipherable chromatogram. Until it was possible to have a specific sequencing, we found that a intron-less sequence of *Ci*FLC was included in the 3kb fragment and what for us was the supposed ATG start codon, was replaced by a TGA codon, usually read as a stop codon. The presence of this sequence into the chicory genome is still without explanation.

The second and third sequence of DNA, were amplified with the same primers combination (5'-GAA CCA GGT TTT GGC TAG CCA GAT-3' and 5'-CAT TTG GAT TGA TTA CAG TTG GG-3') during the same PCR reaction, fig.19B. Both sequences were cloned and full sequenced. They corresponded again to intron-less *Ci*FLC. In particular, one of them lacked 40 aa inside the MADS region (corresponding to *Ci*FLC3), the other was completely corresponding to the *Ci*FLC2 transcript. Contrasting with the primer combination used for this last PCR, we found that the orientation of the two intron-less sequences was inverted in respect to a 5'-3' direction.

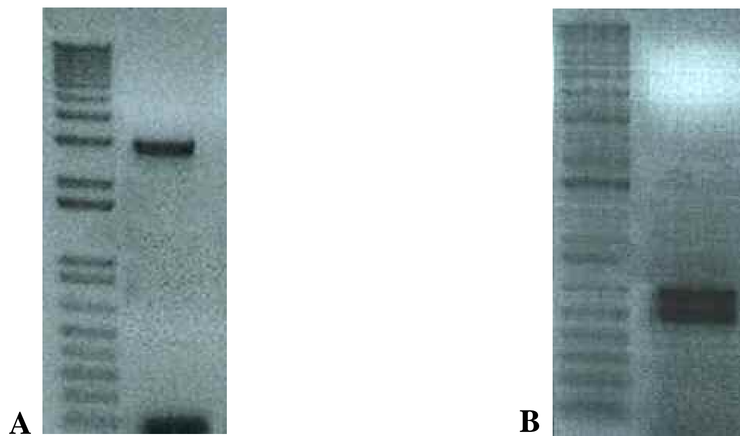


Fig.19 PCR amplification of genomic *Ci*FLC sequences. In **A,** a 3kb amplicon, partially sequenced revealed the presence of an intron-less sequence of FLC where the hypothesized start codon ATG was substituted by a TGA. In **B** two amplicons of FLC genomic sequences. The bigger corresponding to *Ci*FLC2 is around 800bp; the lower is 500 bp and correspond to *Ci*FLC3. The orientation of these sequences does not respect the 5'-3' orientation in relation to the primer combination utilized.

To isolate the full-gene of *Ci*FLC, we decided to use the *genome-walking* strategy. Four DNA libraries were generated and each of them was screened by PCR, using different FLC-specific primers in combination with the adaptor primer provided by the kit (BD-Clontech).

A short sequence has been obtained using the FLC-specific primer 5'CAG TCA CGA CGT TGT AAA ACG ACG GC- 3'. This fragment corresponds to the putative second exon end and beginning of the second intron. We say "putative" because we have evidence that at least another sequence upstream this one ought to exist. This sequence should contain at least part of the MADS domain that we found in the transcript, but not present in this genomic sequence (fig.20-21). The nucleotidic sequence of the region amplified is shown in fig.20, while the nucleotidic alignment with *AtFLC* is depicted in fig.3 appendix1.

TCCGTCGCTCTTCTCGTCGTCTCCGCCTCCGGCAAGCTCTAGCTTCTCCTC
CGGCGATAACCTGGTCAAGATCCTTGATCGATATGGGAAACAGGAAATCG
AATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCAAT
ATTTAAAATATAAATCGTCGCCGTTCAAAAAAATAAAAAGCTCAATGTGTCG

Fig. 20 Nucleotidic sequence of *CiFLC* 438nt long. The red bold character is indicative for the sequence corresponding to a known exon comprising part of the MADS domain.

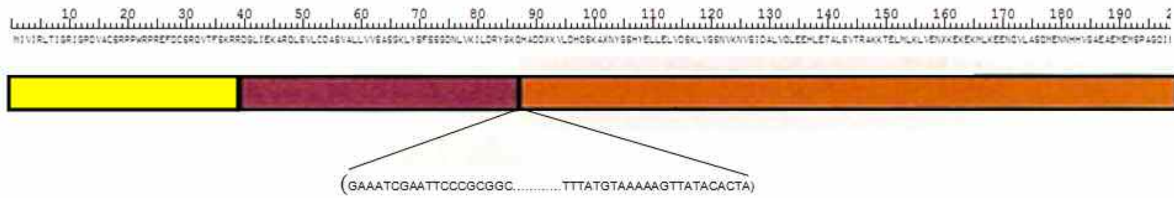


Fig.21.Schematic representation for *Ci*FLC2 transcript and the genomic sequence of FLC isolated by genome walking. The yellow box represent the region of MADS domain not identified in the genomic sequence described in the test. The violet box indicate the exonic region of the MADS present as in the transcript than in the genomic sequence isolated. The orange box represents the rest of the amino acidic sequence of *Ci*FLC2. The sequence of the intron is reported within parenthesis. For graphical exigencies, the sequence of *Ci*FLC2 is truncated at the 3'- end.

In order to exclude a PCR non-specific product of amplification, we tested the effective presence of the sequence isolated by genome-walking in the genome. A southern blotting was performed using this sequence to probe a filter previously used to check the changes in DNA methylation after vernalization, fig.18.

Because the genomic sequence recognized traits in the genome of chicory, we had the confirmation that this sequence was not a PCR artefact.

Many other primer combinations have been used to extend the *CiFLC* sequence but, so far, few sequences have been obtained and these need to be better characterized.

5.6 Functional analyses of *Ci*FLC2

5.6.1 Complementation of *At FRI flc3* by *Ci*FLC2

Arabidopsis Col. FRI flc3 mutant shows an early flowering phenotype due to a FLC null mutation (Michaels, 1999). To assess its biological function, *Ci*FLC2 was ectopically expressed in the *Arabidopsis FRI flc3* background. *Ci*FLC2 was driven by the 35S promoter, into a binary vector containing kanamycin- and hygromicin- resistance genes used for the selection of transformants fig.22. The construct was inserted into Agrobacterium strain C58C1-pMD90 by electroporation and *Arabidopsis* was transformed by the floral dipping method (Clough, 1998).

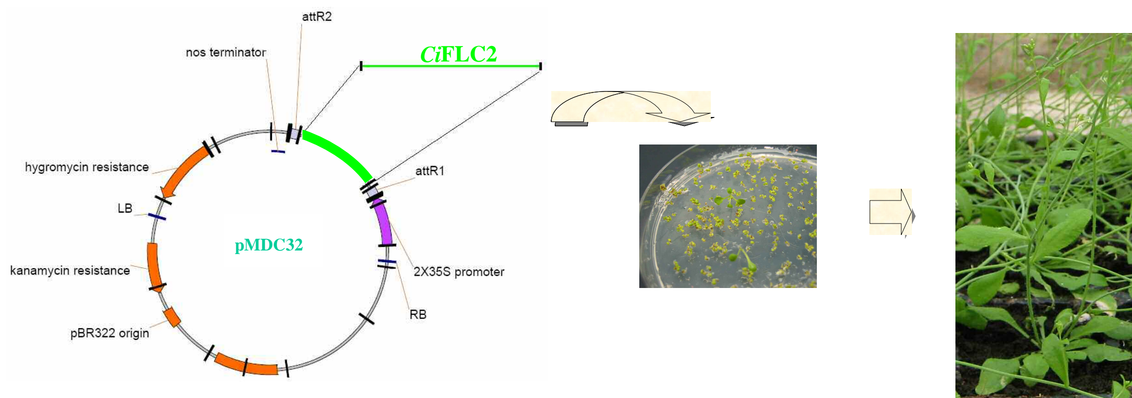
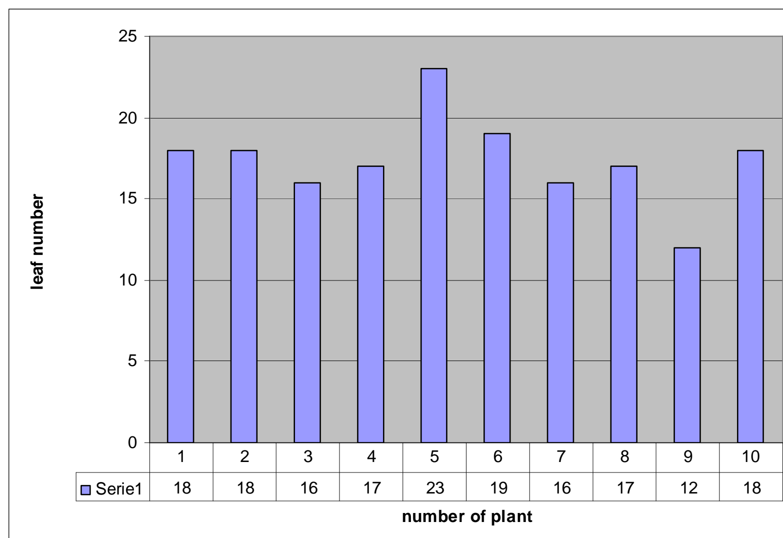


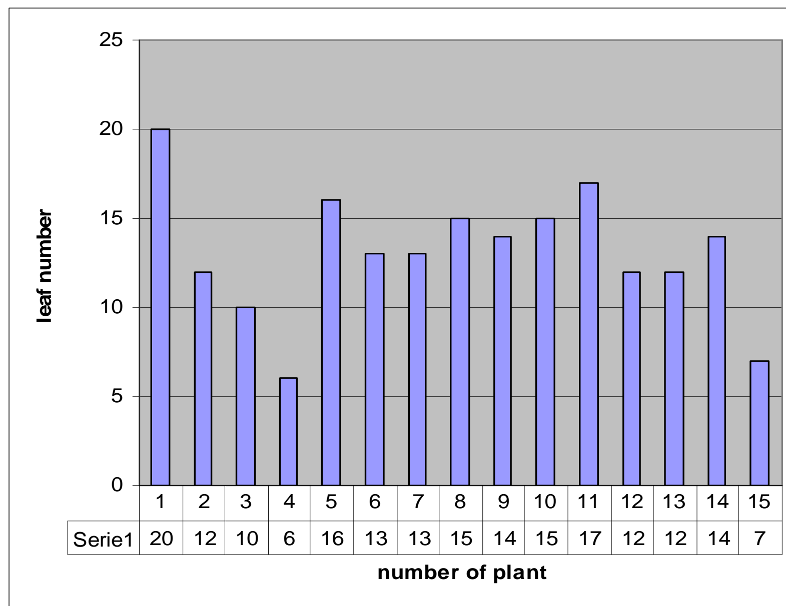
Fig. 22 The vector pMDC32 carrying *Ci*FLC2 was inserted into agrobacterium. At FRI flc3 has been transformed by floral dipping and seeds obtained were plated into Petri dish containing hygromicin for the selection of transformants. Finally, transformants were transplanted in soil.

Transgenic plants were selected by hygromicin resistance and analysed for the transgene presence by PCR using specific primers. The 35S::*Ci*FLC2 lines did not show a typical late-flowering phenotype as we expected. Instead, using the leaf number as a parameter to define the flowering behaviour, they resulted quite early, (Tab.1 and 2).



	1	2	3	4	5	6	7	8	9	10
Serie1	18	18	16	17	23	19	16	17	12	18

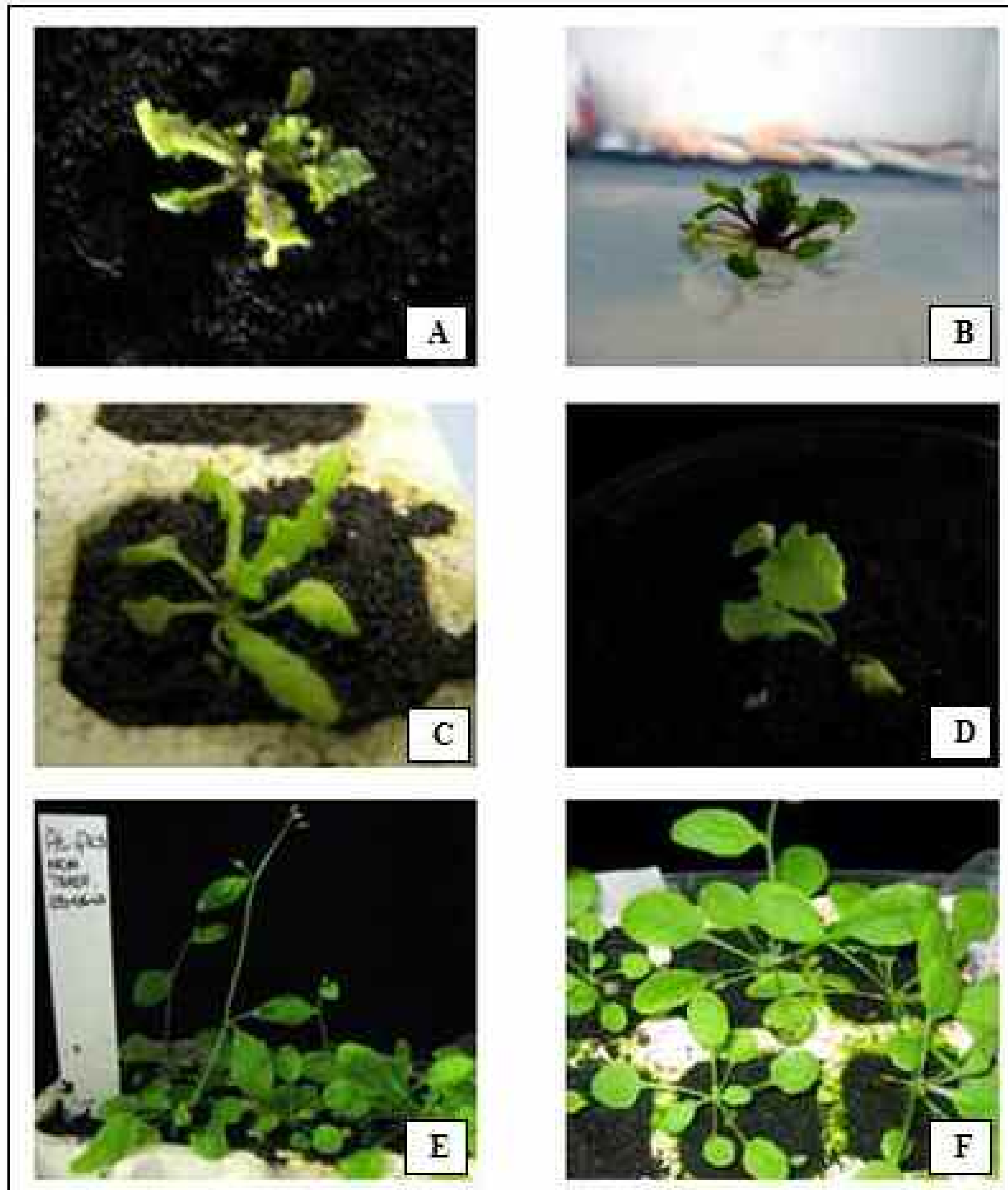
Tab1. Leaf number was counted in 10 plants of *Arabidopsis FRI flc3* and related with flowering behaviour. The majority of plants flowered with a mean of 16.4 leaves.



Tab2. 15 plants of transgenic *At FRI flc3* 35S::*Ci*FLC2 were considered for leaves counting. The mutants flowered with a mean of 13 leaves.

Our attention was captured by the disposition of the leaves and their margins. In fact, as shown in Tav. 3, seedlings of *Arabidopsis* had very unusual curled leaves with irregular margins. Interestingly, these characteristics are peculiar of adult plant of chicory.

Even after transplanting the transformants from agar medium to regular soil, leaf orientation remained quite disorganized. Conversely, the control *At FRI flc3* showed a regular phenotype, Tav.3.



Tav.3. In **A** *AtFRIflc3* 35S::*Ci*FLC2. The phenotype showed very irregular margins characteristic of adult plant of chicory and purple petiole pigmentation. In **B** seedling of *AtFRIflc3* 35S::*Ci*FLC2 cultivated in agarized medium. In **C** another seedling of *AtFRIflc3* 35S::*Ci*FLC2, showing the altered phenotype. In **D** particular of leaf in an *AtFRIflc3* 35S::*Ci*FLC2 mutant. In **E** *AtFRIflc3* control not transformed. It is possible to note a regular leaf phenotype. In **F** rosette of *AtFRIflc3,* control.

Using primers specific for *Ci*FLC2 we looked at the expression of FLC in transgenic *Arabidopsis* plants. Semi-quantitative RT-PCR confirmed that *Ci*FLC2 was downregulated in all positive transgenic lines after its introgression in the arabidopsis genome (fig. 23). The control *At FRI flc3* showed a very low level of FLC whereas the initial level of *Ci*FLC2 was higher, as expected because driven by the 35S promoter, and was only lightly

downregulated. The high level of *Ci*FLC2 expression did not explain the early flowering phenotype. *Arabidopsis thaliana* var. *Columbia* wild type was used as control for the PCR.

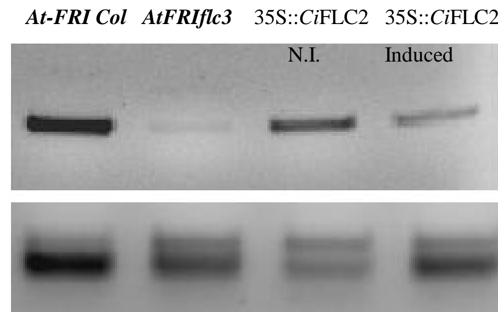


Fig.23. Expression of FLC in At Columbia, *AtFRI flc3* mutant, *At FRI flc3* 35S::*Ci*FLC2 in not inducted plant (35S::*Ci*FLC2 N.I.) and in the last lane the same mutant, but in inducted plant (35S::*Ci*FLC2 Induced). cDNAs were normalized by using the RT-PCR products of 18S as internal control, shown in the lower panel.

5.6.2 Over-expression of *Ci*FLC2 in chicory

We have previously demonstrated that *Ci*FLC2 was not effective to rescue the At*FRI flc3* mutant. In order to verify if in the chicory background *Ci*FLC2 is responsible for a late flowering phenotype, we engineered a gene over-expression experiment using the same construct used to complement the *Arabidopsis flc-3* mutant, but to transform leaf discs of chicory -TVT- by agro-infiltration. The effectiveness of the 35S::*Ci*FLC introgression in the chicory genome will be analysed in term of cold requirement to downregulates *Ci*FLC2 level. If it will require more than 7 days, that means that *Ci*FLC2 is effective only in the chicory background and this feature is probably dependent of some other regulatory factors, present/absent in chicory in respect to *Arabidopsis*.

5.6.3 Silencing of *Ci*FLC2 in chicory

The analyses of flowering time under cold treatment have shown the presence of four *Ci*FLC variants; all of those sequences were repressed by vernalization, but only under LD photoperiod. In order to better understand the mechanism controlling flowering in chicory,

we would analyse what happens when *Ci*FLC is silenced by a RNA interference mechanism. Plants vernalized under SD photoperiod never flowered before two growing seasons, demonstrating that the cold treatment was totally ineffective. In this experiment we would verify which could be the flowering behaviour when a chicory plant is grown under SD in a *Ci flc* background.

