Genetic and molecular mechanisms controlling flowering time of *Arabidopsis thaliana* in response to diverse environmental cues

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Abstract

Abstract

As sessile organisms, plants have acquired the capacity to perceive and integrate a wide range of seasonal and environmental cues, and to respond to these signals by modulating their developmental programs. Seasonal signals detected by plants include changes in day-length (photoperiod) and ambient temperature. Plants also respond to changes in light quality, such as variations in red:far red (R:FR) light ratio characteristic of crowded canopies. Plants detect changes in photoperiod to induce seasonal patterns of flowering. The photoperiodic pathway accelerates flowering of *Arabidopsis thaliana* (*A. thaliana*) under long days (LDs), whereas it is inactive under short days (SDs) resulting in delayed flowering. This delay is overcome by exposure of plants to high temperature (27 °C) under SD (27°C-SD). Previously, high temperature flowering response was proposed to involve either the impaired activity of MADS-box transcription factor floral repressors or PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) transcription factor mediated activation of *FLOWERING LOCUS T* (*FT*), which encodes the output signal of the photoperiodic pathway. Here, these observations were integrated by studying several PIFs, the MADS-box transcription factor SHORT VEGETATIVE PHASE (SVP) and the photoperiodic pathway under 27°C-SD. The results presented show that the mRNAs of *FT* and its paralogue *TWIN SISTER OF FT* (*TSF*) are increased at dusk under 27°C-SD compared to 21°C-SD, and that this requires PIF4 and PIF5 as well as CONSTANS (CO), a transcription factor that promotes flowering under LDs. The CO and PIF4 proteins are present at dusk under 27°C-SD and they physically interact. Although Col-0 plants flower at similar times under 27°C-SD and 21°C-LD the expression level of *FT* is approximately 10-fold higher under 21°C-LD, suggesting that responsiveness to FT is also increased under 27°C-SD, perhaps due to reduced activity of SVP in the meristem. Accordingly, only *svp ft tsf* triple plant plants flowered at the same time under 21°C-

SD and 27°C-SD. Taken together, these results suggest that under non-inductive SDs, elevated temperatures increase the activity and sensitize the response to the photoperiod pathway.

In addition to their role in warm ambient temperature, PIF transcription factors are important components of light signalling pathways. In particular, PIFs participate in the developmental changes associated with the shade avoidance syndrome (SAS). Under R light conditions, the active form of Phytochrome (Phy) photoreceptors interact with PIFs targeting them for degradation. Under shade conditions, the R:FR ratio is reduced and Phys phototransform to the inactive form. This Phy form no longer interacts with PIFs allowing their accumulation and the direct activation of target genes involved in developmental processes. Plants lacking functional PhyB show a phenotype that resembles the SAS. One characteristic of the SAS is the induction of flowering. Here, the early-flowering phenotype of *phyB* mutants is shown to be suppressed by mutations in *PIF4* and *PIF5*. Furthermore, *phyB* mutants require functional CO and FT TSF to promote flowering. Experiments performed under simulated shade, however, demonstrated that *PIF4* and *PIF5* as well as *PIF1*, *PIF3* and *PIF7* do not play a role in the promotion of flowering by shade. In addition, these experiments reveal that photoperiod pathway components, such as *GI*, *CO*, *FT* and *TSF* are required for the induction of flowering under shade.

The study of diverse signals regulating flowering demonstrated a role of photoperiod pathway components not only in the modulation of flowering in response to seasonal changes in day length, but also in the adjustment of the timing of flowering mediated by temperature and light quality signals. Thus, this thesis places CO as a pivotal component in the crosstalk between distinct seasonal and environmental signals orchestrating the regulation of flowering.

Zusammenfassung

Als sessile Lebewesen haben Pflanzen die Fähigkeit erworben, eine Vielzahl von jahreszeit- und umweltabhängigen Signalen wahrzunehmen und ihr Entwicklungsprogram daran anzupassen. Jahreszeitlich schwankende Signale, die von Pflanzen wahrgenommen werden sind zum Beispiel Änderungen der Tageslänge (Photoperiode) und Umgebungstemperatur. Pflanzen reagieren außerdem auf Änderungen des Lichtspektrums, wie beispielsweise Veränderungen des Verhältnis von rotem zu fernrotem Licht (R:FR) wie man es unter einer Blaumkrone findet. Pflanzen erkennen Änderungen in der Photoperiode um die Blühinduktion saisonal zu regulieren. Der Photoperioden-Signalweg fördert die Blühinduktion in *Arabidopsis thaliana* (*A. thaliana*) im Langtag, wohingegen der Signalweg im Kurztag inaktiv ist und somit die Blühinduktion verspätet wird. Diese Verspätung der Blühinduktion wird umgangen, wenn die Pflanzen im Kurztag hohen Temperaturen (27 °C) ausgesetzt sind. Bisher wurde dieser Temperatureffekt auf die Blühinduktion auf die Beeinträchtigung der Aktivität von MADS-box Trankriptionsfaktoren, welche die Blühinduktion unterdrücken, oder auf die Aktivierung von *FLOWERING LOCUS T* (*FT*), dem Ausgangsfaktor des Photoperioden-Signalwegs, durch den PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) Transkriptionsfaktor zurückgeführt.

In dieser Arbeit wurden diese Beobachtungen zueinander in Beziehung gesetzt. Dafür wurden verschiedene PIFs, der MADS-box Transkriptionsfaktor SHORT VEGETATIVE PHASE (SVP) und der Photoperioden-Signalweg bei 27°C untersucht. Die vorgestellten Ergebnisse zeigen, dass die Transkripte von *FT* und seinem Paralog *TWIN SISTER OF FT* (*TSF*) am Abend bei 27°C im Vergleich zu 21°C erhöht sind. Dies benötigt PIF4, PIF5 und CONSTANS (CO), einen Transkriptionsfaktor, welcher die Blühinduktion im Langtag fördert. CO und PIF4 Proteine akkumulieren am Abend bei 27°C und binden aneinander. Obwohl

Col-0 Pflanzen bei 27°C im Kurztag zur gleichen Zeit blühen wie bei 21°C im Langtag, ist *FT* im Langtag circa 10 mal höher exprimiert, was darauf hindeutet, dass die Sensitivität gegenüber FT bei 27°C erhöht ist, möglicherweise durch eine verminderte Aktivität von SVP im Meristem. Dementsprechend blühten lediglich *svp ft tsf* Tripelmutanten zur selben Zeit bei 21°C im Kurztag und bei 27°C im Kurztag. Zusammenfassend suggerieren diese Ergebnisse, dass erhöhte Temperaturen im Kurztag die Aktivität und Sensitivität des Photoperioden-Signalweges erhöhen.

Neben ihrer Rolle unter erhöhten Umgebungstemperaturen sind PIF Transkriptionsfaktoren auch wichtige Komponenten von Lichtsignalwegen. Besonders wichtig sind PIFs für die Schattenvermeidungsantwort. In rotem Licht interagiert die aktive Form des Phytochrom (Phy) Lichtrezeptors mit den PIFs und markiert sie für die Degradierung. Im Schatten ist das R:FR Verhältnis vermindert und die Phys wechseln in ihre inaktive Form. Diese Form interagiert nicht mit den PIFs wodurch diese akkumulieren und Zielgene, welche in Entwicklungsprozesse involviert sind, aktivieren. Pflanzen, die keinen funktionalen PhyB Lichtrezeptor besitzen, haben einen ähnlichen Phänotyp, wie Pflanzen welche die Schattenvermeidungsantwort zeigen. Ein Aspekt dieses Phänotyps ist die Blühinduktion. Hier wird gezeigt, dass dieser Phänotyp von *phyB* Mutanten durch die Mutation von *PIF4* und *PIF5* unterdrückt wird. Desweiteren benötigen *phyB* Mutanten funktionales CO, sowie FT TSF um die Blüte zu induzieren. Experimente, welche unter simuliertem Schatten durchgeführt wurden zeigten jedoch, dass *PIF4* und *PIF5* sowie *PIF1*, *PIF3* und *PIF7* keine Rolle in der Blühinduktion im Schatten spielen. Außerdem zeigen diese Experimente, dass andere Komponenten des Photoperioden-Signalweges, wie *GI*, *CO*, *FT* und *TSF* für die Blühinduktion im Schatten benötigt werden.

Unsere Studie diverser Signale in der Regulation der Blühinduktion zeigte eine wichtige Rolle von Komponenten des Photoperioden-Signalwegs nicht nur in der Anpassung der

Blühantwort an jahreszeitliche Änderungen der Tageslänge sondern außerdem in der Anpassung des Blühzeitpunktes and an Änderungen der Umgebungstemperatur und des Lichtspektrums. Somit postuliert diese Arbeit eine entscheidende Rolle für CO in der Interaktion zwischen jahreszeit- und umweltabhängigen Signalen in der Blühinduktion.

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1. Introduction

1.1 Preamble

Plants are sessile organisms that must adapt to highly variable environments. This is achieved potentially through a high degree of developmental plasticity, so that they sense and predict changes in their environment and modify their developmental programs accordingly. These environmental responses are both trait and resource specific, and vary among genotypes, populations and species. The environmental changes to which plants are exposed through their life cycle are mainly seasonal and involve variations in factors such as day length, light quality and ambient temperature and are subjected to the latitude and altitude in which they grow.

One of the most plastic developmental adaptations to changing environment is the timing of flowering, which is the transition from vegetative to reproductive development. This developmental process contributes to the perpetuation of the species and is especially critical for monocarpic plants, as they flower only once in their life cycle (Amasino 2009). In an agricultural context, flowering time is crucial in determining the production of seeds and fruits in crop plants. Early flowering is of great value in some aspects of agriculture, as it allows shortening of seed production time in plants such as cereals or oil-seed crops and thereby allows the range of latitudes at which they can be productively grown to be extended. Delayed flowering can also enhance yield in forage plants like alfalfa and root or leaf crops like sugar beet and spinach (Jung and Muller 2009, Pin *et al.* 2010).

Flowering time is significantly affected by the environmental conditions, as for example ambient temperature. Thus, the global climate change is predicted to dramatically affect the World plant production. Understanding the molecular basis of flowering in response to seasonal and environmental changes provides the means to breed for flowering time,

increasing the potential for genetic improvement to boost the plant adaptation to global climate changes, and to meet the needs of a growing human population.

In natural environments, temperature varies tremendously among seasons and latitudes, having a remarkable effect on flowering time. The effect of temperature on flowering time is very well described for many plants species that require prolonged periods of cold (vernalization) in order to allow floral transition to occur (Amasino 2004, Kim and Sung 2014). Moreover, slight ambient temperature variations of around 5 °C have also dramatic effects in the timing of flowering (Verhage *et al.* 2014, Capovilla *et al.* 2015). Besides the effect in floral transition, ambient temperatures influence fertility and fruit ripening of essential species in agriculture, such as rice and tomato, respectively (Satake and Yoshida 1978, Adams *et al.* 2001). Records of over the last 150 years have detected yearly increments of the ambient temperatures (Ellwood *et al.* 2013). According to the Intergovernmental Panel on Climate Change (IPCC) fourth assessment report (Kumar 2007), global average surface temperature increased by 0.74 ± 0.18 °C in the last century and an increase of further 1.1 – 6.0 °C is projected to occur in this century. The impact of climate change on plant phenology has been described in recent decades, highlighting a hastening of flowering phenology in response to increasing winter and spring temperatures (Chmielewski and Rotzer 2001, Gordo and Sanz 2005, Menzel *et al.* 2006, Primack *et al.* 2009, Xiao *et al.* 2016). Elevated temperatures are known to affect the physiology of flowering plants in a number of ways, resulting in altered production of flowers, nectar, and pollen (Petanidou and Smets 1996, Saavedra *et al.* 2003, Koti *et al.* 2005). In addition to advancing many phenological events, climate warming is altering the distributions of both, plant and animal species (Hughes 2000). These alterations might compromise the interactions with herbivores, pollinators, and other ecological associates and lead to ecological mismatches (Parmesan 2006, Durant *et al.* 2007, Post and Forchhammer 2008, Both *et al.* 2009, Forrest and Thomson 2011).

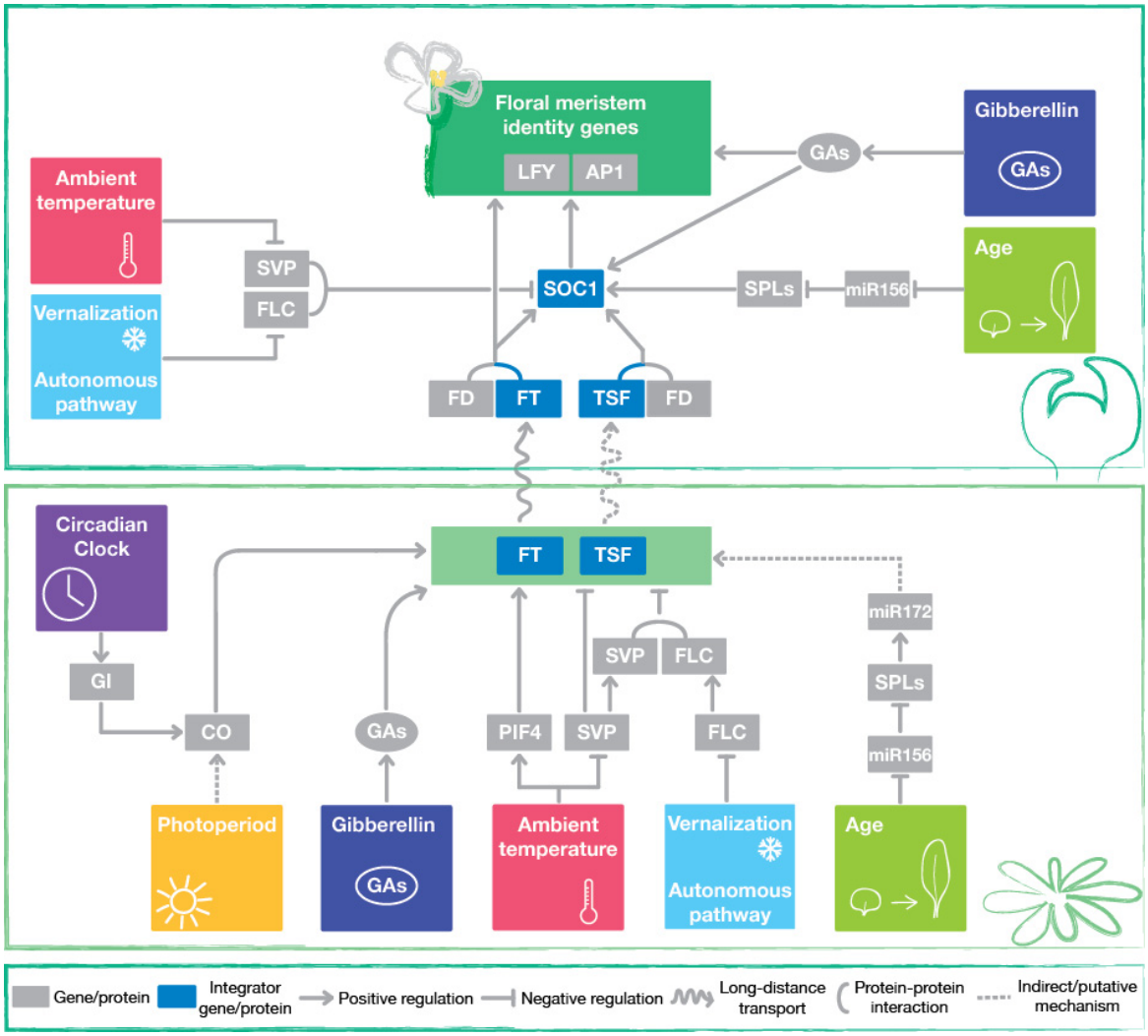

Figure 1.1. Control of flowering time in A. thaliana. Flowering time is regulated in *A. thaliana* by molecular mechanisms that operate in the leaf (bottom panel) and in the shoot apex (top panel). In the leaf, the photoperiodic pathway is articulated by the circadian clock and the light signalling, mainly affecting the activity of GI and CO. Under LDs, CO is activated by light at the end of the day, when it directly induces the expression of *FT* by binding on its promoter. This regulation occurs in the companion cells where *FT* is also transcriptionally activated by the plant hormone GA (GA pathway). The ambient temperature pathway is mainly controlled by the opposite function of the transcription factors PIF4 and SVP. PIF4 binds the promoter of *FT* and induces its expression at warm temperatures (at least under SDs). On the other hand, warm temperatures inhibit the floral repressor activity of SVP allowing *FT* mRNA to be induced. Long exposure to cold treatments (vernalization pathway) and the autonomous pathway contributes to the transcriptional silencing of *FLC*, which subsequently releases its repression on *FT*. SVP and FLC can act as a complex to inhibit the expression of *FT* in the leaf (bottom panel) and *SOC1* in the SAM (top panel). In the age pathway, a gradual reduction on the miR156 levels leads to the upregulation of the *SPLs* and the miR172. Increased levels of miR172 trigger the down-regulation of the *AP2*-like

genes expression (not shown) and the induction of *FT* mRNA. FT protein is transported to the shoot apex (top panel) where it interacts with bZIP transcription factors (e.g. FD) to activate the expression of the floral integrator SOC1 and the floral meristem identity genes *LFY* and *AP1*. These genes are also transcriptionally activated by the GA and the age pathway in the SAM. The transcriptional activation of *LFY* and *AP1* defines the initiation of a series of genetic changes that ultimately lead to the floral transition. Adapted from Bouché *et al.* (2015).

Variations in day-length (usually termed “photoperiod”) due to seasonal changes, especially in temperate and cool-temperate zones, can be perceived by plants to trigger developmental responses. In the North hemisphere, day-length is shortened during winter and prolonged in summer. These photoperiod fluctuation enables plants to flower under favourable day-length conditions whereas it represses flowering under less favourable day-lengths (Garner and Allard 1920). Plants that flower when day-length becomes longer than a certain critical length (usually in summer), are termed “long-day plants”. This group of plants includes the model plant species *Arabidopsis thaliana* (*A. thaliana*) and some crop plants such as wheat, barley and sugar beet. By contrast, “short-day plants” flower when the day is shorter than a critical length (usually in autumn) and these plants include rice and maize. Plants that flower independently of photoperiod are day-neutral plants, many cultivars of tomato and potato are examples of this group of plants (Garner and Allard 1920, Andres and Coupland 2012, Nakamichi 2015). Photoperiodism plays and important role in the natural distribution of the plant species. In general, short-day plants have been originated at low latitudes where temperatures are usually warm, while long-day plants are usually from high and relatively cold latitudes (Rayner 1969). Therefore, the local adaptation of plants to different environments has influenced their developmental behaviour and gave rise to distinct patterns of flowering response to photoperiod and temperature (Izawa 2007, Nakamichi 2015).

In addition to photoperiod and temperature, flowering time is also influenced by the microenvironment where pants grow. In natural and agricultural ecosystems, plants are

exposed to the influence of neighbouring plants. In highly dense vegetation, light intensity perceived by an individual is reduced and its quality affected as a result of absorption and reflection of determined wavelengths by other plants in the vicinity. In crowded environments, light available for photosynthesis is limited, causing important losses in crop production. In order to avoid shading from other plants, individuals growing below a canopy activate specific developmental programs aiming to avoid the degree not only of current but also of future shade by overtopping the competitors or inducing flowering to ensure seed production. These developmental changes, initiated by variations in the proportion of the far-red (FR) light perceived, are collectively called shade avoidance syndrome (SAS) (Casal 2012, Martinez-Garcia *et al.* 2014).

Solar irradiance, temperature and photoperiod changes influence many aspects of plants development causing a wide phenotypic variability across latitudes (Stinchcombe *et al.* 2004, Hopkins *et al.* 2008). Flowering time variation in response to these environmental factors is the result of the activity of different genetic networks controlling flowering time in plants (Figure 1.1). These genetic pathways have been widely studied in *A. thaliana* (Andres and Coupland 2012). Even though mostly of the previous studies have focused on the regulation of flowering time by a single environmental factor (i.e. light and temperature), a strong interaction between genetic pathways related to distinct factors is predicted to control plant development in natural environments.

1.2 *Arabidopsis thaliana* as a model plant

Most of the current knowledge on plant flowering and development originated from studies in the model plant *A. thaliana*. This species has been used as a model since the early 1900s, but it became widely used by the scientific community in the 1980s and 1990s (Meyerowitz

1989). *A thaliana* is a particularly suitable model for genetic, cellular and molecular biology for several reasons. It has a small, diploid genome, of approximately 135 megabase pairs (Mbp), which was fully sequenced in the year 2000 (Arabidopsis Genome 2000), and is one of the smallest among flowering plants. It is a small plant, which makes it easy to cultivate in reduced spaces, and has a rapid lifecycle, taking about six weeks from germination to mature seeds, which accelerates genetic approaches. It is easy to make transgenic lines by "floral-dip", a technique that involves dipping of the flowers in a solution of *Agrobacterium tumefaciens* (*A. tumefaciens*) (Clough and Bent 1998). This method contributed to elucidate the function of many genes in *A. thaliana*. Indeed, it facilitated the rapid generation of a vast collection of *A. thaliana* T-DNA insertion alleles that has extensively been used for reverse genetic studies (Alonso *et al.* 2003). Recently, these methods have been supplemented by new generation genome editing techniques, and especially those based on the CRISPR/Cas9 system (Jinek *et al.* 2012) have shown an extraordinary proliferation during the last years (Feng *et al.* 2013, Feng *et al.* 2014, Hyun *et al.* 2015). Moreover, a powerful Genome Wide Association (GWAS) platform is currently being generated and constantly improved by the 1001 Genomes Consortium. In this consortium, a database containing the genome sequence of over 1,000 *A. thaliana* accessions from a worldwide hierarchical collection is being created with the main motivation to provide insights into the genetic basis of natural variation (Genomes Consortium. Electronic address and Genomes 2016).

Studies in *A. thaliana* have enabled many fundamental and technical advances in plant sciences (Provart *et al.* 2016). Also in areas such as flowering control that are important in crops, many efforts have also been made to transfer the basic knowledge acquired in the model plant to economically important crop species, where the study of this trait is frequently tedious. Partly thanks to the high degree of conservation between flowering plants in distinct plant species, the knowledge acquired in *A. thaliana* has been of great value to reveal the

mechanism controlling flowering time in herbaceous species such as rice, wheat, barley, maize, tomato and sugar beet as well as trees, such as, poplar, apple and citrus (Jung and Muller 2009, Andres and Coupland 2012, van Nocker and Gardiner 2014, Song *et al.* 2015). Thus, the use of *A. thaliana* in basic research not only has generated a broad knowledge about how flowering time is controlled in plants, but also has provided the means to better understand this process in non-model systems.

1.3 Genetic bases of flowering time regulation in *A. thaliana*: a brief historical perspective

Classic genetic studies performed in *A. thaliana* have resulted in the identification of several mutants displaying altered flowering time behaviour. These mutants were classified into distinct genetic groups depending on their response to light (photoperiod and quality), temperature and plat hormone treatments (Figure 1.1). In terms of photoperiod, flowering is promoted in *A. thaliana* under long days (LDs) of spring or summer whereas under short days (SDs) of autumn and winter it is repressed. Mutants impaired in the photoperiodic flowering response were first reported in 1962 (Redei 1962). Among them, the causal genes of three mutants showing late flowering under LDs were mapped at in the *CONSTANS* (*CO*) and *GIGANTEA* (*GI*) loci (Redei 1962). In 1991, these two loci were classified into the same epistatic group known as the photoperiodic flowering pathway, together with *FLOWERING LOCUS T* (*FT*) (Koornneef *et al.* 1991). In this pioneer large-scale genetic screen, Koornneef and collaborators (Koornneef *et al.* 1991) analysed over forty independently induced *A. thaliana* mutants showing late flowering under LDs. These mutants were found to represent mutations at eleven loci, which based on recombination of mutant at different loci and the differential response to environmental factors, were placed in three different epistatic groups

i.e. photoperiodic, vernalization and autonomous pathway (Koornneef *et al.* 1991). Vernalization and autonomous mutants were isolated because of their differential response to vernalization treatment (Martinez-Zapater and Somerville 1990, Koornneef *et al.* 1991, Chandler and Dean 1994). Mutants in the autonomous pathway, including *luminidependens* (*ld*), *fca*, *fve*, *fy* and *fpa*, flowered later than wild-type (WT) controls irrespective of the day-length conditions (Martinez-Zapater and Somerville 1990, Koornneef *et al.* 1991). Characteristically, these mutants exhibited a very marked suppression of late flowering when subjected to vernalization (Martinez-Zapater and Somerville 1990, Koornneef *et al.* 1991). Genes involved in the vernalization pathway were first genetically identified by Napp-Zinn at the University of Cologne (Napp-Zinn 1957, Koornneef 2013). Later genetic analyses demonstrated that this pathway is mainly controlled by the interaction between the two major loci *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) (Napp-Zinn 1979, Clarke and Dean 1994, Koornneef *et al.* 1994). Importantly, allelic variation of these two genes was found to explain a large part of the natural variation in flowering time encounter in *A. thaliana* ecotypes (Clarke and Dean 1994, Levy and Dean 1998).

Floral transition in *A. thaliana* is also regulated by endogenous factors including levels of the phytohormones gibberellins (GAs). Early studies showed that *A. thaliana* plants grown under SDs accelerate flowering upon GA treatment (Zeevaart 1983). Several other studies demonstrated that mutants affected in GA biosynthesis (eg. *gai1-3*) flower late under SDs, suggesting that GA levels are essential for flowering under non-inductive photoperiodic conditions (Wilson *et al.* 1992).

Early genetic studies reported the identification of dozens of loci involved in flowering time. Physiological and epistatic analysis revealed the existence of at least three distinct flowering time pathways: photoperiod, vernalization-autonomous and gibberellin pathways (Koornneef *et al.* 1991, Reeves and Coupland 2001). More recently, genetic and molecular analyses of

mutants in response to ambient temperature (15°C - 27°C) suggested the existence of a thermosensory pathway, which in part is controlled by the autonomous pathway-related genes *FCA* and *FVE* (Blazquez et al. 2003). This information was complemented by molecular studies that pointed to the transcription factors *SHORT VEGETATIVE PHASE* (*SVP*) and *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) as main modulators of flowering time in response to warm temperatures (Lee *et al.* 2007, Kumar *et al.* 2012, Lee *et al.* 2013, Pose *et al.* 2013, Verhage *et al.* 2014, Capovilla *et al.* 2015). In addition, the existence of a pathway that ensures plants eventually flower under non-inductive conditions was recently identified (Schwab *et al.* 2005, Wu and Poethig 2006, Schwarz *et al.* 2008, Wang *et al.* 2009, Jung *et al.* 2011, Yamaguchi and Abe 2012, Zhou and Wang 2013). This pathway, named the "age" pathway, relies on the developmental decline of the micro-RNA156 (miR156) and the control of its targets *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) transcription factors, which mainly function as floral activators (Wu and Poethig 2006, Wang *et al.* 2009, Yamaguchi *et al.* 2009, Wang 2014).

Thus, during the last decades, knowledge in plant development has increased tremendously thanks to the constant advances in new technologies and the concerted effort of plant scientist from different laboratories around the world. In particular, pathways governing flowering time regulation have been described in detail, although how they interact to modulate plant development in response to environmental changes still remain poorly explored.

1.4 Regulation of flowering time in response to day-length

Day length is one of the most widely used cues that regulate flowering time (Garner and Allard 1920). It provides precise seasonal information allowing plants to anticipate changes

in their environment and to distinguish between LDs of spring or summer and SDs of autumn or winter. *A. thaliana* flowers rapidly under LDs, when the ambient conditions are suitable for seed production, and late under SDs, when conditions are hostile for growth (Jung and Muller 2009). Differences in day length are detected in the leaves and its effect is mediated by several proteins that together form the photoperiodic flowering pathway (Figure 1.2). At the core of this pathway is the transcription factor encoded by *CO* (Putterill *et al.* 1995). *CO* encodes for a 373 amino acids-long protein consisting of two zinc binding B-Box motifs and a CO, CO-like, TIMING OF CAB2 EXPRESSION 1 (TOC1) (CCT) domain, which contains nuclear localization signals and a DNA-binding motif (Putterill *et al.* 1995, Robson *et al.* 2001, Tiwari *et al.* 2010). Mutations in either the B-Box or CCT domains were shown to impair CO protein function, as these domains are essential for interaction with other proteins and binding to DNA (Robson *et al.* 2001). *CO* mRNA is expressed in the phloem companion cells of the leaves where it activates the transcription of *FT* and its closest homolog *TWIN SISTER OF FT* (*TSF*), two positive regulators of flowering (Putterill *et al.* 1995, Suarez-Lopez *et al.* 2001, An *et al.* 2004, Yamaguchi *et al.* 2005). *FT* encodes for a small protein of 20 kDa that shares homology with animal phosphatidylethanolamine-binding proteins, also called RAF kinase inhibitor proteins (Kardailsky *et al.* 1999, Kobayashi *et al.* 1999). In plants FT belongs to the CETS protein family, which is named after the three founding members CENTRORADIALIS (CEN), TERMINAL FLOWER 1 (TFL1) and FT (Pnueli *et al.* 2001). Once *FT* mRNA is expressed in the leaves, its small protein product moves though the phloem to the shoot apex, where it causes changes in genes expression that reprogram the shoot apical meristem (SAM) to form flowers instead of leaves (Corbesier *et al.* 2007, Jaeger and Wigge 2007, Mathieu *et al.* 2007, Tamaki *et al.* 2007).

Thus, CO and FT are two positive regulators of flowering time in *A. thaliana* that have been place at the core of the photoperiodic pathway. In this pathway, they act subsequently to

decode and transmit seasonal signals that reprogram the SAM in order to determine precise flowering time. The proper coordination of this process is ensured in part through the transcriptional and post-translational regulation of CO (Suarez-Lopez *et al.* 2001, Valverde *et al.* 2004, Wigge *et al.* 2005, Corbesier *et al.* 2007, Torti *et al.* 2012).

1.4.1 Transcriptional regulation of *CO*

CO is transcriptionally regulated and this regulation is directed by the circadian clock (Suarez-Lopez *et al.* 2001), the endogenous timer with a cycle time of approximately 24 h (Dunlap 1998, McClung 2006, de Montaigu *et al.* 2010). *A. thaliana* as well as other species such as wheat, barley and rice, measure the differences in photoperiod by mechanisms that involve the interactions between circadian clock and light signalling (Fjellheim *et al.* 2014). These mechanisms have been explained by two models. The external coincidence model explains that photoperiodic responses are triggered when a circadian clock-regulated gene is expressed at certain level in the precise time window that favours the activation of its protein product by light (Pittendrigh 1964, Song *et al.* 2010, Andres and Coupland 2012, Song *et al.* 2015). The internal coincidence model also explains the mechanism underlying photoperiodism. In this model, induction of a photoperiodic response occurs only when two (or more) regulators, which have differently entrained expression rhythms depending on day length, show the same phase (Pittendrigh 1960, Pittendrigh 1966, Sawa *et al.* 2008). Under constant conditions, *CO* mRNA levels oscillate with a period of 24 h (Suarez-Lopez *et al.* 2001), but show different expression patterns under LDs and SDs. Under SDs *CO* mRNA accumulates only during the night. Under LDs, an additional peak of *CO* mRNA is detected during the day, around 12 – 16 h after dawn, when plants are still exposed to light (Figure 1.2) (Suarez-Lopez *et al.* 2001). The expression of *CO* mRNA at this time of the day is

essential to ensure the light-activation of CO protein and the promotion of flowering under LDs (see below) (Valverde *et al.* 2004). This mechanism of photoperiodic control of flowering is well explained by the model of external coincidence (Imaizumi *et al.* 2003, Sawa *et al.* 2007).

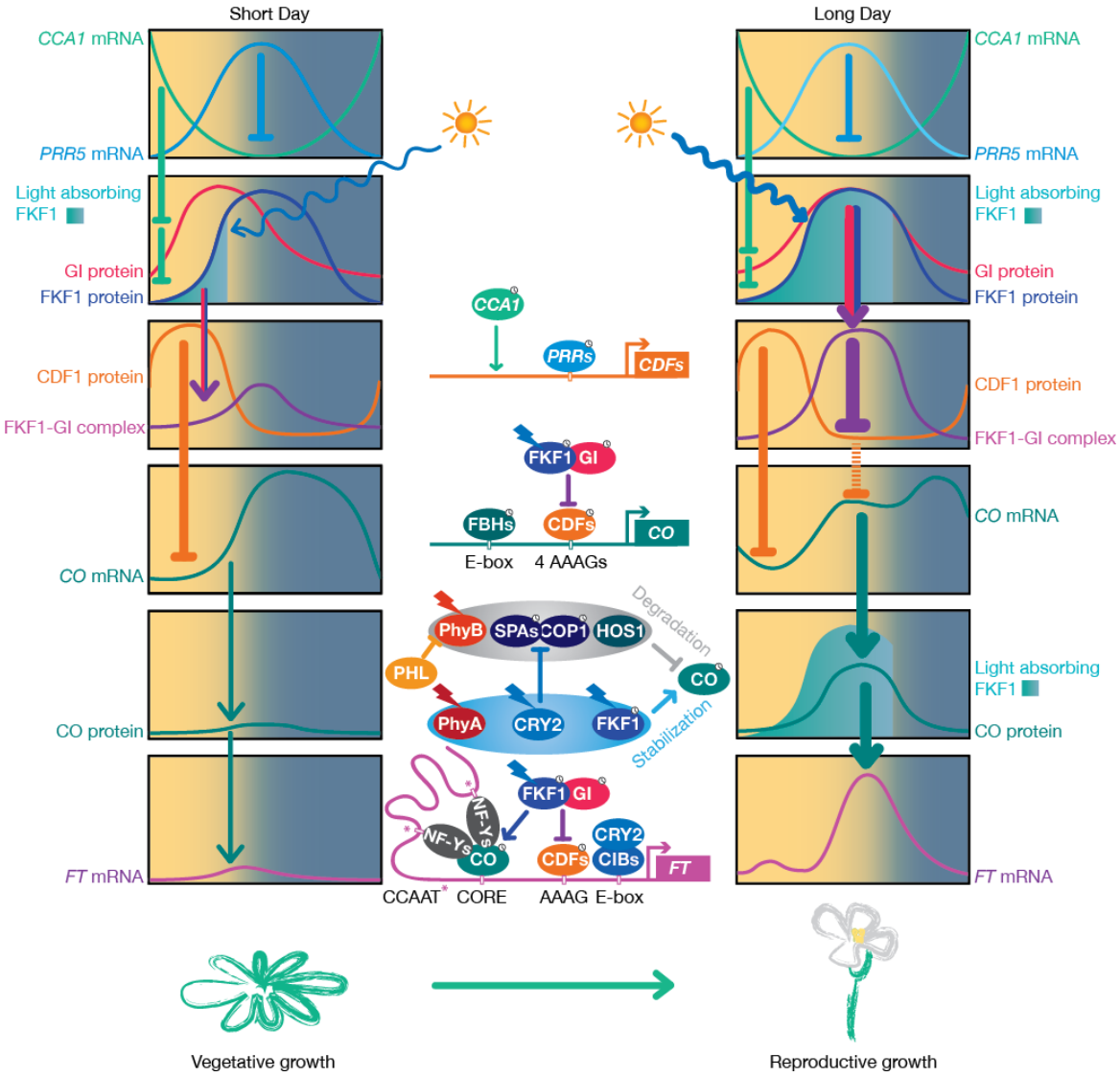

Figure1.2. Photoperiodic regulation of flowering in *A. thaliana*. *CCA1* and *PRR5* mRNA abundance oscillate throughout the day, both under LDs and SDs. *CCA1* mRNA is high in the early morning (top panel). Its protein product and LHY, its homolog, bind to promoters of *PPR5*, *GI* and *FKF1* to repress their expression during the morning. PRR5 protein regulates the transcription of *CCA1* to constitute a feedback loop between morning and evening clock components. *CDF1* expression is induced in the morning by CCA1 and LHY. In the afternoon, *CDF*

transcription is repressed by PRRs. Under LDs the protein expression profiles of GI and FKF1 synchronize in the afternoon (right panel). Blue light-mediated activation of FKF1 enables the formation of the FKF1-GI ubiquitin ligase complex. This complex recognizes and mediates the degradation of CDFs proteins. This degradation alleviates the repression of *CO* transcription by CDFs. Once this repression is relieved, it allows the binding of FBH proteins to the *CO* promoter and the activation of its transcription. In contrast, under SD conditions the FKF1 and GI protein expression are out-of-phase and the formation of the FKF1-GI complex is significantly reduced (left panel). Under these conditions, *CO* mRNA remains low during the day and only peaks at night. CO protein is accumulated only in the late afternoon and its stability is regulated by several factors (central cartoon). COP1-SPA ubiquitin ligase and HOS1 directly bind to and degrade the protein. PhyB is also involved in the degradation of CO. The function of PhyB in this regulation is counteracted by PHL, which interacts with both CO and PhyB under red light. During the night under both LDs and SDs CO is actively degraded by the COP1-SPA complex. By contrast, the photoreceptors PhyA, CRY2 and FKF1 stabilize CO during the day. Blue light-stimulated CRY2 interacts with COP1 and SPAs leading to the accumulation of CO protein by suppression of the COP1-SPA complex activity. Blue-light activated FKF1 interacts with CO and promotes the stabilization of the protein in the late afternoon under LDs. *FT* transcription is regulated by several factors throughout the day. In the morning *FT* transcription is directly repressed by CDFs. CO directly regulated *FT* transcription at dusk by binding to the CORE element in its promoter as well as by interactions with other *FT* regulators, such as NF-Y. NF-Ys bind to CCAAT-box regions located approximately at 2 kb and 5.3 kb of the transcription start site (TSS) of the *FT* gene. These interactions promote the formation of a DNA loop whose timing shows diurnal oscillations under LDs. CIB proteins, which interact with CRY2 under blue light, also bind to E-box located near the TSS in the *FT* promoter, participating in the regulation of *FT* transcription. Thus, the strong activation of *FT* transcription at dusk under LDs promotes de transition from vegetative to reproductive development. Arrows and block lines denote activation and repression respectively. Clock symbol indicates circadian clock regulation. Blue, red and dark red flashes denote blue, red and far red light. Adapted from Song *et al.* (2015).

The restriction of CO activity to the late afternoon under LDs to ensure proper *FT* induction is achieved by both circadian clock regulation of *CO* transcription and photoreceptor regulation of CO protein abundance (Figure 1.2). The transcriptional pattern of *CO* is conferred by genes that act upstream in the photoperiodic pathway and include *CYCLING DOF FACTORs* (*CDFs*), *GI*, *FLAVIN-BINDING, KELCH REPEAT, F-BOX 1* (*FKF1*) and its homologs *ZEITLUPE* (*ZTL*) and *LOV KELCH PROTEIN 2* (*LKP2*). The transcriptional patterns of several of these genes are also regulated by the circadian clock (Fowler *et al.* 1999, Park *et al.* 1999, Imaizumi *et al.* 2003, Imaizumi *et al.* 2005, Fornara *et al.* 2009, Baudry

et al. 2010). *GI* mRNA encodes for a plant specific protein of unknown function that accumulates during the day under LDs with highest abundance 12 h after dawn (David *et al.* 2006). GI activates *CO* transcription by interacting with and stabilizing the F-box ubiquitin ligase FKF1 (Imaizumi *et al.* 2005). FKF1 protein has a similar temporal expression pattern to GI, peaking 12 h after dawn under LDs. The interaction between GI and FKF1 only occurs in the presence of blue (B) light, when FKF1 protein is activated. Thus, the GI-FKF1 complex formation is maximized by the internal coincidence of their protein peaks under LDs and the activation of FKF1 by B light (external coincidence). Once activated, together with GI, FKF1 recognizes CDF protein family members and mediates their ubiquitin-dependent degradation. This degradation releases the repression of the *CO* promoter by CDF transcription factors (Imaizumi *et al.* 2005, Sawa *et al.* 2007), leading to the upregulation of *CO* mRNA around 12 h after dawn only under LDs (Suarez-Lopez *et al.* 2001, Song *et al.* 2015). ZTL and LKP2, which are closely related proteins to FKF1, also interact with GI and contribute to the removal of *CO* transcriptional repression (Kim *et al.* 2007, Fornara *et al.* 2009). Thus, the transcriptional repression of *CO* occurs in the morning when CDFs are expressed, but is relieved by the degradation of CDFs towards the late afternoon under LDs, when plants are exposed to light. The small ubiquitin-related modifier (SUMO)–targeted ubiquitin ligase (STUbL) also plays a role in CDF degradation by targeting SUMOylated CDF for degradation (Budhiraja *et al.* 2009, Elrouby *et al.* 2013). Under SDs, *FKF1* is mainly expressed at night and GI and FKF1 proteins are out of phase. Therefore the formation of the GI-FKF1 complex is reduced, disfavouring the degradation of CDFs and de-repression of *CO* mRNA (Sawa *et al.* 2007). As a consequence, *CO* mRNA expression remains low during the day and increases during the night under SDs. Another group of transcription factors belonging to the basic helix-loop-helix (bHLH) family called FLOWERING BHLH (FBH) and composed by four members (FBH1-4) were recently reported to regulate the amplitude of

daily *CO* mRNA oscillation (Ito *et al.* 2012). Hence, the daily pattern of *CO* mRNA is tightly regulated by several transcription factors that are mostly under control of the circadian clock. In this context, the modulation of the *CO* expression by the FKF1-GI complex-mediated control of CDFs transcription factors is another example of a regulatory mechanism nicely explained by the external and internal coincidence models.

