SECTION I

GENERAL INTRODUCTION

We are all born ignorant, but one must work hard to remain stupid.

Benjamin Franklin

CHAPTER

1 STATE OF THE ART

1.1 • ARABIDOPSIS AS A MODEL PLANT

1.1.1 • A BRIEF HISTORY

The use of *Arabidopsis thaliana* in plant research is not recent. As reviewed in Somerville and Koornneef (2002), one of the first experiments carried on Arabidopsis was published in 1907 in a Ph.D. thesis conducted at the University of Bonn, Germany. In his thesis, Friedrich Laibach carried out cytological analyses of chromosome number in several plant species, including *Arabidopsis thaliana* (Měsíček, 1967). Thirty years later, Laibach went back to studying Arabidopsis, focusing this time on the flowering-time control by light quality and quantity. On this occasion, he proposed Arabidopsis as a suitable model for plant genetics, as it provided several advantages over other plants that were then commonly used in research (Laibach, 1943):

- *Arabidopsis thaliana* has a small number of chromosomes (five pairs);
- its life cycle is short (six to eight weeks under favorable conditions);
- it can be easily grown in a small space;
- it produces large amount of seeds;
- it shows a large natural genetic variation.

Fifteen years later, Arabidopsis had been adopted by several German scientists. Among them, Napp-Zinn was famous for his work on vernalization (*i.e.* the acceleration of flowering by a long period of cold). From the early 1960's, thanks to the possibility to generate mutants using X-rays, Arabidopsis became more and more widely used as a model organism in plant genetics. Indeed, mutagenesis requires a considerable amount of plants, and the small size of Arabidopsis was, therefore, a significant advantage to manage large-scale screening of mutants. The first international Arabidopsis symposium was held in 1965 in Göttingen, marking the willingness to organize a structured community around Arabidopsis. However, during the 1970's, the interest for Arabidopsis declined because of the lack of methods to identify the molecular bases of the characterized mutants. Therefore, in this "pre-molecular" era, plant researchers preferred to study organisms of agricultural and horticultural impor-

tance, such as petunia, snapdragon, tomato, barley, maize, or species regarded as physiological models. Only a few research groups remained focused on the characterization of *Arabidopsis thaliana*.

Later, the fast developments in the molecular techniques participated in the reversal of this trend. During the early 1980's, more and more research groups began to describe the phenotypes of Arabidopsis mutants in many different processes, from the response to light to phytohormone deficiencies. The confirmation of the small size of the Arabidopsis genome, even though slightly underestimated (70 Mb instead of 125 Mb), also contributed to the regain of interest for this species. It indeed opened new perspectives, such as the cloning of the entire Arabidopsis genome in a relatively small number of phage clones (Leutwiler *et al.*, 1984) or the possibility to clone any Arabidopsis gene by positional cloning and thereby integrate genetics and molecular biology in large-scale studies (Meyerowitz, 1987). Such prospects were not conceivable in other plant species since most of them contain large and complex genomes. The genomic resources were then constructed and, in the early 1990's, the first Arabidopsis genes were mapped (Arondel *et al.*, 1992; Giraudat *et al.*, 1992). In parallel, scientists discovered that *Agrobacterium tumefaciens* (now *Rhizobium radiobacter*, the bacteria that is responsible for crown gall disease) was able to transfer its T-DNA into the genome of Arabidopsis seeds (Feldmann and Marks, 1987). This exciting result opened new perspectives and led to the development of highly-effective *Agrobacterium*-mediated plant transformation protocols. This revolutionary tool was used to produce large populations of transformants that were subsequently screened by many labs worldwide, thus bringing Arabidopsis at the forefront of plant research. Among the key research topics that confirmed Arabidopsis as a model plant, the study of flower architecture was fascinating. The elegant ABC model (**described page 20**), built by groundbreaking genetic studies, demonstrated the power of the new molecular tools developed for Arabidopsis (Bowman *et al.*, 1991). The Arabidopsis community became more organized, finally leading to the creation of international committees that would carry worldwide-synchronized initiatives. These consortiums drove coordinated efforts to sequence the Arabidopsis genome (Arabidopsis Genome Initiative, 2000), build Arabidopsis stock centers gathering thousands of natural accessions as well as large insert clone libraries (http://arabidopsis.info/; https://abrc.osu.edu/), and create multiple bioinformatic tools (http://www.arabidopsis.org; http://www.plantgdb.org/AtGDB/; https://www.araport.org/). Nowadays, those resources are essential to perform high-level genetic research on every aspect of Arabidopsis development.

The completion of the Arabidopsis genome sequencing effort was achieved in 2000 (Arabidopsis Genome Initiative, 2000). The entire genetic material of Arabidopsis contains 125 Megabases (Mb) spread over five chromosomes, each of them present in two copies in somatic cells. The diploidy of Arabidopsis was a crucial criterion for its selection as a model organism, since higher ploidy levels make the generation of homozygous mutants very difficult. For example, conducting genetic studies in hexaploid wheat is very tricky, even if those challenges are about to be overcome by the development of new genetic engineering tools (Wang *et al.*, 2014). Scientists working with *Arabidopsis thaliana* do not face such issues. According to TAIR10 annotations (Lamesch *et al.*, 2012), the Arabidopsis genome contains 27,416 protein-coding genes, 1,359 noncoding RNAs loci, and 4,827 pseudogenes. Arabidopsis genome thus contains about the same number of genes than human's genome, although it is more than 20 times smaller.

Currently, Arabidopsis remains the most studied species in plant genetics. However, a keen interest for translational research is arising, with the aim to transfer knowledge acquired on Arabidopsis to other species, especially to crops. Arabidopsis is a member of the Brassicaceae family, which contains several cultivated species, such as rapeseed, mustard, cabbage, etc. However most widely grown crops like rice, maize, or wheat are unrelated to Arabidopsis. One can then anticipate that the pole position of Arabidopsis will be shared in the future with other species. With the improvement of next-generation sequencing techniques and the development of highly efficient methods to perform directed mutagenesis of very complex genomes, the research on crops will likely be more and more supported. However, fascinating challenges are still ahead of the Arabidopsis community. The attribution of a function to each gene - a goal initially planned for 2010 (Chory *et al.*, 2000) - is far from completion. Moreover, the understanding of the genetic bases of natural variation - an objective targeted by the 1,001 Arabidopsis genome project (Gan *et al.*, 2011; Cao *et al.*, 2011; http://1001genomes.org/) - will bring new insights into understanding plant evolution and adaptation to diverse environments.

1.1.2 • ARABIDOPSIS LIFE CYCLE

The short life cycle of *Arabidopsis thaliana* is one of the key features that led to its selection as a model organism (**Figure 1-1**). Under favorable conditions, six to eight weeks are sufficient to grow the plants from seed to seed. Cotyledons are formed during the embryogenesis and are the first photosynthetic structures that emerge from the seed. True leaves are subsequently produced by the shoot apical meristem (SAM). The absence of

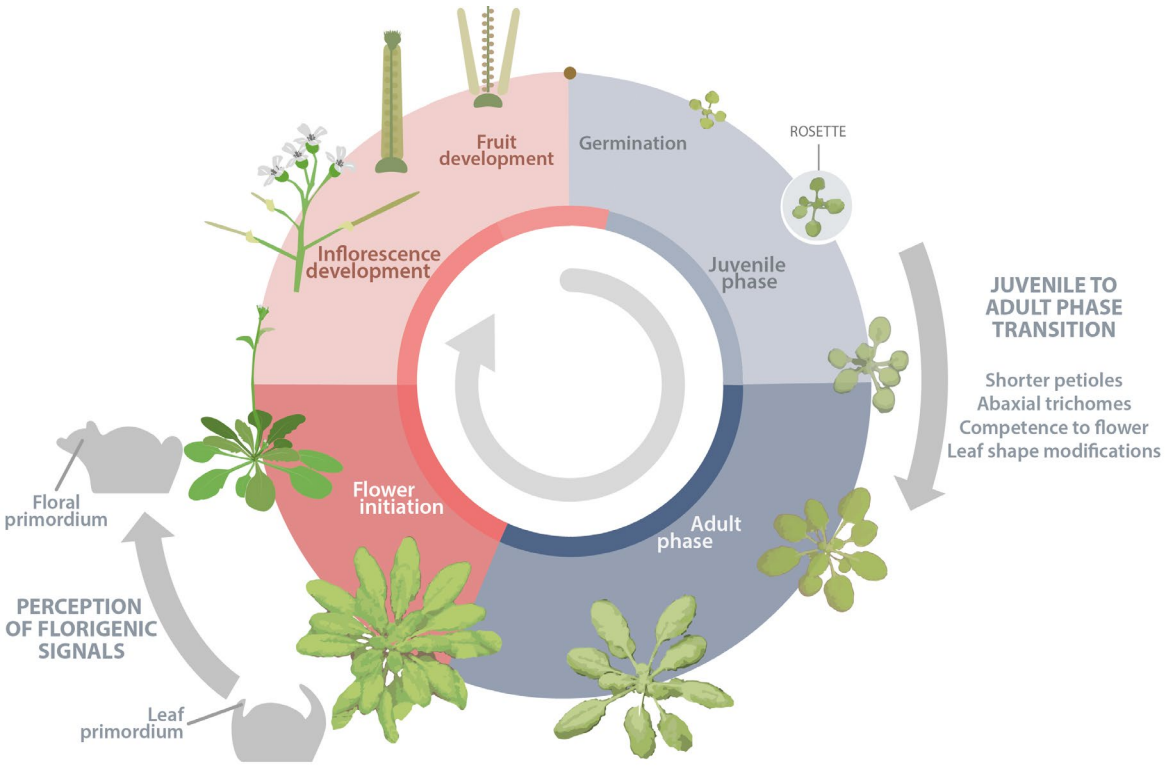

Figure 1-1. Overview of the Arabidopsis life cycle.

internode elongation between the initiation of successive leaves leads to a rosette growth habit, in which the successive leaves are separated by an exact angle of 137.5° (**Figure 1-2A,B**). During early growth, Arabidopsis plants remain juvenile: they are unable to flower even if they perceive inductive signals. The transition from the juvenile to the adult phase is accompanied by several morphological changes, such as the apparition of abaxial trichomes (*i.e.* trichomes developing on the lower face of leaves), the modification of leaf shape and petiole length and, of course, the acquisition of competence to flower (**Figure 1-2C**).

Once at the adult stage, plants can flower if they perceive flowering inductive signals, such as an increase in the photoperiod. However, even in the absence of inductive signals, flowering eventually occurs by an endogenous developmental program called the "aging pathway". Floral induction causes significant changes in the SAM, as it stops producing leaves to initiate flowers. Flowers are initiated on the flanks of the SAM whereas the center remains indeterminate: the inflorescence of Arabidopsis is a raceme. The internodes of the inflorescence and a few internodes below the first flower elongate to form the floral

stem bearing flowers and cauline leaves at the axil of which secondary inflorescences arise (Figure 1-2D). A wild type Arabidopsis plant produces hundreds of flowers with four whorls of organs: sepals, petals, stamens, and carpels (Figure 1-2E). Arabidopsis flowers are mostly fertilized through self-pollination, as a high proportion of them are already fertilized before opening. Self-pollination is a crucial feature in plant genetics since it highly facilitates the production of homozygous lines. However, it is also possible to perform manual cross-pollination between different individuals, thus allowing the production of multiple mutants or transgenic lines within a few months. The fertilized flowers produce siliques, the Arabidopsis fruit. Each silique containing about 50 seeds, a wild type Arabidopsis plant typically produces 10,000-40,000 seeds.

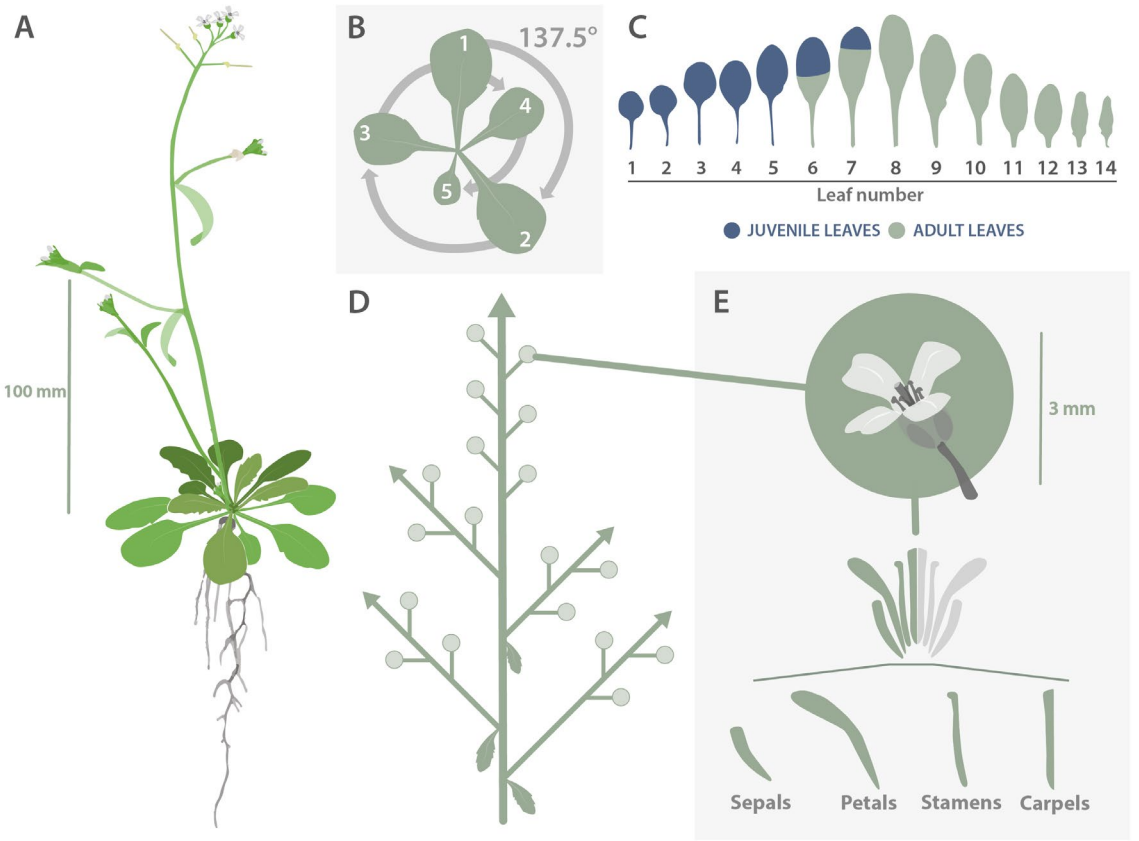

Figure 1-2. Shoot development of *Arabidopsis thaliana*.

A. Overview of a mature Arabidopsis plant. **B.** Phyllotaxy. **C.** Shapes of juvenile and mature leaves. **D.** Simple raceme inflorescence. Arrows represent indeterminate growth, circles represent flowers. **E.** Flower structure.

1.2 • FLOWERING-TIME REGULATION

1.2.1 • OVERVIEW

The correct timing of flowering is critical to ensure reproductive success. In crops, flowering time has a significant impact on yield: premature flowering leads to reduced biomass and seed set, whereas a prolonged vegetative growth results in increased biomass but reduced seed number and filling (Demura and Ye, 2010). The regulation of flowering is thus a topic that has fascinated generations of plant scientists from the beginning of plant physiology research (reviewed in Kobayashi and Weigel, 2007). From the 1990's, molecular genetics studies conducted in Arabidopsis allowed huge advances in the understanding of flowering. Hence, our literature survey will be focused on this species. We will first summarize what occurs in the SAM once flowering has been induced, before dissecting the signalling pathways that regulate flowering time *per se*.

The switch in meristem fate

In Arabidopsis, flowers are initiated on the flanks of the SAM. Because the center of the meristem remains indeterminate, the raceme-type inflorescence can contain a virtually infinite number of flowers. Transition from leaf- to flower-fate in newly initiated primordia requires the activity of the *LEAFY* (*LFY*) and *APETALA1* (*AP1*) genes: mutation of any of them indeed results in the formation of leafy shoots where flowers should develop (Irish and Sussex, 1990; Schultz and Haughn, 1991; Bowman *et al.*, 1993). Abnormal flowers are eventually initiated in the single mutant, but not in the double *lfy;ap1,* in which flowers are replaced by leaf-like structures (Huala and Sussex, 1992). On the opposite, overexpression of *LFY* or *AP1* is sufficient to convert the SAM into flowers, including the center that forms a terminal flower (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). This phenotype is due to the LFY- and AP1-mediated repression of the *TERMINAL FLOWER1* (*TFL1*) gene, whose function is to maintain the SAM indeterminate (Ratcliffe *et al.*, 1999; Parcy *et al.*, 2002; Kaufmann *et al.*, 2010; **Figure 1-3**). Consistently, *tfl1* null mutant produces very few flowers before inflorescence termination (Shannon and Meeks-Wagner, 1991; Alvarez *et al.*, 1992; Shannon and Meeks-Wagner, 1993). *LFY*, *AP1* and *TFL1* are all upregulated in the SAM at floral transition (Schmid *et al.*, 2003; Jaeger *et al.*, 2013) but whereas *LFY* and *AP1* repress *TFL1* and *vice versa*, *LFY* and *AP1* activate each others, installing a positive feedback in the floral meristems, restricting the expression of *TFL1* to the center of the SAM (Wagner *et al.*, 1999; Wellmer *et al.*, 2006; Kaufmann *et al.*, 2010). Both LFY and AP1 act together to induce the expression of transcription factors

involved in the specification of floral organs (reviewed in Wellmer *et al.*, 2014).