The tentative of complementation of the null mutant *At-flc3* has revealed a “leaf” phenotype. In this experiment we would analyse the phenotype of chicory *flc* mutant and verify if in this background could be possible to indicate an involvement of *Ci*FLC in leaves distribution or attribute the “leaf” phenotype to an improper introgression of *Ci*FLC2 in the genome of arabidopsis, which could result in the disruption of the coding frame of some gene involved in leaf shape determining.

A construct pMDC32-35S::*Ci*FLCS+*Ci*FLCA has been made and transformation of leaf discs of chicory has been carried out (fig.24). The results of this experiment will be available in the future.

Fig.24 Leaf discs regenerating seedling from callus. After infection with Agrobacterium, leaf disc were positioned in agar medium supplied of antibiotic and cefotaxime. After 25 days callus formations were visible at the margins of the discs as spherical structures. In the stage ahead seedlings will emerge from each callus.

Chapter VI

Discussion

Several examples of gene co-option or duplication, followed by neo- or sub-functionalization, have been documented within the MADS-box gene family (Irish & Litt 2005). All newly identified members of the FLC-like subfamily are uncharacterized, with the exception of the *Brassica napus FLC* orthologue, it is not known if they have a role similar to *AtFLC* during vernalization. In this work we have examined some regulatory and functional attributes of *Cichorium intybus FLC-* homolog.

We sequenced the chicory FLC-homolog and identified 4 variants. Among these, *CiFLC2*, has been investigated in details. We chose *CiFLC2* for its high identity of sequence with *AtFLC* (72%), the absence of stop codon in the deduced amino acidic sequence and for its intron-exon gene structure similar to the *AtFLC* one. Then, *Ci*FLC2 has been monitored in its expression and putative regulation.

The other transcriptional variants isolated in chicory differ mainly at level of the MADS domain or at the 3'-end. In particular, compared to *CiFLC2*, *CiFLC1* shows 60 additional amino acids at the 3'-end; *CiFLC3* shows a 40 aa deletion within the MADS domain; *CiFLC4* is only 42 aa long and we suppose it represents a form of alternative splicing, because the deduced amino acidic sequence shows 8 amino acids (GLVEKARQ) which belong to the MADS domain, the 3' of the sequence shows homology with the amino acids present at the 3'-end in *CiFLC2* and in *AtFLC* and the central part shows a sequence that cannot be related to any of the known domains of *FLC*.

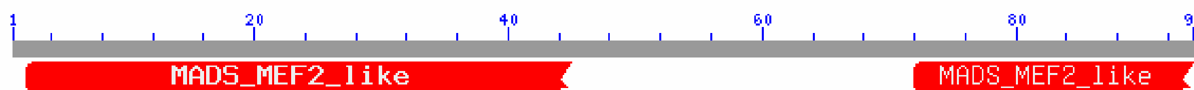
We found that *CiFLC2* and *CiFLC4* are shared between wild chicory and the domesticated cultivar Treviso.

Alignment of *Ci*FLC with sequences *FLC-like* belonging to other species, had underlined a 14 amino acids sequence within the MADS domain which is unique for the *Cichory*

species. Blasting this particular sequence on the NCBI database did not reveal any similarity with possible known regulative regions.

At present, our knowledge about *Ci*FLC does not permit to define if the four variants isolated belong to the same gene or not. Chicory shows a high complex genome and high amounts of carbohydrates and polyphenols which make it difficult to extract a DNA of good quality. Moreover, chicory presents a high auto-incompatibility, which prevents to obtain inbred line, with the disadvantage to find high heterozygosis and diversity among the alleles. During the efforts for the isolation of the genomic sequence or the full open reading frame (ORF) comprising the UTR regions, had difficulties related with the complicate genomic structure of chicory. In fact, when a primer was designed with the intention to amplify in forward direction, it worked in an aspecific direction. Precisely, when used a couple of primers designed on the base of the exon-intron structure of the *Arabidopsis FLC*, with the intent to isolate the first intron in *CiFLC*, could isolate in its place a sequence 3kb long, containing a FLC sequence without any introns (fig.19A). Even if the sequence of 3kb was cloned, the sequencing was quite difficult. In fact, both our specific *Ci*FLC primers and the universal primers supplied with the commercial vectors (such as M13, SP6, T7), recognized sequences both in the vector and in the fragment cloned, so that the resulting chromatogram was indecipherable. Using a different primer combination designed to amplify the full gene, two other sequences were detected (fig.19B): one FLC intron-less oriented in reverse direction and the other, without introns as well, lacking 40 aa inside the MADS-box. The trait of sequence before and ahead that "cDNA-like" sequences did not contain any useful information to characterize it. In addition, when we tried the isolation of the *CiFLC* genomic sequence by making DNA libraries using the genome walker kit (provided by Clontech), we found that primers planned to work in forward direction, amplified even sequences directed in reverse orientation. These evidences suggest that inside the genome of chicory many sequences are repeated and inverted, so that the primers recognize more than a site to begin the PCR amplification, with the result to have a very complicate pattern of amplicons and background to be interpreted. An example of repeated sequence is reported in the figure below. This sequence has not be introduced in the result because need to be better characterized.

TGTAGCCGACAAGTCACCTTCTCCAAACGTCGCGACGGTCTCATCGAGAAAGCTCGTCAGCTTTCTGT
TCTCTGTGACGCATCCGTCGCTCTTCTCGTCGTCTCCGCCTCCGGCAAGCTCTACAGATTCTCCTCCA
CGAAAATCTGATCCGACTCCCTGATCGACAAAGGAAACACGACATCGCATTCTCCCGGCCGCCATGGCGGC
CGCGGGAATTCGAT**TGTAGCCGACAAGTCACCTTCTCCAAACGTCGCGACGGTCTCATCGAGAAAGCT**
CGTCAGCT



Genomic sequence amplified by PCR using the primers combination 5'-GTAGCCGACAAGTCACCTTCTCCAAACGTCG-3' and 5' – CGACGGTCTCATCGAGAAAGCTCGTCAGCT -3'. These primers flank the MADS domain and if used in *Arabidopsis* they would amplify the region of the first intron. In chicory these primers generated 5 amplicons, among those the one showed above shows a partial duplication of the MADS domain. The duplicated region is indicated by bold underlined character.
Below a schematic view of the sequence described above obtained by tBLASTx (NCBI web-site).

The evidence of replicated and sometime inverted regions inside the chicory genome, lead us to think at the involvement of a mechanism of duplication operated by transposable elements. As observed in maize, soybean, rice and arabidopsis, the origin of duplicated gene is mostly referred to the insertional activity of *copy-and-paste* mainly involving DNA transposon, such as *helitrons* and *mutator-like* elements (Morgante et al. 2007). These elements appear to capture and transport host DNA fragments. The gene mobility mediated by retrotransposons constitute a peculiar strategy, involving an RNA intermediate, that is supposed to facilitate the reverse-transcription of spliced host mRNAs and their insertion (retrotransposition) into new genomic positions to form intron-less retrogenes, some of which might be functional (Weiner, et al. 1986). The transduplicated region could contribute to the phenotypic variation by generating small interfering RNAs, which may participate in RNA-mediated silencing of the host genes from which the fragments were duplicated, and which therefore could potentially represent *trans*-acting regulatory factors (Meister et al. 2004).

On the basis of these observations we could address the presence of FLC copies without introns at the result of retrotransposition; the evidences of duplicated, but disorganized exons could be explained as the effect of the "exon-shuffling" phenomenon, where fragments of gene are fused together. The difference of precocity among the cultivar of

chicory could be referred to the effect of *trans*-regulation derived from the event of transduplication.
The difficulties to plan PCR amplifications due to the complexity of the chicory genome organization rend the isolation of the complete *CiFLC* gene not possible yet. However, recently, by means of genome walking, three genomic sequences have been characterized. The first one corresponds to the putative second exon-end and the beginning of the second intron (fig.20, 21). A splice site within this sequence has been assessed (fig.3 appendix 1). The second and third ones confirmed the presence of *Ci*FLC copies conform to the transcript, intron-less (data not shown). Two other sequences have been lately isolated: one match to the region putatively upstream of the second exon and the second localizes at the 3'-end of *CiFLC*. Actually, these sequences need to be better characterized before they can be certainly attributed to the same *CiFLC* gene. Indeed the Southern blotting revealed at least four hybridization bands, then suggesting the presence of more than one copy for this gene, as confirmed by the isolation of repeated sequence of FLC. At the same time the possibility of alternative splice variants of a same gene cannot be ruled out, because alternative splice variants have been observed for all *FLC-like* genes in *Arabidopsis* (Scortecci 2001; Ratcliffe et al. 2003) and in sugar beet (Reeves et al. 2007). At the present state of the work and given the considerations previously explicated, we think that the only possibility to obtain a genomic sequence of *Ci*FLC is to predispose a BAC library.
The expression of *CiFLC* and its regulation by vernalization were also analyzed. *Ci*FLC transcripts analyzed by RT-PCR were expressed in the shoot apex, in leaves and in floral tissues in all the developmental stages considered: immature flower, fully developed flower, but still closed into the sepals and fertilized flower (fig.9). Through *in-situ* hybridization the expression pattern has been confirmed in all the above tissues mentioned (Tav.1). At the early embryonic stage, *Ci*FLC is expressed in the shoot apex, as observed in *Arabidopsis* (Michaels & Amasino, 1999), and all around the cotyledons. In the adult and inducted plants the domain of expression seems to change. Even if *Ci*FLC was still amplified in the apex by RT-PCR, the transcript was undetectable by *in-situ*, while it was well represented at the adaxial face of leaf primordia and in the root apex (Tav.2). In the floral tissues, *Ci*FLC was detected in the ovary and in the mature pollen, here the signal is weaker.

It is known that *AtFLC* expression decreases after vernalization treatment and this repression is maintained through mitosis, but is resetted in an undetermined moment before or during meiosis. Our results confirm the expression of *Ci*FLC after meiosis, as the transcript has been detected in the mature pollen (Tav.1). However, the weakly detection of *Ci*FLC in the ovary and in the leaf primordia (Tav.1-2), whose cells are not generated by meiosis, seems to contrast the hypothesis of a resetting occurring during meiosis. Certainly, there is a phase during flowering induction, in which FLC is not long detectable. In our experiments *Ci*FLC expression is strongly down regulated both in the apex and in leaves of vernalized plants (Tav.2-D). This result demonstrates that changes in *Ci*FLC expression in leaves are an essential component of the vernalization response (Tav.2). A similar conclusion has been recently drawn for *Arabidopsis* (Searle et al. 2006).

A possible explanation for these results could be that *Ci*FLC is inhibited by vernalization through one of the mechanisms known for *Arabidopsis,* but the level of expression is not totally repressed or is only partially and temporary down regulated. In fact, after that the apex changed its fate from vegetative to reproductive and the flower structure began to be determined, *Ci*FLC appeared again (i.e. ovary, leaf primordia). These evidences suggest that a post-transcriptional regulation of *Ci*FLC could be predominant rather than a transcriptional repression. The hypothesis is that when a plant is subject to vernalization, *Ci*FLC decreases because its transcription is epigenetically inhibited or because the transcript is in some way locked away. It was observed that *At*FLC acts in a complex with AP1 and SVP (Ilha Lee, personal communication). AP1 is required for the proper transition from inflorescence to floral meristem and for the formation of sepals and petals. The presence of AP1 in a FLC complex could be justified by supposing that during the vegetative phase FLC holds AP1, but when FLC expression is inhibited, AP1 is "liberated" and could exert its function, maybe constituting a new complex with other MADS proteins. After that the flower is determined, the floral pathway integrators are inhibited (Yu et al 2004). At this point, FLC repression should be released, in fact, FLC expression is again detectable in the floral tissues (i.e. ovary). By then, the mechanism that leads reproduction has been set out.

The expression of FLC in pollen grains has been detected even in transgenic *Arabidopsis* carrying the cabbage *FLC4-1* sequence. In this case the authors concluded that FLC could

have a further role in pollen development or pollination in addition to flowering control (Lin et al. 2005).

To further characterize *Ci*FLC2 expression and, more precisely, its cellular localization, the *Ci*FLC2 sequence has been fused to the GFP reporter gene into a pTZ-U19 vector. Protoplasts of chicory were then transformed with this construct. Analyses of the transformants showed a nuclear localization of *Ci*FLC2, as attended for a transcriptional factor. In order to define if the 40 aa region inside the *Ci*FLC2 MADS-box, but absent in *Ci*FLC3, was necessary for its localization, we repeated the previous experiment by fusing *Ci*FLC3 to the GFP reporter. Even in this case the expression was nuclear, so that we could exclude that the 40 aa sequence is required for the localization of *Ci*FLC.

Steady-state levels of *Ci*FLC have been analyzed during the vernalization treatment to determine, from a molecular point of view, which was the real "cold requirement" chicory needs to be considered induced. From our experience we know that flowering in chicory can be promoted by vernalization of adult plants, passed through the juvenile phase until the third true leaf has been emitted. Seeds and young seedlings do not respond to vernalization.
Monitoring of the transcripts has been carried out by semi-quantitative RT-PCR and the results were compared with cytological data (fig.11-14). *Ci*FLC expression decreased under a level undetectable after only 7 days of cold treatment and the SAM turned into the "flat" apex, indicative of a meristem committed to flowering. It is of our interest to investigate in the near future the level of *Ci*FLC expression in other varieties of chicory. To assess whether the different precocity among cultivars can be related to the *Ci*FLC level before vernalization treatment or attributed to a different degree of *Ci*FLC reduction after vernalization.
Because the vernalization treatment was effective only during LD photoperiod, but not under SD (fig.14), we suppose that *Ci*FLC is the point where photoperiodic and vernalization pathways converge to regulate flowering time. For a biennal crop as chicory, the presence of multiple control points for flowering induction is important to ensure that the switch into a reproductive program occurs in a favourable season. Temperature is one

of these control factors. During our experiments we noted, that a decrease of 2-4°C had remarkably effects on flowering time of vernalized plants (data not shown). Even if the apex presented the peculiarities of an inducted meristem and the photoperiod was favourable, the reproductive program was arrested and restarted only when the temperature increased again. These observations lead us to conclude that *Ci*FLC regulation may be due to a different regulatory process than vernalization, even if the elicited effect is the same. We demonstrated by molecular and cytological data that LD photoperiod itself is not able to induct flowering (fig.14-H) and that *Ci*FLC expression was unaffected by the rhythms of the circadian clock (fig.15), neither vernalization under unfavourable photoperiodic condition seems to down regulate *Ci*FLC (fig.11B). We conclude that vernalization and long day photoperiod first and the maintenance of high temperatures later, are required together to ensure the proper timing of flowering.

In *Arabidopsis* FLC expression is modulated at the transcriptional level by a mechanism triggered by vernalization (Sheldon et al. 2002; Sung et al 2006; Sung & Amasino 2004; Bastow et al. 2004). This regulation comprises epigenetic modifications at histone level - deacetylation of H3K9 and K27- while changes in DNA methylation have been excluded (Finnegan et al 2005).
Similarly to *AtFLC* regulation, we found that *Ci*FLC is downregulated during vernalization (fig.11). To exclude that DNA methylation was involved in the epigenetic control of *CiFLC* expression, we verified by Southern blotting whether the *Ci*FLC sequence was methylated and the pattern of methylation changed after cold treatment (fig. 18- 19). Correspondence between the pattern of hybridization *before* and *after* treatment and between the same group of samples digested alternatively with two enzymes showing different sensitivity to methylation, leads to exclude the methylation as mechanism of control for *CiFLC* expression.

In this study *Ci*FLC has been shown to be a key regulator in the vernalization response pathway. In order to test the degree of functional conservation between *Ci*FLC and *At*FLC in a heterologous context, the ability of *Ci*FLC2 to rescue the *Arabidopsis* FLC null mutant *flc-3* (Michaels and Amasino 1999) was evaluated. A 35S::*Ci*FLC2 construct was inserted

into the genome of the arabidopsis mutant by agro-infection. Even if *AtFLC* and *Ci*FLC2 show a high identity of sequence (74%), nevertheless *Ci*FLC2 was not able to rescue the mutant (Tab.1-2). Moreover, the leaf shape was quite altered in respect to that of *Arabidopsis* wild type (Tav.3). Interestingly, looking at the leaf morphology of this arabidopsis transformant was possible to note a leaf distribution which, actually, reminds the leaf structure of adult plants of chicory. In addition, a weak red pigmentation in the petiole of the leaves was observed in all the transformants before the bloom, after which pigmentation disappeared. We do not know how relate the pigmentation to *Ci*FLC2 expression because in chicory the pigmentation is acquired after the juvenile stage, which is the exact opposite of the observed situation.
As the phenotype of the mutant was not rescued, despite of the verified *Ci*FLC2 presence in the *Arabidopsis* genome, we looked for the possible causes that generated the early flowering phenotype. One hypothesis is that the additional sequence (i.e. 14 aa in the MADS-box) present in *Ci*FLC2 could disturb the FLC-complex formation. Moreover, this sequence could be recognized as a foreign sequence, so that a mechanism of silencing, similar to that observed for viral sequences in *Arabidopsis*, would block *Ci*FLC2 expression. Another possibility is that *Ci*FLC exhibited a new function that act in the regulation of leaf shape-orientation and probably does not affecting the flowering time determination.
In order to test these hypotheses and understand *when* and *if* *Ci*FLC2 inhibition occurred, a semi-quantitative RT-PCR analysis was conducted. cDNA samples from leaves of *Atflc3* not transformed plants, *Atflc3* not inducted and carrying 35S::*Ci*FLC2, *Atflc3* with flower and carrying 35S::*Ci*FLC2 and At FRI *Col-0*, were analysed to test *FLC* expression. *Ci*FLC2 seemed lightly silenced after its introgression (fig.23). In fact, its expression is still high, compared to that in arabidopsis null mutant. Interestingly, the transformants did not manifest a late flowering phenotype. So far, *why* remain to be explicitly tested.

Another approach recently employed to test the hypotheses before mentioned about *Ci*FLC2 function, was the tentative of over-expression and silencing of the endogenous *Ci*FLC. The objective was to create a late and an early flowering phenotype to further

investigate flowering behaviour or obtain a leaf phenotype to exclude the decisive role of *Ci*FLC in flowering determination.

Leaf discs of chicory *cv* TVT were infected using *A. tumefaciens* strain C58C1-pMD90, carrying alternatively pMDC85-35S::*Ci*FLC::GFP and pMDC32-35S::*Ci*FLCS+*Ci*FLCA. As negative control, the infection has been carried out with the corresponding empty vectors.

The results of this most recent work will be become available and will be evaluated in the next future.