1.4.2 Post translational regulation of CO

CO protein is subjected to posttranslational regulation and interacts with many proteins during a period of 24 h which shape the daily pattern of CO accumulation and ultimately define the outcome of the photoperiodic regulation of flowering (Figure 1.2). During the night, *CO* mRNA expression is high under LDs and SDs, however CO protein accumulation is prevented at this time by the action of a complex containing CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and SUPPRESSOR OF PHYTOCHROME A (SPA) (Hoecker *et al.* 1999, Hoecker and Quail 2001, Valverde *et al.* 2004, Laubinger *et al.* 2006, Jang *et al.* 2008, Liu *et al.* 2008b, Zuo *et al.* 2011). *COP1* is a single-copy gene, while SPA proteins are encoded by a family of four partially redundant genes (*SPA1–SPA4*) (Laubinger *et al.* 2004, Laubinger *et al.* 2006). The COP1/SPA complex is a Cullin4-based E3 ubiquitin ligase that catalyses the ubiquitination of CO protein and facilitates its degradation by the 26S proteasome (Laubinger *et al.* 2006, Jang *et al.* 2008, Liu *et al.* 2008b). Additionally, CO protein is subjected to phosphorylation and concomitant degradation, preferentially during the dark, in a COP1-dependent manner (Sarid-Krebs *et al.* 2015). This dark-dependent degradation of CO is particularly important for preventing flowering under SDs.

Exposure to light inactivates the COP1/SPA complex (Balcerowicz *et al.* 2011), thus only the peak of *CO* mRNA that occurs in the light at the end of a LD leads to CO protein accumulation

(Valverde *et al.* 2004). The stabilization of CO protein at the end of the LD relies on the direct interaction of COP1 and SPA with CRYPTOCHROME 2 (CRY2), a B light photoreceptor that inactivates the COP1/SPA complex (Liu *et al.* 2008b, Lian *et al.* 2011, Liu *et al.* 2011, Zuo *et al.* 2011). This complex is also inhibited by activated PHYTOCHROME A (PhyA), a red (R)/FR light photoreceptor (Sheerin *et al.* 2015). The exclusive stability of CO protein during the late afternoon under LDs cannot be explained only by the effect of CRY2 and PhyA as they are expressed throughout the day. In presence of B light, the FKF1 protein, which has a diurnal rhythm of abundance in LDs similar to that of CO, interacts with CO protein through its LOV (light, oxygen, or voltage) domain increasing its stability in the long-day afternoon (Imaizumi *et al.* 2003, Valverde *et al.* 2004, Song *et al.* 2012b).

The E3 ubiquitin ligase, HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1) and PhyB, a R/FR light photoreceptor, promote the COP1-independent CO protein degradation early in the morning (Valverde *et al.* 2004, Lazaro *et al.* 2012). HOS1 interacts directly with CO and contributes to the control of CO protein abundance, promoting the degradation of CO in a proteasome-dependent manner (Lazaro *et al.* 2012). The molecular mechanism by which PhyB destabilizes CO protein remains unclear. Recent evidence show that PhyB-dependent degradation of CO is counteracted in the afternoon by direct interaction of PHYTOCHROME-DEPENDENT LATE-FLOWERING (PHL) with both PhyB and CO, presumably contributing to the accumulation of CO protein at this time of the day (Endo *et al.* 2013).

In summary, two layers of regulation are acting simultaneously in the leaves in order to modulate the abundance and activity of CO protein. As a result of this complex transcriptional and posttranslational regulation, CO protein accumulates only under LDs, activating *FT* mRNA expression in the leaves (Figure 1.2) (Suarez-Lopez *et al.* 2001, Valverde *et al.* 2004, Song *et al.* 2015). Subsequently, FT protein acts as a signalling molecule that is transported

through the phloem to the SAM where it activates flowering (Figure 1.1) (Corbesier *et al.* 2007, Jaeger and Wigge 2007, Mathieu *et al.* 2007, Tamaki *et al.* 2007).

1.4.3 Photoperiodic regulation of flowering induction in the SAM

Under LD conditions, when CO protein is stabilized by light, it directly binds to the CO-responsive element (CORE) in the *FT* promoter through its CCT domain and activates its transcription (Kobayashi *et al.* 1999, Samach *et al.* 2000, Wigge *et al.* 2005, Tiwari *et al.* 2010, Song *et al.* 2012b, Zhang *et al.* 2015). The recruitment of CO to CORE elements proximal to the transcriptional start site of the *FT* promoter is assisted by NUCLEAR FACTOR Y (NF-Y) complexes which bind to CCAAT box in the distal enhancer element of the promoter (Figure 1.2) (Wenkel *et al.* 2006, Adrian *et al.* 2010, Cao *et al.* 2014). The transcriptional activation of *FT* in the phloem companion cells of leaves is followed by transport of its protein to the shoot apex (Yamaguchi *et al.* 2005, Corbesier *et al.* 2007, Jaeger and Wigge 2007, Mathieu *et al.* 2007). Upon arrival of FT protein to the meristem, it is believed to interact with 14-3-3 proteins and the bZIP transcription factors FD and FD PARALOG (FDP) (Abe *et al.* 2005, Wigge *et al.* 2005, Taoka *et al.* 2011) causing transcriptional reprogramming of the shoot meristem and activation of downstream genes such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *FRUITFULL* (*FUL*), *APETALA 1* (*AP1*) and *LEAFY* (*LFY*) (Figure 1.1) (Schmid *et al.* 2003b, Abe *et al.* 2005, Wigge *et al.* 2005, Yoo *et al.* 2005, Searle *et al.* 2006, Torti *et al.* 2012). Activation of these genes leads to the formation of an inflorescence meristem and to the production of flowers. Thus, a suggested role for FT is to form part of molecular complexes that activate floral promoter genes in the SAM (Wigge *et al.* 2005, Andres and Coupland 2012). Additionally, the function of FT has been related to lipids. FT specifically binds *in vitro* to phosphatidylcholine (PC), a phospholipid that promotes

flowering when it is highly accumulated in the SAM (Nakamura *et al.* 2014). Therefore, the binding of FT to PC, and in particular to PC species that are more abundant during the day, might constitute a novel molecular mechanism of flowering control in the SAM mediated by the FT-signalling pathway (Nakamura *et al.* 2014, Romera-Branchat *et al.* 2014).

SOC1 encodes for a MADS-box transcription factor expressed early during floral induction in the shoot apical meristem (Borner *et al.* 2000, Lee *et al.* 2000, Samach *et al.* 2000). Loss of function mutants of *SOC1* strongly delay flowering under LDs and SDs, indicating that *SOC1* is not only involved in the regulation of flowering mediated by photoperiod, but it also participates in other genetic pathways (Borner *et al.* 2000, Lee *et al.* 2000, Samach *et al.* 2000, Lee and Lee 2010). *FUL* encodes for another MADS-box transcription factor whose transcriptional activation also depends on FT and is genetically redundant with *SOC1* (Schmid *et al.* 2003b, Teper-Bamnolker and Samach 2005). Loss of function mutations of *FUL* slightly delay flowering under LDs, however, when combined with *soc1* mutation, these plants flower later than each single mutant and present a strongly attenuated response to *FT* overexpression (Ferrandiz *et al.* 2000, Melzer *et al.* 2008, Torti *et al.* 2012). The late flowering phenotype observed in the *soc1 ful* double mutant demonstrates that these two genes are essential components of the photoperiodic pathway. Once expressed in the meristem, SOC1 forms heterodimers with AGAMOUS-LIKE 24 (AGL24), another MADS-Box transcription factor and floral regulator, and promotes the transcriptional activation of *LFY* (Michaels *et al.* 2003, Lee *et al.* 2008). LFY, together with AP1, another MADS-Box transcription factor, are the main meristem identity regulators and link floral induction with floral development (Yu *et al.* 2002, Michaels *et al.* 2003, Lee *et al.* 2008, Siriwardana and Lamb 2012). Thus, this photoperiod activation of *SOC1* and *FUL* transcription in the SAM is one of the earliest steps of a series of signalling cascades that trigger the flowering initiation in *A. thaliana*.

The LD-promotion of flowering is also modulated by a set of floral repressors that regulate the transcription of floral integrators in the leaf and in the SAM. Among them, the two MADS-Box transcription factors encoded by *SVP* and *FLOWERING LOCUS C* (*FLC*) play a crucial role in this process (Michaels and Amasino 1999, Sheldon *et al.* 1999, Hartmann *et al.* 2000). Genetic and genome-wide analyses of these two genes revealed that they control flowering time and reproductive development through the repression of multiple genes and genetic pathways. Recent studies demonstrated that SVP modulates the transcriptional expression of genes involved in the photoperiodic pathway. Interestingly, this modulation occurs at different plant positions as SVP directly represses the transcription of *FT* in the leaf and *SOC1* in the shoot apex (Lee *et al.* 2007, Li *et al.* 2008, Jang *et al.* 2009). A more recent study showed that SVP also regulates the floral transition by reducing GA levels at the shoot apex through repression of *GA20ox2* transcription, a gene encoding a key enzyme required for GA biosynthesis (Andres *et al.* 2014). *TSF* transcription is also negatively regulated in the phloem by SVP, but there is no evidence so far of a direct regulation (Hepworth *et al.* 2002, Searle *et al.* 2006, Lee *et al.* 2007, Li *et al.* 2008, Jang *et al.* 2009). Thus, SVP was postulated to regulate flowering by acting on the photoperiod pathway and GA levels (Lee *et al.* 2007, Li *et al.* 2008, Jang *et al.* 2009, Andres *et al.* 2014). As shown for SVP, FLC directly binds to *FT* and *SOC1* and represses their transcription. The repression of these two genes by FLC strongly supresses flowering before the plant undergoes vernalization (Hepworth *et al.* 2002, Helliwell *et al.* 2006, Searle *et al.* 2006, Lee *et al.* 2007). When plants are exposed to low temperatures during winter, *FLC* mRNA levels are reduced, releasing the repression on *FT* and *SOC1* and promoting flowering (Michaels and Amasino 1999, Sheldon *et al.* 1999).

Characteristically, MADS-box transcription factors act in homodimers/heterodimers and quaternary complexes that bind to genomic regions containing a specific motif called CArG-box (Theissen and Saedler 2001, de Folter *et al.* 2005). SVP and FLC form a heterodimeric

complex that bind to a large number of target genes predominantly repressing their transcription (de Folter *et al.* 2005, Li *et al.* 2008). Comparative genome-wide studies of direct targets of SVP and FLC demonstrated that these two proteins function together as a complex that regulate genetic networks to confer seasonal patterns of flowering (Deng *et al.* 2011, Tao *et al.* 2012, Gregis *et al.* 2013, Mateos *et al.* 2015).

Thus, the photoperiodic flowering is promoted in the SAM by FT and inhibited through the activity of several floral repressors. This balance between repressors and activators results in a complex regulatory network where different signals converge to modulate seasonal flowering responses (Figure 1.1). These signals are not only produced by fluctuations in day-length, but also by changes in other factors such as temperature and light quality that typically coexist in natural environments.

1.5 Regulation of flowering time by light quality

The spectrum and intensity of sun light varies depending on conditions such as time of day, altitude and local environment. For example, in dense vegetation, the daylight spectrum is dramatically altered compared to open environments (Figure 1.3). Photosynthetic pigments, chlorophylls and carotenoids, absorb light over most of the visible spectrum, such as B and R light and some of the green light is reflected or transmitted. FR light radiation is poorly absorbed by plants, and consequently, the light that is transmitted through or reflected from dense canopies is increased in FR, reducing the R:FR ratio (Franklin and Shinkle 2008, Casal 2012). This ratio is a comparison of photon irradiance in the R spectrum (between 655 and 665 nm), to that in the FR spectrum (between 725 and 735 nm) (Franklin and Whitelam 2005). Underneath vegetational canopies the ration of R:FR decreases from 1.15 to values typically in the range 0.05 - 0.8 (Smith 1982). The extent of this reduction in the R:FR ratio is

quantitatively related to the density and proximity of neighbouring vegetation (Smith and Whitelam 1997). The R:FR ratio is therefore a useful parameter to describe the natural light environment and for plants to detect the presence of competing neighbours. Such reductions in R:FR can be detected by plants as a change in the relative proportions of the R vs. the FR-absorbing forms of Phys (Pr and Pfr, respectively) indicating that potential competitors are nearby.

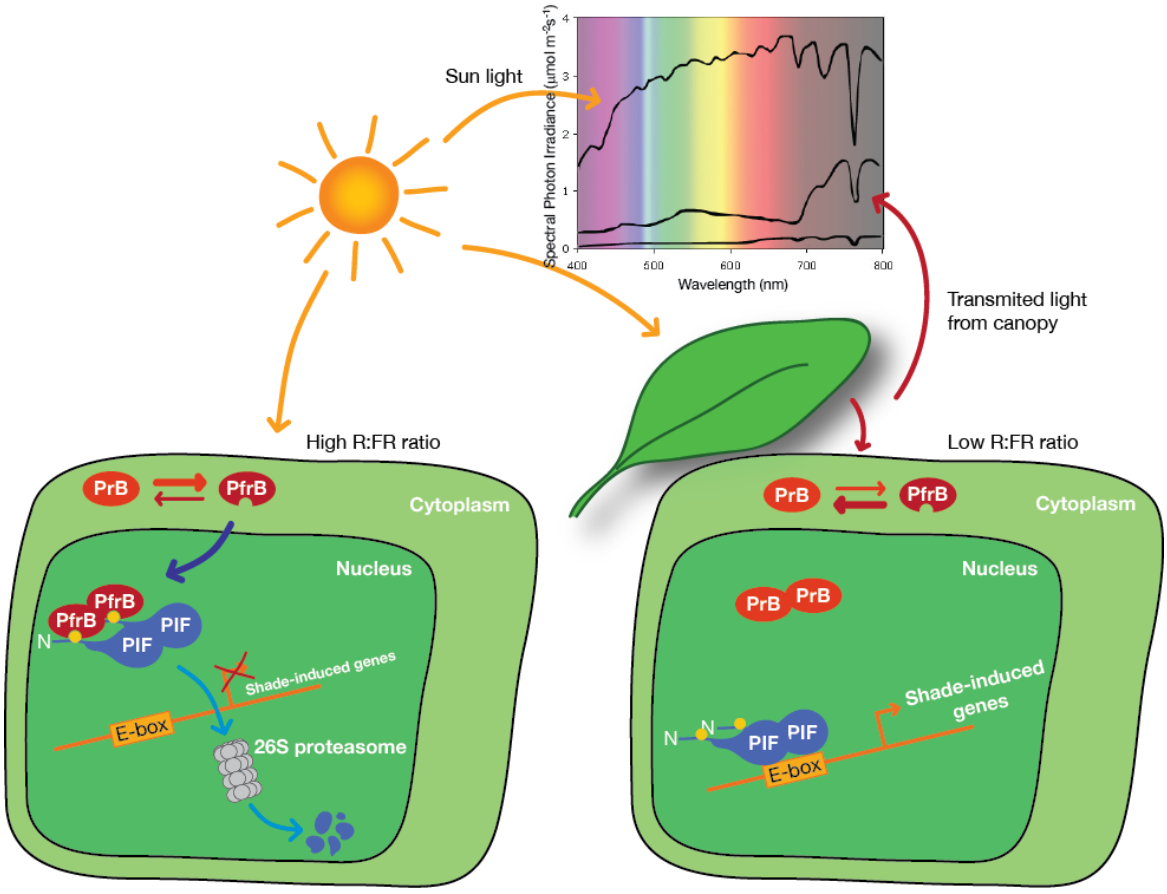

Figure1.3 Model for the control of the shade avoidance through PhyB mediated by PIF4 and PIF5 stability. Upper panel: the spectral energy distribution of incident solar radiation on a clear day under the following conditions. Upper curve = light spectrum at midday; middle curve = midday sun light spectrum filtered through a canopy of mustard seedlings; lower curve = dusk. Lower panel: model for the control of shade regulated genes mediated by PIFs. In sun light conditions (left) PhyB is mainly in the Pfr from (absorption maximum approx. 730 nm). The Pfr form of PhyB accumulates in the nucleus where it interacts with PIFs through the active phytochrome binding domain. This interaction targets PIFs transcription factors for degradation by the 26S proteasome, leading

to deactivation of genes induced by shade. Under a canopy (right), light filtered or reflected by the neighbouring vegetation is enriched in FR light. Under these conditions, Pfr form of PhyB is photoconverted to Pr form (absorption maximum approx. 660 nm). Pr form of PhyB no longer interacts with PIFs, allowing the rapid accumulation of these transcription factors. Under shade conditions, PIFs directly bind to and promote the expression of shade-induced genes involved in cell expansion and hypocotyl growth. Adapted from Franklin and Shinkle (2008) and Lorrain *et al.* (2008).

Four major classes of photoreceptors have been identified in plants. The first ones identified were the Phy photoreceptors (Butler *et al.* 1959) that predominantly absorb the R and FR wavelengths (600–750 nm). Three types of photoreceptors perceive the B and ultraviolet-A (UV-A) wavelengths (320–500 nm). They are cryptochromes (Yu *et al.* 2010), phototropins (Huala *et al.* 1997, Christie *et al.* 1998), and the LOV/F-box/Kelch-repeat proteins ZTL, FKF1, and LKP2 (Zoltowski and Imaizumi 2014). Additionally, the UV-B (282–320 nm) region of the spectrum is perceived by UV RESISTANCE LOCUS 8 (UVR8) photoreceptor (Jiao *et al.* 2007, Li *et al.* 2011, Rizzini *et al.* 2011). These photoreceptors transduce the light signals via different signalling pathways, to modulate light regulated gene expression and ultimately diverse developmental processes, including seed germination, seedling photo-morphogenesis, phototropism, gravitropism, chloroplast movement, shade avoidance, circadian rhythms and flowering induction (Kami *et al.* 2010).

1.5.1 Photoreceptors involved in the regulation of the shade avoidance response

1.5.1.1 Phytochromes

Phy photoreceptors are a family of soluble chromoproteins that can monitor changes in the R:FR light ratio and transduce intracellular signals during light-regulated plant development (Sharrock and Clack 2002, Jiao *et al.* 2007). They exist as homodimers of two independently reversible subunits. Each subunit consists of a polypeptide covalently linked through its N-

terminal domain to a light-absorbing linear tetrapyrrole chromophore, phytochromobilin, via a thioether linkage (Furuya and Song 1994, Fankhauser 2001). The C-terminal part of PHY is required for dimerization and interaction with signalling partners as well as light-dependent nuclear localization (Fankhauser 2001, Jiao *et al.* 2007). In the dark, Phy is synthesized in the inactive R light-absorbing (Pr) form and it is activated upon photo-conversion to the FR light-absorbing (Pfr) form (Quail 2002). Under almost all irradiation conditions, Phy exists as an equilibrium mixture of the two forms (Franklin and Whitelam 2005, Casal 2012). The perception of changes in amounts of R and FR light is accomplished by changes in the equilibrium of Pr and Pfr forms (Figure 1.3). A low R:FR ratio, characteristic of the presence of neighboring vegetation, alters the equilibrium, leading to low levels of Pfr (Franklin and Whitelam 2005). In response to low R:FR ratio signals, many plants display a set of physiological responses characterized by a reallocation of resources to elongation-growth responses in order for the plant to reach unfiltered sunlight (Smith 2000, Franklin and Whitelam 2004, Vandenbussche *et al.* 2005). These responses include the rapid and pronounced increase in the elongation growth rate of stems and petioles, furthermore, reduced chlorophyll content and reduction in leaf thickness is often observed in plants receiving low R:FR ratio signals (McLaren and SMITH 1978). Under shade, plants also develop elevated leaf angles (hyponasty) and an increase in apical dominance leading to reduced branching (Casal *et al.* 1986). These responses are collectively known as the SAS and provide a survival strategy in rapidly growing populations (Franklin and Whitelam 2005). A reduction in the R:FR ration is sensed before plants are actually being shaded, thus the plants can initiate the escape programme before they suffer from competition (Ballare *et al.* 1990). If reduced R:FR ratio persists, and the plant is unable to outgrow the competing vegetation, then flowering is accelerated enhancing the probability of reproductive success (Halliday *et al.* 1994, Smith and Whitelam 1997).

Higher plants contain at least two different forms of Phy (Hillman 1967, Abe *et al.* 1985, Shimazaki and Pratt 1985, Tokuhisa *et al.* 1985). These are referred to as type I or “light-labile” Phy, which predominate in etiolated tissue, and type II or “light-stable” Phy, which predominate in light-grown tissue (Furuya 1993).

In *A. thaliana* there are five isoforms of Phy photoreceptors, PhyA - PhyE (Sharrock and Quail 1989, Clack *et al.* 1994). *PHYA* encodes for a light-labile, type I Phy. It is most abundant in dark-grown (etiolated) seedlings, whereas its level drops rapidly, up to 100-fold, upon exposure to R or WL (Clough and Vierstra 1997, Hennig *et al.* 1999, Sharrock and Clack 2002). PhyB to PhyE are light-stable, type II Phys. Among these, PhyB and PhyC have intermediate light stability compared with the most light-stable PhyD and PhyE forms (Sharrock and Clack 2002). PhyB is the most abundant Phy, whereas PhyC - PhyE are less abundant (Clack *et al.* 1994, Hirschfeld *et al.* 1998, Sharrock and Clack 2002).

Plants mutated in *PHYB* display a constitutive shade avoidance phenotype, typically presenting elongated stems and petioles, reduced leaf size, decreased chlorophyll content and early flowering (Somers *et al.* 1991, Reed *et al.* 1993). These observations therefore, indicated that PhyB plays a predominant function in the shade avoidance response (Franklin and Whitelam 2005). However, PhyB is not the only Phy involved in this response since *phyB* mutants retain a response to low R:FR irradiation (Whitelam and Smith 1991). Indeed, genetic and physiological studies have indicated that PhyD and PhyE act redundantly with PhyB in the control of some aspects of the SAS-driven development (Devlin *et al.* 1998, Devlin *et al.* 1999, Franklin 2008, Martínez-García *et al.* 2010).

1.5.1.2 Cryptochromes

Cryptochromes are photolyase-like B light photoreceptors that regulate light responses in plants. Arabidopsis has two of these photoreceptors, CRY1 and CRY2, which also participate in shade avoidance responses (Keller *et al.* 2011, Casal 2012). Under a canopy, besides changes in the R:FR ratio, the total irradiance is reduced and there is variation in B light caused by absorption of visible wavelengths by photosynthetic pigments (Ballaré *et al.* 1991). CRY1 and CRY2 mediate B light suppression of hypocotyl elongation and photoperiodic responses of flowering time (Ahmad and Cashmore 1993, Guo *et al.* 1998, Lin *et al.* 1998). The activity of cryptochromes is directly proportional to the intensity of B light, therefore, CRY1 and CRY2 act as sensors of irradiance levels (Sellaro *et al.* 2010), and their activity is reduced in shaded conditions.

1.5.2 Acceleration of flowering under enriched FR light

Plants have evolved the ability to detect differences in day-length and light quality, allowing them to adjust their development in anticipation of seasonal and environmental changes. Environments like dense canopies trigger the SAS, which includes physiological and developmental changes in plant architecture. One of the most prominent of these responses is the acceleration of flowering (Smith and Whitelam 1997). Information provided by the circadian clock, day-length and light quality regulates *CO* mRNA and CO protein stability to influence flowering time (Suarez-Lopez *et al.* 2001, Yanovsky and Kay 2002, Valverde *et al.* 2004). Changes in the R:FR ratio affect the stability of CO protein. Activation of PhyB by R light promotes the degradation of CO protein early in the day, delaying flowering. Under shaded conditions, when the R:FR ratio is low, the inactive Pr form of PhyB predominates impeding the degradation of CO protein during the morning hours, facilitating the promotion

of flowering (Valverde *et al.* 2004, Kim *et al.* 2008). Promotion of flowering mediated by stabilization of CO protein is also achieved by photoexcited PhyA and CRY2-interaction with SPA1 which supress the activity of COP1 during the late afternoon (Valverde *et al.* 2004, Laubinger *et al.* 2006, Endo *et al.* 2007, Jang *et al.* 2008, Liu *et al.* 2008b, Balcerowicz *et al.* 2011, Zuo *et al.* 2011). Therefore, the induction of flowering by shade is highly dependent on photoperiod pathway components, explaining why low R:FR ratios have little effect in this process under SDs (Wollenberg *et al.* 2008).

phyB mutants flower early compared to WT under LDs and SDs, as part of their constitutive SAS, and this phenotype is temperature-dependent (Reed *et al.* 1993, Halliday *et al.* 1994, Halliday *et al.* 2003). Conversely, *phyD* and *phyE* single mutant plants do not display a constitutive SAS. However, in both cases double mutants with *phyB* show a stronger flowering and hypocotyl length phenotype than *phyB* single mutants (Aukerman *et al.* 1997, Devlin *et al.* 1998). Similarly, *phyB phyD phyE* triple mutants flower earlier than wild type under LDs and SDs (Wollenberg *et al.* 2008). These facts reflect a high degree of functional redundancy between these three Phys. Shade avoidance responses can be triggered not only by changes in the R:FR ratio, but also by end-of-day (EOD) FR pulses (Devlin *et al.* 1996). PhyB, PhyD and PhyE are involved in the regulation of flowering in response to shade (Devlin *et al.* 1998, Devlin *et al.* 1999, Franklin *et al.* 2003a, Franklin and Whitelam 2005). The *phyB* mutant retains a response to shade flowering early under both LDs and SDs (Whitelam and Smith 1991, Reed *et al.* 1993, Halliday *et al.* 1994, Devlin *et al.* 1999, Wollenberg *et al.* 2008). The *phyB phyD* and *phyB phyE* double mutants also respond to enriched FR and EOD-FR by inducing flowering (Devlin *et al.* 1999, Franklin *et al.* 2003a). However, this response is suppressed in *phyB phyD phyE* triple as well as *phyA phyB phyD phyE* quadruple mutant, indicating that PhyA has little or no effect in the regulation of flowering under shade (Franklin *et al.* 2003a, Wollenberg *et al.* 2008). A role of PhyC in

flowering time under different photoperiod has been shown (Monte *et al.* 2003), but its function in the regulation of flowering mediated by shade remains unclear.

Plants grown under enriched FR light accumulate higher levels of *CO* mRNA during the early hours of the day than plants grown under WL (Kim *et al.* 2008). Furthermore, under these conditions the accumulation of CO protein is higher (Kim *et al.* 2008). Conversely, *co* and *gi* mutants, plants with absence or very low levels of *CO* mRNA transcripts, are relatively insensitive to enriched FR light in terms of flowering time (Suarez-Lopez *et al.* 2001, Kim *et al.* 2008). Thus, *CO* plays a crucial role in the promotion of flowering under enriched FR light (David *et al.* 2006, Kim *et al.* 2008). However, not all the effect on flowering mediated by the Phys can be accounted for by changes in the stability of CO as *phyA phyB phyE co* quadruple mutant plants flower earlier than *phyA phyB co* triple mutants (Devlin *et al.* 1998). Furthermore, the action of PHYTOCHROME AND FLOWERING TIME 1 (PFT1) downstream of PhyB was also proposed to regulate flowering time in response to changes in light quality through the transcriptional activation of *FT*, in a CO-independent manner (Cerdán and Chory 2002).

Plants growing at high dense vegetation have adapted their developmental programs to avoid the negative effects caused by the light interception from the neighbouring vegetation. Acceleration of flowering is one of the usual responses that plants employ to optimize their survival opportunities under shade conditions. For this purpose, plants recognise the reduced R:FR ratio indicative of shade conditions by making use of the Phys, which through conformational changes initiate a series of molecular cascades to induce flowering. This signalling process involves the regulation of components of the photoperiodic pathway, such as *CO* and *FT* genes. However, the detailed mechanism by which R:FR signalling regulate flowering is still largely unknown.

1.5.3 Role of PIFs in light responses and development

PIF proteins belong to a fifteen-member family of bHLH transcription factors (Toledo-Ortiz *et al.* 2003). Originally identified during a yeast-two-hybrid screen for PhyB interactors, PIF3 is the founding member of this family (Ni *et al.* 1998). Seven members of the family have been reported to interact with Pfr-PhyB (PIF1/PIF3-LIKE 5 [PIL5], PIF3, PIF4, PIF5/PIL6, PIF6/PIL2, PIF7 and PIF8), while two of them (PIF1/PIL5, PIF3) also bind PhyA (Huq *et al.* 2004, Khanna *et al.* 2004, Shen *et al.* 2008, Lucas and Prat 2014). All PIF proteins contain a conserved N-terminal sequence, called the Active Phytochrome B-binding (APB) motif which is necessary and sufficient for PhyB-specific binding (Khanna *et al.* 2004). PIF1 and PIF3 also contain a separate domain, called the Active Phytochrome A-binding (APA), necessary for PhyA binding (Al-Sady *et al.* 2006, Shen *et al.* 2008).

Upon perception of light by Phys, photoconversion from the inactive Pr form into the active Pfr form takes place. Once activated they rapidly translocate from the cytosol to the nucleus where they bind directly to APB or APA interaction sites in PIFs, colocalizing in nuclear speckles (Duek and Fankhauser 2005, Al-Sady *et al.* 2006, Castillon *et al.* 2007, Shen *et al.* 2008, Quail 2010). As a result of the interaction, Phys induce rapid phosphorylation of PIFs and this leads to ubiquitination and degradation by the proteasome (Park *et al.* 2004, Shen *et al.* 2005, Al-Sady *et al.* 2006, Oh *et al.* 2006, Al-Sady *et al.* 2008, Lorrain *et al.* 2008). The light-mediated degradation of PIFs does not lead to complete disappearance of the protein, but rather to a low steady state level (Nozue *et al.* 2007, Lorrain *et al.* 2008). Upon removal of Pfr by exposing plants to darkness or shade conditions, PIF degradation ceases and the proteins can accumulate (Figure 1.3) (Shen *et al.* 2005, Nozue *et al.* 2007, Lorrain *et al.* 2008, Soy *et al.* 2012).

PIF transcription factors regulate gene expression by binding to G-box (CACGTG) and PIF-binding E-box (PBE-box) (CACATG) motifs, two variants of the canonical E-box motif (CANNTG) (Figure 1.3) (Hornitschek *et al.* 2012, Zhang *et al.* 2013). RNA sequencing (RNA-Seq) and Chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) analyses showed that PIFs regulate the expression of a large number of target genes related to different developmental processes (Hornitschek *et al.* 2012, Zhang *et al.* 2013, Pfeiffer *et al.* 2014). For example, PIFs play a central role in light signalling during *A. thaliana* photomorphogenesis (Leivar *et al.* 2008b, Leivar and Quail 2011), they regulate growth (Duek and Fankhauser 2005), chloroplast differentiation (Stephenson *et al.* 2009), seed germination (Oh *et al.* 2004, Oh *et al.* 2006, Oh *et al.* 2007), the phototropic response (Sun *et al.* 2013) and flowering (Kumar *et al.* 2012), as well as many aspects of the SAS (Lorrain *et al.* 2008, Li *et al.* 2012a), and the circadian clock (Nusinow *et al.* 2011). Changes in R:FR light occurring under shade condition allow the accumulation of PIF4 and PIF5, which then bind to target genes with roles in cell expansion and hypocotyl growth, such as *ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2* (*ATHB2*), *PIF3-LIKE 1* (*PIL1*), *LONG HYPOCOTYL IN FAR-RED 1* (*HFR1*), *XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7* (*XTR7*) and *PHYTOCHROME RAPIDLY REGULATED1* (*PAR1*) (de Lucas *et al.* 2008, Lorrain *et al.* 2008, Hornitschek *et al.* 2009, Leivar and Quail 2011, Hao *et al.* 2012). Two of these genes, *HFR1* and *PAR1*, encode for bHLH transcription factors that participate in a negative feedback loop avoiding exaggerated shade avoidance response. After a long term exposure to shade HFR1 and PAR1 accumulate, heterodimerize with PIF4 and PIF5 limiting their capacity to bind DNA and to promote shade induced gene expression (Hornitschek *et al.* 2009, Hao *et al.* 2012). Further regulation of the prolonged shade response is performed by the post transcriptional modulation of PhyB abundance mediated by PIF3, PIF4 and PIF7, thereby altering the sensitivity to incoming light signals (Leivar *et al.* 2008a).

In addition to the R:FR ratio, B light is also reduced under canopies. B light induces shade avoidance responses, including stem and hypocotyl elongation (Ballaré *et al.* 1991, Pierik *et al.* 2004, Djakovic-Petrovic *et al.* 2007, Franklin and Shinkle 2008, Sasidharan *et al.* 2008, Keller *et al.* 2011). CRY1 and CRY2 perceive B light changes and respond by directly interacting with PIF4 and PIF5. The bHLH transcription factors and CRYs bind overlapping regions on gene promoters, indicating that B light regulates plant growth by modulating PIF4 and PIF5 activities (Pedmale *et al.* 2016). In contrast to reduced B light intensity, under high B light, CRY1 directly interacts with PIF4 reducing its activity without affecting DNA-binding ability, thus regulating the expression of PIF4 targets and also high temperature-promoted hypocotyl elongation (Ma *et al.* 2016). CRY2 interacts with CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX (CIB1) protein in a B light–specific manner. The expression of the CIB protein is regulated specifically by B light. CIB1 together with additional CIB1-related proteins promote CRY2-dependent floral initiation by inducing *FT* mRNA expression (Liu *et al.* 2008a, Liu *et al.* 2013).

1.5.3.1 PIFs mediate the crosstalk between light and hormone signalling

PIFs play important roles in the integration of hormonal signals, such as GA, auxin and brassinosteroids (BRs) that regulate light-mediated hypocotyl growth.(Jaillais and Chory 2010, Lau and Deng 2010, Depuydt and Hardtke 2011, Franklin *et al.* 2011a, Hornitschek *et al.* 2012, Li *et al.* 2012a, Li *et al.* 2012b, Oh *et al.* 2012, Sun *et al.* 2012). Hypocotyl elongation is promoted by degradation of DELLA proteins triggered by GA. DELLA proteins in *A. thaliana* are encoded by five genes: *REPRESSOR OF ga1-3* (*RGA*), *GIBBERELLIC ACID INSENSITIVE* (*GAI*) and *RGA-LIKE 1-3* (*RGL1-3*) (Schwechheimer 2008, Hauvermale *et al.* 2012). DELLAs interact with and sequester PIFs impairing their ability to bind DNA (de Lucas

et al. 2008, Feng *et al.* 2008, Alabadí and Blázquez 2009). Upon perception of GA, the ubiquitination and degradation of DELLAs is triggered, releasing the PIF transcription factors and inducing the expression of cell elongation genes (Willige *et al.* 2007, Gao *et al.* 2011, Sun 2011).

BRs play a role in light-grown hypocotyl elongation. They act independently of, but cooperatively with, GAs and auxin (Tanaka *et al.* 2003). BR-activated transcription factor BRASSINAZOLE-RESISTANT 1 (BZR1) and PIF4 interact and synergistically regulate many common target genes, including transcription factors required for promoting cell elongation. Both, BZR1 and PIF4 also control a large number of unique targets, allowing differential regulation of various processes by BRs and environmental signals (Oh *et al.* 2012).

In plants growing under dense canopies, auxin contributes to the shade avoidance response. PIF4, PIF5 and PIF7 have been shown to bind the promoters and activate the transcription of *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*)/ *SHADE AVOIDANCE 3* (*SAV3*) and *YUCCA 8* (*YUC8*), genes encoding enzymes involved in the synthesis of Indole-3-acetic acid (IAA) (Franklin *et al.* 2011a, Sun 2011, Hornitschek *et al.* 2012, Li *et al.* 2012a, Li *et al.* 2012b). The expression of *INDOLE-3-ACETIC ACID INDUCIBLE 19* (*IAA19*) and *IAA29*, involved in auxin-mediated hypocotyl phototropic growth, is also regulated by PIF4 and PIF5 (Kunihiro *et al.* 2011, Sun *et al.* 2013). In high R:FR light the expression of these genes is reduced in *pif4 pif5* double mutant, however it is still induced under shade (Hornitschek *et al.* 2009). Thus, the auxin synthesized through a PIF-regulated pathway links directly the perception of a light quality signal to a rapid growth response. However, *PIF4* and *PIF5* are not essential to regulate the transcriptional activation of auxin-mediated hypocotyl growth genes *IAA19* and *IAA29* under shade conditions.

PIF4 and PIF5 also regulate dark-induced leaf senescence mainly mediated by the antagonistic interaction between cytokinins and ethylene, two plant hormones. Ethylene

biosynthesis and signalling are positively regulated by PIF4 (Sakuraba *et al.* 2014, Song *et al.* 2014). *PIF5* is also likely to regulate ethylene biosynthesis since ethylene production is significantly increased in transgenic plants overexpressing this gene (Khanna *et al.* 2007).
PIF transcription factors are key regulators of plant development that act as central hubs in the perception and integration of environmental and internal signals. They modulate light signalling cascades by direct interaction with Phy photoreceptors. Moreover, PIFs play an important role in the crosstalk between light and hormone signalling and directly regulate downstream genes involved in numerous developmental processes. These facts highlight the importance of PIFs in plant fitness and adaptation to the environment in Phys- and hormone- mediated signalling cascades among others.

1.5.3.2 Circadian clock regulation of *PIF* genes transcription

PIF4 and *PIF5* are not only light-regulated but also circadian-controlled genes (Yamashino *et al.* 2003, Nozue *et al.* 2007). Under continuous light, they oscillate robustly with a period of about 24 h showing a peak after dawn (Yamashino *et al.* 2003). The diurnal pattern of *PIF4* and *PIF5* expression is modulated by the action of circadian clock genes, such as *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*), a MYB-related transcription factor, and *PSEUDO-RESPONSE REGULATOR 9* (*PRR9)*, *PRR7*, *PRR5* and *TOC1*, members of a group of atypical variants of two-component signal transducers (Schaffer *et al.* 1998, Wang and Tobin 1998, Makino *et al.* 2000, Matsushika *et al.* 2000, Strayer *et al.* 2000, Mizoguchi *et al.* 2002, Niwa *et al.* 2009). The expression of *PIF4* and *PIF5* is repressed at dusk by the evening complex, composed of the proteins encoded by *EARLY FLOWERING 3* (*ELF3*), *ELF4* and *LUXARRHYTHMO* (*LUX*; also known as *PHYTOCLOCK 1*) (Zagotta *et al.* 1996, Hicks *et al.* 2001, Doyle *et al.* 2002, Hazen *et al.* 2005, Onai and Ishiura 2005, Nusinow *et*

al. 2011). As PIF4 and PIF5 function as a positive regulators of the hypocotyl elongation, the repression of *PIF4* and *PIF5* transcription at dusk leads to the repression of hypocotyl growth. This process combined with light regulated turnover of PIF4 and PIF5 proteins allows maximum hypocotyl growth at dawn (Nozue *et al.* 2007, Lorrain *et al.* 2008, Nusinow *et al.* 2011). The accumulation of *PIF4* and *PIF5* transcripts at dawn under SDs combined with longer night periods in which the protein products are stably accumulated, lead to SD-specific elongation of hypocotyls (Nomoto *et al.* 2012, Yamashino *et al.* 2013a). Specific hypocotyl growth at dawn under SDs is well explained by the coincident accumulation of the active PIF4 and PIF5 proteins during night-time (Nozue *et al.* 2007, Nomoto *et al.* 2012, Yamashino *et al.* 2013a). Thus, the regulation of hypocotyl growth mediated by PIF4 and PIF5 is another example of an external coincidence mechanism, which involves photoperiodic and clock signals.

PIFs act as molecular hubs by interacting with light and hormone signalling cascades to modulate developmental processes. In this context, as clock-regulated proteins, PIFs contribute to the integration of different environmental signals to generate rhythmic patterns of growth. Furthermore, PIFs are involved in flowering time regulation (see below) (Brock *et al.* 2010, Kumar *et al.* 2012). However, whether PIFs act as integrators of distinct environmental signals to modulate flowering requires further studies.

1.6 Regulation of flowering by ambient temperature

During the last few years the effect of ambient temperature in flowering time control has been extensively studied. In particular, much emphasis has been given to the effect of warm ambient temperature. This is thought to be significant in terms of climate change, and its effect on the ecology of natural plant populations as well as on agriculture. Ambient

temperature affects many aspects of plant growth and development, such as clock entrainment, growth, disease resistance and flowering (Gray *et al.* 1998, Stavang *et al.* 2009, Alcázar and Parker 2011, Boikoglou *et al.* 2011, Franklin *et al.* 2011a, Sun *et al.* 2012). In *A. thaliana*, within the range of 12 °C and 27 °C these changes on growth, development and flowering time occur without significant induction of stress responses (Samach and Wigge 2005, Balasubramanian *et al.* 2006, Kumar and Wigge 2010). In terms of flowering, ambient temperatures around 12 - 16 °C delay flowering time (Blazquez *et al.* 2003, Kumar *et al.* 2012) whereas temperatures around 27 °C accelerate flowering even under non-inductive SD conditions (Lee *et al.* 2007, Kumar and Wigge 2010, Kumar *et al.* 2012, Lee *et al.* 2013, Pose *et al.* 2013, Galvao *et al.* 2015). These variations in flowering time are influenced by the action of several molecular factors. These factors involve changes in the expression, activity or stability of floral activators and repressors, such as PIF4, SVP and FLOWERING LOCUS M (FLM), as well as changes in the DNA structure (Figure 1.4) (Balasubramanian *et al.* 2006, Lee *et al.* 2007, Kumar and Wigge 2010, Kumar *et al.* 2012, Lee *et al.* 2013, Pose *et al.* 2013). In addition, genes of the autonomous pathway, which regulate flowering by downregulating the floral repressor FLC (Simpson 2004) are involved in mediating the effects of ambient temperature (Blazquez *et al.* 2003). Collectively, these changes ultimately affect the regulation of the floral integrator *FT* modulating the timing of flowering (Blazquez *et al.* 2003, Halliday *et al.* 2003, Balasubramanian *et al.* 2006, Lee *et al.* 2007, Kumar and Wigge 2010, Kumar *et al.* 2012, Lee *et al.* 2013, Pose *et al.* 2013).