The activation of *AP1* and *LFY* expression is triggered by a small subset of genes responsible for the early events of floral specification. Global analyses of transcriptome modifications occurring in the apex during the photoperiodic induction of flowering uncovered the rapid activation of several transcription factors, including *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE3* (*SPL3*) as well as the MADS-BOX genes *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) and *FRUITFULL* (*FUL*) (Schmid *et al.*, 2003). The characterization of those genes unraveled their role in the regulation of the early events of floral specification (Figure 1-3). SPL3 induces the expression of *AP1*, *LFY* and *FUL* (Yamaguchi *et al.*, 2009; Wang *et al.*, 2009). In parallel, SOC1 interacts with another MADS-box protein AGAMOUS LIKE 24 (AGL24) to activate *LFY* (Moon *et al.*, 2005; de Folter *et al.*, 2005; Liu *et al.*, 2008a; Lee *et al.*,

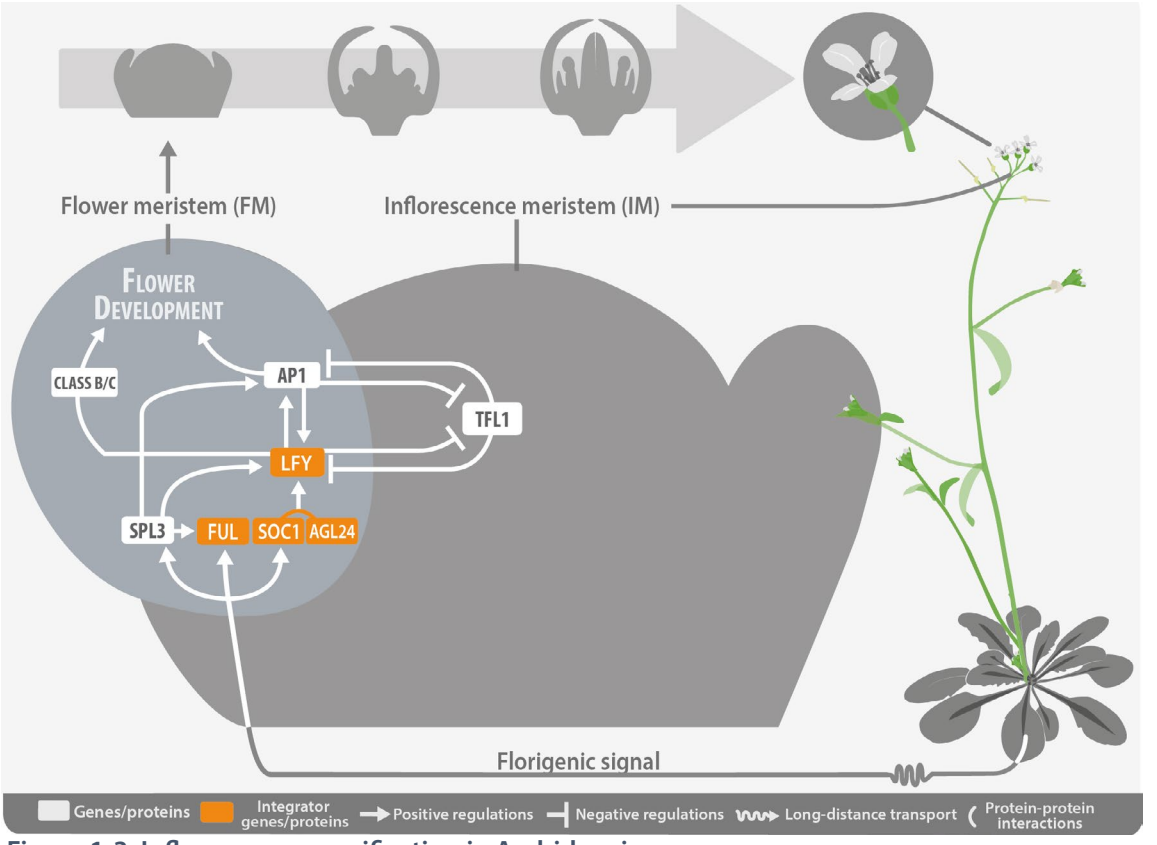

Figure 1-3. Inflorescence specification in Arabidopsis.

The transition to flowering induces a switch in shoot apical meristem (SAM) fate, which becomes inflorescential and initiates flowers on its flanks. The induction of flowering by the photoperiodic pathway induces the expression of *SOC1*, *FUL*, and *SPL3*. SOC1 interacts with AGL24. SPL3 and the SOC1-AGL24 heteroduplex trigger the upregulation of both *LFY* and *AP1* in floral primordia, whereas high TFL1 protein level in the center of the SAM prevents the termination of the inflorescence.

2008). Together, those events converge to the upregulation of both *AP1* and *LFY* to induce the development of floral primordia on the flanks of the SAM.

Flower development

The development of the flower and the genetic determinants involved in the specification of its different organs were among the first topics to be investigated at the beginning of the plant molecular biology era (reviewed in Causier *et al.*, 2010). The gathered knowledge led to the elaboration of the elegant ABC(E) model, illustrated in **Figure 1-4A** (Schwarz-Sommer *et al.*, 1990; Bowman *et al.*, 1991; Coen and Meyerowitz, 1991). This model accounts for the specification of the different whorls of the Arabidopsis flower - sepals, petals, stamens and carpels - by the combination of a small subset of MADS-box transcription factors: class *A* genes are necessary for the specification of the two outer whorls (sepals, petals), while class *C* genes specify the identity of the two inner whorls (stamens, carpels). The products of class *A* and *C* genes repress each other's, so that their expression patterns do not overlap. The class *B* genes are expressed in the central whorls; they are thus necessary for the initiation of petals and stamens.

This model was elaborated from detailed characterization of homeotic mutants:

(i) The mutation in class *A* genes, either *APETALA2* (*AP2*) or *AP1*, results in sepal and petal defects (Bowman *et al.*, 1989; Irish and Sussex, 1990);

(ii) Mutations in class *B* genes lead to the abnormal development of the two central whorls of the flower. The single mutation of either *PISTILLATA* (*PI*) or *APETALA3* (*AP3*) causes the homeotic conversion of petals into sepals and stamens into carpels (Bowman *et al.*, 1989; Bowman *et al.*, 1991; Jack *et al.*, 1992);

(iii) The null mutation of class *C* gene *AGAMOUS* (*AG*) results in the replacement of the six stamens by six petals while carpels are replaced by sepals (Bowman *et al.*, 1989; Yanofsky *et al.*, 1990; Irish and Sussex, 1990).

In situ hybridization confirmed that *A, B* and *C* genes are expressed in the whorls where their mutation causes abnormalities and hence the superposition of the expression patterns of the *ABC* genes is sufficient to explain the specification of floral organs, as shown in **Figure 1-4C** (reviewed in Fornara, 2014). Ten years after the discovery of the floral homeotic genes, another class of factors was identified: the redundant *SEPALLATA1-4* (*SEP1-4*) genes, constituting class E (Pelaz *et al.*, 2000; Honma and Goto, 2001). In the *sep1;2;3* triple mutant (Pelaz *et al.*, 2000), all flower organs develop as sepals, whereas in the *sep1;2;3;4* quadruple mutant, all whorls are converted into leaf-like structures, indicating that *SEP* genes redundantly control sepal identity (Ditta *et al.*, 2004; Pelaz *et al.*, 2000). The ABC model was therefore extended to include the E class genes, thus becoming the ABCE model (Theißen, 2001).

The MADS-box proteins are characterized by a conserved DNA-binding domain that recognizes a conserved motif, the CArG box. The MIKC subfamily of MADS-box proteins, to which belong the ABCE proteins, also displays a conserved protein-protein interaction domain (Riechmann *et al.*, 1996; Fan *et al.*, 1997; Yang *et al.*, 2003). The quartet model explains the requirement of precise combinations of ABCE proteins to specify floral organs (Figure 1-4B; Theißen, 2001; Honma and Goto, 2001). In this model, the ABCE proteins interact together to form quaternary complexes. The specific combination of ABC proteins regulates organ specification while the SEP3 protein acts as a "glue" to bind together the other proteins. Each complex is proposed to bind to two distant CArG boxes in the promoter of target genes, inducing a bending in the DNA that participates in the activation of downstream genes (Melzer *et al.*, 2009).

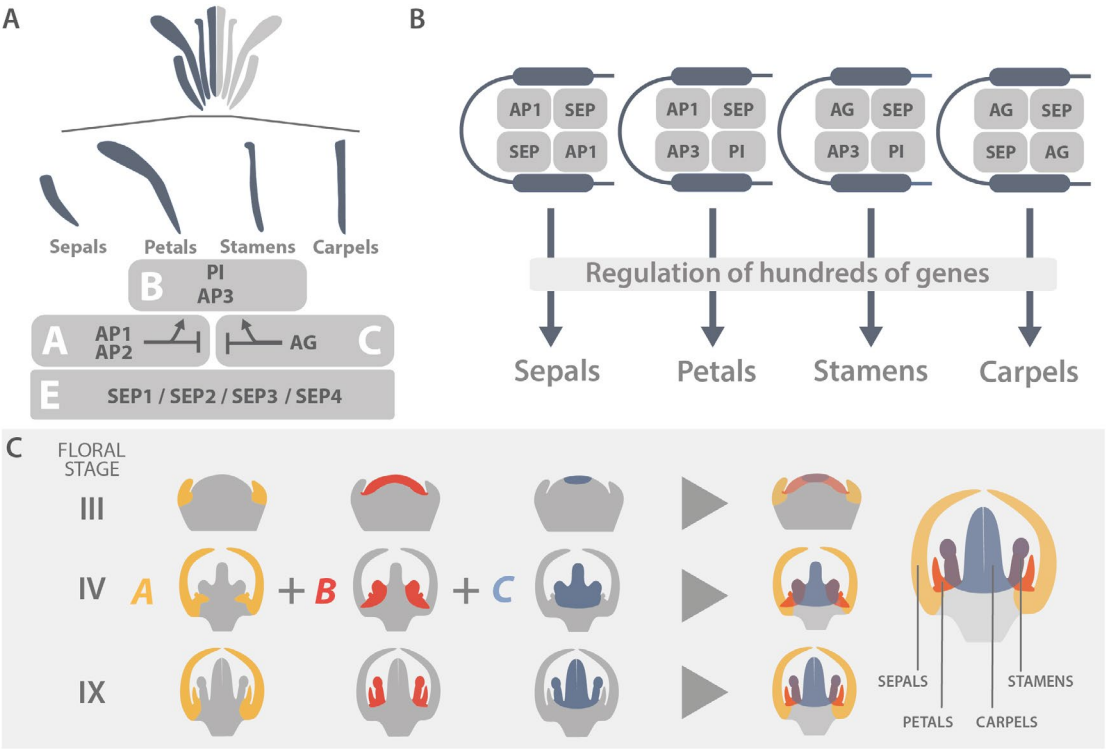

Figure 1-4. The ABC model of flower development.

A. According to the ABC(E) model, *A* and *C* class genes are antagonistic and exclude each other from their expression area. Class *A* genes are responsible for the development of sepals and, together with class *B* genes, for petal formation. Class *C* genes drive both carpel initiation and - with class *B* genes - stamen production. Class *E* genes are expressed throughout flower primordia. **B.** In the quartet model, a quaternary complex is formed in each whorl through the combination of distinct subsets of A, B, C, and E class proteins. Those quartets bind to two distinct regions of their targets, thus inducing a bending of their promoter. **C.** Expression patterns of class *A* [yellow], *B* [red], and *C* [blue] genes at different stages of flower development. The combination of their expression patterns is sufficient to explain the initiation of floral organs. Adapted from Fornara (2014).

Cues controlling flowering

When researchers started to use Arabidopsis as a model plant for genetic studies, the transition from the vegetative phase to the reproductive development was among the first topics to be investigated. Flowering of *Arabidopsis thaliana* is indeed easily achieved in greenhouses or cabinets, due to its low requirement for specific environmental cues. Favorable conditions are long days, warm ambient temperature (usually around 23°C) and, in some accessions, prior vernalization of the seeds (reviewed in Andrés and Coupland, 2012).

Very early, several loss-of-function mutants were identified: *constans* (*co*), *gigantea* (*gi*), and *luminidependens* (*ld*) were late flowering under inductive photoperiod (Rédei, 1962). However, the first large-scale genetic screening for abnormal flowering-time mutants was only performed in the 1990's, by Koornneef and colleagues (Koornneef *et al.*, 1991). The mutants identified in this mutagenesis experiments were all late flowering but responded differently to environmental factors. Some phenotypes were restricted to long-day conditions; some mutants were late flowering under long- and short- days; others showed altered responses to vernalization treatments. Those results suggested that flowering proceeds through different pathways in response to distinct environmental cues. After this pioneering work, flowering was viewed as the outcome of four major pathways (Levy and Dean, 1998; Koornneef *et al.*, 1998) :

- The **photoperiod pathway** induces flowering in response to long days;
- The **vernalization pathway** accelerates flowering in response to a prolonged cold period;
- The **gibberellin pathway** stimulates flowering under non-inductive photoperiod in response to increased levels of gibberellins;
- The **autonomous pathway** regrouped the mutants that are late-flowering under both photoperiods but still responsive to vernalizing treatments.

More recently, additional regulatory mechanisms were discovered and led to the introduction of:

- The **aging pathway**, which induces flowering of plants grown under non-inductive conditions;
- The **ambient temperature** pathway controls the acceleration of flowering at higher growth temperatures;
- The **sugar pathway** - intricately connected to the photoperiod and the aging pathway - induces flowering under favorable endogenous carbohydrate status.

As shown in Figure 1-5, those pathways converge to regulate a small subset of genes, called «flowering-time integrators», which trigger the activation of *LFY*, *AP1* and *TFL1* in the SAM (Reviewed in Bouché *et al.*, 2015).

Because of their primary importance, we will hereafter detail current knowledge acquired on the photoperiod-, vernalization- and plant aging pathways leading to flowering.

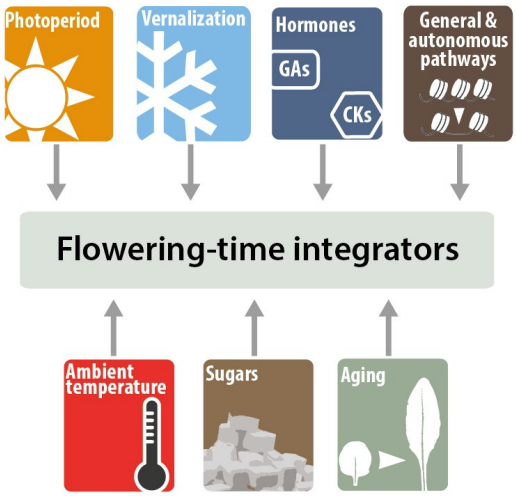

Figure 1-5. Several pathways control flowering time.

1.2.2 • THE PHOTOPERIODIC PATHWAY

Related to Chapter 4 («Rooting the flowering process», page 137).

Photoperiodic control of flowering: a coincidence

The length of the daily light period is a crucial factor that regulates flowering time in many species. The first experiments were performed about 100 years ago, on Maryland mammoth tobacco and soybean plants (Garner and Allard, 1920). To assess the impact of photoperiod on the bolting time of those species, Garner and Allard grew plants in pots in the field. Each day, some plants remained outside, while others were transferred into a windowless shed in the late afternoon until the next morning. This simple experimental work was sufficient to trigger flowering of both species, suggesting that their floral induction relied on the duration of the exposure to light. In these specific cases, plants flowered under short day conditions. The analysis of different species thereafter led to their categorization in three distinct groups, depending on the photoperiod accelerating their flowering: short-day, long-day, and day-neutral plants (reviewed in Thomas and Vince-Prue, 1996). The notion of long- and short- days is relative and varies between species, as it relies on the day length threshold above- or below-which flowering is induced. For instance, in Maryland mammoth tobacco, the critical day length below which the plants flower is 14 hours, a photoperiod that would be sufficient to trigger flowering in most long-day plants.

The nature of photoperiodism has been subject to much debate, and it took decades to establish the external **coincidence model** (Pittendrigh and Minis, 1964), now extensively supported by molecular data. This model suggests that the coincidence between light and an endogenous factor is required for flowering. We will consider the case of a long day plant, like *Arabidopsis thaliana*. Conceptually, the first assumption of this model was that flowering is controlled by a hypothetical enzyme whose level remains constant during the day, but which is only active in the light (**Figure 1-6**). This enzyme catalyzes the transformation of a specific substrate into a florigenic substance. The second assumption is that the abundance of this substrate changes throughout the day, peaking during the dark period under short day conditions. Thus, under non-inductive photoperiod, the substrate and the active enzyme are out of phase. An extension of the light period brings them in phase, providing the substrate to the enzyme, which can then catalyze its transformation into a florigenic signal (reviewed in Song, 2014). We will see that this model, established 50 years ago, accurately reflects the molecular processes involved in the photoperiodic induction of flowering in *Arabidopsis thaliana*.