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Vernalization and cell division at the shoot apex interact to affect flowering time in Raphanus sativus

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Abstract

Raphanus sativus (radish) is a species belonging to the Brassicaceae family. Recently, radish has been studied for its characteristics that make it an excellent plant model for vernalization studies. In this study, we firstly investigated the optimal condition to vernalize radish, defining photoperiodic condition and responsive cold temperature. In Arabidopsis thaliana, vernalization treatment triggers VIN3 expression and then VIN3 represses FLC, a potent repressor of floral transition, thereby allowing induced flowering. Here, we report cloning and functional characterization of radish FLC and VIN3 equivalents. In a comparative study, we analyzed the behaviour of radish and arabidopsis in regards to vernalization response. In the second part of study, we focused our attention on determining the possible characteristics determining the short requirement of cold exhibited by radish. A cytological analysis of the apical meristem was conducted in both vernalized and non-vernalized plants to determine the rate of cell division. Our results suggest that an increase of cell division rate could be responsible for the short cold perception as vernalization signal in radish.

Introduction

In many plants, the transition of the shoot apical meristem from the vegetative to the flowering state occurs in response to environmental cues. Temperature is one such cue and plants can exhibit an obligate (qualitative) or facultative (quantitative) vernalization requirement. Vernalization is defined as the acquisition or acceleration of the ability to

flower by a chilling treatment (Chouard, 1960). Many crop species, such as beet, cabbage, and certain varieties of wheat, require prolonged exposure to cold, such as occurs during winter, in order to achieve competence to flower. The vernalization requirement of certain plant species allows them to occupy a temporal niche in which establishment of vegetative structures occurs in the fall but flowering is delayed until return of favourable growing conditions in the spring.

The effectiveness of the treatment in inducing flowering requires: active growth, oxygen, administration of the appropriate duration of cold temperature and an appropriate developmental stage (Lang, 1965). Optimal temperatures for vernalization range from 1 to 10°C, but the range of responsive temperatures can be quite broad (-6° to 14°C) (Chouard, 1960). The site of perception of cold are leaves and shoot, but in particular dividing cells are necessary for the action of low temperature (Wellensiek, 1962). Wellensiek provided evidences that the cell division during the vernalization is necessary for thermoinduction in *Lunaria annua* and that flowering structures are ultimately derived from the mitotically active cells that were subjected to vernalizing temperatures. Wellensiek concluded that meristematic cells are subject to vernalization and reproduce the vernalized conditions by mitosis to the newly regenerating plant (Wellensiek, 1961).

There are two temporal characteristics of the vernalization response that are noteworthy. First, only prolonged periods of cold are sufficient to generate a vernalization signal in responsive plants. Vernalization is thus distinct from the process of cold acclimation (Thomashow, 2001), which is designed to respond to low temperature exposure as rapidly as possible in order to avoid freezing hazard. The much slower vernalization response is useful in natural conditions because plants should not respond to brief periods of warming, such as might occur in mid-winter, as a signal that winter has passed. Second, once achieved, the vernalized state can be maintained stably during vegetative growth. This aspect of vernalization was demonstrated by the classic experiments of Lang and Melchers (Lang, 1965). Henbane plants exposed to cold for a time sufficient to achieve vernalization continued to grow vegetatively in warmer temperatures under non-inductive photoperiods. However, the transition to flowering occurred rapidly when such plants were exposed to inductive photoperiods. Thus the “memory” of prior cold exposure is stably maintained throughout the course of subsequent cell division cycles.

Genetic analyses of natural variation in *Arabidopsis* have identified accessions that display the vernalization-requiring (winter-annual) growth habit (Napp-Zinn, 1979) and molecular characterization has identified some of the factors responsible for execution of the vernalization response (Burn et al., 1993; Clarke and Dean, 1994; Koornneef et al., 1994; Lee et al., 1993). In particular, it has been shown that dominant alleles of *FRIGIDA (FRI)* and *FLOWERING LOCUS C* (*FLC)* confer synergistically the winter-annual trait (late-flowering without vernalization) (Koornneef et al., 1994; Lee et al., 1994). The cloning of *FLC* provided a clue as to how vernalization affects the competence to flower (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* is a repressor of flowering and FRI elevates FLC expression to levels sufficient to block flowering (Michaels and Amasino, 1999; Michaels and Amasino, 2001; Sheldon et al., 1999). Vernalization promotes flowering by repressing *FLC* expression and this repression is stably maintained after a return to warm conditions (Michaels and Amasino, 2000; Michaels and Amasino, 1999).

A gene that functions in the measurement of the duration of cold exposure and in the establishment of the vernalized state has been identified in *Arabidopsis thaliana* and named as *VERNALIZATION INSENSITIVE 3 (VIN3)* (Sung and Amasino, 2004). *VIN3* has a role in the establishment of *FLC* repression during vernalization; is expressed only after a period of cold exposure that is effective for vernalization, and *FLC* repression does not occur until *VIN3* is induced (Sung and Amasino, 2004). *FLC* repression involves chromatin remodelling which is reflected by a series of histone modifications and these processes are mediated by *VIN3* (Sung and Amasino, 2004).

Much less is known that accounts for the requirement of long-term exposure to cold in order to achieve the vernalized state. One clue to understanding how plants distinguish between long-term and short-term cold exposure can be found in the temporal pattern of *VIN3* expression upon exposure to near-freezing temperatures. In contrast to the rapid induction of genes known to be involved in cold acclimation (Thomashow, 1999), *VIN3* expression is induced only after several weeks of cold, accompanied by a proportionate decrease in *FLC* expression (Sung and Amasino, 2004). However, whereas the change in *FLC* expression level is maintained even after vernalized plants are shifted to warm temperatures, *VIN3* expression rapidly decreases to pre-vernalization levels (Sung and Amasino, 2004).

Raphanus sativus (radish) has been proposed for a unique model to study 'vernalization'. It has vernalization responses but saturation of vernalization occurs only in 6-8 days of cold treatment (Engelen-Eigles and Erwin, 1997), showing extremely rapid vernalization responses compared to arabidopsis, which requires ~40 days. It also has an obligate vernalization requirement when grown under short days (Erwin et al., 2002). *R. sativus* is closely related to *Arabidopsis,* as in the same Brassicaceae family, is seed propagated, and develops as a rosette plant until flowering. Flower induction results in development of a flower stalk which branches and develops racemes on the main and lateral shoots (Nakamura, 1985). Effects of vernalization and photoperiod on inflorescence formation in radish have been investigated. According to a "flower formation index", the inflorescence formation was suppressed under the condition of short photoperiods, and increased under the condition of long photoperiods after the low temperature treatment as compared with that during the low temperature treatment (Cheon and Saito, 2004). Here, research presented in this work further describes the vernalization requirement of *Raphanus sativus*. Cloning and characterization of *Rs*FLC and *Rs*VIN3 has been achieved. In order to understand the difference in duration of vernalization requirement of radish from *Arabidopsis*, a cytological analysis of the shoot apical meristem also has been conducted.

Materials and methods

Vernalization treatment and flowering time analysis

For vernalization treatment, *R. sativus* seeds were imbibed in water for 2 hours and then placed in Petri dish on Whatman n.3 filter papers saturated with distilled water. Petri dishes were sealed with micropore tape and placed at 25°C overnight. Upon germination, seedlings were transferred to a refrigerated chamber at 4° C and 6°C under short-day photoperiod (light 8hrs/ dark 16hrs). In the first set of experiment, seedlings were transplanted to soil under short day or long day (light 16hrs/ dark 8hrs) condition after a series of vernalization treatment (3, 6, 9, 12, 15 and 20 days) for flowering time analysis. In the second set of experiment, seedlings of radish were vernalized for 7 and 18 days at 6°C under short day condition. For arabidopsis, seedlings (Col-*FRI*) were vernalized for 7 and 40 days at 4°C under short day condition and then transplanted to soil for flowering time analysis. In both cases of sets, cold meristem tissues were collected for subsequent RNA analysis before plants being transplanted. For flowering time, total leaf number until anthesis were collected. More than 10 plants were used for all flowering time analyses.

Cloning of *Rs*VIN3 and *Rs*FLC

Seedlings of either vernalized or non-vernalized radish plants were collected for RNA isolation. Total RNA was obtained using TRIZOL® reagent (SIGMA) according to the manufacturer instructions. cDNA were generated from total RNA using Advantage® cDNA PCR kit & polymerase mix (Clontech). Radish *FLC* was cloned by RT-PCR using FLC-F primer and FLC-R primer from non-vernalized sample. For *VIN3* isolation, first, 3’ conserved fragment was amplified using degenerate primers oJY011 and oJY012 from vernalized sample and then amplified fragment was sequenced. Based on identified sequence information, unique primers of oJY017 for 5’ RACE and of oJY016 for 3’ RACE were generated. To identify whole transcription region including 5’ and 3’ UTR, either 5’

or 3' RACE was carried out using GeneRacer® kit (Invitrogen). All PCR fragments were cloned into pGEM®-Teasy vector (Promega) and verified by sequencing. Amino acid alignments of deduced radish FLC between different plant species (*Arabidopsis, Brassica rapa, B. oleracea,* and *B. napus*) and of deduced radish VIN3 between *Arabidopsis* VIN3 were carried out using 'Multalign', a web-based multi-alignment software at http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html (Corpet, 1988).

Semi-quantitative RT-PCR

Total RNAs were extracted from various samples and cDNA were generated as mentioned above. Semi-quantitative RT-PCR were performed on cDNA using gene specific primers for radish and for arabidopsis and then PCR products were analyzed on 1% agarose gel. First, primers of UBQ-F and UBQ-R specific for *Ubiquitin* from *Arabidopsis* were used as an internal control to normalize the arabidopsis RT products, while primers of oJY021 and oJY022 specific for *RsACTIN* from radish were used to normalize radish RT mixtures. The normalized RT products were then used as templates for semi-quantitative RT-PCR of targets genes. The expression of *RsVIN3-1*, *RsVIN3-2*, and *RsVIN3-3* were analyzed using the primer pairs of oJY058 & oJY017, oJY059 & oJY061, and oJY060 & oJY061, respectively. *RsFLC* were tested for its expression using oJY023 & oJY024. For arabidopsis *VIN3* and *FLC* expression, VIN3-RTF & VIN3-RTR and FLC-RTF & FLC-RTR were used for their expressions, respectively. All RT-PCR were repeated at least 3 times using independent biological replicates.

Over-expression construct for functional analyses of *Rs*VIN3

For over-expression construct, radish genomic DNA was isolate from dark-grown 4 day-old seedlings. The genomic *RsVIN3-1* was PCR-amplified using primers oJY075 & oJY076. First, amplified fragment was cloned into D-TOPO® entry vector (Invitrogen) and was subject to Gateway® cloning system (Invitrogen) to introduce into 35S promoter-containing

destination vector, pMDC32 (ABRC stock #: *CD3-738*) to generate transgene containing construct pJY027A. Transformation of pJY027A into *Arabidopsis vin3-4* was performed as described by Clough and Bent. Homozygous T_3 generations of transgenic plants were collected for subsequent vernalization analysis.

Vernalization analyses by cell number counting

Apical meristems from radish and arabidopsis seedlings were collected and fixed in 4% paraformaldheyde (SIGMA) in 0.1M phosphate Buffer pH 7.2, (NaH_2PO_4 5M and Na_2HPO_4 0.5M were mixed to reach the final concentration 0.1M) and 0.1% gluteraldheyde (SIGMA). Samples were then incubated for 16 hours at 4°C. After fixation tissues were dehydrated by washing and incubation in a series of ethanol- xylene solution until the samples were only in xylene (protocol described by Varotto et al., 2003). Then the drops of Paraplast Plus (Sigma-Aldrich) were added to the samples in order to progressively substitute the xylene and finally embed the tissues. The embedded samples were cut in 6 μm sections using a microtome (SPENCER LENS CO.) and collected in SuperFrost® Plus Slides (Menzel-Glazer) to be subsequently deparaffinized with progressively washing in 100% ethanol, 50% ethanol-xylene and last in 100% xylene. After sections were dried, slides were stained for 2 minutes in 0.05 % toluidyne blue. Slides were dried and mounted with DPX Mountant for histology (Fluka Biochemika). Coloured sections were observed with a microscope (LEICA MZ6) under white light. Images were captured by the camera (LEICA DFC480) and processed by using Adobe Photoshop v.6 (Adobe System). 3 SAMs for each time-point of vernalization were collected and analyzed; the experiment has been repeated two times. The values of cell number in the result section represent mean numbers calculated among the samples collected in each experiment.

Table 1. Primers used

Name	Sequences (5'-3')
FLC-F	ACCTGAGGATCAAATTAGGGCACAAAG
FLC-R	CTAATTAAGTAGTGGGAGAGTCACCGG
oJY011	AARACNATHAGRTGYYTI GA
oJY012	AANGTRTCNACIARYTGYTGNCC
oJY016	CATATAGACAAGAGTTTCAGGGAAAG
oJY017	GAGAGGATAGATCGTCCATGAATGTC
UBQ-F	GATCTTTGCCGGAAAACAATTGGAGG
UBQ-R	CGACTTGTCATTAGAAAGAAAGAGAT
oJY021	CCATCGAGAAGAACTACGAG
oJY022	TGGACCTGCCTCATCATACTC
oJY058	TGAGACGAGGTCTACCATCCAAGCAC
oJY059	TGTTTTGAAGTCTGATGATGTTCTGG
oJY060	GTTTCAAGACGAGGTGACATGTTGAG
oJY061	CCTCTGCTGCACTACAGTGTCCAATG
VIN3-RTF	TCATCTTGTCACCTTGTCCTGAAACAC
VIN3-RTR	TGACTTGCTCGGATGCTGGAGAAAAC
FLC-RTF	TTCTCCAAACGTCGCAACGGTCTC
FLC-RTR	GATTTGTCCAGCAGGTGACATCTC
oJY075	CACCCAAAGGGAAAAAAATGCAAGCTGCTTCG
oJY076	AGGTAGTGTTGGCGGAAGTTACATAAGATATCG

Results

Response to photoperiod and vernalization of *Raphanus sativus*

Many varieties of radish are known to be "cold sensitive" (can be vernalized by short-term cold) and therefore flower during the fall when ambient temperatures fall to 5-6℃ (Curtis, 2003). To reproduce this physiology in experimental conditions, the flowering time of radish plants was measured upon vernalization (Fig. 1). Seeds were subject to overnight imbibition in distilled water and were then vernalized either at 6˚C or 4˚C for 0, 3, 6, 9, 12, 15, or 20 days. Seedlings vernalized at 6˚C were transferred to soil and grown under long- (16 h) or short-day photoperiod (8 h). Seedlings vernalized at 4˚C were transplanted to soil under short-day photoperiod. Total leaf number upon flowering was used to measure flowering time. Consistent with previous reports, acceleration of flowering began after less than 10 days of vernalization under both long- or short-day conditions (Fig. 1). Without any exposure to cold treatment, radish failed to flower under short-day growth conditions, indicating that radish has an obligate vernalization requirement when grown under short-day conditions (Fig. 1). Quantitative effect of vernalization at 4˚C has been diminished compared to 6˚C, indicating radish has an optimal vernalization temperature range around 6˚C more than 4˚C (Fig. 1). It is interesting to note that plants vernalized at 4˚C showed a faster acquisition of flowering competence at 6 days of cold treatment while the plants vernalized at 6˚C showed at 9 days of cold treatment (Fig. 1). A common feature is that both *Arabidopsis* and radish do have a vernalization response. However, radish has adapted to respond to extremely shorter exposure to cold and a warmer ambient temperature than *Arabidopsis*.

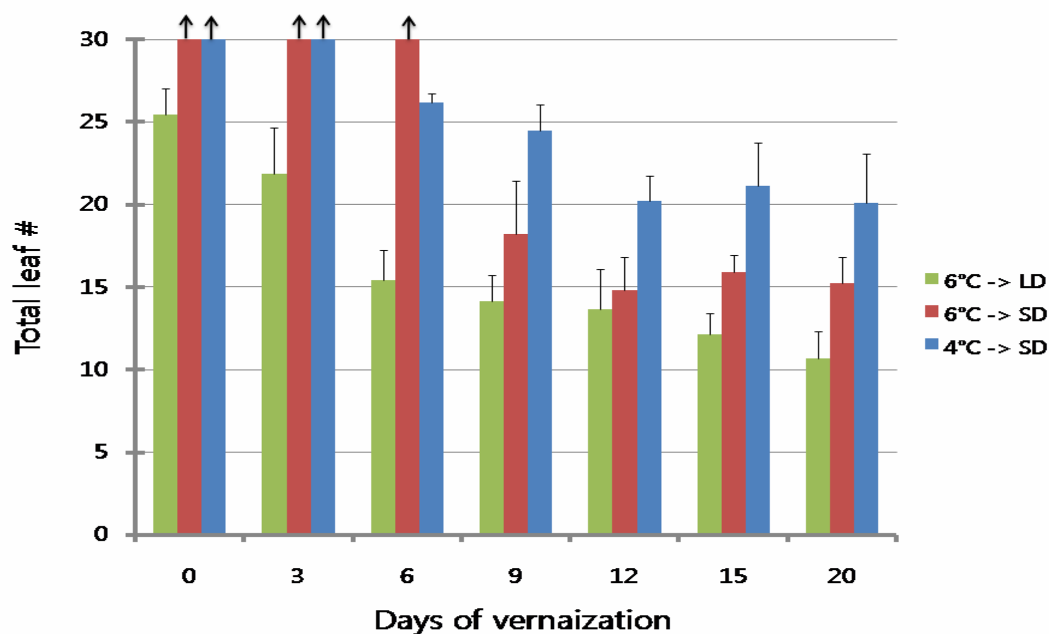

Fig.1 Vernalization responses of radish. Acceleration of flowering starts to be seen after 6 days of cold under LD, but the effect of low temperature seems more conspicuous after 9 days under SD. 6°C cold is more effective for vernalization than 4°C although flowering competence is obtained earlier in 4°C. Arrow indicates that flowering is not initiated when tested.

Cloning of full-coding cDNA of *Raphanus sativus* VIN3 and FLC

Verification of the presence of a unique vernalization response in radish prompted us to identify and isolate *FLC* and *VIN3* orthologs, which are known as a major target and a key mediator respectively in vernalization pathway in *Arabidopsis.* Radish *FLC* was cloned by RT-PCR using primers homologous to conserved sequences of *FLC*s in *B. napus* (another member of the *Crucifers*) and *Arabidopsis*. Like other *FLC* orthologs in *Crucifer* members, radish *FLC* is also highly conserved among them at the amino acid level with sequence identity of ~80% to AtFLC, suggesting a conserved mechanism of *FLC* repression of flowering (Fig. 2).