1.6.1 PIF4-mediated regulation of flowering time at warm ambient temperature

Incorporation of different histones into chromatin can influence transcription (Verhage *et al.* 2014, Weber and Henikoff 2014). H2A.Z is a histone variant that can be incorporated into

chromatin as a replacement of the canonical histone H2A. The deposition of H2A.Z in *A. thaliana* is carried out by the SWR1 complex (Krogan *et al.* 2003, Kobor *et al.* 2004, Mizuguchi *et al.* 2004, March-Díaz and Reyes 2009). Three subunits of the SWR1 complex have been identified and characterized, PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1 (PIE1), ACTIN-RELATED PROTEIN6 (ARP6), and SWR1 COMPLEX6 (SWC6) (Choi *et al.* 2005, Deal *et al.* 2005, Choi *et al.* 2007, Deal *et al.* 2007, Lazaro *et al.* 2008, March-Diaz and Reyes 2009). Both PIE1 and ARP6 are required for the incorporation of H2A.Z throughout the genome (Deal *et al.* 2007). Kumar and Wigge (2010) isolated in a genetic screen two mutant alleles of *ARP6* that display constitutive developmental and architectural phenotypes of warm grown plants. Mutations in *ARP6* had previously been identified as early flowering mutants (Choi *et al.* 2005, Deal *et al.* 2005, Martin-Trillo *et al.* 2006). On exposure to high temperatures, H2A.Z histone-containing nucleosomes are removed from temperature sensitive promoters, including that of *FT* (Kumar and Wigge 2010, Kumar *et al.* 2012), suggesting that the early flowering of *arp6* mutant could be explained by increased *FT* transcription through this mechanism. Removal of H2A.Z histone-containing nucleosomes from these promoters make them more accessible to specific transcription factors that increase the expression of the cognate genes (Kumar and Wigge 2010). For example, PIF4 transcription factor binds to the *FT* promoter more strongly when plants are growing under SDs at 27 °C (hereafter 27°C-SD) compared to 12 °C (Kumar *et al.* 2012). Consistent with this observation, the *pif4* null mutant of *A. thaliana* is later flowering than wild-type under 27°C-SD and flowers with the same number of leaves under 27°C-SD and 22°C-SD, indicating a crucial role for this transcription factor in the thermosensory induction of flowering under these conditions (Kumar *et al.* 2012). The role of PIF4 in warm temperature promotion of flowering is also evident in photoperiods of 12 h light and 12 h dark (Thines *et al.* 2014). By contrast, the *pif4* null mutant was recently described to flower at times similar to Col-0

plants under 27°C-SD (Galvao *et al.* 2015). Similar results were observed under LDs at high temperature, where PIF4 was not required for early flowering (Koini *et al.* 2009).

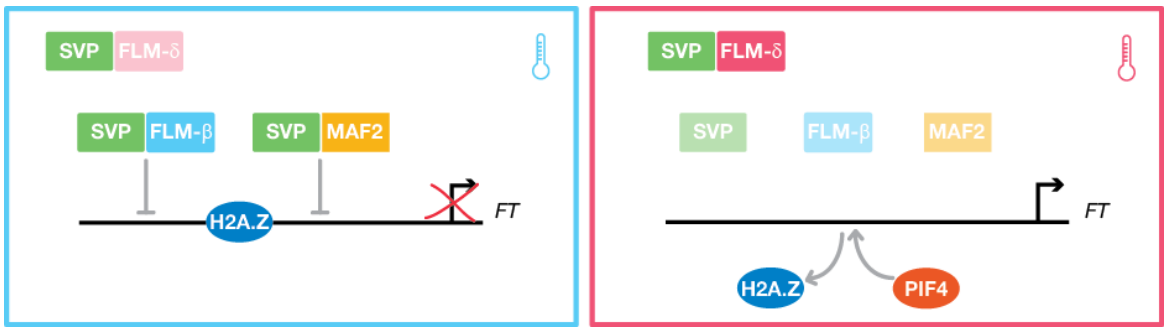

Figure 1.4. Regulation of flowering time by ambient temperature. Distinct mechanisms of regulation of flowering by ambient temperature have been described. At low temperature (left panel), complexes by the MADS-box transcription factors SVP, FLM splicing isoform β (FLM-β) and MAF2 splicing isoform var1 (MAF2-1) are formed (upper part). Under these conditions, the splicing forms FLM-β and MAF2-1 are the most predominant ones and interact with SVP to repress *FT* expression. When the temperature increases (right panel, upper part), different processes inactivating the SVP/FLM-β and SVP/ MAF2-1 complexes have been proposed. One model suggests that SVP is degraded via the 26S proteasome. Alternative models argue that the FLM-β activity is reduced either by degradation of *FLM*-β at the RNA level or by a dominant-negative version of the FLM protein, FLM-δ, that is predominant at warm temperature and poisons the repressive MADS-domain transcription factor complex. MAF2-1 is also reduced at warm temperatures in favour of the MAF2-2, a non-functional splicing isoform. Inhibition of the MADS-box transcription factor complexes by any of these mechanisms leads to the induction of the *FT* expression and flowering. Under SDs, warm temperature promotes the removal of H2A.Z-containing nucleosomes from the DNA, enabling the binding of PIF4 to the promoter and the induction of *FT* (right panel, lower part). Arrows and block lines denote activation and repression, respectively. Intensity of colours indicate protein abundance. Adapted from Capovilla *et al.* (2015), Airoldi *et al.* (2015) and Sureshkumar *et al.* (2016).

The early flowering phenotype mediated by the transcriptional induction of *FT* in the *arp6* mutant was proposed to be due to higher accessibility of PIF4 to the *FT* promoter (Kumar and Wigge 2010, Kumar *et al.* 2012). Therefore, mutations in *PIF4* should strongly delay flowering of *arp6* mutants. Galvao *et al.* (2015), however, showed that the delay of flowering of *pif4 arp6* double mutants compared to *arp6* is very subtle under LDs at 23 °C, and so is the reduction of *FT* mRNA induction detected in *pif4 arp6* mutants under SDs at 23 °C

compared to *arp6*. Furthermore, at 27 °C *arp6* mutants flower earlier than WT (Kumar and Wigge 2010). Therefore, besides the removal of H2A.Z from the temperature sensitive promoters and the role of PIF4 in the activation of *FT* mRNA, there are other factors responsible for the early flowering of *arp6* and the H2A.Z-dependent induction of flowering under warm temperatures (Kumar and Wigge 2010, Verhage *et al.* 2014).

GA signalling acts through DELLA proteins, which interact with and regulate a diverse set of transcription factors. In the presence of GA, DELLA proteins are degraded (Hauvermale *et al.* 2012, Claeys *et al.* 2014). DELLA accumulation strongly suppresses flowering both upstream and downstream of *FT* in leaves and at the SAM, respectively (Galvão *et al.* 2012, Porri *et al.* 2012). PIF4 is sequestered by DELLA though a direct interaction and is one of the transcription factors regulated by these proteins (de Lucas *et al.* 2008). It has been proposed that the DELLA-mediated repression of PIF4 activity, at least partially, represses flowering under low ambient temperatures (12 °C). Indeed, mutant plants impaired in DELLAs function flower early than WT plants at 12 °C (Kumar *et al.* 2012). However, the model that proposes the decrease in occupancy of *FT* promoter by H2A.Z at warm ambient temperatures does not fully explain these results. Under cool ambient temperatures (below 21 °C) the occupancy of the *FT* promoter by H2A.Z is proposed to be high, thus impeding PIF4 binding to *FT* promoter and its transcriptional activation (Kumar and Wigge 2010). In agreement with the repressive role of DELLAs in this process, low GA levels delay the early flowering and repress the *FT* mRNA expression of *arp6* mutants at 23 °C (Galvao *et al.* 2015). However, it is unlikely that the delay of flowering and the reduction of *FT* expression in *arp6* mutants is caused by a DELLA-mediated repression of PIF4 activity since the double mutant *pif4 arp6* is not later than *arp6* single mutant (Galvao *et al.* 2015). Therefore, there might exist a still unidentified PIF4-independent role of DELLA controlling flowering under warm ambient temperatures, as previously proposed (Galvao *et al.* 2015).

Several factors shape the flowering response to ambient temperatures. The accessibility to DNA, especially at the *FT* locus, of PIF4 transcription factor seems to be the most critical. Consequently, the activity of PIF4 controlled by DELLAs (and thereby by GA) also has a great contribution in this regulation. However, the function of PIF4 as well as the DNA occupancy by H2A.Z are not the solely regulators of the flowering response to ambient temperature.

1.6.2 Role of floral repressors in warm temperature induction of flowering

Several MADS-box transcription factors that act as floral repressors are also implicated in the thermosensory flowering pathway. One of these repressors, SVP, delays flowering by reducing transcription of *FT* and *TSF* in leaves and of *SOC1* in the shoot meristem (Hartmann *et al.* 2000, Lee *et al.* 2007, Li *et al.* 2008, Jang *et al.* 2009). The *svp* mutants are early flowering and insensitive to changes in ambient temperature under LDs, flowering at the same time when exposed to 16 °C, 23 °C or 27 °C (Lee *et al.* 2007, Lee *et al.* 2013, Pose *et al.* 2013). Moreover, the stability of the SVP protein is reduced at high temperatures, suggesting that reduction in SVP protein levels at 27 °C contributes to early flowering of WT plants under these conditions (Lee *et al.* 2013). However, under SDs the *svp* mutant retains some responsiveness to warm temperature, suggesting other proteins must contribute to this response (Galvao *et al.* 2015). Indeed, two other MADS-box transcription factors that delay flowering, FLM and MADS AFFECTING FLOWERING 2 (MAF2), were also shown to contribute to the thermosensory pathway (Figure 1.4) (Balasubramanian and Weigel 2006, Pose *et al.* 2013, Airoldi *et al.* 2015, Sureshkumar *et al.* 2016). The mRNA of *FLM* is alternatively spliced producing two protein varieties, FLM-β and FLM-δ. The *FLM-β* mRNA is the more abundant form at 16 °C, the temperature at which FLM represses flowering, whereas at 27 °C its level is decreased (Pose *et al.* 2013). Both protein forms interact with

SVP in yeast and *in vitro* to produce the heterodimers SVP-FLM-β and SVP-FLM-δ, however, their activities differ such that SVP-FLM-β binds DNA and represses flowering, whereas SVP-FLM-δ does not bind DNA. This led to a model whereby alternative splicing of *FLM* mRNA at 27 °C increases the ratio of FLM-δ to FLM-β, inhibiting SVP activity and causing earlier flowering (Pose *et al.* 2013). Alternative models have also been considered since under warm temperatures plants present the same amount of *FLM-δ* and *FLM-β* transcripts and both proteins have similar affinity to SVP. In this context, reduced stability of SVP protein was proposed to be the main determinant of the promotion of flowering under warm temperatures (Hwan Lee *et al.* 2014). Furthermore, the existence of a predominant FLM-β splicing variant at warm temperatures has been questioned by recent publications. Sureshkumar *et al.* (2016) proposed that the formation of the SVP-FLM repressive complex is compromised at warm temperatures by reduced levels of *FLM* mRNA through alternative splicing coupled with nonsense-mediated mRNA decay (AS-NMD). Thus, the formation of the SVP-FLM-δ repressive complex was proposed not to be formed under warm temperatures, but reduction of FLM-β levels was suggested to be the key factor. *MAF2* mRNA is also alternatively spliced at different temperatures and the form of the protein produced at low temperature also interacts with SVP, whereas the one formed at high temperature does not (Airoldi *et al.* 2015). Thus, these different mechanisms involving differential protein stability and splicing are proposed to lead to reduced activity of SVP at elevated temperatures and to accelerated flowering under these conditions.

1.6.3 Regulation of flowering time by cool temperatures

Low temperatures also affect flowering time. Exposure to cold in nature can occur for long periods during winter or for shorter times, such as during the night in spring or autumn. The

long-term exposure response is called vernalization and typically requires several weeks of exposure to cold. Vernalization accelerates flowering by suppressing transcription of the floral repressor, *FLC* (Michaels and Amasino 1999). The requirement for a long cold period ensures that the plant does not flower only after a few days of cold, but rather only flowers after winter. The duration of the cold period required for vernalization varies between accessions of *A. thaliana* (Shindo *et al.* 2006). *FLC* transcription is promoted by FRI, and in natural populations extensive genetic variation at *FLC* and *FRI* has been described. (Johanson *et al.* 2000, Le Corre *et al.* 2002).

Short-term exposure to low temperatures exerts the opposite effect to extended cold by delaying flowering time. These short-term exposures can be due to temperature fluctuations during local weather conditions or to diurnal fluctuations. Intermittent cold treatments (4 °C) trigger the HOS1-mediated degradation of CO in a COP1 and FLC-independent manner (Jung *et al.* 2012b). In *hos1* mutants, *FT* mRNA is misexpressed in the middle of the day under LDs. Thus, under cold temperatures HOS1 is proposed to regulate the timing of *FT* expression by regulating the abundance of CO protein (Jung *et al.* 2012b, Lazaro *et al.* 2012). Hence, the HOS1-CO module contributes to the fine tuning of photoperiodic flowering under-short term temperature fluctuations. This module is proposed to monitor short-term changes in ambient temperature and delay flowering until the appropriate spring season (Jung *et al.* 2012b). HOS1 also controls flowering time in response to ambient temperatures (16 °C and 23 °C) and it appears to do so by interacting with FVE and FLK, proteins that act in the autonomous pathway (Lee *et al.* 2012b). Under 16 °C, COP1 is stabilized and regulates temperature sensitivity by controlling the degradation of GI mediated by the 26S proteasome. GI directly binds to the *FT* promoter to induce flowering in a CO-independent manner (Sawa and Kay 2011). Under cool ambient temperatures the direct binding of GI to the *FT* promoter is reduced and flowering is delayed (Jang *et al.* 2015). The ambient temperature regulation

of flowering is largely dependent on *FT*, rather than *FLC*, indicating that the temperature-dependent flowering is distinct from vernalization (Blazquez *et al.* 2003). However, a *FT*, *TSF* and *PIF* independent pathway also contributes to the flowering regulation under cool ambient temperatures mediated by GA, as GA treatments promote flowering at least partially independently of these genes (Galvao *et al.* 2015).

Variations in temperatures between day and night also affect flowering time. Under standard day temperature conditions (22 °C) and cooler nights (12 °C) the expression of *CO* mRNA at night time is increased through a mechanism dependent on FBH, altering the *FT* expression profile. A recent study showed that *FT* mRNA is increased at the end of cooler nights and reduced at dusk in an SVP dependent manner, delaying flowering time (Kinmonth-Schultz *et al.* 2016). This and another previous work (Thines *et al.* 2014) demonstrated how plants adjust their flowering response to different day and night temperatures. This differential response might contribute to the distinction between typical fluctuations (warmer day vs colder night) and variations associated with seasonal changes (warmer day vs colder day).

In addition to these factors, several miRNAs are also involved in the temperature-mediated regulation of flowering (Jung *et al.* 2012a, Kim *et al.* 2012a). miR156 and miR172 reduce the expression levels of transcription factors that affect flowering, but they have opposite effect on this process (Jung *et al.* 2012a, Kim *et al.* 2012a). At 16 °C, miR156 accumulates to higher levels compared to 23 °C downregulating *SPL3* mRNA. This downregulation of *SPL3* reduces *FT* mRNA levels in leaves, which in turn delays flowering (Kim *et al.* 2012b). Thus, the miR156–SPL3 module plays an important role not only during development (Wu and Poethig 2006), but also in response to ambient temperature (Kim *et al.* 2012b). Ambient temperature regulates miR172 biogenesis primarily at the pri-miR172 processing step, causing miR172 abundance to be elevated at 23 °C but not at 16 °C (Jung *et al.* 2012a). Therefore, at 16 °C the APETALA2-LIKE transcription factors, TARGET OF EAT 1 (TOE1), TOE2, and

SCHLAFMÜTZE (SMZ), potent repressors of flowering whose mRNA are regulated by miR172, are increased in expression delaying flowering (Aukerman and Sakai 2003, Schmid *et al.* 2003a, Lee *et al.* 2010).

Both warm and cold temperatures have strong effects on flowering time. The impact of temperature changes in plant development is evidenced by the numerous factors involved in the complex regulatory network that drives the transition from vegetative to reproductive stage under different ambient temperatures. This intricate regulation ensures that plants flower at the time when the environment is optimal to maximize their reproductive success and plant fitness. The crosstalk between PIF4, SVP and photoperiod related genes might be relevant for regulation of flowering in response to ambient temperature. Thus understanding the molecular and genetic relationship between these genes could shed light on the concerted regulation of flowering time in response to seasonal cues.

2. Aims of the study

Plants are extremely sensitive to changes in their environment. During evolution mechanisms have arisen that rapidly adapt the developmental programs of plants to changing environmental conditions and improve fitness in the context of their sessile life cycle. In particular, the timing of flowering is strongly influenced by environmental factors such as photoperiod, light quality and temperature. These factors are highly variable across different latitudes and seasons, thus plants exhibit an enormous variety of flowering behaviors associated with local adaptation to natural and agronomical ecosystems. However, adaptation to local environments is challenged by rapid changes in ambient temperature caused by global warming. In this context, global climate change is also predicted to dramatically affect crop yield. Therefore, understanding how plants perceive and integrate seasonal and local environmental factors to adjust the timing of flowering to their local environment is necessary to ensure the sustainability of agriculture. Distinct genetic pathways regulate flowering in response to environmental cues. The control of flowering by day length has been extensively studied and detailed information on the function and regulation of the major components of this pathway, such as *CO* and its main transcriptional target *FT* have been deciphered. However, information on how temperature and light quality regulate flowering is less detailed. Furthermore, the interplay between these flowering regulatory pathways has not been thoroughly explored at the molecular level.

Recent studies on the regulation of flowering time under warm ambient temperature in *A. thaliana* highlighted the essential role of two transcription factors in this process. On the one hand, the ability of PIF4 to bind DNA at the *FT* locus is increased under warm ambient temperatures. On the other hand, the repressive function of SVP is impaired under these conditions. As a result, *FT* transcription is activated and flowering is induced. The activity of these two transcription factors partially explains the acceleration of flowering observed in *A. thaliana* plants exposed to warm temperatures, but how they are integrated in the wider

regulatory networks controlling flowering is unknown. One aim of this thesis is to integrate PIF4 and SVP into a single model of temperature regulation of flowering. In addition, as plants integrate multiple environmental signals to regulate flowering, the interactions between the thermosensory and the photoperiodic flowering pathways will be investigated. To achieve these objectives, the genetic bases of flowering control by temperature will be studied through the analysis of the flowering phenotypes of different *A. thaliana* mutants, including those related to the photoperiodic pathway. The molecular mechanisms that trigger flowering under warm ambient temperature in *A. thaliana* will also be investigated.

In addition to the role in the thermosensory pathway, PIFs are central hubs in the light signalling pathway mediated by Phys. As part of this pathway, they facilitate the promotion of the SAS, typical in environments of high dense vegetation. One characteristic of the SAS is the acceleration of flowering. However, no data on the function of PIFs in the induction of flowering as part of the SAS has been reported. Thus, another main objective of this thesis is to explore the role of PIFs in the promotion of flowering mediated by shade. To accomplish this objective, a detailed phenotypic and molecular study of mutants affected in flowering time and light signalling responses will be performed. Furthermore, interactions between the light signalling pathway mediated by PhyB and PIFs with photoperiodic pathway components will be explored.

3. Materials and methods

3.1 Plant material

All mutant lines are in the Columbia background (Col-0), except *co-2* which is in the *Landsberg erecta* background (Ler-0, (Koornneef *et al.* 1991, Putterill *et al.* 1995).

Mutant lines in Col-0 background used were: *ft-10* (Yoo *et al.* 2005), *tsf-1* (Yamaguchi *et al.* 2005), *ft-10 tsf-1* (Jang *et al.* 2009), *co-10* (Laubinger *et al.* 2006), *pif4-101* (Lorrain *et al.* 2008), *pif4-2* (Leivar *et al.* 2008a), *pif5-3/pil6-1* (Fujimori *et al.* 2004), *pif4-2 pif5-3* (Leivar *et al.* 2012), *pif1-1 pif3-3 pif4-2 pif5-3* (Leivar *et al.* 2008b, Shin *et al.* 2009b), *svp-41* (Hartmann *et al.* 2000), *svp-41 co-10*, *ft-10 tsf-1 svp-41* (Jang *et al.* 2009), *gi-2* (Rèdei 1962), p*PIF4:PIF4-citrine-3HA pif4-101* (Hornitschek *et al.* 2012), p*SVP::SVP:GFP svp-41 flc-3 FRI* (Mateos *et al.* 2015), p*KNAT1::SVP svp-41* (Andres *et al.* 2014), p$FT_{1.8\ kb}$*::GUS* lines # 1.6 and # 2.2, p$FT_{1.8\ kb}$*::GUS* (Adrian *et al.* 2010), p*CO::HA:CO co-10* (Sarid-Krebs *et al.* 2015), p*35S::PIF4:HA*, p*35S::PIF5:HA* (Lorrain *et al.* 2008), *pif7-1*, *pif7-2*, *pif7-1phyB-9* (Leivar *et al.* 2008a), p*35S::PIF7:Flash* (9xMyc-6xHis-3xFlag) *pif7-2* (Li *et al.* 2012a), *phyB-9* (Reed *et al.* 1993), *svp-41 pif4-101* double mutant was generated by crossing *svp-41* with *pif4-101*, *svp-41 co-10 pif4-101* was generated by crossing *svp-41 pif4-101* with *svp-41 co-10*. Several of the lines used in this work were previously generated in our lab, i.e. p$FT_{1.8\ kb}$*::GUS* # 1.6 p*35S::PIF4:HA*, p$FT_{1.8\ kb}$*::GUS* # 2.2 p*35S::PIF4:HA*, p$FT_{1.8\ kb}$*::GUS* # 1.6 p*35S::PIF5:HA* and p$FT_{1.8\ kb}$*::GUS* # 2.2 p*35S::PIF5:HA* were obtained by crossing p$FT_{1.8\ kb}$*::GUS* # 1.6 or p$FT_{1.8\ kb}$*::GUS* # 2.2 with p*35S::PIF4:HA* or p*35S::PIF5:HA*, *co-10 pif4-2*, *co-10 pif4-2 pif5-3* mutants were generated by crossing *co-10* with *pif4-2 pif5-3* double mutant , p*SUC2::PIF4* line was previously generated in the lab with *PIF4* cDNA obtained from the REGIA collection in GATEWAY compatible vectors (Paz-Ares and Regia 2002), p*SUC2::PIF4 co-10* line was generated by crossing p*SUC2::PIF4* with *co-10*, *phyB-9 pif4-2*, *phyB-9 pif5-3*, *phyB-9 pif4-2 pif5-3* were generated by crossing *phyB-9* with *pif4-2 pif5-3*, *phyB-9 co-10*, *phyB-9 ft-10 tsf-1* were generated by crossing *phyB-9* with *co-10* and *ft-10 tsf-1*, respectively,

p*SUC2::CO*, p*SUC2::CO pif4-2*, p*SUC2::CO pif5-3*, p*SUC2::CO pif4-2 pif5-3* lines were generated by crossing p*SUC2::CO* with *pif4-2*, with *pif5-3* and with *pif4-2 pif5-3*, respectively.

Primers used to genotype the mutants are listed in Table 3.1.

Table 3.1 Primers for genotyping

Allele	**Name**	**Sequence (5´ - 3´)**	**Annealing temperature**	**Comment**
co-10	CO-1-121-R	GTGGTGAGTAGTGGTCATGGAGC	55 °C	WT allele: CO-1-121-F - CO-1-121-R Mutant allele: CO-1-121-F - SAIL-LB3
	CO-1-121-F	ATGTTGAAACAAGAGAGTAACG		
	SAIL-LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC		
pif4-101	VF11	CTCGATTTCCGGTTATGG	55 °C	WT allele: VF11-VF12 Mutant allele: VF12-VF13
	VF12	CAGACGGTTGATCATCTG		
	VF13	GCATCTGAATTTCATAACCAATC		
svp-41	SVP-41 F	TGTGGGTACCATAACATGAGGA	60 °C	Digestion with NlaIV after amplification. WT allele: 1 band. Mutant allele: 2 bands.
	SVP-41 R	AAAGCTCAACTCTCTACACAGGA		

Table 3.1 Primers for genotyping (continuation)

Allele	Name	Sequence (5´ - 3´)	Annealing temperature	Comment
pif4-2	PIF4-2 F	CAGATCATCTCCGACCGGTTT	58 °C	WT allele: PIF4-2 F - PIF4-2 R Mutant allele: SAIL-LB3 - PIF4-2 R
	PIF4-2 R	CGACGGTTGTTGACTTTGCTG		
	SAIL-LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC		
pif5-3/pil6	PIL6-RP	ATCTTCCATCCATTCAGAGGC	58 °C	WT allele: PIL6-RP - PIL6-LP Mutant allele: SALK-LBb1 - PIL6-RP
	PIL6-LP	TGTTCCTTCCATAGCTGCAACC		
	SALK-LBb1	GCGTGGACCGCTTGCTGCAACT		

3.2 Growth conditions

Before starting every experiments, seeds were stratified on soil or petri dished at 4 °C for 3 days in dark.

For warm temperature experiments, plants were grown on soil under controlled SD conditions (8 h light/16 h dark) at 21 °C or 27 °C, or under LDs (16 h light/8 h dark). For GUS staining experiments, plants were grown on Murashige and Skoog media. The photosynthetically active radiation (PAR) was 100 - 150 $\mu mol \cdot m^{-2}s^{-1}$ in all conditions.

For shade avoidance experiments, growth cabinets with LED technology (Snijder) were used. Plants were grown on soil under controlled LD conditions (16 h light/8 h dark), under SD conditions (8 h light/16 h dark) or under intermediate photoperiods (12 h light/12 h dark) at 21 °C. The PAR provided by fluorescent light tubes was 85 - 100

$\mu mol \cdot m^{-2}s^{-1}$ for experiments performed under 100 % of the growth chamber capacity. For experiments performed under 65 % of the growth chamber capacity, light measurements rendered values around 40 $\mu mol \cdot m^{-2}s^{-1}$. For the enriched FR condition, the white light (WL) was supplemented with FR light from LED technology. WL, R and FR light intensities were measured using SpectraSuit® Spectrometer from Ocean Optics. R light was measured between 650 and 680 nm and FR light between 720 and 750 nm. Table 3.2 provides information of the different ratios between R and FR light used through this work.

Table 3.2 R:FR light ratios

WL / FR	R:FR ratio
100% / 100%	0.06
100% / 65%	0.12
65% / 100%	0.03
65% / 65%	0.05

3.3 Molecular cloning

To generate p*35S::PIF4:CFP* vector to perform FRET assays, *PIF4* cDNA was amplified from *A. thaliana* cDNA with overhangs compatible for GATEWAY cloning using primers listed in Table 3.3. Amplified *PIF4* cDNA was subcloned by a BP reaction into p*DONR201* (Invitrogen), according to manufacturer's instructions. After confirmation by Sanger sequencing, *PIF4* cDNA was recombined into the destination vector p*35S::GW:CFP* by an LR reaction, according to manufacturer's instructions.

To generate p*35S::PIF4:HA* vector used for co-immunoprecipitation experiments, *PIF4* cDNA was recombined by LR reaction into p*35S::GW:HA* destination vector.

The binary vectors were transformed into *A. tumefaciens* GV3101 by electroporation.

p*35S::CO:YFP* construct used for Co-IP and FRET experiments was previously generated in the laboratory.

The constructs for the Y2H experiments were previously generated in the laboratory by Yasuyuki Takahashi.

Table 3.3 Primers for cloning

Gene	Name	Sequence (5´ - 3´)
PIF4	V03-PIF4-GW-FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGG AACACCAAGGTTGGAG
	V04-PIF4-GW-RE-C-term	GGGGACCACTTTGTACAAGAAAGCTGGGTGTGGTC CAAACGAGAACCGTC

3.4 DNA extraction and genotyping

In order to genotype the crosses between plants of different genetic background, the F2 generation was grown in trays of 96 pots, leaf material harvested in 96-well plates, grinded in presence of RLT buffer and DNA extracted using Biosprint 96 (QIAGEN) according to manufacturer's instructions.

Polymerase Chain Reactions (PCR) for genotyping were performed in 96-well plates, according to standard lab protocols using Taq DNA Polymerase (Invitrogen). The annealing temperature for each primer pair is indicated in Table 3.1.

3.5 Flowering time analysis

Flowering time was determined by counting the number of rosette leaves (at bolting time) and cauline leaves on the main stem for the number of plants indicated for each experiment (Total leaf number = Rosette leaves + Cauline leaves). For most flowering time plots of the warm temperature experiments the displayed values were generated by combining the flowering time scoring of several independent experiments. For flowering

time plots of the shade avoidance experiments the graphs shown were performed with data obtained from a single experiment. Statistical analyses were performed with the same pool of data used for flowering time graphs. The software used for the analysis was SigmaStat 3.5.

3.6 Hypocotyl length measurements

Seedlings were grown for 10 days on soil and then transferred to MS-agar plates to perform the measurements. The seedlings were placed horizontally lying on the media and pictures of the plates were taken to perform the measurements using ImageJ. Millimeter paper included on the picture was use as reference. Measurements were done from the junction between the root and the hypocotyl until the point where the cotyledons are placed.

3.7 Analysis of gene expression levels (Quantitative Real-Time PCR)

For gene expression analysis, at least 20 whole seedlings per sample were harvested in 1.5 µL Eppendorf tubes and frozen in liquid N_2. Frozen samples were ground using TissueLyser (Qiagen). Total RNA was extracted using RNAeasy extraction Kit (Qiagen) following the manufacturer's instructions. Isolated RNA was quantified using NanoDrop (Thermo Scientific) and treated with DNA-free DNase (Ambion) to remove residual genomic DNA following the manufacturer's instructions. Approximately 2 and 4 µg of total RNA were used for cDNA synthesis of samples harvested under shade and under 21°C-SD and 27°C-SD, respectively. Oligo-dT primer and SuperScript II or III (Invitrogen) were used for reverse transcription. SuperScript III was used for samples harvested under 21°C-SD and 27°C-SD conditions, as the expression of some of the genes of interest was very low and it has a higher efficiency than SuperScript II and SuperScript II was

used for samples harvested under shade. cDNA was diluted to 200 µL and to 150 µL with water for samples under shade and warm temperature, respectively and 3 µL were used as template for qRT-PCR. iQ SYBR Green Supermix (BIO-RAD) was used for warm temperature samples and *Taq* DNA Polymerase (Invitrogen) and EvaGreen were used for samples harvested under shade. Transcript levels were quantified by in a LightCycler 480 instrument (Roche). The PCR program used for all the measurements was as follows:

95°C	5 min		
95°C	20 s	⎡	
60°C	20 s	⎢	x 45
72°C	20 s	⎣	

In order to convert the florescence intensity measurements in gene expression values, calibration curves were used. Aliquots of random RNA samples of the working set were collected in a single Eppendorf tube after DNase treatment and cDNA was synthesized. A dilution series of this cDNA was used to build the standard curve and obtain a PCR efficiency for each primer pair. Calibration against the standard curve resulted in arbitrary concentration values. Relative expression values were obtained by using PHOSPHATASE 2A (*PP2AA3*) (AT1G13320) as reference gene (Czechowski *et al.* 2005). For every warm temperature experiment, normalization of each expression value to one expression value in the same experiment (usually the highest) was performed. Final plots were obtained by determining the average of the normalized values from several biological replicates. SEs were calculated from averaged values. For shade avoidance experiments, the result of individual experiments is shown.

Primers used for Quantitative Real-Time PCR in this study are listed in Table 3.4.

Table 3.4 Primers for Quantitative Real-Time PCR

Gene	Name	Sequence (5´ - 3´)
CO	CO-qRT-F	TAAGGATGCCAAGGAGGTTG
	CO-qRT-R	CCCTGAGGAGCCATATTTGA
FT	FT-qRT-F	CGAGTAACGAACGGTGATGA
	FT-qRT-R	CGCATCACACACTATATAAGTAAAACA
TSF	TSF-qRT-F	CTCGGGAATTCATCGTATTG
	TSF-qRT-R	CCCTCTGGCAGTTGAAGTAA
PIF4	qRT-PIF4-1F	CGGAGTTCAACCTCAGCAGT
	qRT-PIF4-1R	ACCGGGATTGTTCTGAATTG
PP2AA3	PP2AA3-F	CAGCAACGAATTGTGTTTGG
	PP2AA3-R	AAATACGCCCAACGAACAAA
SOC1	qRT-SOC1_F1	TGATGAAGAGAGTAGCCCAAG
	qRT-SOC1_R1	TGAGAGAGAGAGAGTGAGAGAGAAA
IAA29	qRT-IAA29-F	CCGAATATGAAGATTGCGACA
	qRT-IAA29-R	TGCACACGGTCGATCTCTAA
PIF7	V16-PIF7 qRT-PCR	TGGCCACAGCGTCACTGCAA
	V17-PIF7 qRT-PCR	TGCTCGTCCCCGTCGTCCAT
AP1	Y28	ATGAGAGGTACTCTTACGCCGA
	Y29	CAAGTCTTCCCCAAGATAATGC
FUL	FUL-F	TGCTCCAACTCTTCTTCAGTTCTTC
	FUL-R	TGGAGGAGGTTACGCAGTATTGA

3.8 GUS histochemical analysis

GUS staining was performed as previously described (Adrian *et al.* 2010). Seedlings were harvested and fixed on ice with 90 % acetone for 30 min, then vacuum infiltrated with GUS staining buffer and incubated until detection of signal at 37°C in the same

buffer. GUS staining buffer composition: X-Gluc, 0.5 mg/mL; Triton X100, 0.1 %; ferricianate solution (K3Fe(CN)6.3H2O, 4.22 % and K3Fe(CN)6.3H2O, 3.92 %), 0.5 mM; phosphate buffer (Na2HPO4, 57.7 mM and NaH2PO4, 42.3 mM), 50 mM. Clearing was performed in ethanol (30 % 1 hour and 70 % until clearance of leaves). Samples were preserved in 70 % ethanol.

3.9 Western blot analysis and nuclear protein quantification

Nuclear proteins were isolated from around 30 seedlings per time point. Samples were ground inside a 1.5 ml tube using a TissueLyser (Qiagen) and frozen adaptors. The ground tissue was resuspended in Nuclear Protein Isolation Buffer (NPIB: Tris pH 6.8, 0.02 M; Sucrose, 50 $g \cdot L^{-1}$; Glycerol, 40 %; Triton X100, 0.8 % v/v; MgCl2, 0.02 M; 2-Mercaptoethanol, 0.08 %; Protease Inhibitor Cocktail (PIC); Dithiothreitol (DTT), 1 mM; Phenylmethylsulfonylfluorid (PMSF), 1.34 mM) pre-cooled at 4 °C. After low speed centrifugation at 4 °C, the pellet was washed 5 times with the NPIB or until the supernatant became clear. Pellets were resuspended in 2X Laemmli buffer (Tris-HCl pH 6.8, 0.125 M; 2-Mercaptoethanol, 10 %; Sodium dodecyl sulfate (SDS), 4 %; Sucrose, 10 %; Bromophenol blue, 0.015 %) and boiled for 10 min at 96 °C. Extracted proteins were resolved in 10 % SDS-PAGE and transferred overnight (ON) at 50 mV in Transfer Buffer (190 mM Glycine; 0.25 mM Tris; 0.05 % SDS; 20 % Methanol) to a PVDF membrane (Merck Millipore). The membrane was washed 2 times for 10 min with TBS (20 mM Tris-HCl pH 7.5; 150 mM NaCl) containing sodium azide (0.01 % w/v sodium azide). It was blocked for 1 h with TBS milk (TBS; 5 % w/v skim milk). The membrane was then incubated for 2 h with TBS milk and sodium azide containing the primary antibody. Immunodetection of 3HA:CO and PIF4:3HA was done with anti-HA monoclonal antibody, 1:2000 dilution, from rat (Roche, 11867423001); SVP-GFP was detected with anti-GFP monoclonal antibody, 1:2000 dilution, from mouse (Roche, 11814460001) and

H3 histone was detected with anti-histone H3 polyclonal antibody, 1:30000 dilution, from rabbit (Abcam, ab1791). The membrane was washed 4 times for 15 minutes with TBST (TBS; 0.1 % Tween 20) and incubated for 1 h with TBST containing the secondary antibody. Secondary antibodies used were Horseradish peroxidase conjugates: anti-rat HRP from goat, 1:5000 dilution (Sigma, 9037), anti-rabbit HRP in donkey, 1:20000 dilution, (Abcam ab97064). After washing the membrane 4 times for 15 minutes with TBST, chemiluminescence detection of the proteins was done by using SuperSignal West Femto and SuperSignal West Dura kits (Thermo Scientific) in a LAS-4000 imaging system (Fuji). Technical replicates were loaded on two independent gels. Protein quantification was done with ImageJ Software using the images captured with the lowest possible exposure to avoid saturation. Each time point was normalized to the corresponding histone counterpart. Values were normalized against the highest value in each technical replicate. The averages of 2 technical replicates for each of 2 biological replicates were combined to describe the biological average and variation. SEs were calculated from averaged values.

3.10 Co-Immunoprecipitation assays

For protein interaction analysis, 4 weeks-old transiently transformed *Nicotiana benthamiana* (*N. benthamiana*) leaves were used. 50 mL liquid culture of *A. tumefaciens* harboring p*35S::CO:YFP* or p*35S::PIF4:HA* were grown ON, centrifuged and the cell pellet resuspended in an infiltration buffer (MES-KOH pH 5.6, 10 mM; MgCl2, 10 mM) to final optical density (OD) of 1. Cell suspension supplemented with 1:1000 of Acetosyringone 150 mM was incubated for 1 h at room temperature (RT). Leaves of *N. benthamiana* were infiltrated with a 1 mL syringe. After infiltration, plants were incubated for 3 days at RT and leaf material was collected and frozen in liquid N_2.

For co-immunoprecipitation assay, approximately 400 mg of grinded material were resuspended in cooled Nuclear Extraction Buffer (NEB: Tris-HCl pH 7.4, 0.05 M; $MgCl_2$, 0.02 M; Sucrose, 5 $g \cdot L^{-1}$; Glycerol, 40 %; NP40, 0.5 % v/v; PIC; DTT, 1 mM; PMSF, 2 mM; MG132, 0.05 mM). After centrifugation the pellet was washed 4 times with NEB and resuspended in Sonication Buffer (SB: Tris-HCl pH 7.4, 0.05 M; NaCl, 0.05 M, NP40, 0.5 % v/v; PIC; DTT, 1 mM; PMSF, 2 mM; MG132, 0.05 mM). Samples were sonicated for 5 min on intervals of 15 s sonication with 15 s of break. Samples were diluted 3 times with SB supplemented with NaCl 0.1 M and incubated for 15 min at 4 °C. After centrifugation at maximum speed, 20 % of the sample was concentrated in centrifugal filter unit 30 K (Amicon ultra) and mixed with 2X Laemmli Buffer to use as input. The rest of the sample was incubated with anti-GFP monoclonal antibody from mouse (Roche, 11814460001) at 4 °C for 1 h in circular rotor. Protein G sepharose 4 Fast Flow (GE Healthcare) were equilibrated with SB, added to the samples and incubated for 3 h at 4 °C in circular rotor. Samples were centrifuged at low speed for 30 s and the beads washed 5 times with Washing Buffer (WB: Tris-HCl pH 7.4, 0.05 M; NaCl, 0,15 M, NP40, 0.5 % v/v). Beads were resuspended in 2X Laemmli Buffer and boiled at 96°C for 10 min.

Input and immunoprecipitated samples were loaded in duplicate and resolved in 10 % SDS-PAGE. Transference to a PVDF membrane (Merck Millipore) and western blot analysis were performed as described above. Immunoprecipitated CO:YFP protein was detected with anti-GFP monoclonal antibody from mouse (Roche, 11814460001) and co-immunoprecipitated PIF4:HA was detected with anti-HA monoclonal antibody from rat (Roche, 11867423001). Secondary antibodies used were Horseradish peroxidase conjugates: anti-rat HRP from goat, 1:5000 dilution (Sigma, 9037) and anti-mouse HRP from goat, 1:10000 dilution (Abcam, AB97265). Chemiluminescence detection of the proteins was done by using SuperSignal West Femto and SuperSignal West Dura kits (Thermo Scientific) in a LAS-4000 imaging system (Fuji).

3.11 Yeast two Hybrid assay (Y2H)

For every Y2H assay, newly prepared competent yeast cells were used (yeast strain PJ694). 2 mL of freshly grown yeast was inoculated into 100 mL YPAD media (Bactopeptone, 20 g/L; Yeast extract, 10 g/L; Glucose, 20 g/L; Adenine-hemisulfate, 0.01 %; Agar, 16 g/L) and incubated for 5 – 6 h at 28 °C until OD = 1. The yeast culture was equality divided into 4 falcon tubes and centrifuged at 4000 rpm for 5 min. The cell pellet was washed with water and centrifuged at 4000 rpm for 5 min. The cells were resuspended in 1 mL LiAc (100 mM, pH 7.5), transferred to 1.5 mL Eppendorf tubes and centrifuged at 6000 rpm for 5 min. Cells were then resuspended in 500 µL LiAc (100 mM, pH 7.5). After a new centrifugation at 6000 rpm for 5 min cells were resuspended in 1 mL of transformation mix (40 % PEG 4000; 0.1 M LiAc; TE buffer pH 7.5 - 10 mM Tris, 1 mM EDTA -). 20 µL of salmon sperm DNA (10 mg/mL) was added. 100 µL of this mix was added to a previously prepared plasmid mix. The plasmid mix contained a pair of vectors expressing the proteins to be tested as well as pairs with the plasmids expressing the proteins of interest and the corresponding empty vectors (Table 3.5). The plasmid mix and the transformation mix were incubated 25 min at 30 °C and then 25 min at 42 °C. 1 mL sterile water was added to the tube and centrifuged 5 min at 6.000 rpm. The pellet was resuspended in 200 µL of sterile water and transformed cells plated in drop out medium (N_2 Base 6.7 g/L; Glucose 20 g/L; Drop out mix [-Leucine (-Leu), - Tryptophan (-Trp)] or Drop out mix [-Leu, -Trp, - Histidine (-His)]; Agar, 20 g/L) (-Leu, -Trp) to select transformed cells. Plates were incubated for 3 – 4 days at 30 °C until colonies were visible. A mix of grown colonies in the same plate was done in water and serial dilution of the cell mix performed. The dilutions were then plated in drop out medium -Leu, -Trp; -Leu, -Trp, -His and -Leu, -Trp, -His plus 5 mM 3AT. Plates were incubated for 3 – 4 days

at 30 °C until colonies were visible and colonies growth in plates -Leu, -Trp, -His and -Leu, -Trp, -His plus 5 mM 3AT traduced into protein interaction information.

The DNA fragments cloned into pDEST22 and pDEST32 generate fusion proteins fused to an activation domain (pray) and a DNA binding domain (bait), respectively.