The florigen

Very early, scientists observed that the signal that triggers flowering in response to photoperiod originates from leaves and moves to the SAM where flowers are initiated. This observation was first reported in spinach, a long day plant, as the exposure of leaves to increased day length was sufficient to trigger the development of an inflorescence, even if the SAM remained in the dark (reviewed in Zeevaart, 1976). The concept of a mobile "florigen" was then proposed, supported by many grafting experiments that confirmed the existence of a phloem-transmissible signal involved in the induction of flowering (Correns, 1924; Chailakhyan, 1936; Zeevaart, 1958; King *et al.*, 1968). Indeed, the florigenic signal was found to be transmissible from an induced donor to a vegetative receptor via grafting, even between different species (reviewed in Zeevaart, 1976). For instance in *Perilla*, grafting a single induced leaf on a vegetative plant is sufficient to trigger flowering of the recipient (Zeevaart, 1985). The hunt for this "universal" transmissible florigenic signal, initially thought to be a metabolic product analogous to plant hormones, took decades and regularly fell into dispute.

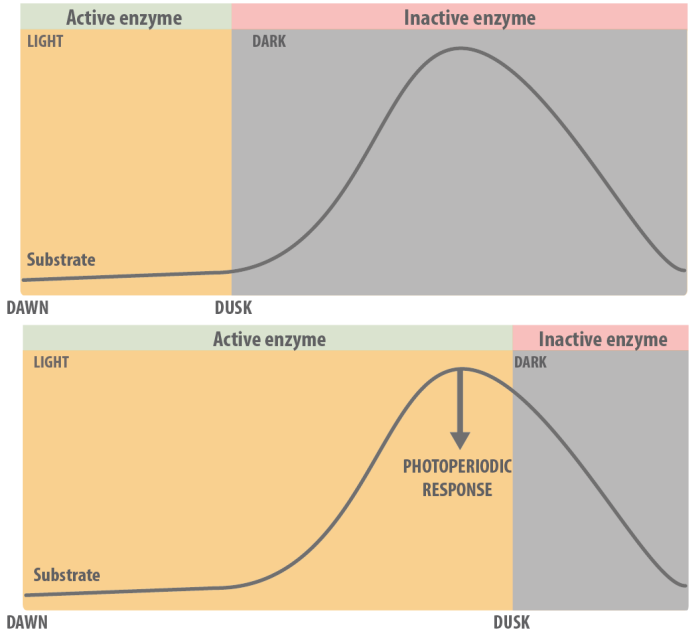

Figure 1-6. The external coincidence model.

In the original external coincidence model described by Pittendrigh and Minis (1964), a flowering-promoting enzyme is only active in the light while its substrate accumulates in the evening [upper panel]. The coincidence between light stimulus and the peak of substrate is responsible for the flowering induction in response to longer light periods [lower panel]. Adapted from Song *et al.* (2014).

While the biochemical and physiological attempts failed to identify the holy grail of flowering time control, molecular genetics allowed breakthrough progress in the understanding of the process. The multiple screenings for Arabidopsis mutants allowed the identification of both early and late-flowering genotypes. Their extensive characterization led to the classification of flowering-time genes into four distinct groups: photoperiod, vernalization, gibberellins, and autonomous flowering pathways (Koornneef *et al.*, 1998; Levy and Dean, 1998; Corbesier and Coupland, 2006). At first, these molecular pathways were thought to act independently and the idea of a single florigen gene vanished. However, more and more pieces of evidence pinpointed to a candidate gene, called *FLOWERING LOCUS T* (*FT*), that had been identified in the first flowering-time screenings (Koornneef *et al.*, 1991). In 2004, the report of its expression pattern in the leaves under favorable photoperiod, and the localization of its biological function in the SAM opened the idea that it is involved in the production of a systemic signal of flowering (An *et al.*, 2004). In 2007, the nature of a crucial component of the florigenic signal was finally confirmed, as the FT protein was shown to be graft-transmissible and responsible for the induction of flowering in *Arabidopsis thaliana* by long days (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007).

FT gene family

FT gene encodes a small protein of 20 kD that belongs to the Phosphatidylethanolamine Binding Protein (PEBP) family (Kobayashi *et al.*, 1999; Kardailsky *et al.*, 1999). These proteins do not contain any DNA-interacting domain and are conserved among all eukaryotes. In animals, they encode Raf kinase inhibitors involved in signalling cascades controlling cell growth (Reviewed in Keller *et al.*, 2004). In plants, PEBPs evolved to regulate distinct developmental processes, including flowering. The Arabidopsis genome contains six PEBPs that can be spread in three categories displaying different functions (**Figure 1-7A**):

(i) The FT-like proteins, including FT and TWIN SISTER OF FT (TSF), are involved in the control of flowering time;

(ii) The TFL1-like factors - TFL1, BROTHER OF FT (BFT), and CENTRORADIALIS (ATC) - act antagonistically to FT-like proteins and repress flowering (Yoo *et al.*, 2010; Mimida *et al.*, 2011; Huang *et al.*, 2012);

(iii) The MOTHER OF FT AND TFL1 (MFT) protein is mainly involved in the regulation of seed germination (Xi *et al.*, 2010).

The occurrence of *FT*-like and *TFL1*-like genes is associated with the evolution of seed-producing plants (gymnosperms and angiosperms) (Hedman *et al.*, 2009). Before the

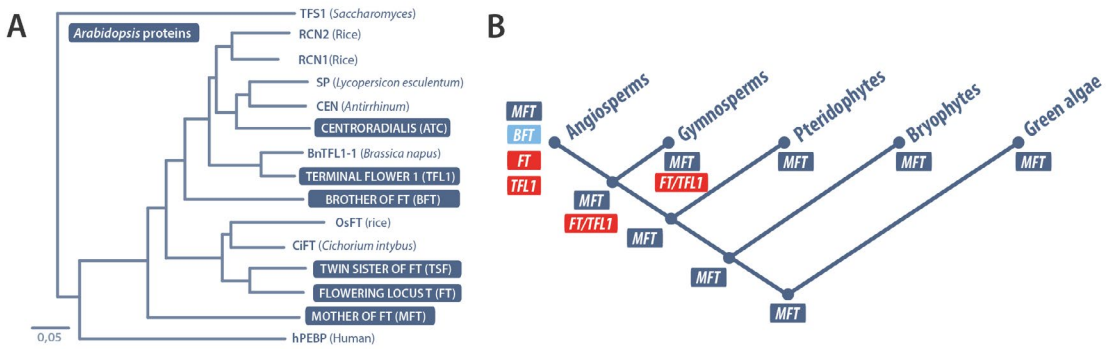

Figure 1-7. Evolution of the *FT* gene family.

A. Phylogenetic tree of 13 representative phosphatidylethanolamine-binding proteins (PEBP) from plants and *Saccharomyces cerevisiae*. Adapted from Kobayashi *et al.* (1999). **B.** Evolution of the *FT*-like genes across plant divisions. Successive genetic duplications lead to four functional paralog forms of FT-family proteins in angiosperms. Further genetic duplication in Brassicaceae resulted in six functional PEBP in *Arabidopsis thaliana*. Adapted from Pin and Nilsson (2012).

appearance of seed plants, a gene duplication event led to the creation of *MFT*- and *FT/TFL1*-like genes (Karlgren *et al.*, 2011). A subsequent duplication occurred only in the angiosperm lineage, giving birth to *FT*- and *TFL1*-like genes, two functionally distinct groups promoting or repressing flowering, respectively. Therefore, all the members of the PEBP family from Brassicaceae arose from the successive duplications of *MFT*-like genes, which are conserved across all plant species (**Figure 1-7B**). The PEBP proteins share structural features. The crystal structures of FT and TFL1 are similar, displaying a ligand-binding pocket as well as an external loop (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006). The sequence of this outer loop is essential for protein function, as the mutation of a single amino acid in this loop is sufficient to convert FT from a promoter into a TFL1-like inhibitor of flowering (Hanzawa *et al.*, 2005). In the context of the photoperiodic control of flowering, we will focus on the regulation of *FT*.

Transcriptional and post-translational mechanisms controlling CO and FT abundance

Early genetic screens for flowering-time mutants identified *constans* (*co*), which is insensitive to photoperiod and flowers as late in long days than in short days (Rédei, 1962; Koornneef *et al.*, 1991). The *CO* gene encodes a transcription factor and is expressed in the companion cells of phloem in the leaves only, not in the SAM (An *et al.*, 2004).

The activity of CO is subject to a very precise temporal regulation. The gene displays a peak of expression in the evening (*i.e.* at night under short day conditions) whereas the protein is

only stable in the light (Suarez-Lopez *et al.*, 2001; Valverde, 2004). Circadian clock-regulated factors control the rhythmicity of *CO* transcription (**Figure 1-8A**, upper panel). During the day, both the CYCLING DOF FACTORs (CDFs) and DAY NEUTRAL FLOWERING (DNF) repress *CO* expression (Fornara *et al.*, 2009; Morris *et al.*, 2010). The expression of *CO* during the night is partially controlled by the FLOWERING BHLHs (FBHs) proteins, which bind to the *CO* locus to induce its expression (Ito *et al.*, 2012).

The transfer of the plants from short days to long days increases the amplitude of the *CO* mRNA expression (**Figure 1-8A**, lower panel). This is due to the release from CDFs, these repressors being targeted for proteolysis by the blue light-induced complex formed by the interaction of FLAVIN KELCH REPEAT F BOX 1 (FKF1), an ubiquitin ligase, with the circadian-clock regulated protein GIGANTEA (GI) (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Fornara *et al.*, 2009; Song *et al.*, 2014). The expression of *CO* thus starts earlier than in short days. However, the later night peak still occurs, probably caused by FBHs; the expression of *CO* in long days thus shows a biphasic pattern (Roden *et al.*, 2002; Ito *et al.*, 2012).

Post-translational regulation of the CO protein accounts for the fact that it is not detected in short days (reviewed in Andrés and Coupland, 2012). During the night, the ubiquitin ligase complex formed by SUPPRESSOR OF PHYA-105 1 (SPA1) and CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) triggers the degradation of CO by the 26S proteasome (Hoecker and Quail, 2001; Laubinger *et al.*, 2006; Liu *et al.*, 2008d; Jang *et al.*, 2008; Sarid-Krebs *et al.*, 2015). During the day, both the PHYTOCHROME B (PHYB) and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1), an E3 ubiquitin ligase, are responsible for the degradation of CO protein (Valverde, 2004; Lazaro *et al.*, 2015). Under short days, CO protein is thus degraded both during the day and during the night.

Extension of the photoperiod not only promotes *CO* transcription as seen above, but also inactivates the protein-degrading complexes. First, PHYB activity is reduced by interaction with the PHYTOCHROME-DEPENDENT LATE FLOWERING (PHL) protein, which accumulates in the nucleus during the afternoon (Endo *et al.*, 2013). Second, the SPA1/COP1 dependent proteolysis of CO is suppressed by blue-light photoreceptors CRYPTOCHROME1 and 2 (CRY 1/2) (Zuo *et al.*, 2011; Liu *et al.*, 2011). Finally, PHYA is also involved in the post-transcriptional stabilization of CO (Valverde, 2004).

Altogether, those processes allow the accumulation of CO protein in long days; the transcription factor can then activate downstream processes. Targets of CO were identified by

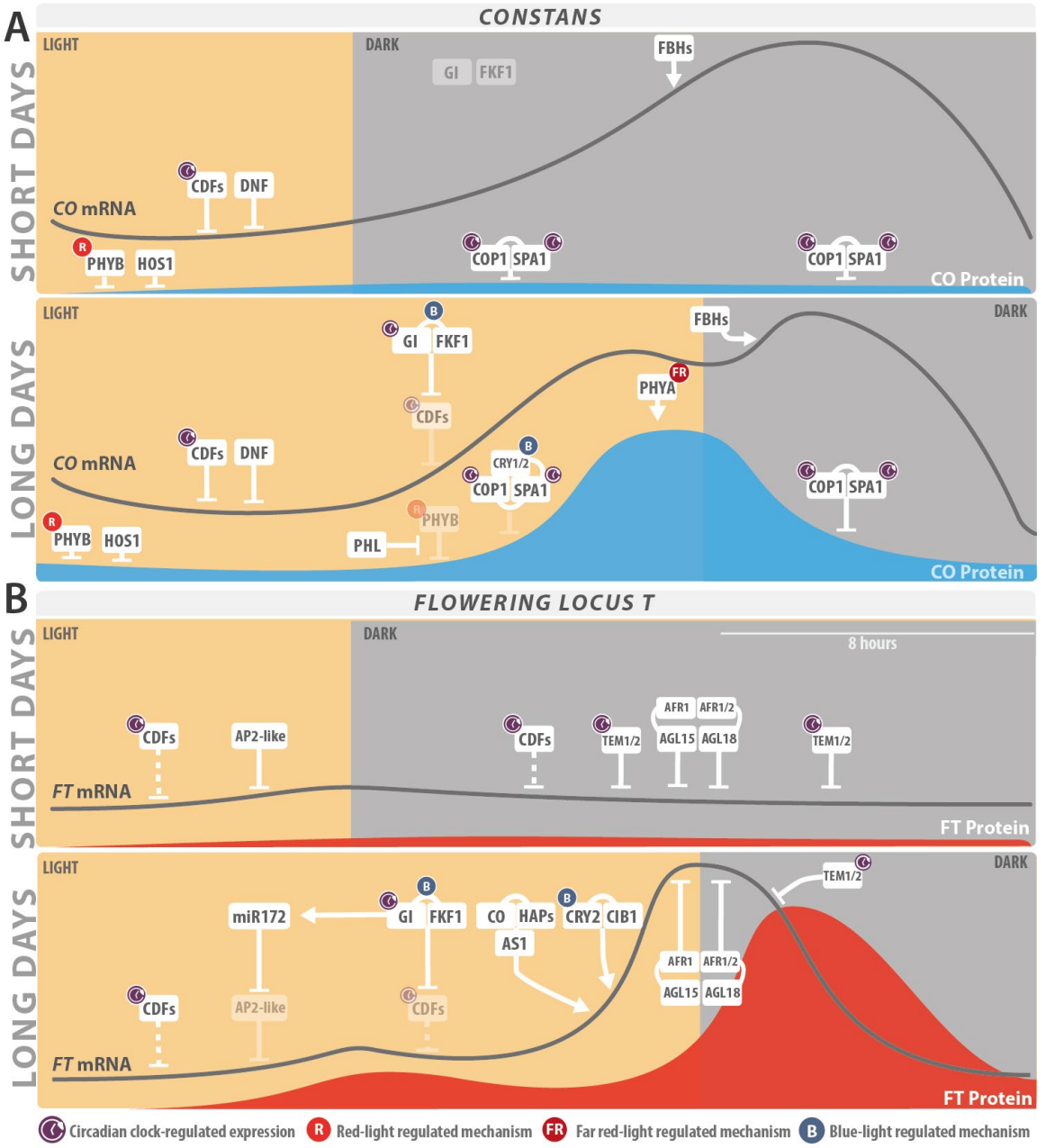

Figure 1-8. Time-course control of *CO* and *FT* expression.

A. Regulation of *CO* transcription and protein stability under SD [upper panel] and LD [lower panel]. Under SD, level of *CO* mRNA peak during the evening and at night. However, in the absence of light, CO protein is degraded by the COP1-SPA1 complex. Under LD, several protein complexes act to stabilize CO and prevent its degradation by the COP1-SPA1 complex. CO thus induces the expression of *FT* in phloem companion cells. Mauve circles specify circadian clock-regulated mechanisms. Blue (B), red (R), and dark red (FR) circles indicate processes controlled by blue, red, and far red light, respectively. **B.** Regulation of *FT* expression and protein stability under both SD [upper panel] and LD [lower panel]. Under SD, CO is absent and does not trigger *FT* expression, whereas several proteins additionally prevent the expression of *FT*. Under LD, CO - together with the CRY2-CIB1 complex - triggers the expression of *FT*. Later during the night, TEM1/2 and other protein complexes repress the expression of *FT* to prevent its ectopic induction. Upon favorable conditions, the amount of FT protein reaches a level sufficient to induce flowering.

suppressor mutagenesis of 35S::*CO* plants (Onouchi *et al.*, 2000) or transcriptomic analyses of inducible *CO* overexpressors (Samach *et al.*, 2000). Most prominent among these targets is the *FT* gene, which is expressed in the phloem of the leaves like *CO* (Takada and Goto, 2003).

The expression of *FT* is constitutively repressed under short days, by AP2-like, CDFs and TEMPRANILLO1/2 (TEM1/2) transcription factors (**Figure 1-8B**; Jung *et al.*, 2007; Castillejo and Pelaz, 2008; Mathieu *et al.*, 2009; Song *et al.*, 2012b; Zhang *et al.*, 2015). During the night, additional repression comes from chromatin deacetylation complexes formed by AGAMOUS-LIKE FACTOR15/18 (AGL15/18) and SAP30 FUNCTION-RELATED 1/2 (AFR1/2) (Gu *et al.*, 2013; Fernandez *et al.*, 2014).