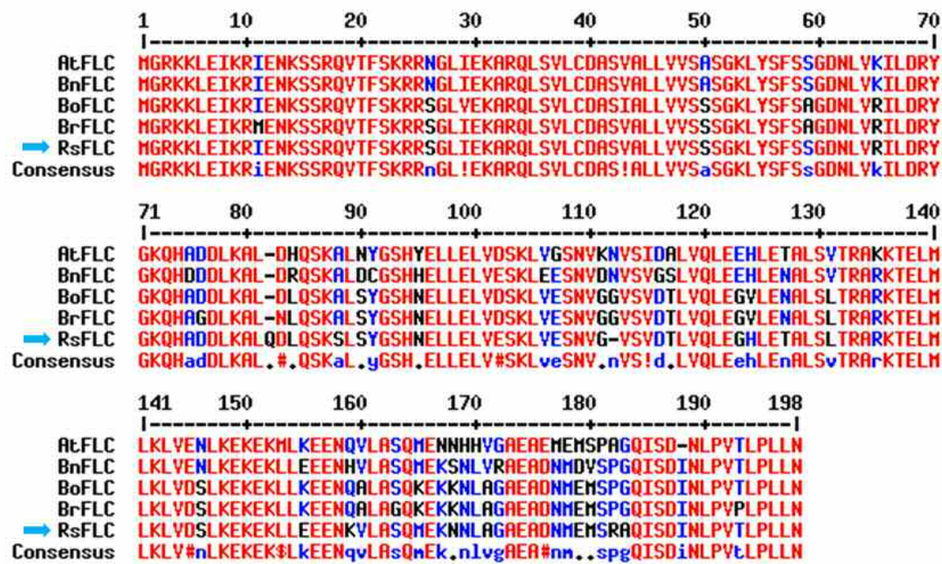

Fig.2 Amino acid sequence of radish FLC (blue arrow) and sequence alignment with other Brassica FLC homologs. All FLCs are well conserved.

For *VIN3* isolation, three polymorphic *VIN3*-like transcripts (*VIN3-1, VIN3-2,* and *VIN3-3*) were identified by 5' RACE (Fig. 3). Differences among these variants can be represented by the presence or absence of two major polymorphic sites. *RsVIN3-2* and *RsVIN3-3* contain 234 bp deletions at their 3' sequences spanning FNIII domain compared to *Arabidopsis VIN3*. *RsVIN3-3* has another polymorphic 57 bp insertion adjacent to 5' proximal end of FNIII domain compared to *AtVIN3* and other *RsVIN3* species. However, overall sequences of 3 radish *VIN3* variants are well conserved to *AtVIN3* that sequence identities show a range of 60~72% (Fig. 4; data not shown for *RsVIN3-2* and *RsVIN3-3*). It was not a surprising result to isolate three distinct *VIN3*-like transcripts since the radish genome is hexaploid. In most cases of polyploidy, some of duplicated genes remain native and others turn to inactive (pseudogene) or altered (Adams et al., 2003; Lynch, 2002; Osborn et al., 2003). None of polymorphic sites identified are predicted to generate any premature stop or aberrant transcripts, suggesting that all 3 loci might be generating functionally active transcripts.

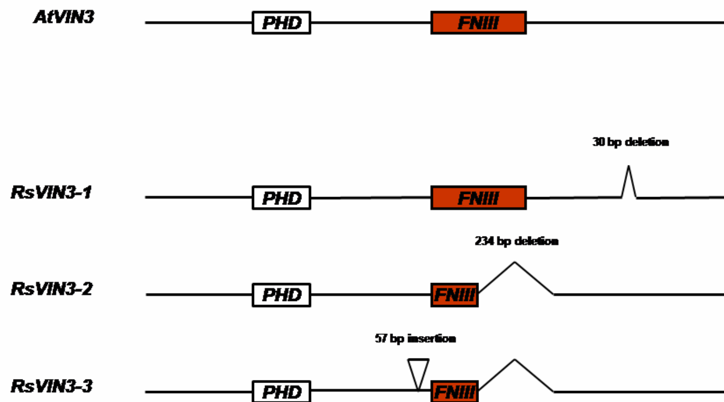

Fig.3 Schematic representation of *AtVIN3*-like loci identified in *R. sativus*. The sequence of the PHD domain is highly conserved in all the variants, while the differences in sequence are mainly at the FNIII and the C-terminal domain, only *VIN3-1* has both intact PHD and FNII domains.

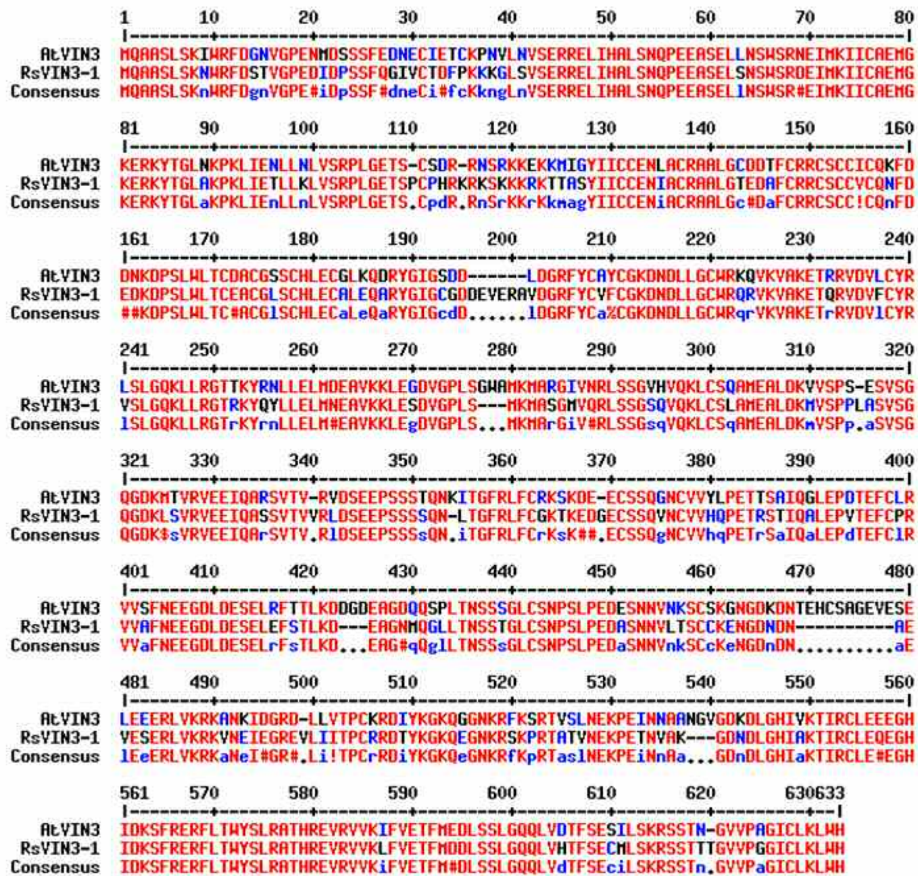

Fig.4 Amino acid sequence of radish *VIN3-1* and sequence alignment with arabidopsis *FLC*.

Analyses of *Rs*VIN3s and *Rs*FLC expression upon vernalization treatment

To determine the functional *VIN3* ortholog in radish, each expression pattern was analyzed. A series of radish RNA samples were prepared depending on the duration of cold treatment and then they were subject to RT-PCR analysis to test which transcript showed vernalization-specific expression pattern similar to that of *Arabidopsis VIN3*. As shown in figure 5, only *VIN3-1* appeared to be regulated in a cold-specific manner and its induction coincided with the downregulation of *FLC*. Additionally, these molecular behaviours exactly reflected physiology of radish vernalization as shown in figure 1. Thus, these data strongly suggest that *VIN3-1* is a native *VIN3* ortholog, functionally equivalent to *Arabidopsis VIN3*.

This radish *VIN3* ortholog is slightly more divergent than *FLC* although it is also significantly conserved at the amino acid level, (Fig. 2 & 4). It is interesting to note that non-native radish *FLC* copies were not found in this cloning, possibly due to very strict functional conservation. This seems reasonable because *FLC* is a central regulator of flowering processes and also a target of several flowering-promotion pathways including vernalization pathway. To adapt certain environmental conditions, plants must have evolved by modulating and altering the sensitivity and responsiveness of such pathways rather than the signal-gathering targets like *FLC*.

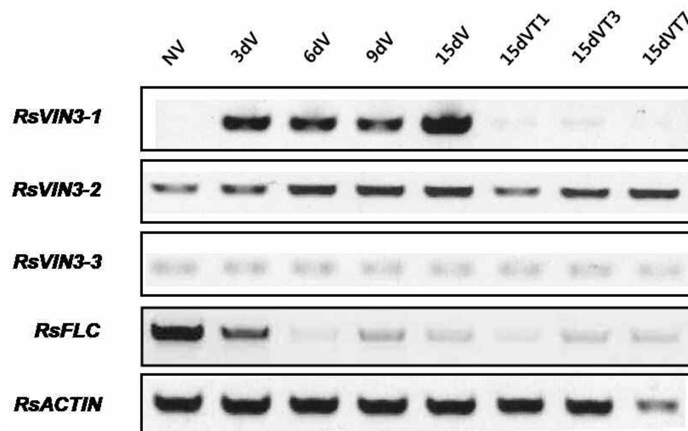

Fig.5 Semi-quantitative RT-PCR analysis of *RsVIN3* and *RsFLC* upon vernalization. Only *RsVIN3-1* is very rapidly expressed specific to cold treatment along with decrease of *RsFLC*. dV represents days of vernalization and T# represents # days in warm condition after vernalization treatment.

Complementation of *Arabidopsis vin3-4* by radish VIN3-1

To further test if radish *VIN3-1* was functional equivalent of *Arabidopsis VIN3*, overexpression construct of *RsVIN3-1* was introduced into *Arabidopsis vin3* mutant. Transgene contained 35S promoter, total 2726 bp nucleotides including coding region of *RsVIN3-1*, and *nos* terminator (Fig. 6). Representative homozygous T3 lines were selected for further analysis. *Arabidopsis vin3-4* mutant seems null based on undetected *VIN3* transcripts with vernalization (data not shown) and therefore does not respond to vernalization (Fig. 7). When *vin3-4* was introduced by *RsVIN3-1* under control of 35S promoter, ectopic expression of RsVIN3-1 was observed (data not shown). 35S promoter-driven ectopic expression of *RsVIN3* was able to rescue normal vernalization response, suggesting *RsVIN3-1* is functional (Fig. 7). It is interesting to note that without cold treatment, ectopic expression of *RsVIN3-1* has no effect on flowering time, suggesting there might be 'cold-induced' post-transcriptional regulation or protein-protein interaction to repress *FLC*.

Fig.6 Schematic representation of transgene containing genomic *RsVIN3-1* under control of 35S-CaMV promoter

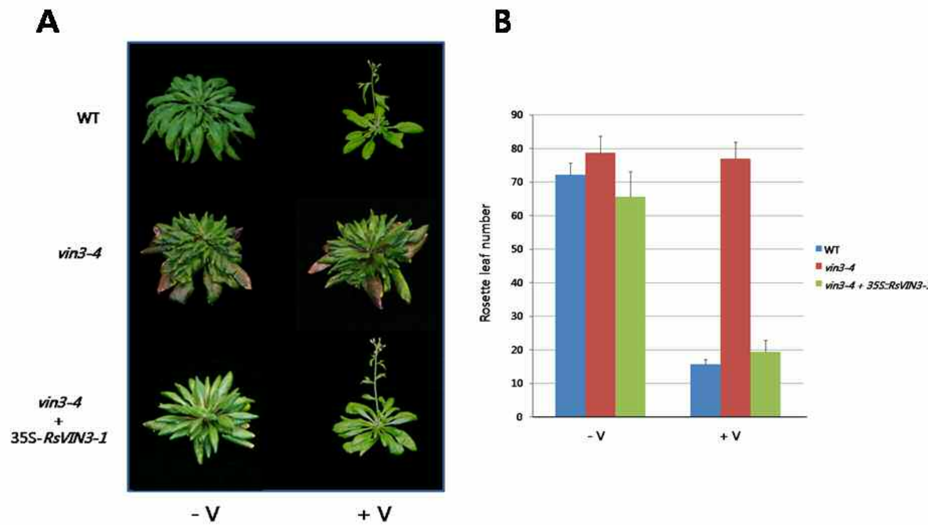

Fig.7 Molecular complementation of *vin3-4* by *RsVIN3-1*. Vernalization response was rescued by introduced transgene containing *RsVIN3-1*.

Kinetics of vernalization in radish and arabidopsis

Once we verified that radish vernalizes very rapidly and the molecular characteristics coincide with such physiology, then we attempted to analyze vernalization 'kinetics' between radish and arabidopsis. RNAs were extracted from non-vernalized, 7 day-vernalized, and 18 day-vernalized radish plants at 6°C. Contemporary, *Arabidopsis* RNAs were collected from non-vernalized, 7 day-vernalized and 40 day-vernalized samples at 4°C. As expected, *RsFLC* was repressed only after 7 days of treatment. Correspondingly, *RsVIN3-1* reached the maximum level of expression after 7 days of cold (Fig. 8). For arabidopsis plants, *AtFLC* was down regulated after 40 days of vernalization at the same time as *At*VIN3 reached its highest level of expression (Fig. 9). This molecular behaviour reflects flowering time after such vernalization treatment as shown Figure 10. Taken together, we confirmed that relatively short-term cold represented by 7 days here is quite enough to affect on radish vernalization response but not in *Arabidopsis*, which requires 40 days of cold treatment.

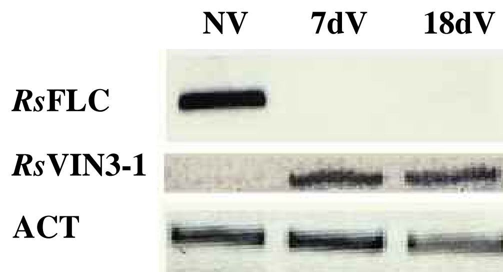

Fig.8 Semiquantitative RT-PCRs showing *Rs*FLC and *Rs*VIN3-1 expression during vernalization treatment. Amplification of actin was used as internal control of the PCR. *Rs*FLC is completely downregulated after 7 days of cold treatment; contemporary, *Rs*VIN3 reached its full-expression.

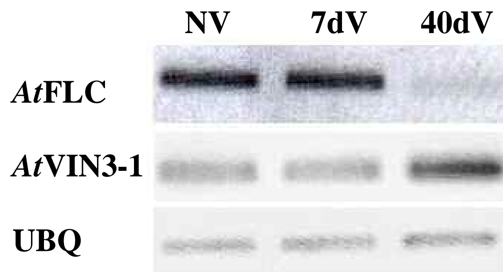

Fig.9 Semiquantitative RT-PCRs showing *At*FLC and *At*VIN3 expression during vernalization treatment. FLC was undetectable after 40 days of cold treatment corresponding to the full-expression of VIN3. Ubiquitin amplification was used as internal control for the reaction.

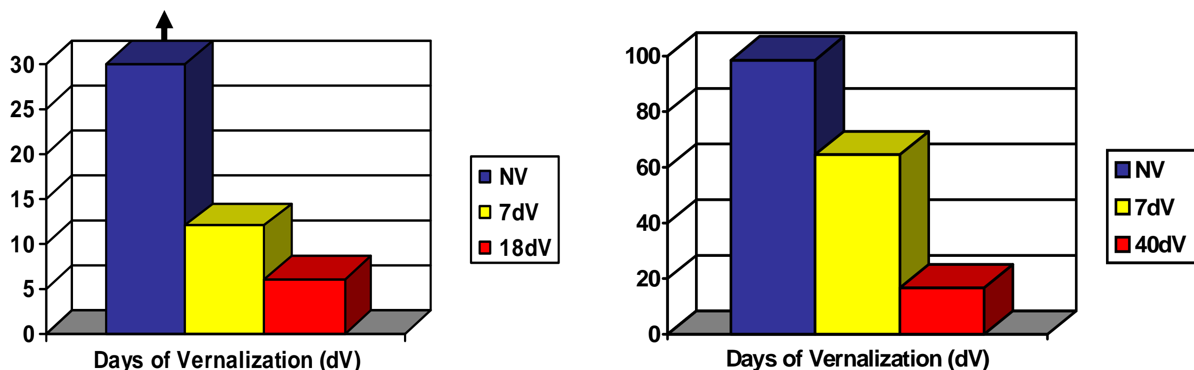

Fig.10 Effect of vernalization on flowering-time of *Raphanus s*. (left) and *Arabidopsis* t. (right), under LD conditions. The *y*-axis indicates total leaf number (number of rosette leaves) formed when plants started to bolt. Vernalization was conducted at 6°C for radish and at 4°C for arabidopsis.

Cell division at the shoot apex of radish and arabidopsis

From the analyses of the response toward cold treatment, resulted that radish required a very short period of treatment to gain the status full-vernalized, while arabidopsis needed a longer period (Fig. 8-10). Interested by the behaviour of radish, we would further investigate the cause that determines a so short requirement of cold treatment respect to arabidopsis plants.

Stimulation of cell division and changes in size and shape in various parts of the shoot apex are known to be common features of the floral transition (Lyndon and Francis 1984; Francis 1992, Francis and Herbert 1993; Lyndon 1998; Dorota 2006).

Moreover, it is known, that at the moment in which plant is vernalized each meristematic cell is subject to vernalization, reproduces the vernalized condition by mitosis to the newly regenerating plant (Wellensiek, 1961), and dividing cells are a pre-requisite for vernalization (Wellensiek, 1962). On the basis of these notions, our hypothesis was that determination of the duration of cold treatment could be in some way related to the cell division rate at the apical meristem.

In order to investigate our hypothesis, plants of radish and arabidopsis, as control, were vernalized; than shoot apical meristems (SAMs) from these plants were excised, fixed, embedded and the cell number in transversal sections of SAM was collected.

Based on the partitioning in layers, as previously reported by Lauf et al. 1998, fig.6, we sectioned the SAMs of radish and arabidopsis both vernalized and not. Our purpose was to evidence differences in cell number at the beginning of the treatment and show evidences of cell division rate from the beginning of the treatment to the complete induction of the meristem.

The data collected are represented in Fig.12. Because identification of the basal section of the meristem was determined by visual aspect at the microscope, count of the mean number of the meristematic cells comprised the cells in the sections over and below the one that we fixed as basal.

Analyses were repeated in two independent experiments, for each experiment three different apical samples were considered.

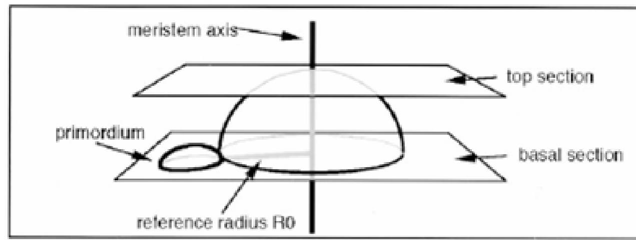

Fig.11 Graphic representation of SAM partitioning. The basal section determine the zone where is meristematic cells are visible in the central part of the section and primordium joins the meristem flank.