Table 3.5 Construct combinations for Y2H experiments

	AD clone #	BD clone #	AD	BD
1	368	382	CO full length/pDEST22	PIF4 full length/pDEST32
2	374	376	PIF4 full length/pDEST22	CO full length/pDEST32
3	368	pDEST32	CO full length/pDEST22	pDEST32
4	374	pDEST32	PIF4 full length/pDEST22	pDEST32
5	pDEST22	376	pDEST22	CO full length/pDEST32
6	pDEST22	382	pDEST22	PIF4 full length/pDEST32

3.12 Förster resonance energy transfer-acceptor photo bleaching assay (FRET-A.PB)

N. benthamiana leaves were infiltrated with *A. tumefaciens* containing p*35S::PIF4:CFP* or p*35S::CO:YFP* as described before for co-Immunoprecipitation assays. Three days after infiltration protein-protein interaction was assayed by FRET - acceptor photobleaching (APB). Transiently transformed *N. benthamiana* cell nuclei were imaged with LSM 780 (Carl Zeiss). After photobleaching of the acceptor (CO:YFP) the change in fluorescence intensity of the donor (PIF4:CFP) was analysed. Pre and post-bleaching images were compared.

4. Regulation of flowering time by warm ambient temperatures in *A. thaliana*

Ambient temperature is one of the environmental factors that are detected by plants as indicator of the changing seasons. In addition, ambient temperature varies on diurnal basis, particularly between day and night. Therefore it is important that plants can adapt and respond to these temperature changes. In *A. thaliana*, variations in ambient temperature affect several aspects of plant development, such as germination, growth rate, and flowering time (Strand *et al.* 1999, Schmuths *et al.* 2006, Lee *et al.* 2007, Toh *et al.* 2008, Koini *et al.* 2009, Kumar *et al.* 2012). Warm ambient temperatures greatly accelerate flowering, even under non-inductive photoperiods (Balasubramanian *et al.* 2006, Lee *et al.* 2007, Kumar and Wigge 2010, Kumar *et al.* 2012, Lee *et al.* 2013, Pose *et al.* 2013, Galvao *et al.* 2015). The regulation of flowering by the photoperiod and thermosensory pathways converge on the transcriptional regulation of the floral integrator *FT*, but the interactions between these two pathways are poorly understood. Here, the intersection between the photoperiodic and ambient temperature pathways are analysed, and the relative contributions in controlling flowering at high temperatures of the transcription factors involved in this response evaluated.

4.1 Timing and amplitude of *FT* transcription under 27°C-SDs

Transcriptional activation of *FT* in warm temperatures accelerates flowering under SDs (Balasubramanian *et al.* 2006, Kumar *et al.* 2012). Under LDs *FT* activation occurs in the light 12-16 h after dawn (Suarez-Lopez *et al.* 2001). To describe the diurnal pattern of *FT* transcription under warm SDs in Col-0 wild-type plants, *FT* mRNA was analysed through a 24 h time course in plants grown at 21 °C or 27 °C under SDs of 8 h light. *FT* mRNA levels peaked 8 h after dawn (Zeitgeber 8 [ZT8]) in the 27°C-SD time course, whereas only very low *FT* mRNA levels were detected in seedlings grown under 21°C-SD (Figure 4.1a). The contribution of *FT* to flowering under 27°C-SD was then assessed using the null *ft-10* allele.

Col-0 plants grown under 27°C-SD flowered much earlier than those grown under 21°C-SD, producing on average 26 and 65 leaves, respectively (Figure 4.1b). In comparison to Col-0, *ft-10* mutants were strongly delayed in flowering at 27 °C although they were still earlier flowering than Col-0 at 21 °C (Figure 4.1b). These results support the idea that *FT* is required for full acceleration of flowering under these conditions, as previously shown (Balasubramanian *et al.* 2006, Kumar *et al.* 2012). By contrast, a null mutant of *TSF* (*tsf-1*) did not show any delay in flowering compared to Col-0 under 27°C-SD (Figure 4.1b). Although *TSF* mRNA abundance was increased at 27°C-SD (Figure 4.1c), the genetic data suggest that *FT* could compensate for loss of *TSF* activity under these conditions. However, the double mutant *ft-10 tsf-1* flowered moderately later than *ft-10* under 27°C-SD (Figure 4.1b), indicating that *TSF* plays a significant role under these conditions in the absence of functional *FT*, as previously observed in plants grown under LDs (Yamaguchi *et al.* 2005, Jang *et al.* 2009). Nevertheless, even the double mutant *ft-10 tsf-1* flowered slightly earlier under 27°C-SD than 21°C-SD, suggesting that these plants retained some responsiveness to 27 °C. In summary, the acceleration of flowering time of Col-0 plants under 27°C-SD largely depends on *FT* and *TSF*, and *FT* mRNA exhibits a diurnal pattern of expression with a peak at ZT8.

Col-0 plants growing under 21°C-LD flowered with a similar number of leaves to those growing under 27°C-SD (Figure 4.1d). Therefore, the levels of *FT* mRNA were directly compared in plants exposed to SDs and high temperatures with those exposed to LDs (Figure 4.1e). As expected, the maximum accumulation of *FT* mRNA in Col-0 seedlings growing under 21°C-LD and 27°C-SD occurred at ZT16 and ZT8, respectively (Figure 4.1a and 4.1e). However, under 21°C-LD the peak level of *FT* mRNA (ZT16) was at least 10 fold higher than under 27°C-SD (ZT8) (Figure 4.1e). Therefore, although under these two conditions flowering time was similar, the absolute levels of *FT* mRNA differed tremendously. This suggests that,

although *FT* is required for early flowering under 27°C-SD, its transcriptional activation alone might not be sufficient to explain the extreme early flowering observed.

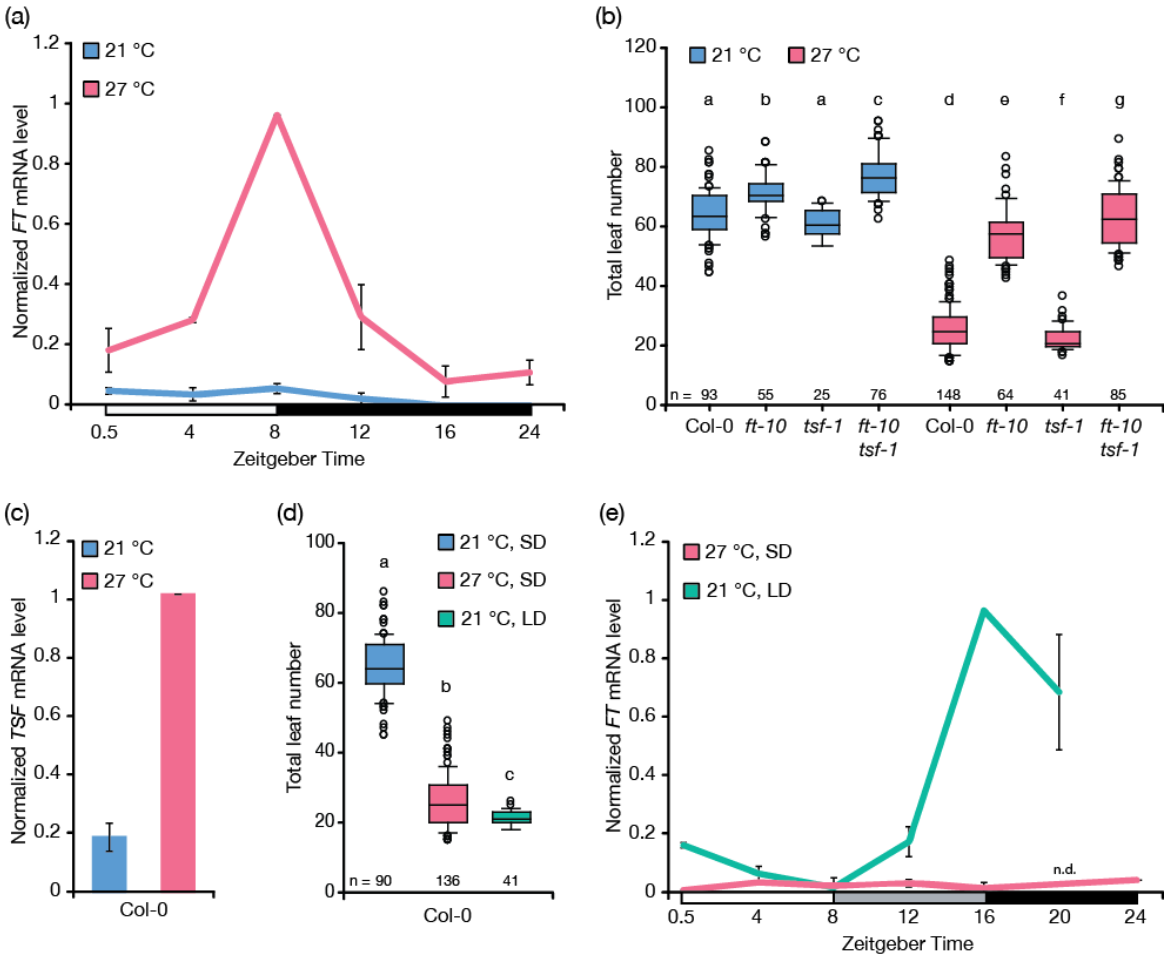

Figure 4.1 Role of *FT* on the flowering response to warm temperature under SDs. (a) *FT* mRNA expression time course in 14-day-old Col-0 seedlings grown under 21°C-SD and 27°C-SD. (b) Flowering time of plants grown under 21°C-SD and 27°C-SD. (c) *TSF* mRNA expression at ZT8 in 14-day-old Col-0 seedlings grown under 21°C-SD and 27°C-SD. (d) Flowering time of Col-0 plants grown under 21°C-SD, 27°C-SD and 21°C-LD. (e) *FT* mRNA expression profile in 14 and 10-day-old Col-0 seedlings grown under 27°C-SD and 21°C-LD, respectively. At this stage seedlings in both conditions were at the same developmental stage. In (a) and (e), seedlings were harvested every 4 h for 24 h and *FT* mRNA expression was measured by qRT-PCR; error bars are standard errors (SEs) of three and two (27°C-SD ZT16, 27°C-SD ZT24, 21°C-SD ZT16) independent biological replicates in (a) and two independent biological replicates in (e). For time points 21°C-SD ZT24 in (a) and 27°C-SD ZT24 in (e) n = 1. Data point 27°C-SD ZT20 in (e) was not determined. In (c) error bars are SEs of eight independent biological replicates. In (b) and (d), letters indicate statistical groups determined with a two-way analysis of variance (ANOVA) and multiple comparisons with the Holm–Sidak method. Multiple comparisons were performed within temperatures and within genotypes. Groups were considered statistically different when $P \leq 0.05$.

4.2 *PIF4* weakly promotes flowering under 27°C-SD through the transcriptional activation of *FT* in the vascular tissue

Under 27°C-SD, PIF4 induce flowering by binding to the *FT* promoter and activating transcription, while plants homozygous for the *pif4-101* allele growing in the same environment were strongly delayed in flowering compared to Col-0 (Kumar *et al.* 2012). Under our 27°C-SD and 21°C-SD conditions, the flowering times of *pif4-101* and *pif4-2* mutants were measured in several experiments that included large numbers of plants of each genotype (Figure 4.2a). Both mutants were significantly later flowering than Col-0 controls under 27°C-SD (Figure 4.2a and 4.2b), but flowered much earlier than Col-0 or the *pif4* mutants grown under 21°C-SD (Figure 4.2a). Because of the variability of the flowering response under 27°C-SD, occasional *pif4* mutants flowered within the range of Col-0 under 21°C-SD (Figure 4.2a). However, under our conditions neither *pif4-101* nor *pif4-2* mutants showed as strong a suppression of the early-flowering response under 27°C-SD as previously described (Figure 4.2a) (Kumar *et al.* 2012). The effect on flowering time of the loss of function of other members of the *PIF* family was tested, because of their potential functional redundancy with *PIF4*. Interestingly, the *pif5-3* single mutant flowered at a similar time to *pif4* mutants, whereas the double mutant *pif4-2 pif5-3* flowered later than the single *pif4-2* (Figure 4.2a), suggesting that *PIF5* also has a role in the promotion of flowering under warm SD. By contrast, *PIF1* and *PIF3*, two other members of the family, did not seem to play a role in flowering regulation under SDs at warm temperature, because flowering of the quadruple mutant *pif1-1 pif3-3 pif4-2 pif5-3* was not significantly delayed compared to *pif4-2 pif5-3* (Figure 4.2a).

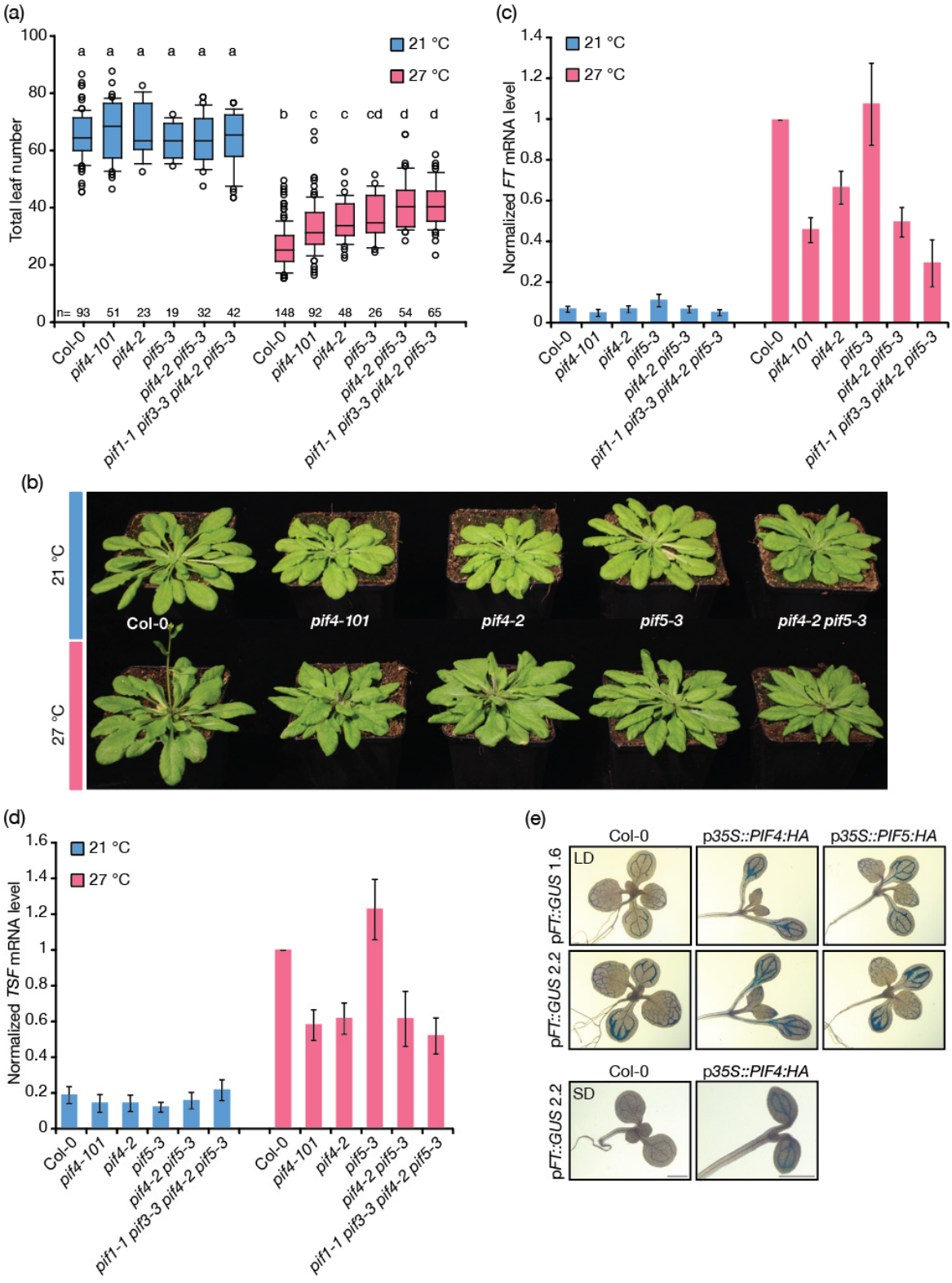
(a)
Total leaf number
21 °C
27 °C
a a a a a a
b c c cd d d
n= 93 51 23 19 32 42 148 92 48 26 54 65
Col-0
pif4-101
pif4-2
pif5-3
pif4-2 pif5-3
pif1-1 pif3-3 pif4-2 pif5-3
(c)
Normalized FT mRNA level
21 °C
27 °C
(b)
21 °C
27 °C
Col-0
pif4-101
pif4-2
pif5-3
pif4-2 pif5-3
(d)
Normalized TSF mRNA level
21 °C
27 °C
(e)
Col-0
p35S::PIF4:HA
p35S::PIF5:HA
pFT::GUS 1.6
LD
pFT::GUS 2.2
SD

Figure 4.2 Mutations in *PIFs* affect flowering time and *FT* and *TSF* expression under 27°C-SD. (a) Flowering time of plants grown under 21°C-SD and 27°C-SD. (b) Pictures of representative plants of each genotype grown under 21°C-SD and 27°C-SD. (c) *FT* mRNA expression in seedlings grown under 21°C-SD and 27°C-SD. (d) *TSF* mRNA expression in seedlings grown under 21°C-SD and 27°C-SD. (e) GUS staining of two independent transgenic lines harboring the p*FT::GUS* fusion construct (1.6 and 2.2) in Col-0, p*35S::PIF4:HA* or p*35S::PIF5:HA* backgrounds. Plants were grown for 10 days under LDs or SDs and harvested at ZT16 and ZT4, respectively, before GUS staining (7 h staining time) (the GUS-staining experiment pictures shown were taken by Yasuyuki Takahashi). In (c) and (d), seedlings were grown for 14 days and harvested 8 h after dawn (Zeitgeber 8, ZT8). *FT* and *TSF* mRNA expression was measured by qRT-PCR; error bars are SEs of at least six and five independent biological replicates, respectively. Statistical analysis as described in Figure 4.1.

Whether the flowering behaviour of these single and higher order *pif* mutants is correlated with changes in *FT* mRNA level was examined by quantifying the abundance of *FT* mRNA at ZT8, when the maximum peak in mRNA expression is observed, in seedlings grown under 21°C-SD and 27°C-SD (Figure 4.2c). As expected, a significant reduction in *FT* mRNA level at ZT8 was observed in *pif4-2* and *pif4-101* single mutants compared to Col-0 at 27 °C but not 21 °C. Although *pif5-3* mutants were late flowering, no reduction in *FT* mRNA levels was detected in single mutants, suggesting that PIF5 might mainly affect *FT* mRNA at other stages of development or that it affects flowering by another route. However, the reduction in *FT* mRNA observed in *pif4-2* was enhanced in seedlings of the double mutant *pif4-2 pif5-3* (Figure 4.2c) and was enhanced slightly further in the quadruple mutant *pif1-1 pif3-3 pif4-2 pif5-3* (Figure 4.2c), although this reduction was apparently not sufficient to cause a significant difference in flowering time (Figure 4.2a).

TSF mRNA was expressed in a similar pattern to *FT* mRNA, and the peak observed in Col-0 plants was suppressed in *pif4-101* and *pif4-2* single mutants (Figure 4.2d). *TSF* mRNA levels were also suppressed in *pif4-2 pif5-3* double mutants as well as *pif1-1 pif3-3 pif4-2 pif5-3*, where it was present at similar levels to those observed in *pif4* single mutants. Similar to *FT*, the levels of *TSF* mRNA expression were not reduced in *pif5-3* mutants compared to

Col-0 (Figure 4.2d), supporting the idea that the expression of *FT* and *TSF* could be affected by *PIF5* at other stages of development or that *PIF5* affects flowering by another route.

In order to define the effect of *PIF4* overexpression on the spatial pattern of *FT* expression, a construct that facilitates misexpression of *PIF4* from the constitutive *Cauliflower mosaic virus 35S* promoter (p*35S*; p*35S::PIF4:HA*) (Lorrain *et al.* 2008) was introduced into plants harbouring p$FT_{1.8\ kb}$::GUS (Adrian *et al.* 2010) (p*35S::PIF4:HA;* p*FT::GUS*). Under SDs and LDs, GUS expression was detected only in the vascular tissue (Figure 4.2e).A similar GUS expression pattern was observed in *PIF5* over-expressing lines (p*35S::PIF5:HA*) (Lorrain *et al.* 2008) under LDs (Figure 4.2e). These results indicated that although *PIF4* is broadly expressed in leaves of wild-type plants (Kumar *et al.* 2012) and presumably p*35S::PIF4:HA* transgenics, its effect on *FT* mRNA induction occurs only in the vascular tissue, where *FT* is normally expressed. The effects of warm temperature on *FT* mRNA induction and the spatial pattern of expression were also evaluated. Seedlings containing p$FT_{1.8\ kb}$::GUS and grown under 27°C-SD and 21°C-SD were used. However, no GUS expression was detected under either condition (data not shown). As the qPCR analysis indicated (Figure 4.1e), the levels of expression of *FT* under 27°C-SD are very low, therefore, the GUS staining was probably not sensitive enough to detect p$FT_{1.8\ kb}$::GUS expression under these conditions.

4.3 CO is required for full activation of *FT* transcription and promotion of flowering under 27°C-SD

The data presented above suggested that, besides *PIF4* and *PIF5*, there must be other factors promoting early flowering and *FT* transcription under 27°C-SD. CO induces *FT* transcription specifically in the vascular tissue under LDs (An *et al.* 2004, Adrian *et al.* 2010), as was observed for p*35S::PIF4:HA* (Figure 4.2e). Therefore, the contribution of CO to *FT*

mRNA expression under 27°C-SD was tested. To this end, the flowering times of *co-10* mutant plants and Col-0 growing under 27°C-SDs were compared (Figure 4.3a).

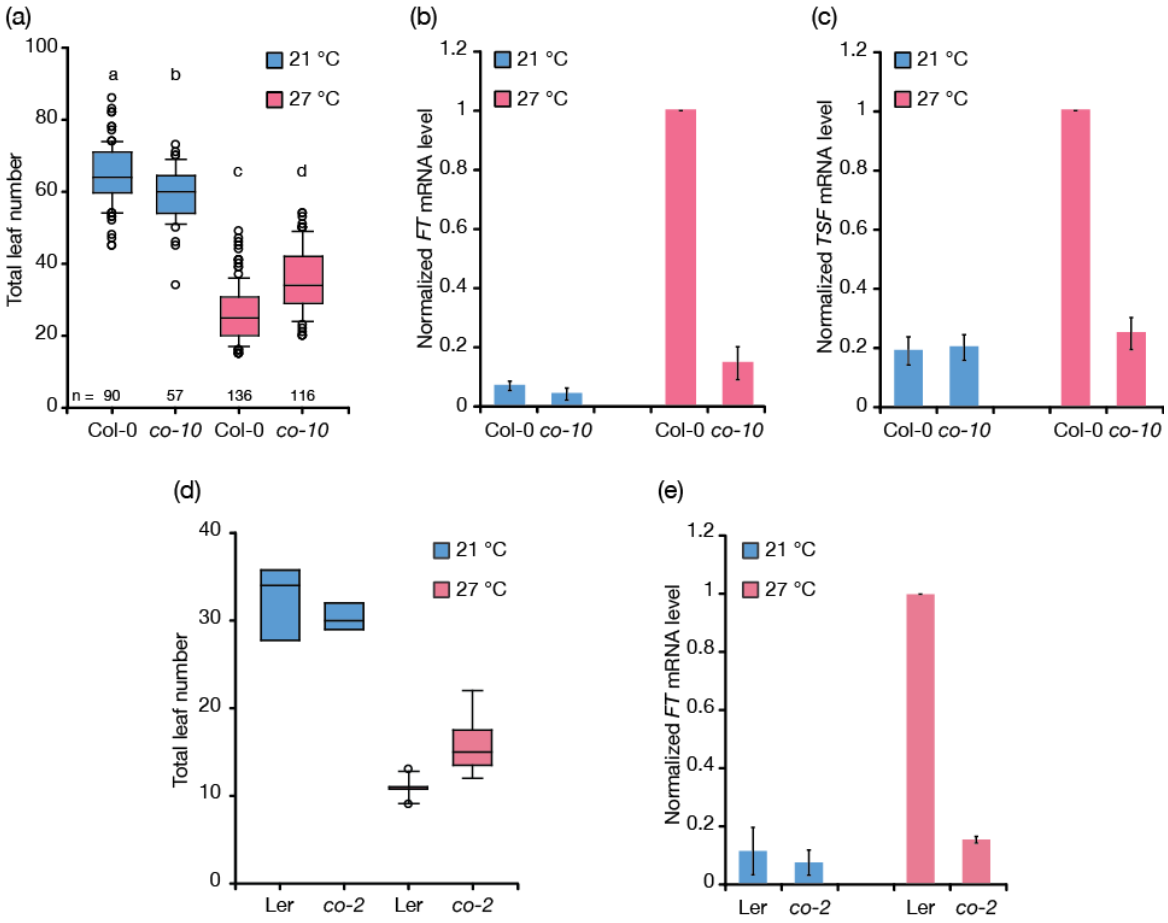

Figure 4.3 Mutations in *CO* affect flowering time and *FT* and *TSF* mRNA expression under 27°C-SD. (a) Flowering time of Col-0 and *co-10* plants grown under 21°C-SD and 27°C-SD. (b) *FT* mRNA expression in Col-0 and *co-10* seedlings grown under 21°C-SD and 27°C-SD. (c) *TSF* mRNA expression in Col-0 and *co-10* seedlings grown under 21°C-SD and 27°C-SD. (d) Flowering time of Ler and *co-2* plants grown under 21°C-SD and 27°C-SD. (e) *FT* mRNA expression in Ler and *co-2* seedlings grown under 21°C-SD and 27°C-SD.In (b), (c) and (e), seedlings were grown for 14 days and harvested at ZT8. *FT* and *TSF* mRNA expression was measured by qRT-PCR; error bars are SEs of at least six independent biological replicates in (b) an (c) and two independent biological replicates in (e). Statistical analysis as described in Figure 4.1.

A significant delay in flowering of the *co-10* mutants was observed, although they still flowered much earlier than plants of the same genotype grown under 21°C-SDs. Therefore,

as observed for two *pif4* mutant alleles, *pif5-3* and *pif4-2 pif5-3*, the *co-10* mutation partially reduced the flowering response to 27°C-SD. Similarly, *FT* mRNA levels in *co-10* mutants were significantly reduced compared to Col-0 under 27°C-SD at ZT8, but not under 21°C-SD (Figure 4.3b). A similar result was observed for *TSF* mRNA levels (Figure 4.3c). To demonstrate that the results obtained using *co-10* mutants were independent of the allele or the ecotype used, a second *co* mutant allele (*co-2*) in *Landsberg erecta* (Ler) background was studied. The *co-2* mutant plants showed late flowering and a strong suppression of *FT* mRNA expression compared to Ler at warm ambient temperature (Figure 4.3d and 4.3e), confirming the effect of CO on flowering time and *FT* mRNA expression under 27°C-SD. Therefore, *CO* contributes to the activation of *FT* and *TSF* mRNA expression during the thermosensory induction of flowering under SDs.

4.4 Simultaneous accumulation of CO and PIF4 proteins overlap with the peak in *FT* transcription at ZT8 under 27°C-SD

Our data support the idea that both CO and PIF4 are required for full induction of flowering at warm temperature under SD. Therefore, the timing of expression of these proteins was tested under 27°C-SD and compared to the timing of the peak in *FT* transcription. Transgenic p*CO::HA:CO co-10* seedlings were used (Sarid-Krebs *et al.* 2015) and HA:CO protein abundance followed through 24 h time courses. A diurnal peak of CO protein accumulation was observed at ZT8, both at 21 °C and 27 °C (Figure 4.4a), although this is of much lower amplitude than under LDs (Sarid-Krebs *et al.* 2015). Quantification of CO protein revealed that it is slightly more abundant (less than 2 fold) at 27 °C compared to 21 °C (Figure 4.4b), suggesting that warm temperature might favour the accumulation of CO protein at ZT8. The

analysis of *CO* mRNA indicated that this increase in CO protein at ZT8 might be due to the presence of more *CO* mRNA at this time under 27 °C compared to 21 °C (Figure 4.4c).

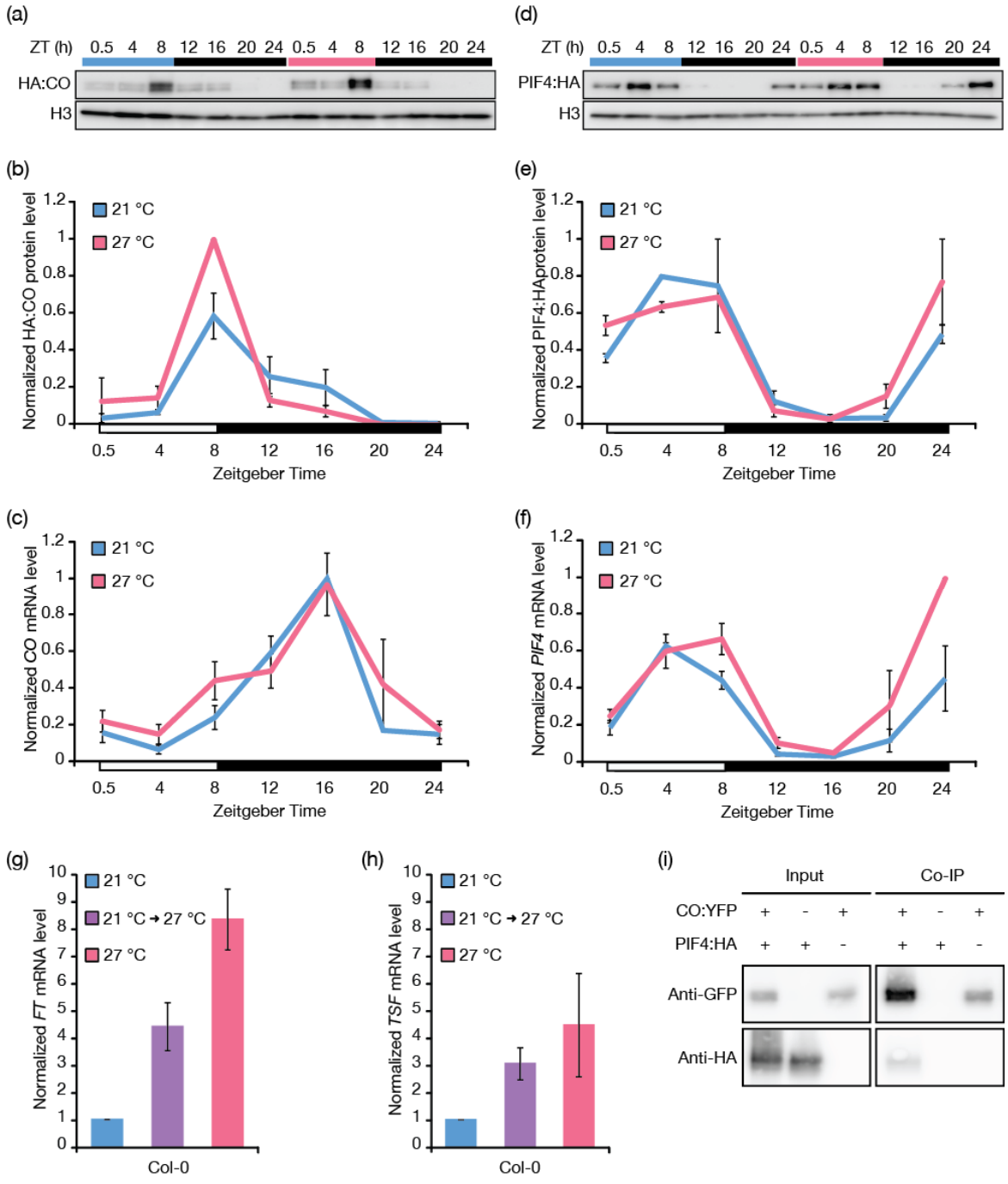

Figure 4.4 High levels of CO and PIF4 protein overlap ZT8. (a) Western blot from nuclear protein extracts of p*CO::HA:CO co-10* plants. Time course showing HA:CO protein accumulation over 24 h. (b) HA:CO protein

quantification. (c) *CO* mRNA expression in Col-0 seedlings grown under 21°C-SD and 27°C-SD. (d) Western blot from nuclear protein extracts of p*PIF4::PIF4:HA pif4-101* plants. Time course showing PIF4:HA protein accumulation over 24 h. (e) PIF4:HA protein quantification. (f) *PIF4* mRNA expression in Col-0 seedlings grown under 21°C-SD and 27°C-SD. (g) *FT* and (h) *TSF* mRNA expression in Col-0 seedlings grown under 21°C-SD, 27°C-SD or 21°C-SD and shifted to 27°C-SD. (i) In vivo co-immunoprecipitation of PIF4:HA protein with CO:YFP. CO:YFP was precipitated with anti-GFP antibody and co-precipitation of PIF4:HA was detected by western blot using anti-HA antibody. In (a) and (d), nuclear proteins were extracted from seedlings grown for 14 days under 21°C-SD or 10 days under 21°C-SD, and shifted to 27°C-SD for 4 days. The western blot images shown are representative results from three (a) and (d) two independent biological replicates. In (b) and (e), error bars are SEs of two independent biological replicates. In (c) and (f), 14-day-old seedlings were harvested every 4 h for 24 h. *CO* and *PIF4* mRNA expression was measured by qRT-PCR; error bars are SEs of three independent biological replicates for all time points, except for: 27°C-SD ZT20 and 21°C-SD ZT24 in (c) and 27°C-SD ZT20 and 21°C-SD ZT20 in (f), where two independent biological replicates were performed. For time point 21°C-SD ZT20 in (c) n =1. In (g) and (h) seedlings where grown for 14 days under 21°C-SD, 27°C-SD or for 10 days under 21°C-SD and transferred to 27°C-SD for 4 days. *FT* and *TSF* mRNA expression was measured by qRT-PCR; error bars = SE of 2 independent biological replicates.

To test accumulation of PIF4 protein under 21°C-SD and 27°C-SD, p*PIF4::PIF4:HA pif4-101* seedlings were used (Hornitschek *et al.* 2012). Figure 4.4d and 4.4e show that PIF4:HA was present during the day in a relatively unchanged and similar level at both temperatures, whereas it rapidly disappeared in darkness. At the end of the night, PIF4 protein levels increased, reaching higher levels at 27 °C compared to 21 °C. Analysis of *PIF4* mRNA showed that differences in the protein expression pattern between these temperatures correlated with changes in mRNA expression (Figure 4.4f). Both protein time courses were performed using seedlings grown for 14 days under 21°C-SD, for the 21 °C samples, or 10 days under 21°C-SD and shifted to 27°C-SD for 4 days, for the 27 °C samples. This strategy was chosen because the very long hypocotyls of the p*PIF4::PIF4:HA pif4-101* transgenic lines prevented proper development of the seedlings if grown continuously under 27°C-SD. The effect of the shift to high temperature and the accumulated CO and PIF4 proteins on *FT* and *TSF* mRNA expression was verified. Figure 4.4g and 4.4h show that four days of

exposure to warm temperature after 10 days of growth in non-inductive SD conditions are enough to trigger increased expression of *FT* and *TSF* mRNA. The results presented above showed that CO and PIF4 proteins accumulate at the same time as the peak of *FT* mRNA under 27°C-SD. Moreover, CO and PIF4 directly bind to proximal regions of the *FT* promoter (Tiwari *et al.* 2010, Kumar *et al.* 2012, Song *et al.* 2012b, Zhang *et al.* 2015). Thus, a possible hypothesis is that CO and PIF4 proteins might physically interact as a part of the mechanism promoting *FT* induction under warm SDs. In order to test this possibility, several molecular techniques were used. Given the technical simplicity and the availability of material, Yeast Two Hybrid (Y2H) assays were the first choice. *PIF4* and CO full length (FL) open reading frames cloned into Gateway pDEST22 and pDEST23 vectors were used. Open reading frames of both genes cloned in each vector were used, therefore, the interaction between CO and PIF4 proteins could be tested in both directions. As shown in Annex a.1, a positive interaction demonstrated by yeast growth in the absence of Histidine (-His) was observed in both cases. However, the presence of auto activation in the controls expressing only one of the proteins made it difficult to properly interpret the results. A second attempt to study the CO/PIF4 interaction was done by making use of confocal imaging in transient assays in *N. benthamiana*. CO and PIF4 were fused to YFP and CFP fluorescent proteins, respectively. *A. tumefaciens* transformed with vectors containing the fusion constructs were co-infiltrated in *N. benthamiana* leaves. Three days after infiltration FRET-A.PB was performed. Positive FRET-A.PB signals were detected, suggesting an interaction between CO:YFP and PIF4:CFP fusion proteins (Annex A.2). However, the detection of false positive FRET-A.PB signal in control experiments using CO:YFP and an empty vector expressing CFP prevented positive conclusions on the existence of an interaction between CO and PIF4 proteins (Annex A.2). Finally, co-immunoprecipitation (Co-IP) assays were carried out in *N. benthamiana* leaves. *A. tumefaciens* carrying CO:YFP or PIF4:HA constructs were co-infiltrated in leaves.

After three days of incubation to allow protein synthesis, leaf proteins were extracted. Immunoprecipitation of CO:YFP protein from leaf extracts using anti-GFP antibody allowed the co-immunoprecipitation of PIF4:HA protein (Figure 4.4i). This experiment demonstrated the existence of a physical interaction between CO and PIF4 that might be important for the activation of *FT* transcription under 27°C-SD.

Thus, CO and PIF4 proteins are both present at ZT8 (Figure 4.4a and 4.4d) when the peak of *FT* mRNA under 27°C-SD was observed (Figure 4.1a) and both are required for full induction of *FT* mRNA expression (Figure 4.2c and 4.3b). In addition, CO and PIF4 proteins physically interact (Figure 4.4i). These results suggest that the presence of both proteins at ZT8 allows each of them to contribute in activating *FT* and probably *TSF* transcription and thereby accelerating flowering at warm ambient temperature under SD.

4.5 Genetic analysis demonstrates additivity of *CO* and *PIF4* in promoting flowering under 27°C-SD

CO and PIF4 bind to the *FT* promoter, both are co-expressed when *FT* is transcribed at peak levels under 27°C-SD and the two proteins physically interact. The significance of these observations was tested genetically by making use of the *co-10 pif4-2* and *co-10 pif4-2 pif5-3* double and triple mutants. Under 27°C-SD, *co-10 pif4-2* and *co-10 pif4-2 pif5-3* plants flowered later than the corresponding single mutants, so that the triple mutant flowered after producing on average 24 leaves more than Col-0 (Figure 4.5a). Whether the genetic interaction between *co-10* and the *pif* mutations was additive or whether there was a synergistic effect was then tested. Three-Way ANOVA ($p < 0.5$) of the flowering-time data of single mutants and combinations indicated that there is not a synergistic interaction between the *co-10*, *pif4-2* and *pif5-3* mutations, but rather that the later-flowering phenotype of the

triple mutant can be explained by an additive effect. Therefore, the *co-10 pif4-2 pif5-3* triple mutant is strongly impaired in its responsiveness to 27°C-SD, although it still flowered earlier under 27°C-SD than under 21°C-SD (Figure 4.5a).

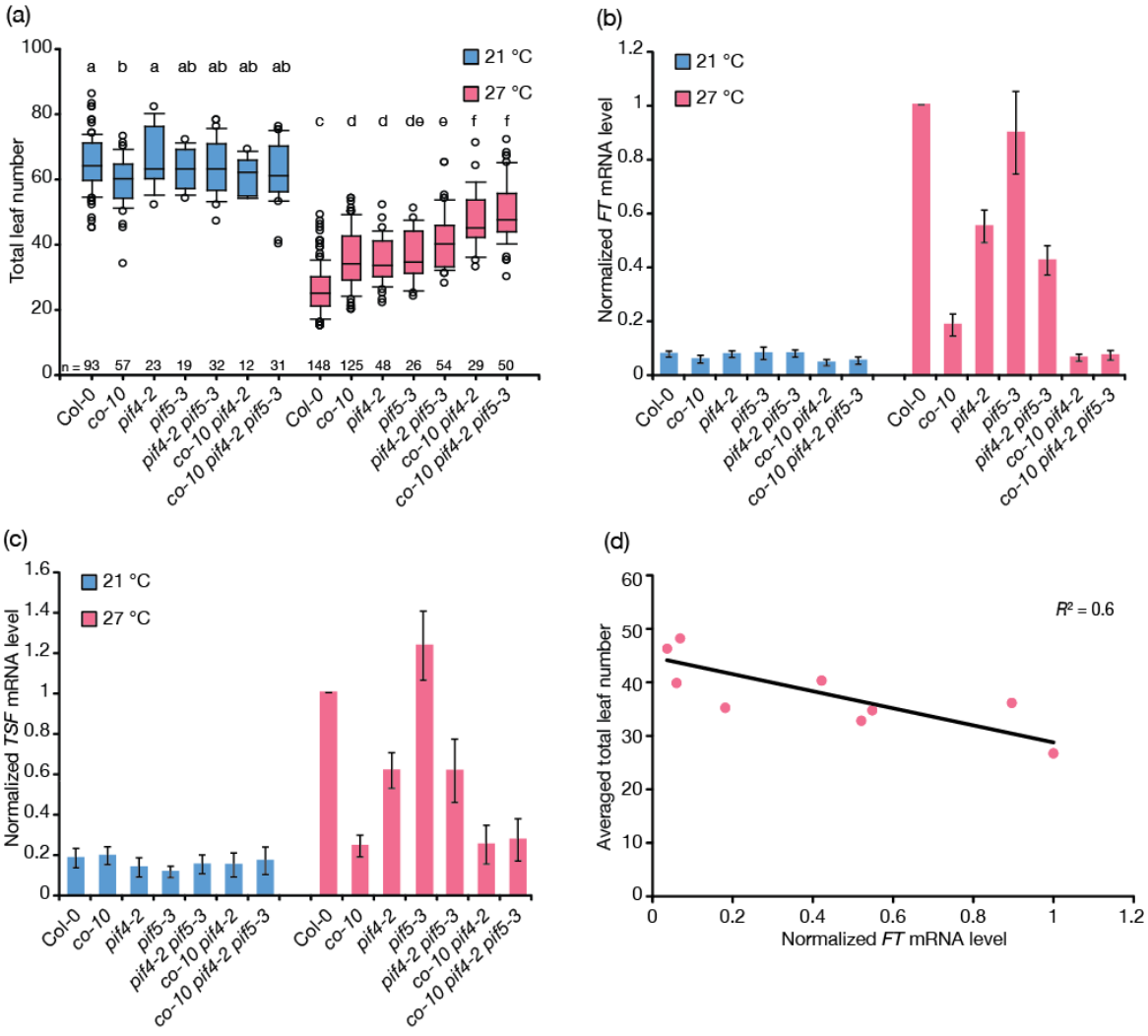

Figure 4.5 *CO* and *PIF4* have an additive effect on flowering time under 27°C-SD. (a) Flowering time of plants grown under 21°C-SD and 27°C-SD. (b) *FT* and (c) *TSF* mRNA expression of plants grown under 21°C-SD and 27°C-SD. (d) Pearson correlation analysis between normalized *FT* mRNA expression values and averaged flowering time data of plants grown under 27°C-SD. In (b) and (c) seedlings were grown for 14 days and harvested at ZT8. *FT* and *TSF* mRNA expression was measured by qRT-PCR; error bars are SEs of at least four independent biological replicates. Statistical analysis as described in Figure 4.1. In (d) correlation was performed with data presented in figures 4.2, 4.3 and 4.5, P = 0.01.

