PIF3 (phytochrome•interaeting factor 3) and Circadian Clock interaction in the regulation of plant growth

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Als meus pares

“You can't study the darkness by flooding it with light.”
Edward Abbey

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SUMMARY

Arabidopsis thaliana seedling growth and development is influenced by both external (such as light and temperature) and internal (like hormones or circadian clock) factors. In short-day (SD) growing conditions, it is known that both light and clock have a pivotal role in the regulation of hypocotyl elongation, as mutations in members of the two pathways result in altered lengthening of the hypocotyl.

The main objective of the thesis was to determine the function of PIF transcription factors, as light-signaling pathway intermediates, in the regulation of hypocotyl elongation of SD-grown seedlings, together with the possible interplay between them and TOC1 protein, a central component of the circadian clock.

Phenotypic and molecular data obtained along the thesis allowed us to conclude that PIF1 and PIF3 proteins, together with the other members PIF4 and PIF5 which had previously been related with the process, act as positive regulators of hypocotyl growth at the end of the dark period in SD conditions. Moreover, several phenotypic studies, gene expression and protein accumulation analysis, together with chromatin immunoprecipitation assays performed in multiple combinations of PIFs and TOC1 mutants allows us to determine that there is a previously unknown direct crosstalk point between light and circadian clock in the regulation of hypocotyl elongation. Together, the work indicates the existence of a new regulatory mechanism by which clock gates hypocotyl elongation at the end of the dark period in SD-grown seedlings by repressing the transcriptional activity of PIF3 protein during the rest of the night.

El desenvolupament i creixement de plàntules *d'Arabidopsis thaliana* està influenciat tant per factors ambientals, com la llum o la temperatura, com per factors interns, com per exemple les hormones o el rellotge circadià. Es coneix que, en condicions de dia curt (cicles de 8h de llum i 16h de foscor), tant la llum com el rellotge circadià realitzen un paper clau en la regulació de l'elongació de l'hipocòtil ja que mutacions en components de les dues vies tenen com a conseqüència alteracions en la llargada d'aquest òrgan.

L'objectiu principal d'estudi en aquesta tesi ha estat determinar la funció dels factors de transcripció PIF, intermediaris de la via de senyalització lumínica, en la regulació de l'elongació de l'hipocòtil en plàntules crescudes en dia curt i la connexió que existeix entre aquestes proteïnes i la proteina TOC1, component central del rellotge circadià.

Les dades fenotípiques i moleculars obtingudes al llarg de la tesi ens han permès concloure que les proteïnes PIF1 i PIF3, conjuntament amb PIF4 i PIF5 les quals ja havien estat prèviament involucrades en el procés, actuen com a reguladors positius de l'elongació de l'hipocòtil al final del període de foscor en condicions de dia curt. A més, diferents estudis fenotípics i anàlisis genòmics i bioquímics, conjuntament amb experiments d'immunoprecipitació de la cromatina, realitzats en diferents combinacions de mutants *PIFs* i *TOC1* han permès determinar que existeix un nou punt de relació directa entre la llum i el rellotge circadià en la regulació de l'elongació de l'hipocòtil. Aquest treball ha permès demostrar l'existència d'un nou mecanisme de regulació pel qual el rellotge circadià restringeix el creixement de l'hipocòtil just al final del període de foscor en plàntules crescudes en condicions de dia curt. Tal mecanisme regulatori es basa en la repressió feta per la proteïna TOC1 sobre l'activitat transcripcional de la proteïna PIF3 durant la primera meitat del període de foscor.

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I. INTRODUCTION

"What we know is a drop, what we don't know is an ocean"
Isaac Newton

Plant growth and development is influenced by both external (such as temperature and presence of neighbors/competitors) and internal (for example hormones and circadian clock) factors along the plant life cycle, from early germination stages to plant senescence: through seedling development, stationary to vegetative transition, flowering and new seed formation and maturation [1,2,3].

Like other organisms, plants are able to perceive specific environmental and internal signals, interpret them and respond according to the received information. As plants are sessile organisms, the perception of any environmental change that occurs in the surrounding ambient it is crucial for their survival. The continuous monitoring (of both external and internal stimuli) allows them to adequate all physiological responses in an optimal way, according to the received information, to enhance their growth and survival and, ultimately, get a reproductive success.

1. Light Perception

There are several external factors that influence plant growth and development, such as temperature, humidity, salinity and nutrient availability. Among all these parameters, light is one of the most important because of its dual function as a source of energy (for photosynthesis) and as an information signal (like season and presence of neighbors/competitors). In nature, plants have to face frequent variations in the intensity and spectral quality of the light and the detection of these changes is decisive for plants to survive. For this reason, plants have evolved to be able to detect different aspects of light such as quality (wavelength in the light spectrum), quantity and direction and dispose of a multilevel network that allows them to adapt and cope with light fluctuations.

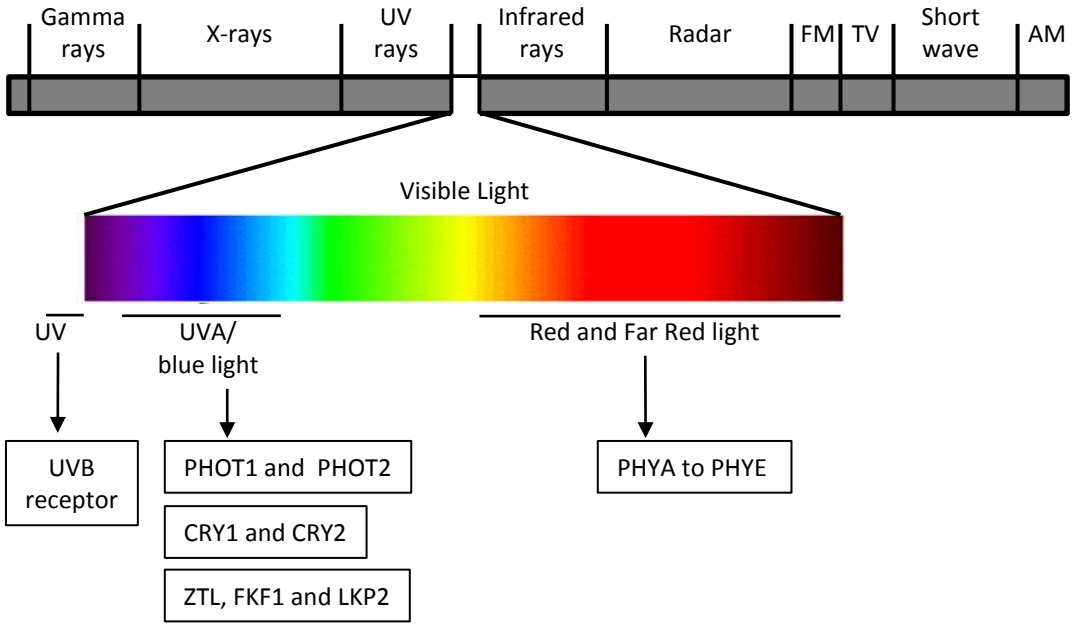

Figure 1.Diagram of light spectrum.In grey, non-visible wavelengths light.Colored, spectrum of visible light.In black squares, acronyms of the different photoreceptors found in *Arabidopsis thaliana.*UVR8 from Ultraviolet Resistance Locus 8, CRY from cryptochrome, PHOT from phototropin, ZTL from Zeitlupe, FKF1 from Flavin-binding Kelch F-box1, LKP2 from LOV (light, oxygen or voltage) Kelch Protein 2 and PHY from phytochrome

1.1 Photoreceptors

At a molecular level, plants contain a bunch of light sensitive proteins, the photoreceptors, which are involved in sensing and transduce the light signal. There are different kinds of photoreceptors which are able to perceive light from different wavelengths of the visible light spectrum (Figure 1) which allows plants to integrate the entire photosensory signal.

1.1.1. Groups of photoreceptors

- Ultraviolet B Light (280-320 nm) Photoreceptors

The last photoreceptor discovered in plants is the one involved in Ultraviolet B (UV-B, 280-320nm) light perception, named UV Resistance Locus 8 (UVR8) [4]. Although this type of light is a minor component of sunlight, it must be taken into account as it has been reported that it has an impact in stress and early seedling development responses in plants [5,6,7,8].

- Ultraviolet A and Blue Light (320-500 nm) Photoreceptors

In higher plants, several photoreceptors are involved in the perception of blue light (B) and Ultraviolet A (UV-A) (320-500nm): cryptochromes (cry1 and cry2) [9,10] and a group containing LOV (light, oxygen or voltage) domain. In *Arabidopsis thaliana*, LOV containing group comprises phototropins, phot1 and phot2 [11,12], and Zeitlupe (ZTL) family: ZTL[13], LOV Kelch Protein 2 (LKP2) [14,15] and Flavin-binding Kelch F-box1 (FKF1)[16,17].

- Red and Far Red Light (600-750 nm) Photoreceptors

The family of phytochromes (phys) is in charge of Red (R) and Far Red (FR) light perception (600-750nm) [17]. These molecules were initially discovered in plants [18] but nowadays it is known that they are also present in in cyanobacteria and eubacteria [19,20,21] as well as some filamentous fungi [22].

In *Arabidopsis thaliana* five different phytochromes have been identified (PHYA to PHYE) and categorized in two different groups according to their stability in light: photolabile or type I (PHYA) and photostable or type II (PHYB to PHYE) [19,23]. Because of their importance on the work presented on this thesis, phytochromes will be further explained later in this introduction.

1.1.2. The structure of photoreceptors

All photoreceptors carry a molecule, named chromophore, that is able to capture light (of different wavelength depending on its nature), with the exception of UVR8 in which two tryptophan residues work as chromophore [20]. This light absorption causes a conformational change on the photoreceptors' structure that leads to their activation or inactivation, which allows that the light signal is propagated. A part from the chromophore, each type of photoreceptor contains several different domains (Figure 2) that confer them specific biological activity [21].

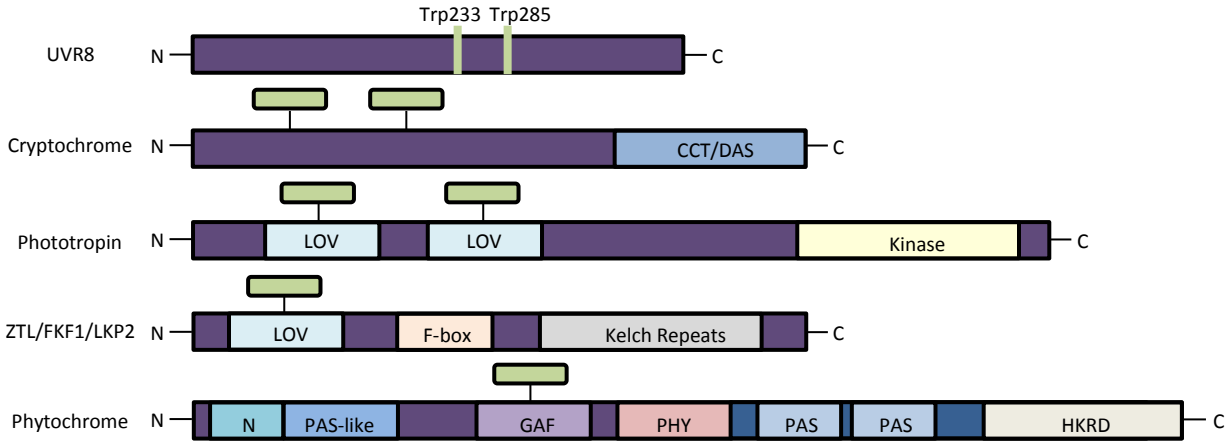

Figure 2.Diagram of different domains of photoreceptors. Each colored rectangle represents a different domain. Green vertical bars in UVR8 are for the two tryptophan residues that act as cromophores. Green rectangles associated to the other photoreceptors represent the chromophore molecule. Acronyms' meaning: Trp (Tryptophan), CCT/DAS (cry carboxyterminal), LOV (light, ozygen or voltage), N (non-conserved amino-terminal extension), PAS (PER, ARNT, SIM), GAF (cGMP-specific phosphodiesterase, adenylate cyclase, FhIA), PHY (phytochrome) and HKRD (His-kinase related domain).

1.2.Phytochromes

As mentioned, in *Arabidopsis thaliana* there are five phytochromes, from PHYA to PHYE. The relative abundance of each phytochrome depends on light growing conditions. In dark grown seedlings, phyA is the most abundant photoreceptor, but it is rapidly degraded after exposure to red and/or white light. The other phytochromes are more abundant in light-grown seedlings, and phyB was demonstrated to be the most abundant of all of them [22,23]. All phytochromes together regulate multiple light responses along all life cycle of the plant, such as seed germination, seedling and chloroplast development, plant architecture, shade avoidance responses and flowering [19,24,25]. In some cases they do it in a unique way and in other cases acting several phytochromes together [26], for example, phyA plays a major role in far red light enriched growing conditions [27], while phyB and phyD seem to redundantly detect changes in the ratio of red and far red ratio (R:FR) [28].

Phytochromes are soluble proteins that are functional when acting as dimers [29].The sensory function of phytochromes resides in their capacity to switch between two different photoreversible protein conformations, the inactive Pr (Phytochrome Red-absorbing) and the active Pfr (Phytochrome Far Red-absorbing) [30] forms. Upon red light irradiation, the Pr changes its conformation and is converted to the Pfr form which, in turn, is able to absorb far red (FR) light that transforms it back to the biological inactive form (Figure 3).

In plants, phytochrome molecule is synthesized in the inactive Pr form [31], and the photoconvertion to the Pfr form is a signal to the plant that it has been exposed to light. In continuous light growing conditions, the two conformers establish a photoequilibrium in which the relative levels of each conformer depends on the ratio of red and far red light (R:FR). When light conditions change and the organism is exposed to prolonged darkness, a process known as dark reversion takes place [32]. In this process Pfr phytochrome is slowly converted to the Pr form (Figure 3).

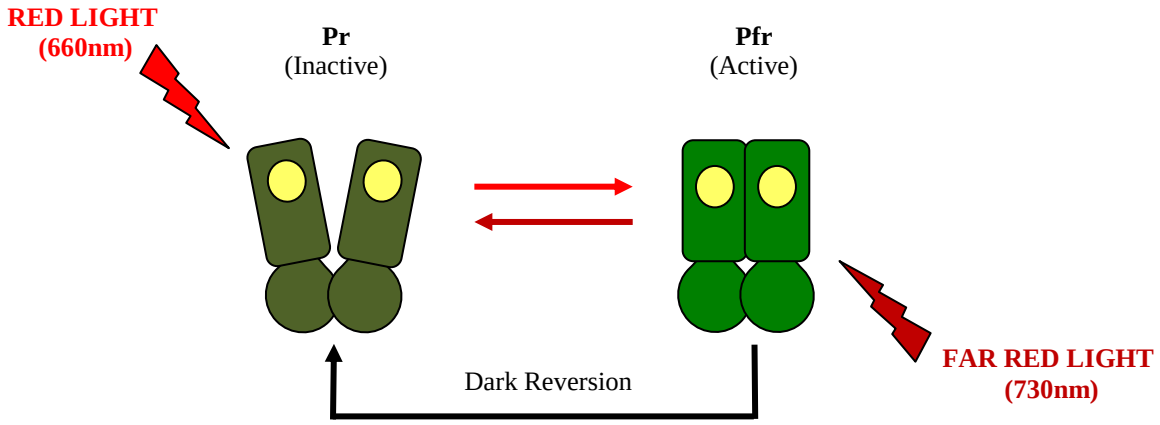

Figure 3. Scheme of photoconversion of phytochromes. In dark green the Pr inactive form, able to absorb red light. After light absorption, Pr is photoconverted to Pfr biological active form, represented with a pale green in the scheme. Yellow circles represent the associated chromophore to each polypeptide.

Photoconvertion and activation of phytochromes is associated to a change in the subcellular localization. Pr form is synthesized and accumulates in the cytoplasm and activation into Pfr by a red light exposure causes a translocation into the nucleus [31,33,34]. This event is different between phyA and phyB; translocation of phyA is very rapid (within minutes) and requires the presence of two other small proteins: FHY1 (FAR RED ELONGATED HYPOCOTYL1) and FHL (FHY1 LIKE). These two proteins contain two conserved domains, the nuclear localitzation signal (NLS) and the nuclear exclusion signal (NES). NLS and NES are used by phyA to translocate into the nucleus and back to the cytoplasm respectively [35]. For phyB, translocation is a relatively slower process and does not require other proteins because the photoreceptor itself contains a putative NLS used for its nuclear translocation [36].

Confocal microscopy analysis showed that phytochromes accumulate in subnuclear regions of the nucleus, called nuclear bodies (NBs) or speckles [37]. Some components of the phytochrome signaling are also found in these NBs, which suggest that the co-localization of phytochromes in these specific areas could play an important role for phytochrome signaling [38] but the exact function of NBs existence is still unknown. Once in the nucleus, the activated phytochrome induces a cascade of events that lead to the modification of the transcriptome and adaptation of the plant physiology to the changing light environment.

Phytochromes transduce the perceived light signal by interacting with other intermediate proteins, mostly transcription factors that can act as positive or negative regulators of gene expression [39]. About 10% of the total *Arabidopsis* genes are affected by light pathway [40,41].

2. The Phytochrome-Interacting Factors

One of the most important families of proteins that interact with phytochromes is the PIF (PHYTOCHROME-INTERACTING FACTOR) subfamily, that belong to a basic/helix-loop-helix (bHLH) transcription factors superfamily [42,43,44].

In figure 4, there is a summary of the members that form the subfamily 15 of the bHLH superfamily, that consist in seven PIFs and eight other members. At a polypeptide level, PIF proteins contain several conserved domains such as the bHLH domain that provides them the capacity to bind DNA (basic domain) and to dimerize (HLH domain) [43,45,46] and a nuclear localization signal (NLS) responsible for the constitutive nuclear localization of these proteins [42]. Most of the members have also an binding to photoactivated phyB (APB) domain that allows the interactions to the active form of the phytochrome B [47]. To date, 7 members of the family have been shown to interact with phyB: PIF1 and PIF3 to PIF8 [48]. In addition to bind phyB, two of them, PIF1 and PIF3, have also a phytochrome A binding domain (APA) [47,49].

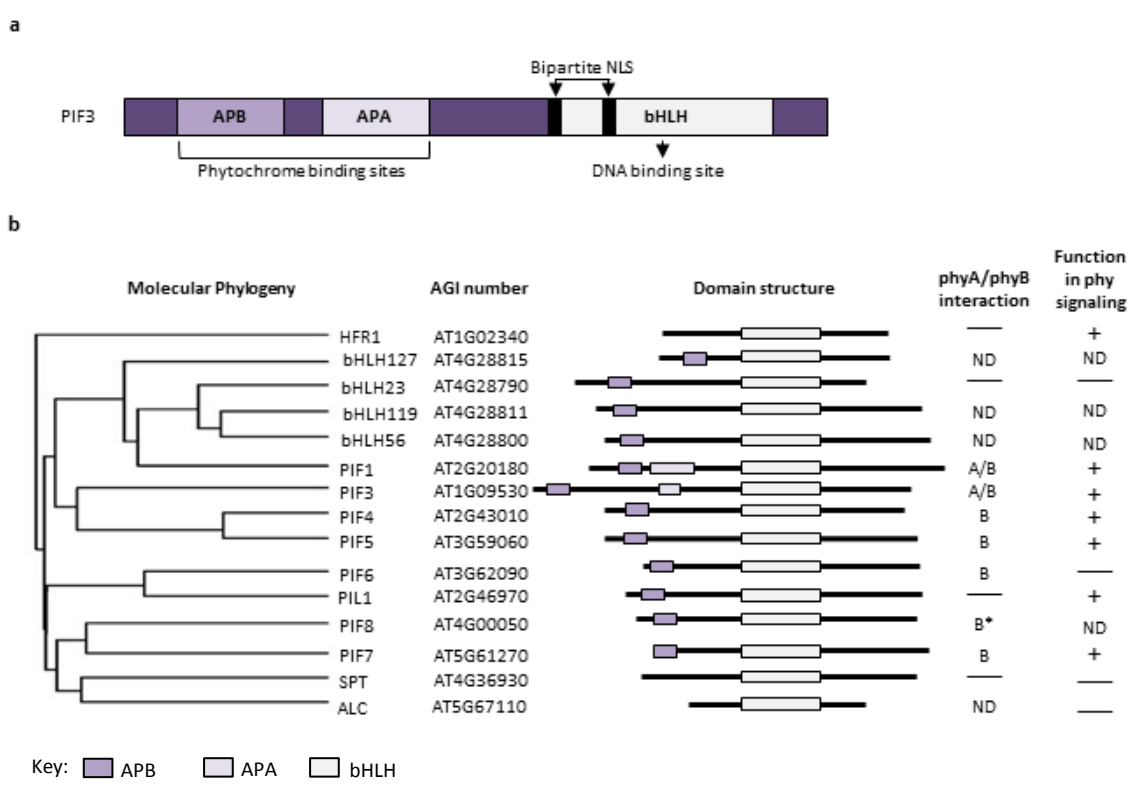

Figure 4.Subfamily 15 of the bHLH superfamily of transcription factors. (a), Protein structure of PIF3, showing the four different domains: bHLH domain (responsible of dimerization and DNA-binding), NLS (nuclear localization site) and APA and APB (binding to photoactivatedphyA and phyB). (b) Summary of subfamily 15 of the bHLH superfamily of transcription factors. For each member, molecular phylogeny, nomenclature, polypeptide structure, capacity to interact to phyA and/or phyB and implication in the phy signaling are shown. Specific interaction with phyA and /or phyB is indicated as A and/or B and lack of interaction as (-). The asterisk indicates evidence of binding (Y.Oka and P. H. Quail, unpublished). Evidence of functional involvement in phy signaling in vivo is indicated as (+) and (-) indicates no evidence. ND: not determinate. Adapted from [48].

2.1 Function of PIFs in seedling growth

PIF proteins are involved in multiple physiological processes, acting in several stages of the plant development and growth such as seed dormancy and germination, seedling growth and development, chlorophyll and chloroplast biosynthesis and stomatal development [48,50,51,52,53,54,55]. All PIFs function as negative regulators of light signaling, with the exception of PIF6 that acts as a positive regulator [56]. The study of single and multiple *pif* mutants have indicated that they have shared and distinct functions [57]. Some responses are regulated basically for one single PIF such as PIF1

in the regulation of seed germination [51], PIF3 in the promotion of hypocotyl elongation in response to ethylene [58] or PIF4 in the adaptation to high-temperature conditions [49,59,60]. In other cases PIFs can also act together to regulate an specific response, as it is the case of PIF1 and PIF3 that commonly control the expression of genes involved in chlorophyll biosynthetic [61].

Our laboratory is mainly interested in the study of PIF function in *Arabidopsis thaliana* seedling growth and development, a complex physiological process that is extremely influenced by light and in which seedlings adequate the pattern of development depending on light conditions.
When a seed geminates in the soil in underground darkness, the seedling follows a developmental pattern known as skotomorphogenesis or etiolation. This process is characterized by an elongation of the hypocotyl, closed cotyledons and presence of folded apical hook (figure 5). Etiolation is a strategy for seedlings to reach the soil surface and light as soon as possible, keeping the apical meristem of the seedling protected from possible damage. When seedling emerges to the soil surface and is exposed to light, developmental pattern switches to a photomorphogenic development, process known as de-etiolation. Seedling undergoes a light grown phenotype, hypocotyl elongation is inhibited, hook is unfolded and cotyledon separate, expand and starts the development of chloroplast, accumulating chlorophyll and starting photosynthesis.

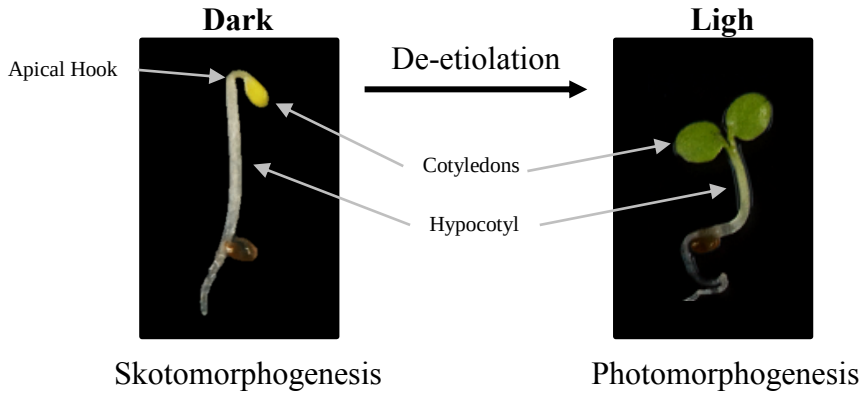

Figure 5. Phenotype of two seedlings grown in dark and light conditions. On the left a dark-grown seedling is shown (with elongated hypocotyl, folded hook and closed cotyledons). Seedling on the right is a light-grown seedling grown under continuous light conditions (with short hypocotyl, separated and chlorophyll accumulating cotyledons and primary leaves developed).

Gene expression analysis and mutant phenotypic studies have revealed that the default developmental pattern is photomorphogenesis; skotomorphogenesis is achieved by an active constant repression of the photomorphogenic process [41,62].
Analysis of a quadruple mutant (*pifq*) that lacks four PIF family members (PIF1, PIF3, PIF4 and PIF5) established the primordial function of PIF proteins in this repression; *pifq* grown in the dark phenocopies a wild-type seedling grown in the light (Figure 6), not only at a phenotypical level but also at a gene expression level [41,62], indicating their function is achieved by a direct regulation of gene expression.
After light exposure, there is a light-induced PIF degradation (mechanism that will be explained in more detail below) which leads to a reversion of the repression and the change of skotomorphogenesis to a photomorphogenic developmental pattern.

In constant light growing conditions, even if the levels of PIFs are very low, these proteins can still promote growth. This is explained by the fact that, in this continuous light situation, the few PIF molecules that are present in the cell are able to induce the degradation of active phytochrome [63]. Hence, the promotion of growth in continuous light done by the PIFs it is due to their capacity to modulate phytochrome abundance and not their intrinsic transcriptional activity (phy-activated signaling intermediates) as it is happens in darkness, diurnal conditions or shade ambient.

In other seedling growing conditions such as diurnal conditions or in shade environments, PIFs also participate in the promotion of growth, inducing the expression of multiple genes related with growth [64,65,66,67,68] (Figure 6).

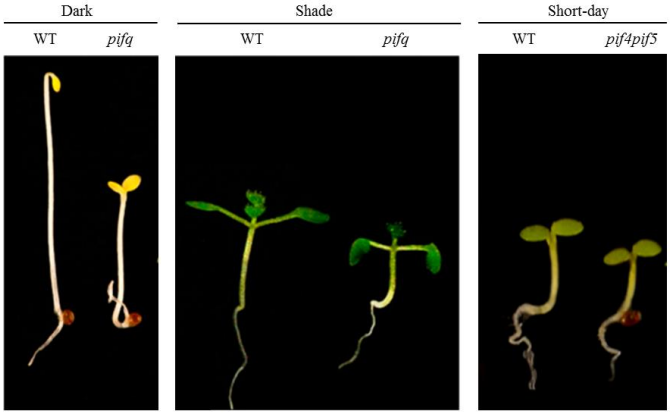

Figure 6. Phenotype of wild-type (WT) and *pif* mutant seedlings grown in different light conditions. Left and middle panel, WT and *pifq* seedlings grown for 3 days in continuous dark or simulated shade (2d light + 5d shade) respectively. Right panel, 3-day-old SD-grown WT and *pif4pif5* seedlings. Adapted from [69].

2.2. Regulation of PIFs

The crucial role of PIFs in seedling development requires the existence of very fine and continuous regulation of these transcription factors' activity. Such regulation is done directly regulating the expression of *PIFs* transcripts and/or regulating at a posttranslational level.

2.2.1 Transcriptional regulation of the PIFs

Studies reported in the last years have shown that *PIFs* gene expression is regulated by several different transcription factors that belong to diverse cellular pathways. Some examples of *PIF* transcription regulations are summarized below.

- Feedback regulation

PIF genes are bound by themselves, suggesting the existence of an autoregulatory mechanism [69].

- Hormones

The small molecules hormones, or phytohormones, are regulators of growth in plants. Six different hormones regulate growth: gibberellins (GA), auxin (IAA) andbrassinosteroids (BR) that promote cell expansion in a longitudinal way, abscisic

acid (ABA) that inhibits the longitudinal growth and cytokinin (CK) and ethylene (ACC) that promote growth in the transverse axes [70]. Several hormone such as ethylene and BRs and the hormone-related molecule nitric oxide (NO) are able to regulate *PIF* transcript levels [58,71,72].

- Temperature

In high temperature ambient, the expression of *PIF*, especially *PIF4*, is increased and therefore hypocotyl growth is induced [73,74,75].

- Circadian Clock

In diurnal conditions, *PIF4* and *PIF5* transcription is regulated by proteins of the circadian clock [66,76]. This regulation and the concept of circadian clock itself will be further explained below on the introduction because of their importance on hypocotyl elongation in short-day conditions.

- Combination of transcription factors

Comparison of different transcriptomic analysis has shown that each PIF is targeted by different combinations of transcription factors [69].