In long days, repression of *FT* by the CDFs transcription factors is relieved by the GI/FKF1 complex, as found for *CO* (Song *et al.*, 2012b). The same complex upregulates synthesis of miR172 which in turn post-transcriptionally downregulates the AP2-like repressors of *FT* (Jung *et al.*, 2007).

Upregulation of *FT* in long days also occurs by activation, the major activator being CO (Samach *et al.*, 2000; Yoo *et al.*, 2005; Song *et al.*, 2012a). Furthermore, CRY2 interacts with CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX1(CIB1) in a blue-light-dependent manner to induce the expression of *FT* (Liu *et al.*, 2008b). *FT* mRNA level thus peaks late in the evening under long day conditions. Later, during the night, TEM1/2 and histone deacetylase repress *FT* to avoid its constitutive expression (Castillejo and Pelaz, 2008; Gu *et al.*, 2013; Fernandez *et al.*, 2014).

Although the activation of *FT* by the CO protein proved to be the key of the external coincidence model, the process appeared much more complex than initially thought. In the "promoter bending" model, the stabilization of CO by the extended light period allows its interaction with two partners: ASYMMETRIC LEAVES1 (AS1), forming a complex that binds the core elements of the *FT* promoter, and HEME ACTIVATOR PROTEINs (HAPs), which interact with distal regions of the promoter of *FT* (**Figure 1-9**; Wenkel *et al.*, 2006; Cai *et al.*, 2007; Kumimoto *et al.*, 2010). These distant interactions cause conformational changes in the *FT* promoter, which participate in *FT* activation (Cao *et al.*, 2014). Additionally, the HAP proteins are involved in the recruitment of complexes regulating H3K27 trimethylation at the *FT* locus (Hou *et al.*, 2014). It has been shown indeed that *FT* expression is partially mediated by chromatin modifications (reviewed in He, 2012). H3K27 and H3K9 di- and trimethylation are involved in the repression of *FT*. Conversely, the expression of *FT* increases upon H3 acetylation as well as H3K4 and H3K36 trimethylation. Those modifications create a chromatin environment appropriate for the induction of *FT*.

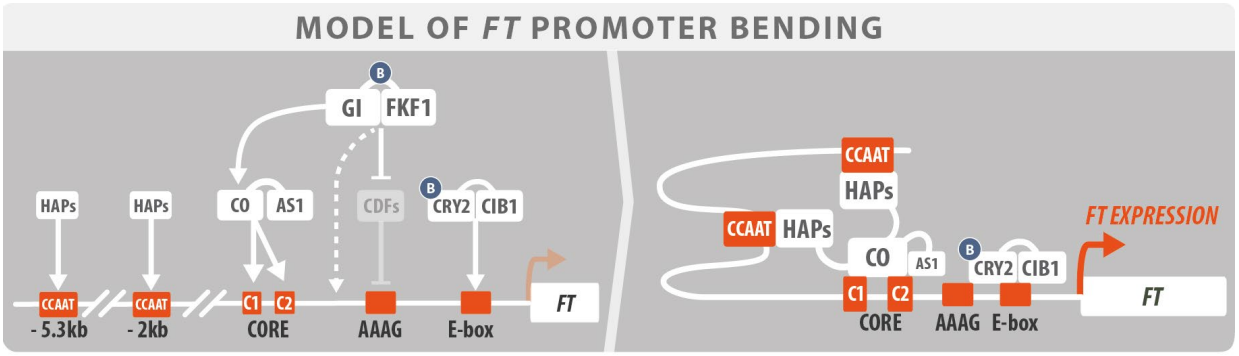

Figure 1-9. Current model of the induction of *FT* expression by CO and HAPs proteins.

The HAPs bind to the distal region of the *FT* promoter while CO binds to a proximal motif. The interaction of CO with HAPs modifies the conformation of the *FT* promoter, thus providing a positive local chromatin environment ensuring the expression of *FT*.

Systemic activity of FT

Once produced, the FT protein moves through the phloem towards the SAM (Corbesier *et al.*, 2007). The loading of FT from the companion cells to the sieve elements requires the FT-INTERACTING PROTEIN 1 (FTIP1) (Liu *et al.*, 2012). Besides, *FE*, a gene that was identified in the very first flowering-time mutant screenings but that was only recently characterized, positively regulates the expression of both *FTIP1* and *FT*, thus coordinating the production of FT and its transport from phloem companion cells (Abe *et al.*, 2015). *TSF*, the closest paralogue of *FT*, plays a redundant role with *FT*, even if its impact on the promotion of flowering is weaker (Yamaguchi *et al.*, 2005; Jang *et al.*, 2009). TSF is also supposed to move through the phloem, as proteomic analysis detected its presence in the phloem sap of *Brassica napus* (Giavalisco *et al.*, 2006). However, a recent study combining micro-grafting experiments and the generation of chimera proteins built from different domains of FT and TSF, showed that TSF has a lower mobility than FT (Jin *et al.*, 2015).

FT lacks a DNA-binding domain and hence it cannot induce in the SAM the floral meristem identity genes such as *LFY* and *AP1*. The upregulation of *AP1* has been shown to be mediated by the interaction between FT and FD, a bZIP transcription factor, albeit the direct binding of the FT-FD duplex to the promoter of *AP1* could not be demonstrated, suggesting an indirect activation (Abe *et al.*, 2005; Wigge *et al.*, 2005; Benlloch *et al.*, 2011). *FD* expression domain is restricted to the SAM and the RAM, thus spatially restricting FT function. In rice and Arabidopsis, 14-3-3 proteins are necessary for the interaction between FT and FD through the formation of an hetero-hexameric complex (Taoka *et al.*, 2011; Ho and Weigel, 2014). 14-3-3 proteins regulate a broad range of signalling pathways through their interaction

with phosphorylated proteins (reviewed in Denison *et al.*, 2011) and, accordingly, the phosphorylation of FD is necessary for the formation of the florigenic complex (Kawamoto *et al.*, 2015). Once formed, this complex is able to induce the expression of *SOC1*, *SPL3*, and *FUL*, thus initiating the development of floral primordia on the flanks of the SAM (Michaels *et al.*, 2005; Moon *et al.*, 2005; Yoo *et al.*, 2005; Teper-Bamnolker and Samach, 2005; Jung *et al.*, 2012).

Flowering-independent roles of *FT*

FT is the principal determinant of the induction of flowering by photoperiod. However, *FT* is also involved in the control of other processes in Arabidopsis (reviewed in Pin and Nilsson, 2012), such as shoot branching (Hiraoka *et al.*, 2013), the maintenance of floral commitment of the inflorescence (Müller-Xing *et al.*, 2014), and stomata movement (Kinoshita *et al.*, 2011). Loss-of-function *ft* mutant or *FT* overexpressors display closed and opened stomata, respectively. The phenotype of the overexpressors was associated with a higher H^+-ATPase activity, suggesting that FT could be involved in the post-translational activation of these transporters (Kinoshita *et al.*, 2011). Other flowering-time genes were subsequently linked to the regulation of this process since the light-induced stomata opening is reduced in the Col *FRI* lines and partially restored by vernalization. Additionally, *SOC1* overexpressors show constitutively opened stomata (Kimura *et al.*, 2015). However, the mechanistic reason behind this phenotype remains poorly understood.

In other species, *FT*-like genes may have diverged to regulate various processes. In poplar, *FT* is involved in the control of short-day induced growth cessation as well as bud-set (Böhlenius *et al.*, 2006; Hsu *et al.*, 2011). In legumes, several *FT*-like genes evolved to regulate organ storage differentiation (reviewed in Navarro *et al.*, 2015). In onion, a biennial crop, bulb-formation at the end of the first growing season is controlled by two antagonistic *FT*-like genes, *AcFT1* and *AcFT4*. Flowering occurs the second year after vernalization in winter and is regulated by AcFT2, which have the florigenic role of Arabidopsis' FT (Lee *et al.*, 2013b). *AcFT1* encodes a mobile factor promoting the formation of the bulb. Vernalization indirectly induces the expression of *AcFT2* in the SAM, located in the center of the bulb. Interestingly, AcFT2 shows only limited mobility, suggesting that - unlike in Arabidopsis - the florigenic protein acts locally in onion. In potato, tubers develop from underground stolons that stop growing longitudinally to initiate radial expansion and starch storage. This developmental switch, initiated under short days, is caused by a mobile signal originating from leaves and transported to the stolon. This signal was identified as StSP6a, an FT-like

protein (Navarro *et al.*, 2011). Interestingly, the regulation of the FT-like tuberization signal is controlled both by cytokinins and miR156/miR172. However, the expression patterns of those microRNAs are different from Arabidopsis, as they are both highly expressed during tuber formation (reviewed in Navarro *et al.*, 2015).

Overall, the PEBP gene functions have evolved to synchronize several crucial aspects of plant development, such as flowering or tuberization, with the seasonal changes in photoperiod.

1.2.3 • CIRCADIAN CLOCK

Related to Chapter 4 («Rooting the flowering process», page 137).

In most organisms, many behavioral and physiological processes are regulated at specific times of the day. The existence of diurnal-regulated processes in plants is known since about 2500 years but the first thorough description of an endogenous process controlling physiological outputs was published in 1729. A French scientist, de Mairan, showed that the movements of the leaves of Mimosa continued when plants were grown 24-hours in darkness, revealing an endogenous time-keeping mechanism (reviewed in Gardner *et al.*, 2006). Such rhythms were subsequently observed in many types of organisms and are qualified as "circadian" when their periodicity matches the periodicity of earth's rotation. They provide a selective advantage allowing to phase specific reactions with the time of the day and anticipate regular changes in the environment (Dodd *et al.*, 2005).

Measuring time

The circadian rhythms are controlled by an internal timekeeper called the **circadian clock**. Its oscillation is controlled by complex interlocking feedbacks. The circadian clock components are not well conserved among kingdoms, suggesting that they evolved independently (Young and Kay, 2001), but this point of view is still subject to debates (Rosbash, 2009). Together, the clock components regulate the timing of several processes - called the outputs - so that they occur at an appropriate time of the day. Those rhythms do not have an exact 24-hour period under constant conditions. Specific inputs, called zeitgebers, reset the clock to ensure its synchronization with the surrounding environment. Those signals may be of different nature: light/dark cycles, diurnal temperature variations, or - in some organisms - nutrient availability (reviewed in Gardner *et al.*, 2006).

The first conceptualization of the plant circadian clock originated in 1930, with the Bünning model (Bünning and Stern, 1930). From his analyses of the diurnal movements of soybean leaves, Bünning concluded that plants have a "biological clock" that is synchronized by red light but partially independent of light/dark cycles (Bünning and Stern, 1930). The clock runs endogenously with a period slightly different from 24h, and light thus acts as a signal that resets the clock to keep a 24-hour cycle. In his theory, a 24-hour cycle is divided into two distinct phases: the photophile phase, when plants are sensitive to light, and a scotophile phase, which is dark-sensitive. Plants thus distinguish daylength based on whether the light period coincides with the scotophile or photophile phase. This principle was the basis of the "external

coincidence" model, in which light entrains both the circadian oscillation of the photo- and scotophile phases and mediates the production of the flowering-inductive signal. In this model, the duration of the light exposition is not the key to the induction of flowering. The significant factor is the timing of the light period. Thus, the total duration of the light exposure is not important whereas the occurrence of the light signal at a particular time is important, as shown previously with the external coincidence model (**Figure 1-6**) .

The Arabidopsis endogenous rhythms oscillate with a period between 22 and 29 hours, depending on the ecotype and the growth conditions (Michael *et al.*, 2003). The regulation of this endogenous clock is complex and involves dozens of genes that display interlocked feedbacks controlled both at the post-transcriptional and post-translational levels. In Arabidopsis, light and temperature are the principal zeitgebers (Millar, 2004). Every day, at dusk and dawn, the clock is reset. Changing the day length thus triggers adjustments in the clock. Such a modification occurs in the external coincidence model described in the previous section (**page 24**), as the increase in day length modifies the period of *CO* expression. The light period is thus important, but its intensity also has an impact, as higher light intensities shorten the free-running period of the clock (Aschoff, 1979). Both the red light sensors - the PHYTOCHROMES (PHYs) -, and the blue light photoreceptors - the CRYPTOCHROMES (CRYs) -, act in the input pathways that synchronize the clock (Somers *et al.*, 1998). In return, these photoreceptors are controlled by the clock (Bognár *et al.*, 1999; Harmer *et al.*, 2000; Toth *et al.*, 2001).

Molecular mechanisms of the clock

The molecular mechanisms governing the Arabidopsis circadian clock have been extensively studied during the last 20 years, and significant advances were obtained by combining large-scale analyses and mathematical modeling. However, the exact mechanisms of circadian regulation at the transcriptome level as well as the signalling cascades between the core clock components and output genes are still poorly understood. We will thus give an overview of the current model of the circadian clock regulation, without unveiling all its complexity. We can divide the progression of the clock into four distinct phases (**Figure 1-10A**, reviewed in Hsu and Harmer, 2014):

(i) The morning phase. During this phase, *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), two genes encoding interacting MYB transcription factors, are expressed at high levels. Those factors repress the expression of the evening gene *TIMING OF CAB EXPRESSION 1* (*TOC1*) via the binding to a motif called «evening element» (EE) in the promoter. TOC1 in turn inhibits the expression of *CCA1*, thus creating the first circadian

clock feedback loop identified in plants. CCA1 and LHY also repress the expression of the components of the evening complex, *EARLY FLOWERING3/4* (*ELF3/4*) and *LUX ARRHYTHMO* (*LUX*).

(ii) The day phase components. Around noon, there is an increased expression of two *PSEUDO-RESPONSE REGULATORs (PRRs)* genes, *PRR7* and *PRR9*, which are induced by CCA1 and LHY. PRR7/9 in turn repress *CCA1* and *LHY*, thus restricting their expression to the morning. PRR7/9 also repress *REVEILLE8* (*RVE8*).

(iii) The afternoon phase. During the subjective afternoon, *RVE8* is highly expressed. RVE8 regulates the induction of both the evening complex components (*ELF3/4*, *LUX*), *PRR5* and *TOC1*. PRR5 thus forms a negative feedback with RVE8.

(iv) The evening phase. During the evening, the induction of *TOC1* and *PRR5* is preceded by the activation of the evening complex, composed of three interacting proteins (ELF3, ELF4, and LUX). The evening complex inhibits *PRR9*, while TOC1 and PRR5 repress the transcription of the morning genes *CCA1* and *LHY*. The expression of the evening complex components is controlled by the other circadian clock genes expressed at every time point of the day. In turn, they regulate the expression of *TOC1*, *PRR5*, *PRR9*, and *RVE8*. In addition, the expression *GI* - a gene involved in the photoperiodic control of flowering - peaks during the evening (**Figure 1-10B**). Those core clock components thus form intricately interconnected loops that ensure a circadian oscillation. The **Figure 1-10C** shows the period of several of those genes according to data obtained in 12-light/12h-dark photoperiod by James *et al.* (2008).

The circadian clock components display an internal oscillation that is reset each day by external clues. Several inputs (temperature, sucrose, etc.) are able to modulate the clock phase (reviewed in Hsu and Harmer, 2014) but we will limit our description to the light. Light influences the clock in many ways, regulating transcription, mRNA stability, and splicing, as well as post-transcriptional degradation of core clock components. Here are the main light inputs controlling the circadian oscillations (**Figure 1-10A**) :

- The exposition of GI to light triggers the formation of the GI-ZEITLUPE (ZTL) complex, which in turn induces the degradation of TOC1 and PRRs proteins by the 26S proteasome (Kim *et al.*, 2007). Conversely, the evening complex, through ELF3, acts to induce the degradation of GI (Yu *et al.*, 2008).
- Light triggers the expression of *CCA1* and *LHY* (Wang and Tobin, 1998; Schaffer *et al.*, 1998). However, their peak is only observable at the end of the night and early in the morning (**Figure 1-10C**) (Wang and Tobin, 1998; Schaffer *et al.*, 1998). The *CCA1* transcripts are unstable in the light, and their level thus diminishes after dawn (Yakir *et al.*, 2007b).