FT mRNA levels were then tested in the *co-10 pif4-2* and *co-10 pif4-2 pif5-3* backgrounds at ZT8 under 27°C-SD. The abundance of *FT* mRNA was strongly reduced in the *co-10 pif4-2* and *co-10 pif4-2 pif5-3* mutant plants compared to Col-0, and was similar to that observed in Col-0 growing under 21°C-SD (Figure 4.5b). This result indicates that the increased *FT* expression under 27°C-SD is almost entirely explained by the activity of CO, PIF4 and PIF5. Similar results were obtained for *TSF* mRNA levels in double and single mutants (Figure 4.5c). These observations were supported by a significant correlation (R^2 = 0.6; P = 0.01) between flowering time and the levels of *FT* mRNA of plants mutated in *CO* or the *PIF* genes and the corresponding double and triple mutants (Figure 4.5d).

4.6 Misexpression of *PIF4* in the companion cells accelerates flowering and activates transcription of *FT*

PIF4 and *CO* act as positive regulators of flowering by inducing the transcriptional activation of *FT* under 27°C-SD. In agreement with this, transgenic plants misexpressing *PIF4* from a companion cell-specific promoter (p*SUC2*) were very early flowering under 21°C-SD as well as 27°C-SD (Figure 4.6a). In p*SUC2::PIF4* plants *FT* mRNA levels were much higher than in Col-0 (Figure 4.6b), and this increase was stronger under 27°C-SD. Similarly, *TSF* mRNA levels were also induced in p*SUC2::PIF4* plants compared to Col-0 (Figure 4.6c). The higher levels of *FT* and *TSF* mRNA under 27°C-SD were associated with earlier flowering of p*SUC2::PIF4* plants under these conditions. This acceleration of flowering was observed despite the very high levels of *FT* and *TSF* mRNA present in p*SUC2::PIF4* plants under 21°C-SD. In addition, p*SUC2::PIF4* overexpression strongly accelerated flowering of *co-10* mutant plants (Figure 4.6a). This acceleration again correlated with a strong induction of *FT* mRNA levels under both 21°C-SD and 27°C-SD (Figure 4.6b). By contrast, the induction of *TSF*

mRNA expression in p*SUC2::PIF4* plants was suppressed by the *co-10* mutation (Figure 4.6c). However, the lack of *TSF* mRNA induction does not affect the flowering time of p*SUC2::PIF4 co-10* plants, presumably because of the high levels of *FT* mRNA present. Thus, *PIF4* overexpression in the vasculature can overcome the requirement for *CO* in the induction of flowering under 27°C-SD.

Similarly, requirement for *PIF4* for the induction of flowering under 27°C-SD was overcame by over-expression of *CO* driven by p*SUC2*. p*SUC2::CO* construct was able to accelerate flowering regardless of *pif4-2*, *pif5-3* and *pif4-2 pif5-3* mutants under 27°C-SD (Figure 4.6d). The early flowering response of p*SUC2::CO pif4-2*, p*SUC2::CO pif5-3* and *pSUC2::CO pif4-2 pif5-3* lines to warm ambient temperature was not significantly different to the response of p*SUC2::CO* (Figure 4.6d). These observations suggest that, the requirement of *PIF4* and *PIF5* for the promotion of flowering under 27°C-SD becomes negligible when high levels of CO are present. In addition, the induction of flowering by p*SUC2::CO* transgene was statistically similar under SDs compared to LDs and no delay in flowering time due to mutations in *PIF4* and *PIF5* was caused under either photoperiod (Figure 4.6e).

4.7 *SVP*, *FT* and *TSF* are essential for thermosensory induction of flowering under SD

At 27 °C, the stability of SVP protein is reduced compared to 21 °C (Lee *et al.* 2013). To test the abundance of SVP protein under our growing conditions, SVP:GFP protein levels in pSVP::SVP:GFP *svp-41 flc-3 FRI* seedlings (Mateos *et al.* 2015) through 24 h time course under 21°C-SD and 27°C-SD were monitored. SVP:GFP protein level decreased under 27°C-SD compared to 21°C-SD, however the protein was not absent at high temperature and SVP:GFP was still detected at all time points (Figure 4.7a and 4.7b).

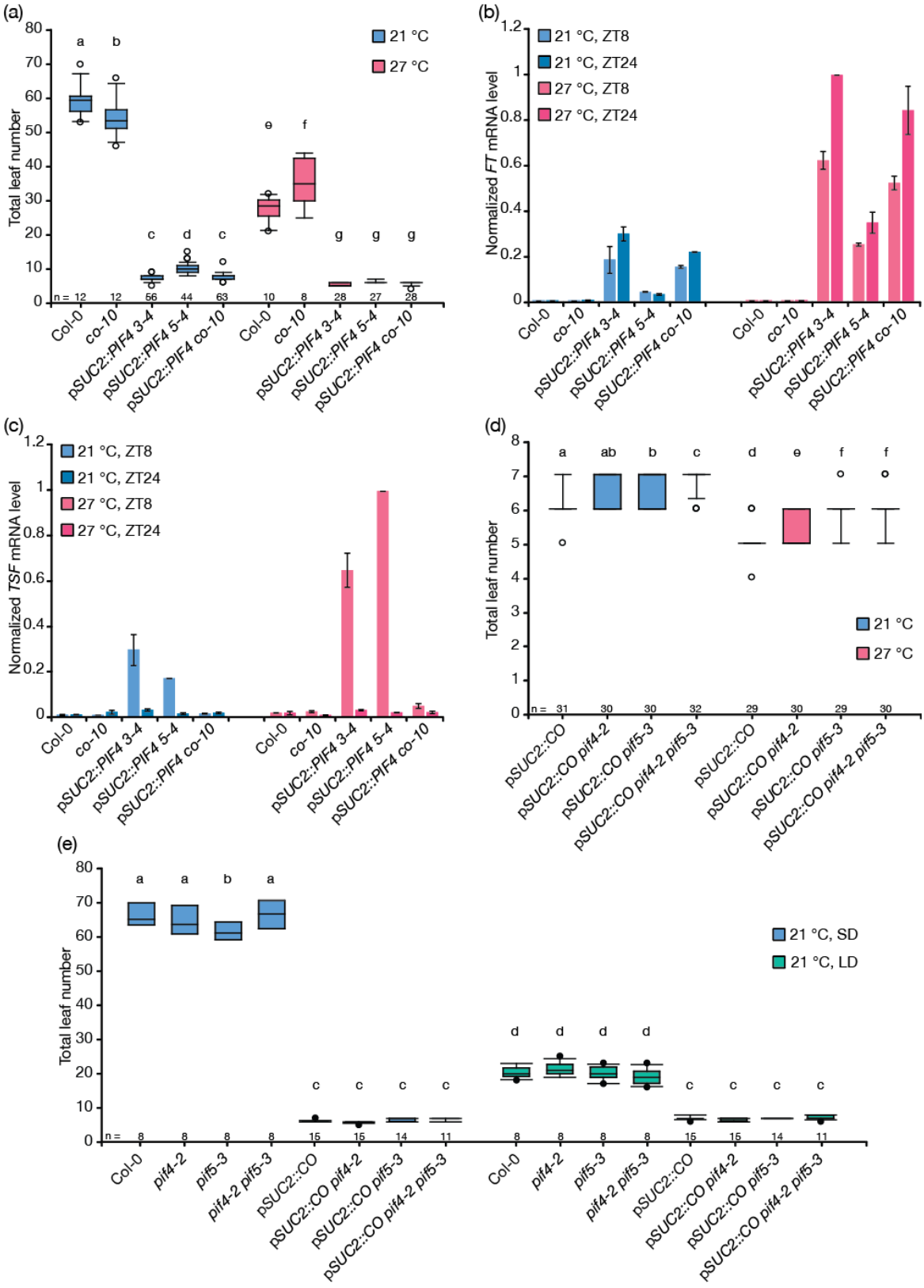
(a)
Total leaf number
21 °C
27 °C
n = 12 12 56 44 63 10 8 28 27 28
Col-0
co-10
pSUC2::PIF4 3-4
pSUC2::PIF4 5-4
pSUC2::PIF4 co-10
(b)
Normalized FT mRNA level
21 °C, ZT8
21 °C, ZT24
27 °C, ZT8
27 °C, ZT24
(c)
Normalized TSF mRNA level
21 °C, ZT8
21 °C, ZT24
27 °C, ZT8
27 °C, ZT24
(d)
Total leaf number
21 °C
27 °C
n = 31 30 30 32 29 30 29 30
pSUC2::CO
pSUC2::CO pif4-2
pSUC2::CO pif5-3
pSUC2::CO pif4-2 pif5-3
(e)
Total leaf number
21 °C, SD
21 °C, LD
n = 8 8 8 8 15 15 14 11 8 8 8 8 15 15 14 11
Col-0
pif4-2
pif5-3
pif4-2 pif5-3
pSUC2::CO
pSUC2::CO pif4-2
pSUC2::CO pif5-3
pSUC2::CO pif4-2 pif5-3

Figure 4.6 Misexpression of *PIF4* and *CO* in the companion cells can overcome the requirement of *CO* and *PIF4*, respectively, for the acceleration of flowering. (a) Flowering time of plants grown under 21°C-SD and 27°C-SD. (b) *FT* and (c) *TSF* mRNA expression of plants grown under 21°C-SD and 27°C-SD. (d) Flowering time of plants grown under 21°C-SD and 27°C-SD. (e) Flowering time of plants grown under 21°C-SD and 21°C-LD. In (b) and (c) seedlings were grown for 10 days and harvested at ZT8 and ZT24; error bars are SEs of two independent biological replicates. Statistical analysis as described in Figure 4.1.

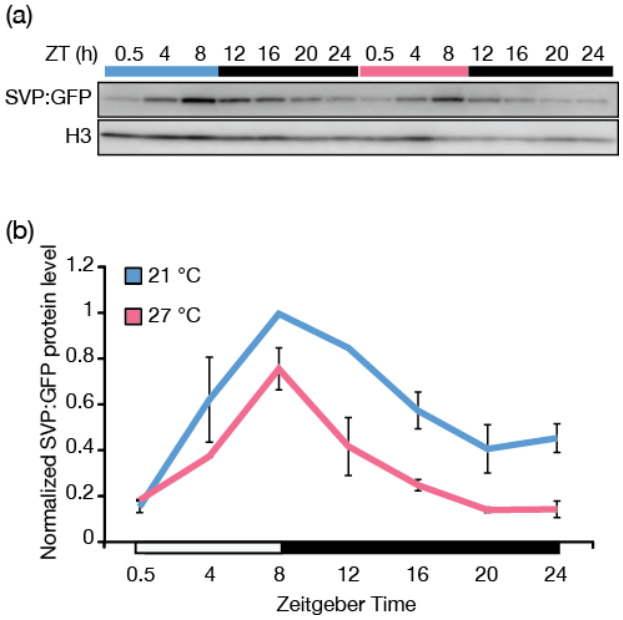

Figure 4.7 SVP protein stability decreases under 27°C-SD. (a) Western blot from nuclear protein extracts of p*SVP::SVP:GFP svp-41 flc-3 FRI* plants showing SVP:GFP protein accumulation over 24 hours. (b) SVP:GFP protein quantification. Nuclear proteins were extracted from seedlings grown for 14 days at 21°C-SD or 10 days at 21°C-SD and shifted to 27°C-SD for 4 days. The western blot image shown in (a) is a representative result from two independent biological replicates. In (b), error bars are SEs of two independent biological replicates. For time point 21°C-SD ZT12, n = 1.

Under LDs, *svp* mutants are insensitive to warm ambient temperature (Lee *et al.* 2007, Lee *et al.* 2013, Pose *et al.* 2013). By contrast under 27°C-SD *svp-41* flowered with around 6 leaves fewer than under 21°C-SD (Figure 4.8a). The *svp-41* mutant also shows increased expression of *FT* and *TSF* mRNA at ZT8 under 27°C-SD compared to 21°C-SD, as observed in Col-0 (Figure 4.8b and 4.8c). To test for a genetic interaction between the *SVP*-dependent and the *CO* and *PIF*-dependent thermosensory flowering pathways, the *svp-41 pif4-101* line

was generated and the flowering times of *svp-41 pif4-101* and *svp-41 co-10* double mutants were determined. Mutations in *SVP* strongly reduced the delay in flowering of *co-10* and *pif4-101* mutants grown under 27°C-SD (Figure 4.8a). To further study the effect on flowering time of *svp-41* mutant plants under warm ambient temperatures in the absence of floral promoters, the triple mutant *svp-41 co-10 pif4-101* was generated. The *svp-41 co-10 pif4-101* plants were significantly later flowering than the double mutants *svp-41 co-10* and *svp-41 pif4-101* (Figure 4.8a), although they retained a flowering response to 27°C-SD. Mutations in *CO* and *PIF4* also reduced the expression of *FT* mRNA in the *svp-41* mutant at 27°C-SD (Figure 4.8b).

CO and PIF4 promote transcription of *FT* and *TSF* under 27°C-SD, therefore the flowering response of the *svp-41 ft-10 tsf-1* mutant to warm ambient temperatures was also tested. In contrast to *svp-41 co-10 pif4-101*, the *svp-41 ft-10 tsf-1* triple mutant showed no flowering response to warm temperatures under SD, and was the only genotype tested that did not flower earlier under these conditions (Figure 4.8d). Additionally, *svp-41 co-10 pif4-101* and *svp-41 ft-10 tsf-1* flowered at similar times under 21°C-SD but *svp-41 co-10 pif4-101* flowered earlier under 27°C-SD, suggesting that in *svp-41 co-10 pif4-101* plants *FT TSF* transcription still responds to higher temperature, perhaps through PIF5 (Figure 4.8d). Taken together, these results demonstrate that the thermosensory induction of flowering occurs in the leaves through the promotion of *FT* and *TSF* transcription by CO and PIF4 PIF5 as well as the inactivation of the repressor SVP, while reduction of SVP activity has an additional effect in the *ft-10 tsf-1* double mutant that likely occurs in the meristem. Additional evidence of the role of SVP in the thermosensory flowering response in the meristem was obtained using p*KNAT1::SVP svp-41* plants (Andres *et al.* 2014), which showed delayed flowering under 27°C-SDs compared to *svp-41* mutants (Figure 4.8d). Nevertheless, p*KNAT1::SVP svp-41*

plants showed a greater response to temperature than the *svp-41* mutants, probably due to impairment of the function of the SVP in the SAM caused by the warm temperatures

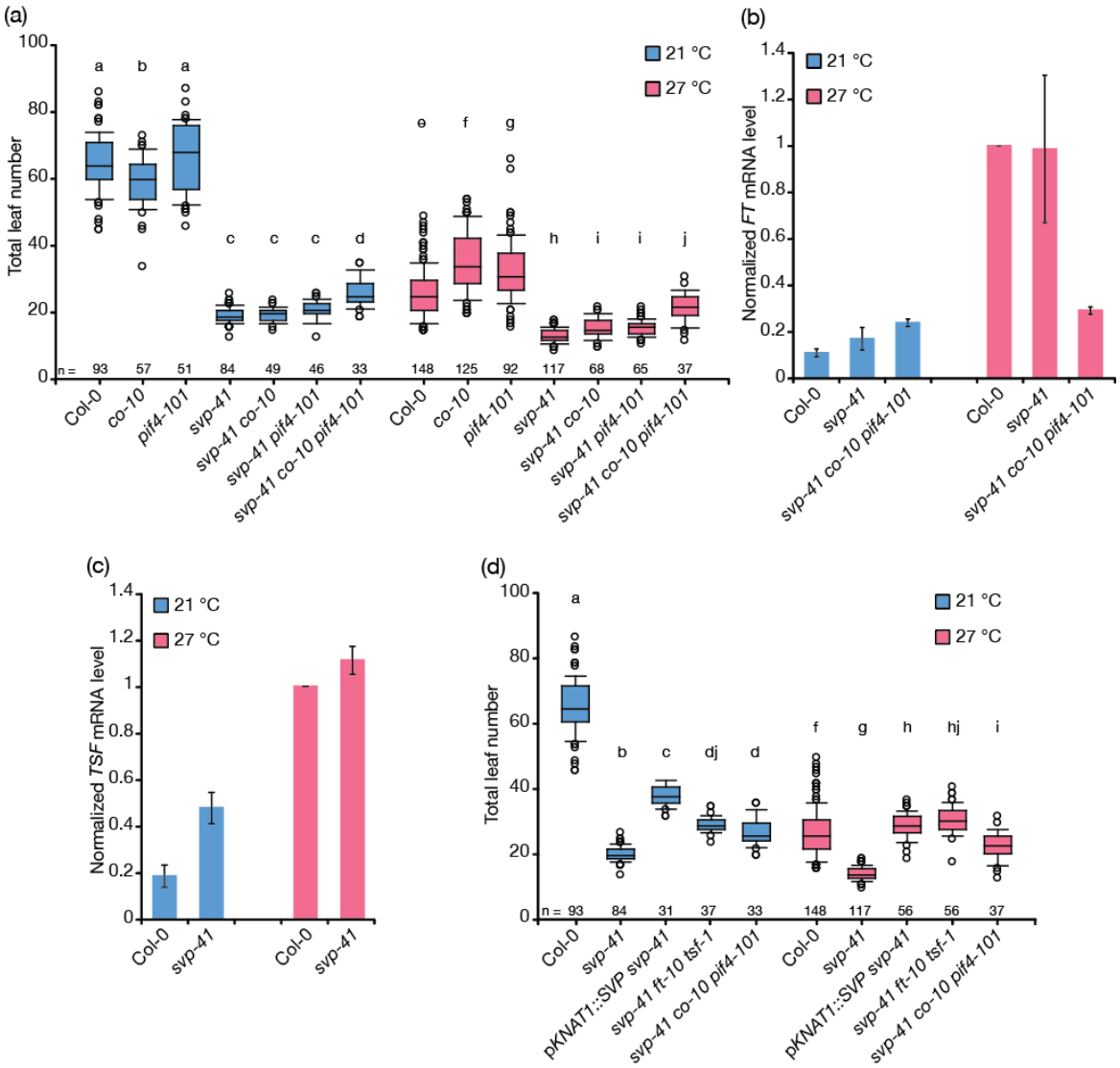

Figure 4.8 Convergent function of *SVP*, *CO* and *PIF4* in the regulation of *FT* and the relevance of *SVP* in the meristem on the flowering response to warm temperatures under SDs. (a) Flowering time of plants grown under 21°C-SD and 27°C-SD. (b) *FT* and (c) *TSF* mRNA expression in seedlings grown under 21°C-SD and 27°C-SD. (d) Flowering time of plants grown under 21°C-SD and 27°C-SD. In (b) and (c) seedlings were grown for 14 days and harvested at ZT8. *FT* and *TSF* mRNA expression was measured by qRT-PCR; error bars are SEs of at least two and three independent biological replicates, respectively. Statistical analysis as described in Figure 4.1.

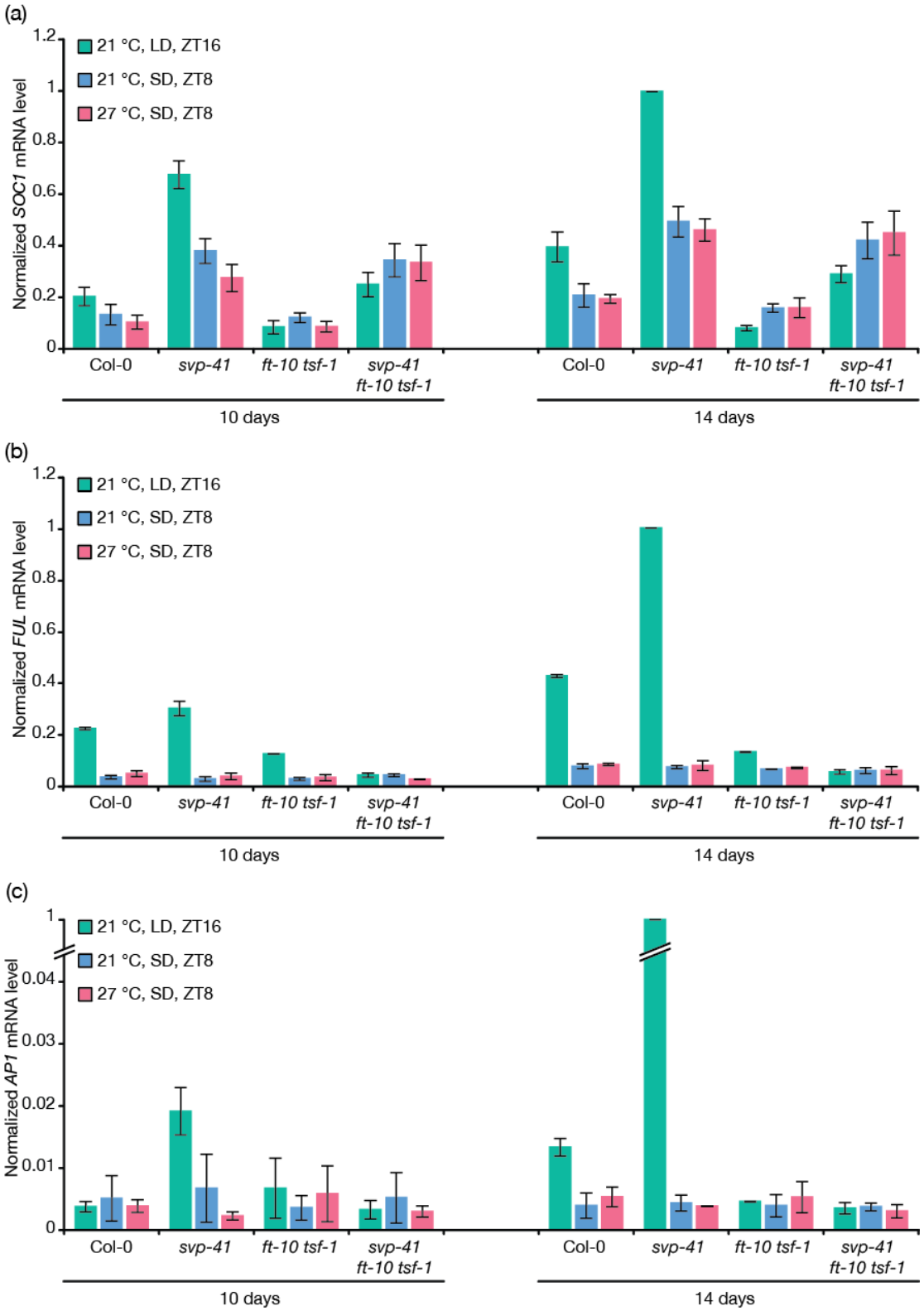
(a)
Normalized SOC1 mRNA level
21 °C, LD, ZT16
21 °C, SD, ZT8
27 °C, SD, ZT8
Col-0
svp-41
ft-10 tsf-1
svp-41 ft-10 tsf-1
10 days
14 days
(b)
Normalized FUL mRNA level
(c)
Normalized AP1 mRNA level

Figure 4.9 *SOC1*, *FUL* and *AP1* transcript levels are not increased by warm temperature under SDs. (a) *SOC1*, (b) *FUL* and (c) *AP1* mRNA expression in apices of plants grown under 21°C-LD, 21°C-SD and 27°C-SD. Apices of 10 and 14 days old where harvested at ZT16 and ZT8 and *SOC1*, *FUL* and *AP1* mRNA expression measured by qRT-PCR; error bars are SEs of two independent biological replicates.

An alternative possibility to reduced SVP activity at the meristem is that floral promoter genes, such as *SOC1*, *FUL* and *AP1* might respond directly to warm temperatures in the meristem. To test this, the expression of these genes was measured in apices of plants grown under different photoperiodic and temperature conditions. As expected, under 21°C-LD increased expression of *SOC1*, *FUL* and *AP1* mRNA was detected as plants proceeded to flowering, so plants of 14 days old showed higher expression levels than plants of 10 days old (Figure 49a, 49b and 49c). Furthermore, in apices of *svp-41* mutant plants the increase in *SOC1*, *FUL* and *AP1* mRNA expression was larger, suggesting the apex was in a more advanced floral stage. High expression levels of *SOC1*, *FUL* and *AP1* mRNA were observed in plants grown under 21°C-LD and were suppressed by *ft-10 tsf-1* mutations. However, analysis of *SOC1*, *FUL* and *AP1* mRNA under 21°C-SD and 27°C-SD detected no increase at the higher temperature in any genotype or time point tested (Figure 4.9a, 4.9b and 4.9c). Furthermore, even although in plants of 14 days old the induction of *FT* and *TSF* mRNA in leaves can be detected, no changes on *SOC1*, *FUL* and *AP1* mRNA were found in apices. Given that the role of *FT* and *TSF* is crucial under 27°C-SD, it can be speculated that the induction of the expression of genes expressed at the apex would be evident later in development. Therefore, reduction of SVP activity in the meristem under 27°C-SD, and not a direct effect of temperature on the expression of floral integrator genes, is proposed to increase sensitivity to the low level of *FT* and *TSF* expressed under these conditions.

4.8 Concluding remarks

Several transcription factors contributing to the photoperiod and thermosensory flowering pathways converge on the transcriptional regulation of the floral integrator *FT* (Figure 1.1), but the interactions between them and their relative contributions in controlling flowering at high temperatures are poorly understood.

The results presented in this work show that although CO is stabilized by LDs and promotes flowering in response to photoperiod, it is required under 27°C-SD for the activation of *FT*. Genetic analyses indicated that the response to 27°C-SD in the leaves depends on the coordinate functions of CO, PIF4 and PIF5 as well as SVP, and that loss of function of the *PIF4 PIF5* genes alone has a weak and variable effect on flowering. Furthermore, the activation of *FT* mRNA in the leaves occurs at much lower levels under 27°C-SD than under 21°C-LD. Thus, the removal of repressors from the meristem at 27 °C would be also necessary for responsiveness to such low levels of *FT* expression. In support of this, genetic analysis shows that *svp-41 ft-10 tsf-1* triple mutants are insensitive to increased temperature under SDs. These data provide a genetic and molecular framework for interaction between the photoperiod and thermosensory pathways.

5. Regulation of flowering time by enriched FR light in *A. thaliana*

Light is not only the primary source of energy for plants, but also one of the most important environmental signals that control plant growth and development (Li *et al.* 2011). Plants monitor several aspects of light, such as duration, intensity and quality, by means of a number of photoreceptors. In *A. thaliana*, a family of five photoreceptors (PhyA - PhyE) function as sensors of R and FR light (Casal *et al.* 2003). One of the functions of the Phy photoreceptors is to act as the first line of perception of neighboring plants in a crowded environment. Under a dense canopy, light quality differs greatly from the unfiltered light to which plants are exposed in open environments (Figure 1.3). The spectrum is strongly shifted to FR in a crowded environment and varies according to the nature of the vegetation and the density of the canopy. The green leaves of the surrounding plants absorb R and B light, whereas FR light is transmitted or reflected. Phys detect these changes in light quality by modifying the ratio between the inactive Pr form and the active Pfr form (Franklin and Whitelam 2005). When the environment is enriched in FR light, the Phy equilibrium is shifted towards the inactive Pr form. One Phy isoform, PhyB is the principal sensor of these R:FR ratio changes, however PhyD and PhyE also play important roles (Devlin *et al.* 1998, Devlin *et al.* 1999, Franklin *et al.* 2003a, Franklin and Whitelam 2005). This decrease in the R:FR ratio and concomitant inactivation of Phys triggers important developmental changes collectively named as the SAS. These changes involve elongation of the hypocotyl, petioles and stems to reach more open areas in the canopy enhancing the light capturing capacity and if the shade persists, acceleration of flowering (Smith 2000, Franklin and Whitelam 2004, Vandenbussche *et al.* 2005). *phyB* mutants display a constitutive shade avoidance response, including hypocotyl and petiole elongation, reduced chlorophyll content and acceleration of flowering (Somers *et al.* 1991, Devlin *et al.* 1992, Franklin *et al.* 2003b). As previously mentioned (see Introduction), some of these responses depend on the transcriptional activation of growth-promoting genes by PIFs, predominantly PIF4, PIF5 and PIF7 (Franklin

et al. 2011b, Hornitschek *et al.* 2012, Li *et al.* 2012a, Zhang *et al.* 2013, Lucas and Prat 2014). Here, we analysed the early-flowering phenotype of *phyB* mutants and inquired whether this response is mediated by PIFs. Furthermore, we study the early-flowering phenotype of plants grown under simulated shade and examine the roles of PIFs in this response.

5.1 Early-flowering phenotype of *phyB-9* mutant requires *CO*, *FT* and *TSF*

Plants with mutations in *phyB* show a constitutive SAS, and early flowering is one of the most notable phenotypes of these plants (Somers *et al.* 1991, Reed *et al.* 1993, Smith and Whitelam 1997).

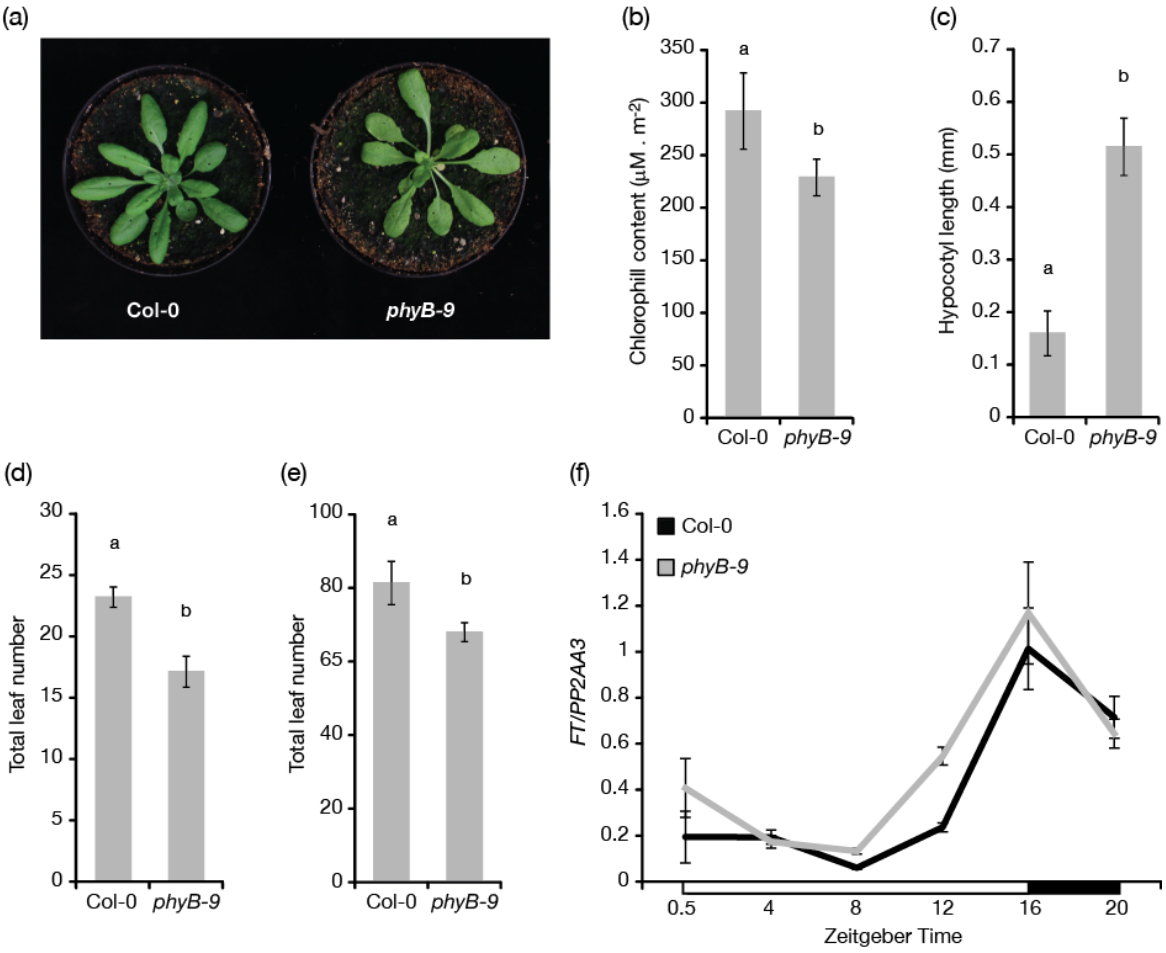

Figure 5.1 The *phyB-9* mutant displays phenotypic traits characteristics of the shade avoidance syndrome. (a) Picture of Col-0 and *phyB-9* mutant grown under LDs in WL. (b) Chlorophyll content in leaves of Col-0 and *phyB-9* mutant plants grown under LDs in WL. (c) Hypocotyl length of 10 days-old Col-0 and *phyB-9* mutant seedlings grown under LDs in WL. Flowering time of Col-0 and *phyB-9* mutant plants grown under (d) LDs and (e) SDs in WL. (f) *FT* mRNA expression profile in 12-day-old Col-0 and *phyB-9* mutant seedlings grown under LDs in WL. Seedlings were harvested every 4 h for 24 h and *FT* mRNA expression was measured by qRT-PCR; error bars are standard deviation (StD) of three technical replicates. In (b), (c), (d) and (e) letters indicate statistical groups determined with a Student's t-test. Groups were considered statistically different when $P \leq 0.05$. Error bars are StD. In (b) n = 5, in (c) n = 20, in (d) n = 10-11, in (e) n = 6-10.

Some of the characteristic phenotypes displayed by *phyB* were validated using the *phyB-9* mutant plants (Figure 5.1a - 5.1f). As part of the constitutive SAS, under LD conditions, these mutants showed lower chlorophyll content (Figure 5.1b) and longer hypocotyls compared to Col-0 (Figure 5.1c). Also, *phyB-9* mutants flowered earlier than Col-0 under LDs and SDs (Figure 5.1d and 5.1e). Twelve day old *phyB-9* mutants showed an increase in *FT* mRNA level compared to Col-0 under LDs (Figure 5.1f and Annex A.3a). Previous experiments performed in our laboratory also showed that similar to *FT*, *TSF* mRNA level in *phyB-9* mutants is higher than in Col-0 under LDs (Annex A.3b). Under SDs, the levels of *FT* and *TSF* mRNA also correlated with the observed flowering phenotypes, showing *phyB-9* mutants higher expression of these floral integrators than Col-0 (Annex A.3d and A.3e). These results indicate that *phyB* negatively regulates the expression of *FT* and *TSF* mRNA, both under LDs and SDs conditions, consistent with previous reports (Cerdán and Chory 2002, Halliday *et al.* 2003, Endo *et al.* 2005, Yamaguchi *et al.* 2005).

To genetically confirm that the early flowering of the *phyB-9* mutant depends on *FT* and *TSF*, the flowering time of *phyB-9 ft-10 tsf-1* triple mutants were tested under LDs and SDs. The *phyB-9 ft-10 tsf-1* triple mutant flowered as late as the *ft-10 tsf-1* double mutant under both photoperiods suggesting that *phyB-9* mutant plants require functional *FT* and *TSF* to display the early-flowering phenotype (Figure 5.2a and Annex A.4). PhyB regulates CO protein

stability by promoting its degradation early in the day, just after dawn (Valverde *et al.* 2004). Under LDs, when CO protein is stable in the afternoon, it promotes the expression of *FT* and *TSF* mRNA and consequently flowering is accelerated (Suarez-Lopez *et al.* 2001). Therefore, the promotion of flowering observed in *phyB-9* mutants might be due to an effect on *FT* and *TSF* transcription that is dependent on *CO*. To test the role of *CO* in the early flowering of *phyB-9* mutants, the flowering time of *phyB-9 co-10* double mutants was determined. Plants carrying mutations in *CO* are late flowering under LDs (Figure 5.2a) (Putterill *et al.* 1995).

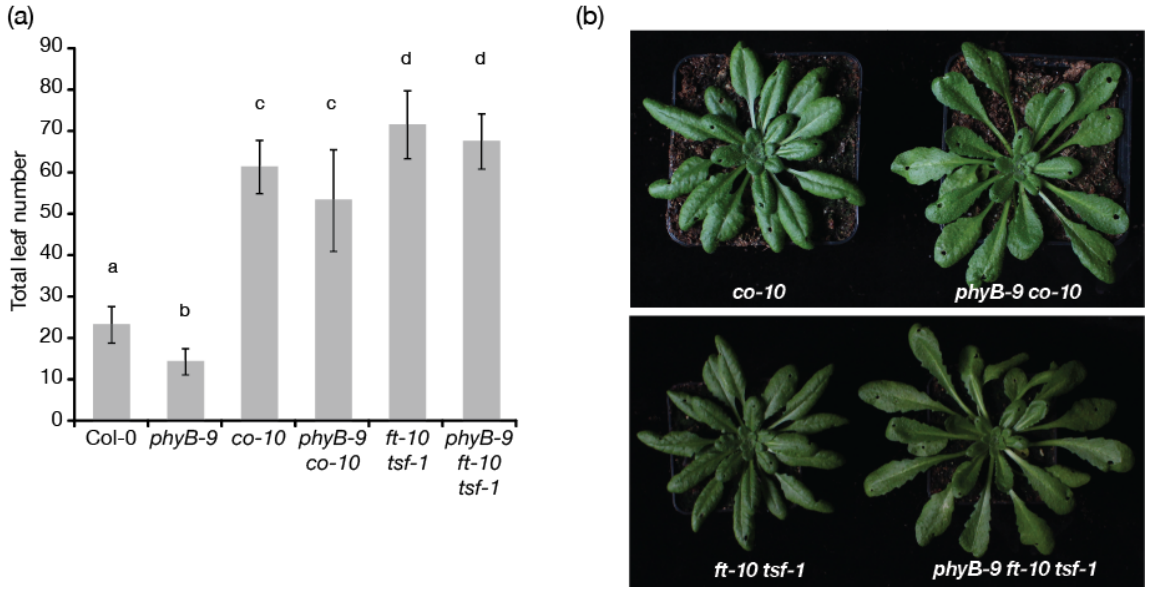

Figure 5.2 *CO*, *FT* and *TSF* are required for the early flowering of *phyB-9* mutant. (a) Flowering time of plants grown under LDs in WL. (b) Picture of *co-10* and *phyB-9 co-10* mutants (upper panel) and of *ft-10 tsf-1* and *ft-10 tsf-1 phyB-9* mutants (lower panel) grown under LDs in WL. In (a) letters indicate statistical groups determined with a Student's t-test. Groups were considered statistically different when $P \leq 0.05$. N = 17-21.

Double mutants *phyB-9 co-10* flower as late as *co-10* single mutants under LDs and SDs (Figure 5.2a and Annex A.4), indicating that *CO* is essential for the early flowering phenotype of *phyB-9* mutants. Taken together, these results indicate that *phyB-9* mutants require *CO*, *FT* and *TSF* to accelerate flowering under LDs and SDs. These photoperiod pathway

components seem not to be required, however, for the petiole elongation of the *phyB-9* mutant (Figure 5.2b). *CO* mRNA expression measurements revealed only slight changes under both photoperiods (Annex A.3c and A.3f), suggesting that the dependence on *CO* for the early flowering time of *phyB* mutant does not depend on transcriptional changes of this gene.

5.2 *PIF4* and *PIF5* are required for the early flowering of *phyB-9* mutant

PIF family members are central regulators in the Phy-mediated light signalling pathway (Lorrain *et al.* 2008). Although they are crucial regulators of growth and germination (Lucas and Prat 2014), several members of the family (*PIF1*, *PIF3*, *PIF4* and *PIF5*) appear not to have a role in flowering under LDs and SDs at standard growth temperatures (21 °C) (Figure 5.3a, 5.3b and 5.3c) (Shin *et al.* 2009a). *PIF4* and *PIF5* act downstream of PhyB in the regulation of hypocotyl elongation, as the elongated hypocotyl phenotype of *phyB* mutants is suppressed by *pif4* and *pif5* mutations (Lorrain *et al.* 2008). Therefore, given the central role played by the PIF transcription factors in Phy-mediated regulation of growth, their requirement for the early flowering of *phyB-9* was tested. Analysis of flowering time under LDs showed that the early flowering of *phyB-9* mutants is suppressed in *phyB-9 pif4-2 pif5-3* triple mutants but not in *phyB pif4* or *phyB pif5* double mutants (Figure 5.3c). The suppression of the early flowering of *phyB-9* mutants by *pif4-2 pif5-3* is also observed under SDs (Annex A.4). Other features of the SAS constitutively displayed by *phyB* mutants, such as elongation of petioles, seem to be also suppressed by the *pif4 pif5* mutations (Figure 5.3d). These results suggest that *PIF4* and *PIF5* are required for the early flowering phenotype of *phyB* mutant plants as well as for other phenotypes also associated with the SAS. Nevertheless, *PIF4* and *PIF5* seem to play redundant roles in conferring flowering acceleration in *phyB-9* mutants, as

neither *phyB-9 pif4* nor *phyB-9 pif5* double mutants flowered later than *phyB-9* single mutant (Figure 5.3c).