2.2.2 Posttranslational regulation of the PIFs

2.2.2.1 Regulation of PIFs by phytochrome interaction

The interaction between PIFs and phys is very important because it is intimately related with PIFs degradation and/or regulation of their activity in the cell [77]. In dark conditions, PIF quartet proteins can accumulate in the nucleus where they regulate the expression of genes associated with light pathway. The degradation of this type of proteins is a light-induced process. Upon exposure to light, Pr phytochrome is rapidly photoconverted to the Pfr, as explained above. Photoactivation is associated with translocation of the dimers into the nuclear speckles where they physically interact with the accumulated PIF proteins [46,47,78]. Interaction between phytochromes and PIF results in a rapid phosphorylation, ubiquitylation and subsequent degradation of the transcription factor via the ubiquitin-proteasome system. The degradation process, particularly in the PIF quartet members (PIF1, PIF3, PIF4 and PIF5) is a fast event, with half-lives of 5 to 20 min [50,77], that brings PIF protein to a new low steady-state level [50,77,78,79]. Interestingly, although the main steps of this degradation process are well known, both the kinase(s) and the E3 ubiquitin ligase(s) responsible for phosphorylation and ubiquitination of PIFs have still to be identified.
Phy-regulation of PIF levels is a highly dynamic process that not only occurs upon first seedling exposure to light but also in other light changing conditions like diurnal cycles or in shade environments. Moreover, the reduction of PIF activity through their proteolysis induced by phytochromes is not the only way these photoreceptors can regulate them. In the case of PIF7, the physical interaction with phyB does not lead to a proteolysis of the transcription factor but to an accumulation of a relatively stable phosphorylated form under constant light, form that is dephosphorylated when there is a phy inactivation (under simulated shade), facilitating the binding of PIF7 of its target genes [63,80,81].

It is important to comment that the interaction of phytochromes and PIFs is not only important for regulation of PIFs degradation and activity but also in the reciprocal way. Under prolonged light conditions the transcription factors induce a reduction of phytochrome levels, enhancing the binding of the photoactivated phyB of the nuclear bodies to the protein COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1), an E3-ligase that promotes the degradation of phyB by the proteasome [45,50,63,82,83,84].

2.2.2.2 Regulation of PIFs by interaction with other proteins

A part from the well-known light regulation of PIF protein stability, in the last few years, other posttranslational mechanisms that regulate PIFs activity have been discovered. These regulations are based on the fact that PIFs, a part from homo- and heterodimerize, can also interact with other proteins.

- Inhibition of PIF binding to DNA by protein interaction

So far at least three different groups of proteins have been described to inhibit the binding of PIF to DNA. One of them is the phytochrome B, which, a part from destabilizing PIF proteins, is able to regulate PIF1 and PIF3 binding to DNA [85].

Another group of PIF DNA-binding inhibitors is the group of DELLA proteins which belong to the gibberellin hormone signaling pathway. DELLAs are growth repressors that physically interact with PIFs to avoid them to bind DNA. In light conditions, DELLAs bind to PIFs (through the bHLH domain of PIFs) [64,65], hence the transcription factors cannot bind to DNA and induce the expression of growth related genes. In the absence of light, gibberellin (GAs) hormones accumulate, destabilize DELLAs and PIFs are released from the inhibition, so they can induce gene expression of growth promoting genes.

The third group of PIF DNA-binding inhibitors is the named HLH (Helix-Loop-Helix). These proteins are lacking the basic (b) DNA-binding domain of the bHLH motif but have the HLH dimerizing domain. HLH heterodimerize with PIFs (creating a HLH-bHLH interaction) and PIFs DNA-binding is blocked. Examples include the protein HFR1 (that interacts with several PIFs [86,87]) and the proteins PAR1 and PAR2 (involved in shade responses) [88].

- Inhibition of PIF transcriptional activity

Regulation of PIFs is complex, and as it was recently reported, their capacity to be active does not just depend on the presence/absence of a certain inhibitors but also in the relative amount and equilibrium of these other proteins. An example of this regulatory mechanism is the inhibition of HY5/HYH on PIF1/PIF3 activity to repress the expression of genes related to ROS (reactive oxygen species). In the dark, the amount of PIFs is very high, and they act over ROS-related genes to repress their expression. In the light, HY5 and HYH increase their abundance while PIF1 and PIF3 levels are lower in comparison to dark. In this situation, HY5/HYH and PIF1/PIF3 form heterodimers, generating an inactive complex that keeps the expression of ROS-responsive genes in a basal level. When light conditions change and light intensity increases, PIF1 and PIF3 are totally degraded whereas HY5/HYH are increased, bind to

the promoter of these genes and activate their expression to adequate the cellular response to a high-light ambient [89].

- Transcriptional co-regulators

The interaction of PIFs with other proteins does not always result in an inhibition of PIF protein. There are some cases in which the interaction of PIF to other transcription factors is what allows them to regulate in a coordinated way the expression of common set of genes. An example of this mechanism is the reported interaction between PIF4 with BZR1, a member of the brassinosteroid-hormone (BR) signaling pathway, that together co-regulate multiple genes (the two transcription factors share about 50% of their targets genes) [90].

Clearly, there are multiple factors that have a strong impact on PIF protein function. At the same time, last years' discoveries have shown that PIF can directly regulate multiple different pathways in the cell that affect growth responses. This entire complex network highlights the importance of PIFs as integrators of several pathways, all of them crucial for a proper seedling growth and development (recent and extended reviews on the role of PIFs as integrators are found in Leivar and Monte (2014) and de Lucas and Prat (2014) [69,91].

2.3. PIFs regulation of gene expression

As mentioned above, PIF proteins are transcription factors that are able to bind DNA through their bHLH domain. In the last years, lots of efforts have been done in order to decipher PIF-regulated transcriptional networks; several microarrays, RNA-seq, ChIP-chip and ChIP-seq analysis of individual and multiple PIFs have been performed by different research groups (some of the references:[41,50,57,68,92,93,94]). All these studies have given enough data to have a wide list of PIF-regulated genes and potential direct targets of PIFs. These studies have defined high confidence binding sites, mostly located in the promoter of target genes and strongly enriched in the DNA motives named G-box (CACGTG) and PBE-box (for PIF-binding E-box, an E-box variant CACATG and CATGTG).

The comparison of the different gene regulated lists obtained by transcriptomic analysis using different *pif* mutants and growing conditions, together with the lists of PIF-bound genes list obtained by ChIP-seq data has given a better understanding of how PIFs function [68,69]. Broadly, the comparison of different transcriptomic analysis done in different light conditions (deetiolation, diurnal and shade) shows that, although there is a significant overlap, a large number of PIF targets are specific of one light growing condition. For a particular light condition, analysis done so far seem to indicate that there could be some gene specificity in PIF binding, which could explain, at least in part, the shared and distinct roles in different PIFs function. However, much more studies need to be done in order to determine a possible specificity of PIFs action on gene expression.

The analysis of upregulated and downregulated genes in the three growing conditions (deetiolation, diurnal and shade) to know how PIFs regulate gene expression, seemed to indicate that in general PIF proteins mainly act as transcriptional activators [69].

However, the action of PIFs as transcriptional repressors is also very important as it has been reported for some seedling development processes. For example, PIF1 represses the expression of the carotenoid biosynthesis gene *PSY* [95] or PIF1 and PIF3 are repressors of chlorophyll biosynthesis genes [96]. Additionally, it has also been shown that PIFs can act as coactivators of other transcription factors, meaning that the presence of PIF proteins is necessary for this other transcription factor to be active [45].

PIF proteins regulate the expression of thousands of genes; among them several different classes of transcription factors are found: bHLH, homeobox, bZIP, ARF (AUXIN RESPONSIVE FACTORS), AUX/IAA, AP2-EREBP (APETALA2/ethylene-responsive element binding protein), BBX (B-BOX PROTEIN) and TCP. PIFs also control the expression of genes related with cell wall expansion (like *XTR7*) and growth, photosynthesis/chloroplast-related genes [41,61,90] and also the expression of hormone-related genes (like *YUCCA8*, *IAA19*, *PAR1*, etc). The plethora of genes that are regulated by PIFs and the fact that among them there are genes very important for seedling growth and development, underscores the complex network existing downstream of the PIFs and demonstrates the relevant importance that these proteins have acting as hubs in this developmental process.

3. Circadian clock

It has already been mentioned in this introduction that not only external factors influence plant behavior. Internal factors have also an impact on plant responses, as for example, changes in the relative abundance of different types of hormones or alteration in the concentration of ions [97]. Among them, a very important internal cue is the Circadian Clock. The circadian clock is a self-sustaining endogenous timekeeping mechanism that generates oscillations/rhythms of about 24 hours (h) (in Latin, circadian is "about a day"). This mechanism is ubiquitous in eukaryotes, existing also in organisms such as cyanobacteria, fungi, flies and mammals. The functioning bases of the mechanism are similar in all organisms, even the specific molecular components differ from one organism to other. The circadian clock enables organisms to anticipate and synchronize the biological processes with environmental cycles such as day/night cycles and changes in temperature along the day, and this anticipation benefits to plant fitness, adaptation and survival [98]. The circadian clock allows that each specific biological response is accomplished in the moment of the day that is more beneficial for the plant, changing the sensitivity of the plant to a determined factor and/or capacity to respond to external cues, process known as gating.

Circadian rhythms are defined by three parameters: amplitude (difference between peak and trough), phase (time of the day for a specific event, often defined as *zeitgeber* time, ZT) and period (time to complete one cycle) (Figure 7).

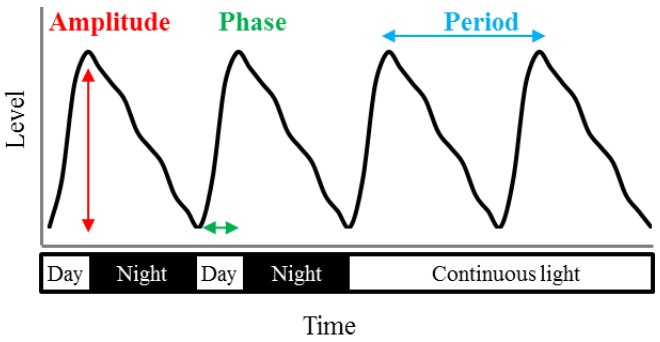

Figure 7. Representation of a circadian oscillation. Red arrow shows the amplitude of the rhythm, green arrow the phase and the blue arrow the period. Because of the sustaining of the clock, even when day-night cycling conditions change to continuous light conditions, the oscillations continue.

The daily oscillations generated by the circadian clock, also known as biological rhythms or circadian rhythms, are seen at different levels, from cellular level (like changes in transcription pattern [99]) to organism level (like movements of leaves and flowers [100]) and persist the entire life cycle, from early seed germination events to flowering [101,102,103]. One important oscillatory biological process, which has actually been focus of study of this thesis, is the hypocotyl elongation of young seedlings [66,104,105].

3.1. Circadian clock system

Conceptually, the circadian clock is a complex system that consists in three main components (Figure 8). First, the central oscillator or clock, a self-sustained mechanism that generates and maintains the rhythmicity. The sustaining of the clock is based on a complex network of transcriptional/translational regulatory loops. In order to function correctly and to match the internal time with external one, there is a continuous daily resetting of the clock, process known as entrainment, which permits to adapt to the time cues from the environment. Such resetting is done by the second components of the circadian clock, named input pathways (also known as *zeitgebers*, German word for "time givers"). The third component is the output pathways, consisting in the conversion of the oscillation into physiological and behavioral rhythms, as for example the stem growth.

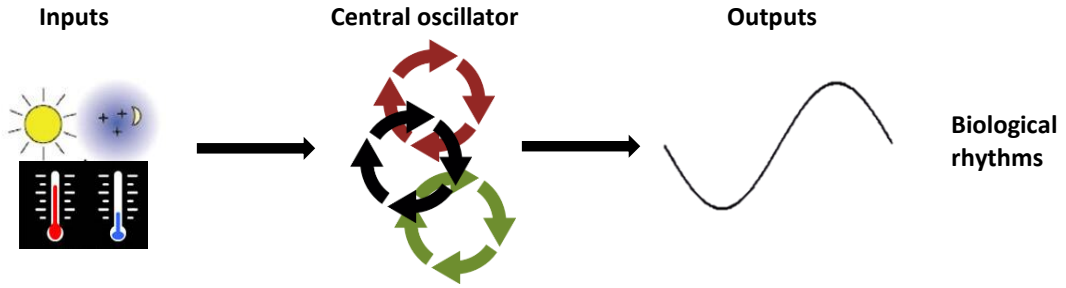

Figure 8.Scheme of the three components that form the circadian clock. Two of the main inputs of clock are shown: light and temperature. The three interconnected circles (red, black and green) represent the central oscillator loops. Oscillatory line represents all the circadian output rhythms generated by the clock. At a transcriptional level, about one third of the *Arabidopsis* genes present rhythmic expression.

3.1.1 Inputs

Several factors can entrain the central clock acting as inputs but, among them, the most studied entraining signals are light and temperature [106,107,108]. As input signals, they both have the capacity to regulate the abundance of the clock components.

Light input is one of the factors with higher influence on circadian oscillations. Periodical changes in the number of light hours or light intensity, is a direct reflection of, for example, the season of the year. Changes in light are continuously entraining clock, which modifies biological responses to perfectly fit/adapt to the environment, for example to induce flowering at the appropriate time of the year.

Temperature is also an important input of the circadian clock. Under constant light conditions, cyclic variations of temperature are able to generate circadian oscillations in

clock proteins and, therefore, generate biological rhythms [109]. However, it has been shown that clock periodicity is maintained over a large range of temperatures. This phenomenon, known as Temperature Compensation, acts as a kind of buffer guaranteeing that there are not important changes in biological rhythms as a consequence of, for example, an accentuated increase or decrease in temperature induced by an isolated atmospherically event.

3.1.2. Central oscillator

To date, more than 20 clock or clock associated components have been identified in *Arabidopsis thaliana.* You can find detailed information about them in Hsu and Harmer, 2013 [110].

Different clock proteins are present at different times of the day and, a part from regulating the outputs of the clock, they also regulate the expression of other, differentially phased, clock components. The central oscillator of the clock is composed of three main regulatory loops: a core loop, a morning loop and an evening loop (Figure 9).

The central core mechanism is based in reciprocal regulation between three proteins [111]: two morning-expressed MYB transcription factors, CCA1 and LHY (CIRCADIAN CLOCK ASSOCIATED 1 and LATE ELONGATED HYPOCOTYL) [112,113], and the evening-phased TOC1/PRR1 (TIMING OF CAB EXPRESSION 1/PSEUDORESPONSE REGULATOR 1) [114,115]. *CCA1* and *LHY* genes are highly expressed and protein accumulated specifically in the morning. Their action is partially redundant [116,117], and act directly repressing the expression of *TOC1*. The binding of CCA1 and LHY to the promoter of *TOC1* is through a conserved motif known as Evening Element (EE), that is also present in the promoter of other evening clock-regulated genes [111]. When TOC1 accumulates represses, in turn, the expression of *CCA1* and *LHY*, closing the loop. Because of the importance of TOC1 in the thesis project, this protein will be explained below on the introduction.

CCA1, LHY and TOC1 are connected to the other two loops: the morning loop and the evening loop. In each loop, multiple proteins have been identified and each one has a specific function, although some components are redundant with other clock components. Most of these proteins have an exclusive role in the circadian clock network itself but some few clock components have been reported to have a role in other biological processes as for example the proteins ZTL, FKF and LKP2, which were already introduced above as plant blue-light photoreceptors and are also clock components.

Most of the clock components act as transcriptional repressors [118,119,120,121] of output genes. Even so, some components can act as transcriptional activators as for example seen in the protein BOA that induces the expression of *CCA1* [122].

The complex network of reciprocal regulations present in the central oscillator ensures that there is an ordered expression of the different clock components, essential for a correct functioning of the circadian clock. Alterations in the expression or relative abundance of clock components can lead to changes in the expression pattern

(amplitude, phase or period) of clock outputs and, consequently, effect on the fitness of the plant. A scheme of several of the transcriptional loops of the central oscillator is shown in figure 9.

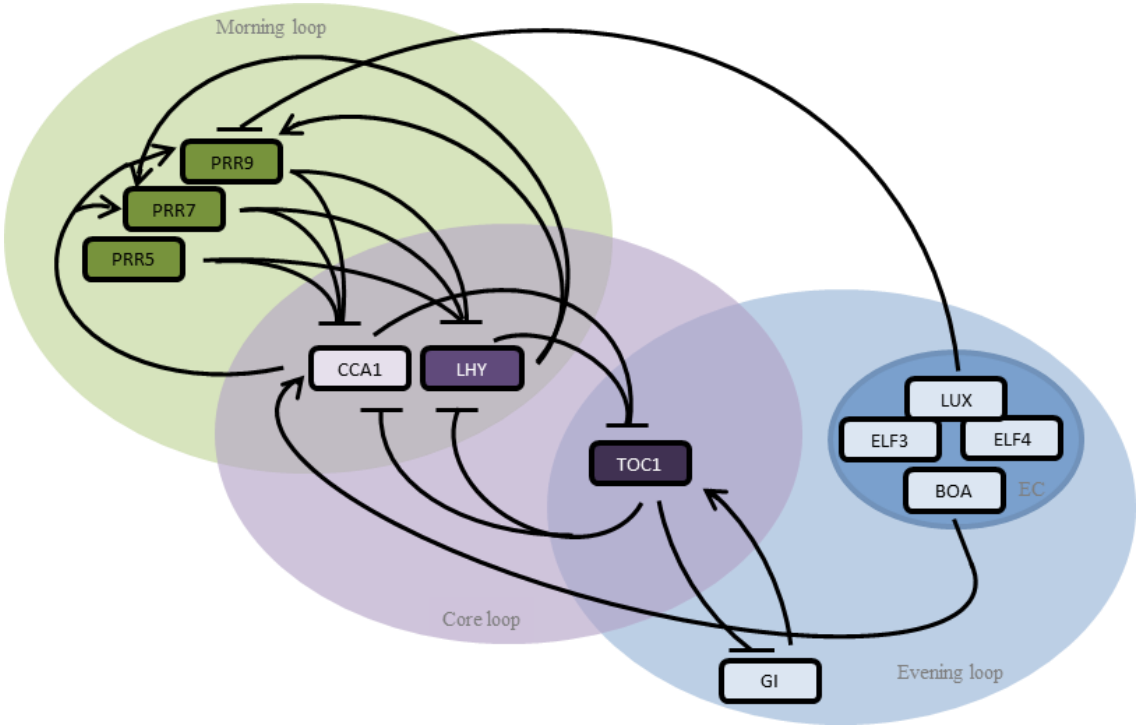

Figure 9. Scheme of the central oscillator of the *Arabidopsis* circadian clock. Only transcriptional regulatory loops are shown. Three big differentially colored circles show the three main interlocking loops that conform the central oscillator: in green the morning loop, in purple the core and in blue the evening loop. Darker blue circle drawn inside the blue evening loop represents a protein complex known as Evening Complex (EC). Arrows indicate activation. Perpendicular bars indicate repression. Acronyms: CCA1 (CIRCADIAN CLOCK-ASSOCIATED1), LHY (LATE ELONGATED HYPOCOTYL), TOC1 (TIMING OF CAB EXPRESSION 1), PRR (PSEUDO-RESPONSE REGULATOR), GI (GIGANTEA), LUX (LYX ARRHYTHMO), BOA (BOTHER OF LUX ARRHYTHMO) and ELF (EARLY FLOWERING).

3.1.2.1. TOC1

As mentioned, TOC1/PRR1 is one of main components of the central core of the clock and it is the founding member of the PRR (PSEUDORESPONSE REGULATORS) family. All members of the family (TOC1/PRR1, PRR3, PRR5, PRR7 and PRR9) contribute to the circadian clock function, each of them being expressed at a different moment of the day. Concretely, *TOC1* is an evening-phased gene meaning that its expression and subsequent protein accumulation peak at dusk. Then it is progressively degraded during the dark period of the diurnal cycle [123]. TOC1 degradation is associated to the action of the clock proteins ZTL, FKF and LKP2 that physically interact with TOC1 and are involved in its proteasomal degradation [124,125].

TOC1 localizes in the nucleus where, as it was recently reported, it binds to the promoter of multiple clock-regulated genes and functions as a transcriptional repressor [118,119].

TOC1 have been shown to interact with other proteins, including other members of the clock system, for example PRR5 [124], and also proteins of other pathways, for example the PIF proteins [115,126].

Interestingly, a part from having a pivotal role in the maintenance of a proper clock function and in the direct regulation of output genes expression, TOC1 have been also related to seedling growth responses and light pathway [127].

3.1.3. Outputs

Output pathways of the clock refer to the plant rhythmic biological responses that are generated by the clock and that keep oscillating even if plants are transferred to non-cycling growing conditions, like continuous light. Examples of output physiological responses are: hypocotyl elongation, leaf movement and stomata opening.

3.2. Circadian clock and seedling growth development

Several works using circadian clock mutants have shown that alterations in clock oscillator components result in changes in the hypocotyl length under diurnal conditions, shortening or lengthening. For example, double deficient mutant *lhycca1* have shorter hypocotyls than wild-type while *toc1* and *prr9prr7prr5* mutants present longer hypocotyls [128,129,130,131,132]. This fact underlines an important role of the circadian clock in the regulation of hypocotyl length.

3.3. Regulation of the circadian clock

Clearly, transcriptional regulation is a key molecular regulatory process that ensures a suitable circadian clock function; however, it is not the only existing regulatory mechanism. Distinct regulatory mechanisms are explained below:

- Histone and chromatin remodeling

In the last recent years, chromatin remodeling process has been reported as a new level of regulation that controls many biological processes. In the circadian clock, chromatin modifications in the promoter region of circadian components have been shown to modulate the binding of transcriptional regulatory proteins and to affect their expression. Some of these chromatin modifications are: histone acetylation in the promoter of TOC1 [133], histone acetylation and methylation in the promoter of CCA1, LHY and TOC1[134,135,136]; histone monoubiquitination to facilitate transcriptional elongation [137], histone deacetylation [138] and histone demethylation [139].

- Alternative splicing

RNAseq technology development has served as a tool to provide supplementary information of non-annotated genes or splice variants that before was not possible to analyze by microarrays. The impact of alternative splicing on multiple biological processes is a field of study that has increased in the last years. Concretely, it has been reported that this molecular mechanism can influence on clock function [140,141]. Different splice variants have been found in several of the clock main components [142]. For example, CCA1, LHY and PRR9 can be present in the cell in different splicing isoforms. The presence of one and/or other isoform has been shown to depend on the environmental conditions and can, for example, control the stability/functionality of the "reference" one, which refers to the most studied one. An example of this regulatory mechanism is seen in *CCA1*. A part from the "reference" isoform, two more

splicing variants have been identified, CCA1α and CCA1β, that accumulate differentially in function of growing temperature conditions [143].

- Protein turnover, post-translational modifications and protein-protein interactions

As for most cellular pathways, the activity of clock proteins can be regulated at polypeptide level. The regulation can affect either their stability (there are different proteins in charge of clock-proteins turnover) or altering their activity by modifying the structural/chemical composition or through interaction with other proteins that can reduce or enhance their activity.

Posttranslational modifications like phosphorylation play an important role in the regulation of proteins' activity, acting as activation or inactivation mark. For example, phosphoraylation of PRRs makes them more susceptible to be degraded [144].

Finally, the role of protein-protein interactions is also crucial to control several clock components. For example, homo- and heterodimerization are seen in CCA1 and LHY [145,146]. Sometimes, the interaction between two or more clock proteins is strictly necessary to from an active complex. An example is the case of the Evening Complex (EC), which will be explained below. For more details of protein-protein interactions reported for clock proteins you can check in Chow and Kay, 2013 [147].

It is also important to mention that clock components are not only able to interact with other clock components but they can also interact with proteins from other molecular pathways providing important crosstalk points between the circadian clock and other biological processes.

- Subcellular localization

Some clock proteins are present in both the cytoplasm and the nucleus and their subcellular location can affect their functional activation or inactivation. Moreover, the localization of a protein to one specific organelle can also induce the translocation of another protein to that organelle (for example, nuclear localization of GI is modulated by ELF4 [148]).

It is clear that circadian clock is very complex. The existence of so many interlocking loops that regulate clock components at transcriptional and posttranslational level, and the functional redundancy between some members, makes it very difficult to have a complete view of the clock network and full understanding of the mechanism. However, the emergence of multiple tools available for research, allowed in the last decade significantly increase the knowledge of clock components and their function, All this new information showed that rather than be a unidirectional system as it was originally described (as shown in figure 7 of this introduction), circadian system is a complex network in which inputs are also influenced by clock and that some clock outputs have an impact on clock function acting as inputs.

4. Hypocotyl growth regulation in diurnal growing conditions

Seedling developmental pattern is different depending on environmental conditions, especially light conditions which have a direct impact on hypocotyl elongation and the development of the apical part of the seedling. Even so, in all possible light conditions, the elongation of the hypocotyl is achieved by an expansion of the cells that form part of the embryo [149].

As mentioned above, hypocotyl elongation is a biological process that follows circadian oscillations [66,104,105]. Moreover, the phase of the growth rate changes depending on the growing conditions. In continuous light growing conditions, the peak of growth is at dusk [129]. In diurnal short-day conditions (8 h of light and 16h of dark), peak of growth is shifted towards the end of the night [66].

Different pathways regulate stem elongation and among them clock and light are two of the most important. It is essential for plant to coordinate the multiple regulatory pathways to ensure an optimal hypocotyl growth. The following is a more detailed explanation of some of the regulatory processes that control the timing of hypocotyl growth.

- Concerted action of light and clock in the regulation of PIF4 and PIF5 in SDs (Coincidence model)

The oscillating growth rate pattern seen in short-day grown seedlings can, in part, be explained by the presence of PIF4 and PIF5 proteins towards the end of the night, achieved by the coordinated action of clock and light, mechanism known as the coincidence model [66,74]. Circadian clock directly generates oscillations in *PIF4* and *PIF5* transcript levels. The expression of these two genes is repressed during the beginning of the night period and peaks at the end of the night and early morning. Such transcriptional repression is done by a protein complex, that peaks at dusk, known as Evening Complex (EC) [149], formed by ELF3 (EARLY FLOWERING 3), ELF4 and LUX (LUX ARRHYTHMO) proteins. Then, active phytochrome induces PIF degradation when the light period starts. Hence, it is only at the end of the night when there is transcription of these genes and the protein is stable, being able to promote growth and induce hypocotyl elongation.

- Clock, PIFs and Growth-promoting hormones in diurnal growth

The hormonal control of hypocotyl elongation is basically restricted to GAs, BRs and IAA. Because of having a crucial function in growth, it is not surprising then that both circadian clock and light regulate at different levels plants hormones, like controlling the expression of hormone biosynthetic genes [150] or affecting plant responsiveness to a specific hormone [151]. Transcriptional analysis done in different growing conditions and mutant backgrounds showed that GA, IAA and BR genes are, indeed, expressed at the time of the day corresponding to the maximum growth rate. Promoter sequence analysis of these hormone-related genes have showed an overrepresentation of a CACATG motif or HUD (Hormone Up at Dawn) [150] which is actually the same motif defined as PBE described above for PIFs, underscoring that light pathway and clock converge to regulate the expression of these genes.

Specific cases for hypocotyl growth-promoting hormone are explained below.

- Gibberellins (GAs)

Clock controls the expression of two growth-related GA biosynthetic and receptor (*GID1*) genes [152], inducing the presence of the complex GA-GID1 at the end of the night. GA-GID1 complex formation leads to the degradation of DELLA family proteins that act as growth repressors. In the absence of GA, DELLAs interact with PIFs and avoid their binding to DNA so that they cannot induce growth [64,65]. At the end of the night, clock-induced accumulation of GA-GID1 causes the destabilization of DELLAs and PIFs are then able to bind DNA gene promoters and induce growth. During the light hours of the cycle, *GID1* expression is reduced, and, moreover, light induces the reduction of GA levels [153].

- Auxin

The role of auxin hormone in the elongation of hypocotyl has recently been reported in several different growing conditions, such as deetiolation, SAS (Shade avoidance syndrome) and diurnal growth [59,68,94,154,155]. In the case of IAA, both light and clock have been reported to directly regulate the expression of auxin biosynthetic genes. An example is the gene *YUCCA8* which is a direct target of both PIFs and also REV1 (a member of the clock component) [156,157]. Moreover, circadian clock modulates through a gating mechanism the responsiveness of the plant to auxin levels, making it greater at the end of the night [151].

- Brassinosteroids (BRs)

It was mentioned above that PIF4 and BZR1 transcription factors can directly interact and act together as coactivators, representing an important point of the integration of light and hormones.

The crosstalk between different hormones is also of great relevance in terms of regulation because the action of a certain group of hormones can be modified by the presence/absence of another phytohormone group. An example is the crosstalk that exists between brassinosteroids and gibberellins; DELLA proteins can physically interact with BES1 and BZR1 proteins, blocking their capacity to bind DNA and, hence, suppressing their activity as transcription factors [93,158].

Considering the different molecular events explained above, it becomes evident that the fine regulation of hypocotyl elongation in short day conditions is actually achieved by the coordinated action of light, clock and also hormones. Phytohormones are able to regulate growth by directly regulate the expression of growth-related genes (some references: [159,160,161,162]). Additionally, hormones can also modulate growth by acting on PIFs, both regulating their transcription levels [58,71,72] and affecting their activity [64,65,90] and/or altering the circadian clock function by changing the period, amplitude or phase of clock components [163]. Although the role of specific types of hormones was not an objective of study of this thesis, it is important to do not forget that the role of these molecules is crucial for hypocotyl elongation and their participation on the regulation of the process has to be kept in mind.

This introduction tried to highlight the multiple regulatory mechanisms that plants have evolved in order to control such a crucial developmental stage as it is seedling growth, and more specifically, the elongation of the hypocotyl. Importantly, it is worth to mention that much of the current knowledge have been reported in the last recent years, while the PhD thesis was carried out. Together, data indicate that seedling diurnal development under diurnal conditions is a complex process controlled by three components: PIFs (as intermediates of light signaling pathway), circadian clock and hormones. At a transcriptional level it is already evident that there is a high degree of crosstalk between the three pathways, since reciprocal transcriptional regulation is observed between them. Connection points between the three pathways are also seen at a protein level, in some cases by acting together to regulate the gene expression of common target genes, and in other cases by affecting the activity of a component of another pathway.

Although the knowledge in the field of diurnal seedling hypocotyl elongation has considerably increased in the last recent years, there were still unknown aspects of PIF proteins function that could help to better understand the regulatory mechanisms that allow a proper seedling growth and development in short-day conditions. In figure 10, there is a scheme of the reported data about PIFs in the moment the thesis started.