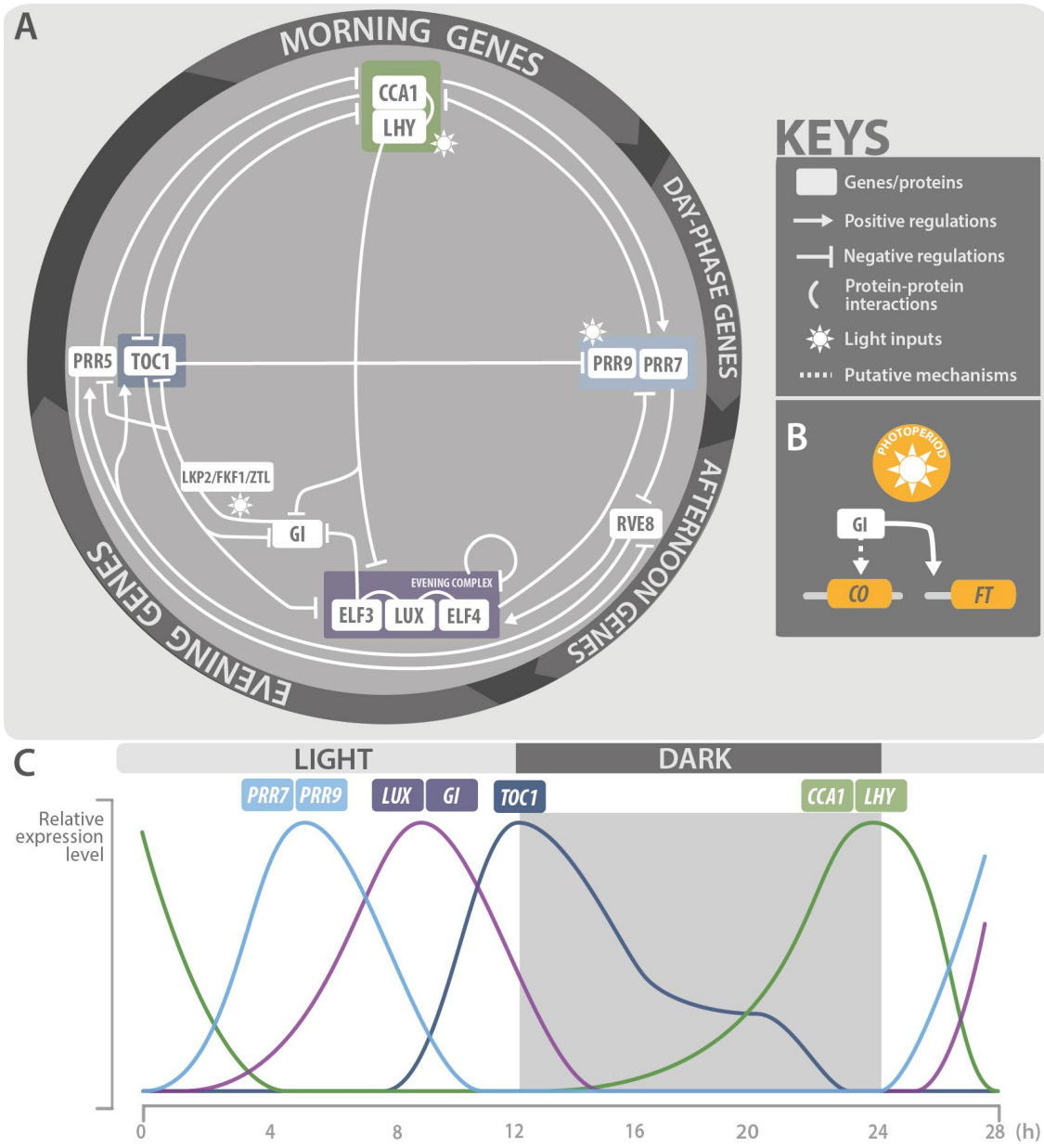

Figure 1-10. Overview of the Arabidopsis circadian clock.

A. The Arabidopsis clock is composed of several interlocked feedback loops. Details are provided in the main text. **B.** The principal flowering-related output of the circadian clock is the control of *CO* and *FT* expression by GI. **C.** Time-course expression levels of core circadian clock genes in the shoot. As represented data are transcript levels, the actual protein peak of those factors occurs slightly later. Data from James *et al.* (2008).

- Light induces the expression of *PRR7* and *PRR9* (Farré *et al.*, 2005).

Collectively, those regulatory inputs allow the synchronization of the clock with light signals, ensuring a periodicity adapted to the surrounding daily environmental changes.

Outputs mechanisms controlled by the clock

In Arabidopsis, the circadian clock controls a high proportion - above 30 % - of the shoot transcriptome (Covington *et al.*, 2008; Michaels, 2009). Its role is to provide a temporal coordination between different physiological processes to maximize their efficiency. Here are some examples of processes regulated by the circadian oscillator (reviewed in Yakir *et al.*, 2007a and de Montaigu *et al.* 2010; **Figure 1-11**):

- In several species including Arabidopsis, the clock regulates the **germination** through the control of hormone biosynthesis. The clock is not functional in dry seeds, but starts oscillating while they are hydrated during imbibition.
- The **elongation of the hypocotyl** is maximal during the evening and minimal during the day. The modulation of the hypocotyl growth participates in the shade avoidance response.
- **Cotyledon and leaf movements** are circadian clock-regulated and allow the plants to maximize its photosynthetic area during the day.
- The initiation of the **reproductive development** is also controlled by the clock. The external coincidence model, described previously, relies on the existence of endogenous rhythms regulating *CO* expression and protein stability.
- In several species, the clock controls the **opening of the flowers** to occur when pollinator are the more active, thus maximizing the balance between pollination and potential damages. For example, Arabidopsis petals open during the morning and close at midday. In several species, the emission of volatile compounds attracting pollinator is also governed by the circadian rhythm.
- The **photosynthesis** is also regulated by the clock through complex mechanisms not yet fully unraveled (reviewed in Müller *et al.*, 2014). Besides, during the night, the mobilization of starch increases to avoid sucrose starvation and growth penalties during the dark periods. Therefore, carbon assimilation, as well as starch metabolism, are under circadian regulation. In turn, the photosynthetic products, mainly sucrose, are involved in the entrainment of the circadian clock (Haydon *et al.*, 2013).

- Circadian oscillations control many other processes, such as **stomatal opening, response to cold temperatures, response to biotic stresses** as well as the **regulation of Ca^{++} cytosolic levels.**

Many questions are still ahead of plant chronobiologists, as the regulation of the circadian clock and its outputs are still not yet fully understood. Nagel *et al.* (2015) showed that the core clock protein CCA1 binds to more than 1000 genomic regions, thus regulating many biological processes, most of which remain to be identified. GI is another crucial output of the clock, as it regulates multiple targets involved in different biological processes. Recently, de Montaigu *et al.* (2015) showed the existence of natural variation in the circadian expression pattern of *GI* for different Arabidopsis accessions. Those allelic variations, caused by alterations in GI sensitivity to light, affect the expression of important developmental regulators such as *PIF4*. Thus, the fitness of Arabidopsis accessions to their environment partially relies on circadian clock adaptations. A recent review focuses on the influence of the clock on agricultural traits (Bendix *et al.*, 2015), showing that the circadian clock response to day length is crucial for maximizing biomass, flowering and yields in many crop species.

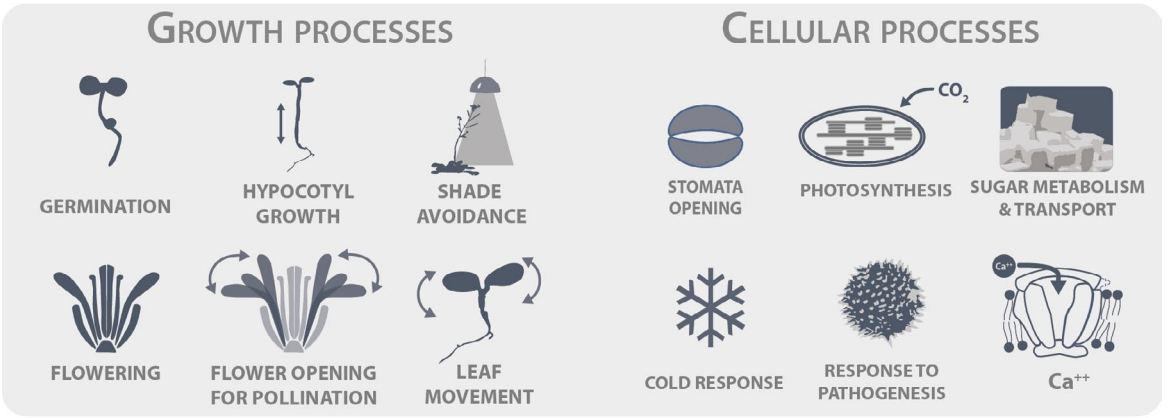

Figure 1-11. Processes controlled by the clock.

Processes regulated by the circadian clock, both at the organism [left] and at the cellular level [right]. Adapted from Yakir *et al.* (2007).

1.2.4 • THE VERNALIZATION PATHWAY

Related to :

- **Chapter 3 (« The growing substrate affects plant development and root transcriptome», page 97);**
- **Chapter S1 («An *FLC* ortholog from root chicory», page S3);**
- **Chapter S2 (« Heat can erase epigenetic marks of vernalization», page S33).**

Overview of the vernalization

The first reports suggesting the need for a long cold period to induce flowering were published more than 150 years ago. Initially observed in cereals, vernalization requirement was later observed in many other species (reviewed in Chouard, 1960). The extensive physiological characterization of cold-responsiveness in different plant species led to the categorization of monocarpic plants into three functional groups: **biennials** have an obligate vernalization requirement, **winter annuals** show a facultative vernalization response, while **summer annuals** are only weakly sensitive to a cold period. In the first case, plants are unable to flower unless exposed to a prolonged cold period of 1-10°C.

The effect of vernalization is quantitative: flowering is progressively accelerated as plants are exposed to longer cold periods, but the optimal duration varies with species (Amasino, 2010; Duncan *et al.*, 2015). This feature ensures that flowering does not occur after short cold spells but after winter. Once the cold period ends, the vernalized state is stable under normal growth conditions but is not transmitted to the progeny, as the cold-induced changes are reset during meiosis. It is noteworthy that, in most of the winter species, vernalization is necessary but not sufficient to induce flowering upon return to normal growth temperatures (Amasino, 2004). The induction of flowering requires other endogenous and/or environmental cues, most often long days (reviewed in Kim *et al.*, 2009). In those species, the cold period thus provides the competence to flower, not its induction.

Most of the *Arabidopsis thaliana* accessions that are commonly used in genetics studies are rapid-cycling summer annuals, which do not require cold treatment. However, among the numerous ecotypes collected worldwide, some need a vernalization treatment to flower. The first clues to the molecular basis of vernalization were obtained by studying this natural variation. By crossing winter and summer accessions, researchers identified the molecular markers segregating with the late-flowering phenotype (Lee *et al.*, 1993a; Burn *et al.*, 1993; Clarke and Dean, 1994). The major determinant of the vernalization requirement was named *FRIGIDA* (*FRI*). The introgression of *FRI* into the Col-0 summer accession is sufficient to convert it intro a

vernalization-requiring plant (Lee *et al.*, 1994). The *FRI*-mediated delay of flowering relies on another locus called *FLOWERING LOCUS C* (*FLC*), which is quantitatively downregulated by cold (Lee *et al.*, 1994; Koornneef *et al.*, 1994; Sheldon *et al.*, 1999; Michaels and Amasino, 1999). *FLC* is thus another major determinant of winter accessions, and rapid-cycling summer annuals carry mutations in *FRI* and/or *FLC* (Johanson *et al.*, 2000; Gazzani *et al.*, 2003; Michaels *et al.*, 2003). Epistasis and molecular analyses subsequently showed that FRI is a direct activator of *FLC* (Johanson *et al.*, 2000; Choi *et al.*, 2011). The *FRI-FLC* module thus constitutes the core determinant of vernalization requirement in Arabidopsis (**Figure 1-12A**): FRI activates the expression of *FLC*, which in turn represses flowering. During exposure to non-freezing cold temperatures, *FLC* is downregulated and this repression is maintained upon return to warmer growth temperatures (Sheldon *et al.*, 1999; Michaels and Amasino, 1999).

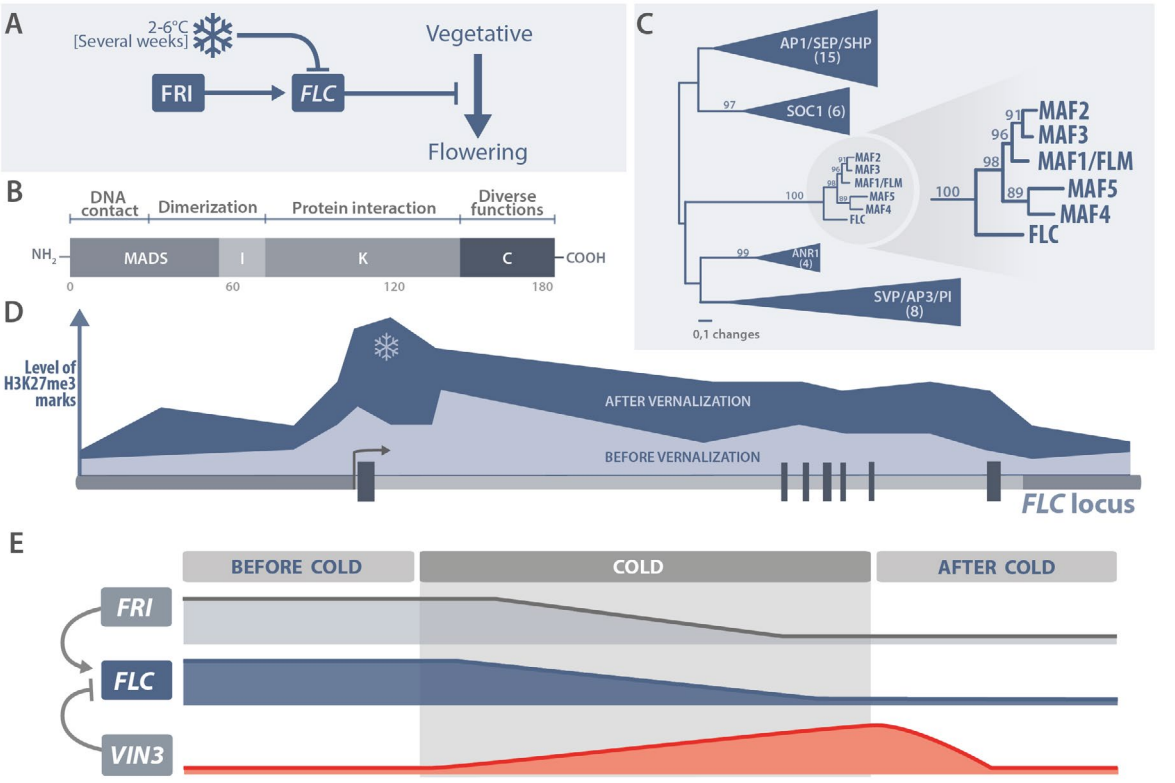

Figure 1-12. Role of *FLC* in the vernalization pathway.

A. Overview of the *FRI*/*FLC*-mediated response to vernalization. **B.** Structure of the FLC protein showing the conserved domains of MIKC-type MADS box transcription factors. **C.** Phylogenetic tree of a subset of MADS-box genes showing the *FLC* subfamily, which contains six genes. Adapted from Parenicová *et al.* (2003). **D.** Profile of H3K27me3 repressive marks levels before [light blue] and after [dark blue] vernalization (data from Angel *et al.*, 2011). **E.** Time-course expression of key regulators of the vernalization response. *FLC* is not the only cold-responsive flowering-time gene, as the expression of both activator (*FRI*) and repressor (*VIN3*) of *FLC* are modified upon cold.

General mechanisms regulating *FLC*

FLC encodes a MADS-box transcription factor from the MIKC subfamily (Sheldon *et al.*, 1999; Michaels and Amasino, 1999), which contains both a DNA-binding region and a protein-protein interaction domain (**Figure 1-12B**). Phylogenetic analysis of the MADS-box genes showed that *FLC* is part of a subfamily containing five closely related genes, called *MADS AFFECTING FLOWERING 1-5* (*MAF1-5*) (**Figure 1-12C**). *MAF1-5* genes participate - albeit marginally - in the response to vernalization, and *MAF1/2* are also involved in the acceleration of flowering by increased ambient temperatures (Scortecci *et al.*, 2001; Scortecci *et al.*, 2003; Posé *et al.*, 2013; Lee *et al.*, 2013a; Airoldi *et al.*, 2015).

One of the key steps in our understanding of *FLC* regulation was achieved in 2004 when Bastow and colleagues showed that vernalization induces an increase in H3K9 and H3K27 di- and tri-methylation of chromatin at the *FLC* locus (Bastow *et al.*, 2004). Those marks, previously characterized in Drosophila and human cells, are associated with silenced chromatin states. The cold period thus induces chromatin changes that prevent *FLC* expression (**Figure 1-12D**).

In parallel, several screenings were performed to identify negative regulators of *FLC* in winter accessions. Late-flowering mutants insensitive to vernalization allowed the identification of genes controlling the cold-mediated repression of *FLC*, including *REDUCED VERNALIZATION1* (*VRN1*), *VRN2* (Chandler *et al.*, 1996; Gendall *et al.*, 2001; Levy *et al.*, 2002), and *VERNALIZATION INSENSITIVE3* (*VIN3*) (Sung and Amasino, 2004). *VRN1* and *VRN2* are constitutively expressed whereas *VIN3* is activated upon cold exposure, suggesting its participation in the early steps of the vernalization response (Sung and Amasino, 2004) (**Figure 1-12E**). However, the activation of *VIN3* is not maintained after the end of the cold period. The role of VIN3 in the repression of *FLC* family members has been extensively demonstrated (Wood *et al.*, 2006; Sheldon *et al.*, 2009; Kim and Sung, 2013), but other processes are required to maintain the repressed state of *FLC* after return to warm temperatures. Concomitantly to the increase of *FLC* repressors, recent results suggests that cold causes proteolysis of FRI (Hu *et al.*, 2014). Vernalization thus controls the abundance of several positive and negative regulators of *FLC* to ensure a fine-tuned response to prolonged cold.