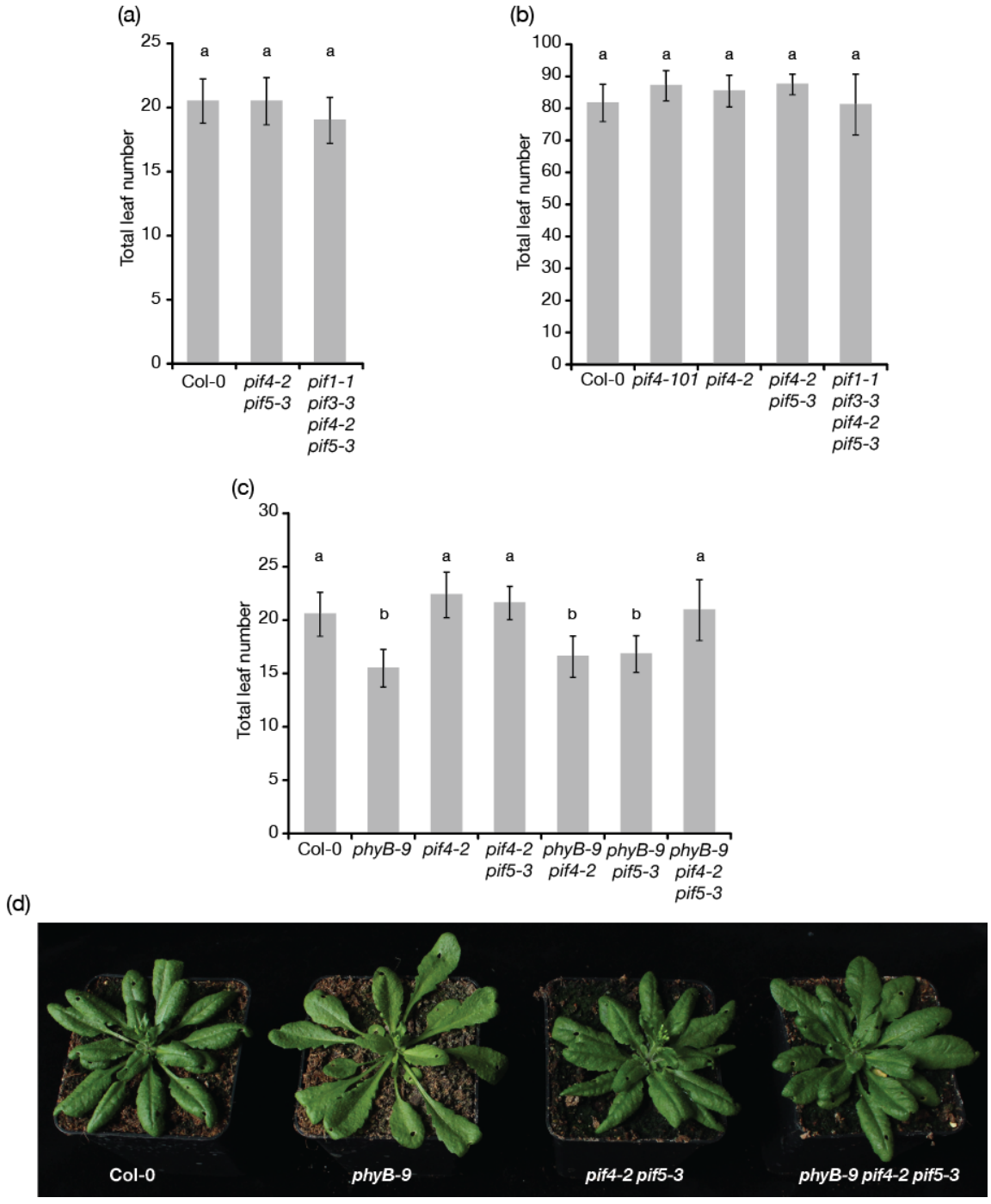

Figure 5.3 *PIF4* and *PIF5* are required for the early flowering of *phyB-9* mutant. Flowering time of plants grown under LDs (a) and (c), and SDs (b) in WL. (d) Picture of Col-0, *phyB-9*, *pif4-2 pif5-3* and *phyB-9 pif4-2 pif5-3* mutant plants grown under LDs in WL. In (a), (b) and (c) letters indicate statistical groups determined with a one-way ANOVA. Groups were considered statistically different when $P \leq 0.05$. Error bars are StD. In (a) n = 10-11, in (b) n = 8-10, in (c) n = 5-12.

5.3 Enriched FR light triggers the SAS in Col-0 and *phyB-9* mutants

PhyB activity is reduced by enriched FR light characteristic of vegetative shade and under these conditions shade avoidance responses are triggered (Rockwell and Lagarias 2006, Jiao *et al.* 2007, Martinez-Garcia *et al.* 2014). Similarly, reduction of PhyB activity in *phyB* mutants causes a phenotype that resembles the SAS (Figure 5.1a – 1f) (Somers *et al.* 1991, Reed *et al.* 1993, Smith and Whitelam 1997).

To analyze the flowering-time response of plants growing under shade and to compare it with the phenotype of *phyB* mutant plants, shade conditions were simulated by supplementing the standard white light (WL) growth conditions with FR light (and therefore named as enriched FR light). This condition resembles the vegetative shade encountered by plants that grow under a canopy. Indeed, light spectra measurements under these experimental conditions showed an increase of the light intensity at wavelengths between 700 and 750 nm compared to WL control conditions (Figure 5.4a and 5.4b).

In order to set up the experimental shade conditions several light intensities were assessed, including changes in WL and FR light intensities and the response of Col-0 plants was evaluated. Two WL conditions were used (100% and 65% of the maximum WL light intensity) and combined with two FR light intensities (100% and 65% of the maximum FR intensity) (Figure 5.4a and 5.4b; Materials and methods). Thus, four different enriched FR light conditions were tested and two WL control conditions were used. Col-0 plants responded in similar ways to each simulated shade condition tested. They all showed similar acceleration

of flowering, to around 50% of the flowering time under control conditions (Figure 5.5a and 5.5b). Furthermore, plants grown under enriched FR light developed typical characteristics of the SAS, such as elongation of hypocotyls and petioles (Figure 5.5c, 5.5d and 5.5e).

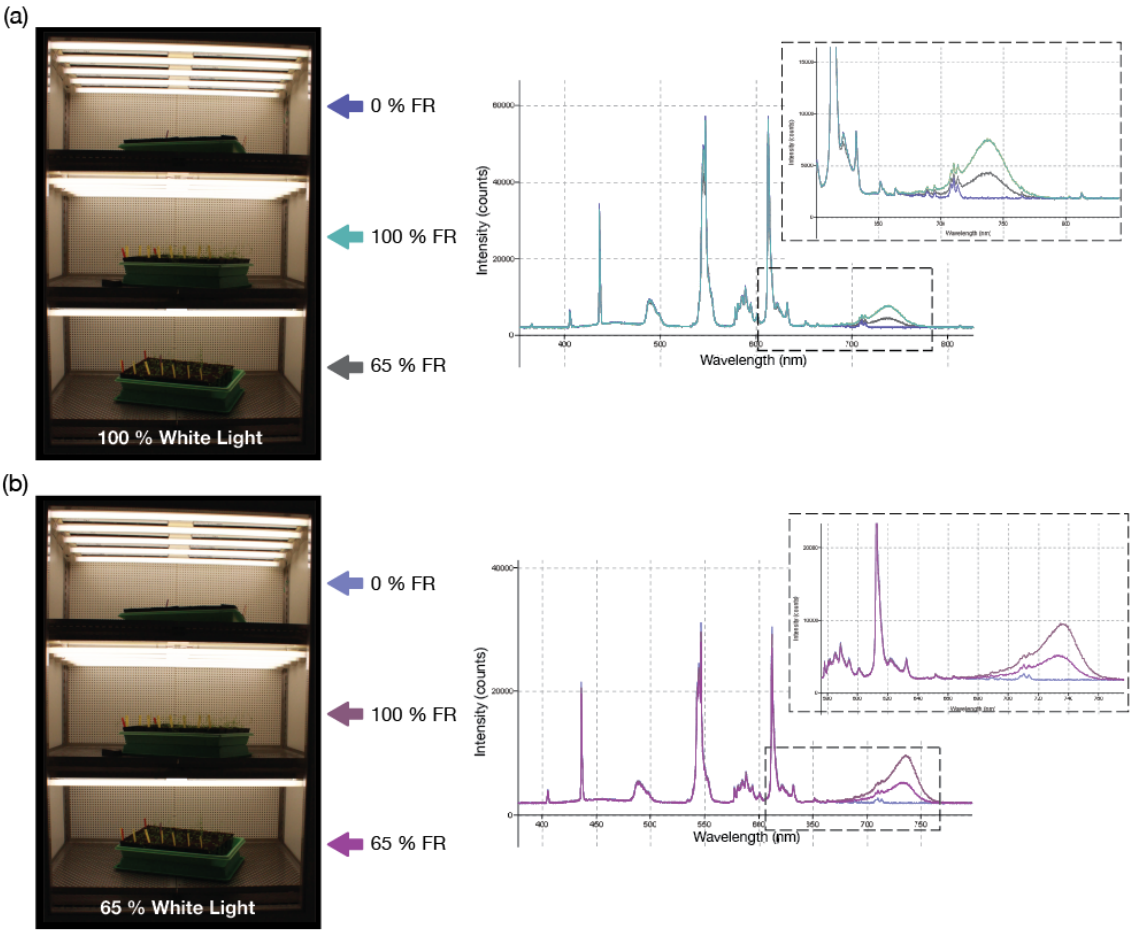

Figure 5.4 Establishment and measurements of the different simulated shade conditions used in this work. (a) Growth cabinet with 100 % WL and 0 %, 65 % and 100 % FR light (left); light spectrum in every condition under 100 % WL (right). (b) Growth cabinet with 65 % WL and 0 %, 65 % and 100 % FR light (left); light spectrum in every condition under 65 % WL (right).

These observations indicate that every condition tested can trigger the SAS in Col-0 plants. In addition, *phyB*-9 mutant plants responded to enriched FR light as much as Col-0 (Figure 5.6a, 5.6b and 5.6c), suggesting that under these conditions there might be functional

redundancy among PhyB, PhyD and PhyE for this response. Therefore, accelerated flowering under enriched FR light might be promoted by PhyD and PhyE when PhyB is not functional. Consistent with this idea, in every light condition tested, Col-0 under enriched FR light flowered earlier than *phyB* mutants under WL (Figure 5.5a, 5.5b, 5.6a and 5.6b). These results suggest that under enriched FR light not only the function of PhyB is affected but also PhyD and PhyE functions.

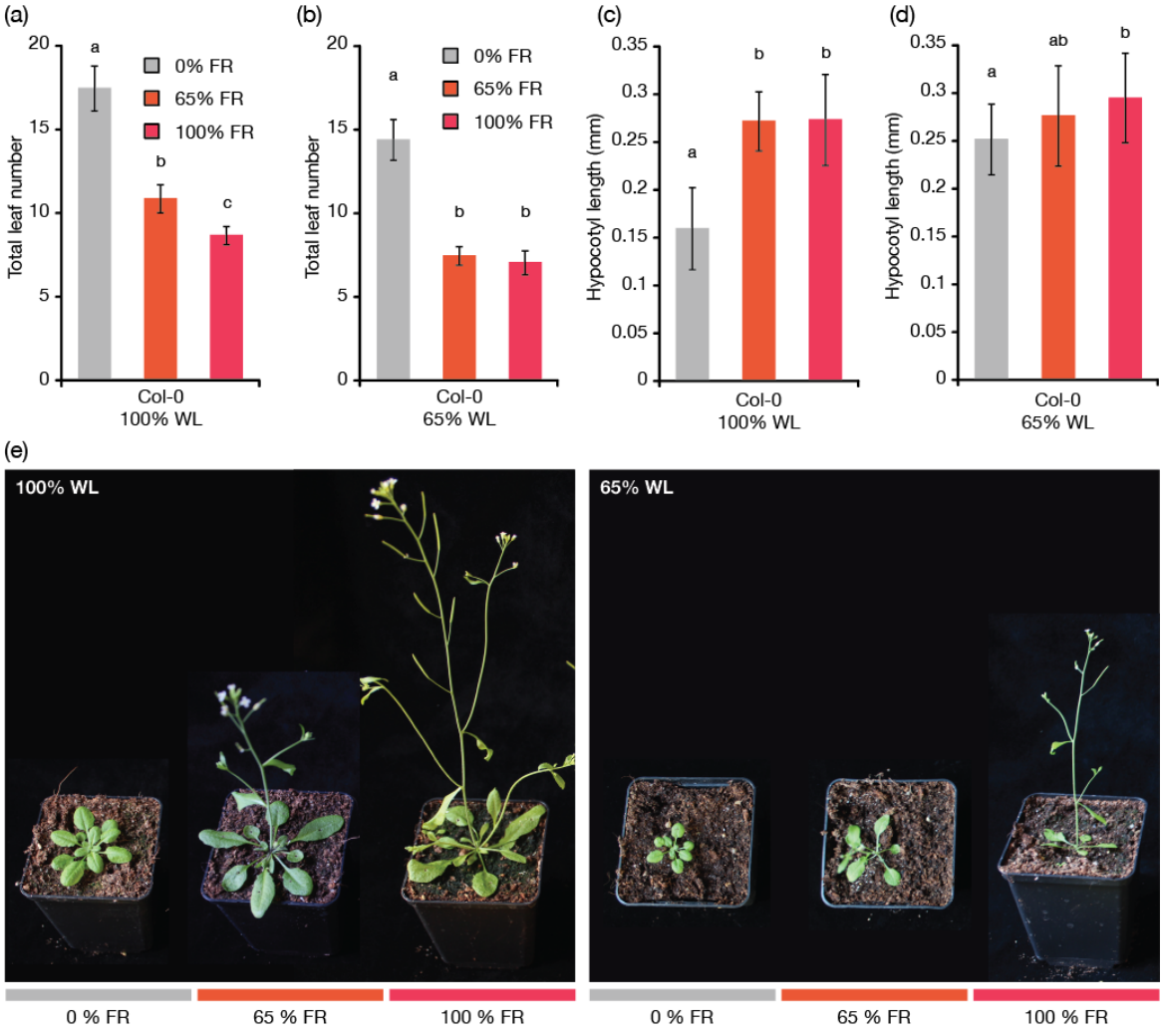

Figure 5.5 All simulated shade conditions used can trigger the shade avoidance syndrome in Col-0 plants. Flowering time of Col-0 plants grown under simulated shade (100 % and 65 % of FR light enrichment) with (a) 100 % WL and (b) 65 % WL under LDs. Hypocotyl length of 10-day-old Col-0 seedlings grown under simulated shade (100 % and 65 % of FR light enrichment) with (c) 100 % WL and (d) 65 % WL under LDs. (e) Picture of

Col-0 plants grown under LDs in simulated shade (100 % and 65 % of FR light enrichment) with 100 % WL (left) and 65 % WL (right). In (a), (b), (c) and (d) letters indicate statistical groups determined with a Student's t-test. Groups were considered statistically different when P ≤ 0.05. Error bars are StD. In (a) n = 5, in (b) n = 5-6, in (c) n = 21-22, in (d) n = 20. In (c) and (d), color code as in (a) and (b).

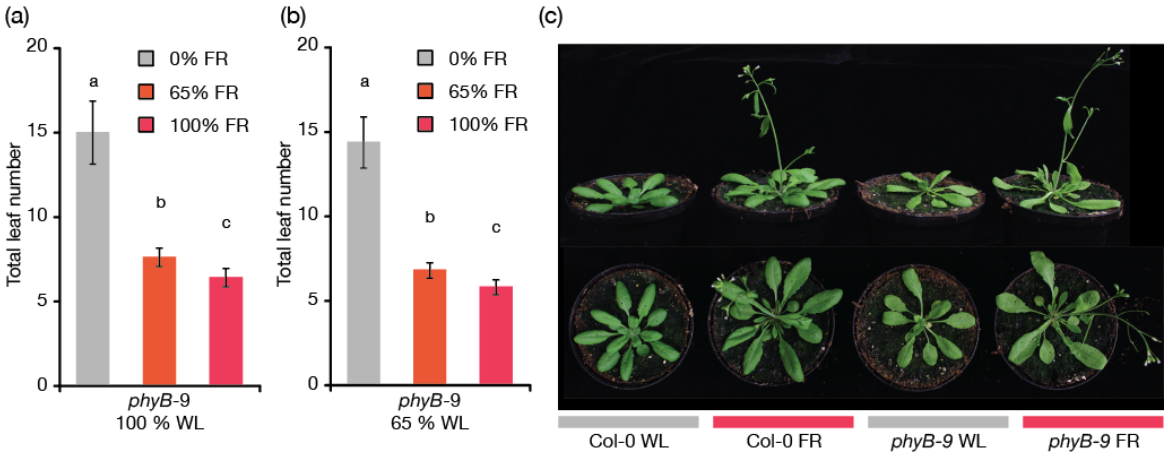

Figure 5.6 All simulated shade conditions used can accelerate flowering in *phyB-9* mutant plants. Flowering time of *phyB-9* mutant plants grown under simulated shade (100 % and 65 % of FR light enrichment) with (a) 100 % WL and (b) 65 % WL under LDs. (c) Picture of Col-0 and *phyB*-9 mutant plants grown under LDs in simulated shade (100 % FR light enrichment) with 100 % WL. In (a) and (b) letters indicate statistical groups determined with a Student's t-test. Groups were considered statistically different when P ≤ 0.05. Error bars are StD. In (a) and (b) n = 5.

5.4 Enriched FR light stabilizes PIF4 protein

PIF4 and *PIF5* are part of the PhyB signalling pathway that promotes, at least, part of the shade avoidance response (Huq and Quail 2002, Lorrain *et al.* 2008). PIF proteins stability is light regulated (Nozue *et al.* 2007, Lorrain *et al.* 2008). Under high R:FR light ratio, PhyB is active and translocates to the nucleus where it binds PIF4 and targets the transcription factor for degradation by the 26S proteasome. Under low R:FR light ratio, PhyB becomes predominantly inactive, allowing the stabilization of PIF proteins and to bind to the promoters of shade induced genes (Lorrain *et al.* 2008). To test if the simulated shade conditions affected PIF protein stability, the abundance of PIF4 through a 24 h time course under WL

and enriched FR light was evaluated. To perform this experiment *pPIF4::PIF4:HA pif4-101* transgenic plants were used (Hornitschek *et al.* 2012). Given the similarities in the flowering responses of Col-0 to all the conditions tested, the PIF4 abundance was tested in only one of the simulated shade conditions (100% of the WL intensity and 65% of FR light). After growing 12 days on soil under WL or enriched FR light conditions, seedlings were harvested every 4 h. Figure 5.7a and Annex A.5 show that early in the day there is no difference in PIF4 protein accumulation under enriched FR light compared to WL. However, at ZT12 and ZT16 PIF4 protein increases in abundance under shade compared to WL condition.

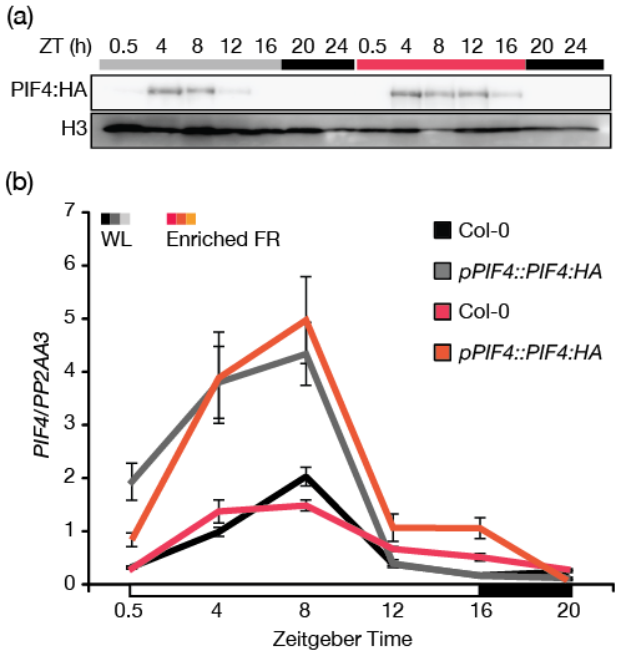

Figure 5.7 PIF4 protein is stabilized under enriched FR light. (a) Western blot from nuclear protein extracts of p*PIF4::PIF4:HA pif4-101* seedlings. Time course showing PIF4:HA protein accumulation over 24 h under WL and enriched FR. (b) *PIF4* mRNA expression in Col-0 and p*PIF4::PIF4:HA pif4-101* seedlings grown under WL and enriched FR. In (a), nuclear proteins were extracted from seedlings grown for 12 days under WL or enriched FR. In (b), 12-day-old seedlings were harvested every 4 h for 24 h. *PIF4* mRNA expression was measured by qRT-PCR; error bars are StD of three technical replicates.

Analysis of *PIF4* mRNA expression in Col-0 and *pPIF4::PIF4:HA pif4-101* plants under WL and shade showed that under enriched FR light the expression of *PIF4* mRNA in both genotypes is slightly higher than under WL at ZT12 and ZT16 (Figure 5.7b). The increase in *PIF4* mRNA expression coincides with the increase in PIF4 protein (Figure 5.7a), but at ZT12 and ZT16 the small difference in mRNA abundance is probably not sufficient to explain the difference in protein level.

5.5 Flowering induction under enriched FR light conditions does not require *PIF1*, *PIF3*, *PIF4*, *PIF5* and *PIF7*

Early flowering and other aspects of the constitutive shade avoidance response displayed by *phyB* mutants are suppressed by mutations in *PIF4* and *PIF5* (Figure 5.3c and 5.3d, Annex A.4). Also, PIF4 protein accumulation is higher under enriched FR light conditions compared to WL (Figure 5.7a). The accumulation of PIF4 protein under simulated shade conditions might be due to FR light inactivation of PhyB (Lorrain *et al.* 2008). Taken together, these observations suggest that *PIF4* and *PIF5* might also play a role downstream of PhyB in the shade-dependent acceleration of flowering. To test this hypothesis, the flowering time of single and higher order *pif* mutants was scored under enriched FR light conditions and compared to plants growing under WL. For this experiment, 100% WL plus 100% FR light was used to simulate the shade condition. Two single mutant alleles of *PIF4*, *pif4-101* and *pif4-2*, as well as *pif4-2 pif5-3* and *pif1-1 pif3-3 pif4-2 pif5-3* double and quadruple mutants, respectively, were tested. The single and higher order *pif* mutants did not show any delay of flowering compared to Col-0 in simulated shade conditions under LDs and SDs (Figure 5.8a, 5.8b and 5.8c).

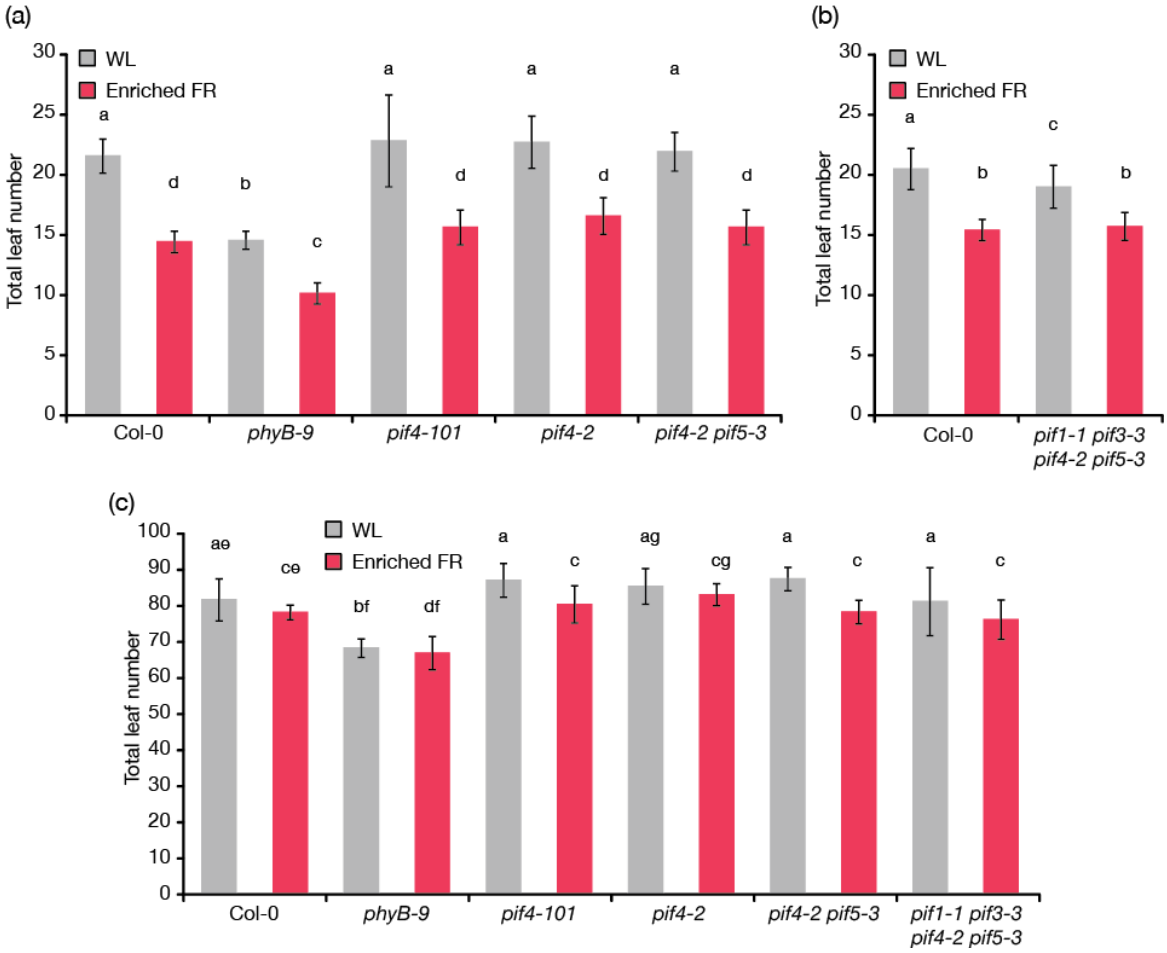

Figure 5.8 *PIF1*, *PIF3*, *PIF4* and *PIF5* are not required for the acceleration of flowering by simulated shade under LDs and SDs. (a) and (b) Flowering time of plants grown under LDs in WL or enriched FR light. (c) Flowering time of plants grown under SDs in WL or enriched FR light. In (a), (b) and (c) letters indicate statistical groups determined with a two-way ANOVA and multiple comparisons with the Holm–Sidak method. Multiple comparisons were performed within conditions and within genotypes. Groups were considered statistically different when $P \leq 0.05$. Error bars are StD. In (a) n = 7-10, in (b) n = 10-11, in (c) n = 6-10.

To exclude the possibility that under different shade conditions the early-flowering response to shade of the *pif* mutants differs from the response of Col-0, a study of the flowering time responses of *pif4-2 pif5-3* and *pif1-1 pif3-3 pif4-2 pif5-3* mutants under the four previously tested shade conditions was performed (Figure 5.9a and 5.9b).

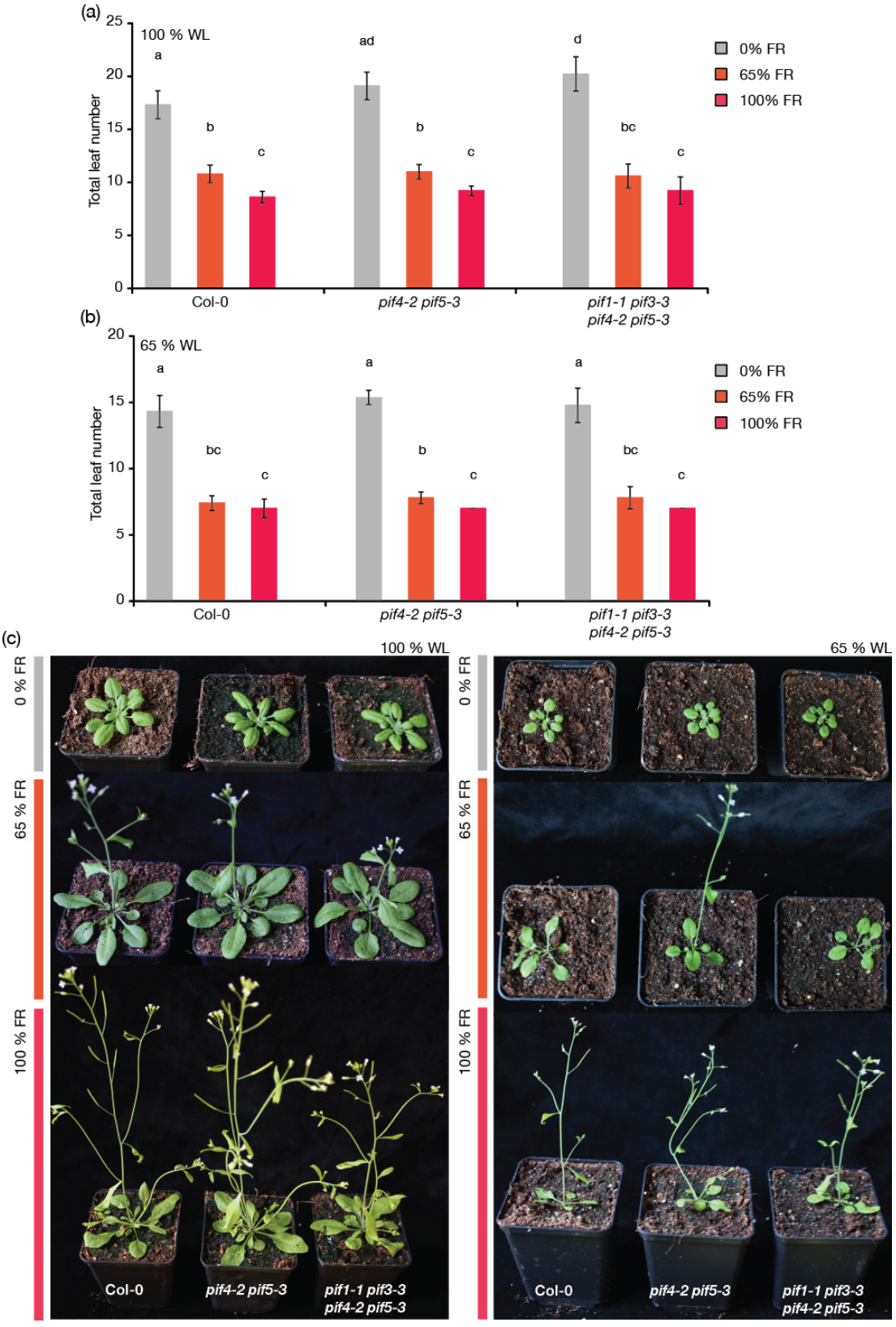
(a)
100 % WL
Total leaf number
0% FR
65% FR
100% FR
a
b
c
ad
b
c
d
bc
c
Col-0
pif4-2 pif5-3
pif1-1 pif3-3
pif4-2 pif5-3
(b)
65 % WL
Total leaf number
0% FR
65% FR
100% FR
a
bc
c
a
b
c
a
bc
c
Col-0
pif4-2 pif5-3
pif1-1 pif3-3
pif4-2 pif5-3
(c)
100 % WL
65 % WL
0 % FR
65 % FR
100 % FR
Col-0
pif4-2 pif5-3
pif1-1 pif3-3
pif4-2 pif5-3

Figure 5.9 All simulated shade conditions tested can accelerate flowering of *pif4-2 pif5-3* double *pif1-1 pif3-3 pif4-2 pif5-3* quadruple mutants. (a) and (b) Flowering time of plants grown under simulated shade (100 % and 65 % of FR light enrichment) with (a) 100 % WL and (b) 65 % WL under LDs. (c) Picture of Col-0, *pif4-2 pif5-3* and *pif1-1 pif3-3 pif4-2 pif5-3* mutant plants grown under LDs in simulated shade (100 % and 65 % of FR light enrichment) with 100 % WL (left) and 65 % WL (right). In (a) and (b) letters indicate statistical groups determined with a Student's t-test within genotypes and within conditions. Groups were considered statistically different when $P \leq 0.05$. Error bars are StD. In (a) and (b) n = 5-6.

Similar to the previous experiment (Figure 5.8a and 5.8b), no differences in the early-flowering response of *pif* mutants under enriched FR light compared to Col-0 were detected. These results suggest that the higher abundance of PIF4 protein detected at ZT12 and ZT16 under enriched FR light does not have an effect on flowering time under the tested conditions. Furthermore, *pif* mutants developed some of the characteristics of the SAS under enriched FR conditions, such as elongated petioles (Figure 5.9c). These responses to simulated shade indicate that *PIF1*, *PIF3*, *PIF4* and *PIF5* are not essential for the promotion of certain phenotypic traits associated to the SAS. This idea was also corroborated at the molecular level. In *pif4-2 pif5-3* and *pif1-1 pif3-3 pif4-2 pif5-3* mutants, the expression of *IAA29* mRNA, a direct target of *PIF4* and *PIF5* (Hornitschek *et al.* 2012) is lower than in Col-0 under WL, however, its expression is induced under enriched FR light conditions to levels similar to the ones observed for Col-0 (Figure 5.10). Taken together, these results support the idea that *PIF1*, *PIF3*, *PIF4* and *PIF5* are not essential for the flowering response to shade as well as other SAS-related traits.

Mutations in *PIF4* and *PIF5* suppress the acceleration of flowering of *phyB-9* mutants (Figure 5.3c). To test whether *pif4-2 pif5-3* mutations affect the response to simulated shade in a *phyB-9* mutant background compared to Col-0, the flowering time of *phyB-9 pif4-2 pif5-3* triple mutant was evaluated in WL and simulated shade conditions under LDs. Triple mutant *phyB-9 pif4-2 pif5-3* induced flowering to similar levels as Col-0 plants in simulated shade

(Figure 5.11). These results support previous observations suggesting that when PhyB is not functional, flowering might be promoted by PhyD and PhyE under enriched FR light. Furthermore, this promotion of flowering probably mediated by PhyD and PhyE seems to be independent of *PIF4* and *PIF5*.

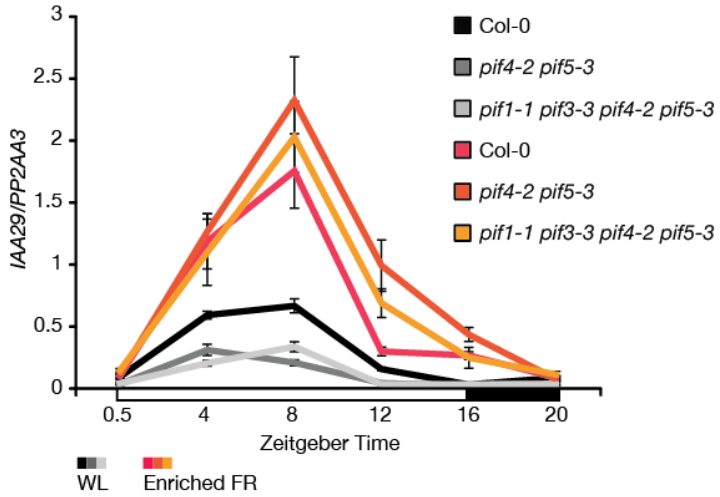

Figure 5.10 Expression of *IAA29* mRNA is induced in *pif4-2 pif5-3* and *pif1-1 pif3-3 pif4-2 pif5-3* mutant seedlings grown under enriched FR light. *IAA29* mRNA expression in Col-0, *pif4-2 pif5-3* and *pif1-1 pif3-3 pif4-2 pif5-3* mutant seedlings grown under WL and enriched FR. Twelve-day-old seedlings were harvested every 4 h for 24 h. *IAA29* mRNA expression was measured by qRT-PCR; error bars are StD of three technical replicates.

The p*PIF4::PIF4:HA* transgene caused an exaggerated SAS, showing the plants extremely long hypocotyls (Yamashino *et al.* 2013a) compared to Col-0. The flowering time of the p*PIF4::PIF4:HA pif4-101* transgenic plants under enriched FR light was, therefore, also assessed. No early flowering was displayed by these plants in WL under LDs and SDs (Figure 5.12a and 5.12b). Furthermore, under enriched FR light, where the PIF4:HA protein was more abundant than under WL at ZT12 and ZT16 in LDs, the shade mediated induction of flowering observed was similar to the flowering response to shade of Col-0 in both photoperiods (Figure 5.12a and 5.12b). Taken together, *pif* mutant plants as well as *pif4-101*

plants harboring p*PIF4::PIF4:HA* construct, which trigger a constitutive SAS phenotype (Yamashino *et al.* 2013a), do not show a flowering time phenotype WL or enriched FR light.

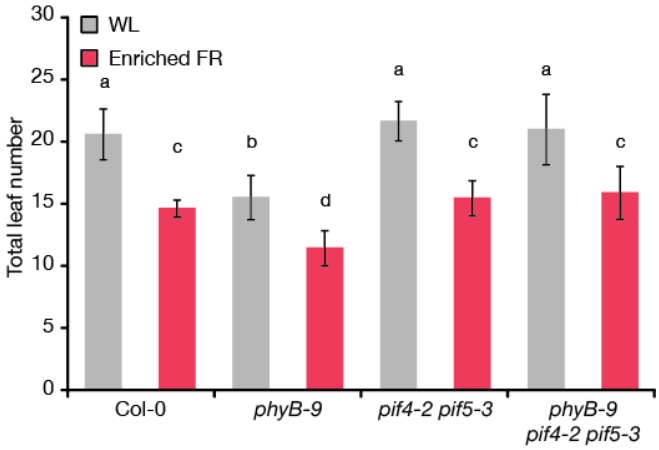

Figure 5.11 Mutations in *PIF4* and *PIF5* do not affect the flowering response to shade of *phyB-9* mutant plants. Flowering time of plants grown under SDs in WL and enriched FR light. Letters indicate statistical groups determined with a two-way ANOVA and multiple comparisons with the Holm–Sidak method. Multiple comparisons were performed within conditions and within genotypes. Groups were considered statistically different when P ≤ 0.05. Error bars are StD, n = 9-10.

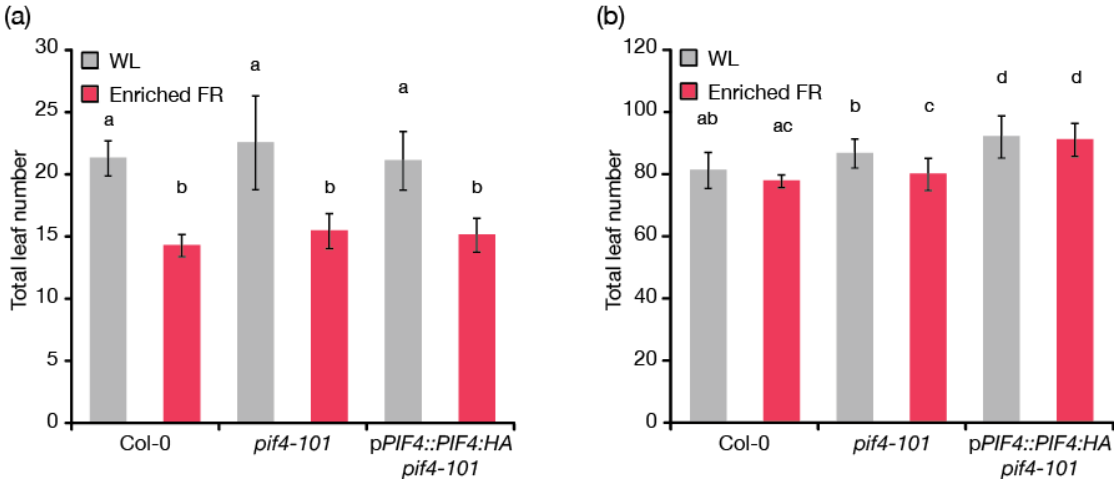

Figure 5.12 The flowering response to enriched FR is not modified by the p*PIF4::PIF4:HA* transgene. Flowering time of plants grown under LDs (a) and SDs (b) in WL and enriched FR light. Letters indicate statistical groups determined with a two-way ANOVA and all pairwise multiple comparisons within genotypes and within conditions with the Holm–Sidak method. Groups were considered statistically different when P ≤ 0.05. Error bars are StD. In (a) n = 8-10 and in (b) n = 4-10.

As shown above, there is functional redundancy among PIF transcription factors in conferring the shade avoidance response. *PIF7* is also implicated in the response to shade conditions. Mutants in *PIF7* suppress the elongation of hypocotyls promoted by enriched FR light and by the absence of PhyB (Li *et al.* 2012a). Thus, the role for *PIF7* in flowering induction under enriched FR light was tested. Two different alleles of *pif7* mutants were tested (*pif7-1 and pif7-2*) as well as the double mutant *phyB-9 pif7-1* and a transgenic line overexpressing PIF7::FLASH under the *35S* promoter. Under enriched FR conditions, *pif7* mutants flowered with the same leaf number as Col-0 plants (Figure 5.13a, 5.13b and 5.13c). Furthermore, *phyB-9 pif7-1* double mutants also showed a suppression of the early flowering time of *phyB* single mutants in WL and responded like Col-0 to enriched FR light in terms of flowering time (Figure 5.13a, 5.13b and 5.13c). Thus *PIF7* does not affect shade-induced early flowering, in contrast to its effect on shade-induced hypocotyl elongation (Li *et al.* 2012a). Transgenic plants harboring the p*35S:PIF7::FLASH* construct have a stronger response to shade than Col-0, producing longer hypocotyls than Col-0 (Li *et al.* 2012a). However, the flowering time of the p*35S:PIF7::FLASH* transgenic plants was not different from Col-0 under WL and enriched FR light (Figure 5.13a). *PIF7* mRNA expression analysis in a 24 h time course under WL and enriched FR light showed similar *PIF7* mRNA expression under enriched FR compared to WL (Figure 5.13d). Taken together, all these results suggest that PIF1, PIF3, PIF4, PIF5 and PIF7 transcription factors do not play a role in the regulation of flowering in response to shade, although to exclude redundancy between these factors analysis of a quintuple mutant (*pif1 pif3 pif4 pif5 pif7*) would be required. Also, PIF4, PIF5 and PIF7 appear to promote early flowering of *phyB* mutants, identifying a difference to the simulated shade.