Considering that in other growing conditions, several PIFs members act collectively to regulate one same biological process (as for example in etiolated seedlings [62]), we decided to explore the possibility that other members of the PIF family, a part from PIF4 and PIF5, also participate in the regulation of hypocotyl elongation under SD and, in case they do it, study at a molecular level how they do it. Indeed, supporting that idea, preliminary results in the laboratory had shown a possible role for PIF3 in the regulation of hypocotyl growth in SD. Moreover, it was known that other PIFs like PIF3 are not transcriptionally clock-regulated in diurnal conditions [164], in contrast to *PIF4* and *PIF5*. It was therefore of interest to understand the mechanisms underlying the accumulation and activity of PIF3 under these conditions and to explore the possibility that circadian clock regulates PIF3 through a different mechanism than the one reported for PIF4 and PIF5.

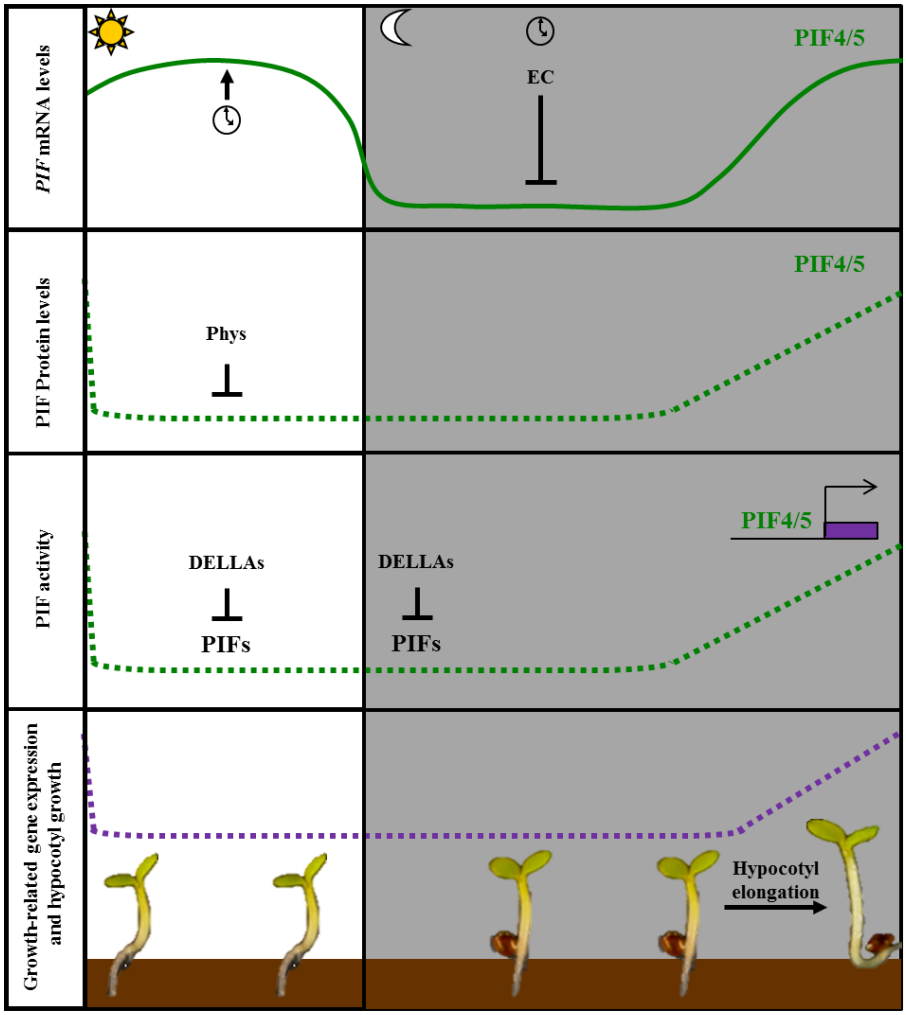

Figure 10. Simplified model depicting the knowledge about hypocotyl growth regulation in SDs at the moment the PhD thesis was starting. Acronym EC is for Evening Complex. Drawing of a clock represents the Circadian clock. Already confirmed data is depicted in a solid line. Predicted data (endogenous PIF4 and PIF5 protein oscillation, PIF4 and PIF5 protein activity and gene expression pattern) is depicted in a dashed line.

II. OBJECTIVES

"If there is something you know, communicate it.
If there is something you don't know, search for it."
Encyclopédie, 1772

The aim of this work was:

1.- To determine the role of PIF3 protein in diurnal growth

1.1-To determine if PIF3 regulates growth under short-day conditions

1.2- To understand how PIF3 protein is accumulated in short-day conditions

1.3- To define how PIF3 regulates hypocotyl growth in short-day conditions

1.4- To check the relative importance of PIF3 in the regulation of hypocotyl growth in comparison to PIF4 and PIF5

2.- To evaluate the relevance of PIFs levels and the involvement of PIF1 in hypocotyl elongation in short-day conditions

2.1- To check how hypocotyl growth correlates with PIF protein levels

2.2- To test the role of PIF1 in the regulation of hypocotyl growth in short-day

3.- To understand the relationship of PIF3 (and other PIFs) with the circadian clock.

3.1- To determine the possible role of TOC1 circadian clock protein in the regulation of hypocotyl elongation in different light conditions and its connection with PIFs proteins

3.2- To check if TOC1 and PIFs share common targets genes.

3.3- To study the relationship between TOC1 and PIF3 on the regulation of gene expression in short-day conditions.

III. RESULTS

"It is in doing things and not reading about them that results come about."
Stephen Richards

Barcelona, 2 May 2014

Judit Soy has co-authored 3 publications during her PhD, all 3 with high Impact Factor:

1-Author(s): **Soy, Judit**; Leivar, Pablo; Gonzalez-Schain, Nahuel; Sentandreu, Maria; Prat, Salome; Quail, Peter H.; Monte, Elena
Title: Phytochrome-imposed oscillations in PIF3 protein abundance regulate hypocotyl growth under diurnal light/dark conditions in Arabidopsis
Journal: **PLANT JOURNAL**, 2012. 71: 390
Impact Factor in the Year of Publication: 6.582
Quartile within the Area and the Year: Q1
ISI Journal Citation Reports © Ranking: 2012: 7/197 (Plant Sciences)

2-Author(s): **Soy, Judit**; Leivar, Pablo; Monte, Elena
Title: PIF1 promotes phytochrome-regulated growth under photoperiodic conditions in Arabidopsis together with PIF3, PIF4, and PIF5.
Journal: J EXP BOTANY, 2014. Jan 13. [Epub ahead of print].
Impact Factor in the Year of Publication: 5.242 in 2013.
Quartile within the Area and the Year: Q1
Named one of the top 100 most influential journals in Biology and Medicine over the last 100 years (DBIO 100).

3-Author(s): Sentandreu, Maria; Martin, Guiomar; Gonzalez-Schain, Nahuel; Leivar, Pablo; **Soy, Judit**; Tepperman, James M.; Quail, Peter H.; Monte, Elena
Title: Functional Profiling Identifies Genes Involved in Organ-Specific Branches of the PIF3 Regulatory Network in Arabidopsis
Source: **PLANT CELL**, 2011. 23: 3974
Impact Factor in the Year of Publication: 8.987
Quartile within the Area and the Year: Q1
Five-Year Impact Factor: 10.125. Highest impact factor of primary research journals in plant biology.

Best regards,

Elena Monte

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Barcelona, 2 May 2014

The contribution of the PhD candidate Judit Soy in the 3 publications presented in this thesis is as follows:

1- Phytochrome-imposed oscillations in PIF3 protein abundance regulate hypocotyl growth under diurnal light/dark conditions in Arabidopsis.

Soy, Judit; Leivar, Pablo; Gonzalez-Schain, Nahuel; Sentandreu, Maria; Prat, Salome; Quail, Peter H.; Monte, Elena. **PLANT JOURNAL**, 2012. 71: 390

In this paper, Judit Soy performed most of the experiments presented in the Figures. In addition, Judit prepared all the data for publication and participated in the process of writing the manuscript.The co-author Maria Sentandreu, who was also a PhD student in the Department at the time this work was performed and obtained her PhD in January 2013, contributed the data presented in Fig 5A.

2- PIF1 promotes phytochrome-regulated growth under photoperiodic conditions in Arabidopsis together with PIF3, PIF4 and PIF5.

Soy, Judit; Leivar, Pablo; Monte, Elena. **J EXP BOTANY**, 2014. Jan 13. [Epub ahead of print].

In this paper, Judit Soy performed all the experiments presented in the Figures. In addition, Judit prepared all the data for publication and participated in the process of writing the manuscript.

3- Functional Profiling Identifies Genes Involved in Organ-Specific Branches of the PIF3 Regulatory Network in Arabidopsis

Sentandreu, Maria; Martin, Guiomar; Gonzalez-Schain, Nahuel; Leivar, Pablo; **Soy, Judit**; Tepperman, James M.; Quail, Peter H.; Monte, Elena **PLANT CELL**, 2011. 23: 3974

This paper is the main body of research done by Maria Sentandreu, who performed and prepared most of the work presented in the Figures. Maria Sentandreu obtained her PhD in January 2013 (Departament de Genètica, UB). As a co-author, Judit Soy participated in the initial identification of some of the mutant lines characterized (Figure 2). Guiomar Martin, who is also a PhD candidate in the Department, participated in the identification and characterization of additional mutant lines. Guiomar Martín has not defended her PhD thesis yet.

Best regards,

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Publication 1.

Phytochrome-imposed oscillations in PIF3 protein abundance regulate hypocotyl growth under diurnal light/dark conditions in Arabidopsis

Judit Soy, Pablo Leivar, Nahuel González-Schain, Maria Sentandreu, Salomé Prat, Peter H. Quail and Elena Monte.

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SEB Society for Experimental Biology

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Phytochrome-imposed oscillations in PIF3 protein abundance regulate hypocotyl growth under diurnal light/dark conditions in Arabidopsis

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SUMMARY

Arabidopsis seedlings display rhythmic growth when grown under diurnal conditions, with maximal elongation rates occurring at the end of the night under short-day photoperiods. Current evidence indicates that this behavior involves the action of the growth-promoting bHLH factors PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PHYTOCHROME-INTERACTING FACTOR 5 (PIF5) at the end of the night, through a coincidence mechanism that combines their transcriptional regulation by the circadian clock with control of protein accumulation by light. To assess the possible role of PIF3 in this process, we have analyzed hypocotyl responses and marker gene expression in *pif* single- and higher-order mutants. The data show that PIF3 plays a prominent role as a promoter of seedling growth under diurnal light/dark conditions, in conjunction with PIF4 and PIF5. In addition, we provide evidence that PIF3 functions in this process through its intrinsic transcriptional regulatory activity, at least in part by directly targeting growth-related genes, and independently of its ability to regulate phytochrome B (phyB) levels. Furthermore, in sharp contrast to *PIF4* and *PIF5*, our data show that the *PIF3* gene is not subject to transcriptional regulation by the clock, but that PIF3 protein abundance oscillates under diurnal conditions as a result of a progressive decline in PIF3 protein degradation mediated by photoactivated phyB, and consequent accumulation of the bHLH factor during the dark period. Collectively, the data suggest that phyB-mediated, post-translational regulation allows PIF3 accumulation to peak just before dawn, at which time it accelerates hypocotyl growth, together with PIF4 and PIF5, by directly regulating the induction of growth-related genes.

Keywords: PIF3, hypocotyl elongation, short day, phytochrome-mediated degradation, transcriptional regulation, Arabidopsis.

INTRODUCTION

Light is fundamental for plants as a source of energy as well as an indicator of their living environment. Plants perceive and respond to ambient light signals through informational photoreceptors that include the phytochrome family (phyA–phyE in Arabidopsis) (Rockwell *et al.*, 2006; Schafer and Nagy, 2006; Quail, 2010). The phytochromes perceive red (660 nm) and far red (720 nm) light of the solar spectrum, and monitor changes in light quality, quantity and duration to control developmental and growth responses such as germination, seedling de-etiolation, shade avoidance and flowering time (Franklin and Quail, 2010; Strasser *et al.*, 2010). phyA is the only receptor for continuous far red light, but both phyA and phyB contribute to perception of continuous red light during early de-etiolation, with phyB being the dominant if not exclusive regulator of the hypocotyl-elongation response to continuous red light (Rockwell *et al.*,

2006; Schafer and Nagy, 2006; Tepperman *et al.*, 2006; Franklin and Quail, 2010). The phytochromes reversibly photoconvert between two conformers: the inactive red light-absorbing Pr form and the biologically active far red light-absorbing Pfr form. Pr to Pfr photoconversion takes place within seconds upon absorption of red light photons (Linschitz and Kasche, 1966), and reversion of Pfr to Pr occurs in far red light-enriched environments (Franklin, 2008), and also in the dark. In seedlings grown in the light, Pfr remains active upon initial transfer to the dark, but slowly reverts, at least partially, back to Pr with a half-life of approximately 60 min (Sweere *et al.*, 2001; Rausenberger *et al.*, 2010).

Phytochromes are synthesized in the cytoplasm in the inactive Pr form, and, upon photoactivation to Pfr, are translocated into the nucleus (Nagatani, 2004), where they associate with a subset of basic helix-loop-helix (bHLH) transcription factors called phytochrome-interacting factors (PIFs). The PIFs (PIF1, PIF3, PIF4, PIF5, PIF6 and PIF7 in Arabidopsis) accumulate in the dark and interact photoreversibly and conformer-specifically with the active Pfr phytochrome in the light (Leivar and Quail, 2011). This light-induced interaction between the Pfr phytochrome and PIF initiates a cascade of transcriptional changes that allows the seedling to adjust to the new light environment (Castillon *et al.*, 2007; Jiao *et al.*, 2007; Bae and Choi, 2008; Leivar and Quail, 2011). For a subset of these PIFs (PIF1, PIF3, PIF4 and PIF5), interaction with phyA and/or phyB triggers rapid phosphorylation and degradation of the PIF proteins within minutes (Bauer *et al.*, 2004; Park *et al.*, 2004; Shen *et al.*, 2005; Oh *et al.*, 2006; Nozue *et al.*, 2007; Shen *et al.*, 2007; Lorrain *et al.*, 2008; Shen *et al.*, 2008), establishing a new lower steady-state level of PIFs in continuous light (Monte *et al.*, 2004). Concomitantly, exposure to light induces rapid phyA degradation (with a half-life of <2 h), and a slower and more modest degradation of phyB (Hennig *et al.*, 1999; Khanna *et al.*, 2007; Al-Sady *et al.*, 2008), which remains relatively abundant in the light, together with phyC–phyE (Hirschfeld *et al.*, 1998). During prolonged growth in continuous light, the PIFs induce phyB proteolytic degradation through the proteasome system using COP1 as an E3 ligase (Khanna *et al.*, 2007; Al-Sady *et al.*, 2008; Leivar *et al.*, 2008a; Jang *et al.*, 2010), suggesting the existence of a mutually negative feedback loop between the phyB and PIF proteins (Leivar and Quail, 2011). This light-induced phyB degradation is expected to contribute to the progressive decline in Pfr levels during the dark period under diurnal conditions (light/dark cycles). In addition, the PIFs re-accumulate in light-grown seedlings upon exposure to darkness (such as under diurnal conditions) (Monte *et al.*, 2004; Shen *et al.*, 2005; Nozue *et al.*, 2007) or far red light-enriched environments (such as vegetational shade) (Lorrain *et al.*, 2008) through a process that depends on the activation state (or Pfr/Pr ratio) of the phytochromes.

Hypocotyl elongation is a well-established light-regulated response that is maximal in seedlings grown in continuous dark. In post-germinative darkness, the PIF proteins promote hypocotyl elongation through their intrinsic transcription factor capacity, regulating a transcriptional network that sustains etiolated growth (Leivar *et al.*, 2009; Shin *et al.*, 2009). This conclusion is supported by the observation that a quadruple mutant deficient in PIF1, 3, 4 and 5 (*pifq*) exhibits a partial constitutively photomorphogenic phenotype in the dark, characterized by a short-hypocotyl phenotype (Leivar *et al.*, 2008b). In continuous light, under which PIFs induce phyB degradation, PIF-deficient mutants display a hypersensitive short-hypocotyl phenotype that is interpreted to be, at least partially, the result of enhanced photosensitivity of the seedling due to elevated photoreceptor levels (Khanna *et al.*, 2007; Al-Sady *et al.*, 2008; Leivar *et al.*, 2008a).

Under diurnal conditions, with an alternating light/dark cycle, the extent of hypocotyl elongation depends on the duration of the dark period (Niwa *et al.*, 2009). During dark hours, the hypocotyl elongation rate is maximal at the end of the night in seedlings grown under short-day (SD) photoperiods (Nozue *et al.*, 2007). Studies have indicated that PIF4 and PIF5 are positive regulators of this response (Nozue *et al.*, 2007; Niwa *et al.*, 2009). The precise regulation of their time of action at the end of the dark period has been proposed to involve a coincidence mechanism that combines regulation of *PIF4* and *PIF5* transcript levels by the circadian clock, superimposed on the control of PIF protein accumulation by light (Nozue *et al.*, 2007; Nusinow *et al.*, 2011). In addition to PIF4 and PIF5, the current model predicts that additional, yet to be identified, factors are involved in the regulation of seedling growth under SD conditions.

In this study, we have used single and multiple *pif3*, *pif4* and *pif5* mutants, combined with analyses of PIF3 protein accumulation and target gene expression, to define the role of PIF3 in the regulation of hypocotyl elongation in seedlings grown under diurnal conditions, and have examined the relative contributions of PIF3, PIF4 and PIF5 to this response. Our results suggest that phytochromes generate an oscillation of PIF3 abundance under SD conditions such that it peaks just before dawn, at which time PIF3 plays a prominent role in promoting elongation growth, in conjunction with PIF4 and PIF5, at least in part by directly regulating the expression of growth-related genes.

RESULTS

The pattern of PIF3 accumulation under SD conditions is regulated by phyA and phyB and is independent of transcriptional regulation by the clock

To establish the pattern of *PIF3* expression under diurnal SD conditions [8 h white light + 16 h darkness], we analyzed *PIF3* transcript levels over 24 h during the third day of seedling growth under SD conditions (Figure 1a), and

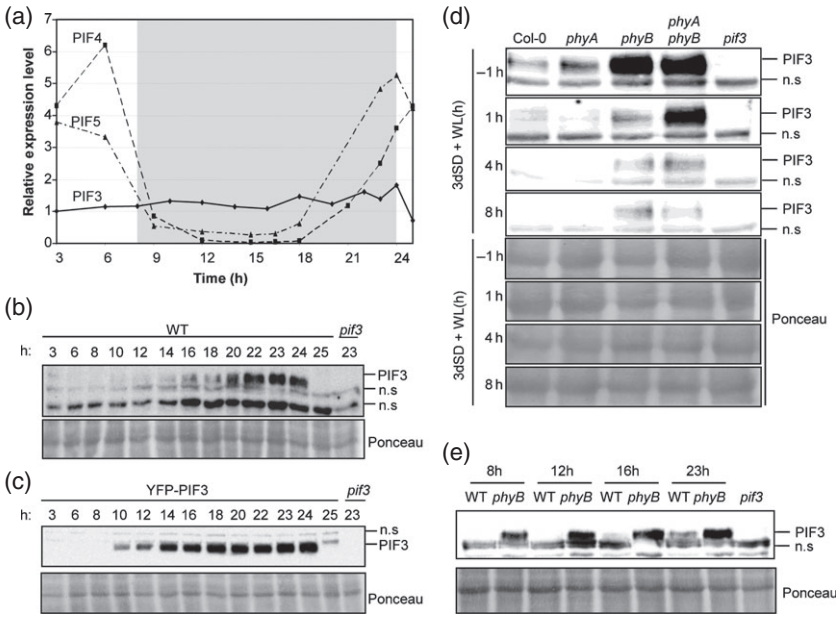

Figure 1. PIF3 protein accumulation under SD conditions.
Seedlings were grown under SD conditions for 2 days (a–c, e) or 3 days (d), and samples were taken during the following day at the specified time points.
(a) The expression of *PIF3*, *PIF4* and *PIF5* was analyzed by quantitative RT-PCR. Values were normalized to *PP2A*, and expression levels relative to *PIF3* at 3 h are shown. Values are means of technical triplicates.
(b) Immunoblot of protein extracts from WT seedlings.
(c) Immunoblot of protein extracts from seedlings over-expressing YFP–PIF3.
(d) Immunoblot of protein extracts from WT, *phyA-211*, *phyB-9* and *phyA phyB* seedlings.
(e) Immunoblot of protein extracts from WT and *phyB-9* seedlings.
For (b–e), a PIF3-specific polyclonal antibody was used as the probe (top). As an antibody specificity control, a protein extract from *pif3-3* harvested at time 23 h was included. Ponceau staining was used as a loading control (bottom). n.s., non-specific cross-reacting bands.

compared them to the expression pattern of *PIF4* and *PIF5*. *PIF3* transcript levels remained fairly constant over the 24 h photoperiod (Figure 1a). In sharp contrast, *PIF4* and *PIF5* transcript levels decreased during the day, stayed low during most of the dark period, and increased again to peak at the end of the night (Figure 1a), consistent with the previously reported circadian clock regulation of *PIF4* and *PIF5* transcript levels (Yamashino *et al.*, 2003; Nozue *et al.*, 2007; Niwa *et al.*, 2009). These results indicate that, in contrast to *PIF4* and *PIF5*, *PIF3* transcript levels do not oscillate under diurnal conditions, and suggest that the circadian clock does not regulate *PIF3* transcription under SD conditions.

We next examined the pattern of accumulation of the endogenous PIF3 protein under diurnal light/dark cycles. To do this, we grew seedlings under SD conditions and tested the levels of endogenous PIF3 protein every 1–3 h over a period of 24 h. PIF3 protein started to accumulate at the start of the dark period, as early as 2 h after the transition from light to dark (10 h time point, Figures 1b and S1), and kept accumulating progressively to reach a maximum at the end of the night, after 14–16 h of darkness (22, 23 and 24 h time points, Figure 1b). PIF3 protein levels then dropped to below the detection limit after exposure of seedlings to white light for 1 h (25 h time point, Figure 1b). Transgenic plants over-expressing a YFP–PIF3 fusion (Al-Sady *et al.*, 2006) showed a similar pattern of YFP–PIF3 accumulation under SD conditions, with low levels during the light period and a progressive increase during the dark to peak at the end of the night (Figure 1c). A similar pattern was also observed in transgenic lines over-expressing PIF4:HA and PIF5:HA, although in these experiments the seedlings were grown under SD/3 conditions (i.e. 8 h light/dark cycles comprising 160 min light + 320 min dark) (Nozue *et al.*, 2007).

Together, the above experiments indicate that, under SD conditions, PIF3 protein levels are very low during the light period, but increase progressively during the night (Figure 1b) through post-transcriptional regulation (Figure 1a). In order to examine the role of phytochrome activity in regulation of this pattern of PIF3 accumulation, we measured PIF3 levels at the end of the night (-1 h) and after 1, 4 and 8 h of light exposure in *phyA* and *phyB* single and double mutants (Figure 1d). Wild-type (WT) seedlings accumulated PIF3 protein during the dark period, and light induced a rapid reduction in these levels within 1 h. Compared to WT, *phyA phyB* double mutants accumulated higher levels of PIF3 both at the end of the night and during the light period (Figure 1d), suggesting that phyA and/or

phyB act to reduce PIF3 levels under SD conditions. Detailed single *phyA* and *phyB* mutant analysis at various time points suggests that the two photoreceptors contribute differentially to this activity. First, *phyA* mutants showed similar levels of PIF3 at the end of the night compared to WT (Figure 1d), but the levels in *phyB* and *phyA phyB* mutants were much higher (Figure 1d). Second, in contrast to the rapid light-induced degradation of PIF3 observed in WT, *phyA* and *phyB* seedlings, PIF3 levels remained relatively constant in *phyA phyB* double mutants after 1 h of illumination (Figure 1d). Finally, PIF3 levels further decreased and remained below the detection limit in *phyA* mutants during the day (4 and 8 h time points), similar to the WT (Figure 1d). In *phyA phyB* mutants, PIF3 levels also decreased between 1 and 4 h of illumination but PIF3 was still detectable after 4 and 8 h of light. In contrast, PIF3 levels in the *phyB* mutant did not further decrease after 1 h of illumination, and its levels were similar during the rest of the light period. Together, these results suggest that phyA and phyB act redundantly to rapidly reduce PIF3 levels within 1 h of illumination, and that at least one other photoreceptor is involved in the decrease in PIF3 levels at later time points (between 1 and 4 h). This scenario is similar to that reported during seedling de-etiolation, where phyD was shown to act together with phyA and phyB to induce PIF3 degradation in etiolated seedlings transferred to light (Bauer *et al.*, 2004; Al-Sady *et al.*, 2006). In addition, phyB activity is required to induce complete PIF3 degradation during the light period, and to prevent re-accumulation of PIF3 during the dark hours, in a process that requires little or no participation of phyA.

To obtain further insight into the role of phyB in preventing re-accumulation of PIF3 during the night in SD-grown seedlings, we performed a more detailed comparison of PIF3 levels in WT and the *phyB* mutant during the dark period. Figure 1(e) shows that PIF3 levels at the start of the night (8 h time point) were higher in *phyB* compared to WT seedlings, and rapidly increased in *phyB* during the first 4 h of darkness (8–12 h time points), reaching a new steady-state level that remained relatively constant until the end of the night (23 h). In contrast, PIF3 re-accumulation in the WT was slower during the first hours of darkness, and much lower levels were observed at the end of the night (Figure 1e). Together, our results suggest that the induction of PIF3 degradation by photoactive phyB Pfr during the light period extends into the first hours of the subsequent dark period. This possibility is in accordance with previous data showing that a far red light pulse given at the start of a 12 h dark period (removing the Pfr phytochrome pool from the cell) induced faster re-accumulation of GUS activity in GUS:PIF3 over-expressing seedlings grown under day-neutral conditions (Monte *et al.*, 2004), and with the observation that the Pfr form of the photoreceptor continues to function in the dark to induce PIF3 degradation (Al-Sady *et al.*, 2006).

PIF3 is necessary for hypocotyl growth under SD conditions

To examine the role of PIF3 during seedling growth under SD conditions, we measured hypocotyl elongation in seedlings lacking PIF3 (Monte *et al.*, 2004). Hypocotyls of 3-day-old SD-grown *pif3* mutants were approximately 40% shorter than the Col-0 control under these conditions (Figure 2a,b). In detailed time-course analyses, we found that WT hypocotyls elongated from 2 days onwards after germination under SD conditions, but the growth rate was severely reduced in the *pif3* mutants (Figure 2c). The impact of PIF3 deficiency on growth was already apparent 48 h after initial exposure to SD, the first time point at which it was possible to measure seedling length (Figure 2c). In comparison to SD conditions, WT seedlings were shorter when grown under continuous white light (Figure 2b,d), and *pif3* mutants grown under continuous white light were only slightly shorter than the WT (Figure 2d). These data indicate that the 16 h dark period in SD-grown seedlings accelerates hypocotyl elongation, consistent with previous reports (Niwa *et al.*, 2009). Together with the PIF3 protein accumulation pattern (Figure 1), our data suggest that PIF3 is an important component of the cellular machinery that induces growth during the night hours.

In contrast to the short phenotype of *pif3* (Figure 2b), *phyB* mutant seedlings had more elongated hypocotyls than WT under SD conditions (Michael *et al.*, 2008; Niwa *et al.*, 2009), indicating an antagonistic functional relationship between phyB and PIF3 in regulating this response. Characterization of *phyB* and *pif3* single and double mutants showed that *phyB* seedlings grown under SD conditions were approximately 1.5 mm taller than the corresponding WT (Figure 2b,e), and that genetic removal of PIF3 partially and significantly suppressed the *phyB* phenotype by 1 mm (Figure 2b, *phyB* versus *pif3 phyB*). These data suggest that the increased levels of PIF3 (Figure 1d) are at least partially responsible for the elongated hypocotyl phenotype of *phyB* mutant seedlings. In addition, compared to SD conditions, *phyB* mutant seedlings grown under continuous white light displayed a much reduced tall-hypocotyl phenotype (Figure 2d) and reduced suppression of this phenotype by the *pif3* mutation (Figure 2d, *phyB* versus *pif3 phyB*). These data suggest that the dark period is necessary for full expression of the *phyB* mutant phenotype, probably by allowing higher accumulation of PIF3 protein under SD conditions compared to continuous white light (Figure 1d,e). Correlation between PIF3 levels and hypocotyl elongation was further observed in *phyA phyB* mutants (Figure S2). Compared to *phyB*, the double *phyA phyB* mutant had slightly longer hypocotyls under SD conditions, in agreement with the higher PIF3 levels detected at the start of the day in *phyA phyB* compared to *phyB* (Figure 1d).