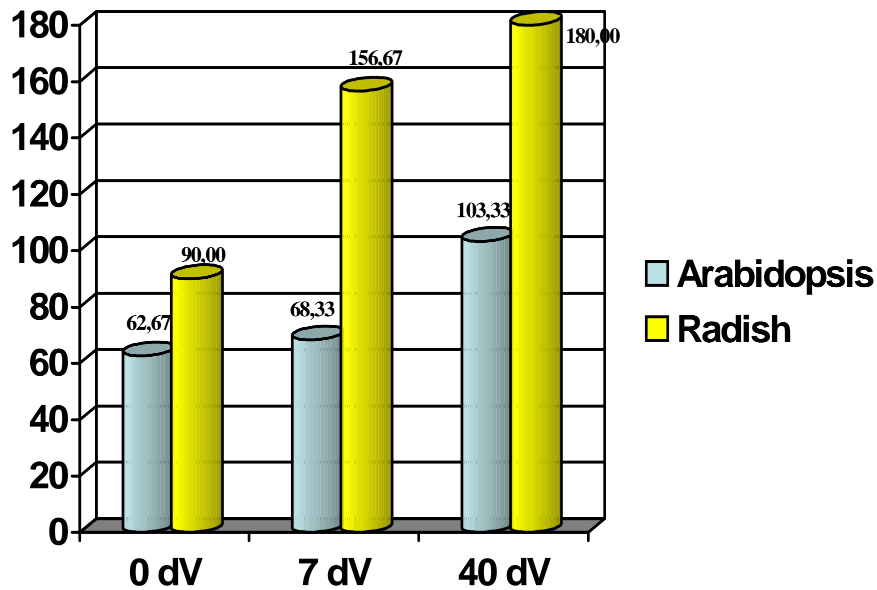

Fig.12 Graph shows meristematic cell number collected in SAM from plants of *Arabidopsis* and *Raphanus* vernalized and not. dV= days of Vernalization; in the *y*-axis cell number. For each sample the mean number of cells is indicated.

Images at the microscope of representative samples are indicated in fig. 13. In these pictures is possible to note that the cell number in arabidopsis apices remained quite the same as in not vernalized than in 7 days treated plants. Correspondingly, in radish apices the number of cells appears clearly increased.

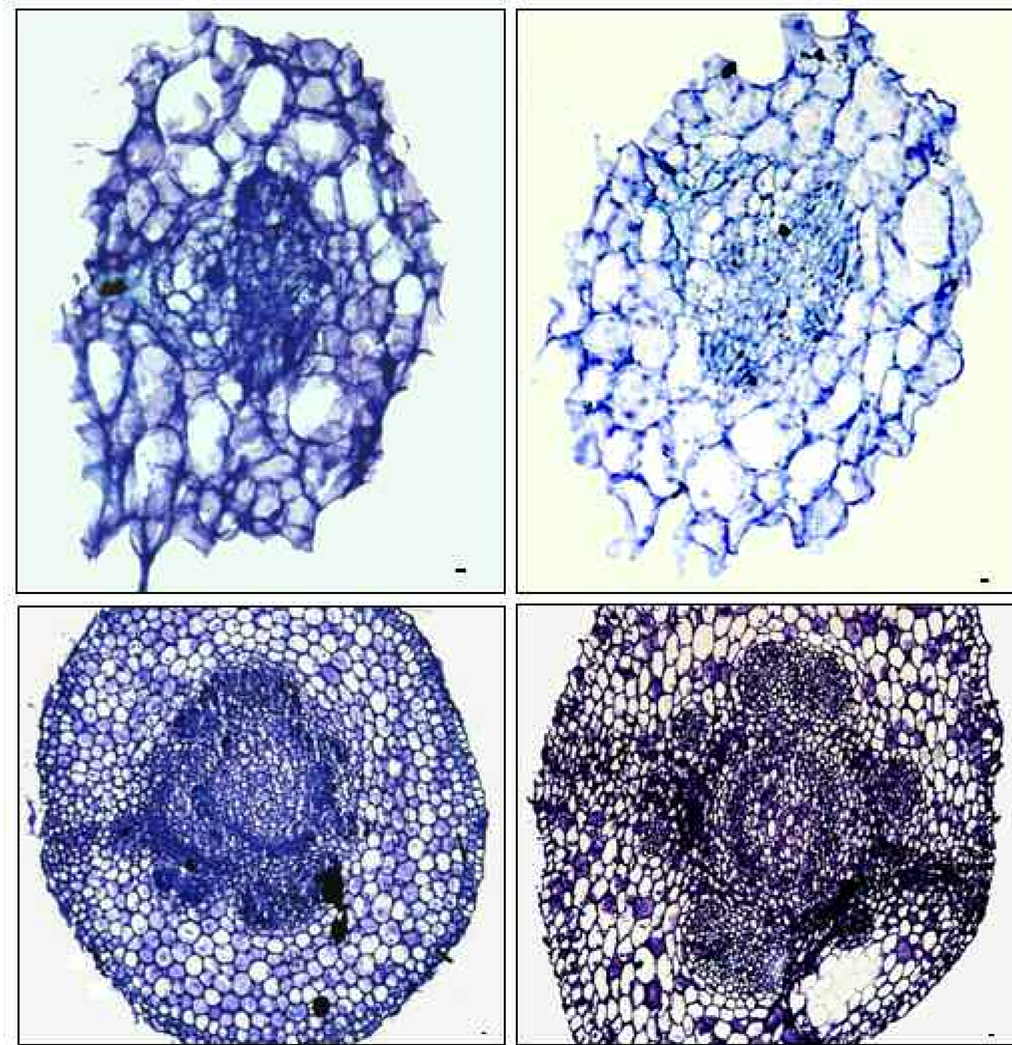

Fig.13 Transversal sections of apical meristems from arabidopsis and radish seedlings. Cell number is appreciable from comparing the four samples. From the top, arabidopsis meristem in a not-vernalized plant (left), beside apical meristem of a 7 days vernalized plant (right); on the bottom SAM of radish in a not-vernalized plant (left) and in a 7 days vernalized radish (right). Primordia at the flank of the meristem are appreciable in all the sections. Bars=100µm.

Discussion

Radish sativus is a crop with useful characteristics for vernalization studies. In fact, it is completely vernalized in a short-time, is propagated by seed, seeds can be vernalized in a short time and flowering, after cold treatment, is relatively fast (2 months).

Nevertheless, radish has not been deeply studied from this point of view, but more for its response toward gibberellin treatment and interaction with photoperiod on determining of flowering time (Erwin et al. 2002).

Interested by the deeply difference of behaviour manifested by *Raphanus* in respect to *Arabidopsis,* we investigated phenotypic and cytological aspects of the vernalization response of radish, in a parallel analyses with arabidopsis plants.

A previous work from Engelen-Eigles and Erwin in 1997, demonstrated that *Raphanus sativus* var. Chinese Radish Jumbo Scarlet can be completely vernalized in 6-8 days as an imbibed seed. In this work we analysed the response toward vernalization of seedlings of *Raphanus sativus* (Chinese Radish Jumbo Scarlet), whose seeds were not vernalized, considering at first, two different photoperiodic conditions (long day vs. short day); then, we tested the optimal temperature upon which plants can be vernalized.

Our results demonstrated that requirement of cold for radish was very short even in the stage of seedlings. In fact, 7 days were enough to obtain full-vernalized plants. A short-day photoperiod seemed more effective than a long-day. However, plants responded to cold even in long-day, where the first response is manifested after only 6 days of treatment (fig. 1).

It is known that the more the temperature varies from the temperature optima for vernalization, the longer plants need to be treated for complete flower induction; this characteristic make the vernalization a quantitative process (Metzger 1996). In our work we tested that the optima temperature to vernalize radish plants was 6°C.

The previous results were obtained by analyzing phenotypic aspects of the cold treatment (i.e. total leaf number at the time of which flower has been emitted). Because *Raphanus*

belongs to the *Brassicaceae* family, as *Arabidopsis thaliana* and responds to vernalization, there was reason to suppose that Flowering Locus C (FLC), a strong repressor of flowering, and Vernalization Insensitive 3 (VIN3), a negative regulator of FLC, were not present even in radish and that probably they could share biological function with the genes isolated in *Arabidopsis*.

In this study we described for the first time the cloning and characterization of VIN3 and FLC form *Raphanus sativus*, designed as *RsVIN3* and *RsFLC*. *RsVIN3* is present in three variants, that we named VIN3-1, VIN3-2 and VIN3-3 respectively. By mean of RT-PCR, we tested that only VIN3-1 responded to vernalization. Comparing the nucleotidic sequences of the *Rs*VIN3s isolated by RACE against *At*VIN3, we could verify a range of 60-72% of identity. The major differences among the sequences comprise a lack in 30 nucleotides at the C-terminal region of VIN3-1; a lack in 234 nucleotides in a region comprising part of the FNIII domain and the C-end in VIN3-2 and a 54 nucleotides insertion in the region before the FNIII domain and the same 234 nucleotides deletion evidenced in VIN3-2 for VIN3-3. Further investigations need to characterize the role of VIN3-2 and VIN3-3, which probably could be derived from event of gene duplication, given that radish is a hexaploid specie. Moreover, none of polymorphic sites identified are predicted to generate any premature stop or aberrant transcripts, suggesting that all 3 loci might be generating functionally active transcripts .

*Rs*FLC has been identified as a single copy gene, which share an 80% of identity with *At*FLC.

Molecular expression of *Rs*VIN3-1 and *Rs*FLC was respectively tested by RT-PCR. In our experiment VIN3-1 resulted full-expressed after 7 days of cold (fig.5) and its expression was completely down-regulated after the plant returned to the warmer temperature (fig.5), demonstrating that VIN3-1 expression is activated by the cold. *Rs*FLC expression was completely down-regulated after 7 days of treatment and its expression was maintained low even when plants were transferred to the warmer temperature, demonstrating that analogously to *At*FLC, *Rs*FLC was down regulated by the vernalization pathway and that its expression is stably controlled even after that the treatment has ended.

Shoot apical meristems (SAMs) are constituted by small groups of dividing cells that initiate all of the aerial parts of the plant. SAM can be dissected in functionally distinct zones (Kerstetter and Hake 1997; Medford 1992; Clark 1997). In dicots, three layers can be distinguished and they are called L1, L2 and L3. The majority of the cells derived from the L1 layer form the epidermis, whereas L2 and L3 provide cells for the inner parts of the organs (Steeves and Sussex 1989). Genetic analyses have shown that an intricate framework exists between a number of gene expressed in the SAM and that the different time and area of expression, is responsible for the change of fate of the meristem from vegetative to reproductive. Recently, cellular parameters of the shoot apical meristem (Lauf et al 1998) and the pattern of cell proliferation (Traas and Bohn-Courseau 2005; Dorota 2006) have been defined in *Arabidopsis thaliana*.

The role of the cell cycle in shaping the plant is linked to the capacity of cell cycle regulators to influence growth, but how this system works has not been unequivocally defined.

Many cell cycle regulators have been studied and their expression has been related with environmental cues, such as temperature and light quality, other than with the stage of develop of the plant.

The transition of the vegetative to reproductive form involves geometry changes, especially surface expansion, accompanying flower primordium formation. It was proposed that an increase in the cell division rate is a prerequisite for the subsequent morphological changes at the SAM floral transition (Francis 1992; Bernier 1997). Studies have been conducted to determine where the mitotic frequency start to increase in the SAM; when this increase happen and when the SAM change in size and shape. The results showed that increase in the rate of cell division happen in the central zone and peripheral zones of the SAM (Jacquemard et al 2003). The mitotic and labelling indices exhibit increases of the order of 250-300% in *Arabidopsis* against of 400-1000% in *Sinapis alba* and *Xanthium strumarium* SAMs (Bernier et al 1967; Jacquemard et al 1976). This lesser activation in arabidopsis has been explained as probably due to the fact that this species is a facultative LD plant which is progressing slowly with time to the floral state even in unfavourable SDs (Gocal et al 2001).

With the purpose to investigate the requirement of vernalization on radish respect to that of arabidopsis, and on the basis of the information about cell rate division and area of increased cell proliferation during floral transition in arabidopsis, we decided to monitor the cell number in the central zone of the radish SAM. Because we would test, approximately, cells proliferation at the SAM of radish during floral induction, we chose a representative section of the meristem, defined as "basal" in arabidopsis. This layer comprises the beginning of the central zone and show primordia formation at its flanks. We chose this layer because it was the easiest one to individuate and representative for the cellular proliferation at the meristem and then useful to compare the different SAMs analysed. An increase in cell proliferation was observed in radish at the time of flower induction. One consideration emerged from the data: the rate of cell division seemed higher in radish than arabidopsis. In fact, we observed that the mean cell number was twice in radish after only 7 days of treatment (90 *vs*. 157), while it was slowly increased in arabidopsis (68 *vs*. 63). After this moment, the rate of division became slower in radish (180 vs. 156 after 33 days of cold), while increased in arabidopsis (103 vs. 68 after 33 days of cold), fig.12.

Because full-activation of VIN3-1, complete down-regulation of FLC and a significant increase of cell division have been observed after only 7 days of cold exposition, we hypothesize that rapidity of cell cycle is strongly related with the acquisition of the competence to flower by the SAM. We know that vernalized status is mitotically transmitted to the daughter cells, so that, if more cells are present in the moment of which cold is supplied, the effect of the treatment would be faster and that the minimum number of cells required for flower primordium initiation is raised in a short time.

The evolutionary reason of the behaviour manifested by *Raphanus sativus* in respect to that of *Arabidopsis thaliana*, could be that this specie evolved under adverse environmental conditions, so that the duration of the time favourable interposed between flower induction and seeds production was too short and the plant had to organize a genetic program to overcome the risks of premature death. Further experiments are needed to confirm this hypothesis.

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Appendix 1

Fig. 1. Nucleotidic alignment between partial cDNA sequences of *Ci*FLC isolated in wild chicory F2. population. Identity is indicated by red box. The alignment has been produced by ClustalV V algorithm.

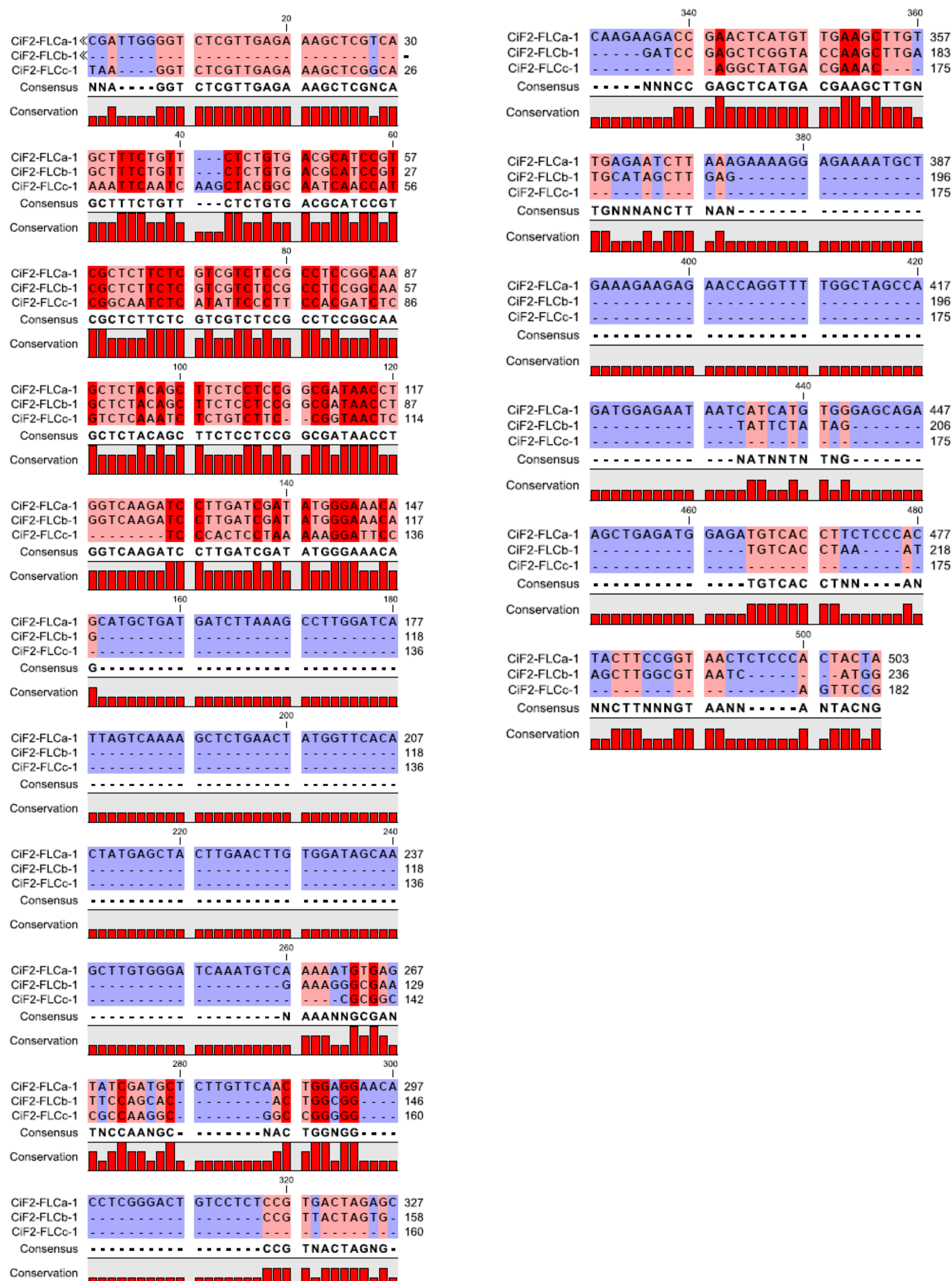
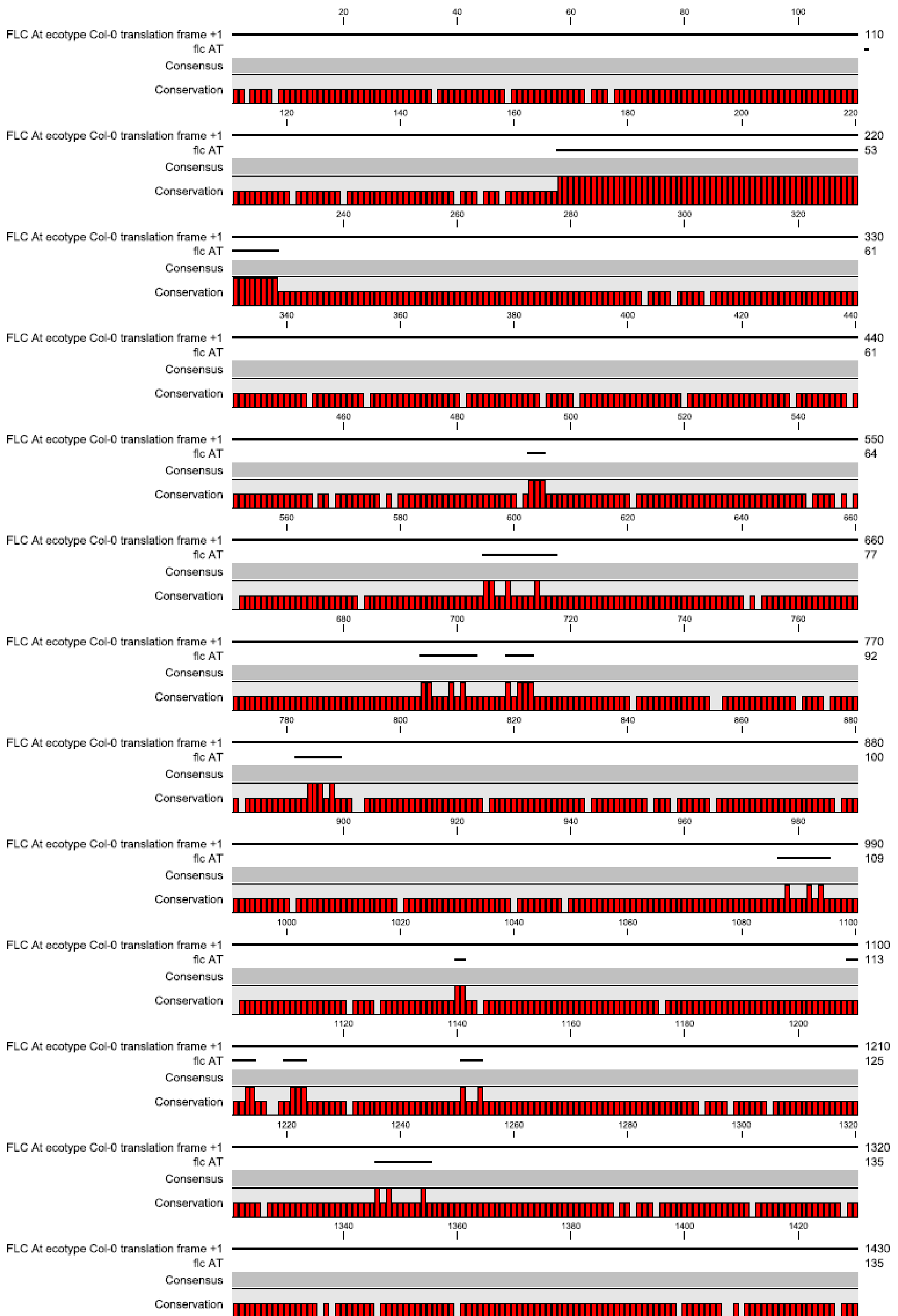