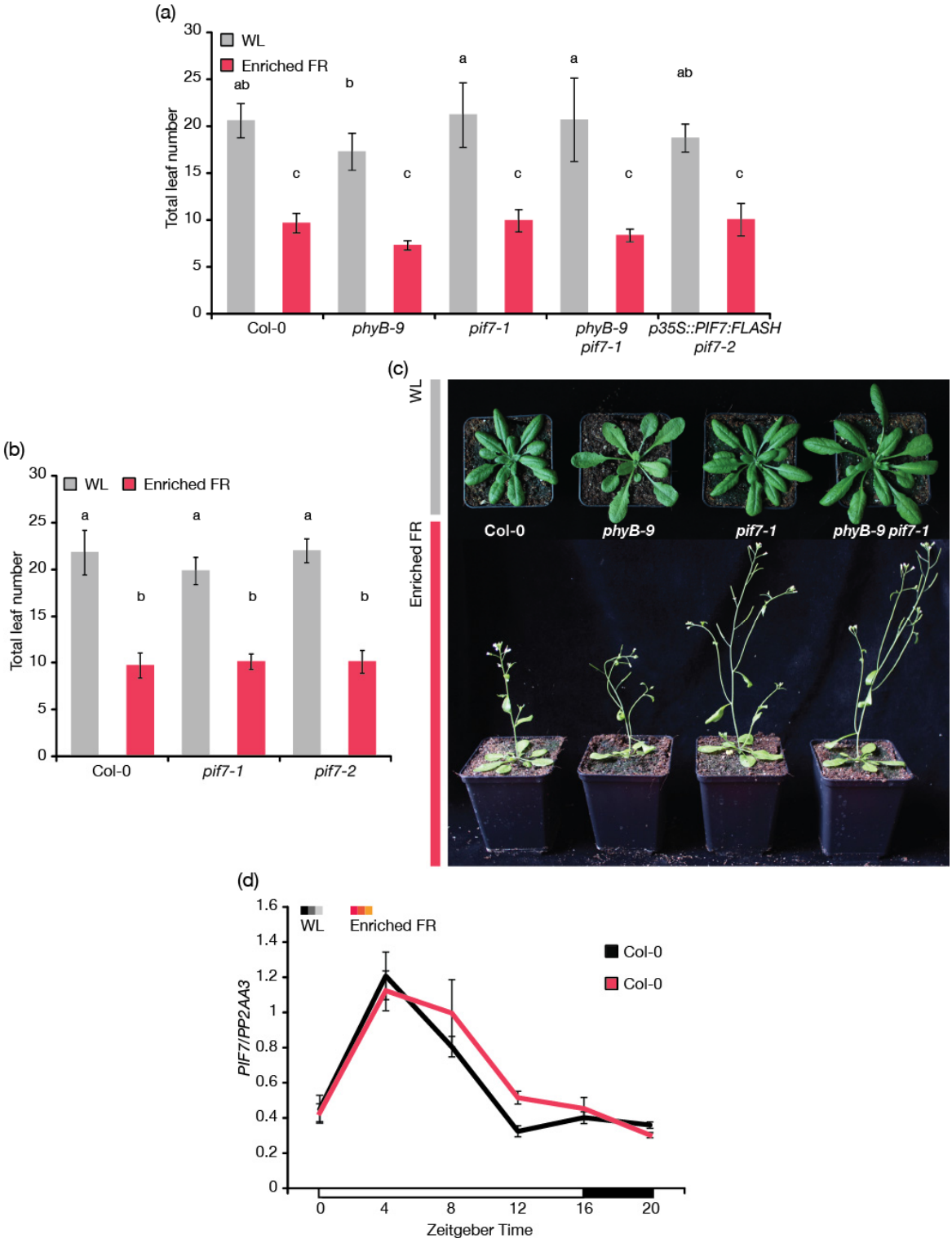

Figure 5.13 *PIF7* is not required for the acceleration of flowering under enriched FR light. (a) and (b) Flowering time of plants grown under LDs in WL and enriched FR light. (c) Picture of plants grown under LDs in

WL and enriched FR light. (d) *PIF7* mRNA expression in Col-0 seedlings grown under WL and enriched FR under LDs. Twelve-day-old seedlings were harvested every 4 h for 24 h. *PIF7* mRNA expression was measured by qRT-PCR; error bars are StD of three technical replicates. In (a) and (b) letters indicate statistical groups determined with a two-way ANOVA and all pairwise multiple comparisons within genotypes and within conditions with the Holm–Sidak method. Groups were considered statistically different when $P \leq 0.05$. Error bars are StD. In (a) n = 3-10 and in (b) n = 4-10.

5.6 Enriched FR light induces increased *FT* and *TSF* mRNA expression

Plants carrying mutations in *PHYB* mimic the SAS and display higher mRNA levels of *FT* and *TSF* compared to Col-0 (Figure 5.1f, Annex A.3a, A.3b, A.3b and A.3d). To evaluate the response of *FT* and *TSF* mRNA expression to enriched FR light conditions, the expression of these floral integrators in Col-0 plants grown under shade conditions was analysed in LDs through a 24 h time course. *FT* and *TSF* mRNA levels showed an increase in response to enriched FR light (Figure 5.14a and 5.14b).

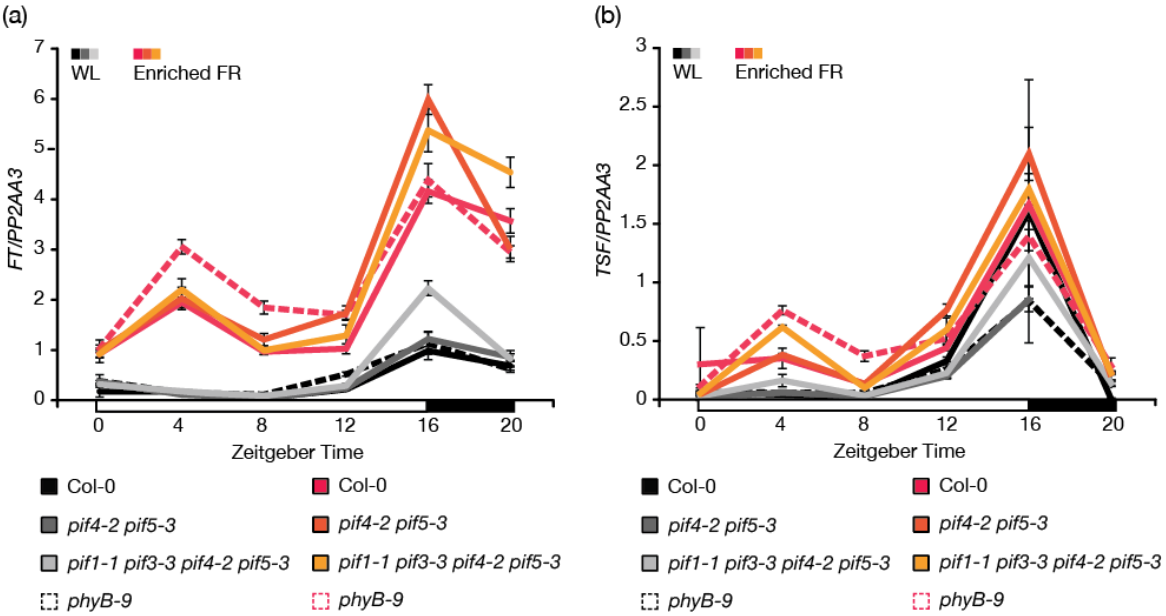

Figure 5.14 *FT* and *TSF* mRNA expression are induced in response to enriched FR light. *FT* (a) and *TSF* (b) mRNA expression in Col-0, pif4-2 pif5-3, pif1-1 pif3-3 pif4-2 pif5-3 and *phyB-9* mutant seedlings grown under

WL and enriched FR light. In (a) and (b) 12-day-old seedlings were harvested every 4 h for 24 h. *FT* and *TFS* mRNA expression were measured by qRT-PCR; error bars are StD of three technical replicates.

The peak of mRNA expression of *FT* and *TSF* (albeit to a lesser extent) at ZT16 is higher in plants grown under enriched FR light compared to plants grown under WL. Furthermore, an additional peak of *FT* and *TSF* mRNA level is observed at ZT4 in plants exposed to simulated shade (Figure 5.14a and 5.14b). This increase in the expression of *FT* and *TSF* mRNA under enriched FR light is displayed not only by Col-0 plants but also by *phyB* mutants, *pif4-2 pif5-3* double mutants and *pif1-1 pif3-3 pif4-2 pif5-3* quadruple mutants (Figure 5.14a and 5.14b). The similarities in the expression changes of the floral integrators observed in the mutants compared to Col-0, are consistent with the flowering phenotypes of *phyB-9*, *pif4-2 pif5-3* and *pif1-1 pif3-3 pif4-2 pif5-3* mutants observed under the same conditions. The flowering responses of these mutants to enriched FR light were similar to those of Col-0 (Figure 5.8a and 5.8b). Thus, these results show that the expression of *FT* and *TSF* mRNA is higher under enriched FR light conditions compared to WL, showing peaks of expression at ZT4 and ZT16 (Figure 5.14a and 5.14b). Therefore, the induction of flowering observed in plants growing under enriched FR light is driven at least partly by increased expression of *FT* and *TSF* mRNA. Furthermore, *phyB* mutants as well as *pif* mutants respond to shade in a similar manner to Col-0, inducing *FT* and *TSF* mRNA expression and causing comparable acceleration of flowering.

5.7 Photoperiod pathway components are required for the shade induction of flowering

Col-0 plants are highly sensitive to enriched FR light conditions, flowering with around half the number of leaves of plants under WL (Figure 5.5a and 5.5b). This early-flowering response is mediated, at least partly, by *FT* and *TSF* (Figure 5.14a and 5.14b). Results obtained with *phyB* mutants, together with previous reports (Figure 5.2a) (Kim *et al.* 2008, Wollenberg *et al.* 2008), suggest that photoperiodic pathway components play fundamental roles in the promotion of flowering by enriched FR light and are regulated by the PhyB signalling pathway. Therefore, the contribution of *GI*, *CO*, *FT* and *TSF* to the acceleration of flowering under simulated shade conditions was assessed. Mutants in *GI* or *CO* and double mutants in *FT TSF* were highly insensitive to enriched FR light conditions, flowering with a similar leaf number under WL compared to simulated shade (Figure 5.15). Under simulated shade, *ft-10* single mutant plants flowered later than *tsf-1* single mutants, as they do in WL under LDs. However, the response of *ft-10* or *tsf-1* single mutants to enriched FR light compared to WL is the same as Col-0 (all genotypes produce under FR light half the number of leaves as under WL). In terms of absolute number of leaves *tsf-1* mutant plants flower under both WL and enriched FR light at the same time as Col-0. Therefore, *TSF* is not essential for the flowering response to shade of Col-0, probably because *FT* is able to compensate for the lack of *TSF*. In *ft-10* mutant plants flowering is delayed compared to Col-0, both under WL and enriched FR light conditions. However the response to simulated shade conditions compared to WL is the same as in Col-0 and *tsf-1* mutants (Figure 5.15). These data indicate that, although *FT* seems to play a more important role in defining the time of flowering of plants under WL or enriched FR light than *TSF*, the early flowering response to the enriched FR light can be driven equally by the two genes.

Taken together, the results presented above indicate that the photoperiod pathway components *CO* and *GI* as well as *FT*, *TSF* play a redundant role and are essential for the early-flowering response to enriched FR light.

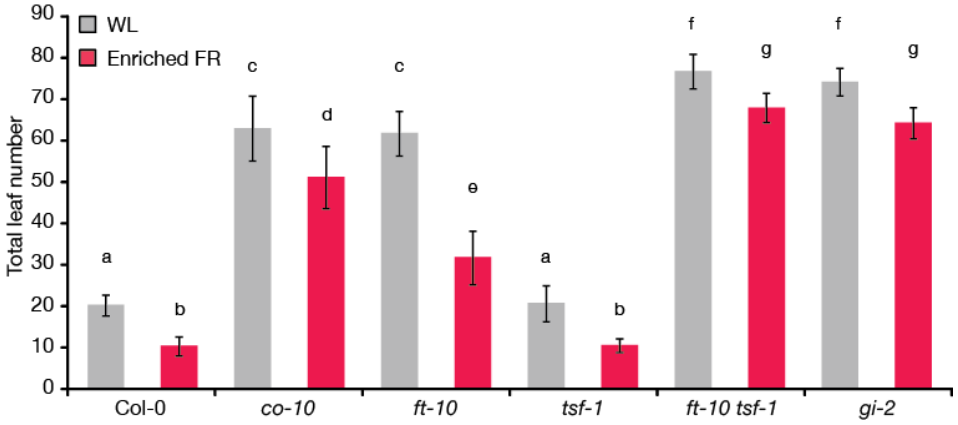

Figure 5.15 Photoperiodic pathway components are required for the promotion of flowering by enriched FR light. Flowering time of plants grown under LDs in WL and enriched FR light conditions. Letters indicate statistical groups determined with a two-way ANOVA and all pairwise multiple comparisons within genotypes and within conditions with the Holm–Sidak method. Groups were considered statistically different when $P \leq 0.05$. Error bars are StD, n = 7-11.

5.8 CO activity does not mask the response to shade in plants lacking *PIF4* and *PIF5*

CO plays an important role in inducing flowering under enriched FR light, because the flowering time of *co-10* mutant plants is strongly delayed under these conditions (Figure 5.15). In order to test whether *PIFs* have an effect on flowering under enriched FR light that could be masked by the strong effect of CO, we studied the flowering time of *co-10 pif4-2 pif5-3* triple mutants. In the triple mutant the early-flowering response to shade is strongly suppressed, similar to the effect observed in *co-10* single mutants. More importantly, there is no significant difference between the flowering time of *co-10* and *co-10 pif4-2 pif5-3* under WL or simulated shade (Figure 5.16a). *FT* and *TSF* mRNA levels were not increased in *co-10* mutants under simulated shade (Figure 5.16b and 5.16c).

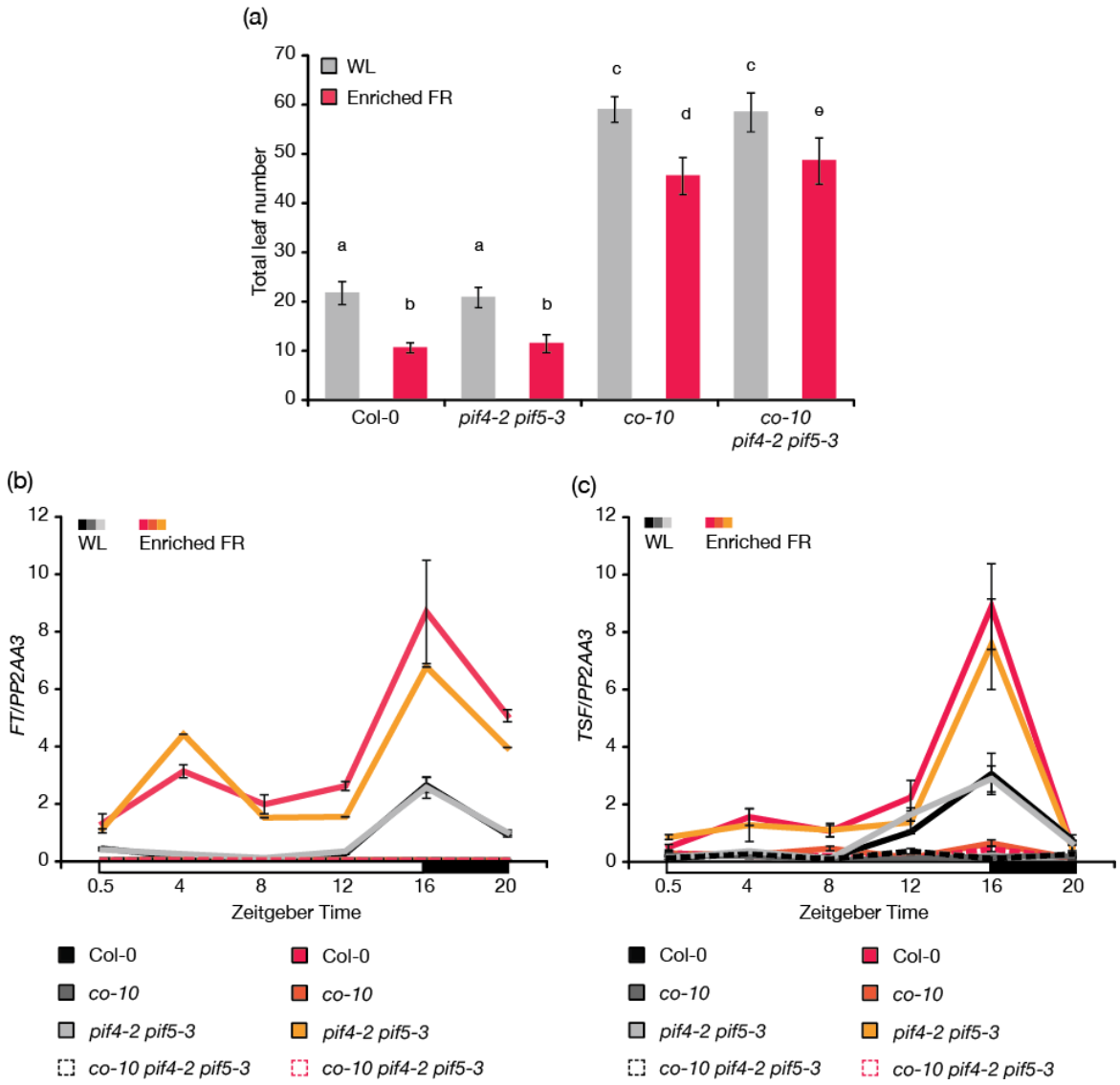

Figure 5.16 *PIF4* and *PIF5* do not have effect on the flowering response to shade in absence of *CO*. (a) Flowering time of plants grown under LDs in WL and enriched FR light conditions. *FT* (b) and *TSF* (c) mRNA expression in Col-0, *co-10*, *pif4-2 pif5-3* and *pif1-1 pif3-3 pif4-2 pif5-3* mutant seedlings grown under WL and enriched FR light. In (b) and (c) 12-day-old seedlings were harvested every 4 h for 24 h. *FT* and *TFS* mRNA expression were measured by qRT-PCR; error bars are StD of three technical replicates. In (a) letters indicate statistical groups determined with a two-way ANOVA and all pairwise multiple comparisons within genotypes and within conditions with the Holm–Sidak method. Groups were considered statistically different when $P \leq 0.05$. Error bars are StD, n = 14-17.

Thus, both *co-10* and *co-10 pif4-2 pif5-3* mutants showed strongly reduced expression of *FT* and *TSF* mRNAs under WL or enriched FR light. By contrast *pif4-2 pif5-3* double mutants retained a strong increase in *FT* and *TSF* mRNA levels under simulated shade (Figure 5.16b and 5.16c). In agreement with previous results, these experiments support the conclusion that *PIF4* and *PIF5* as well as *PIF1*, *PIF3* and *PIF7* do not play a role in the induction of flowering mediated by enriched FR light.

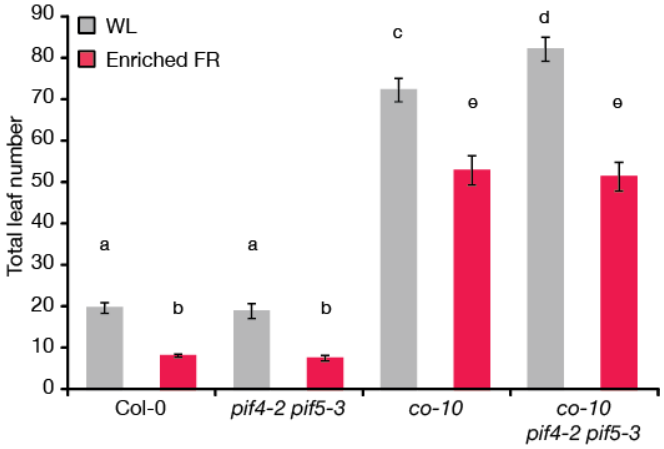

Figure 5.17 Reduction in the photosynthetic active radiation (PAR) does not affect the response of *pif4-2 pif5-3* mutants to shade. Flowering time of plants grown under reduced WL (reduced PAR) and enriched FR light conditions. Letters indicate statistical groups determined with a two-way ANOVA and all pairwise multiple comparisons within genotypes and within conditions with the Holm–Sidak method. Groups were considered statistically different when $P \leq 0.05$. Error bars are StD, n = 10.

Given that light induces the degradation of PIF proteins, the stability of these proteins and their potential role in flowering under shade, might be increased under low light intensities. Therefore, *pif4-2 pif5-3*, *co-10* and *co-10 pif4-2 pif5-3* mutants were grown under low WL (65 %) and low WL plus FR light (65 % WL, 65 % FR, see Methods) and their flowering time measured. *pif4-2 pif5-3* double mutant plants flowered with a similar number of leaves as Col-0, both under low WL and low WL plus FR light (Figure 5.17). Furthermore, no delay in

flowering time was detected for *co-10 pif4-2 pif5-3* compared to *co-10*. Thus, even under these low PAR conditions, *PIF4 PIF5* had no detectable effect on flowering.

PIF4 protein interacts with CO perhaps to facilitate binding to DNA and activation of gene expression (Chapter 4). This interaction could be relevant for an induction of flowering under shade conditions. In order to detect possible effects of this interaction on flowering response to shade, conditions in which CO activity is reduced compared to LDs were used. To achieve this, plants were grown under photoperiods of 12 h light with 12 h dark (12:12). Under this condition, CO protein stability would be reduced, since the peak of *CO* mRNA expression detected in the evening that facilitates accumulation of the protein at this time would overlap for longer with darkness, and CO would be targeted for degradation by the COP1/SPA complex. Col-0 plants grown under 12 h photoperiods flower with around 30 leaves, about 10 leaves more than Col-0 plants grown under 16 h photoperiods (Figure 5.18). From these results, a reduction of the *FT* and *TSF* mRNA expression levels would be expected under 12:12 photoperiods, however, these changes in expression remain to be tested. Flowering time experiments performed with WL and enriched FR light under a 12:12 photoperiod show an induction of flowering time of Col-0 under FR light compared to WL (Figure 5.18). The *pif4-2* and *pif4-2 pif5-3* mutants flowered at similar times to Col-0 under both light conditions. The *co-10* mutants flower very late under WL and the induction of flowering under FR is strongly suppressed. The same suppression of the early flowering under enriched FR is observed for *co-10 pif4-2* and *co-10 pif4-2 pif5-3*. These two genotypes flower as late as *co-10* mutant under WL. Taken together, these results support that even in conditions where the activity of CO is impaired, *PIF4* and *PIF5* are not able to flowering under enriched FR light.

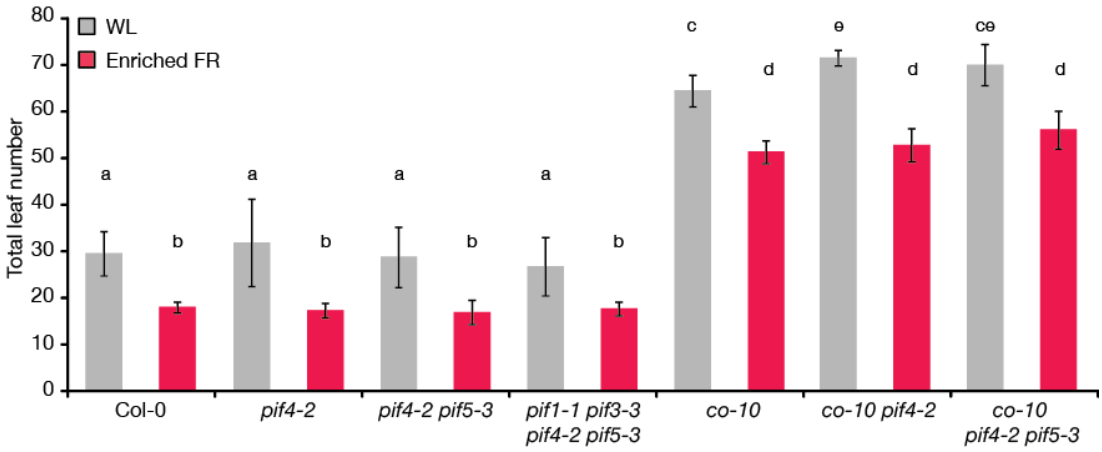

Figure 5.18 *pif4-2 pif5-3* mutations do not affect the flowering phenotype of Col-0 or *co-10* plants under shade in shorter photoperiods. Flowering time of plants grown under 12:12 photoperiods and enriched FR light conditions. Letters indicate statistical groups determined with a two-way ANOVA and all pairwise multiple comparisons within genotypes and within conditions with the Holm–Sidak method. Groups were considered statistically different when $P \leq 0.05$. Error bars are StD, n = 6-11.

5.9 Concluding remarks

Light quality plays a very important role in the regulation of flowering time. The FR light enrichment encountered in crowded canopies accelerates flowering as part of the shade avoidance response. Light quality changes are perceived by Phy photoreceptors that transmit the signals ultimately regulating the timing of flowering.

This work shows that the early flowering of *phyB* mutant requires components of the photoperiodic pathway, such as CO and FT TSF as well as transcription factors involved in light signalling pathways, such as PIF4 and PIF5. Furthermore, this work demonstrates that the promotion of flowering by simulated shade is dependent on CO, FT TSF and GI, but does not depend on PIFs transcription factors. However, as the PIF family has a very high degree of functional redundancy between members, it cannot be ruled out the possibility that additional PIFs play a role in the flowering response to shade.

6. Discussion

In this thesis, the genetic and molecular mechanism that control flowering of *A. thaliana* in response to ambient temperature changes and differences in light quality were examined. In particular, common molecular mechanisms in the regulation of flowering time by the photoperiodic, ambient temperature and light quality pathways were defined.

6.1 Flowering time is promoted by warm ambient temperatures under SDs

In *A. thaliana* the photoperiodic pathway promotes flowering specifically under LDs, so that flowering occurs much more rapidly under these conditions than under SDs. However, surprisingly, this delay under SDs can be overcome by growing plants at high temperatures, so that they flower with similar numbers of leaves under 27°C-SD and 21°C-LD. Therefore, the effect of high temperature on the activity of the photoperiodic pathway under SDs was examined. Consistent with previous data (Balasubramanian *et al.* 2006), this work shows that *FT* and *TSF*, the output genes of the photoperiodic pathway (Kardailsky *et al.* 1999, Kobayashi *et al.* 1999, Valverde *et al.* 2004, Yamaguchi *et al.* 2005, Sun *et al.* 2012), are essential for extreme early flowering under 27°C-SD and that the abundance of their transcripts is increased under these conditions compared to 21°C-SD. Also, under 27°C-SD, CO, a central component of the photoperiodic pathway (Putterill *et al.* 1995, Suarez-Lopez *et al.* 2001, Valverde *et al.* 2004), contributes additively with PIFs to increase expression of *FT* mRNA. Nevertheless, although plants flower at similar times under 27°C-SD and 21°C-LD, even the additive effect of CO and PIFs on *FT* mRNA under 27°C-SD causes it to accumulate to much lower levels than in plants exposed to 21°C-LD. Thus there must be additional factors that contribute to the early flowering under 27°C-SD. Reduced activity of floral repressors such as SVP in the shoot meristem of plants grown at 27 °C (Lee *et al.* 2013, Pose *et al.* 2013) might cause greater responsiveness to the FT signal and thereby contribute to the

early flowering under 27°C-SD. Consistent with this idea, *svp-41 ft-10 tsf-1* triple mutants exhibited no earlier flowering response to 27°C-SD compared to 21°C-SD, and indeed this was the only genotype tested that did not flower earlier when exposed to these conditions. Taken together, these data indicate that under higher temperatures the threshold level of photoperiodic pathway components required to activate *FT TSF* transcription is lowered so that the genes are expressed under non-inductive photoperiods, and that the amount of FT TSF required to induce flowering is reduced.

6.2 Low levels of *FT* mRNA under 27°C-SD are sufficient to promote early flowering

Compared to plants grown under 21°C-SD, exposure of plants to 21°C-LD or to high temperatures under SDs causes a dramatic acceleration of flowering, by approximately 43 and 39 leaves respectively (Figure 4.1d). *FT* strongly contributes to both responses (Kardailsky *et al.* 1999, Kobayashi *et al.* 1999, Samach *et al.* 2000, Suarez-Lopez *et al.* 2001, Balasubramanian *et al.* 2006, Kumar *et al.* 2012, Galvao *et al.* 2015), but the level of transcriptional activation of *FT* under 27°C-SD or 21°C-LD is not proportional to the degree of acceleration of flowering time under these conditions, as *FT* mRNA is over 10 fold more abundant under 21°C-LD than under 27°C-SD (Figure 4.1e). This discrepancy might be explained if similar amounts of FT protein accumulate under each condition despite the differences in the levels of *FT* mRNA. To test this possibility, *A. thaliana* seedlings expressing tagged version of FT (Corbesier *et al.* 2007) could be used to compare the levels of FT protein by western blot analysis under both conditions.

The exacerbated response to low *FT* levels might be more evident in genetic backgrounds impaired in flowering pathways independent of *FT* and *TSF*. *spl15* mutants flower very late under SDs. However, under LDs the flowering-time of this mutant is indistinguishable from

that of the WT plants, indicating that the *FT*-signalling pathway can overcome the lack of *SPL15* function (Hyun *et al.* 2016). It is therefore predicted that the increased response to FT at warm temperatures would bypass the requirements of *SPL15* under SDs. In such a scenario, the very late-flowering of *slp15* mutants under SDs would be strongly suppressed under warm temperatures, showing even a stronger response compared to WT plants.

On the other hand, exposure to high temperatures reduces the activity of floral repressors (Lee *et al.* 2013, Pose *et al.* 2013, Airoldi *et al.* 2015, Sureshkumar *et al.* 2016), suggesting that floral induction might be more sensitive to lower amounts of *FT* under 27°C-SD. An alternative hypothesis is that floral integrators expressed in the meristem are induced directly by high temperature. To test this possibility, the expression of *SOC1*, *FUL* and *AP1* mRNA at 21°C-SD and 27°C-SD was measured. No changes in expression of these genes mediated by warm ambient temperature were detected (Figure 4.9a, 4.9b and 4.9c). By contrast, the activity of the MADS box transcription factors SVP, FLM and MAF2 is reduced at high temperature. The role of these repressors has been compared between 16 °C and 23 °C (Lee *et al.* 2007, Gu *et al.* 2013), a different temperature range than used here, and usually under LDs (Lee *et al.* 2007, Gu *et al.* 2013, Lee *et al.* 2013, Pose *et al.* 2013). Although some studies have tested the function of these proteins under 27°C-LD (Lee *et al.* 2013, Pose *et al.* 2013, Airoldi *et al.* 2015) their activity under 27°C-SD has rarely been examined (Balasubramanian *et al.* 2006, Kumar *et al.* 2012, Galvao *et al.* 2015). Differential splicing of *FLM* and *MAF2* mRNAs at high temperature leads to reduction in the activity of floral repressive complexes involving SVP (Balasubramanian *et al.* 2006, Pose *et al.* 2013, Rosloski *et al.* 2013, Airoldi *et al.* 2015). The stability of SVP is also reduced at higher temperature, although the protein is not completely depleted (Figure 4.7a and 4.7b) (Lee *et al.* 2013). This is in agreement with the flowering phenotype of p*KNAT1::SVP svp-41* plants, which responded to temperature by inducing flowering, but showed a strong delay under

27°C-SD compared to *svp-41* mutants (Figure 4.8d). These combined results suggest that floral repression by SVP is, at least partially, impaired at higher temperatures. In the leaves, SVP acts to repress *FT* and *TSF* transcription, while in the meristem it represses the transcription of *SOC1* and the accumulation of GAs (Lee *et al.* 2007, Li *et al.* 2008, Jang *et al.* 2009, Andres *et al.* 2014). Therefore, reduced activity of SVP and MAF repressors at high temperature under SDs likely contributes to the capacity of low levels of CO to activate transcription of *FT* in the leaves as well as to allowing the meristem to respond when *FT* mRNA is expressed only at low levels. The importance of the role of SVP in the meristem was supported by analysis of the *svp-41 ft-10 tsf-1* triple mutant, where *svp-41* prevented the residual flowering response to 27°C-SD detected in *ft-10 tsf-1* double mutants. That high temperature increases sensitivity of the meristem to FT signalling might be more directly testable using recently described *FT* inducible systems (Krzymuski *et al.* 2015). However, the proposed increased sensitivity of the meristem under 27°C-SD is detected when *FT* mRNA is expressed at very low levels, and whether the inducible system can be tuned to express *FT* at such levels remains to be established.

Alterations in *FT* chromatin structure at higher temperatures may also contribute to the increased activation of transcription by low levels of CO, and particularly removal of H2A.Z under 27°C-SD has been proposed to increase binding of PIF4 to the *FT* promoter and thereby the transcriptional activation of the gene (Kumar and Wigge 2010, Kumar *et al.* 2012). In support of the importance of *FT*, *TSF* and *SVP* for a flowering response under 27°C-SD, the *svp-41 ft-10 tsf-1* triple mutant, but not *svp-41* or *ft-10 tsf-1* double mutants, flowered at the same time under 21°C-SD and 27°C-SD. This differs from LDs, where the *svp* mutation alone is sufficient to cause insensitivity to ambient temperatures (Lee *et al.* 2007, Pose *et al.* 2013), presumably because compared to SDs the photoperiodic pathway is much more

active under LDs, and therefore quantitative modulation of its activity is less likely to cause phenotypic changes under these conditions.

6.3 Roles of CO and PIFs in activation of *FT* under SD at high temperature

Early flowering under 27°C-SD depends on *FT* activity, and increased transcription of *FT* under 27°C-SD compared to 21°C-SD is conferred by CO and PIFs. Analysis of *co-10 pif4-2* double mutants as well as the respective single mutants demonstrated that CO and PIF4 act additively to promote *FT* transcription and flowering under 27°C-SD (Figure 4.5a and 4.5b). Furthermore, both PIF4 and PIF5 contribute to this process, because mutations in either gene delayed flowering under 27°C-SD. Also, the double mutant *pif4-2 pif5-3* was later flowering than the *pif4-2* single mutant, indicating some genetic redundancy. PIF4 was studied as representative of their activity because of the availability of tools to assay the protein when expressed from its own promoter.

Both CO and PIF4 bind to similar regions of the *FT* promoter (Kumar *et al.* 2012, Song *et al.* 2012b, Zhang *et al.* 2015), and a physical interaction between them was detected. The importance of the CO-PIF4 interaction for *FT* activation under 27°C-SD is uncertain, as the two proteins can activate *FT* transcription independently of each other when expressed at high levels. Overexpression of *PIF4* caused earlier flowering of *co-10* mutants (Figure 4.6a), while when *CO* was accumulated at high levels in Col-0 under LDs, PIF4 was not required for *FT* transcription and flowering (Figure 4.6e, 5.14 and 5.16). Similarly, mutations in *PIF4* and *PIF5* did not delay flowering of p*SUC2::CO* plants under 27°C-SD (figure 4.6d). Moreover, under 27°C-SD, the *co-10* and *pif4* single mutants expressed *FT* mRNA at higher levels than the double mutant (Figure 4.5b), suggesting that each protein can act independently of the other in *FT* activation. This capacity to act independently presumably

explains why an additive rather than an epistatic genetic interaction was detected between *pif4* and *co*, and might be explained by genetic redundancy both for *CO* and *PIF4*. However, the diurnal pattern of PIF4 accumulation under 27°C-SD suggests that it can only activate *FT* transcription at times when CO is expressed (Figure 4.4e and 4.4d). PIF4 protein was present from before dawn, when the expression of many of its target genes such as *ATHB2* and *IAA29* occurs (Yamashino *et al.* 2013b), throughout the photoperiod until dusk. However, no peak in *FT* mRNA was reproducibly detected until both CO and PIF4 were expressed at dusk. Therefore, the physical interaction between the proteins might contribute to the activation of *FT* transcription at dusk under 27°C-SD.

The effect of temperature on CO and PIF4 activity is striking, because in wild-type plants neither activates *FT* transcription at 21°C-SD, but they do at 27°C-SD. No increase in PIF4 levels was detected at dusk, when *FT* is expressed, under 27°C-SD compared to 21°C-SD, suggesting that changes in PIF4 levels at high temperature do not contribute to the early-flowering phenotype. By contrast CO was slightly, less than 2 fold, more abundant under 27°C-SD than 21°C-SD, so that an increase in its level might contribute to higher *FT* induction under 27°C-SD. This appears to differ under LDs where no difference in CO abundance was observed between 27 °C and 22 °C (Seaton *et al.* 2015). The much higher levels of CO protein present under LDs compared to SDs might prevent exposure to high temperature from causing a further increase. Modulation of CO protein levels by cold is mediated by the E3 Ubiquitin ligase HOS1, which promotes degradation of CO in response to short periods of cold (Jung *et al.* 2012b, Lee *et al.* 2012a, Joon Seo *et al.* 2013). Similarly, Blazquez *et al.* (2003) showed that at 23 °C, p*35S::CO* induces flowering as dramatically as p*35S::FT*, whereas at 16 °C, p*35S::CO* plants flower later than p*35S::FT*, supporting the idea that the capacity of CO to activate *FT* is regulated by temperature at the post-transcriptional level. If the range of temperatures in which this system operates extends to 27 °C, then reduction in

HOS1 activity could contribute to the increased level of CO under 27°C-SD compared to 21°C-SD. Alternatively, one of the other mechanisms that influence CO protein stability might be affected by high temperatures (Jang *et al.* 2008, Liu *et al.* 2008b, Song *et al.* 2012b).

The activation of *FT* by CO and PIF4 under 27°C-SD but not 21°C-SD might also depend on other aspects of *FT* regulation. For example, SVP, FLM and MAF2 are negative regulators of *FT* transcription, so impairment of their activities at high temperature might allow low levels of CO and PIF4 to additively activate *FT*. However, under 27°C-SD, the *svp-41* mutant did not show more *FT* transcript than Col-0 at dusk (Figure 4.8b), suggesting that under these conditions the early flowering of *svp-41* mutants might mainly be conferred by impairment of its role in the meristem. A similar conclusion can be drawn from the observation that the residual flowering response to 27°C-SD in *ft-10 tsf-1* mutants was abolished in *svp-41 ft-10 tsf-1* (Figure 4.1b and 4.8d), which is likely due to removing SVP activity in the meristem as *ft-10 tsf-1* already blocks the leaf response. Alternatively, the *svp-41* mutation might cause increases in *FT* transcript at other times, or alteration of *FT* chromatin, for example by removal of H2A.Z, might be critical in allowing CO and PIF4 to activate *FT* under 27°C-SD.

In conclusion, the delay in flowering normally observed under SDs is overcome by exposing plants to high temperatures. This depends on photoperiodic flowering pathway components, CO and FT TSF. The present study demonstrates that the levels of these components that induce flowering under 27°C-SD are much reduced compared to inductive LDs. The early flowering response under 27°C-SD therefore likely depends on a shift in the threshold of CO and FT proteins required to activate flowering. The conclusion that the flowering response under 27°C-SD depends on threshold levels of photoperiodic flowering components can also explain the greater variability in flowering time observed under these conditions compared to inductive LDs. Such a threshold effect might be expected to be more sensitive to natural genetic variation among accessions, as observed in other signalling pathways (Polaczyk *et*

al. 1998) and in gene expression responses to high temperature in other systems (Chen *et al.* 2015). In this sense, models that predict flowering-time in crops growing in their normal growing season have been developed. These models (i.e. Decision Support System for Agrotechnology Transfer [DSSAT] and Agricultural Production Systems sIMulator [APSIM]) assume that photoperiod only affects rate of development at and below a certain temperature (T_0), above T_0, the rate is affected only by temperature (Craufurd and Wheeler 2009). In *A. thaliana*, T_0 seem to be just below 27 °C and the effect of temperature in flowering time might be determined by increments in the levels of *FT* beyond a molecular threshold.

The flowering response to temperature varies widely between species. For example, flowering has been shown to be delayed at 25 °C compared to 18 °C in *Boechera stricta*, a perennial relative of *A. thaliana* (Anderson *et al.* 2011). Interestingly, Anderson and collaborators (Anderson *et al.* 2011) found that *CO* was near a QTL associated to the temperature response and not to photoperiod, since the QTL was significant in plants grown under cool but not under elevated temperatures. Similarly, *Fragaria vesca* flowers at low temperatures irrespective of photoperiod (Heide 1977). In *Chrysanthemum morifolium* flowering is delayed at temperatures warmer than 20 °C, and this is due to a delay in the expression of the *FT* homologue *FT*-like *3* (*FTL3*) (Oda *et al.* 2012, Nakano *et al.* 2013). In *Narcissus tazetta* the *FT* homologue (*NtFT*) is induced at 25 °C promoting flowering. At 12 °C, *NtFT* levels remain basal and florogenesis is suppressed (Noy-Porat *et al.* 2009, Noy-Porat *et al.* 2013). In wheat and barley, the reproductive development is controlled by an interaction between temperature and day-length. Under LDs, warm ambient temperatures induce reproductive development in these two cereals, while under SDs warm temperatures have the opposite effect on early reproductive development. Unlike in *A. thaliana*, transcript levels of the barley orthologue of *FT* (*HvFT1*) are not influenced by temperature. Furthermore, no evidence that any other known regulators of the LD flowering response are temperature

sensitive, at least at the transcript level (Hemming *et al.* 2012). These examples highlight the variety of mechanisms governing the regulation of flowering time by ambient temperature in nature. Interestingly, there is some degree of conservation in the thermosensory flowering pathway between *A. thaliana* and several other species, as *FT*-like genes have been identified as integrators in this response. However, the gene regulatory networks that control the expression of *FT*-like genes in many plant species have not been characterized in detail (Capovilla *et al.* 2015).