Previously, PIF3-deficient mutants were shown to have increased phyB levels under continuous red light,

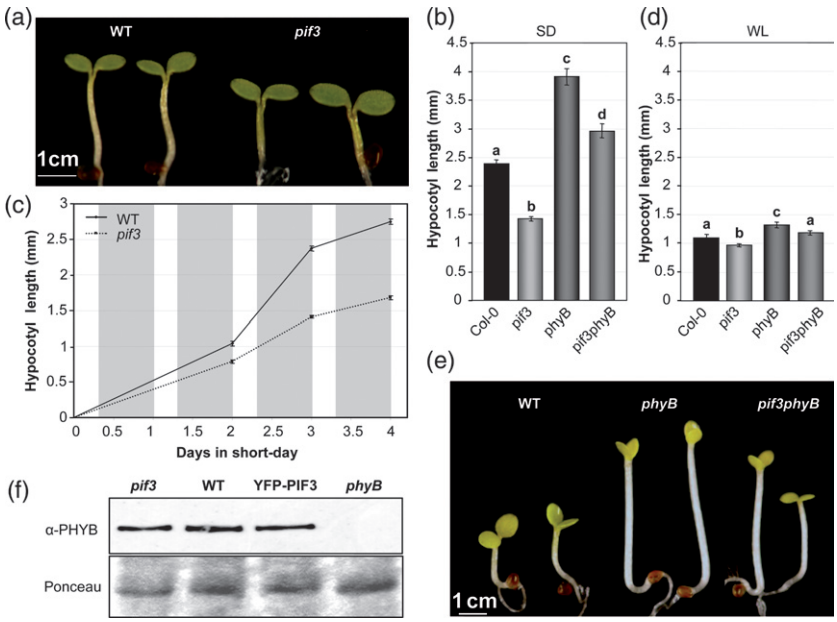

Figure 2. PIF3 promotes hypocotyl growth under SD conditions (8 h light/16 h dark).
(a) Visual phenotype of 3-day-old SD-grown WT and *pif3* mutant seedlings.
(b) Hypocotyl length in 3-day-old SD-grown WT and *pif3* seedlings.
(c) Growth curves for hypocotyl length in WT and *pif3* seedlings grown under SD for 4 days.
(d) Hypocotyl length in 3-day-old WT and *pif3* seedlings grown under continuous white light (WL).
(e) Visual phenotype of 3-day-old SD-grown WT, *pif3*, *phyB* and *pif3 phyB* mutant seedlings.
(f) Immunoblots of protein extracts from 3-day-old WT and *pif3* seedlings. Seedlings were grown under SD conditions for 2 days, and samples were harvested during the third day at the specified time points. Extracts were probed using phyB-specific monoclonal antibodies (top). Ponceau staining was used as a loading control (bottom).
For (b–d), data are means ± SE of at least 30 seedlings. For (b, d), different letters indicate significant differences among means ($P < 0.05$).

contributing to their hypersensitive hypocotyl phenotype (Monte *et al.*, 2004; Al-Sady *et al.*, 2008; Leivar *et al.*, 2008a). To examine whether the described negative feedback modulation of phyB photoreceptor levels by PIF3 under prolonged continuous red light and continuous white light conditions (Leivar *et al.*, 2012a) operates under SD conditions, we measured phyB levels in the *pif3* mutant and in YFP–PIF3 over-expressing lines at the end of the light period (8 h time point). Figure 2(f) shows that there were no significant differences in phyB levels between genotypes after 8 h of illumination, suggesting that PIF3-induced down-regulation of phyB requires more extended periods of light exposure. Together with our observation that PIF3 promotes growth under SD conditions in the absence of phyB (Figure 2b), these results indicate that PIF3 function under SD conditions is not exerted indirectly through regulation of phyB levels, and instead suggest that the PIF3 contribution to hypocotyl length under SD conditions is exerted through its intrinsic transcriptional activity, in accordance with previous data in etiolated seedlings (Al-Sady *et al.*, 2008; Leivar *et al.*, 2008b; Moon *et al.*, 2008; Leivar *et al.*, 2009; Shin *et al.*, 2009; Sentandreu *et al.*, 2011) and during shade avoidance (Hornitschek *et al.*, 2009).

Expression of phytochrome-regulated, growth-related genes peaks at the end of the night under SD conditions and requires PIF3

To test whether PIF3 regulates growth-related genes under SD conditions, we measured the expression of *PIL1* (*PHYTOCHROME-INTERACTING FACTOR 3-LIKE 1*), *HFR1* (*LONG HYPOCOTYL IN FAR-RED 1*) and *XTR7* (*XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7*) in WT and *pif3* seedlings. These genes are repressed by the phytochromes and are up-regulated under conditions in which hypocotyl elongation is induced (Salter *et al.*, 2003; Lorrain *et al.*, 2008; Hornitschek *et al.*, 2009; Leivar *et al.*, 2009; Nozue *et al.*, 2011), and have been proposed to be direct targets of transcriptional regulation by PIF4 in dark-adapted plants (de Lucas *et al.*, 2008) and/or by PIF5 during shade avoidance (Hornitschek *et al.*, 2009). *PIL1* and *HFR1* are PIF-related transcription factors (Leivar and Quail, 2011), and *XTR7* encodes a xyloglucan endotransglycosylase-related protein that is potentially involved in cell-wall growth (Sasidharan *et al.*, 2010).

Time-course expression analysis indicated that the expression levels of these three genes under SD conditions remain low during the light period in the WT, and start accumulating during the dark, peaking at the end of the night

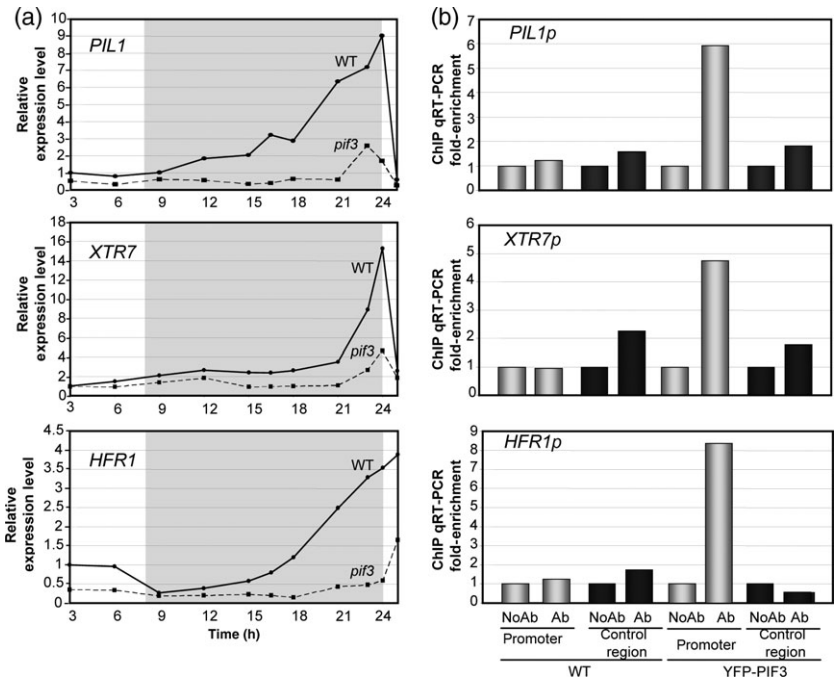

Figure 3. PIF3 regulates gene expression under SD conditions.
(a) Expression of *PIL1*, *XTR7* and *HFR1* was analyzed by quantitative RT-PCR in 2-day-old SD-grown seedlings. Samples were taken during the third day under SD conditions at the specified time points. Values were normalized to *PP2A*. Expression levels relative to WT at 3 h are shown. Data are the means of technical triplicates.
(b) Chromatin immunoprecipitation (ChIP) from 3-day-old SD-grown WT and YFP–PIF3 seedlings. Immunoprecipitated DNA was quantified by quantitative RT-PCR using primers in promoter regions containing G-boxes (promoter) or control regions without G-boxes (control region). Experiments include samples processed with anti-GFP antibody (Ab) and controls processed without antibody (NoAb). Data shown correspond to one representative ChIP experiment. The results of an additional ChIP experiment are shown in Figure S4. Data are the means of at least two technical replicates.

(Figure 3a). The expression levels at the end of the night were 4-, 10- and 16-fold greater than the levels during the light period for *HFR1*, *PIL1* and *XTR7*, respectively (Figure 3a). Interestingly, the pattern of expression of these genes parallels the accumulation pattern of PIF3 protein (Figure 1b,c), rendering them good candidate genes for regulation by PIF3. Expression analysis by quantitative RT-PCR showed that their transcript levels are clearly reduced in the *pif3* mutant, with the amplitude of the peak at the end of the night reduced by 80–90% for the three genes tested (Figure 3a). These data indicate that PIF3 induces expression of *PIL1*, *HFR1* and *XTR7* during the dark period under SD conditions, and suggest that PIF3 promotes growth under diurnal conditions by regulating expression of growth-related genes.

PIF3 directly binds to G-box-containing promoters of growth marker genes *in vivo*

HFR1, *PIL1* and *XTR7* genes harbor G-boxes in their promoters (Hornitschek *et al.*, 2009) (Figure S3), suggesting that they are possible direct targets of PIF3 (Martínez-García *et al.*, 2000; Shin *et al.*, 2007). We analyzed the binding of PIF3 to the promoters of *HFR1*, *PIL1* and *XTR7* by chromatin imunoprecipitation (ChIP) using plants expressing PIF3 tagged with YFP (YFP–PIF3). ChIP was performed using an anti-GFP antibody, and immunoprecipitated G-box-containing and control DNA fragments were quantified by quantitative RT-PCR. Control DNA regions included non-G-box-containing regions of tested or unrelated genes. As controls, we used YFP–PIF3 plants processed without anti-GFP antibody, and Col-0 plants subjected to the same process with and without antibody. We performed these experiments in seedlings grown under SD conditions for 3 days and harvested at the end of the night (time point 23 h), when the maximum levels of PIF3 (Figure 1b) and the peak of expression of these genes coincide (Figure 3a). We observed significant enrichment of binding of PIF3 to the regions of *HFR1*, *PIL1* and *XTR7* promoters containing the G-box (Figures 3b and S4). These data indicate that PIF3 directly binds to the promoter regions of *HFR1*, *PIL1* and *XTR7*, presumably through the G-box motif, and suggest that these genes are direct targets of transcriptional regulation by PIF3 under SD conditions.

PIF3 regulates hypocotyl growth and gene expression under SD conditions, together with PIF4 and PIF5

The observation that *pif3* seedlings exhibit a reduced but still significant growth response to SD conditions compared to

WT (Figure 2b,c) indicates that factors other than PIF3 are involved in the induction of hypocotyl elongation under SD conditions. Evidence obtained using *pif4* and *pif5* mutants indicates that these additional factors are probably PIF4 and PIF5 (Nozue *et al.*, 2007; Niwa *et al.*, 2009). To obtain insight into the contribution of PIF3 to the promotion of hypocotyl elongation under SD conditions relative to that of PIF4 and PIF5, we first analyzed the hypocotyl length of 3-day-old SD-grown *pif3*, *pif4*, *pif5*, *pif4 pif5* and *pif3 pif4 pif5* mutant seedlings. Under these conditions, *pif4* and *pif5* single mutants showed a quantitatively similar short-hypocotyl phenotype compared to the WT, an effect that was additive in the *pif4 pif5* mutant, in accordance with previous reports (Figure 4a) (Nozue *et al.*, 2007). In comparison, *pif3* seedlings had more prominent short-hypocotyl phenotype than either *pif4* or *pif5*, and this phenotype was similar in magnitude to that of the double *pif4 pif5* mutant (Figure 4a). Moreover, the triple *pif3 pif4 pif5* mutant had slightly shorter hypocotyls compared to *pif3* (Figure 4a), confirming that PIF4 and PIF5 promote at least part of the residual growth of *pif3* seedlings under SD conditions. We also compared the hypocotyl lengths of *pif3 phyB* and the *pif3 pif4 pif5 phyB* quadruple mutant. Our data indicate that removal of PIF4 and PIF5 in *pif3 pif4 pif5 phyB* had an additive effect over removal of PIF3 in *pif3 phyB*, and further suppressed the *phyB* tall phenotype (Figure 4b). Altogether, these results suggest that PIF3, PIF4 and PIF5 collectively function in the promotion of hypocotyl length under SD conditions, with the role of PIF3 probably being more prominent.

To examine the interactions between PIF3, PIF4 and PIF5 in regulating gene expression under SD conditions, we analyzed *PIL1* expression in various *pif3*, *pif4* and *pif5* mutant combinations at the end of the night when expression of this gene peaks in the WT (23 h time point) (Figure 3a). The data show that individual deficiencies in PIF4 or PIF5 marginally reduced the level of *PIL1* transcript (WT versus *pif4* and *pif5* single mutants), but a greater (and significant) reduction was observed in *pif3* seedlings (Figure 4c). In addition, double mutant combinations including *pif3* (*pif3 pif4* and *pif3 pif5*) showed a further dramatic reduction in *PIL1* expression (Figure 4c), indicating synergistic interactions between these factors for induction of *PIL1* expression under SD conditions. Similar to the phenotypic analysis (Figure 4a), the magnitude of the reduction in *PIL1* gene expression in *pif3* mutants was similar to that of the double *pif4 pif5* mutant (Figure 4c), and a further reduction in *PIL1* expression was observed in *pif3 pif4 pif5*. These results suggest that PIF3 dominates the induction of *PIL1* under SD conditions at the 23 h time point, and PIF4 and PIF5 are responsible for the residual *PIL1* expression observed in *pif3* single mutant seedlings at the end of the night (Figures 3a and 4c). Consistent with this observation, time-course analysis of *XTR7* expression over the night showed that the peak of expression detected in *pif3*

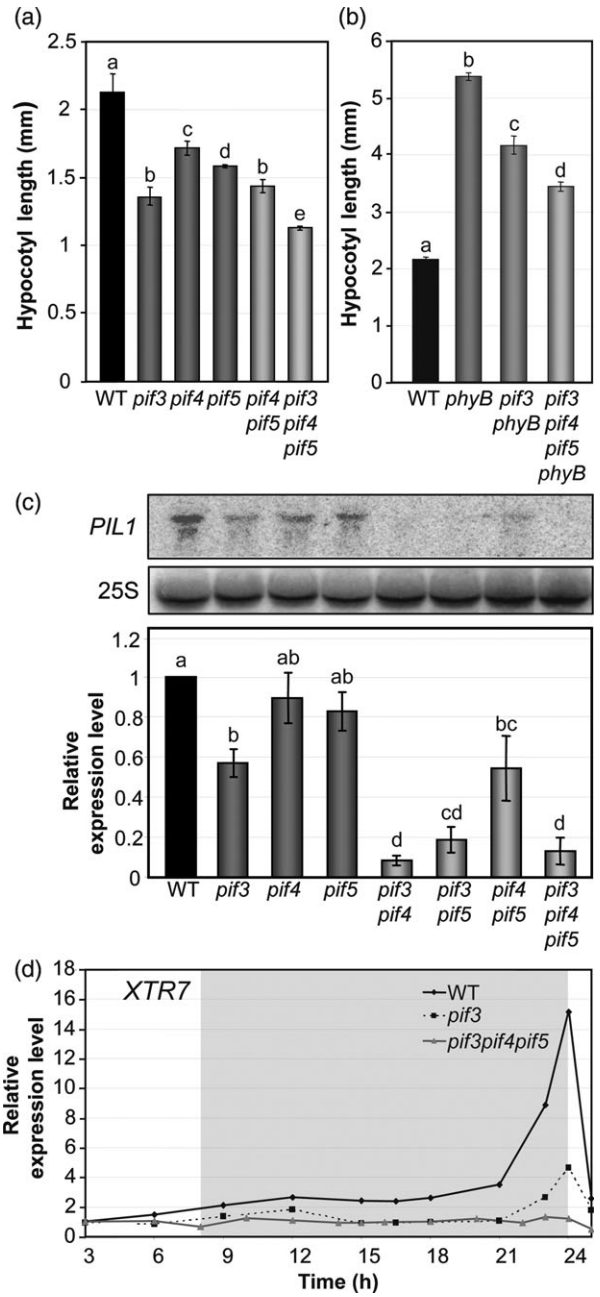

Figure 4. PIF3, PIF4 and PIF5 collectively regulate hypocotyl length and gene expression under SD conditions.
(a) Hypocotyl length in 3-day-old SD-grown WT, *pif3*, *pif4*, *pif5*, *pif4 pif5* and *pif3 pif4 pif5* seedlings.
(b) Hypocotyl length in 3-day-old SD-grown WT, *phyB*, *pif3 phyB* and *pif3 pif4 pif5 phyB* seedlings.
(c) The expression of *PIL1* was analyzed by RNA blots of 3-day-old SD-grown WT, *pif3*, *pif4*, *pif5*, *pif3 pif4*, *pif3 pif5*, *pif4 pif5* and *pif3 pif4 pif5* seedlings. A representative blot is shown (top). Quantitative data (bottom) are means of three biological replicates; bars represent SE. Values were normalized to 25S rRNA.
(d) Expression of *XTR7* was analyzed by quantitative RT-PCR in 2-day-old SD-grown WT, *pif3* and *pif3 pif4 pif5* seedlings during the third day of SD conditions. Values were normalized to *PP2A*. Expression levels relative to WT at 3 h are shown. Data are the means of technical triplicates.
For (a, b), data are means ± SE of at least 30 seedlings. For (a–c), different letters indicate significant differences among means ($P < 0.05$).

(Figures 3a and 4d) is essentially absent in *pif3 pif4 pif5* (Figure 4d), again suggesting that PIF4 and PIF5 are responsible for the residual *XTR7* expression observed in *pif3* single mutant seedlings at the end of the night. Together, the morphological (Figure 4a,b) and gene expression analyses (Figure 4c,d) suggest that PIF3, in conjunction with PIF4 and PIF5, plays a prominent role in induction of growth-related genes at the end of the night to promote growth under SD conditions.

PIF3 is required to promote growth at the end of the night under SD conditions

When grown under SD conditions, seedlings display rhythmic growth, with maximal elongation rates occurring at the end of the night (Nozue *et al.*, 2007). To test whether the *pif3* mutant shows an impaired growth pattern, we monitored seedling growth during a 24 h cycle, and calculated the growth rate of *pif3* seedlings compared to WT. Our data show that WT seedlings, in accordance with previously published results (Nozue *et al.*, 2007), maintain low growth rates during the day and the first half of the night, and the growth rate peaks at the end of the night (Figure 5a). In contrast, *pif3* seedlings show a strong reduction in this growth peak (Figure 5a). These results are in accordance with the progressive pattern of PIF3 protein accumulation in the dark (Figure 1b,c) and the occurrence of PIF3-induced gene expression at the end of the night (Figure 3a), and suggest that the short hypocotyls in the *pif3* mutant under SD conditions (Figure 2a,b) are mainly the result of a reduced growth rate at dawn. Together, these results indicate that PIF3 is required for hypocotyl elongation under diurnal conditions by promoting growth at the end of the night, as has been previously shown for PIF4 and PIF5 (Nozue *et al.*, 2007).

DISCUSSION

For Arabidopsis seedlings grown under SD conditions, the growth rate peaks at the end of the dark period. This rhythmic growth is implemented in part by the growth-promoting factors PIF4 and PIF5, and coincidence of both light and the circadian clock regulation determines their time of action just before dawn (Nozue *et al.*, 2007; Niwa *et al.*, 2009). The experiments presented here examine whether and through what mechanism PIF3 contributes to seedling growth under diurnal conditions. The data indicate that PIF3 protein accumulates progressively during the night under the control of phyB through a mechanism that does not involve transcriptional regulation by the clock, and provide evidence that PIF3, in conjunction with PIF4 and PIF5, is a major component of the cellular machinery that promotes hypocotyl elongation at dawn during growth under SD conditions, functioning at least in part through direct regulation of expression of growth-related genes (Figure 5b).

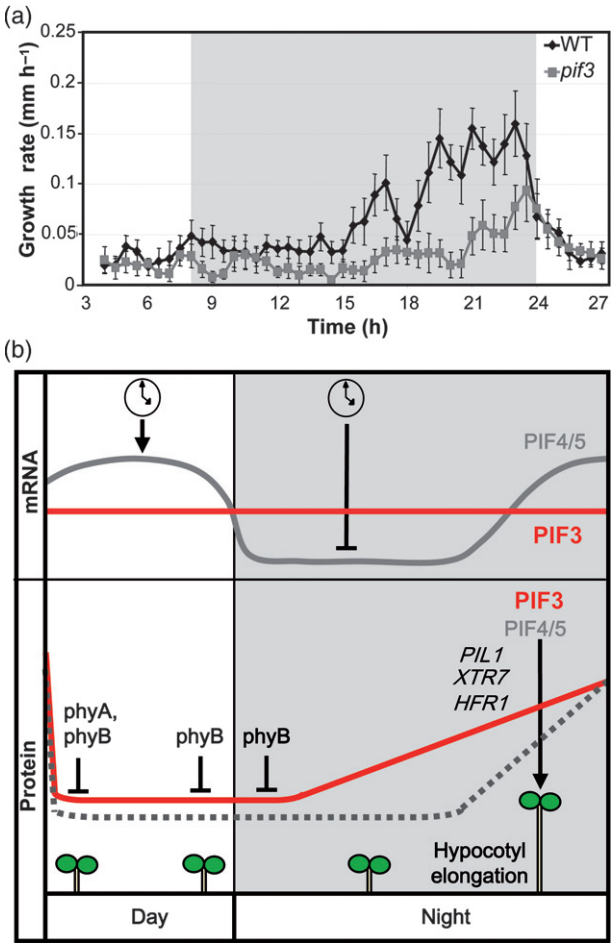

Figure 5. PIF3 is required to promote growth at the end of the night under SD conditions.
(a) Hypocotyl elongation rate under SD conditions. Infrared imaging was used to monitor seedling growth from 2 days onwards. The growth rate is plotted as a function of time. Values are means ± SE of seven seedlings.
(b) Simplified model depicting PIF3- and PIF4/5-mediated hypocotyl growth under SD conditions. Top: the circadian clock regulates oscillation of *PIF4* and *PIF5* transcript abundance in SD-grown seedlings, whereas *PIF3* remains constant throughout the day. Bottom: phyA and phyB activities induce degradation of PIF3 during the day (probably with an additional contribution from phyD), and phyB is active during the first part of the night to keep PIF3 levels low. As the night proceeds, phyB activity decreases and PIF3 progressively accumulates, peaking at the end of the night. For PIF4 and PIF5, coincidence of the circadian clock and light regulation ensures that protein accumulation peaks at the end of the night (Nozue *et al.*, 2007). Endogenous PIF3 protein oscillation is indicated by a solid line, and the predicted endogenous PIF4/5 protein oscillation is indicated by a dashed line. PIF3 directly induces the expression of growth-related genes at the end of the night (exemplified by *PIL1*, *HFR1* and *XTR7*), in conjunction with PIF4 and PIF5, to induce hypocotyl growth before dawn.

Our phenotypic and marker gene expression analyses of *pif* single and higher-order mutants provide evidence that PIF3 is necessary for seedling growth under SD conditions in conjunction with PIF4 and PIF5, and suggest that the PIF3 contribution is comparable to that of PIF4 and PIF5 combined. Various lines of evidence support this conclusion.

First, the *pif3* mutant displays a more prominent short-hypocotyl phenotype than either *pif4* or *pif5* under SD conditions, and this phenotype is similar in magnitude to that of the *pif4 pif5* double mutant (Figure 4a). Second, the *PIL1* expression level in *pif3* shows a greater reduction with respect to WT than in either single *pif4* or *pif5* mutants, and a similar level of reduction to *pif4 pif5* (Figure 4c). Finally, the *pif3 pif4* and *pif3 pif5* mutants show even more reduced *PIL1* gene expression compared to *pif3* or *pif4 pif5*, and this is similar to the triple mutant *pif3 pif4 pif5* (Figure 4c). These data thus suggest that PIF3, PIF4 and PIF5 act together to promote hypocotyl elongation under diurnal conditions, and that PIF3 appears to play a more prominent role. Interestingly, the relative contributions of PIF3 and PIF4/PIF5 to the promotion of seedling growth under diurnal conditions appear to be different from the relative contribution of each PIF under other growth conditions. For example, the roles of PIF3, PIF4 or PIF5 in induction of hypocotyl growth in etiolated seedlings are mainly apparent in the absence of PIF1, the PIF with the strongest contribution to the hypocotyl response in post-germinative growth in the dark (Leivar *et al.*, 2012b). In contrast, although no individual PIF appears to dominate the growth response to a continuous low red/far red ratio (Leivar *et al.*, 2012b), PIF3 makes a greater contribution to afternoon shade events under diurnal conditions (Sellaro *et al.*, 2012), and PIF4 is the strongest contributor to high-temperature effects (Koini *et al.*, 2009; Stavang *et al.*, 2009; Franklin *et al.*, 2011). Together, these data suggest that the contribution of a given PIF to hypocotyl elongation varies between growth situations.

Previous results have shown that PIF3 demonstrates dual functioning during seedling de-etiolation: (i) as a transcriptional regulator during development in the dark and in the initial dark-to-light transition, and (ii) as a regulator of phyB homeostasis during sustained growth under prolonged light conditions. Evidence presented here suggests that, under SD conditions, a growth regime that alternates dark and light periods, PIF3 does not regulate phyB levels (Figure 2). Given the slow dynamics of PIF-induced phyB degradation in response to the initial light signal (Khanna *et al.*, 2007; Al-Sady *et al.*, 2008), it is likely that the short length of the light period (only 8 h) under SD conditions is not enough to promote a detectable effect. Instead, the role of PIF3 as promoter of hypocotyl growth appears to be mediated through its intrinsic transcriptional activity directly regulating the expression of growth-related genes (Figure 3). Our results show that, under SD conditions, PIF3 binds to the promoters and probably directly regulates expression of target genes that were previously reported to be growth-related during etiolation and shade avoidance, such as *PIL1*, *HFR1* and *XTR7* (Figure 3). These genes have been previously shown to be direct targets of PIF4 and/or PIF5 in dark-adapted plants (de Lucas *et al.*, 2008) and under shade conditions (Hornitschek *et al.*, 2009), respectively, and therefore it was not unexpected to find that PIF4 and PIF5 regulate their expression also under SD conditions (Figure 4), in accordance with recent data from Nozue *et al.* (2011) for *HFR1* and *XTR7*. However, as indicated by the results for *PIL1*, the contribution of each of these PIFs to full induction appears to vary between growth conditions: whereas PIF3 is the strongest contributor under SD conditions (Figure 4b), PIF5 dominates in shade (Lorrain *et al.*, 2008; Leivar *et al.*, 2012b). These results suggest different target affinity and/or different relative levels of each PIF depending on the growth conditions.

The results presented here indicate that phyA and phyB are redundant in the rapid phytochrome-mediated degradation of PIF3 within 1 h after transition from darkness to light under SD conditions (Figure 1d), mirroring the phytochrome-mediated degradation of PIF3 during early stages of illumination of etiolated seedlings (Bauer *et al.*, 2004; Al-Sady *et al.*, 2006). These data suggest that PIF3 degradation under SD conditions may also require direct interaction with the phytochrome photoreceptor, leading to rapid phosphorylation of the transcription factor and degradation via the ubiquitin–proteasome system, as described for etiolated seedlings (Bauer *et al.*, 2004; Al-Sady *et al.*, 2006). In addition, our results show that the absence of phyB in *phyB* and *phyA phyB* mutants results in over-accumulation of PIF3 during the dark period, and that these elevated levels are reduced to a certain extent in response to prolonged light conditions (after 4 h), indicating that another photoreceptor is also involved in regulation of PIF3 degradation during the day. These results again mirror those observed in de-etiolation experiments, suggesting that this additional photoreceptor may be phyD (Bauer *et al.*, 2004; Al-Sady *et al.*, 2006). However, adding to previous data for dark-grown seedlings exposed to light (Bauer *et al.*, 2004; Al-Sady *et al.*, 2006), our evidence that the pool of PIF3 protein is not degraded in the absence of phyB indicates that phyB is necessary to mediate complete degradation of PIF3 during the light period under diurnal conditions (Figure 1e). This result provides evidence that phyB regulates degradation of PIF3 under SD conditions during the last part of the day. In addition, the observed re-accumulation of PIF3 in the absence of phyB during the first part of the night (Figure 1e) provides evidence that phyB also targets PIF3 for degradation at the start of the dark period. The extent of phyB action during the night is presumably determined by its dark reversion rate, which has been estimated to have a half-life of 1 h (Sweere *et al.*, 2001; Rausenberger *et al.*, 2010), as well as potentially via selective degradation of the Pfr form.

Our observation that phytochrome regulation keeps PIF3 protein levels low during the day and the first part of the night, with subsequent progressive accumulation, provides evidence for a phytochrome-mediated mechanism of PIF3 oscillation under SD conditions. Although phytochrome-imposed regulation of PIF3 protein accumulation may be

sufficient to ensure timing of action of PIF3 at the end of the night, without additional transcriptional regulation by the circadian clock, a scenario in which the clock post-translationally regulates or fine-tunes PIF3 accumulation and/or activity indirectly cannot be completely discounted. DELLA proteins have been shown to interfere with PIF3 and PIF4 binding to DNA (de Lucas *et al.*, 2008; Feng *et al.*, 2008), and a recent report showed that DELLA proteins accumulate at the start of the night in seedlings grown under diurnal conditions (Arana *et al.*, 2011). Therefore, DELLA proteins could represent a mechanism to prevent PIF3 from binding and inducing its target genes when its levels start to increase during the first part of the night. Further investigation is required to address this possibility.

Taken together, the data presented here indicate that PIF3 has a prominent role as a promoter of hypocotyl elongation under SD conditions, at least in part by directly regulating the expression of growth-related genes. Our work also reveals that phyA, phyB and possibly phyD induce degradation of PIF3 during the dark-to-light transition and the light period of diurnally grown seedlings, and residual photoactivated phyB prevents re-accumulation of PIF3 during the first part of the night. Our findings imply that PIFs regulating growth under diurnal conditions do not necessarily have to be transcriptionally regulated by the clock as previously shown for PIF4 and PIF5, and that phytochrome-mediated regulation may be sufficient. However, the existence of other more indirect layers of regulation of PIF3 by the clock and/or factors such as DELLA proteins (or other unknown mechanisms) cannot be excluded, and these may fine-tune the timing of PIF3 action under SD conditions.

EXPERIMENTAL PROCEDURES

Seedling growth and hypocotyl measurements

Wild-type and mutant *Arabidopsis thaliana* seeds used in these studies were all in the Columbia (Col-0) ecotype and have been described elsewhere, including *pif3-3* (Monte *et al.*, 2004), *pif4-2* and *pif3 pif4* (Leivar *et al.*, 2008a), *pif5-3* (Khanna *et al.*, 2007), *pif3 pif5*, *pif4 pif5* and *pif3 pif4 pif5 phyB* (Leivar *et al.*, 2012a), *pif3 pif4 pif5* (Leivar *et al.*, 2008b), *phyB-9* (Reed *et al.*, 1993), *pif3 phyB* (Al-Sady *et al.*, 2008), *phyA-211* (Nagatani *et al.*, 1993), *phyA phyB* (Cerdan and Chory, 2003) and pif3::YFP-PIF3 (Al-Sady *et al.*, 2006).