FIG. 2. Nucleotidic alignment between *Ci*FLC2 and *At*FLC. The consensus derived from this alignment shows the high degree of conservation between the nucleotidic sequence of the AtFLC ORF and the partial cDNA of *Ci*FLC2. Alignment has been produced by using ClustalV.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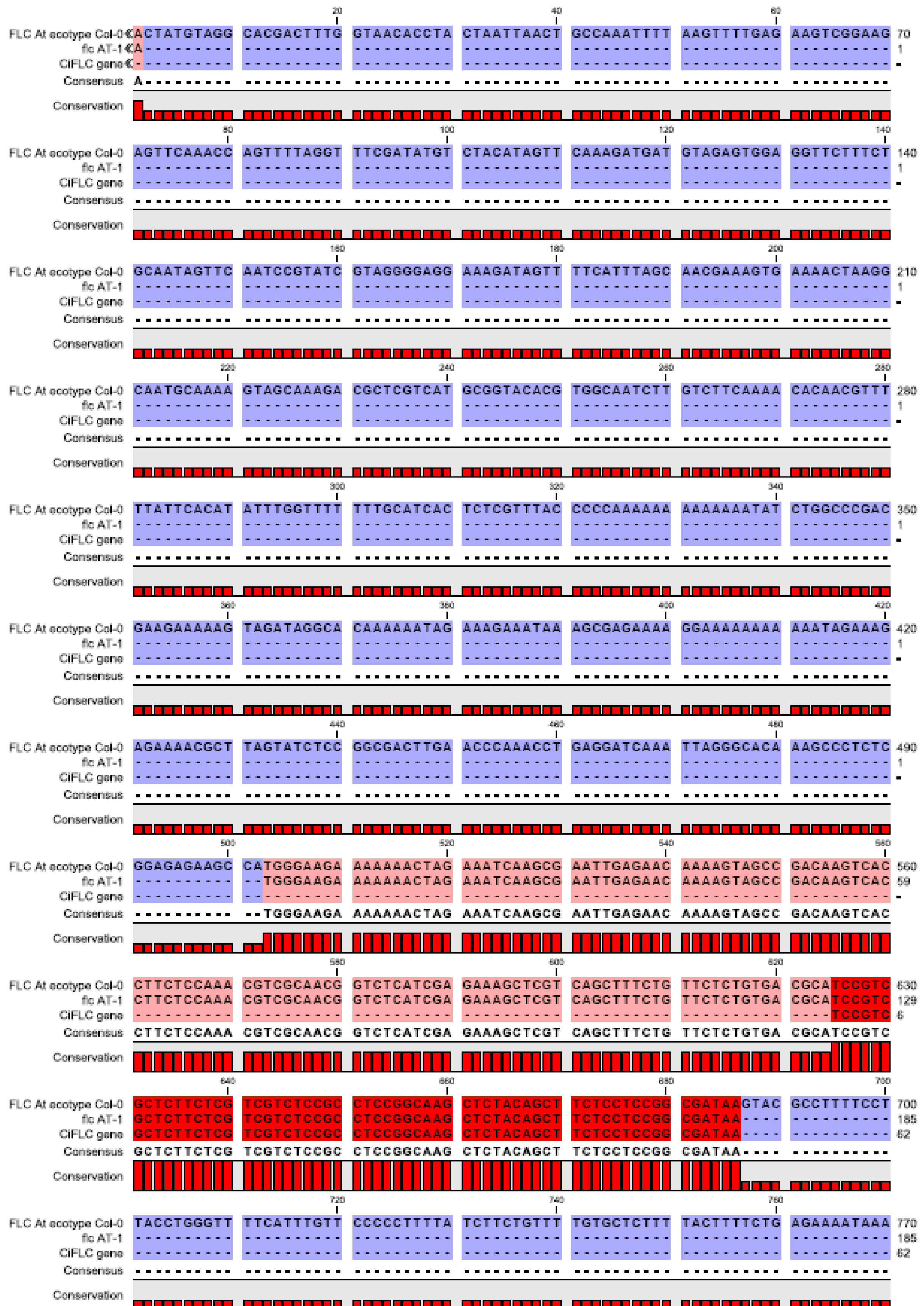
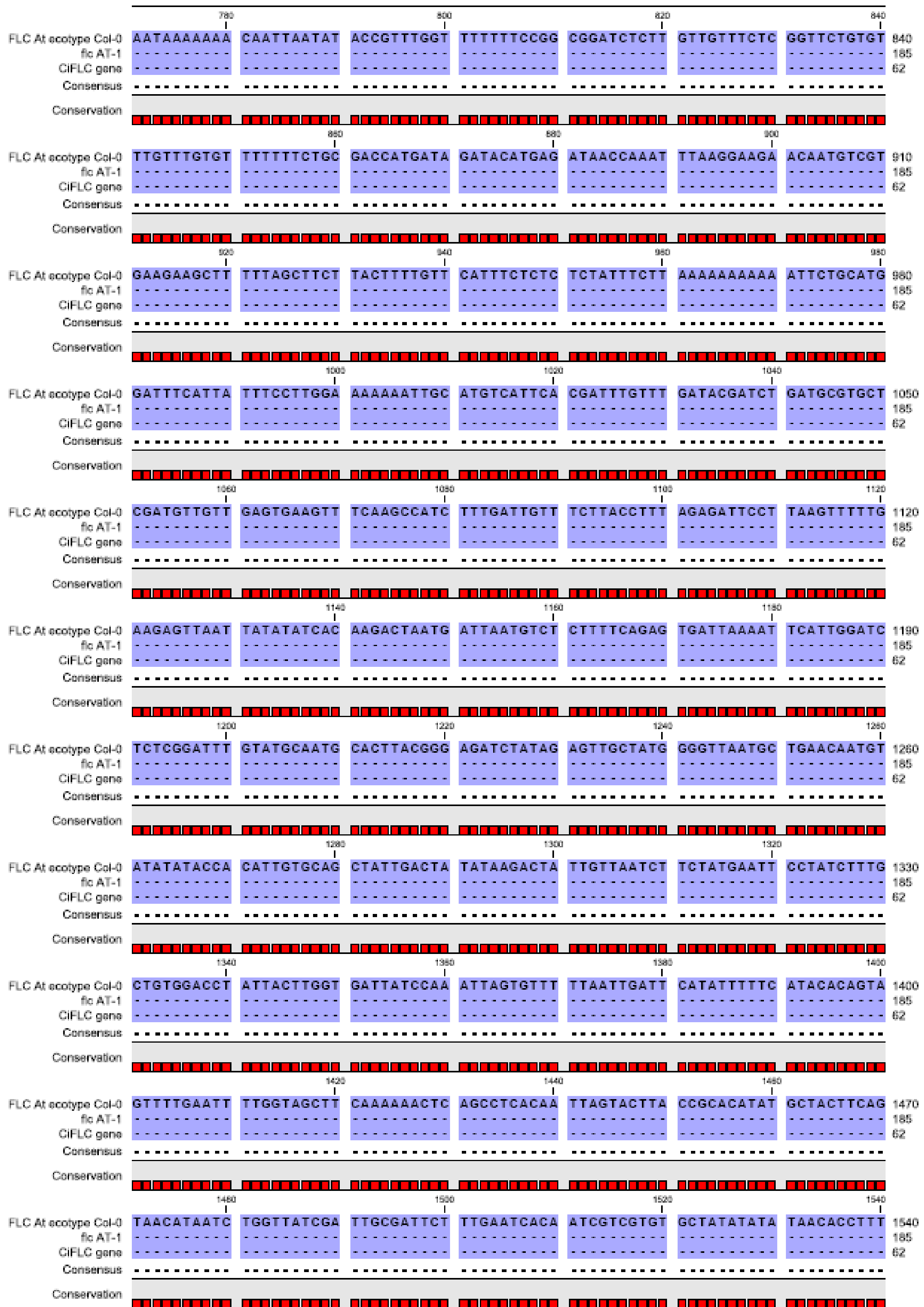
flc AT
Consensus
Conservation

Fig. 3. Nucleotidic alignment between the genomic sequence of FLC from *Arabidopsis* and chicory. Alignment has been produced by ClustalV. The yellow box indicates the splice site identified inside the *CiFLC* genomic sequence.





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Conservation
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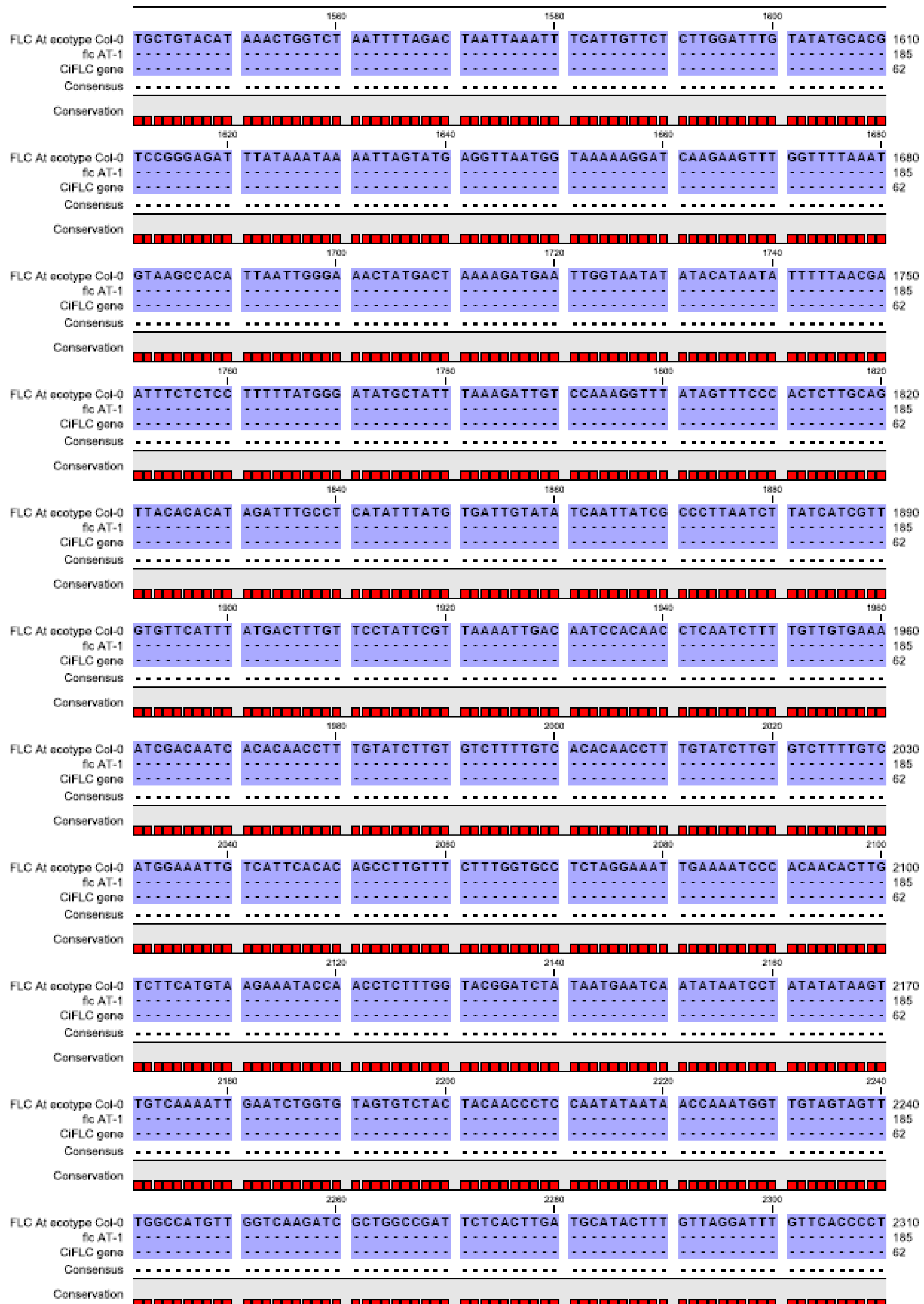