6.4 Photoperiod components are necessary for the induction of flowering under shade

In dense vegetation, plants sense the presence of neighbours by monitoring changes in light quality. These light quality changes are perceived by phytochrome photoreceptors (Figure 1.3). Underneath dense canopies, the R:FR ratio decreases from 1.15 to values typically in the range 0.05 - 0.8 (Smith 1982) and the phytochromes phototransform into the inactive form. This triggers phenotypical changes that together are called the SAS (Smith 1982, Ballaré 1999, Franklin and Whitelam 2005, Franklin 2008, Casal 2012). In *A. thaliana*, PhyB has a predominant function regulating these responses, although PhyD and PhyE also contribute. The results presented here as well as other reports demonstrated that mutations in *PHYB* cause a constitutive shade avoidance phenotype, including long hypocotyl, apical dominance, long petioles and early flowering (Somers *et al.* 1991, Reed *et al.* 1993, Smith and Whitelam 1997). This study shows that *CO*, *FT* and *TSF*, components of the photoperiod pathway, are important contributors to the early flowering phenotype of *phyB* mutants (Figure 5.2a), because mutations in any of these genes significantly supress this phenotype. Similar results were obtained in previous reports (Putterill *et al.* 1995, Endo *et al.* 2005). In support

of this requirement, *FT* and *TSF* mRNA levels were higher in *phyB* mutants compared to Col-0 (Figure 5.1f, Annex A.3a and A.3b (Cerdán and Chory 2002, Endo *et al.* 2005). The increment on *FT* levels in the *phyB* mutants was shown to depend, at least in part, on the activity of a nuclear protein called PFT1 (Cerdán and Chory 2002). Vegetational shade and the characteristic phenotypic responses triggered by this condition were successfully reproduced by supplementing the WL growth condition of the growth chambers with FR light (enriched FR light). Col-0 plants grown in the simulated shade conditions displayed a shade avoidance phenotype, including long hypocotyls and acceleration of flowering (Figure 5.5a – 5.5e). This acceleration of flowering of Col-0 was due ultimately to increases in the expression of *FT* and *TSF* mRNA, typically at ZT16 under LDs compared to Col-0 (Figure 5.14a, 5.14b, 5.16b and 5.16c). Under enriched FR light the expression of these floral-integrator genes was also increased at ZT4, causing a peak in mRNA level characteristic of this condition (Figure 5.14a, 5.14b, 5.16b and 5.16c). The expression peak of *FT* at ZT4 under enriched FR has been previously described (Kim *et al.* 2008, Wollenberg *et al.* 2008) and is likely due to an increase in *CO* mRNA transcription during the early hours of the day. Consistent with the conclusion that early flowering under enriched FR is dependent on *FT* and *TSF*, *ft-10 tsf-1* double mutants were almost insensitive to simulated shade. However, the presence of either functional *FT* or *TSF* is sufficient to trigger a flowering response of approximately the same intensity, causing plants to flower with about 50% of the leaves displayed in WL (Figure 5.15) (Kim *et al.* 2008).

The residual sensitivity of *ft-10 tsf-1* plants to shade is at least partly due to a flowering response driven by *SOC1* (Kim *et al.* 2008). Also, the levels of DELLA proteins are reduced under shade in hypocotyls and petioles and some phenotypic responses to this condition, such as elongation of hypocotyl and petiole, require GA (Djakovic-Petrovic *et al.* 2007). As *SOC1* transcription is induced by GA, the slightly early flowering time of *ft tsf* double mutants

under shade conditions could be influenced by changes in the levels of this hormone (Moon *et al.* 2003, Kim *et al.* 2008). Alternatively, *SOC1* expression could be induced by reduced levels of floral repressors, such as SVP.

Consistent with the above information, plants without a functional *CO* allele were also highly insensitive to enriched FR light conditions, supporting the idea that CO contributes to the early flowering phenotype under shade by activating *FT TSF* (Figure 5.15) (Kim *et al.* 2008). CO is a key transcription factor in the regulation of flowering time under LDs. A PhyB-mediated stabilization of CO protein is postulated to promote the acceleration of flowering under enriched FR light conditions (Valverde *et al.* 2004, Kim *et al.* 2008). Under these conditions, PhyB is predominantly in its inactive form preventing the degradation of CO protein and allowing its accumulation (Kim *et al.* 2008). Increases in *CO* mRNA level are also promoted by enriched FR light as well as by the absence of PhyB (Cerdán and Chory 2002, Kim *et al.* 2008). Thus, the acceleration of flowering mediated by enriched FR light greatly depends on the transcriptional and post translational regulation of *CO*. The requirement for *CO* in the promotion of flowering by shade has also been observed in shorter photoperiods. Under 12:12, *CO* was essential for floral induction mediated by enriched FR light (Figure 5.18). Under these conditions, *co* mutant plants flowered much later than Col-0. However, under SDs *CO* did not have an effect on flowering under shade as its protein is absent or almost undetectable under such short photoperiods (Valverde *et al.* 2004). *GI* is also a key player in the induction of flowering under enriched FR as *gi-2* mutant plants presented a strong insensitivity to these light conditions (Figure 5.15), supporting the idea that enriched FR light accelerates flowering trough the photoperiod pathway. Alternatively, part of the shade-induced acceleration of flowering could be mediated by PFT1 though the activation of *FT* mRNA in a CO-independent manner. However the lack of *FT* expression in *co-10* mutants growing under shade conditions (Figure 5.16b) does not support this hypothesis. Taken

together, these results show that the induction of flowering by shade is highly dependent on photoperiod pathway components. These results are in agreement with previous reports (Kim *et al.* 2008, Wollenberg *et al.* 2008) that showed the attenuated flowering response to low R:FR light ratio of photoperiodic pathways mutants. Consequently, under non-inductive SD photoperiods, enriched FR light did not accelerate flowering (Figure 5.8c) (Kim *et al.* 2008, Wollenberg *et al.* 2008).

Many aspects of the shade avoidance response are conserved in different plant species. The promotion of stem elongation in response low R:FR light ratio is the most conspicuous response. For example, low R:FR and/or EODFR has been demonstrated to promote stem elongation in many crop species including beans (Downs *et al.* 1957), mustard (*Sinapis alba*) (Morgan *et al.* 1980), tobacco (*Nicotiana tabacum*) (Kasperbauer 1971), sunflower (*Helianthus annus*) (Libenson *et al.* 2002) and tomato (*Solanum lycopersicon*) (Selman and Ahmed 1962). The acceleration of flowering in response to shade has also been reported in several *A. thaliana* accessions (Botto and Smith 2002) as well as in other species. In barley, for example, flowering is accelerated by enriched FR light in a phytochrome dependent-manner (Deitzer *et al.* 1979). However, flowering acceleration seems to be less widely conserved than other shade avoidance responses among species (Casal 2012). In this sense, domestication of crops over the years could have affected the conservation of flowering responses to shade, as early flowering might not have been always an advantage for high production in the crowded and therefore, shaded fields.

6.5 PIFs do not play a role in the induction of flowering under shade conditions

PIFs play an important role in elongation growth. PIF4 and PIF5 are positive regulators of these responses, they directly activate the expression of *YUC* and *IAA/AUX*, genes that are

important for auxin biosynthesis and signalling, respectively, thereby controlling auxin-mediated growth (Lorrain *et al.* 2008, Hornitschek *et al.* 2012). Under shade conditions, PIF4 and PIF5 directly activate shade-induced genes, such as *YUC8*, *IAA29*, *CKX5*, *FHL* as well as *PIL1* and *ATHB2* (Lorrain *et al.* 2008, Kunihiro *et al.* 2011, Hornitschek *et al.* 2012). The induction of some of these genes under shade, for instance *PIL1*, *ATHB2*, *YUC8* and *YUC9* is also controlled by PIF7 (Li *et al.* 2012a). Indeed, the control of *YUC8* and *YUC9* by PIF7 is through direct binding to G-box elements in their promoters (Li *et al.* 2012a). The present study shows that under enriched FR light, PIF4 protein accumulates to higher levels during the evening (ZT12 – ZT16) of LDs compared to WL (Figure 5.7a). This accumulation might be the result of an increase in the levels of *PIF4* mRNA detected in the evening under simulated shade conditions compared to WL (Figure 5.7b). Nonetheless, the increase in the expression levels of *PIF4* mRNA must be accompanied by a reduction in the turnover rate of PIF4 protein, because low levels of expression of *PIF4* mRNA in the evening under shade produced comparable quantities of protein made by high *PIF4* mRNA expression earlier in the day. The increase in PIF4 protein under enriched FR light might directly promote the expression of shade-induced genes, such as *IAA29* (Figure 5.10) as well as *FHL*, *CKX5*, *PIL1*, *ATHB2*, *XTR7*, *YUC8* (Lorrain *et al.* 2008, Hornitschek *et al.* 2009, Hornitschek *et al.* 2012) . Although the expression of several of these genes is reduced in the *pif4 pif5* double mutant under high R:FR, the expression levels of *FHL*, *CKX5*, *IAA29*, *PIL1*, *ATHB2*, *XTR7*, *YUC8* are still induced by shade in this genetic background (Figure 5.10) (Lorrain *et al.* 2008, Hornitschek *et al.* 2009, Hornitschek *et al.* 2012). This suggests that another transcriptional regulator controls the expression of these genes under shade. Other members of the PIF family are candidates for such a function since they can bind similar DNA sequences and can act additively (Martínez-García *et al.* 2000, Huq and Quail 2002, Huq *et al.* 2004, Shin *et al.* 2009b, Hornitschek *et al.* 2012). Even after the simultaneous removal of *PIF1*, *PIF3*, *PIF4*

and *PIF5* the quadruple mutant plants can still respond to shade showing high levels of *IAA29* mRNA (Figure 5.8b and 5.10). These observations indicate that PIF1 and PIF3 are either not implicated in this regulation or that their function can be taken over by other PIFs, at least for the control of *IAA29* expression. These results are consistent with previous reports that higher order *pif* mutants still respond to enriched FR light through hypocotyl elongation (Leivar *et al.* 2012).

The hypocotyl elongation displayed by *phyB* mutants, characteristic of the constitutive shade avoidance phenotype, as well as the induction of shade marker genes, such as *PIL1*, *HFR1* and *ATHB2*, are partially suppressed by mutations in *PIF4* and *PIF5* (Lorrain *et al.* 2008). Consistent with these observations, the early flowering phenotype of *phyB-9* mutants was suppressed by *pif4-2 pif5-3* mutations (Figure 5.3c), supporting the idea of a possible role of PIFs in the PhyB-mediated early flowering and consequently in the early-flowering promoted by shade. However, neither *phyB-9* nor *pif4-2 pif5-3* mutants displayed a reduced flowering-response under shade conditions, perhaps because of functional redundancy with other Phys and PIFs. PIF4 and PIF5 interact with PhyB, but no binding to PhyD and PhyE has been reported to date (Leivar and Quail 2011). Thus, the interaction between PhyB and PIF4 PIF5 might be relevant for the PhyB-mediated acceleration of flowering, but not for a more general Phytochrome-mediated flowering response to shade. The effect of PIF4 and PIF5 in flowering of *phyB* mutants might be exerted by increasing the expression of *FT* and *TSF*. However, the expression levels of *FT* and *TSF* mRNA in *phyB* mutant increase around the second half of the day under LDs, when at least PIF4 protein is not present (Figure 5.1f, Annex A.3a and A.3d). Nevertheless, previous publications show that there is some increase of *FT* mRNA compared to Col-0 earlier in the morning (Cerdán and Chory 2002) when PIF4 protein can be detected. Measurements of the expression levels of *FT* and *TSF* in the *phyB-9 pif4-2 pif5-3* triple mutant would more clearly determine the role of PIF4 and PIF5 in the *phyB* mutant.

Alternatively, PIF4 and its interaction with CO might be relevant for the stabilization of CO protein in the *phyB* mutant, explaining why PIF4 and PIF5 are required for the induction of flowering of this mutant. The lack of a flowering phenotype of the *pif4-2 pif5-3* double mutant and higher order *pif* mutants under LDs suggest, however, that PIFs are not required for the regulation of CO protein stability and flowering time in the presence of functional PhyB. CO protein stability assays in *phyB*, *phyB pif4* and *phyB pif4 pif5* mutant backgrounds would further test the roles of PIFs in determining CO protein stability in *phyB* mutants.

Flowering-time experiments performed under enriched FR light demonstrated that mutations in single *PIF* genes (*PIF1*, *PIF3*, *PIF4*, *PIF5* and *PIF7*) as well as higher order mutations did not suppress the early flowering promoted by enriched FR light (Figure 5.8a, 5.8b, 5.9a 5.9b, 5.13a and 5.13b). These results suggest that PIFs do not play a role in flowering under simulated shade conditions. Accordingly, the *FT* and *TSF* mRNA levels of *pif* mutants did no differ from those observed for Col-0 plants under enriched FR, both at ZT4 and ZT16 (Figure 5.14a and 5.14b). Although PhyB is the principal photoreceptor involved in the shade avoidance response, it is not the sole mediator of this process because *phyB* mutant plants retain flowering and elongation responses to enriched FR light (Figure 5.6a, 5.6b and 5.6c) (Goto *et al.* 1991, Somers *et al.* 1991, Whitelam and Smith 1991, Reed *et al.* 1993, Halliday *et al.* 1994, Devlin *et al.* 1996, Smith and Whitelam 1997). PhyD and PhyE are also implicated in this response, so that triple mutant plants *phyB phyD phyE* are insensitive to low R:FR irradiation, excluding role for PhyC in the shade avoidance response (Aukerman *et al.* 1997, Devlin *et al.* 1999, Franklin *et al.* 2003b). The effect of the PIFs under enriched FR light could be masked by the role of PhyD and PhyE in this response, which might act independently of PIF4 and PIF5 (Leivar and Quail 2011). PhyD and PhyE could therefore induce early flowering in shade through other mechanisms by modulating the function of other floral regulators. Generation and further experiments with *phyD phyE pif4 pif5* quadruple, *phyB*

phyD phyE pif4 pif5 quintuple and higher order *pif* mutants would help to elucidate the role of PIFs in flowering time. On the other hand, given the similarity between the *PIF*-like genes, a high level of redundancy between these genes could also explain why a role for *PIFs* in flowering under shade cannot be demonstrated genetically. Even though high order mutants were tested, a genotype with complete loss of *PIFs* function has not yet been generated and tested for flowering. Flowering-time analysis of such a *PIFs* loss of function genotype would be conclusive in determining their function in this process.

6.6 The role of CO in the induction of flowering by shade

CO plays a central role in the promotion of flowering under LDs. Upon CO protein stabilization in long photoperiods, it strongly activates *FT* and *TSF* expression and promotes flowering (Putterill *et al.* 1995, Suarez-Lopez *et al.* 2001, Valverde *et al.* 2004, Michaels *et al.* 2005, Yamaguchi *et al.* 2005). The results presented here show that *CO* is essential for *A. thaliana* to respond to enriched FR light conditions, as in *co-10* mutant plants the response to shade is highly suppressed. This suppression is most likely caused by the strong reduction of *FT* and *TSF* expression in *co-10* mutants under shade (Figure 5.16b and 5.16c) (Kim *et al.* 2008, Wollenberg *et al.* 2008). The role of *CO*, *PIF4* and *PIF5* is also important for the early flowering of *phyB* (Figure 5.2a and 5.3c). This suggests that the interaction between CO and PIF4 is relevant for the promotion of flowering in *phyB* mutants. Hence, given the phenotypic and molecular similarities between the *phyB* mutant and the shade avoidance of Col-0 plants, the CO-PIF4 interaction could play a role in the induction of flowering under shade conditions. Single and higher order mutations in *PIFs*, however, had no effect on flowering time under enriched FR light. One reason for the absence of a flowering phenotype in these mutants under shade could be the strong effect that *CO* has on flowering. The strong promotion of

flowering by *CO* could mask any effect that PIFs might have in flowering time under shade. Therefore, quantitatively reducing rather than abolishing the activity of CO might reveal a role for PIFs and the CO-PIF4 interaction in the induction of flowering by shade. To achieve this situation, experiments under shorter LD-photoperiods, for example 12:12, were performed. Using 12:12 photoperiods failed to demonstrate a role for PIF4 and PIF5 in the induction of flowering under shade, as *pif4-2 pif5-3* double mutants did not flower later than Col-0 under shade (Figure 5.18). Thus, mutations in *PIF4* and *PIF5* do not cause changes in shade response even in environmental and genetic contexts of reduced CO activity. These data suggest that the interaction between PIF4 and CO might not be relevant for the control of flowering under enriched FR conditions and therefore the biological significance of this interaction under these conditions remains unclear.

The expression of direct target genes of PIF4 and PIF5 involved in auxin biosynthesis and signalling, such as, *YUC8* and *IAA29*, are directly regulated by PIF4 and PIF5 under high R:FR in low PAR and their expression increases in low compared to high PAR (Hornitschek *et al.* 2012). Furthermore, differences in traits such as hypocotyl length between Col-0 and the *pif* single and higher order mutants are larger in low compared to high PAR (Hornitschek *et al.* 2012). Under several simulated shade conditions, mutations in *PIF1*, *PIF3*, *PIF4* and *PIF5* genes do not show a flowering phenotype, however preliminary results under low PAR show a delay in flowering time of *co-10 pif4-2 pif5-3* compared to *co-10* (Figure 5.17). This suggests that PIF4 and PIF5 could have a role in promoting flowering under low PAR. However, this should be investigated more thoroughly since *pif4-2 pif5-3* double mutants did not show a flowering phenotype under low PAR. Under these conditions and as postulated previously, the strong induction of flowering mediated by CO might mask any effect that PIFs have on flowering. These results enable a possible scenario where the interaction between

PIF4 (and maybe other PIFs) and CO could play a regulatory role in flowering time under low PAR.

6.7 Conclusion

Striking similarities exist among the phenotypic responses that *A. thaliana* develops to escape from high ambient temperature, such as, leaf hyponasty, hypocotyl elongation and early flowering with those triggered by vegetational shade. The results showed here and several other previous reports describe the existence of common regulatory components in the pathways that regulate these developmental responses (Proveniers and van Zanten 2013). This thesis aimed to increase our understanding on the interaction between environmental signals affecting flowering time. The most remarkable contribution of this study is the finding that CO is not only a key component of the photoperiodic flowering pathway under LDs, but also an essential player in the induction of flowering mediated by warm ambient temperatures under SDs, as well as a central regulator of the promotion of flowering under enriched FR light. CO is thus a molecular hub in the interplay between ambient temperature, light quality and day length contributing to the proper interpretation of environmental signals and rendering therefore the optimal timing of flowering. In addition to CO, other CO-like proteins (COL) have been isolated and their functions related to light signalling, flowering and circadian clock regulation described (Ledger *et al.* 2001, Cheng and Wang 2005, Datta *et al.* 2006). This suggests that during evolution, COL proteins have been recruited to distinct genetic pathways controlling flowering and other processes. In this sense, the *A. thaliana* CO might be an example of a master regulator whose function has diverged to contribute to the responses to several environmental signals and mediate the activation of *FT* and *TSF* (Simon *et al.* 2015). However, this hub function of CO seems not to be conserved

in other species. For example in rice, the orthologue of CO (Hd1) regulates flowering in the photoperiod pathway, but it has been suggested that it does not participate in flowering acceleration dependent on temperature (Song *et al.* 2012a) In *A. thaliana*, the central role of CO in the interpretation of distinct environmental signals is accomplished by its interaction with PIFs, more importantly PIF4. The interaction between these two proteins serves probably as an extra layer of regulation for the proper timing of flowering.

In an ecological context, the ability of plants to regulate flowering in response to temperature changes irrespective of photoperiod might be more important in latitudes near the Equator, where the day-length is approximately constant during the year, than in more extreme northern or southern latitudes, where the changes in photoperiod are more abrupt throughout the year. *A. thaliana* is found in nature in rather northern latitudes where the difference between day-length in winter and summer is usually big. Therefore it has developed a very specialized mechanism to sense the changes in day-length and this has become the predominant signal that drives the timing of flowering. In this sense, the exposure of young *A. thaliana* seedlings to a few days of long photoperiods is enough for the plant to interpret this signal as a seasonal change and activate the developmental programs that will promote flowering (Torti *et al.* 2012).

Changes in temperature highly influence the decision to flower, triggering the reproductive development even under non-inductive photoperiods. However, it is not surprising that these events require long and sustained exposures to warm temperatures (Galvao *et al.* 2015), taking double the amount of time to flower under warm temperatures in non-inductive photoperiods than under LDs. One possible reason for this behaviour is that *A. thaliana* plants consider fluctuations in photoperiod as the most robust indicator of seasonal changes and interpret the warm temperatures under SDs as a sporadic event in nature. Nevertheless, it is common in nature to find intermediates photoperiods in a growing season. Therefore it seems

reasonable that an additive combination of stimuli of photoperiod and temperature determine the timing of flowering (Craufurd and Wheeler 2009). The ability to accelerate flowering under warm temperatures and sub-optimal photoperiods might serve as a strategy to rapidly colonize ecological niches during years of warm transitions from winter to spring. Furthermore, being able to flower rapidly in late winter or early spring might give *A. thaliana* the chance to complete more than one life cycle in those years when the climate is permissive. These mechanisms might allow *A. thaliana* plants to adapt to exceptional environments in which the day-length is short but temperatures are already high. This type of rare environmental situations could be achieved more often in future years when the effect of the global warming is predicted to be more notorious. Thus, the findings obtained in this thesis on the molecular mechanisms controlling flowering under warm temperature and SDs in *A. thaliana* are instrumental to breed crops for a better adaptation to the future climate conditions.

The information on light quality changes that a plant perceives allows it to interpret the environment by which it is surrounded. However, light quality changes give very little or no information about seasonal changes. Therefore, plants can distinguish between “robust” environmental changes and perturbations in the micro-environment that surround them. It seems relevant for plants to prioritize the responses driven by seasonal changes (robust ambient changes) over fluctuations in the micro-environment surrounding them. For example, *A. thaliana* does not accelerate flowering under shade conditions unless the photoperiod is sufficiently prolonged. This is probably explained by the fact that plants, including *A. thaliana*, have developed multiple other strategies in order to escape from the shade produced by neighbouring individuals. In agreement with this, a previous report showed that distinct shade avoidance responses are controlled by independent molecular mechanisms (Kim *et al.* 2008). In this thesis, it has been shown that PIFs, which are involved in several shade avoidance

responses, are not related to the flowering acceleration observed as part of the SAS. Instead, under shade and optimal day-length conditions, key components of the photoperiod pathway are recruited to regulate flowering time. Similar results were previously described by other groups (Kim *et al.* 2008, Wollenberg *et al.* 2008).

In summary, the genetic networks that govern the regulation of plant development, and in particular, flowering time, are highly influenced by seasonal and environmental factors. In nature, plants are simultaneously exposed to a combination these factors whose degree of intensity and duration are characteristic of particular seasons. In *A. thaliana* these factors are perceived by distinct genetic pathways and integrated into regulatory networks to modulate flowering time. In the core of these networks *CO* is a molecular hub which regulates the transcription of *FT* and flowering in response to photoperiod, temperature and light quality changes. The fact that a single transcription factor, with a function that is well conserved among species, integrates and regulates multiple genetic pathways has an extraordinary potential in order to manipulate flowering time in cultivated plant species. This could be especially useful in the context of the global climate change that is expected to have a dramatic effect on plant phenology

7. Literature

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8. Annex

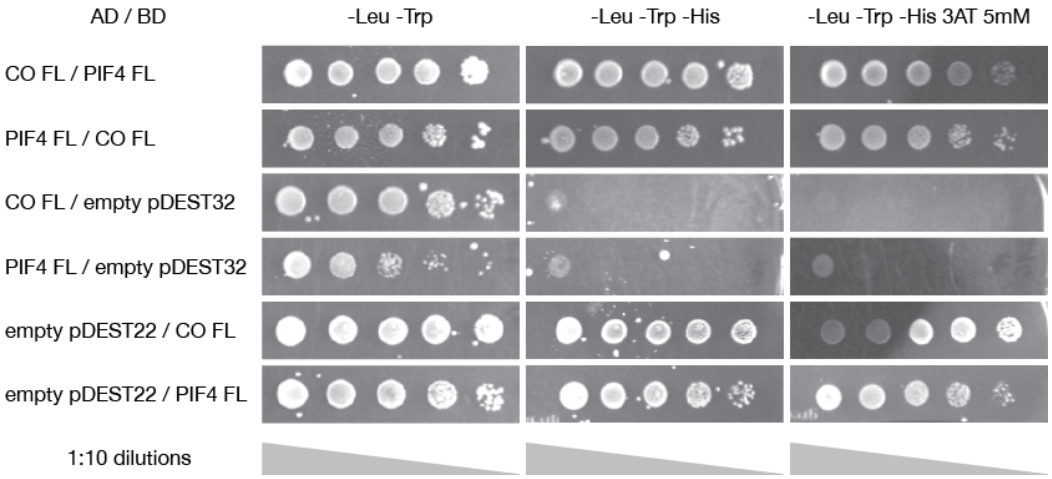

Figure A.1 Y2H experiment to test the interaction between CO and PIF4 proteins. Yeast growth in the first two lines in -Leu -Trp -His and -Leu -Trp -His + 3AT media suggest interaction between CO and PIF4 proteins. Auto activation controls in rows 3 and 4 using CO full length (FL) and PIF4 FL fused to the activation domain show no yeast growth in -Leu -Trp -His and -Leu -Trp -His + 3AT media. Auto activation controls in rows 5 and 6 using CO full length (FL) and PIF4 FL fused to the DNA binding domain show yeast growth in -Leu -Trp -His and -Leu -Trp -His + 3AT media.

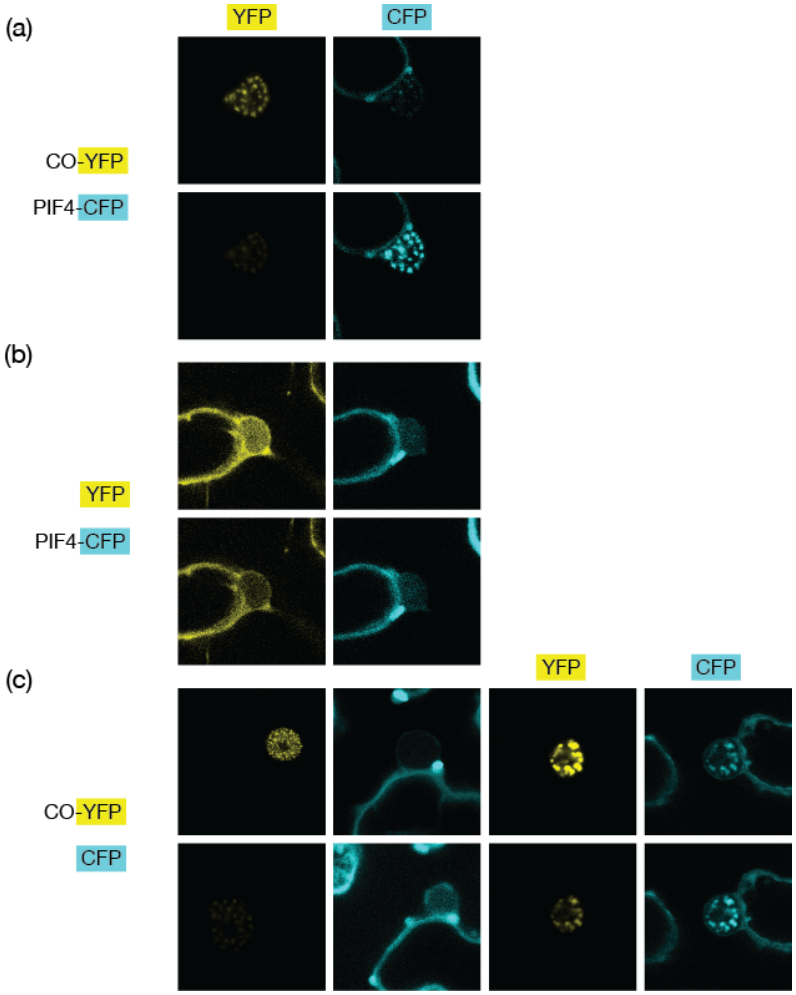

Figure A.2 FRET-A.PB experiment to test the interaction between CO and PIF4 proteins. (a) After photobleaching of the acceptor (CO-YFP) the fluorescence intensity of the PIF4-CFP increase, suggesting interaction between CO and PIF4. (b) PIF4-CFP fluorescence intensity does not increase after photobleaching of YFP fluorescence protein. (c) CFP fluorescence intensity slightly increase after photobleaching of CO-YFP. In (a), (b) and (c), upper panel: image before photobleaching; lower panel: image after photobleaching

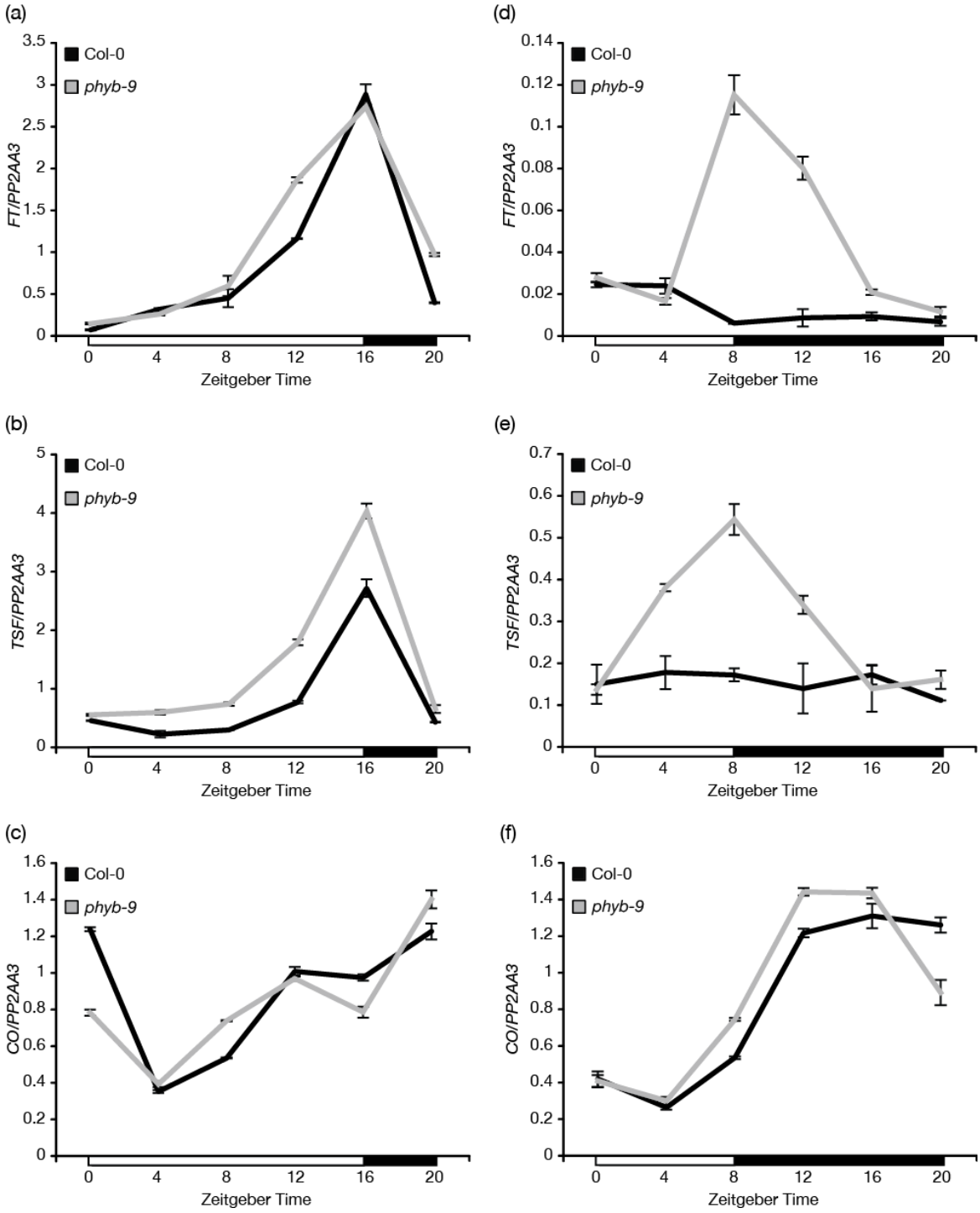

Figure A.3 The *phyB-9* mutant displays higher levels of *FT* and *TSF* mRNA compared to Col-0 under LDs and SDs. (a) and (d) *FT*, (b) and (e) *TSF* and (c) and (f) *CO* mRNA expression profile in 10-day-old Col-0 and *phyB-9* mutant seedlings grown in WL under LDs (a), (b) and (c) and SDs (d), (e) and (f). Seedlings were

harvested every 4 h for 24 h and *FT*, *TSF* and *CO* mRNA expression was measured by qRT-PCR; error bars are StD of three technical replicates. Results provided by Yasuyuki Takahashi.

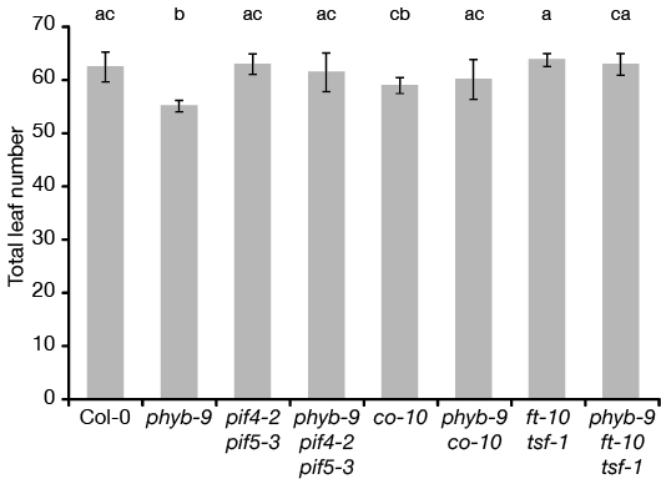

Figure A.4 *PIF4 PIF5*, *CO* and *FT TSF* are required for the early flowering phenotype of *phyB-9* mutants under SDs. Flowering time of plants grown under SDs in WL. Letters indicate statistical groups determined with a one-way ANOVA and all pairwise multiple comparisons with the Holm–Sidak method. Groups were considered statistically different when P ≤ 0.05. Error bars are StD, n = 6. Results provided by Yasuyuki Takahashi.

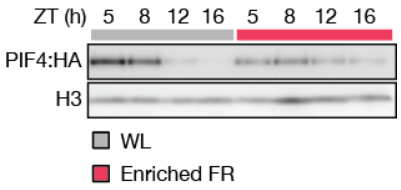

Figure A.5 PIF4 protein is stabilized under enriched FR light. Western blot from nuclear protein extracts of p*PIF4::PIF4:HA pif4-101* seedlings. Time course showing PIF4:HA protein accumulation under WL and enriched FR. Nuclear proteins were extracted from seedlings grown for 12 days under WL or enriched FR.

9. Abbreviations

General

%	percentage
°C	degree Celsius
µ	micro
12:12	photoperiod of 12 h light and 12 h dark
21°C-SD	21 ºC and short days
27°C-SD	27 ºC and short days
3AT	3-amino-1,2,4-triazole
A. thaliana	*Arabidopsis thaliana*
A.PB	acceptor photobleaching
ANOVA	analysis of variance
APA	active Phytochrome A-binding
APB	active Phytochrome B-binding
APSIM	agricultural production systems simulator
AS-NMD	alternative splicing coupled with nonsense-mediated mRNA decay
B	blue (light)
bHLH	basic helix-loop-helix
bp	base pairs
BRs	brassinosteroids
CaMV	*Cauliflower mosaic virus*
Cas9	CRISPR-associated protein-9 nuclease
cDNA	complementary DNA
CETS	*CENTRORADIALIS* (*CEN*), *TERMINAL FLOWER 1* (*TFL1*) and *FT*
CFP	cyan fluorescent protein
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP sequencing
Col-0	Columbia
CORE	CO-responsive element
CRISPR	clustered regularly interspaced short palindromic repeats
cry	cryptochrome
DNA	deoxyribonucleic acid
DSSAT	decision support system for agrotechnology transfer

DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EOD	end-of-day
Flash	9xMyc-6xHis-3xFlag
FR	far-red (light)
FRET	fluorescence resonance energy transfer
g	gram
GA	gibberellin
GFP	green fluorescent protein
GUS	β-glucuronidase
GW	gateway
GWAS	genome wide association
h	hour
HA	hemagglutinin
His	histidine
HRP	horseradish peroxidase
IAA	indole-3-acetic acid
IPCC	intergovernmental panel on climate change
kb	kilobase pair
kDa	kilodalton
L	litre
LDs	long days
LED	light-emitting diode
Ler	*Landsberg erecta*
Leu	Leucine
LOV	light, oxygen, or voltage (domain)
m	meter
MADS-box	MCM1 AGAMOUS DEFICIENS SRF-box
Mbp	megabase pairs
MES	2-(N-morpholino)ethanesulfonic acid
miR	MicroRNA
mol	mole
mRNA	messenger RNA

MS	Murashige and Skoog (medium)
MYB	myeloblastosis (transcription factors)
N. benthamiana	*Nicotiana benthamiana*
N_2	nitrogen
nm	nanometre
NP-40	nonyl phenoxypolyethoxylethanol
NPIB	nuclear protein isolation buffer
O/N	over night
OD	optical density
PAR	photosynthetically active radiation
PBE-box	PIF-binding E-box
PC	phosphatidylcholine
p*CO*	promoter of *CONSTANS*
PCR	polymerase chain reaction
PEG	polyethylene glycol
Pfr	Phy- chromoprotein inactive form
phot	phototropin
phy	phytochrome
PIC	protease inhibitor cocktail
PMSF	phenylmethylsulfonylfluorid
Pr	Phy- chromoprotein active form
PVDF	polyvinylidene fluoride
qRT	quantitative reverse transcription
QTL	quantitative trait locus
R	red (light)
RAF	rapidly accelerated fibrosarcoma
RNA	ribonucleic acid
RNA-seq	RNA sequencing
rpm	revolutions per minute
RT	room temperature
s	second
SAM	shoot apical meristem
SAS	shade avoidance syndrome

SB	sonication buffer
SDS	sodium dodecyl sulfate
SDs	short days
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
StD	standard deviation
STUbL	SUMO–targeted ubiquitin ligase
SUMO	small ubiquitin-related modifier
Taq	*Thermus aquaticus*
TBS	tris-buffered saline
TBST	tris-buffered saline tween 20
T-DNA	transferred DNA
Tris	tris(hidroximetil)aminometano
Trp	Tryptophan
UV-B	ultraviolet (light) B
V	volts
w/v	weight/volume
WB	washing buffer
WL	white light
WT	wild-type
Y2H	yeast-two-hybrid
YPAD	yeast extract-peptone-adenine-dextrose (medium)
ZT	Zeitgeber time

Gene names

AP1	*APETALA 1*
AP2-Like	*APETALA2-LIKE*
ARP6	*ACTIN-RELATED PROTEIN6*
ATBH2	*ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2*
BRZ1	*BRASSINAZOLE-RESISTANT 1*
CCA1	*CIRCADIAN CLOCK-ASSOCIATED 1*
CCT	CO-like, TIMING OF CAB2 EXPRESSION 1 (TOC1)

CDF	*CYCLING DOF FACTOR*
CIB1	*CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX*
CO	*CONSTANS*
COL	*CO-like*
COP1	*CONSTITUTIVE PHOTOMORPHOGENIC 1*
CRY(1,2)	*CRYPTOCHROME (1,2)*
ELF (3,4)	*EARLY FLOWERING* (*3,4*)
FBH	*FLOWERING BHLH*
FDP	*FD PARALOG*
FKF1	*FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*
FLC	*FLOWERING LOCUS C*
FLM	*FLOWERING LOCUS M*
FRI	*FRIGIDA*
FT	*FLOWERING LOCUS T*
FUL	*FRUITFULL*
GA20OX2	*GIBBERELLIN 20 OXIDASE 2*
GAI	*GIBBERELLIC ACID INSENSITIVE*
GI	*GIGANTEA*
H2A.Z	*HISTONE H2A.Z*
Hd1	*Heading date1*
HFR1	*LONG HYPOCOTYL IN FAR-RED 1*
HOS1	*HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1*
HvFT1	*Hordeum vulgare FT*
IAA (19,29)	*INDOLE-3-ACETIC ACID INDUCIBLE (19,29)*
LFY	*LEAFY*
LKP2	*LOV KELCH PROTEIN 2*
LUX	*LUXARRHYTHMO*
NF-Y	*NUCLEAR FACTOR Y*
NtFT	*Narcissus tazetta FT*
PAR1	*HYTOCHROME RAPIDLY REGULATED1*
PFT1	*PHYTOCHROME AND FLOWERING TIME 1*
PHL	*PHYTOCHROME-DEPENDENT LATE-FLOWERING*
PHYA	*PHYTOCHROME A*

PHYB	*PHYTOCHROME B*
PHYC	*PHYTOCHROME C*
PHYD	*PHYTOCHROME D*
PHYE	*PHYTOCHROME E*
PIE1	*PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1*
PIF (1,3,4,5,7,8)	*PHYTOCHROME INTERACTING FACTOR (1,3,4,5,7,8)*
PIL	*PIF-like*
PP2AA3	*PHOSPHATASE 2A*
PRR (5,7,9)	*PSEUDO-RESPONSE REGULATOR (5,7,9)*
RGA	*REPRESSOR OF ga1-3*
RGA-LIKE 1-3	*RGL1-3*
SAV3	*SHADE AVOIDANCE 3* (*TAA1*)
SMZ	*SCHLAFMÜTZE*
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
SPA (1-4)	*SUPPRESSOR OF PHYTOCHROME A (1-4)*
SUC2	*SUCROSE TRANSPORTER 2*
SVP	*SHORT VEGETATIVE PHASE*
SWC6	*SWR1 COMPLEX6*
TAA1	*TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1*
TOC1	*TIMING OF CAB EXPRESSION 1*
TOE1(1,2)	*TARGET OF EAT (1,2)*
TSF	*TWIN SISTER OF FT*
XTR7	*XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7*
YUC8	*YUCCA 8*
ZTL	*ZEITLUPE*

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11. Erklärung

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Köln, Januar 2018 _____

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