Epigenetic marks controlling *FLC* expression

Histones are subject to many different types of post-translational modifications that affect the chromatin structure. Different types of modifications may occur, according to the nature of the modified structure (reviewed in Li *et al.*, 2007).

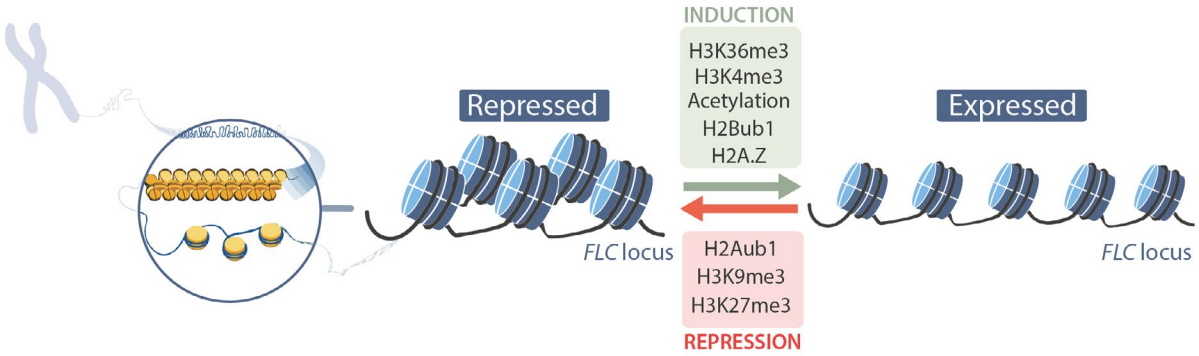

Figure 1-13. Chromatin regulation at the *FLC* locus.

Different types of chromatin marks control both the compaction of chromatin and the recruitment of protein complexes at the *FLC* locus.

Known modifications affecting chromatin structure at the *FLC* locus (Figure 1-13) are:

- **Histone lysine trimethylation**. The histone are globular proteins bearing tails in which many lysine residues may be mono-, di-, or tri-methylated. According to the lysine residue that is modified, the trimethylation has different effects on gene transcription: H3K4me3 and H3K36me3 induce *FLC* expression while H3K9 and H3K27 trimethylation are involved in its repression.
- **Histone acetylation**. The acetylation of histone, catalyzed by histone acetyltransferase complexes, occurs essentially at lysine residue (K). The acetylation of histones is generally associated with an active chromatin state.
- **Histone ubiquitination**. Two antagonistic types of histone monoubiquitinations can be deposited on the nucleosome: H2Bub1 is involved in the activation of transcription while H2Aub1 is associated with a repressed chromatin state.
- **Exchange of histone variant**. The exchange of histone variant is the most evolutionary conserved chromatin remodeling process. It involves the exchange between the histone H2A and its variant H2A.Z, a mechanism essential for the control of multiple developmental aspects, including flowering (Jarillo and Piñeiro, 2015). The deposition of H2A.Z variant is involved in the positive regulation of *FLC* expression.

The epigenetic regulation of *FLC* is complex

As discussed above, the expression of *FLC* is turned off during cold and the silenced state is maintained afterwards. This mitotically stable repression is mediated by epigenetic changes of chromatin structure. Since the molecular complexes regulating chromatin modifications are

highly conserved among eukaryotes, the study of the "memory of winter" became a model for the understanding of epigenetics. Much effort was carried to identify and decipher the molecular processes responsible for the regulation of epigenetic mark deposition/modifications at the *FLC* locus. Those processes can be classified as *FLC*-specific or ubiquitous (reviewed in Berry and Dean, 2015; Summarized in **Figure 1-14**).

Before cold, several complexes act synergistically to maintain high level of *FLC* expression:

- The **FRIGIDA complex** (FRIc) is the major *FLC*-specific transcriptional activator. The FRI protein acts as a scaffold for the formation of a macromolecular complex that includes H3K4 and H3K36 methyltransferases involved in the deposition of activating marks at the *FLC* locus (Ko *et al.*, 2010; Choi *et al.*, 2011). Additionally, FRIc recruits other macromolecular complexes to ensure activation of *FLC* transcription (Choi *et al.*, 2011).
- The **SWR1 complex**, recruited by FRIc, is involved in the activation of *FLC* through the exchange of histone variants (Choi *et al.*, 2011).
- The **RNA polymerase II-Associated factor 1 complex** (PAF1c), which interacts with RNA polymerase II, facilitates the action of chromatin-remodeling complexes involved in the activation of *FLC*. The PAF1c is necessary for the ubiquitination of histone H2B and the deposition of activating H3K4 and H3K36 trimethylation marks. It does not have any histone modification capacities *per se*, but probably acts as a docking complex for histone-modifying enzymes (reviewed in He, 2012).
- The **Compass complex**, recruited by the PAF1c, is involved in the deposition of H3K4me3 activating marks at the *FLC* locus (Krogan *et al.*, 2003; Jiang *et al.*, 2011).
- The **RAD6-Bre1** complex participates in H2B monoubiquitination, a prerequisite for the subsequent deposition of H3K4 trimethylation marks (Gu *et al.*, 2009).
- Finally, the **FACT complex** facilitates the elongation of the nascent transcript by promoting the dissociation of the H2A-H2B dimer from the nucleosome (Belotserkovskaya *et al.*, 2003).

Collectively, those protein complexes activate the expression of *FLC*, establishing the requirement for vernalization. Vernalization stimulates degradation of FRI (Hu *et al.*, 2014), and in parallel triggers negative *FLC* regulators:

- The **Polycomb Repressive Complex 2** (PRC2), widely conserved among higher eukaryotes, catalyzes deposition of both H3K27me3 and H3K9me3 repressive marks. This complex interacts with several PHD finger proteins, including VIN3,

to repress the expression of *FLC* during the cold period (De Lucia *et al.*, 2008). However, since the expression of *VIN3* rapidly decreases upon return to warm temperatures, other mechanisms must be involved in maintenance of the repressed state.

- The maintenance of the repressed chromatin state is achieved by a complex, called **PRC1-like** (Simon and Kingston, 2009), whose component LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) protein was shown to be essential (Sung *et al.*, 2006).
- Finally, the **HDAC complex** is involved in the repression of *FLC* expression through histone deacetylation (He *et al.*, 2003).

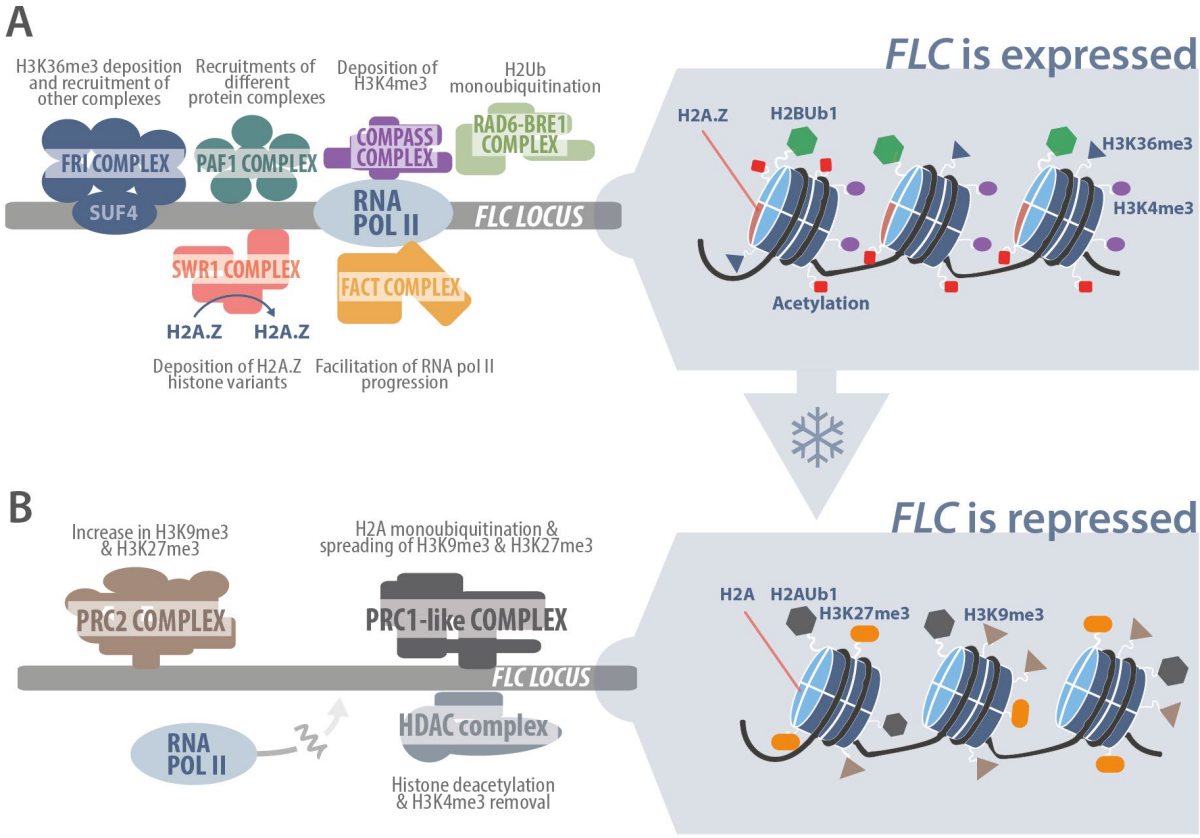

Figure 1-14. Complexes controlling *FLC* expression.

A. Before vernalization, *FLC* is expressed at high levels, thus repressing flowering. The expression of *FLC* is triggered by several complexes (FRIc, PAF1c, SWR1c, COMPASSc, FACTc, and RAD6-BRE1c). Together, they build a local chromatin environment favorable for the transcription of *FLC* by the RNA polymerase II. **B.** The vernalization-mediated repression of *FLC* is mainly achieved through the deposition of repressive marks at the *FLC* locus. The PRC2 complex is involved in the deposition of H3K9me3 and H3K27me3 repressive marks while the PRC1-like complex participates in the spreading of those marks. The HDAC complex removes H3K4me3 activating marks and erases acetylation marks, thus preventing the expression of *FLC*. Additional details are provided in the main text.

Other regulatory mechanisms

In addition to the regulation of *FLC* by chromatin modifications, cold induces the expression of a long antisense transcript of *FLC*, called *COOLAIR* (Swiezewski *et al.*, 2009) and of an alternative noncoding sense transcript, called *COLDAIR* (Heo and Sung, 2011; **Figure 1-15A**). Both *COOLAIR* and *COLDAIR* are thought to regulate the deposition of H3K27me3 repressive marks at the *FLC* locus (Heo and Sung, 2011; Csorba *et al.*, 2014; Marquardt *et al.*, 2014). The antisense transcript of *FLC* is only transiently expressed upon cold exposure, suggesting that it would be involved in the early cold-mediated events.

According to the current model (Berry *et al.*, 2015), the repression of *FLC* occurs through three successive phases:

- First, prior to vernalization, the whole *FLC* locus is in an active state. All the transcripts arising from the *FLC* locus can be expressed but the synthesis of the coding *FLC* RNA is favored.
- At the beginning of the cold period, repression of *FLC* is initiated by the PRC2 complex in a specific domain - the nucleation region - located near the transcription start site. The expression of the *FLC* coding transcript is thus repressed by epigenetic modifications while the transcription start sites of both *COOLAIR* and *COLDAIR* are still active (Finnegan, 2015).
- Finally, the repressive marks spread all over the locus, repressing *FLC* sense as well as *COOLAIR* and *COLDAIR* expression.

In addition to those processes, the regulation of *FLC* is also modified by physical interactions occurring between different regions of its locus. Gene loops were identified in yeast and involve the interaction between the promoter and the 3' regions of the same loci (Ansari and Hampsey, 2005). This structure favor the transcription of the gene, probably through the recycling of RNA polymerase from the 3' end to the promoter region (Lainé *et al.*, 2009). The importance of such three-dimensional chromatin conformation changes in the regulation of genes has been established recently in several model organisms (reviewed in Zhu *et al.*, 2015). The *FLC* gene loop, whose structure is unaffected in chromatin remodeling mutants, is disrupted as one of the first events occurring upon cold treatment and is correlated with a decrease in *FLC* sense transcription (Crevillén *et al.*, 2013; Jégu *et al.*, 2014) (**Figure 1-15B**). However, the functional relevance of this mechanism is not yet fully understood.

All the mechanisms described above account for the repression of *FLC* at the cellular level. At the tissue or organism levels, repression of *FLC* is progressive as the number of cells that have

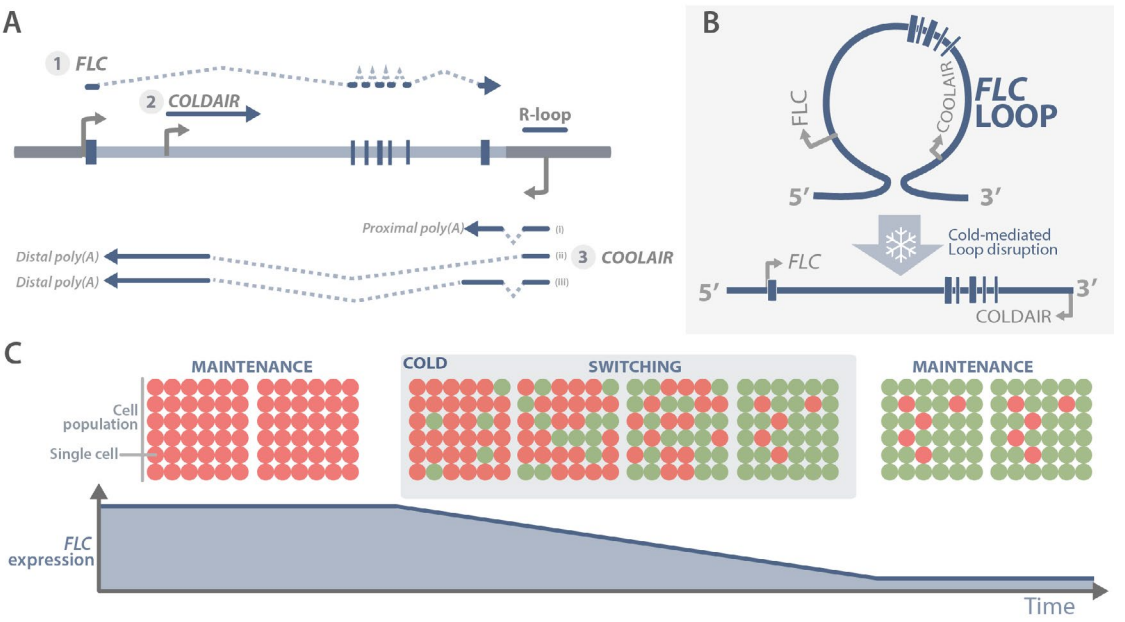

Figure 1-15. Additional mechanisms regulating the expression of *FLC*.

A. The *FLC* locus produces several transcripts: (1) the *FLC* mRNA; (2) a non-coding RNA generated from an alternative transcription start site (*COLDAIR*); (3) an antisense transcript initiated from the 3′ end of the *FLC* gene (*COOLAIR*). Several forms of *COOLAIR* exist. During vernalization, the expression of *COLDAIR* and *COOLAIR* increases. Adapted from Baulcombe and Dean (2014). **B.** At the *FLC* locus, the chromatin is arranged in a complex tridimensional structure, the *FLC* loop, which prevents the expression of *COLDAIR*. The loop is disrupted by vernalization, thus relieving the expression of antisense transcripts of *FLC*. Adapted from Zhu *et al.* (2015). **C.** Digital model of *FLC* repression. In this model, the expression of FLC is either "ON" or "OFF" in each single cell. Upon cold, the population of cells expressing *FLC* gradually switches" from *FLC*-ON [red] to *FLC*-OFF [green]. *FLC* inhibition is stable upon return to warm conditions. Adapted from Berry and Dean (2015).

switched from an "*FLC* ON" to an "*FLC* OFF" state increases (Figure 1-15C) (reviewed in Berry and Dean, 2015). This model suggests that each cell responds independently to a cold period and that the quantitative response to vernalization reflects the whole-population cell average.

FLC targets

FLC encodes a MIKC MADS-box transcription factor whose overexpression leads to the repression of *FT* and *SOC1*, indicating that these flowering regulators are probable downstream targets (Hepworth *et al.*, 2002; Michaels *et al.*, 2005). Chromatin immunoprecipitation confirmed that FLC can bind to the promoters of both *FT* and *SOC1* (Searle *et al.*, 2006; Helliwell *et al.*, 2006) suggesting that *FLC* acts in the leaves, where *FT* is expressed, and in the SAM, where *SOC1* is active. Albeit this pathway seems straightforward, we saw that the regulation of *FLC* occurs through very complex mechanisms and, additionally, recent pieces of evidence indicate

that FLC activity may also be regulated through post-translational modifications, such as sumoylation (Son *et al.*, 2014).