Seeds were sterilized and plated on germination medium (GM) (Valvekens et al., 1988) without sucrose as previously described (Monte *et al.*, 2003). Seedlings were then stratified for 4 days at 4°C in darkness, and then placed under short-day (SD) conditions [8 h white light (85 μmol m^{-2} sec^{-1}) + 16 h dark] for the time indicated in each experiment. For hypocotyl measurements, seedlings were arranged horizontally on a plate, photographed using a digital camera (Nikon D80, http://www.nikon.com/) and measured as described previously (Monte *et al.*, 2003). At least 30 seedlings for each line were measured to calculate the mean and standard error. For time-lapse photography, seedlings were grown on vertical plates, and, after 2 days of growth, photographs were taken at 30 min intervals for 24 h. To acquire images in the dark, 5 sec illumination was provided by an infrared light-emitting diode, and photographs were taken using an infrared-sensitive digital camera (Nikon D80). Hypocotyls of seven Col-0 and *pif3* seedlings were measured, and the growth rate was calculated for each individual seedling.

Protein extraction and immunoblots

Protein extracts were prepared from 2- and 3-day-old seedlings grown under SD conditions as indicated. Tissue samples were collected and frozen in liquid nitrogen, and samples were manually ground under frozen conditions before resuspension in extraction buffer. The extraction buffer used and protein quantification were as previously described (Leivar *et al.*, 2008a). Total protein extracts were subjected to SDS–PAGE (7.5%) for immunodetection of phyB and YFP–PIF3 protein (80 μg) or endogenous PIF3 (200 μg). Proteins were transferred to Hybond C membrane (Amersham Biosciences), and the membrane was stained with Ponceau S as a loading control. Immunodetection of PIF3 and YFP–PIF3 was performed using a rabbit anti-PIF3 polyclonal antibody (Al-Sady *et al.*, 2006), incubated overnight with Hikari solution (Nacalai Tesque), and immunodetection of phyB was performed using mouse monoclonal anti-phyB (B1 and B7) antibodies (Somers *et al.*, 1991). Peroxidase-linked anti-rabbit secondary antibody (Amersham Biosciences) for PIF3 and anti-mouse secondary antibody for phyB (Amersham Biosciences) and a SuperSignal West Femto chemiluminescence kit (Pierce) were used for detection of luminescence using a LAS-4000 Image imaging system (Fujifilm).

Gene expression analysis

For RNA blots, total RNA was extracted from 4-day-old SD-grown seedlings as described by Monte *et al.* (2003) (see Table S1 for primer sequences used to amplify the *PIL1* probe). Hybridization signal was quantified using a Storm 860 PhosphorImager (Molecular Dynamics) and normalized to 25S rRNA levels.

For quantitative RT-PCR analysis, RNA extraction, cDNA synthesis and quantitative RT-PCR were performed as described previously (Sentandreu *et al.*, 2011). Gene expression was measured in three technical replicates for each biological sample. *PP2A* (*AT1G13320*) was used as a normalization control as described previously (Shin *et al.*, 2007). Table S1 lists primer sequences.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using 3-day-old SD-grown pif3::YFP-PIF3 and Col-0 seedlings as described previously (Gendrel *et al.*, 2002). After sonication, protein was quantified, and the inputs used in the subsequent immunoprecipitation step were equivalent for all samples. Antibody samples were immunoprecipitated by overnight incubation with GFP antibody-bound resin (GFP Agarose Beads, MBL). Mock ChIP reactions were performed without antibody to measure non-specific binding to target sequences. After immunoprecipitation, purified DNA was subjected to quantitative RT-PCR using promoter- and control-specific primers (Table S1) for each gene of interest. Quantitative RT-PCR results in the presence or absence of antibody for each genotype were first normalized to their input, and fold enrichment was then calculated for each antibody-containing sample relative to the corresponding sample lacking antibody.

Statistics

Data were analyzed by one-way ANOVA, and the differences between means were evaluated using Duncan's *post-hoc* multiple comparison test (SPSS statistics software, IBM). Statistically significant differences were defined as those with a P value < 0.05.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Accumulation of PIF3 in SD-grown seedlings.

Figure S2. Hypocotyl phenotype of *phyA*, *phyB* and *phyA phyB* seedlings under SD conditions.

Figure S3. Relative position of ChIP primers.

Figure S4. Additional biological replicate for the ChIP experiment shown in Figure 3(b).

Table S1. List of primer sequences.

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Supplemental Figures

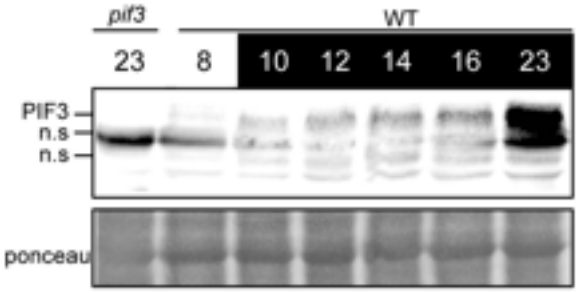

Figure S1

Figure S1. Accumulation of PIF3 in short-day (SD) grown seedlings

Immunoblot of protein extracts from wild-type Col-0 seedlings. Seedlings were grown in SD for 2 days and samples were taken during the third day at the specified time points. A PIF3-specific polyclonal antibody was used as probe (top). As antibody specificity control, a protein extract from *pif3-3* harvested at time 21 h is included. Ponceau staining was used as a loading control (bottom). n.s., nonspecific, cross-reacting bands.

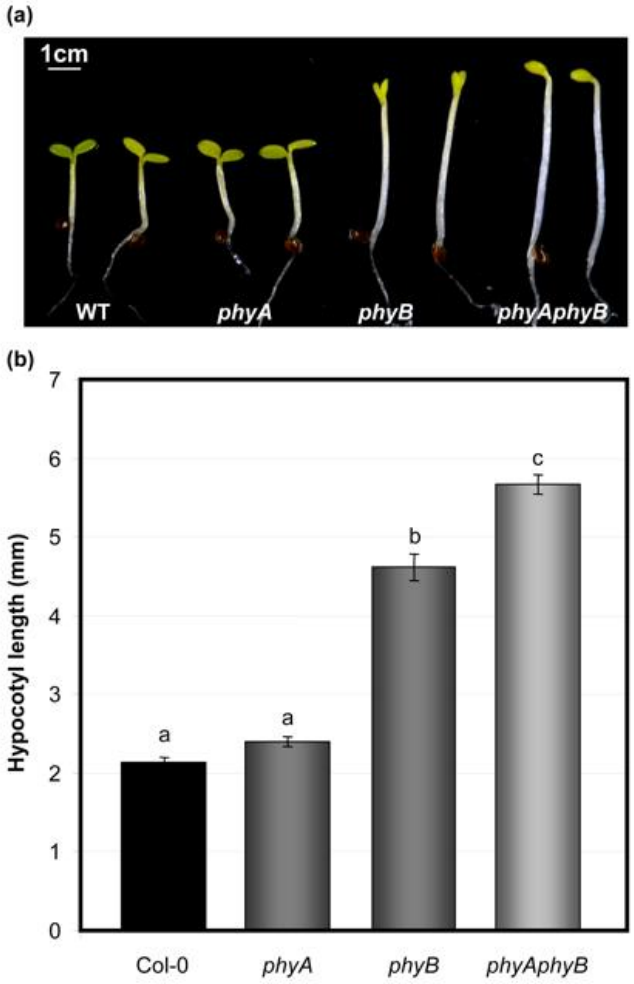

Figure S2. Hypocotyl phenotype of *phyA*, *phyB,* and *phyAphyB* seedlings in SD conditions

(A) Visual phenotype of 3-day-old SD-grown WT, *phyA, phyB and phyAphyB* mutant seedlings.

(B) Hypocotyl length in 3-day-old SD-grown WT, *phyA, phyB and phyAphyB* seedlings. Data are means and s.e. of at least 30 seedlings. Different letters denote significant differences among means (P <0.05).

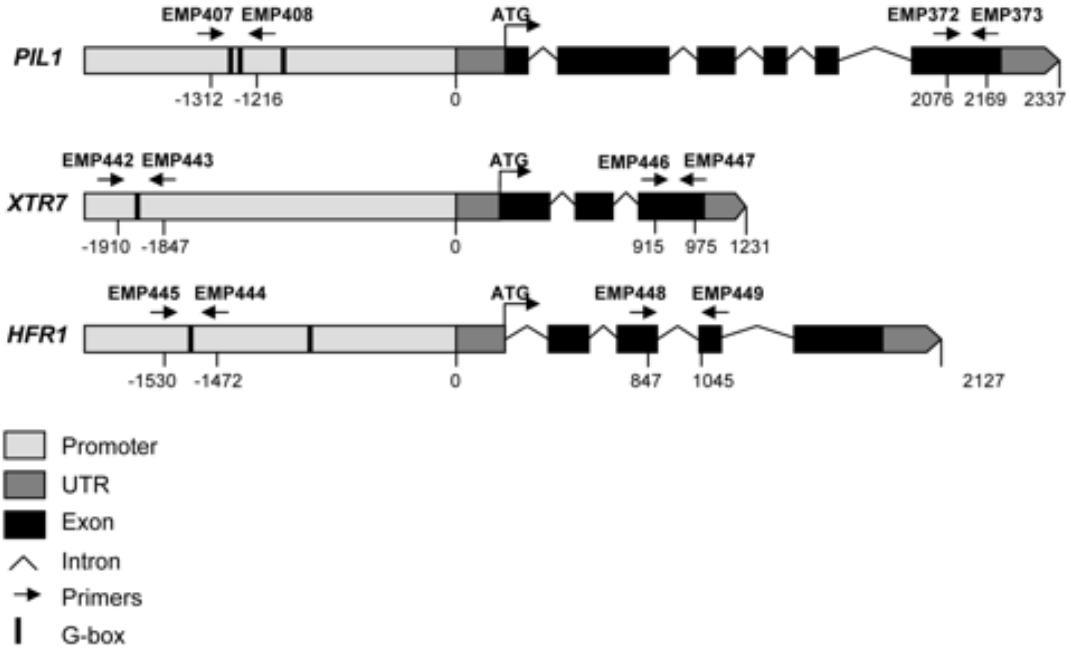

Figure S3. Relative position of ChIP primers

Relative position of the primers used to amplify promoter and control regions in the ChIP experiments. G-boxes are indicated with vertical black lines.

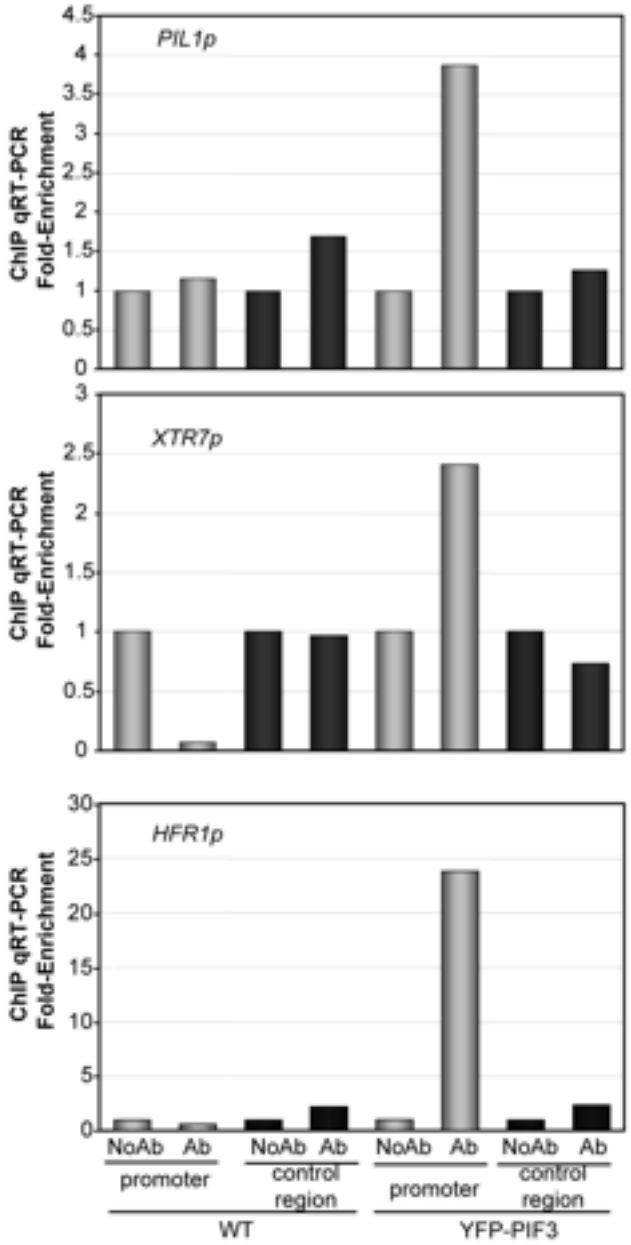

Figure S4. Additional biological replicate for the ChIP experiment shown in Figure 3b.

Chromatin immunoprecipitation (ChIP) from 3 day-old SD-grown wild-type and YFP-PIF3 seedlings. Immunoprecipitated DNA was quantified by qRT-PCR using primers in the promoter region containing G-boxes (promoter) or control regions without G-boxes (control region). Experiments include samples processed with anti-GFP antibody (Ab) and controls processed without antibody (NoAb). Data are average of at least two technical replicates.

Reference					Sequence (from 5' to 3')
Use	**Gene**	**AGI number**	**Code**	**Original Code (*)**	
RNA blots	*PIL1*	*AT2G46970*	EMP3		GATGAAGATTATATGGAGCTGGTG
			EMP4		CGAAGTTCCTCGAGAAAACTTCG
qRT-PCR	*PIL1*	*AT2G46970* Promoter	EMP407		ACAAGAAAGAAGGGAGGGAGACA
			EMP408		TTCTCTTTAAATGGGACCCACAAT
		Coding region	EMP372		TGCCTTCGTGTGTTTCTCAG
			EMP373		AACTAAAACCGTTGCTTCCTC
	XTR7	*AT4G14130* Promoter	EMP442	pPH120	CGCATGCCGGCTGGAATAGATAG
			EMP443	pPH121	CGACGTGTCACTTCCCTCGTACC
		Coding region	EMP446	pPH130	CGGCTTGCACAGCCTCTT
			EMP447	pPH131	TCGGTTGCCACTTGCAATT
	HFR1	*AT1G02340* Promoter	EMP444	pPH112	ACGTGATGCCCTCGTGATGGAC
			EMP445	pPH113	GTCGCTCGCTAAGACACCAAC
		Coding region	EMP448	pPH126	GATGCGTAAGCTACAGCAACTCGT
			EMP449	pPH127	AGAACCGAAACCTTGTCCGTCTTG
	PIF3	*AT1G09530*	EMP417		GGT ATG GGA ATG CCT TAT GCA
			EMP418		TGG AAC TGT GGT CCG TGG TTA
	PIF4	*AT2G43010*	EMP419		GCG GCT TCG GCT CCG ATG AT
			EMP420		AGT CGC GGC CTG CAT GTG TG
	PIF5	*AT3G59060*	EMP421		TCG GAG CAG CTC GCT AGG TA
			EMP422		TTG TTG CAC GGT CTG CAT CT
	PP2A	*AT1G13320*	EMP338		TATCGGATGACGATTCTTCGTGCAG
			EMP339		GCTTGGTCGACTATCGGAATGAGAG

(*) **Hornitschek, P., Lorrain, S., Zoete, V., Michielin, O., Fankhauser, C**. (2009*)*. Inhibition of the shade avoidance response by formation of non-DNA binding bHLH heterodimers. *EMBO J***. 28**:3893-3902.

Table S1. List of primer sequences

Publication 2.

PIF1 promotes phytochrome-regulated growth under photoperiodic conditions in Arabidopsis together with PIF3, PIF4 and PIF5

Judit Soy, Pablo Leivar and Elena Monte.

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RESEARCH PAPER

PIF1 promotes phytochrome-regulated growth under photoperiodic conditions in *Arabidopsis* together with PIF3, PIF4, and PIF5

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Abstract

Seedlings growing under diurnal conditions display maximal growth at the end of the night in short-day (SD) photoperiods. Current evidence indicates that this behaviour involves the action of PHYTOCHROME-INTERACTING FACTOR 3 (PIF3) together with PIF4 and PIF5, through direct regulation of growth-related genes at dawn coinciding with a PIF3 accumulation peak generated by phytochrome-imposed oscillations in protein abundance. Here, to assess how alterations in PIF3 levels impact seedling growth, the night-specific accumulation of PIF3 was modulated by releasing SD-grown seedlings into continuous light, or by exposing them to a phytochrome-inactivating end-of-day far-red pulse (EOD-FRp). The data show a strong direct correlation between PIF3 accumulation, PIF3-regulated induction of growth-related genes, and hypocotyl elongation, and suggest that growth promotion in SD conditions involves factors other than PIF3, PIF4, and PIF5. Using a *pif1* mutant, evidence is provided that PIF1 also contributes to inducing hypocotyl elongation during the dark period under diurnal conditions. PIF1 displayed constitutive transcript levels in SD conditions, suggesting that phytochrome-imposed oscillations in PIF1 protein abundance determine its accumulation and action during the night, similar to PIF3 and in contrast to PIF4 and PIF5, which oscillate diurnally due to a combination of circadian clock-regulated transcription and light control of protein accumulation. Furthermore, using single and higher order *pif* mutants, the relative contribution of each member of the PIF quartet to the regulation of morphogenesis and the expression of selected growth marker genes under SD conditions, or under SD conditions supplemented with an EOD-FRp, is defined. Collectively, the data indicate that PIF1, PIF3, PIF4, and PIF5 act together to promote and optimize growth under photoperiodic conditions.

Key words: *Arabidopsis*, EOD-FRp, growth-related gene expression, hypocotyl elongation, PIF1, short day.

Introduction

Light is a fundamental environmental cue for plants, and photoreceptors of the phytochrome family (phyA–phyE in *Arabidopsis*) perceive the red (R) (660 nm) and far red (FR) (720 nm) light of the solar spectrum (Schafer and Nagy, 2006). Phytochromes are synthesized in the cytoplasm in the inactive R-absorbing Pr form, and upon R absortion reversibly convert to the active FR-absorbing Pfr form that is rapidly translocated to the nucleus (Nagatani, 2004). Pr to Pfr photoactivation occurs within seconds after absorption of R photons (Linschitz and Kasche, 1966), whereas Pfr to Pr inactivation takes place in FR-enriched environments (Franklin, 2008) and also in the dark. Pfr to Pr reversion in light-grown seedlings transferred to darkness is slow, with a Pfr half-life of ~60 min (Rausenberger *et al.*, 2010). Owing to

these properties, phytochromes are able to monitor changes in light quality, quantity, and duration to mediate the adaptation of plant growth and development to changes in ambient light conditions, and regulate processes such as germination, de-etiolation, shade avoidance, or diurnal growth (Franklin and Quail, 2010; Casal, 2013).

The central role of the phytochromes (predominantly phyA and phyB) in growth and development is achieved in large part by regulating the abundance of members of the phytochrome-interacting factor (PIF) family of basic helix–loop–helix (bHLH) transcription factors (Bae and Choi, 2008; Leivar and Quail, 2011). The PIFs (PIF1, PIF3, PIF4, PIF5, PIF6, and PIF7 in *Arabidopsis*) accumulate in the nucleus in the dark, and, upon light exposure, associate photoreversibly and specifically with the Pfr form of the phytochromes. This interaction initiates a cascade of transcriptional changes that allows the implementation of the necessary morphological changes to adapt to the new light environment (Castillon *et al.*, 2007; Jiao *et al.* 2007). For some of the PIF members (PIF1, PIF3, PIF4, and PIF5), light-induced interaction with the Pfr phytochrome triggers their rapid phosphorylation, which in turn induces their ubiquitylation and proteolytic degradation via the proteasome system within minutes (Bauer *et al.*, 2004; Park *et al.*, 2004; Shen *et al.*, 2005; Al-Sady *et al.*, 2006; Oh *et al.*, 2006; Nozue *et al.*, 2007; Shen *et al.*, 2007; Lorrain *et al.*, 2008; Shen *et al.*, 2008), establishing a new lower steady-state level in continuous light of ~10% the amount in the dark for PIF3 (Monte *et al.*, 2004). Exposure to light also induces rapid concomitant phyA degradation (half-life of <2 h) and a slower and more modest degradation of phyB, which remains relatively abundant and stable in the light (Sharrock and Clack, 2002; Khanna *et al.*, 2007; Al-Sady *et al.*, 2008). phyB degradation has recently been shown to require PIF3 phosphorylation, which establishes a mutually negative feedback loop between phyB and PIF3 potentially through co-degradation of both proteins (Leivar *et al.*, 2012a; Ni *et al.*, 2013). The distinct light stability properties of phyA and phyB underlie their differential roles in the regulation of PIF abundance: whereas phyA and phyB function mostly redundantly in dark to light transitions, phyB is more important in continuous light and during the first dark hours in light to dark transitions (Al-Sady *et al.*, 2006; Monte *et al.*, 2007; Shen *et al.*, 2008; Leivar *et al.*, 2012a; Soy *et al.*, 2012). Under diurnal conditions where light and dark periods alternate, the progressive decline in phyB Pfr during the night period due to dark reversion allows for the progressive re-accumulation of the PIFs in light-grown seedlings upon exposure to darkness (Monte *et al.*, 2004; Shen *et al.*, 2005; Nozue *et al.*, 2007; Soy *et al.*, 2012). Exposure to FR light-enriched environments, such as vegetational shade, low R/FR ratios, or an end-of-day FR pulse (EOD-FRp), also triggers re-accumulation of the PIFs due to phyB Pfr inactivation (Lorrain *et al.*, 2008; Leivar *et al.*, 2012a, *b*; Casal, 2013).

Hypocotyl elongation is maximal in seedlings grown in continuous darkness. Under diurnal conditions with alternating light/dark cycles, the extent of hypocotyl elongation in *Arabidopsis* seedlings depends on the duration of the dark period in a non-linear fashion (Niwa *et al.*, 2009). In short-day (SD) photoperiods, seedlings display rhythmic growth, with maximal elongation rates at the end of the night (Nozue *et al.*, 2007). Elongation in SD is largely due to the combined actions of PIF3, PIF4, and PIF5, which promote growth specifically at the end of the night (Nozue *et al.*, 2007; Niwa *et al.*, 2009; Soy *et al.*, 2012). Precise regulation of their accumulation and time of action under diurnal conditions has been proposed to involve at least two different mechanisms. For PIF4 and PIF5, a coincidence mechanism has been described that combines regulation of *PIF4* and *PIF5* transcript levels by the circadian clock, superimposed on the control of their protein accumulation by light (Nozue *et al.*, 2007; Nusinow *et al.*, 2011; Yamashino *et al.*, 2013). For PIF3, transcript levels are relatively constant, and oscillations of PIF3 protein abundance are imposed by the action of the phytochromes (Soy *et al.*, 2012). The effects of PIF3, PIF4, and PIF5 on diurnal hypocotyl elongation involve the direct regulation of the growth-related genes *PIL1* (*PHYTOCHROME-INTERACTING FACTOR-3 LIKE 1*), *HFR1* (*LONG HYPOCOTYL IN FAR-RED 1*), and *XTR7* (*XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7*) (Soy *et al.*, 2012), which are up-regulated in conditions where hypocotyl elongation is induced (Salter *et al.*, 2003; Lorrain *et al.*, 2008; Hornitschek *et al.*, 2009; Leivar *et al.*, 2009; Nozue *et al.*, 2011), and the regulation of auxin-related genes that oscillate in phase with hypocotyl growth (Michael *et al.*, 2008; Nozue *et al.*, 2011).

The role of PIF3 as a positive regulator of growth under diurnal conditions has been defined previously, and it has been described how phytochrome-imposed oscillations ensure that PIF3 protein progressively accumulates during the dark period to peak just before dawn, at which time it accelerates growth together with PIF4 and PIF5 (Soy *et al.*, 2012). Despite these advances, a complete understanding of how phytochrome-mediated regulation of PIF abundance under diurnal conditions impacts the expression of growth-related genes and hypocotyl elongation, and whether factors other than PIF3, PIF4, and PIF5 might be involved is still lacking. To address these questions, and based on the current model, here PIF3 protein accumulation has been altered specifically during the night hours in SD conditions by treating seedlings with an EOD-FRp, or by substituting the dark period by a continuous white light treatment. These treatments have allowed PIF3 abundance to be correlated with gene expression and growth, and a new role for PIF1 as a contributing factor to the phytochrome-mediated regulation of growth under diurnal conditions has been unveiled.

Materials and methods

Seedling growth and hypocotyl measurements

Wild-type and mutant lines used in these studies were in *Arabidopsis thaliana* Columbia ecotype and described elsewhere, and included *pif1-1* (Huq *et al.*, 2004), *pif3-3* (Monte *et al.*, 2004), *pif4-2* (Leivar *et al.*, 2008a), *pif5-3* (Khanna *et al.*, 2007), *pif1pif3*, *pif3pif4pif5*, and *pif1pif3pif4pif5* (Leivar *et al.*, 2008b), *pif4pif5* and *pif1pif4pif5* (Leivar *et al.*, 2012b), *pif3pif4pif5phyB* (Soy *et al.*, 2012), and *phyB-9* (Reed *et al.*, 1993).

Seeds were sterilized and plated on GM medium without sucrose as previously described (Monte *et al.*, 2003). Seedlings were stratified for 4 d at 4 °C in darkness, and then placed in SD conditions [8 h white light (85 µmol m^{-2} s^{-1}) + 16 h dark] for 2 d at 21 °C. During the third day of growth, seedlings were either kept in SD conditions, transferred to continuous white light conditions (WL), or exposed to a pulse of FR (30 µmol m^{-2} s^{-1}) (FRp) for 15 min before the dark period.

For hypocotyl measurements, seedlings were arranged horizontally on a plate, photographed using a digital camera (Nikon D80), and measured using NIH Image software (ImageJ, National Institutes of Health). At least 30 seedlings for each line were measured to calculate the mean and standard error (SE).

Protein extraction and immunoblots

Protein extraction and immunoblots were done as described before in Soy *et al.* (2012). Immunodetection of PIF3 was performed using a rabbit anti-PIF3 polyclonal antibody (Al-Sady *et al.*, 2006), incubated overnight with Hikari solution (Nacalai tesque). Peroxidase-linked anti-rabbit (Amersham) secondary antibody and a SuperSignal West Femto chemiluminescence kit (Pierce) were used for detection using a Las4000 Image (Fujifilm).

Gene expression analysis

For quantitative reverse transcription–PCR (RT–PCR) analysis, RNA extraction, cDNA synthesis, and qRT–PCR were done as described (Sentandreu *et al.*, 2011). Gene expression data in Figs 1, 2, 3, 4E, and 5 represent the mean of three biological replicates (each one measured in three technical replicates), and bars represent the SE. Gene expression data in detailed kinetics in Fig. 4D represent the mean of three technical replicates of one representative biological replicate. *PP2A* (*AT1G13320*) was used as a normalization control as described (Shin *et al.*, 2007). Gene expression analysis of *PIF3*, *PIF4*, *PIF5*, *XTR7*, and *PIL1* was done using the primers described in Soy *et al.* (2012). For *PIF1*, 5′-ATCCAACCTCGGGCCAGCCT-3′ and 5′-TTGGGTCGGGTGGAGACCGC-3′ were used as forward and reverse primers, respectively.

Statistics

Gene expression and hypocotyl length data shown in Figs 1D, 2D, 3 A–D, 4E, 5B, C, and E were analysed by one-way analysis of variance (ANOVA) and the differences between means were evaluated by using Tukey-b post-hoc multiple comparison test (IBM SPSS Statistics Software). Data shown in Fig. 4B and C were submitted to a Student's *t*-test analysis, as well as comparison between *pif3* and *pif3pif4pif5* ZT24D samples in Fig. 1D, and *XTR7* expression between ZT8 and ZT24D in Fig. 3B to complement the analysis by Tukey-b. In all cases, statistically significant differences were defined as those with a *P*-value <0.05.

Results

Exposure of SD-grown seedlings to constant light prevents PIF3 accumulation and leads to growth arrest

It has previously been described that phytochrome-imposed oscillations in PIF3 protein abundance regulate hypocotyl growth under SD conditions (Soy *et al.*, 2012). Under this photoperiodic growth regime, PIF3 levels stay low during the day and progressively accumulate during the dark hours to peak at the end of the night, coinciding with the maximum growth rate.