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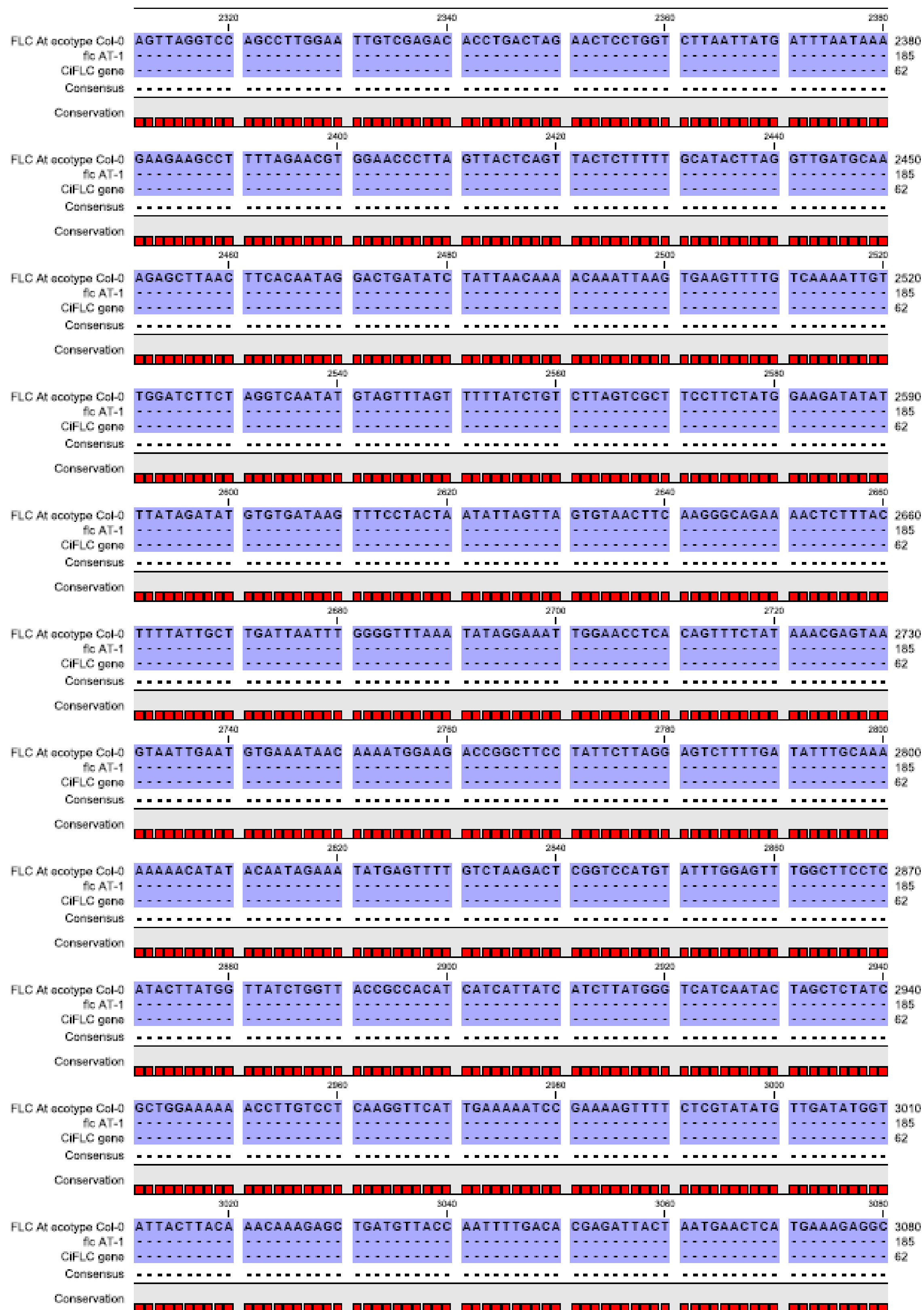
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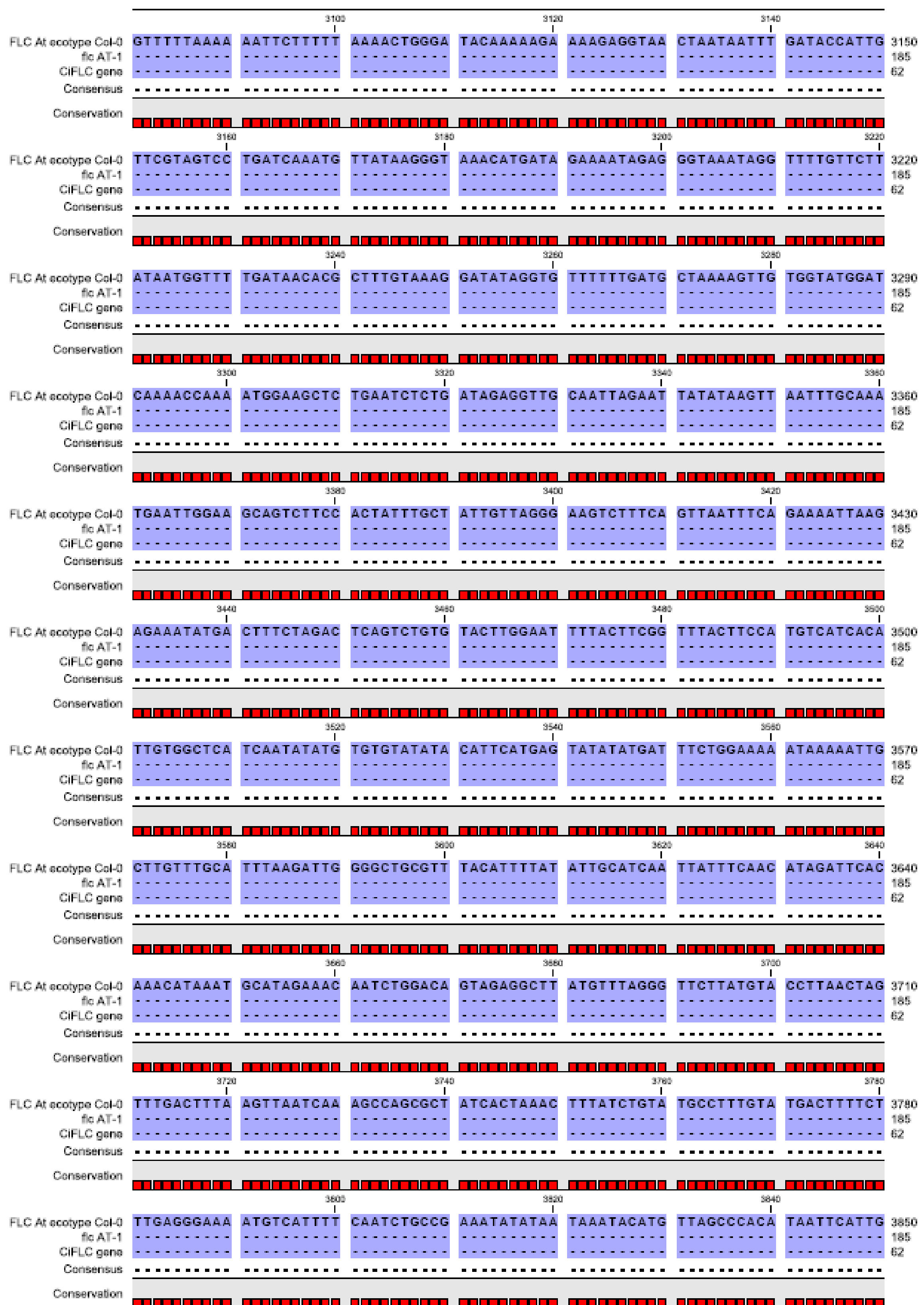
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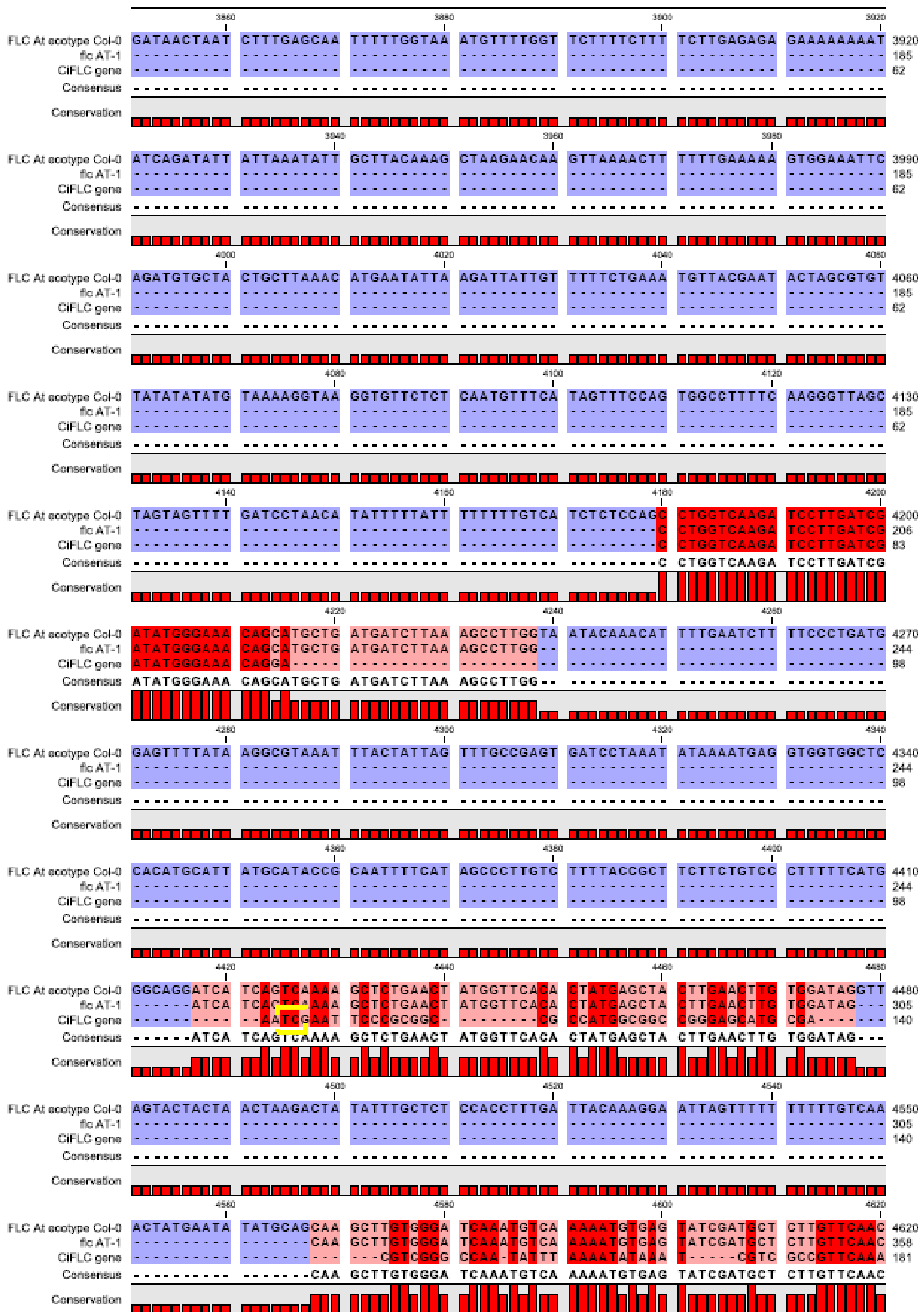

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CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 62
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
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CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 62
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CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 62
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
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CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 62
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
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Consensus ---------- ---------- ---------- ---------- ---------C CTGGTCAAGA TCCTTGATCG
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flc AT-1 ATATGGGAAA CAGCATGCTG ATGATCTTAA AGCCTTGG-- ---------- ---------- ---------- 244
CiFLC gene ATATGGGAAA CAGGA----- ---------- ---------- ---------- ---------- ---------- 98
Consensus ATATGGGAAA CAGCATGCTG ATGATCTTAA AGCCTTGG-- ---------- ---------- ----------
Conservation
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flc AT-1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- 244
CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 98
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
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CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 98
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
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flc AT-1 ------ATCA TCAGTCAAAA GCTCTGAACT ATGGTTCACA CTATGAGCTA CTTGAACTTG TGGATAG--- 305
CiFLC gene ---------- --AATCGAAT TCCCGCGGC- --------CG CCATGGCGGC CGGGAGCATG CGA------- 140
Consensus ------ATCA TCAGTCAAAA GCTCTGAACT ATGGTTCACA CTATGAGCTA CTTGAACTTG TGGATAG---
Conservation
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Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
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flc AT-1 ---------- -------CAA GCTTGTGGGA TCAAATGTCA AAAATGTGAG TATCGATGCT CTTGTTCAAC 358
CiFLC gene ---------- ---------- ---CGTCGGG CCAA-TATTT AAAATATAAA T-----CGTC GCCGTTCAAA 181
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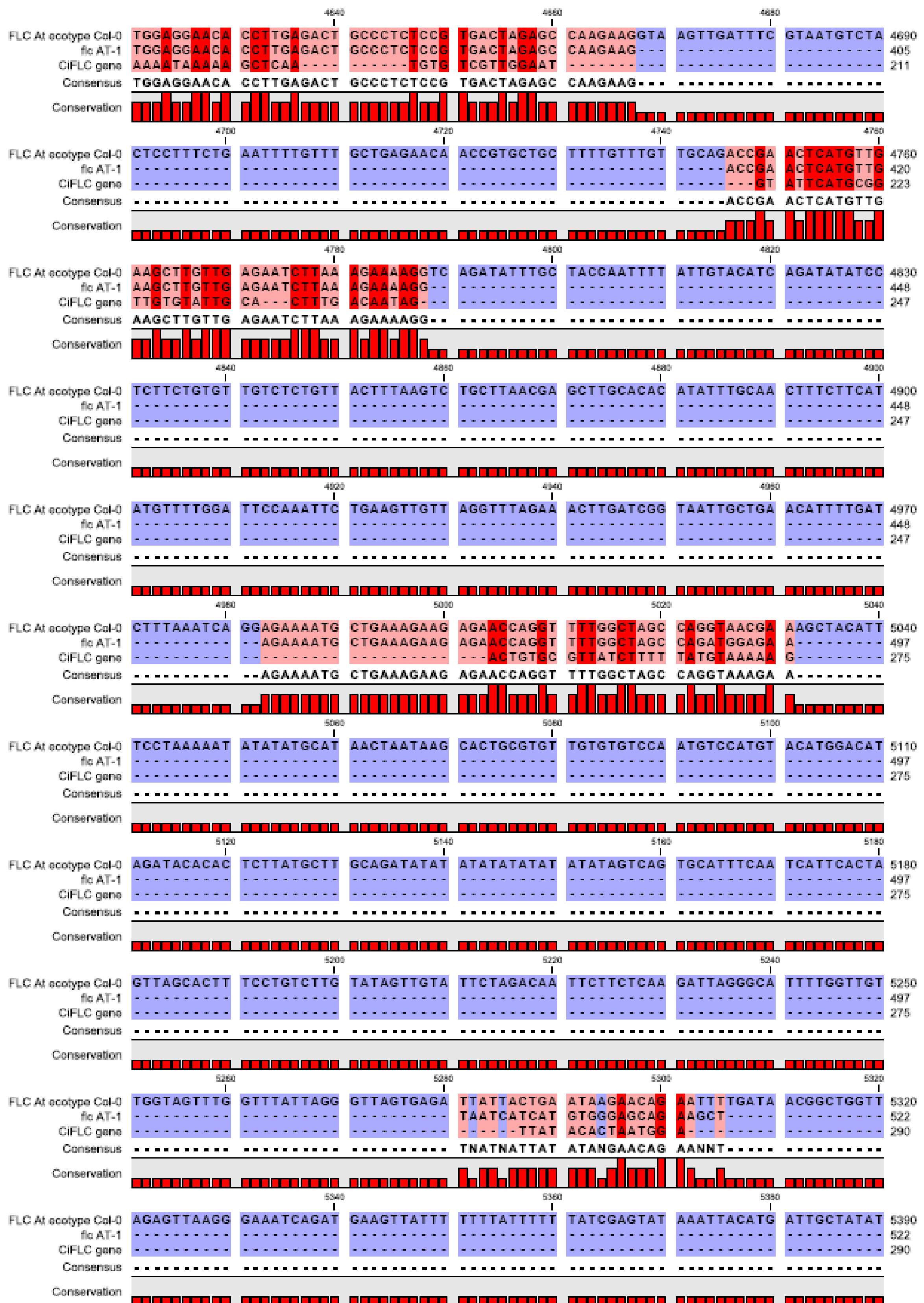
Conservation



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CiFLC gene AAAATAAAAA GCTCAA---- ------TGTG TCGTTGGAAT ---------- ---------- ---------- 211
Consensus TGGAGGAACA CCTTGAGACT GCCCTCTCCG TGACTAGAGC CAAGAAG--- ---------- ----------
Conservation
4700 4720 4740 4760
FLC At ecotype Col-0 CTCCTTTCTG AATTTTGTTT GCTGAGAACA ACCGTGCTGC TTTTGTTTGT TGCAGACCGA ACTCATGTTG 4760
flc AT-1 ---------- ---------- ---------- ---------- ---------- -----ACCGA ACTCATGTTG 420
CiFLC gene ---------- ---------- ---------- ---------- ---------- --------GT ATTCATGCGG 223
Consensus ---------- ---------- ---------- ---------- ---------- -----ACCGA ACTCATGTTG
Conservation
4780 4800 4820
FLC At ecotype Col-0 AAGCTTGTTG AGAATCTTAA AGAAAAGGTC AGATATTTGC TACCAATTTT ATTGTACATC AGATATATCC 4830
flc AT-1 AAGCTTGTTG AGAATCTTAA AGAAAAGG-- ---------- ---------- ---------- ---------- 448
CiFLC gene TTGTGTATTG CA---CTTTG ACAATAG--- ---------- ---------- ---------- ---------- 247
Consensus AAGCTTGTTG AGAATCTTAA AGAAAAGG-- ---------- ---------- ---------- ----------
Conservation
4840 4860 4880 4900
FLC At ecotype Col-0 TCTTCTGTGT TGTCTCTGTT ACTTTAAGTC TGCTTAACGA GCTTGCACAC ATATTTGCAA CTTTCTTCAT 4900
flc AT-1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- 448
CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 247
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
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FLC At ecotype Col-0 ATGTTTTGGA TTCCAAATTC TGAAGTTGTT AGGTTTAGAA ACTTGATCGG TAATTGCTGA ACATTTTGAT 4970
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Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
4980 5000 5020 5040
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Consensus ---------- --AGAAAATG CTGAAAGAAG AGAACCAGGT TTTGGCTAGC CAGGTAAAGA A---------
Conservation
5060 5080 5100
FLC At ecotype Col-0 TCCTAAAAAT ATATATGCAT AACTAATAAG CACTGCGTGT TGTGTGTCCA ATGTCCATGT ACATGGACAT 5110
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CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 275
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
5120 5140 5160 5180
FLC At ecotype Col-0 AGATACACAC TCTTATGCTT GCAGATATAT ATATATATAT ATATAGTCAG TGCATTTCAA TCATTCACTA 5180
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CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 275
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
5200 5220 5240
FLC At ecotype Col-0 GTTAGCACTT TCCTGTCTTG TATAGTTGTA TTCTAGACAA TTCTTCTCAA GATTAGGGCA TTTTGGTTGT 5250
flc AT-1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- 497
CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 275
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
5260 5280 5300 5320
FLC At ecotype Col-0 TGGTAGTTTG GTTTATTAGG GTTAGTGAGA TTATTACTGA ATAAGAACAG AATTTTGATA ACGGCTGGTT 5320
flc AT-1 ---------- ---------- ---------- TAATCATCAT GTGGGAGCAG AAGCT----- ---------- 522
CiFLC gene ---------- ---------- ---------- ------TTAT ACACTAATGG A--------- ---------- 290
Consensus ---------- ---------- ---------- TNATNATTAT ATANGAACAG AANNT----- ----------
Conservation
5340 5360 5380
FLC At ecotype Col-0 AGAGTTAAGG GAAATCAGAT GAAGTTATTT TTTTATTTTT TATCGAGTAT AAATTACATG ATTGCTATAT 5390
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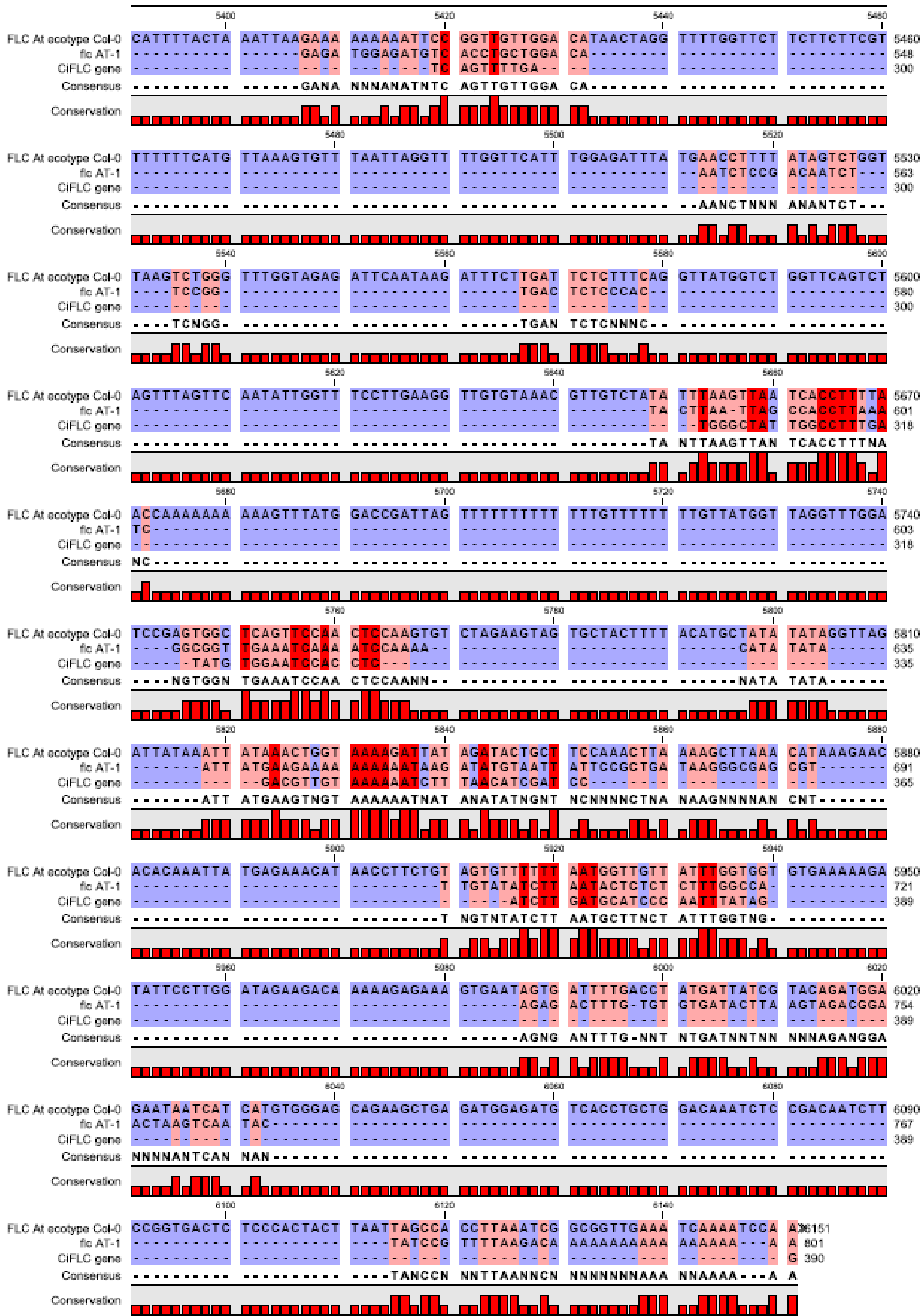
CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 290
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation



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CiFLC gene ---------- ---------- --------TC AGTTTTGA-- ---------- ---------- ---------- 300
Consensus ---------- ------GANA NNNANATNTC AGTTGTTGGA CA-------- ---------- ----------
Conservation
FLC At ecotype Col-0 TTTTTTCATG TTAAAGTGTT TAATTAGGTT TTGGTTCATT TGGAGATTTA TGAACCTTTT ATAGTCTGGT 5530
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CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 300
Consensus ---------- ---------- ---------- ---------- ---------- --AANCTNNN ANANTCT---
Conservation
FLC At ecotype Col-0 TAAGTCTGGG TTTGGTAGAG ATTCAATAAG ATTTCTTGAT TCTCTTTCAG GTTATGGTCT GGTTCAGTCT 5600
flc AT-1 ----TCCGG- ---------- ---------- ------TGAC TCTCCCAC-- ---------- ---------- 580
CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 300
Consensus ----TCNGG- ---------- ---------- ------TGAN TCTCNNNC-- ---------- ----------
Conservation
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flc AT-1 ---------- ---------- ---------- ---------- --------TA CTTAA-TTAG CCACCTTAAA 601
CiFLC gene ---------- ---------- ---------- ---------- ---------- --TGGGCTAT TGGCCTTTGA 318
Consensus ---------- ---------- ---------- ---------- --------TA NTTAAGTTAN TCACCTTTNA
Conservation
FLC At ecotype Col-0 ACCAAAAAAA AAAGTTTATG GACCGATTAG TTTTTTTTTT TTTGTTTTTT TTGTTATGGT TAGGTTTGGA 5740
flc AT-1 TC-------- ---------- ---------- ---------- ---------- ---------- ---------- 603
CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 318
Consensus NC-------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
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flc AT-1 ----GGCGGT TGAAATCAAA ATCCAAAA-- ---------- ---------- ------CATA TATA------ 635
CiFLC gene ------TATG TGGAATGCAC CTC------- ---------- ---------- ---------- ---------- 335
Consensus ----NGTGGN TGAAATCCAA CTCCAANN-- ---------- ---------- ------NATA TATA------
Conservation
FLC At ecotype Col-0 ATTATAAATT ATAAACTGGT AAAAGATTAT AGATACTGCT TCCAAACTTA AAAGCTTAAA CATAAAGAAC 5880
flc AT-1 -------ATT ATGAAGAAAA AAAAAATAAG ATATGTAATT ATTCCGCTGA TAAGGGCGAG CGT------- 691
CiFLC gene ---------- --GACGTTGT AAAAAATCTT TAACATCGAT CC-------- ---------- ---------- 365
Consensus -------ATT ATGAAGTNGT AAAAAATNAT ANATATNGNT NCNNNNCTNA NAAGNNNNAN CNT-------
Conservation
FLC At ecotype Col-0 ACACAAATTA TGAGAAACAT AACCTTCTGT AGTGTTTTTT AATGGTTGTT ATTTGGTGGT GTGAAAAAGA 5950
flc AT-1 ---------- ---------- ---------T TGTATATCTT AATACTCTCT CTTTGGCCA- ---------- 721
CiFLC gene ---------- ---------- ---------- -----ATCTT GATGCATCCC AATTTATAG- ---------- 389
Consensus ---------- ---------- ---------T NGTNTATCTT AATGCTTNCT ATTTGGTNG- ----------
Conservation
FLC At ecotype Col-0 TATTCCTTGG ATAGAAGACA AAAAGAGAAA GTGAATAGTG ATTTTGACCT ATGATTATCG TACAGATGGA 6020
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CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 389
Consensus ---------- ---------- ---------- ------AGNG ANTTTG-NNT NTGATNNTNN NNNAGANGGA
Conservation
FLC At ecotype Col-0 GAATAATCAT CATGTGGGAG CAGAAGCTGA GATGGAGATG TCACCTGCTG GACAAATCTC CGACAATCTT 6090
flc AT-1 ACTAAGTCAA TAC------- ---------- ---------- ---------- ---------- ---------- 767
CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- ---------- 389
Consensus NNNNANTCAN NAN------- ---------- ---------- ---------- ---------- ----------
Conservation
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flc AT-1 ---------- ---------- ----TATCCG TTTTAAGACA AAAAAAAAAA AAAAAA---A A 801
CiFLC gene ---------- ---------- ---------- ---------- ---------- ---------- G 390
Consensus ---------- ---------- ----TANCCN NNTTAANNCN NNNNNNNAAA NNAAAA---A A
Conservation

Appendix 2

Posters produced and presented at congresses

50° Annual Congress SIGA, Ischia 10th-14th September 2005

Regulation of flowering time by vernalization in *Cichorium intybus* L.

Antonella Locascio*, Richard Amasino**, Margherita Lucchin*, Serena Varotto*

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In biennial and winter annual ecotypes of *Arabidopsis thaliana*, flowering is typically blocked in the first growing season. Exposure to prolonged cold temperature, in a process called vernalization, is required to remove this block and permit flowering in the next growing season (R. Amasino, Curr. Opin. Biotec. Vol.16 2005). In late-flowering ecotypes of *Arabidopsis*, a flowering repressor, *FLOWERING LOCUS C (FLC)*, is expressed at such high level as to inhibit flowering in the first growing season. The delayed flowering is due to dominant alleles of *FRIGIDA (FRI)* and *FLC*. *FRI* elevates expression of *FLC* to levels that suppress flowering (S.D. Michaels, The Plant Cell vol.11, 1999). *FLC* inhibits the transition to flower by repressing the expression of the genes often referred to as Floral Pathways Integrators (such as *LFY, FT* and *SOC1*). These genes are able to integrate a balance of stimulations originating from the different pathways inducing flowering and convert these inputs into an induction of FMI (Floral Meristem Identity) genes, thereby initiating the production of the first floral meristems. (F. Parcy, Int. J. Dev. Biol. 49, 2005).

Vernalization is the main process promoting flowering by the repression of FLC. The repression involves epigenetically stable modifications in FLC chromatin that include trymethylation of histone H3 at Lys9 (H3K9) and Lys27 (H3K27). (data recently submitted from S. Sung et al). Summer-annual accessions of *Arabidopsis* flower rapidly without vernalization, due to a mutation in an active FRI allele or due to the presence of a weak FLC allele (Gazzani et al. Plant Physiol. 132 2003; J. Lin et al., Genes &Develop. 18, 2004); in both cases the levels of FLC expression is low compared to the wild type.

Wild chicory (*Cichorium intybus* L.) is a biennial species which requires vernalization to flower. Chicory is economically important for its use as vegetable and as an industrial raw material to obtain inulin from roots. In Italy different types of chicory (the so called italian red and variegate types) have been selected by farmers. These types show quite different classes of precocity in relation to flowering.

We are investigating the molecular mechanism that regulates the switch to flower in chicory, to verify whether such mechanism is the same that controls flowering in *Arabidopsis*, and finally address the diversity of the classes of precocity to one of the cases known for this model plant.

Fig. 1 RNA prep was made from young leaf of wild chicory; through RT-PCR FLC transcripts were isolated

A) Up to now three FLC sequences were isolated through RT-PCR.

B) Aminoacidic alignment of two FLC transcripts with similar lenght, isolated from the same sample of wild chicory. The major difference between the two sequences is the lack of 40 aminoacids within the MADS-box in the incomplete ORF; other minor aminoacidic substitutions are showed. The third sequence of FLC (not showed on the alignment above) is 200bp long. It lack the central corpus of the protein, but a trait of MADSbox and a trait of the end of the protein are still present.

Future perspectives and advances

We are currently investigating if there is a real need of cold in chicory by checking the decreasing of FLC expression at different time point during the process of vernalization.

A construct FLC::GFP was made for the transient transformation of chicory protoplast, to verify the subcellular localization of FLC; the preliminary data shows an expression localized at the nucleo of the cell.

A construct 35S:FLC will be used to transform the mutant flc-3 of arabidopsis to test if the mechanism by which chyFLC is regulated by vernalization is conserved. The trasformation will be performed also with the incomplete ORF to check its functionality.

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Analysis of flowering mediated by vernalization in *Cichorium intybus* L.

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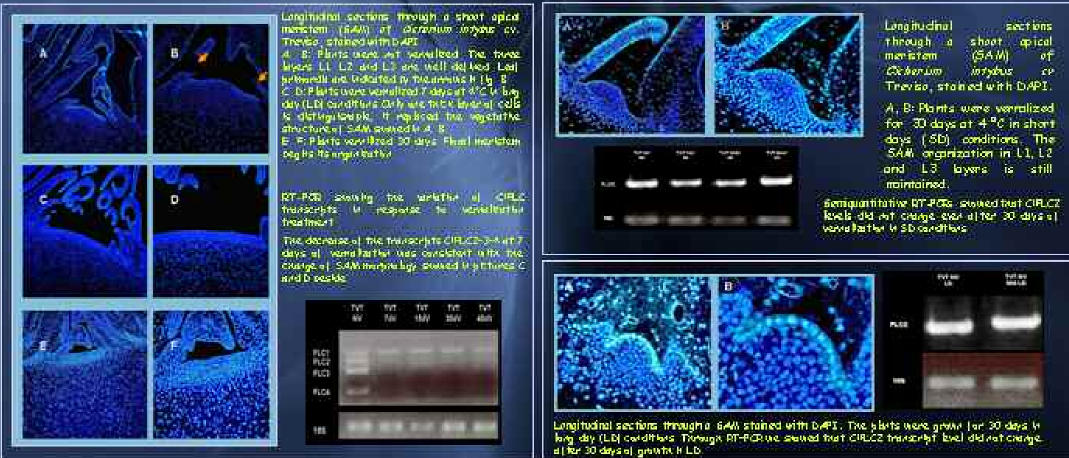
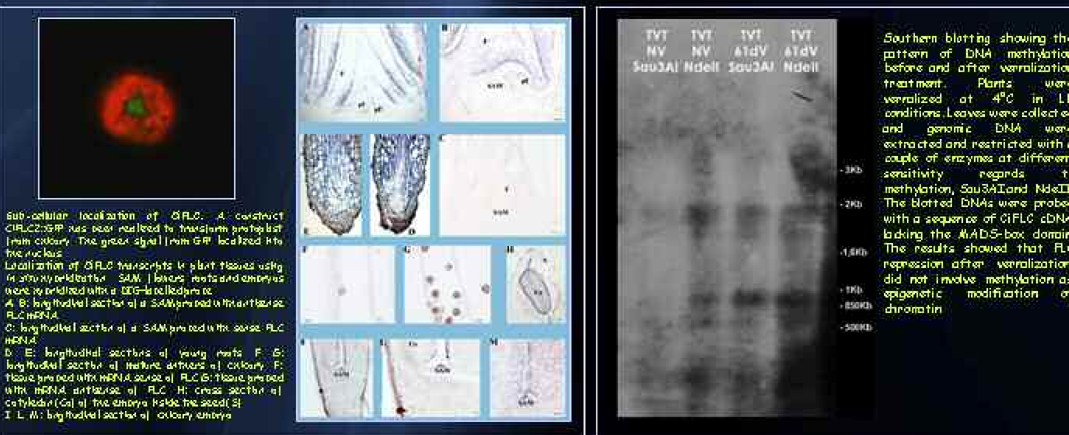
Since proper timing of flowering is critical for the survival of plant species, plants have evolved a complex genetic network to regulate their transition to flowering in response to endogenous signals and environmental cues. In winter annuals ecotypes of *Arabidopsis*, a flowering repressor, *FLOWERING LOCUS C (FLC)*, a MADS box transcription factor, is expressed at such levels as to inhibit flowering in the first growing season. FLC expression is enhanced by *FRIGIDA (FRI)* to levels that inhibit the transition to flowering by repressing the expression of the genes often referred to as Floral Pathways Integrators.
The main process promoting flowering by the repression of *FLC* is the vernalization and the duration of cold has been shown to be proportional to the degree of down-regulation of *FLC*; such repression is maintained for the rest of the plant life even after cold exposure ends, but is restored after meiosis. The repression involves epigenetically stable modifications in *FLC* chromatin that include a H3 Lys27 trimethylation (H3K27me3) and a H3 Lys9 trimethylation, (Sung et al, 2006).
Interestingly, for the light-dependent, autonomous and GA integration and meristematic pathways, comparative genetic approaches show that flowering time genes are conserved between *Arabidopsis* and a large range of crop species, including legumes and cereals. By contrast, the vernalization pathway seems to be only partially conserved, since FLC and FRI were not characterized in dicots other then Brassicaceae.
Wild chicory (*Cichorium intybus* L.) is a biennial species which requires vernalization to flower. In Italy different types of chicory (the so called Italian red and variegate types) have been selected by farmers as leafy vegetable. These types show quite different classes of precocity in relation to flowering.
In our study, we are investigating the molecular basis that regulate the switch to flower in chicory by vernalization, to verify whether such mechanism is the same that controls flowering in *Arabidopsis*, and, finally, to address the diversity of the classes of precocity to one of the cases known for this model plant. We isolated FLC homologues from chicory and characterized their expression patterns in plant tissues and in response to vernalization. We also studied the pattern of cytosine methylation in chicory genomic DNA in response to vernalization. In addition, the vernalization-mediated decrease of FLC transcript was related with changes in SAM morphology. Up to now our results indicate that arabidopsis and chicory share homologies in regulating *FLC* expression in the vernalization response. Further analysis will be conducted to define if the machinery in FLC regulation is shared between the two species.





Plant Biology and Botany 2007-Joint Congress, Chicago-USA, 7th-11th July 2007 and 51st Annual Congress SIGA, Riva del Garda, 26th-29th September 2007.

FLC and the REGULATION OF FLOWERING TIME in *Chicory*

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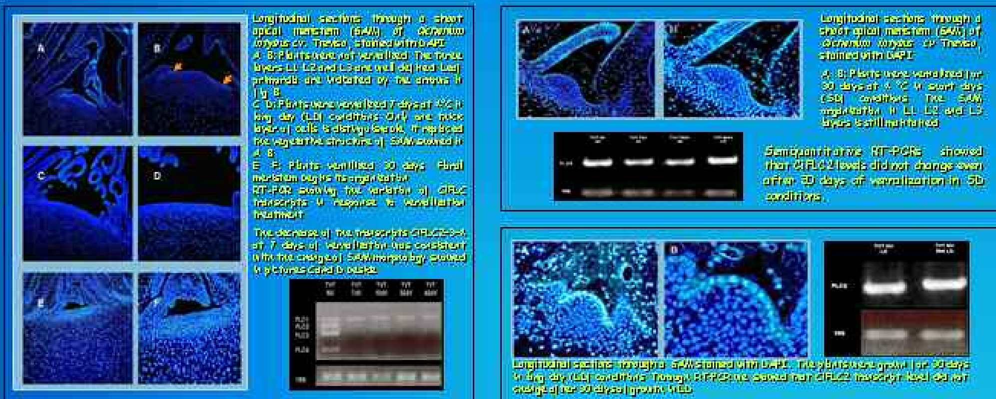
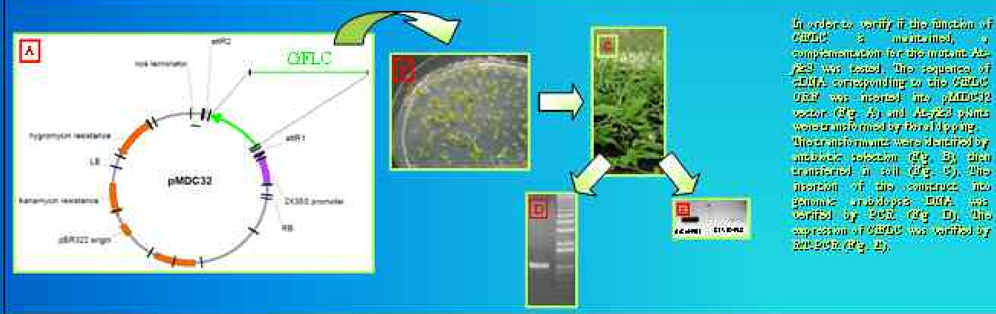
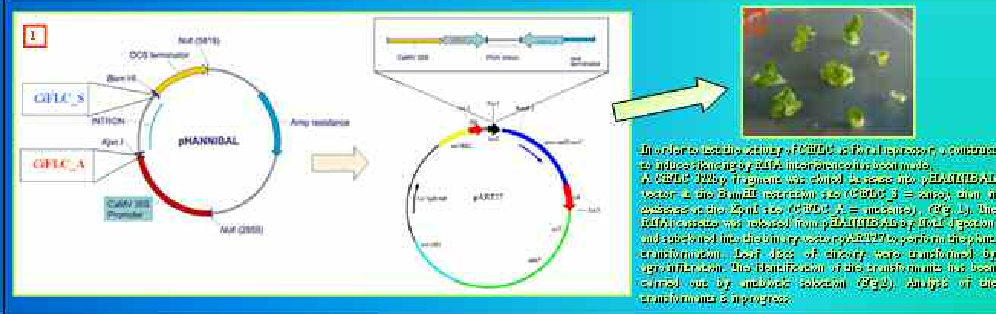
The transition from vegetative to reproductive development for a plant is a highly regulated process sensitive to environmental cues, as day length and temperature. Winter annuals and biennales typically require a prolonged exposure to cold to flower in the spring. The process by which the meristem gains the competence to flower after the experience of low temperatures is known as *vernalization*. In the model plant *Arabidopsis thaliana*, the ability to flower is related with the silencing of a floral repressor named *Flowering Locus C*. FLC is negatively regulated by vernalization, instead its expression is inducted by the gene *FRIGIDA*. The silencing of FLC is an epigenetic process, mitotically stable, but it seems to be resetted after meiosis. Up to now FLC has been isolated only from species belonging to *Brassicaceae* family and from sugar beet.
Wild chicory (*Cichorium intybus* L.) is a biennial species, belonging to *Asteraceae* family. Chicory is a crop mainly cultivated in North Eastern of Italy and it shows a quite obligate request of cold to flower.
In our study, we are investigating the molecular bases that regulate the flowering in chicory by vernalization.
We isolated FLC homologues from chicory and characterized their expression patterns in plant tissues and in response to vernalization. Given the similarities of sequence, pattern of expression and localization of FLC observed between arabidopsis and chicory, a construct 35S::FLC was made to transform the mutant *flc-3* of arabidopsis var. Columbia, with the purpose to complement the repressive mutation flc-3 and rescue the phenotype.
Furthermore the knock-down of FLC in chicory could be useful to verify if FLC is involved in the process of flowering repression mediated by vernalization as in Arabidopsis. RNA interference mediated by miRNA could be the strategy to induce the silencing of FLC. A specific construct to induce interfering was produced and the transformation of chicory has been carried out through Agrobacterium infection of leaf disks. Plant regeneration via organogenesis will be achieved and the selection of the transgenic plants carried out.







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