FLC contains a protein-protein interaction domain, suggesting that it may form complexes with other MADS-box protein(s) (reviewed in Kaufmann *et al.*, 2005). Yeast two-hybrid screenings however failed to identify FLC interactors, possibly because the interaction of FLC with other proteins would require post-translational modifications or additional partners lacking in yeast (de Folter *et al.*, 2005). By contrast, *in vivo* interaction analyses showed that FLC can form homodimers that are part of high-molecular-weight complexes (Helliwell *et al.*, 2006). Interestingly, FLC is also able to interact with SHORT VEGETATIVE PHASE (SVP), another MADS-box protein of the MIKC subfamily that inhibits flowering by direct repression of *FT* expression (Hartmann *et al.*, 2000; Lee *et al.*, 2007; Li *et al.*, 2008). SVP is also involved in the control of flower development (Gregis *et al.*, 2006; Gregis *et al.*, 2008). According to Li and colleagues (2008), the interaction between FLC and SVP proteins is responsible for the repression of flowering-time genes. However, a recent study shows that both FLC and SVP can bind *FT* and *SOC1* independently from each other, while the FLC-SVP complex binds distinct subset of genes, mainly involved in gibberellin-related processes (Mateos *et al.*, 2015). *FLC* is thus a major determinant of flowering time, but it may control additional processes through yet unknown mechanisms.

1.2.5 • THE AGING PATHWAY

Related to Chapter 3 (« The growing substrate affects plant development and root transcriptome», page 97).

The aging pathway has a central role as it regulates transitions between developmental phases: from juvenile to adult phase, and from adult vegetative to reproductive phase. These changes are regulated by a balance between two microRNAs: miR156 and miR172. In this chapter, we will first discuss the general characteristics of microRNAs. We will then tackle their involvement in the control of aging in *Arabidopsis thaliana*.

1.2.5A • MicroRNA biogenesis and characteristics

Discovery

Twenty-five years ago, the first microRNA was identified in the nematode *Caenorhabditis elegans* (Lee *et al.*, 1993; Wightman *et al.*, 1993) but the publication of the genome sequences of different species later revealed that microRNAs are widespread among living organisms, as they are found in mice, humans, and plants (Lagos-Quintana, 2001; Llave *et al.*, 2002; Rhoades *et al.*, 2002). With the improvement of bioinformatic algorithms, the number of predicted microRNAs is continuously increasing. Currently, the microRNA database "miRbase" (http://www.mirbase.org) contains more than 2000 human microRNAs and about 400 Arabidopsis microRNAs (Kozomara and Griffiths-Jones, 2013), most of which remain uncharacterized.

Biogenesis and mechanisms

MicroRNAs are 20 to 22 nucleotide-long noncoding RNA sequences involved in the post-transcriptional downregulation of target genes. As illustrated in **Figure 1-16**, the microRNAs are transcribed by the RNA polymerase II in the same way than protein-coding transcripts (Lee *et al.*, 2004). Some microRNAs are encoded by multiple genes and hence their transcription can be controlled by different promoters containing various response elements. For instance, miR156 is encoded by eight different loci, some of which display various response elements to both biotic and abiotic stresses (Liu *et al.*, 2008c).

Before reaching their mature size, microRNAs undergo successive maturation steps. Once transcribed, the pri-miRNAs are first capped and polyadenylated in the same way than protein-coding mRNAs. The distinctive feature of pri-miRNAs is the presence of an imperfect hairpin-like stem loop structure. Each hairpin gives rise to a unique mature microRNA. Most

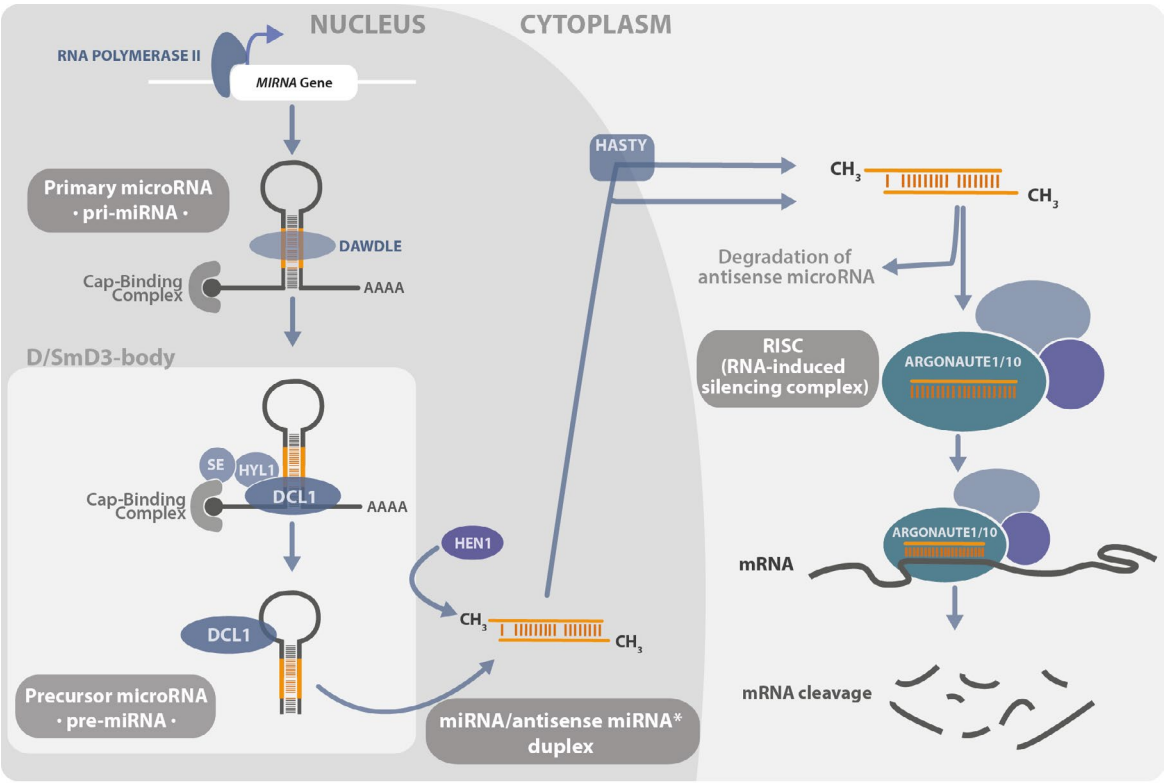

Figure 1-16. MicroRNA biogenesis, processing and functions.

After their transcription by the RNA polymerase II, pri-microRNAs are processed by DCL1 to produce precursor microRNA. Pre-miRNAs are then spliced into miRNA/miRNA* duplexes, which are subsequently methylated and transported to the cytoplasm. The duplex dissociates and one of the strands is selected as a template by the RISC complex, whereas the other strand is, in most cases, degraded. The RISC complex uses the sense microRNA as a template to target complementary mRNA and trigger its degradation or, in certain instances, inhibit its translation. Adapted from Spanudakis and Jackson (2014).

of the pri-miRNAs contain only one loop, but some endogenous pri-microRNAs bear several loops corresponding to distinct mature Arabidopsis microRNAs (Merchan *et al.*, 2009). The DAWDLE protein stabilizes the pri-miRNA loop and recruits DICER-LIKE1 (DCL1), an RNase III endonuclease (Morris *et al.*, 2006). DCL1 catalyzes two successive cleavages of the pri-miRNA sequence. The first step leads to the creation of the pre-microRNA, a stem-loop structure of ± 150 nucleotides. This pre-microRNA is processed to produce an heteroduplex containing the microRNA sense and antisense strands (miRNA/miRNA* duplex) (Papp *et al.*, 2003). The duplex is methylated - a crucial step for its stabilization - and subsequently exported to the cytoplasm (Yu *et al.*, 2005; Park *et al.*, 2005). One of the two strands, called the guide strand, is loaded in the RNA-INDUCED SILENCING COMPLEX (RISC), while the other strand is, in most cases, degraded (Shao *et al.*, 2013). The choice of the guide strand is essentially driven by its thermodynamic features. The strand showing lower thermostability at the 5' end is preferentially loaded in the RISC complex to

guide the cleavage of its targets (Figure 1-17A; Khvorova *et al.*, 2003; Eamens *et al.*, 2009). The RISC complex is composed of several subunits, including proteins from the ARGONAUTE family. In the case of microRNA-induced RISC complex formation, AGO1 and AGO10 directly interact with the guide microRNA (Song *et al.*, 2003; Ma *et al.*, 2005). Those proteins are essential for the microRNA-mediated silencing of the target mRNA, as they both display endonuclease activity (Vaucheret *et al.*, 2004; Baumberger and Baulcombe, 2005; Ji *et al.*, 2011).

A thorough analysis of the sequences of endogenous Arabidopsis microRNAs identified the essential features shared by plant microRNAs (Figure 1-17B; Ossowski *et al.*, 2008). The main characteristics of the microRNA-target duplex could be established:

- Only a few mismatches are allowed at the 5' end of the microRNA;
- The cleavage zone does not admit any mismatch;
- The position 10 is always an adenine;
- The 3' end of the microRNA allows more mismatches.

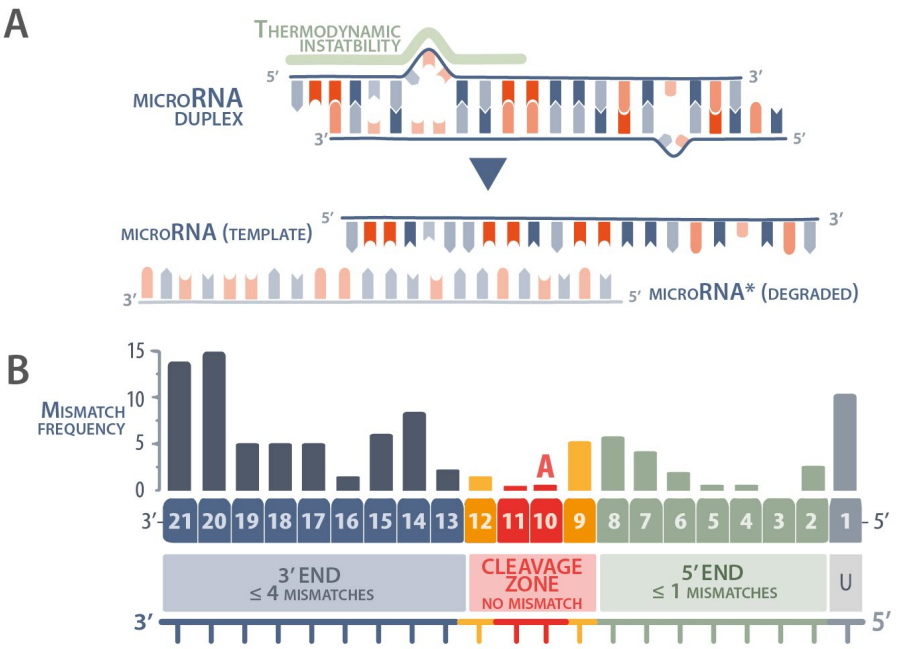

Figure 1-17. Characteristics of plant microRNAs.

A. The selection of the microRNA strand used to target mRNAs seems to be mediated by a thermodynamic instability in the 5′ end of the sense strand. The other strand is, in most cases, degraded. **B.** Endogenous microRNA sequences show conserved features. The RISC complex uses the microRNA to bind target mRNAs and induce their cleavage in front of the position 10 of the microRNA [red region]. Adapted from Ossowski *et al.* (2008).

The adenine conservation in the cleavage zone is crucial for the degradation of the target mRNA as endonucleases preferentially cleave their target after a uridine (Donis-Keller, 1979). Actually, the mode of action of microRNAs is defined by the degree of complementarity with their target. In most cases, a perfect match triggers the degradation of the targeted mRNA while an imperfect match blocks its translation (Hutvágner and Zamore, 2002). In plants, early *in silico* analyses suggested that microRNAs were mostly involved in the degradation of their targets, as endogenous microRNAs typically show high level of complementary with their targets (Rhoades *et al.*, 2002; Wang *et al.*, 2004). However, this paradigm was contradicted by several studies showing the existence of microRNA-mediated translational repression mechanisms in plants (reviewed in Huntzinger and Izaurralde, 2011). Yet, the degradation of the target is probably more efficient, as the guide strand may be used to degrade several successive mRNAs.

1.2.5B • The genetic mechanisms controlling the aging pathway

The developmental transitions of Arabidopsis are controlled by the balance between two microRNAs, miR156 and miR172, whose relative abundances show an opposite pattern: as miR156 decreases with plant age, miR172 increases (Aukerman and Sakai, 2003; Wu and Poethig, 2006; Wang *et al.*, 2009). miR156 is a negative post-transcriptional regulator of several *SPL* transcription factors while miR172 prevents the translation of several *AP2*-like transcription factors (reviewed in Wang, 2014).

The *SPL* transcription factor family contains 16 genes that could not be identified in the first Arabidopsis screenings because of their redundancy. *SPLs* were initially identified in Snapdragon, for their ability to bind the promoter of *SQUAMOSA*, an ortholog of *AP1* (Klein *et al.*, 1996). Afterwards, Cardon and colleagues (1997) used the conserved region of *SQUAMOSA PROMOTER BINDING PROTEIN* (*SBP*) from Snapdragon as a probe to screen Arabidopsis cDNA libraries. They identified seven *SPL* genes in Arabidopsis, and the number of members later increased to 16 (Cardon *et al.*, 1999; Guo *et al.*, 2008).

SPLs belongs to two main functional categories, whether they display a miR156-binding element in their sequence or not (**Figure 1-18A**). miR156-regulated *SPLs* can be divided into subgroups, as *SPL2/10/11*, *SPL9/15* and *SPL3/4/5* are closely related to each other and display partially redundant functions (**Figure 1-18B**). *SPL2/10/11* control leaf serration (Wang *et al.*, 2008; Shikata *et al.*, 2009; Wu *et al.*, 2009). *SPL9* and *SPL15* regulate leaf shape, abaxial trichome initiation, and participate in the induction of flowering (Schwarz *et al.*, 2008; Wu *et al.*, 2009). Finally, *SPL3/4/5* were shown to redundantly control the apparition of abaxial

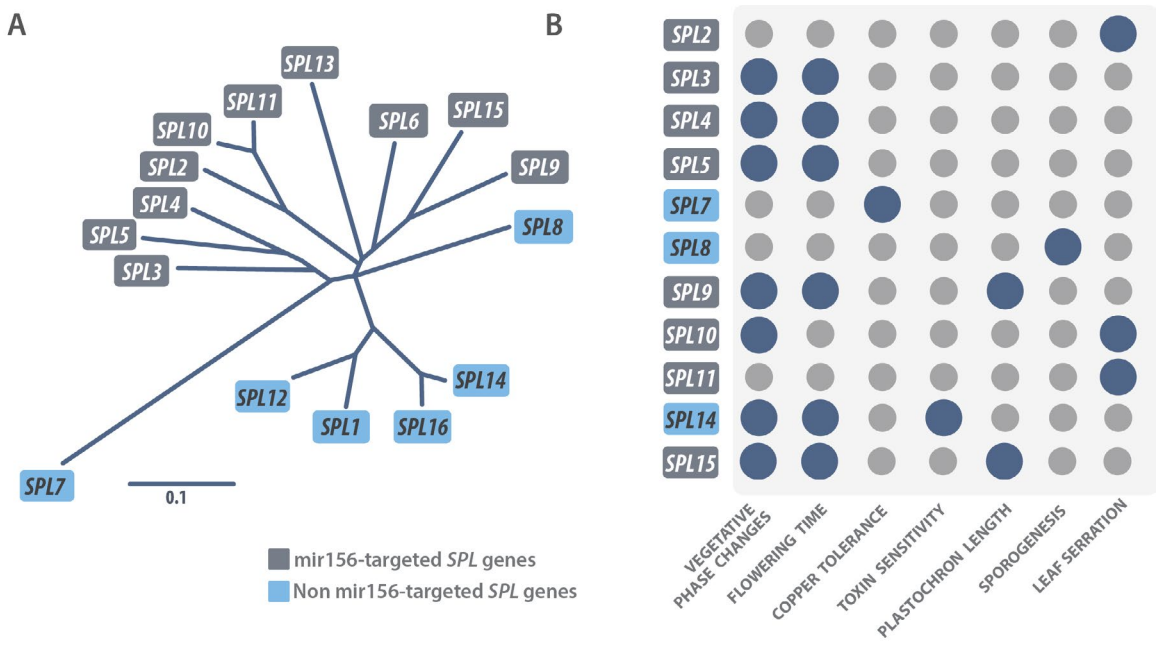

Figure 1-18. *SPL* gene family.

A. Unrooted phylogram of the *SPL* genes based on the conserved SBP domain. Grey boxes indicate miR156-targeted *SPLs*, blue boxes non-targeted *SPLs*. Adapted from Xing *et al.* (2010). **B.** Developmental processes regulated by SPL proteins. Adapted from Preston and Hileman (2013).

trichomes as well as the onset of flowering, and the overexpression of *SPL3* accelerates the transition to the reproductive phase (Cardon *et al.*, 1999). Consistently, *SPL3* mRNA level increases rapidly in the SAM in response to the induction of flowering by long days (Schmid *et al.*, 2003).