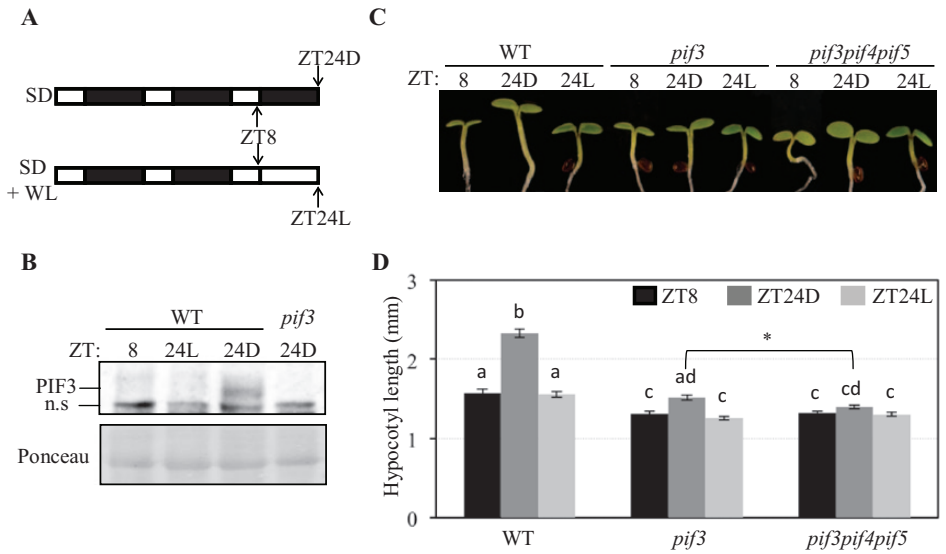

Fig. 1. Exposure to WL prevents PIF3 accumulation and growth under SD conditions. (A) Diagram of the growth regime used for B–D. Seedlings were grown under SD conditions for 2 d and 8 h, at which time (ZT8) they either were kept under SD and experienced a 16 h night (ZT24D), or were moved to WL for the subsequent 16 h (ZT24L). (B) Immunoblot of protein extracts from WT seedlings at the specified time points. A PIF3-specific polyclonal antibody was used as probe. As an antibody specificity control, a protein extract from *pif3-3* harvested at ZT24D was included. Ponceau staining was used as a loading control (bottom). n.s., non-specific cross-reacting bands. (C) Visual phenotype of WT, *pif3*, and *pif3pif4pif5* seedlings grown as detailed in A. (D) Hypocotyl length in WT, *pif3*, and *pif3pif4pif5* seedlings grown as detailed in A. Data represent the mean and SE of at least 20 seedlings. Different letters denote statistically significant differences among means defined by Tukey-b's multiple comparison test ($P < 0.05$). Comparison between *pif3* and *pif3pif4pif5* genotypes in short-day conditions (ZT24D) fell short of statistical significance under the stringent Tukey-b statistical test but showed a statistically significant difference ($P < 0.05$) by Student's *t*-test (indicated with an asterisk). (This figure is available in colour at *JXB* online.)

Based on these results, it was hypothesized that alterations in PIF3 protein accumulation during the night period would have an impact on growth under SD conditions. To test this possibility, wild-type (WT) seedlings were first grown under SD conditions for 2 d, and then transferred to WL at the end of the third day (ZT8) for 16h (Fig. 1A). Controls were kept under SD conditions and experienced a subsequent 16h long night (Fig. 1A). The accumulation of endogenous PIF3 under these two conditions was then examined. PIF3 was below the detection level at ZT8, but was clearly detectable after 16h of darkness (ZT24D) (Fig. 1B) in accordance with previously reported data (Soy *et al.*, 2012). In contrast, PIF3 levels in seedlings transferred to WL remained below the detection level (ZT24L) (Fig. 1B). These results suggest that, in SD-grown seedlings, the night period is necessary to allow for accumulation of PIF3.

Next seedling growth in these conditions was monitored by comparing the hypocotyl length of SD-grown seedlings at ZT8 with the length of seedlings that were subsequently exposed to 16h darkness (ZT24D) or WL (ZT24L). As shown in Fig. 1C and D, 2-day-old SD-grown WT seedlings experienced significant hypocotyl elongation during exposure to the third night (between ZT8 and ZT24D), in accordance with previous reports (Nozue *et al.*, 2007; Soy *et al.*, 2012). In contrast, WT seedlings kept under WL did not exhibit any significant hypocotyl growth during the same 16h period (compare ZT8 with ZT24L). PIF3-deficient seedlings were shorter at ZT8 compared with the WT, and growth activity in the dark between ZT8 and ZT24D was also significantly reduced compared with the WT (Fig. 1C, D), in accordance with previous data (Soy *et al.*, 2012). Growth activity was also below detection when *pif3* seedlings were transferred to WL (compare ZT8 with ZT24L) (Fig. 1C, D). Together, these results support the notion that, under SD conditions, PIF3 accumulation during the night is necessary to induce growth, and substitution of the dark period by WL prevents PIF3 accumulation and leads to growth arrest.

PIF4 and PIF5 are positive regulators of growth under SD conditions together with PIF3 (Nozue *et al.*, 2007; Niwa *et al.*, 2009; Soy *et al.*, 2012). In accordance with this, *pif3pif4pif5* seedlings were slightly shorter at ZT24D compared with *pif3* (Fig 1D, and also see below Figs 2D and 5B), whereas exposure of *pif3pif4pif5* seedlings to 16h of WL instead of darkness did not lead to detectable growth, as observed for *pif3* (compare ZT24L with ZT8) (Fig. 1C, D). Together with previous data showing that accumulation of PIF4 and PIF5 under SD conditions occurs in the dark (Nozue *et al.*, 2007; Yamashino *et al.*, 2013), these data suggest that, under SD conditions, PIF4 and PIF5 accumulation during the night is also necessary to induce growth.

Inactivation of phytochrome activity by an EOD-FRp increases PIF3 accumulation and leads to enhanced growth under SD conditions

To examine further how alterations in PIF3 protein accumulation during the night period have an impact on growth under SD conditions, and based on previous results showing that active Pfr phyB operates during the first hours of the night to prevent accumulation of PIF3 (Monte *et al.*, 2004;

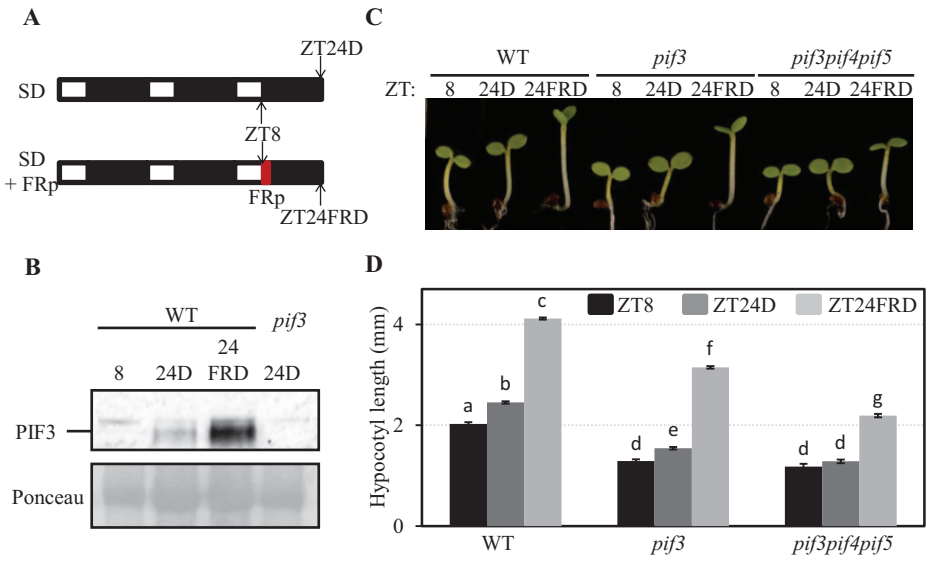

Fig. 2. Inactivation of phytochrome activity by an EOD-FRp increases PIF3 accumulation and hypocotyl growth under SD conditions. (A) Diagram of the growth regime used for B–D. Seedlings were grown under SD conditions for 2 d and 8h, at which time (ZT8) they either were kept under SD and experienced a 16h night (ZT24D), or were given an EOD-FRp before the night period (ZT24FRD). (B) Immunoblot of protein extracts from WT seedlings at the specified time points. A PIF3-specific polyclonal antibody was used as probe. As an antibody specificity control, a protein extract from *pif3-3* harvested at ZT24D was included. Ponceau staining was used as a loading control (bottom). n.s., non-specific cross-reacting bands. (C) Visual phenotype of WT, *pif3*, and *pif3pif4pif5* seedlings grown as detailed in A. (D) Hypocotyl length in WT, *pif3*, and *pif3pif4pif5* seedlings grown as detailed in A. Data represent the mean and SE of at least 20 seedlings. Different letters denote significant differences among means ($P < 0.05$). (This figure is available in colour at *JXB* online.)

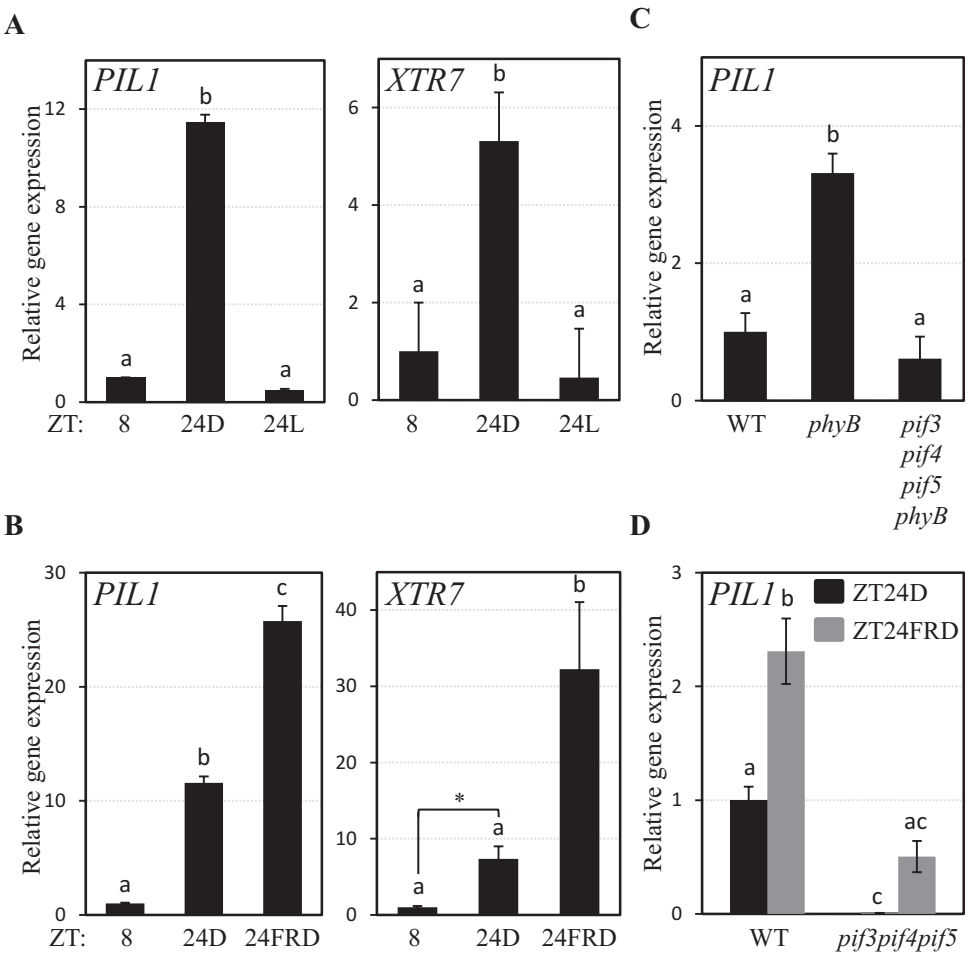

Fig. 3. Correlation of growth marker gene expression with hypocotyl growth and levels of PIF3 under SD conditions. (A) Expression of *PIL1* and *XTR7* in seedlings grown as detailed in Fig. 1A. (B) Expression of *PIL1* and *XTR7* in samples grown as detailed in Fig. 2A. (C) Expression of *PIL1* in 3-day-old SD-grown WT, *phyB*, and *pif3pif4pif5phyB*. (D) Expression of *PIL1* in seedlings grown as detailed in Fig. 2A. The numerical value for *pif3pif4pif5* at ZT24D is <0.01. Expression of *PIL1* and *XTR7* was analysed by quantitative RT–PCR, and values were normalized to *PP2A*. Different letters denote significant differences among means ($P < 0.05$). Comparison between *XTR7* expression at ZT8 and ZT24D in B fell short of statistical significance under the stringent Tukey-b statistical test but showed a statistically significant difference ($P < 0.05$) by Student's *t*-test (indicated with an asterisk).

Soy *et al.*, 2012), seedlings were next treated with a saturating 15 min FRp at the end of the third day (ZT8 time point) (Fig. 2A). It was expected that this EOD-FRp would lead to an increase in PIF3 levels during the night by rapidly inactivating phyB. Indeed, compared with control seedlings under SD conditions (ZT24D) (Fig. 2A), it was observed that PIF3 levels were increased by at least 2-fold in seedlings exposed to SD conditions supplemented by an EOD-FRp (ZT24FRD) (Fig. 2B).

Next the effect of the EOD-FRp on growth was examined by comparing the hypocotyl length at ZT8 with that at ZT24FRD. Control seedlings (ZT24D) were exposed to 16 h of dark after ZT8. As shown in Fig. 2C and D, WT seedlings exposed to a 15 min EOD-FRp and then kept in the dark for 16 h exhibited an increase in hypocotyl length (from 2 mm to 4 mm) (compare ZT24FRD with ZT8) that was much more pronounced than the elongation observed during the same time period in WT control seedlings kept under SD conditions without an EOD-FRp (from 2 mm to 2.4 mm) (compare ZT24D with ZT8). Together, these data show a strong correlation between the increase in PIF levels during the night hours and the increase in hypocotyl growth during the same long-night period, and suggest that, under SD conditions, inactivation of phyB by an EOD-FRp leads to an increase in PIF3 accumulation during the night (and possibly other PIFs such as PIF4 and PIF5) that accelerates growth.

To determine whether and to what extent PIF3 and/or other PIF factors mediate this accelerated growth in response to an EOD-FRp, the effect of an EOD-FRp was examined in *pif3* and *pif3pif4pif5* mutants. Figure 2C and D shows that *pif3* mutants exhibited a robust growth response to EOD-FRp that was only slightly reduced in magnitude compared with that displayed by the WT (from 1.3 mm to 3.1 mm) (compare ZT24FRD with ZT8). In contrast, *pif3pif4pif5* seedlings had a significantly reduced hypocotyl response to the EOD-FRp compared with the WT or *pif3* (from 1.2 mm to 2.2 mm) (Fig. 2C, D). Together, these results suggest that an EOD-FRp triggers an increase in PIF3 protein accumulation during

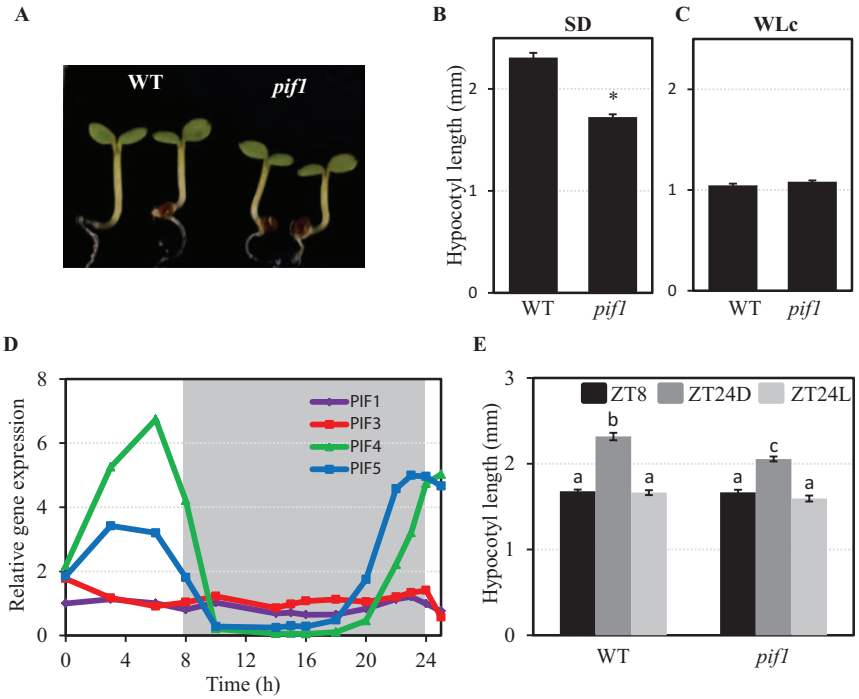

Fig. 4. PIF1 promotes growth under SD conditions. (A) Visual phenotype of 3-day-old SD-grown WT and *pif1* seedlings. (B) Hypocotyl length in 3-day-old SD-grown WT and *pif1* seedlings. The asterisk indicates statistically significant differences (*P*-value <0.05). (C) Hypocotyl length in 3-day-old WT and *pif1* seedlings grown under continuous WL (WLc). (D) Expression of *PIF1*, *PIF3*, *PIF4*, and *PIF5* was analysed by quantitative RT–PCR. Values were normalized to *PP2A*, and expression levels relative to *PIF1* at 0 h are shown. Data represent the mean of technical replicates. (E) Hypocotyl length of 2-day-old SD-grown WT and *pif1* grown as detailed in Fig. 1A. Data represent the mean and SE of at least 20 seedlings. Different letters denote significant differences among means ($P < 0.05$).

the night (as shown in Fig. 2B) and probably also in PIF4 and/or PIF5, and support the notion that the PIFs function redundantly to mediate phytochrome-regulated growth under SD conditions. Interestingly, triple *pif3pif4pif5* mutants still exhibited a significant elongation in response to EOD-FRp (Fig. 2C, D), indicating that at least one additional factor participates in the phytochrome-regulated growth response under these conditions.

Expression of growth marker genes correlates with hypocotyl growth and with levels of PIF3 under SD conditions

Previously it was shown that the phytochrome-regulated growth-marker genes *PIL1* and *XTR7* (Salter *et al.*, 2003; Lorrain *et al.*, 2008; Leivar *et al.*, 2009; Nozue *et al.*, 2011) are direct targets of PIF3 under SD conditions, and are specifically induced at the end of the night with an expression peak that coincides with the moment of maximum growth (Soy *et al.*, 2012). To test whether the expression of these genes is affected under SD conditions when the levels of PIF3 (and probably other PIFs) are altered (see above, Figs 1, 2), the expression of *PIL1* and *XTR7* was analysed in 2-day-old SD-grown seedlings exposed to WL or to an EOD-FRp during the third day of growth (following the light protocols shown in Figs 1A and 2A). As presented in Fig. 3A, the expression levels of *PIL1* and *XTR7* were induced at the end of the night in response to 16 h of darkness (ZT24D) compared with levels at the beginning of the night (ZT8 time point), in accordance with published data (Soy *et al.*, 2012). However, when seedlings were instead kept in WL during the same period of time (ZT24L), the expression levels of *PIL1* and *XTR7* were similar to the levels at the beginning of the night (ZT8) (Fig. 3A). Together with the PIF3 protein data shown in Fig. 1B, these results suggest that PIF3 accumulation during the night is necessary to induce expression of target genes such as *PIL1* and *XTR7*. Next the expression of *PIL1* and *XTR7* was examined 24 h after an EOD-FRp (ZT24FRD) (Figs 2A, 3B). In these conditions, *PIL1* and *XTR7* expression is induced with respect to ZT8, and to levels ~3-fold higher compared with the controls without an EOD-FRp (ZT24D) (Fig. 3B). Together with the higher PIF3 protein accumulation shown in Fig. 2B, these results suggest that an increase in PIF3 protein accumulation following an EOD-FRp during the night (and possibly other PIFs such as PIF4 and PIF5) leads to enhanced expression of target genes such as *PIL1* and *XTR7*.

An antagonistic functional relationship in the regulation of hypocotyl growth under SD conditions has been described between PIF3, PIF4, PIF5, and phyB (Niwa *et al.*, 2009; Soy *et al.*, 2012). In contrast to the short hypocotyl of *pif3*, *pif4pif5*, and *pif3pif4pif5*, *phyB* mutant seedlings exhibit more elongated hypocotyls than the WT. The *phyB* tall phenotype was partially suppressed by genetic removal of PIF3, and further suppressed by additional genetic removal of PIF4 and PIF5, suggesting that higher PIF protein accumulation

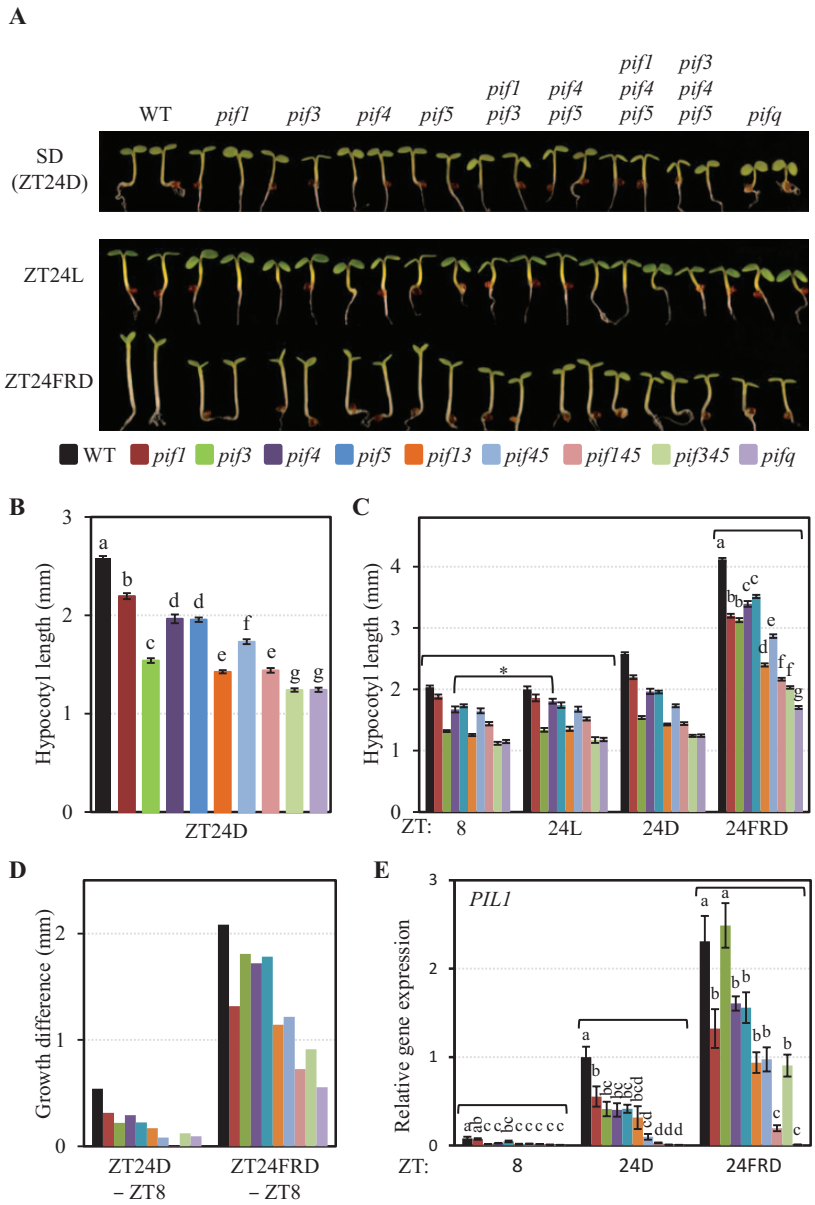

Fig. 5. Morphological and gene expression analysis of *pif1* and higher order *pif* mutant combinations under SD conditions provides evidence for differential contributions of individual PIF quartet members. (A) Visible phenotypes of WT, *pif1*, *pif3*, *pif1pif3*, *pif4pif5*, *pif1pif4pif5*, *pif3pif4pif5*, and *pifq* mutant seedlings grown for 3 d in SD conditions (ZT24D) (top), or in ZT24L and ZT24FRD as specified in Fig. 1A and 2A, respectively (bottom). (B) Quantification of the hypocotyl elongation of 3-day-old SD-grown (ZT24D) WT and *pif*-mutant seedlings shown in A (top). Data represent the mean and SE of at least 20 seedlings. Different letters indicate significant differences among means (*P*-value <0.05). (C) Quantification of the hypocotyl elongation of 3-day-old ZT24FRD- and ZT24L-grown WT and *pif*-mutant seedlings shown in A (bottom), compared with SD-grown (ZT24D) seedlings and seedlings grown under SD conditions for 2 d and 8 h (ZT8). Data represent the mean and SE of at least 20 seedlings. For ZT24FRD, different letters denote significant differences among means ($P < 0.05$). In the case of ZT8 and ZT24L, the genotypes that show a statistical difference in ZT24L compared with ZT8 are indicated with an asterisk. (D) Differential growth responsiveness during the night period was calculated from data shown in C. Mean hypocotyl length at ZT8 was subtracted from the mean hypocotyl length at ZT24D or ZT24FRD for each genotype. The growth difference of *pif1pif4pif5* in ZT24D was nearly zero. (E) *PIL1* gene expression in WT, and *pif1*, *pif3*, *pif1pif3*, *pif4*, *pif5*, *pif4pif5*, *pif3pif4pif5*, and *pifq* mutant seedlings grown under SD conditions for 2 d and 8 h (ZT8), 3 d (ZT24D), or under ZT24FRD conditions as specified in Fig. 2A. Different letters denote significant differences among means ($P < 0.05$). Most mutant combinations at ZT8, *pif1pif4pif5*, *pif3pif4pif5*, and *pifq* at ZT24D, and *pifq* at ZT24FRD had expression levels near zero.

during the night in the absence of phyB is necessary for full expression of the *phyB* phenotype (Soy *et al.*, 2012). To examine whether the described correlation between PIF levels and *phyB* hypocotyl elongation was also observed at the gene expression level, gene expression analysis was performed in 3-day-old SD-grown *phyB* and WT seedlings at ZT24D. The

expression level of *PIL1* was increased by >3-fold in *phyB* compared with the WT (Fig. 3C), an increase that was similar in magnitude to that observed in the WT after an EOD-FRp compared with WT seedlings kept in SD conditions without an EOD-FRp (compare ZTD and ZTFRD time points in Fig. 3B). In addition, this *PIL1* increase was suppressed in *pif3pif4pif5phyB* mutants (Fig. 3C). Together, these data indicate that the increased accumulation of PIF3, PIF4, and PIF5 in the absence of photoactive phyB (when removed either genetically or by an EOD-FRp) induces overexpression of *PIL1*, and suggest a correlation between elevated levels of PIF proteins, increased expression levels of growth-related genes, and the elongated hypocotyl of *phyB*.

To examine further the role of the PIFs in inducing growth-related gene expression, *PIL1* induction was next examined in 3-day-old SD-grown triple *pif3pif4pif5* mutants in response to an EOD-FRp (Fig. 3D). A significantly reduced response in comparison with the WT was detected, confirming the role of PIF3, PIF4, and PIF5 as positive regulators of *PIL1* expression after an EOD-FRp. Interestingly, although reduced in magnitude with respect to the WT, *pif3pif4pif5* mutant seedlings still responded to an EOD-FRp for *PIL1* expression (compare ZT24FRD with ZT24D), suggesting that at least one additional factor participates in the phytochrome-regulated gene induction response under these conditions.

PIF1 regulates hypocotyl growth under SD conditions

The observation that *pif3pif4pif5* seedlings exhibit a reduced but still significant growth response and expression of growth marker genes after an EOD-FRp (Figs 2D, 3D) suggests that factors other than PIF3, PIF4, and PIF5 might be involved in the regulation of hypocotyl growth at night. This prompted the testing of whether PIF1 could be participating in this response under SD conditions. Hypocotyls of 3-day-old SD-grown *pif1* mutant seedlings were ~20% shorter compared with the WT (Fig. 4A, B). In comparison with SD conditions, WT seedlings exhibited shorter hypocotyls when grown under continuous WL for 3 d (WLc) (Fig. 4B, C), and WLc-grown *pif1* mutants were not significantly shorter than the WT (Fig. 4C). These data suggest that PIF1 is a component of the cellular machinery that induces growth during the night hours in SD conditions.

To establish the pattern of *PIF1* expression under diurnal SD conditions, *PIF1* transcript levels were analysed over 24 h during the third day of seedling growth under SD conditions and compared with the expression patterns of *PIF3*, *PIF4*, and *PIF5*. PIF1 levels remained fairly constant over the 24 h photoperiod (Fig. 4D), similarly to the previously reported *PIF3* expression pattern under SD conditions, and in contrast to the oscillating levels of *PIF4* and *PIF5* (Fig. 4D) (Yamashino *et al.*, 2003; Nozue *et al.*, 2007; Soy *et al.*, 2012). This pattern of expression indicates that *PIF1* is not regulated by the circadian clock under SD conditions, in contrast to *PIF4* and *PIF5*, and instead suggests that PIF1 protein abundance is probably regulated post-transcriptionally by the phytochromes as described for PIF3 (Soy *et al.*, 2012). Accordingly, phytochrome-imposed post-transcriptional regulation would keep PIF1 levels in SD-grown seedlings very low during the light hours, and would allow progressive accumulation during the night. This possibility is in accordance with previous data showing that transfer to the dark induced re-accumulation of LUC activity in LUC–PIF1-overexpressing seedlings under day-neutral conditions (Shen *et al.*, 2005). In agreement with this, a contribution of PIF1 to growth was detected during the 16 h dark period in SD-grown seedlings, with *pif1* seedlings displaying reduced hypocotyl growth compared with the WT (ZT24D, Fig. 4E), but not when seedlings were kept in WL (ZT24L, Fig. 4E). These results are similar to those for PIF3 (Fig. 1), and support the notion that the night period is necessary for PIF1 accumulation.

Phenotypic analysis of pif *mutant combinations provides evidence for overlapping and differential contributions of individual PIFs to growth under SD conditions*

To obtain insight into the contribution of PIF1 to the promotion of hypocotyl elongation under SD conditions relative to that of PIF3, PIF4, and PIF5, the hypocotyl length of 3-day-old SD-grown *pif1*, *pif3*, *pif4*, *pif5*, *pif1pif3*, *pif4pif5*, *pif1pif4pif5*, *pif3pif4pif5*, and *pif1pif3pif4pif5* (*pifq*) mutant seedlings was first analysed (Fig. 5A top, B). Under these conditions, *pif1* showed a significantly shorter hypocotyl than the WT (as also shown in Fig. 4), whereas *pif4* and *pif5* were similar and shorter than *pif1*, and *pif3* displayed the strongest phenotype of all four single mutants. Double *pif4pif5* mutants showed a short-hypocotyl phenotype similar to *pif3*, although slightly less robust (Fig. 5A top, B). Genetic removal of PIF1 in *pif3* and *pif4pif5* resulted in marginally shorter hypocotyls in both *pif1pif3* and *pif1pif4pif5* mutants (Fig. 5A top, B). Moreover, triple *pif3pif4pif5* seedlings had shorter hypocotyls than *pif1pif3* or *pif1pif4pif5*, and were similar to *pifq* (Fig. 5A top, B). Together, these results suggest that PIF1, PIF3, PIF4, and PIF5 collectively function in the promotion of growth under SD conditions, with the role of PIF3 probably being more prominent and similar to that of PIF4 and PIF5 combined, and with PIF1 contributing to a lesser extent.

As shown above, the 16 h night period is necessary to induce growth under SD conditions, as WT seedlings arrested their hypocotyl growth when they were transferred to 16 h of WL during the night h (Figs 1, 4), whereas an EOD-FRp given before the dark period accelerated WT growth during the subsequent 16 h of darkness (Fig. 2). Comparison of the hypocotyl elongation at ZT8 and ZT24L in the WT and *pif* mutant seedlings examined in Fig. 5B (see Fig. 1A for a description of the experimental design) showed that WL treatment arrested seedling growth in all genotypes as expected (except for *pif4*, where growth was statistically significant although marginal) (compare ZT24L with ZT8, Fig. 5C), whereas an EOD-FRp (see Fig. 2A for a description of the experimental design) induced hypocotyl elongation to various degrees depending on the genotype (compare ZT24FRD with ZT8, Fig. 5C). To determine the contribution of PIF1 to the regulation of growth following an EOD-FRp-SD, and the possible

interaction of PIF1 with the other PIF members under these conditions, the hypocotyl length of the various *pif* mutant combinations was analysed after the EOD-FRp treatment (ZT24FRD) (Fig. 5A bottom, C). Under these conditions, *pif* mutant seedlings displayed attenuated responses of different magnitude with respect to the WT (Fig. 5A bottom, C). All *pif* single mutants showed short hypocotyls compared with the WT, and this attenuated response to EOD-FRp was further reduced in the *pif1pif3* and *pif4pif5* double mutants, and even more in the triple *pif1pif4pif5* and *pif3pif4pif5* mutants (Fig. 5A bottom, C). These results suggest that all PIFs contribute to the promotion of growth in response to an EOD-FRp under SD conditions. In addition, given that the *pif1* mutant shows a phenotype similar to the other *pif* single mutants at ZT24FRD, and that the hypocotyl phenotype of *pif1* at ZT8 and ZT24D compared with the WT is only modest compared with the other *pif* single mutants, these data suggest that the relative contribution of PIF1 might be quantitatively more important after an EOD-FRp compared with its contribution under regular SD conditions (Fig. 5B, C). Indeed, growth difference measurements between ZT24FRD and ZT8 to quantify the elongation growth experienced during the 16 h night after the EOD-FRp indicate that the *pif1* single mutant has a more attenuated response in comparison with *pif3*, *pif4*, and *pif5* (Fig. 5D). These results thus suggest that PIF1 might have a more prominent relative contribution to growth compared with PIF3, PIF4, and PIF5 after an EOD-FRp, compared with under SD conditions (Fig. 5B–D).

PIF1 regulates expression of the growth-related PIL1 *gene under SD conditions, together with PIF3, PIF4, and PIF5*

The observed contribution of PIF1 to seedling growth in SDs (Figs 4, 5 A–D) suggests that PIF1 might also contribute to the promotion of expression of growth-related genes targeted by PIF3, PIF4, and PIF5 under these conditions, such as *PIL1* (Soy *et al.*, 2012). Expression analyses in 3-day-old SD-grown seedlings (ZT24D) indicated that the promotion of *PIL1* transcript levels observed in the WT during the night hours is reduced in *pif1* similarly to *pif3*, *pif4*, and *pif5*, whereas *PIL1* levels in *pif1pif4pif5*, *pif3pif4pif5*, and *pifq* at ZT24D were all below the level of detection, indicating possible additive effects of the contribution of PIFs in higher order mutants (Fig. 5E). Together, this expression pattern supports the conclusion that PIF1 contributes to growth under diurnal conditions by promoting the expression of growth-related genes together with PIF3, PIF4, and PIF5.

Next the role of PIF1 in promoting gene expression in response to an EOD-FRp under SD conditions was examined. Compared with *PIL1* expression levels in WT seedlings, the expression in *pif1* was significantly reduced (Fig. 5E), and this effect was more robust compared with that in *pif3* (which showed no difference compared with the WT), and similar to that of *pif4* and *pif5*, *pif4pif5*, and *pif1pif3* double mutants, and *pif3pif4pif5* (Fig. 5E). Significantly, expression levels in *pif1pif4pif5* were greatly reduced compared with *pif4pif5*, and removal of PIF1 from *pif3pif4pif5* in the *pifq* mutant resulted in *PIL1* levels below detection (Fig. 5E). Together, this expression pattern is broadly consistent with the morphological phenotypes of the various *pif* mutant combinations after an EOD-FRp presented in Fig. 5A–D (although PIF3 seemed to have a less important role in the regulation of *PIL1* expression compared with its contribution to hypocotyl growth), and supports the conclusion that PIF1, PIF3, PIF4, and PIF5 collectively contribute to growth after an EOD-FRp by promoting the expression of growth-related genes, with PIF1 having a relatively more important role in these conditions compared with under SD conditions.

Discussion

Previously the role of PIF3 as a prominent promoter of rhythmic growth under diurnal conditions together with PIF4 and PIF5 was defined, through direct regulation of growth-related genes at dawn coinciding with a PIF3 accumulation peak generated by phytochrome-imposed oscillations in protein abundance (Soy *et al.*, 2012). The experiments presented here examine the correlation under diurnal conditions between the levels of PIF3 during the night and the promotion of growth, by comparing PIF3 accumulation and hypocotyl elongation in SD conditions, and SD-grown seedlings released into WL or exposed to an EOD-FRp for 1 d. The data indicate a direct correlation between phytochrome activity during the night period, PIF3 levels (and possibly levels of other PIFs), and the extent of the growth response, and suggest that it occurs at least in part through the regulation of growth-related gene expression. In addition, combination of EOD-FRp and SD experiments uncovered PIF1 as a novel contributor to growth under light–dark conditions. Moreover, morphogenic and marker gene expression evidence is provided that individual members of the PIF quartet (PIF1, PIF3, PIF4, and PIF5) contribute differentially to the promotion of seedling growth, suggesting that they act together with partially redundant functions to optimize growth under diurnal conditions.

The observation that substitution of the 16 h dark period by WL led to seedling growth arrest under SD conditions provides evidence that night-induced inactivation of phytochromes and subsequent accumulation of the PIFs are necessary to promote growth (Fig. 1), although additional involvement of other photoreceptors such as cryptochromes, which have been previously shown to participate in the control of photoperiodic growth (Mazzella and Casal, 2001), cannot be discarded. Further support for a direct correlation between PIF levels and the magnitude of the growth response was observed when giving an EOD-FRp before the beginning of the 16 h night period. This treatment promoted overaccumulation of PIF3 and possibly other PIF proteins, increased the expression of PIF-regulated growth-related genes, and enhanced hypocotyl growth by 3-fold during the night period (Figs 2, 3). Based on previous results (Soy *et al.*, 2012), it was expected that this EOD-FRp acted primarily through inactivation of the phytochrome system (mainly of phyB) at the start of the dark period. In agreement, *phyB* mutant seedlings grown under SD conditions, which display a tall phenotype

and accumulate higher amounts of PIF3 during the night (Niwa *et al.*, 2009; Soy *et al.*, 2012), had increased expression of growth-related genes that were suppressed by genetic removal of PIF3, PIF4, and PIF5 (Fig. 3). These data thus provide additional support for a strong correlation between increased PIF levels during the night hours under SD conditions and enhanced hypocotyl growth, and are in agreement with previous data in seedlings exposed to FR light-enriched environments such as vegetational shade, low R/FR ratios, or an EOD-FRp, where inactivation of the phytochromes triggers an increase in PIF abundance and a promotion of growth (Lorrain *et al.*, 2008; Leivar *et al.*, 2012a, *b*; Sellaro *et al.*, 2012).

The results presented here revealed that factors other than PIF3, PIF4, and PIF5 participate in the promotion of phytochrome-regulated growth under diurnal conditions, because the *pif3pif4pif5* triple mutant still responded both morphologically and molecularly to an EOD-FRp treatment given at the beginning of the night in SD conditions (Figs 2, 3), consistent with previous results in shade conditions (Leivar *et al.*, 2012a). The present phenotypic and marker gene expression analyses of *pif1* single and higher order mutants identify PIF1 as an additional factor that contributes to the promotion of growth under SD conditions together with PIF3, PIF4, and PIF5, albeit to a lesser extent, possibly by direct regulation of growth-related genes such as *PIL1* (Figs 4–6). Analyses of *pif1pif4pif5* and *pif3pif4pif5* hypocotyl length compared with *pifq* indicated that PIF3 alone was able partially to complement the *pifq* phenotype, whereas PIF1 was not, suggesting that PIF1 is required but not sufficient to promote growth in SD conditions in the absence of the other three PIFs, although a significant additive effect was observed when PIF1 was removed from *pif3* or *pif4pif5* mutants (Fig. 5). Examination of marker gene expression revealed a picture where the four PIFs collectively induce the expression of the growth marker gene *PIL1* (Fig. 5).

Interestingly, in contrast to SD conditions, PIF1 appears to have a more robust contribution to the promotion of hypocotyl elongation after an EOD-FRp, whereas PIF3, PIF4, and PIF5 contribute to a lesser extent (Fig. 5D). Under these conditions, the role of PIF1 was similar to the combined action of PIF4 and PIF5 (Fig. 5D). Analyses of *pif3pif4pif5* hypocotyl length compared with *pifq* indicated that PIF1 was able partially to complement the *pifq* phenotype at ZT24FRD but not at ZT24D (Fig. 5C), in agreement with the notion that PIF1 has a more predominant role after an EOD-FRp compared with SD conditions. Intriguingly, the *pifq* mutant still retained some ability to grow after an EOD-FRp (Fig. 5), suggesting that additional factors might contribute to the regulation of growth under SD conditions as previously described in shade (Leivar *et al.*, 2012b), and consistent with the possible participation of additional PIFs such as PIF7 (Leivar *et al.*, 2008a; Li *et al.*, 2012; EM and PL, unpublished). Examination of marker gene expression revealed a picture for relative PIF contribution broadly similar to that for hypocotyl elongation, with the four PIFs collectively inducing the expression of *PIL1*, with a more predominant contribution of PIF1 compared with PIF3 (Fig. 5). The data presented here show that treatment of SD-grown seedlings with an EOD-FRp induced exaggerated hypocotyl elongation and a robust increase in growth marker genes such as *PIL1* and *XTR7* (Figs 2, 3), with PIF1 having a prominent contribution in regulating these responses (Fig. 5). These characteristics resemble those of etiolated seedlings (Leivar *et al.*, 2009; Shin *et al.*, 2009), and suggest that SD-grown green seedlings exposed to an EOD-FRp might experience a partial reversal to the etiolated state, similar to what has been previously suggested for shade-induced responses (Leivar *et al.*, 2012b). In agreement with this possibility, SD-grown WT seedlings exposed to an EOD-FRp displayed partially closed cotyledons typical of etiolated seedlings (Fig. 5A). This response was absent in SD conditions or in SD-grown seedlings transferred to WL, and was dependent on PIF activity (Fig. 5A). Overall, the data support the notion that an increase in PIF levels in SD conditions after an EOD-FRp induces a partial reversion to the etiolated state and favours a more important relative contribution of PIF1. This change in PIF relative contribution between SDs and SDs supplemented with an EOD-FRp might include a change in relative activity, abundance, and/or binding affinity for target genes. Additional experiments will be required to elucidate the mechanisms involved.

Taken together, the data presented here indicate that, under SD conditions, there is a strong correlation between PIF protein levels and the levels of marker gene expression and hypocotyl growth. The present work suggests that phytochrome-regulated abundance of PIF levels is a central regulatory pathway that determines the magnitude of growth under diurnal conditions, in good agreement with the previously described role for the PIFs during seedling etiolation or shade avoidance (Bae and Choi, 2008; Leivar *et al.*, 2008b, 2012b; Lorrain *et al.*, 2008). How PIFs implement these growth responses is an active area of research. Current evidence indicates that PIFs directly regulate a subset of genes enriched in transcription factors and in synthesis and responses to auxin during seedling de-etiolation and responses to shade (Hornitschek *et al.*, 2012; Zhang *et al.*, 2013). Under modified SD conditions, the PIF4- and PIF5-regulated transcriptional network has been defined and also includes auxin-related genes (Nozue *et al.*, 2011), although the direct targets in these conditions have not yet been determined. Further experiments are required to define the transcriptional network targeted by the PIF quartet under diurnal conditions. Comparative analysis of the PIFq-regulated transcriptome in SD conditions with that in de-etiolation or shade will establish whether regulation of diurnal growth involves targeting of SD-specific genes, or whether, and to what extent, these different phytochrome/PIF-dependent responses are implemented through a shared transcriptional network that drives common downstream facets of morphogenesis such as hypocotyl growth.

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Light-regulation of diurnal growth is gated by the circadian clock

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Contribution: In this work, Judit Soy performed most of the experiments presented in the Figures. In addition, Judit prepared all the data for publication and participated in the process of writing the manuscript.

Impact Factor: Because of the relevance of the results, we expect that this work will be published in a high impact factor journal.

ABSTRACT

Hypocotyl growth rate in seedlings grown in short-day conditions follows clear oscillations, with a maximum peak of growth at dawn. Such oscillating pattern is implemented by the concerted action of internal (such as clock and hormones) and external factors (like light and temperature). Both light and circadian clock have been reported to act on the growth-promoting PIF (Phytochrome-interacting Factors) subfamily of bHLH transcription factors, either directly controlling their transcription or affecting their protein stability, to get a peak of protein accumulation at the end of the night period. At that precise moment PIFs induce the expression of growth-related genes and there is an induction of hypocotyl growth. Here we establish that TOC1 binds to the promoter region and represses the expression of a subset of PIF-induced growth-related genes at night under short days. This repression occurs at early night, coinciding when TOC1 levels are high, and is relieved at the end of the night when TOC1 levels decline. This direct TOC1 repression of PIF transcriptional activity at early night gates the expression of PIF-induced growth related genes towards the end of the night. This repression-activation regulatory module represents a novel regulatory mechanism by which the circadian clock gates hypocotyl growth at dawn under diurnal conditions.

INTRODUCTION

Hypocotyl elongation is a light-dependent biological process that follows diurnal oscillations, with a growth peak phase that changes depending on the photoperiodic growing conditions [1][2]. In day-night cycling growing conditions, the extent of elongation depends on the number of dark hours in a nonlinear manner, presenting an acceleration of growth in the long nights of short-day (SD) conditions [3]. In such SD conditions (8h of light and 16h of dark), maximum stem elongation occurs at dawn [2][4]. It is well-established that several pathways regulate hypocotyl growth, triggered by both external (basically light and temperature) and internal (such as circadian clock and hormones) cues [1][5][6][7]. Key regulators of the process are the growth-promoting group of bHLH transcription factors PIFs (Phytochrome-interacting Factors), that function as integrators of different regulatory pathways [8][9].

In SD conditions PIF proteins accumulate at the end of the dark period, moment in which they regulate the expression of growth-related genes and induce hypocotyl growth. A coordinated action of light and clock pathways ensures that a peak of PIF proteins accumulation occurs at the appropriate time of the diurnal cycle. PIF quartet (PIFq) members (PIF1, PIF3, PIF4 and PIF5) are differentially regulated at a transcriptional level: *PIF1* and *PIF3* are constantly expressed during all the diurnal cycle [4][10] while *PIF4* and *PIF5* present diurnal oscillations [2]. Circadian clock regulates the expression of *PIF4* and *PIF5*; it represses their expression during the beginning of the night period by the action of the Evening Complex (EC) [11] and allows their expression during the end of the night and the light hours. However, in all cases the accumulation of PIFq proteins is mostly restricted to the end of the dark period by the action of the Red (R) and Far Red (FR) light sensing phytochrome (phy) photoreceptors [2][4]. Light signals act to repress hypocotyl elongation [12]; in the presence of light, phys are activated and translocated to the nucleus where they bind to PIFq members and induce their rapid proteolytic degradation. In SDs, phy-induced PIFq

degradation occurs during the light and first hours of the night (as a consequence of the still active phy, mainly phyB, present in the cells during that period) [4]. Hence, PIFs can only accumulate towards the end of the night and promote growth. Moreover, in order to fine-adjust PIFs action at dawn, hormonal-related regulatory events have been reported to participate in the process by affecting PIFs transcriptional activity, as it is the case of DELLA proteins. The group of DELLA proteins (central components of the gibberellin signaling pathway) inhibit the transcriptional activity of PIFs by binding to the bHLH domain of PIFs during the day and first hours of night [13][14]. Additionally, DELLAs influence on hypocotyl growth by regulating the activity of the transcription factor BZR1 [15] (a component of the brassinosteroid hormone signaling pathway), which recently has also been reported to directly interact with PIF4 and together regulate the expression of common target genes [16].

Moreover, other mechanisms contribute to fine-regulate stem growth and to ensure that it takes place at a very specific moment of the dark period. Circadian clock, a part from regulating the expression of *PIF4* and *PIF5*, has been shown to control the oscillatory expression of growth-promoting genes, among multiple hormone-associated genes are found and whose peak of expression is at dawn [17]. At a transcriptional regulation level, it was recently reported that the clock member TOC1 is a transcriptional repressor that can bind to the promoter of genes [18][19][20].

Even it is known that clock and light regulate hypocotyl elongation in diurnal conditions and, although the direct transcriptional networks of TOC1 and PIFs have been studied individually, no studies have previously focused in determine a possible interplay between these transcription factors in the regulation of growth-related genes, and consequently, in the regulation of hypocotyl elongation in SDs. Here, in order to decipher a possible novel crosstalk point between light and clock pathways, we performed phenotypic studies and gene expression analysis in *toc1* and *pifs* deficient mutant combinations, together with ChIP analysis and comparison of ChIPseq available data [18] (Anne Pfeiffer and Peter H. Quail, unpublished). Data suggest that, TOC1 gates stem elongation to the end of the night in SDs by repressing the transcriptional activity of PIF3 protein during the rest of the dark period.

RESULTS

TOC1 mediates gating of PIF-promoted diurnal growth

In order to establish whether the level of hypocotyl growth induction was the same at all moments of the night period, we took advantage of the previously described hypocotyl elongation induction produced by a FRp treatment [10][21][22]. Seedlings previously entrained under SD and released into WLc were exposed to a 15 min FRp at different time points during the subjective night (ZT8, ZT14, ZT18, and ZT24) and then kept in the dark for 8h (Supplementary Fig. 1a). Measurements of hypocotyl length showed that the extend of the FRp-induced hypocotyl elongation was different at each time point with a growth maximum at ZT8 that was reduced significantly at ZT14 and ZT18, with a recovery at ZT24 (Fig. 1a and 1b). Protein accumulation analysis discarded that these differences were due to different accumulation of the growth promoter PIF3 after the FRp (Supplementary Fig. 1b). Same experiments done in *PIFs* deficient mutant,

revealed that FRp-induced growth in the WT was dependent on the PIFs as expected based on previous reports (Supplementary Fig. 1c). A TOC1-deficient mutant responded to a FRp similarly to the WT at ZT8 and did not display the reduction in growth at ZT14, ZT18, and ZT24 (Fig. 1a and 1b). These results suggest that TOC1 is acting to gate FRp-induced growth under these conditions acting as repressor at ZT14 and ZT18. In addition, *pif3toc1*, *pif4pif5toc1*, and *pif3pif4pif5toc1* mutants displayed incrementally reduced responses compared to *toc1* (Fig. 1a), indicating that PIF3, PIF4 and PIF5 are necessary for the full expression of the *toc1* phenotype.

We examined the expression of the growth marker genes *PIL1* and *HFR1* that rapidly respond to accumulation of PIF3 and other PIFs [10][23] in seedlings treated with a FRp and a subsequent 15 min of darkness (Supplementary Fig. 1d). Expression levels in the WT showed that there is a high induction at ZT8 while at ZT14 and ZT18 the induction is much lower and it increases again at the end of the night, mirroring what happens in growth and, indicating that expression of these genes is gated during the subjective night (Fig. 1c). This response was also mediated by TOC1, as induction of expression was sustained along the subjective night in *toc1* (Fig. 1c). Examination of *PIL1* and *HFR1* induction after a FRp in *pif3toc1*, *pif4pif5toc1*, and *pif3pif4pif5toc1* compared to *toc1* mutants at ZT8 and ZT14 showed incremental suppression of the *toc1* phenotype, suggesting that PIF3, PIF4, and PIF5 are necessary for the full induction of *PIL1* and *HFR1* in *toc1* after a FRp (Fig. 1d).

To understand the relevance of these observations in a photoperiodic framework, we grew WT and *toc1* seedlings under different photoperiods: constant light conditions (LL, 24h of white light), long-day conditions (LD, 16h of white light + 8h of dark) or short-day conditions (8h of white light + 16h of dark). As described previously, increased dark hours induce elongation in a non-linear fashion, and seedlings in SD are longer compared to LD (Fig. 1e) [3].
Deficiency in TOC1 did not affect growth in LL and only marginally in LD, but resulted in longer seedlings in SD (Fig. 1e, Supplementary Fig. 2a), indicating that the repressive action of TOC1 is only relevant under SD. Under these SD conditions, growth is promoted by the PIFs, and *toc1* tall phenotype was incrementally suppressed with the removal of PIF3, PIF4 and PIF5, (Fig. 1f, Supplementary Fig. 2a), suggesting that TOC1 function in SD requires PIF3, PIF4 and PIF5. Expression of *PIL1* and *HFR1* in the WT was higher in 3dSD-grown seedlings compared to 3dLD-grown seedlings, in agreement with the hypocotyl phenotypes, and was elevated in both conditions in *toc1*, also to higher levels under SD coinciding with the *toc1* hypocotyl length phenotype. The de-repression of *PIL1* and *HFR1* in *toc1* was incrementally suppressed in *pif3toc1*, *pif4pif5toc1*, and *pif3pif4pif5toc1*, indicating that TOC1 function as a repressor of *PIL1* and *HFR1* requires PIF3, PIF4, and PIF5 function (Fig. 1g, Supplementary Fig. 2b).

Previously, PIF4 and PIF5 have been shown to promote growth together with PIF3. *PIF4/5* are transcriptionally regulated by the clock and directly targeted by TOC1 [2][11][24]. In accordance to the coincidence model, *PIF4/5* transcript levels are higher in *toc1* mutant in SD at night (as shown for *PIF4* in Supplementary Fig. 3a), which

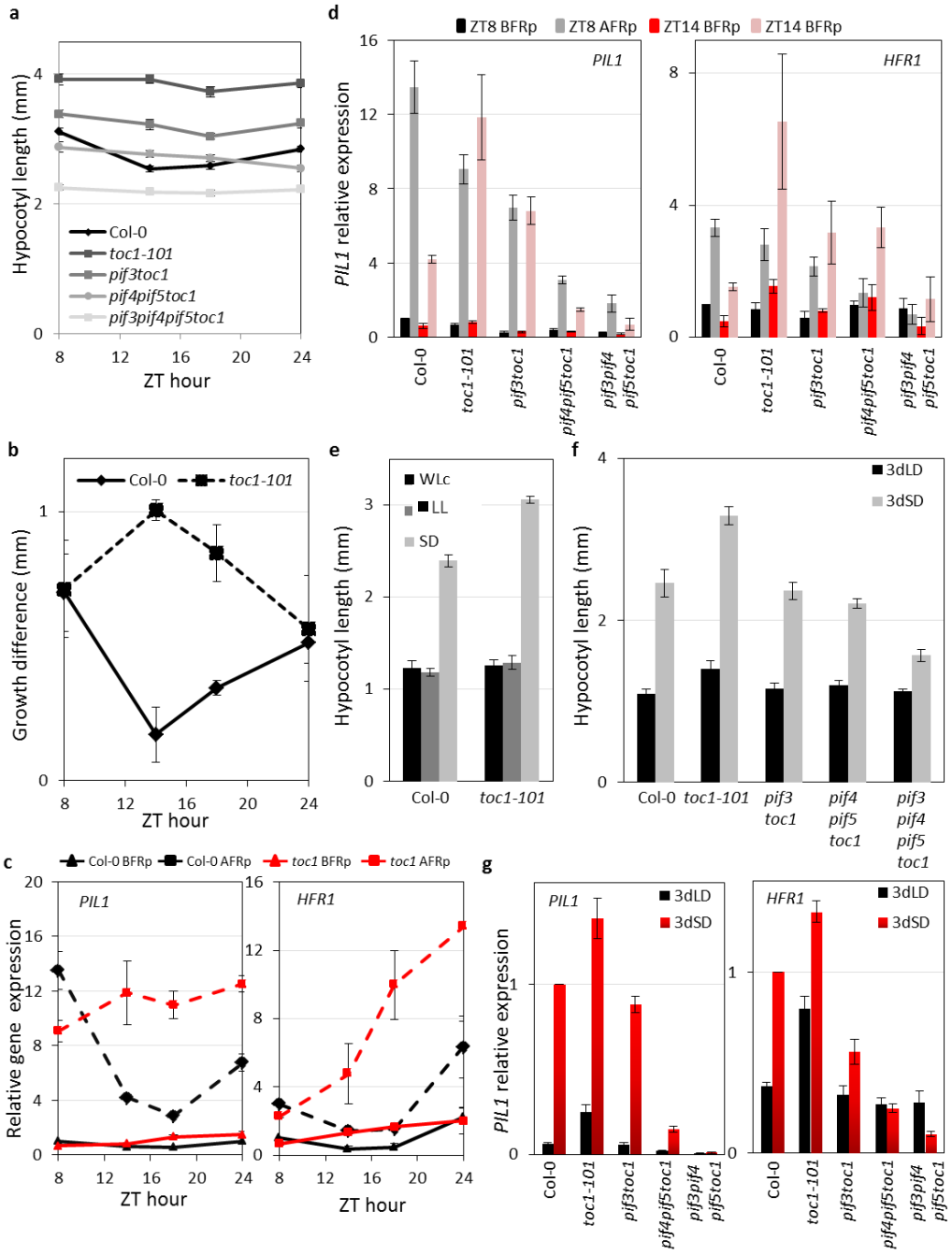


Figure 1. TOC1-mediated Gating of PIF-induced Diurnal Growth

a) and b) Hypocotyl length of 3-day-old seedlings. Seedlings were grown for 2 days in SD conditions and at specific ZT time-points during the third day of growth a picture was taken. At the same time, a second plate was treated with a 15 min Far Red pulse and then kept in dark for 8h, when a second picture was taken. a) Hypocotyl length after a FRp + 8h dark. b) Growth difference value obtained by calculating the difference in the hypocotyl length after FRp + 8h dark and the average length before FRp (average length value is the average of the 4 hypocotyl length measures ZT8, ZT14, ZT18 and ZT24 in the non-Far red treated plates.

c) and d) Expression of *PIL1* and *HFR1* analyzed by qRT-PCR. Seedlings were grown for 2 days in short-day conditions and at specific ZT time-points during the third day of growth tissue sample were harvested. At the same time, a second plate was treated with a 15 min FRp and then kept in dark for 15 min, when tissue sample was harvested. Values were normalized to *PP2A*. c) Expression of *PIL1* and *HFR1* in Col-0 wild-type and *toc1-101* mutant seedlings. d) Expression of *PIL1* and *HFR1* at ZT8 and ZT14 in FRp non-treated and treated seedlings.

e) Hypocotyl length measurements of 3-day-old seedlings grown under constant light conditions (LL, 0 hours of dark), long-day conditions (LD, 8 hours of dark) or short-day conditions (SD, 16 hours of dark).

f) Hypocotyl length measurements of 3dLD and 3dSD grown seedlings.

g) Expression of PIL1 and HFR1 analyzed by qRT-PCR in 3dLD and 3dSD grown seedlings. Values were normalized by *PP2A*.

could explain the contribution of PIF4/5 to the *toc1* elongated phenotype (Figs.1a, 1d, 1f and 1g). However, as shown by the comparisons of *toc1* and *pif3toc1*, and also by the comparison of *pif4pif5toc1* and *pif3pif4pif5toc1*, where the role of PIF3 is strongly manifested, the repressive function of TOC1 requires PIF3. *PIF3* transcript levels are not affected in *toc1* (Supplementary Fig. 3a) and PIF3 protein levels were not found to be significantly different in the *toc1* mutant (Supplementary Fig. 3b). These data suggest that a yet uncharacterized mechanism that might involve regulation of PIF3 transcriptional activity underlies the antagonistic action of TOC1 and PIF3 in the regulation of growth under diurnal conditions.

TOC1 repression of growth under SD involves binding to direct PIF target genes and inhibition of their early expression during the night

Comparison of available TOC1 and PIF ChIP-seq experiments that previously defined 772 TOC1 targets [18] and 2699 PIF targets (Anne Pfeiffer and Peter H. Quail, unpublished) identified a common gene set comprising 229 putative direct targets of both proteins, of which 154 were considered bona fide after eliminating those identified using different criteria in the two experiments (Supplementary Fig. 4a, Table 1). Analysis of the phases of 133 of these 154 genes for which there exists information at the Phaser website (Table 2), indicated that a subset of them were statistically significantly overrepresented in SD at phases 18 (14 genes), 20 (7 genes), and 23 (12 genes)(Fig. 2a, Table 2). When including the genes with the intermediate phases 19 (6 genes), 21 (7 genes), and 22 (6 genes) (Table 2), a total of 52 genes (Gene Set 1) with a phase between 18 and 23 in SD were considered for further analyses (Table 3) as genes that might be co-regulated by TOC1 and PIFs are candidates to promote growth at the end of the night. The phase of Gene Sets 1 (Table 3) in LD or LL was different to SD (Supplementary Fig. 4b, Table 3): in LD statistically significantly overrepresented genes peak at phases 2 (10 genes) and 3 (10 genes), whereas no significantly overrepresented gene was detected in LL. Together with the observation that Phaser analysis of TOC1 target genes that did not overlap with PIFs (441/543 of the 772) showed that statistically significantly overrepresented genes peak at phase 3 (26 genes) and 4 (41 genes) (Table 5), we conclude that this analysis has identified 52 putative TOC1 and PIF target genes with an expression peak at the end of the night in SD that might be relevant to growth induction under diurnal conditions.