SPLs were among the first genes to be predicted as microRNA-targets in Arabidopsis (Rhoades *et al.*, 2002). This was rapidly demonstrated experimentally, as all the *SPLs* bearing a potential miR156 target site were downregulated in *MIR156* overexpressing lines (Kasschau *et al.*, 2003; Schwab *et al.*, 2005). In the wild type accession, the abundance of miR156 decreases as the plant ages (**Figure 1-19A**), while the expression levels of *SPLs* increase. The artificial overexpression of *MIR156* extends the juvenile phase and delays the transition to flowering (Wu and Poethig, 2006). Interestingly, the late-flowering phenotype of *MIR156*-overexpressing lines could be overcome by the expression of a microRNA-resistant form of either *SPL3*, *SPL4*, or *SPL5* (Wu and Poethig, 2006, **Figure 1-19B**) indicating that these redundant *SPLs* mediate most of the miR156-dependent effects on flowering time.

miR172 abundance increases over time, thus exhibiting an expression pattern opposite to

miR156 (Aukerman and Sakai, 2003) (**Figure 1-19A**). miR172 is involved in the post-transcriptional downregulation of the *AP2*-like transcription factors, which include *AP2*, *SCHLAFMUTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*), *TARGET OF EAT1* (*TOE1*), *TOE2*, and *TOE3* (Aukerman and Sakai, 2003; Chen, 2004; Wu *et al.*, 2009; Mathieu *et al.*, 2009). The overexpression of miR172 leads to a very early flowering phenotype as well as flower defects (Aukerman and Sakai, 2003). Interestingly, the sextuple *ap2-like* mutant phenocopies the *MIR172* overexpressor, suggesting that the function of miR172 is only mediated by regulation of the AP2-like transcription factors (Yant *et al.*, 2010). Conversely, the downregulation of miR172 activity by target mimicry resulted in a late-flowering phenotype, confirming its role in the control of flowering time (Todesco *et al.*, 2010). AP2-like transcription factors are directly involved in the control of flowering. Chromatin immunoprecipitation experiments have shown that SMZ and TOE1 proteins can bind the *FT* locus to repress its expression (Mathieu *et al.*, 2009; Zhang *et al.*, 2015) (**Figure 1-20**). Therefore, in the leaves, miR172 indirectly promotes the expression of *FT* via the downregulation of *AP2-like* genes.

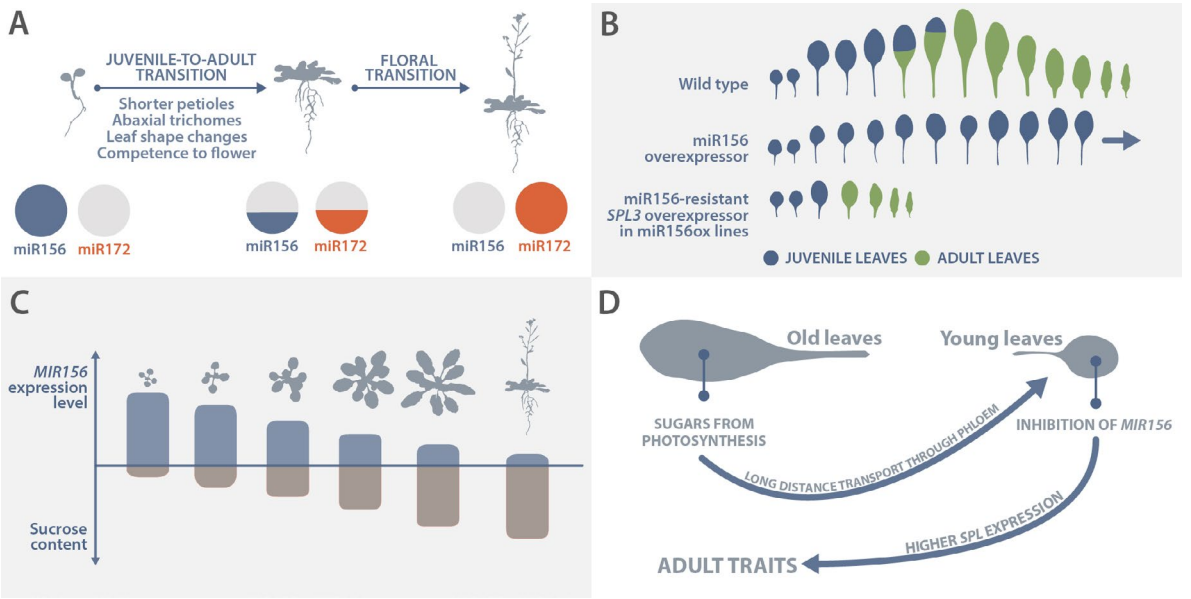

Figure 1-19. Involvement of microRNAs, *SPLs*, and sugars in plant development.

A. The development of Arabidopsis is characterized by a shift in the balance between miR156 and miR172; miR156 level decreases throughout plant development, whereas mir172 abundance increases. The transition from the juvenile to the adult phase is accompanied by several phenotypic changes, including the acquisition of the competence to flower. **B.** The miR156/*SPL3* module is involved in the switch from the juvenile to the adult phase. The transition is delayed in miR156-overexpressing lines and accelerated in plants overexpressing a miR156-resistant *SPL3* gene. Adapted from Wu and Poethig (2006). **C.** The sucrose content in leaves is inversely proportional to the expression level of *MIR156A* and *MIR156C* genes. **D.** Working model of the sugar-mediated juvenile-to-adult phase transition.

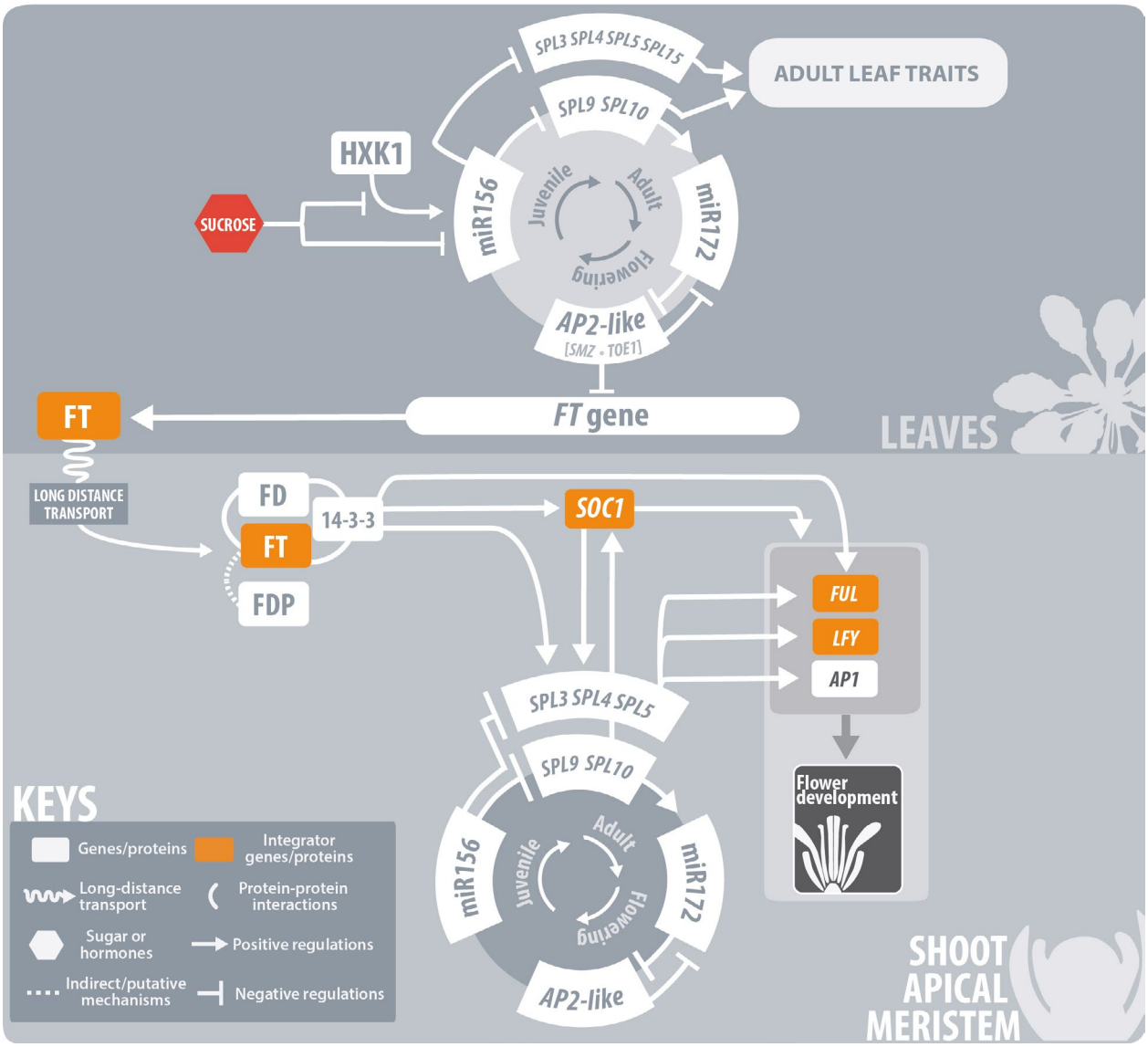

Figure 1-20. Overview of the aging pathway.

The aging pathway is mediated by a progressive modification of the balance between miR156 and miR172, which occurs both in the leaves [upper panel] and in the SAM [lower panel]. During plant development, sucrose represses miR156 in leaves, thus relieving the expression of *SPL*s. SPL proteins subsequently promote the transition from the juvenile to the adult phase and induce miR172 expression, which in turn inhibits the translation of the *AP2*-like *FT* repressors. *FT* is thus upregulated, and its protein moves to SAM to induce flowering. In the apex, SPLs can also bypass the FT signal to induce the expression of floral integrators such as *SOC1*, *LFY* and *FUL* in the absence of florigenic signals. Additional information is provided in the main text.

Wu and colleagues (2009) discovered the missing link between miR156 and miR172, as they showed that the miR156-targeted SPL9 and SPL10 can bind the promoter of *MIR172b* to induce its expression. This link provided a mechanistic connection between the different modules of the aging pathway: the progressive decrease in miR156 level indirectly triggers the concomitant increase in miR172 expression through SPL9/10. However, the nature of the signal responsible for the decline in miR156 level had been a long-standing question. The answer was recently found: as plants age, their photosynthetic area increases, thus boosting the production of photoassimilates. Hence, the sugar content of plants rises over time, whereas miR156 level decreases (**Figure 1-19C**). Sugars regulate miR156 abundance both at the transcriptional and the post-transcriptional level (Yang *et al.*, 2013; Yu *et al.*, 2013). Those results led to the elaboration of the model presented in **Figure 1-19D**: sugars (*i.e.* glucose, sucrose, and maltose) produced by photosynthetically-active mature leaves are transported to younger leaves, where they downregulate miR156. This decrease in miR156 leads to a concomitant increase of *SPLs* transcript levels, which consequently control the apparition of adult leaf traits and trigger the expression of *MIR172* in leaves.

Additionally, *SPLs* control flowering-related molecular mechanisms in the SAM (**Figure 1-20**). When expressed in the apex, *SPLs* induce the expression of several floral identity genes, including *SOC1*, *LFY*, *AP1*, and *FUL* (Yamaguchi *et al.*, 2009; Wang *et al.*, 2009). Therefore, the age-mediated pathway regulates flowering through two distinct outputs:

(i) The upregulation of miR172 allows the indirect activation of *FT* in leaves;

(ii) Increased SPL activity in the SAM triggers the expression of floral meristem identity genes.

Those processes control the floral transition of plants grown in non-inductive conditions. However, the miR156-*SPL*s module is also influenced by some external factors, such as ambient temperature (Kim *et al.*, 2012), CO_2 concentration (May *et al.*, 2013), gibberellins (Yu *et al.*, 2012; Yamaguchi *et al.*, 2014), and stress-induced transcription factors (Megraw *et al.*, 2006; Naqvi *et al.*, 2012). The aging pathway is, therefore, an endogenous program that acts as a hub for external cues that indirectly control flowering.

1.3 • OBJECTIVES OF THE THESIS

As extensively reviewed in the introduction, the control of flowering is very complex and involves hundreds of genes entangled in intricate regulatory networks. Those networks are sensitive to several environmental cues, so that flowering of Arabidopsis is induced in spring, when conditions are favorable for reproductive success. The signalling pathways converge onto a subset of key regulators of flowering, the so-called «flowering-time integrators» that control the switch of the shoot apical meristem from leaf initiation to flower production. Since environmental cues are perceived by different parts of the plant, long-distance signalling participates in the regulation of the integrator genes. Prominent among these signals is the FT protein, which is exported by the leaves during the photoperiodic induction of flowering. Whereas movement of FT and other phloem components towards the shoot apical meristem has been addressed in much detail, the roots remain largely ignored in the systemic view of flowering. However, several flowering-time genes were found to be expressed in root tissues and physiological experiments indicated that roots provide important flowering signals, possibly cytokinins (Bernier and Périlleux, 2005). The purpose of this thesis is to integrate roots in the landscape of flowering-time regulation in Arabidopsis. We will tackle this topic by addressing the following questions:

- Which flowering gene networks are expressed in the roots?
- What is the impact of root environment on flowering?
- What happens in the roots during the induction of flowering through the photoperiodic pathway?

Which flowering gene networks are expressed in the roots?

A prerequisite to answering this first question was to acquire a detailed knowledge on the genetic control of flowering. From the very beginning of my thesis, I've been confronted to the complexity of the literature, even if limited to the case of Arabidopsis. How could I have a correct overview of the current knowledge on this topic? Reviews, even if very informative, often focus on recently published data and, moreover, become rapidly outdated. Thus, I wondered if I could take advantage of the recent developments in online interactive tools to build a database that would gather the accumulating information and display it in a convenient way. This is the purpose of FLOR-ID (Bouché *et al.*, 2015), the online database presented in the Chapter 2 (page 77). I was then able to cross a list of flowering-time genes with

public databases containing lists of genes expressed in the roots in order to evaluate which flowering-time genes and networks are active in the roots (see below).

What is the impact of root environment on developmental phase transitions?

As described above, several flowering-time genes were known to be expressed in the roots, although their functional analyses remained focused on the shoot (Bernier and Périlleux, 2005). One reason is that most genetic studies of Arabidopsis are carried on with plants grown *in vitro* or on soil. In the first case, root growth is «biased» by the facts that the medium provides all kinds of nutrients including sugar and vitamins and that roots are illuminated (Silva-Navas *et al.*, 2015). In the second case, the roots of plants grown on soil are difficult to harvest, and nearly impossible to observe. Hydroponics is more and more used as an alternative since it provides an easy access to the roots and allows a precise control of the growing medium composition (Tocquin *et al.*, 2003). One cannot exclude however that the nature of the growing media affects plant development. It was found for example that plants of *Arabidopsis thaliana* grown in hydroponics were more sensitive to a flower-inducing treatment and at a younger age than plants grown on soil (Corbesier *et al.*, 1996; Tocquin *et al.*, 2003). We were therefore interested to compare these two growing media in terms of genetic regulation of plant development and expression of flowering-time genes in the roots. In order to estimate the variation of the root transcriptome caused by the root environment, we performed a global transcriptomic profiling of plants grown on both media. This analysis pointed out the differential expression of a flowering-time gene, *FLC*. In this part of the thesis (**Chapter 3, page 97**), we aimed at addressing the following questions:

- Is plant development altered by the growing medium?
- What are the root transcriptome differences between plants grown in hydroponics and on soil?

What happens in the roots during the induction of flowering?

Several pieces of evidence suggest a possible link between roots and the control of flowering time. First, the florigenic signals transported in the phloem, including the FT protein, reach the roots (Corbesier *et al.*, 2007). Second, physiological experiments conducted in a relative of Arabidopsis, white mustard *Sinapis alba*, showed that sucrose translocated by the phloem during an inductive photoperiodic treatment triggers the export of cytokinins from the roots, which is necessary for flowering (Lejeune *et al.*, 1994; 1988; Havelange *et al.*, 2000). An in-

crease in the cytokinin transport towards the shoot apical meristem is also observed in Arabidopsis during the induction of flowering by a single 22-hour long day (Corbesier *et al.*, 2003). In order to investigate in more detail the involvement of the roots in flowering, we performed transcriptomic analyses of the roots at two time points during the photoperiodic induction of flowering by a 22-hour long day (**Chapter 4, page 137**). The results lead to the selection of mutants that were characterized in order to identify new regulators of either flowering time and/or root architecture. Using an opposite and complementary approach, we performed a wide data mining analysis of the flowering-time genes expressed in roots. The purpose of these analyses was to answer the following questions:

- Are flowering-time genes expressed in roots?
- What are the root transcriptomic changes triggered by the induction of flowering?
- Are the differentially expressed genes involved in the control of flowering time and/or root architecture?

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