The 52 genes in Gene Set 1 with phases between 18-23 displayed an average diurnal pattern of expression in SD characterized by low expression during the day and increasing expression during the night period (Fig. 2b). This pattern resembles the characteristic pattern of PIF-dependent growth marker genes [4]. The amplitude of the oscillations is diminished in LD and even further reduced in LL (Supplementary Fig. 4c), suggesting a correlation of higher oscillation amplitude with growth in accordance with the PIFs having a role in SD as inducers of the expression of these genes at the end of the night.

In addition, 8 additional PIF-bound genes showing similar phase and diurnal pattern of expression (and of special interest because they encode transcription and hormone-related factors) were identified in the databases (Table 4), including the previously characterized PIF-induced in SD *PIL1* and *XTR7*.

Rather than being a complete comprehensive list of co-targets, these genes represent a tool to understand the mechanism by which TOC1 and PIF are regulating growth under diurnal conditions.

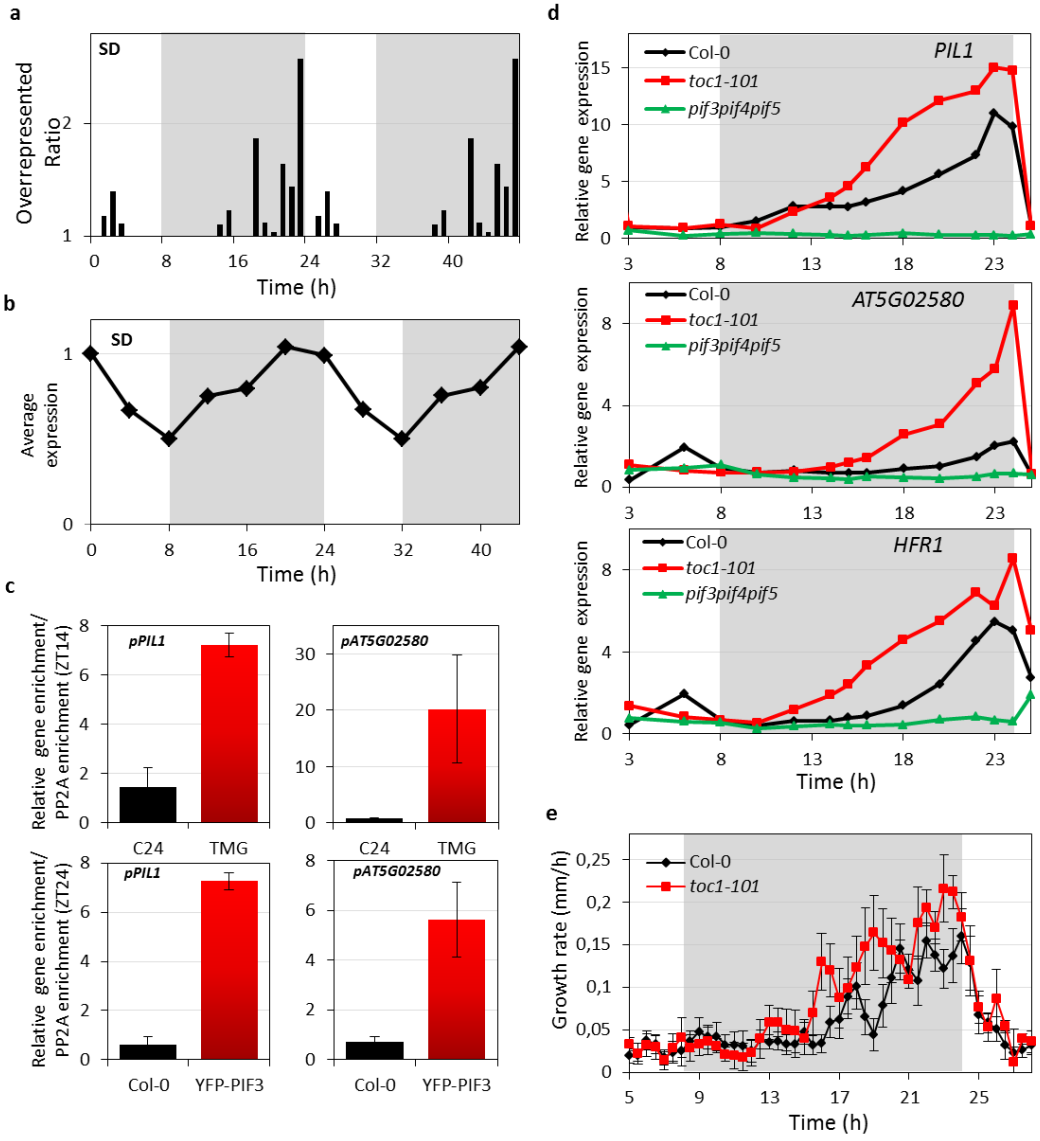


Figure 2. TOC1 repression of growth under SD involves binding to direct PIF target genes and inhibition of their early expression during the night

a) Overrepresentation ratio obtained using Phaser Website of the 133 predicted co-target genes of TOC1 and PIFs in short-day conditions (SD)

b) Average expression of the 52 predicted co-target genes (gene Set 2) of TOC1 and PIFs in short-day conditions (SD). Expression data of each gene (obtained from Diurnal Website) was used to calculate fold change values relative to ZT0. Average expression value represented in the graph was calculated using the fold change data of all 52 genes.

c) Chromatin immunoprecipitation (ChIP) from 3-day-old SD-grown seedlings. Samples were harvested during the third day of growth: at ZT14 for C24 and TMG genotypes or ZT24 for Col-0 and YFP-PIF3. Immunoprecipitated DNA was quantified by quantitative RT-PCR using primers in promoter regions containing G-boxes. Enrichment values shown were obtain by normalizing specific gene promoter enrichment values to PP2A enrichment value and normalizing samples processed with anti-GFP antibody to controls processed without antibody. Data shown correspond to mean of three biological replicates. Error bars indicate s.e.

d) Gene expression analysis done by qRT-PCR of *PIL1*, *AT5G02580* and *HFR1* in 3-day-old SD-grown seedlings. Seedlings were grown for 2 days in SD conditions and samples were harvested during the third day of growth. Values were normalized by *PP2A*.

e) Hypocotyl elongation rate under SD conditions. Infrared imaging was used to monitor seedling growth from 2 day onwards every half an hour. Values are the mean of seven seedlings. Error bars indicate s.e.

As shown for *PIL1* and *AT5G02580* (Fig. 2c), ChIP analysis confirmed that putative co-target genes encoding potentially regulatory factors are indeed direct targets in SD of both TOC1 at ZT14 (when levels of TOC1-TMG are maximum in SD (Supplementary Fig. 5a and 5b), and PIF3 at ZT24 (when PIF3 levels are maximum in SD, [4]).

Time course analysis indicated that expression of these genes peak at the end of night in a PIF3- and PIF4/5-dependent manner (Fig. 2d, Supplementary Fig. 6a and 6b), as in the triple mutant *pif3pif4pif5*, these genes are barely expressed. On the contrary, expression analysis showed that these genes start to be expressed earlier and in higher levels in *toc1*. Such results revealed that TOC1 acts as a direct repressor of early expression of these genes at night (Fig. 2d, Supplementary Fig. 6a and 6b). In order to discard the possibility that the early induction of these genes seen in *toc1* in SD was due to his short-period phase phenotype, we analyzed gene expression in seedlings grown in diurnal cycles of 21 hours (7h of light and 14 hours of dark). In 21h cycles, an early induction of *PIL1* expression was still observed in *toc1* while the very-well defined clock output gene *CAB2* lost the early induction phenotype (Supplementary Fig. 6c), reinforcing the idea that TOC1 is actually acting as direct repressor of early gene expression in SD.

Growth rate analysis indicated that *toc1* starts growing earlier than WT in SD, in accordance to *toc1* tall phenotype, suggesting that TOC1 is repressing growth at early night (Fig. 2d). Together, these results suggest that TOC1 and PIF3 function antagonistically in the regulation of growth under SD conditions by directly regulating the expression of common growth-related target genes. Our data suggest that TOC1 prevents early growth during the night hours by repressing the early expression of PIF-induced growth-related genes to ensure that elongation takes place precisely at dawn.

Temporal analysis of binding of TOC1 and PIF3 to common target genes during the night in short days

We next analyze the interaction of TOC1 and PIF3 with the co-target genes by performing ChIP assays time-course with TMG (toc1::TMG-YFP) and pif::YFP-PIF3 seedlings during the night period in SD. The results showed a peak of TOC1 binding to the promoter of these genes at ZT14, and lower levels of binding at ZT8 or ZT24 (Fig. 3a), in accordance with the oscillation of the protein under these conditions (Supplementary Fig. 5b) and the timing of TOC1 function (Fig. 2d).

The binding analysis for YFP-PIF3 seedlings showed low levels of binding at ZT8, but similar levels of binding at ZT14 and ZT24 (Fig. 3b), also in accordance with the protein accumulation pattern of YFP-PIF3 [4]. This is noteworthy for two reasons. First, it suggests that TOC1 and PIF3 can simultaneously bind to the promoters of these genes at ZT14. Second, binding of YFP-PIF3 to the promoter of these genes does not correlate with the levels of expression of these genes seen at ZT14 and ZT24 (Fig. 3c, Supplementary Fig. 7). At the two time-points, similar levels of YFP-PIF3 binding are observed, whereas levels of expression are very low at ZT14 (similar to that of *pif3*) and high at ZT24. Together, our results suggest that the transcriptional activity of YFP-PIF3

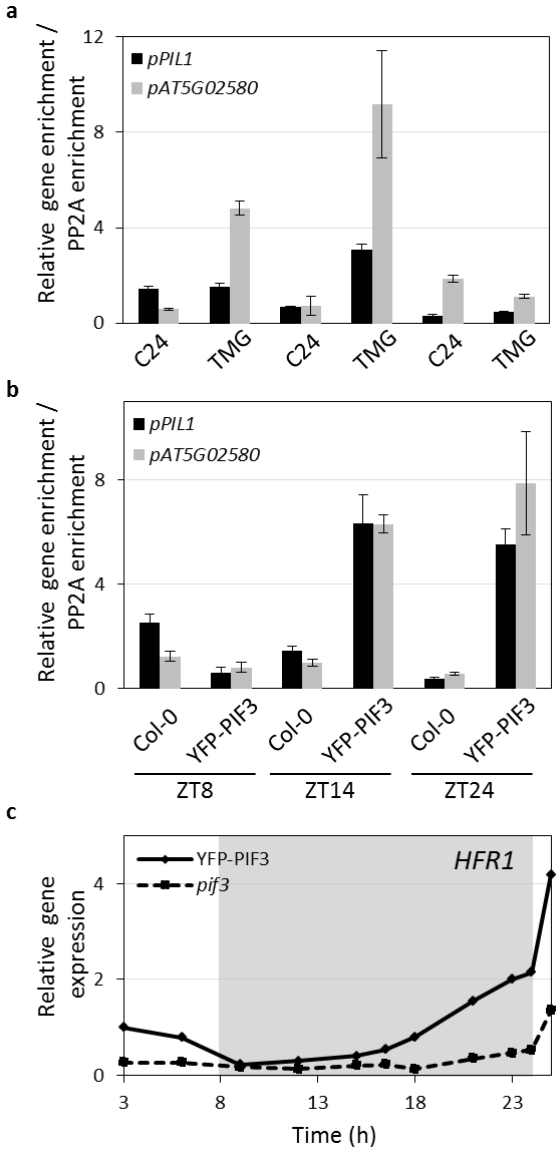


Figure 3. Temporal analysis of binding of TOC1 and PIF3 to common target genes during the night in short days

a) and b) Chromatin immunoprecipitation (ChIP) from 3-day-old SD-grown seedlings. Samples were harvested during the third day of growth at ZT8, ZT14 and ZT24. Immunoprecipitated DNA was quantified by quantitative RT-PCR using primers in promoter regions containing G-boxes. Enrichment values shown were obtain by normalizing specific gene promoter enrichment values to PP2A enrichment value and relativizing samples processed with anti-GFP antibody to controls processed without antibody. Data shown correspond to one representative biological experiment. Error bars indicate s.e of three technical replicates. a) C24 wild-type and TMG genoytpes included. b) Col-0 and YFP-PIF3 genotypes.

c) Expression of *HFR1* analyzed by qRT-PCR in 3-day-old SD-grown seedlings. Seedlings were grown for 2 days in SD conditions and samples were harvested during the third day of growth. Values were normalized by *PP2A*.

might be reduced at ZT14, coinciding with the peak of TOC1 binding to the same promoters. Together with previous reports showing that TOC1 presents transcriptional repressing activity [18][19][20], our results here suggest that PIF3 might be a target for TOC1 repressive activity in the middle of the night under SD

TOC1 is a repressor of PIF3 transcriptional activity in the middle of the night during growth in SD condition

To test this possibility, we next generated double transgenic plants overexpressing TOC1 under a 35S constitutive promoter in the YFP-PIF3 background (TOC1oxYFP-PIF3). These lines display a loss in TOC1 rhythmicity and accumulate similar amounts of TOC1 during the night in SD (Supplementary Fig. 8a). We then analyze the interaction of TOC1 with its target genes by performing ChIP assays with TOC1oxYFP-PIF3 seedlings during the night period in SD. In contrast to TMG (Fig. 3a), TOC1-OX shows a significant binding to the promoters at ZT24, (Fig. 4a), in accordance to previous results showing that TOC1 binding is dictated by its protein abundance [18]. Binding analysis of YFP-PIF3 in these TOC1oxYFP-PIF3 lines was not significantly affected compared to YFP-PIF3 at ZT24 (Fig. 4b), further suggesting that binding of TOC1 and PIF3 to these promoters can take place simultaneously.

We next examined the induction of expression of co-target genes of PIF3 and TOC1 in TOC1oxYFP-PIF3 lines. In this TOC1 overexpressing line, expression induction of co-

target genes such as *PIL1, AT5G02580 or YUCCA8* was completely abolished (Fig. 4c). These results are consistent with a function of TOC1 as repressor of PIF3 transcriptional activity. The relevance of this repression was revealed by the short phenotype of these lines under SD (Fig. 4d and 4e).

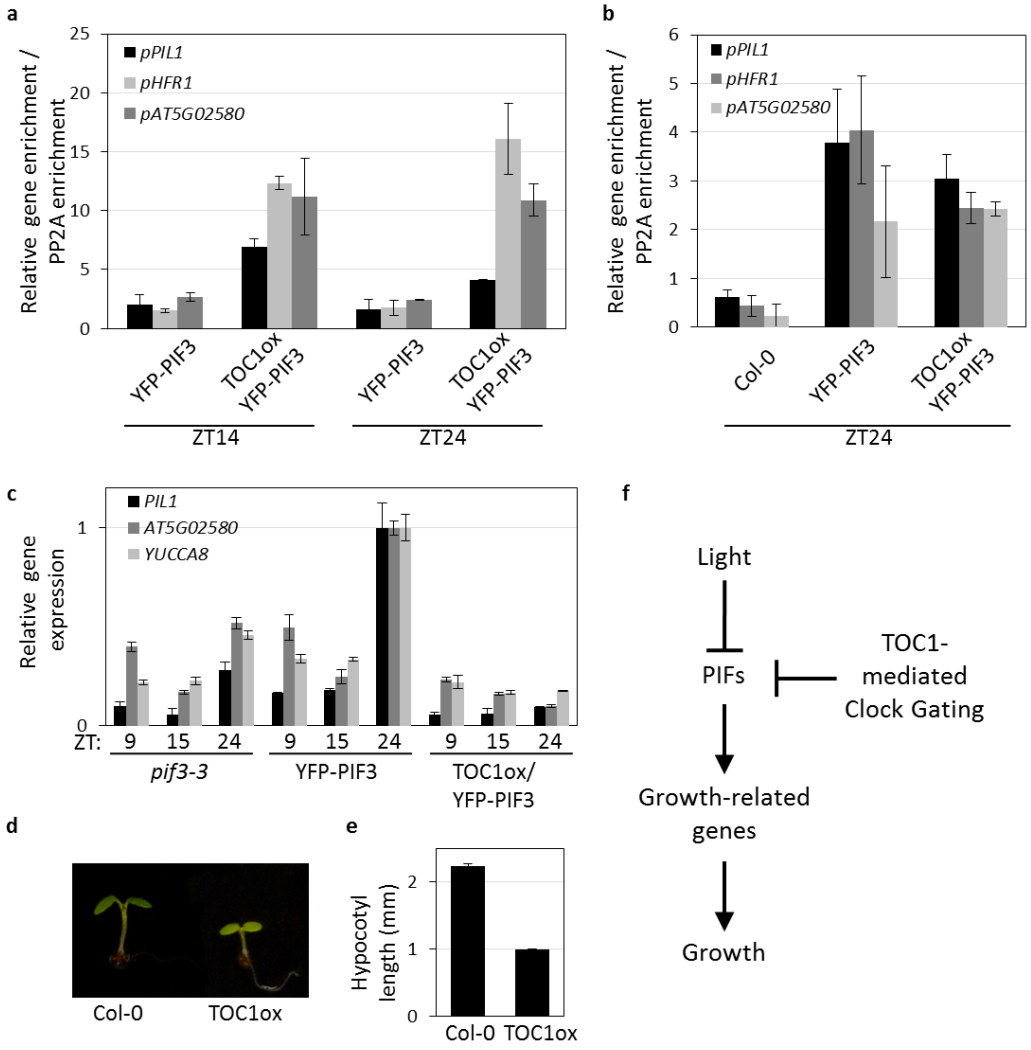


Figure 4. TOC1 is a repressor of PIF3 transcriptional activity in the middle of the night during growth in SD conditions

a) Chromatin immunoprecipitation (ChIP) from 3-day-old SD-grown seedlings. Samples were harvested during the third day of growth at ZT14 and ZT24. Immunoprecipitated DNA was quantified by quantitative RT-PCR using primers in promoter regions containing G-boxes. Enrichment values shown were obtain by normalizing specific gene promoter enrichment values to PP2A enrichment value and relativizing samples processed with anti-MYC antibody to controls processed without antibody. Data shown correspond to one representative biological experiment. Error bars indicate s.e of three technical replicates.

b) Chromatin immunoprecipitation (ChIP) from 3-day-old SD-grown seedlings (samples harvested at ZT24). Immunoprecipitated DNA was quantified by quantitative RT-PCR using primers in promoter regions containing G-boxes. Enrichment values shown were obtain by normalizing specific gene promoter enrichment values to PP2A enrichment value and relativizing samples processed with anti-MYC antibody to controls processed without antibody. Data shown correspond to one representative biological experiment. Error bars indicate s.e of three technical replicates.

c) Visual phenotype of 3-day-old SD-grown Col-0 wild-type and TOC1ox seedlings.

d)Hypocotyl length in 3-day-old SD-grown Col-0 wild-type and TOC1ox seedlings.

e) Expression of *AT5G02580, IAA19 and YUCCA8* analyzed by qRT-PCR in 3-day-old SD-grown seedlings. Seedlings were grown for 2 days in SD conditions and samples were harvested during the third day of growth at ZT9, ZT15 and ZT24. Values were normalized by *PP2A*.

f) Simplified model of the repressing action of TOC1 on PIFs action. Arrows indicate induction. Perpendicular bars indicate repression.

DISCUSSION

In order to determine a possible novel interplay between clock and light in the regulation of hypocotyl growth in SD conditions, *toc1* and *pifs* mutant combinations were used to perform several different phenotypic, genetic and biochemical analysis. Together, results indicate the existence of a new regulatory mechanism in which TOC1 protein represses PIF3 transctiptional activity during approximately the first half of dark period and this repression gates hypocotyl growth to the end of the night.

We provide phenotypic and molecular data that TOC1 gates growth to the end of the night through PIFs based on the following points. 1) a gating regulatory mechanism is controlling hypocotyl elongation (the extend of elongation in the wild-type is different depending on the moment of the subjective night the FRp treatment is done) and seems that TOC1 is the protein in charge of the growth repression during approximately the first half of the dark period (in *toc1* mutant a similar level of hypocotyl elongation was observed at all different time points) (Fig. 1a and 1b). 2) long-hypocotyl phenotype of *toc1* is suppressed in any condition in which PIFs are absent, either by a light-induced PIFs degradation (continuous light growing conditions) or by genetic removal of PIFs in the *toc1* background. PIFs suppression of the long-hypocotyl is seen in different photoperiodic conditions and in end-of-day-FRp treatments (Fig. 1a, 1e and 1f). 3) TOC1 repressive action is also observed in the expression of PIF-target marker genes such as *PIL1* and *HFR1* (Fig. 1c, 1d and 1g). This results indicate that TOC1 gates growth by repressing the expression of growth-related genes, in accordance with the recently reported transcriptional repressive function of TOC1 [18][19][20]. 4) The mechanism we are proposing has to be different from the already reported clock repression of PIF4/5 transcription [11] because neither PIF3 transcription nor protein levels are affected in *toc1* (Supplementary Fig. 3).

Considering that other clock components have also been reported to present altered hypocotyl length compared to wild-type when grown in diurnal conditions, as it is the case of PRR proteins (*prr9prr7prr5* have also a long-hypocotyl phenotype [3]), we cannot discard the other clock components are acting in combination with TOC1 to gate growth in SDs.

Interestingly, phenotypic studies of hypocotyl length done in different growing conditions (continuous light, long-day and short-day) showed that maximum difference in hypocotyl length between Col-0 and *toc1* was seen in SD (Fig. 1e. These results are in accordance with a previous work done in the *toc1-2* mutant in which was shown that the difference in hypocotyl length between the wild-type and the mutant changed depending on the number of hours that the diurnal cycle had [3]. Together, give evidence that the repressive role of TOC1 over stem growth needs that seedlings are exposed to long-night periods to be manifested.

Gene expression analysis indicated that in the *toc1* mutant there was a clear deregulation in the expression of well-known PIFs-target genes. Given that both TOC1 and PIFs are transcription factors that are able to bind to the promoter of genes to regulate their expression [13][14][18][19][23] we decided to explore the possibility that both types of proteins are regulating growth by controlling the expression a common

subset of genes. Indeed, the comparison of available lists of bound genes of TOC1 [18] and PIFs (Anne Pfeiffer and Peter H. Quail, unpublished) showed that a significant amount of TOC1 direct targets are also PIF-bound genes (154 genes) (Supplementary Fig. 4a, Table 1). Interestingly, the analysis by Phaser Website revealed that about one third of these putative co-target genes phase at the end of the night period in SDs (Table 3), coinciding with the moment in which there is a maximum elongation rate[2][4]. Additionally, the analysis of the resulting average expression pattern of the 52 co-target genes using Diurnal Website data clearly showed that these genes present an oscillatory expression profile with a peak of expression at dawn specifically in short-day conditions (Fig. 2a),very similar to that reported for PIFs targets in these growing conditions [4]. By contrast, the average expression of the 52 genes in LD or LL conditions revealed that the amplitude of the oscillations decreased as the number of hours was reduced (Supplementary Fig. 4b). These results indicate that, even these genes are targets of the circadian clock, long periods of dark are needed in order to present oscillations with higher amplitudes in the expression pattern and to peak exactly at dawn. Interestingly, the dark-hours dependence seen at a transcriptional level in the expression of the co-targets correlates with the dark dependence observed at a growth level. Importantly, even the clear diurnal oscillation pattern of the common targets genes of PIF and TOC1, they cannot be considered as outputs of the clock, at least not in a classical view, because all these genes lose the oscillation pattern when seedlings are transferred to continuous light after being entrained in SDs (data not shown).

Expression analysis of selection of these genes in *toc1* and in the triple *pif3pif4pif5* mutants, in comparison to wild-type, showed that TOC1 and PIFs are acting in an antagonistic manner to regulate the expression of the co-target genes, TOC1 acting as a repressor and PIFs as activators (Fig. 2d and Supplementary Fig. 6a and 6b). *toc1* mutant shows overexpression of the target genes during the first hours of the dark period, demonstrating a repressive action of TOC1 on the expression of these genes. On the contrary, there is almost no expression of these genes in the *pif3pif4pif5*, indicating that PIFs induce the expression of these genes and their presence is essential for a proper gene expression. Interestingly, another work had previously related TOC1 protein with the protein PIF7 in the regulation of the expression of the gen *DREB1* [25]. However, the mechanism we are proposing is different than the one because in that work, both transcription factors seem to act as repressors of gene expression while in our work PIFs and TOC1 act antagonistically. Additionally, the fact that there is a direct correlation between the levels of expression of the co-target genes and the hypocotyl growth, underscores the important role that these genes seem to have in hypocotyl elongation. Indeed, several of these genes, such *HFR1*, *IAA29*, *YUCCA8* and *ATHB2*, are hormone-related genes that have been shown to participate in different growing responses in which there is an induction of hypocotyl elongation, as for example shade [26][27][28].

Furthermore, the detailed study of hypocotyl growth rate revealed that the elongation rate peak starts before in *toc1*mutant than in the WT (Fig. 2e). The fact that the absence of TOC1 results in an anticipated peak of growth, give evidence that TOC1 is acting as a repressor of growth. While PIFs were previously shown to promote growth by inducing the expression of growth-related genes [2][4], here we give evidence that

TOC1 gates hypocotyl elongation to the end of the night by repressing growth during the first half of the dark period. Our results indicate that TOC1 carries out gating of growth by repressing the expression of growth-related genes.

The analysis of the binding of both TOC1 and PIF3 to the promoter of the co-targets along the night period showed that in both cases the level of binding roughly correlates to the amount of protein present in the cell (Fig. 3a, 3b and Supplementary Fig. 5b) [4]. These results show that both proteins can be present in the promoter of the co-targets at ZT14. Given that the distance between predicted binding sites of PIFs and TOC1 is less than 50bp for most of the co-targets (Data not shown) and that direct protein-protein interaction was already reported for these proteins [29][30], both proteins might interact in the middle of the night in SDs, a possibility that needs to be tested.

Analysis of PIF3-binding showed that similar levels of binding of YFP-PIF3 are seen in the middle of the night (ZT14) and at the end of night period (ZT24) (Fig. 3b). Intriguingly, levels of expression of the co-targets genes in these two time-points are very different; there is almost no expression at ZT14 while there is a clear induction of expression at the end of the night (Fig. 3c). Such results revealed that there is a lack of correlation between levels of PIF3 binding and gene expression of those genes at ZT14 and suggest that an inhibitory event is happening in the middle of the night over PIF3 activity. The fact that at that same time (ZT14) there are high levels of TOC1 bound to the promoters suggest that both proteins could be present in the promoter of the co-target genes and TOC1 could be repressing the transcription of these genes by inhibiting PIF3 activity. Indeed, in a TOC1 overexpressing line (that accumulates TOC1 in similar levels along the night period (Supplementary Fig. 8)), a high level of binding of TOC1 is seen at ZT24 and then the expression of the genes is abolished (Fig. 4c). These findings indicate that the binding of TOC1 to the promoter is what suppresses the expression. Interestingly, in the TOC1oxYFP-PIF3 line in which the genes are barely expressed, a considerable amount of PIF3 is still bound to the promoter of the genes (Fig. 4b) but now it is unable to induce their expression. This result reinforces the idea that the inhibitory effect of TOC1 over PIF3 seems to be done by affecting its transcriptional activity rather than over its capacity to bind to DNA.

Importantly, the increased repressive action of TOC1 over PIF3 activity in a TOC1ox line has a direct impact on hypocotyl growth, presenting a very short-hypocotyl phenotype (Fig. 4c and 4d) (phenotypically resembling a multiple *pif* deficient mutant).

Together the data is consistent with a model where TOC1 gates hypocotyl growth precisely at dawn in SDs by repressing the transcriptional activity of PIF3 and probably also PIF4 and PIF5 (Fig. 4f) and, as a consequence, restricting the expression of growth-related genes at the end of the night.

EXPERIMENTAL PROCEDURES

Seedling growth and hypocotyl measurements

Arabidopsis thaliana seeds used in the experiments showed in this manuscript were from Columbia and C24 ecotype. Wild-type Col-0 and the previously described *pif3-3* [31], *pif4-2* [32], *pif5-3* [33], *pif4pif5* [34], *pif3pif4pif5* [35], *toc1-101*[36][37],

pif3::YFP-PIF3 [38] and 35S::cMYC-TOC1 [18] were Columbia ecotype. Triple *pif4pif5toc1* and quadruple *pif3pif4pif5toc1* mutants were generated by crossing *pif3pif4pif5* and *toc1-101* lines. Double mutant TOC1ox/YFP-PIF3 was obtained by crossing 35S::cMYC-TOC1 and pif3::YFP-PIF3 lines. Wild-type C24 and toc1::TMG-YFP [18] are C24 ecotype.

Seeds sterilization and stratification were done as previously described in [4]. Seedling growth was done in short-day (8h white light ($85\mu mol \cdot m^{-2} \cdot ^{s-1}$) and 16h dark), long-day (16h white light ($85\mu mol \cdot m^{-2} \cdot s^{-1}$) and 8h dark) or continuous light (24h white light ($85\mu mol \cdot m^{-2} \cdot s^{-1}$)) for the time indicated in each experiment. Far Red pulses were done as previously described [10].Hypocotyl measurements were done as previously described [31]. For hypocotyl growth rate measurements, seedling growth and image acquisition were done as previously described [4].

Gene expression analysis

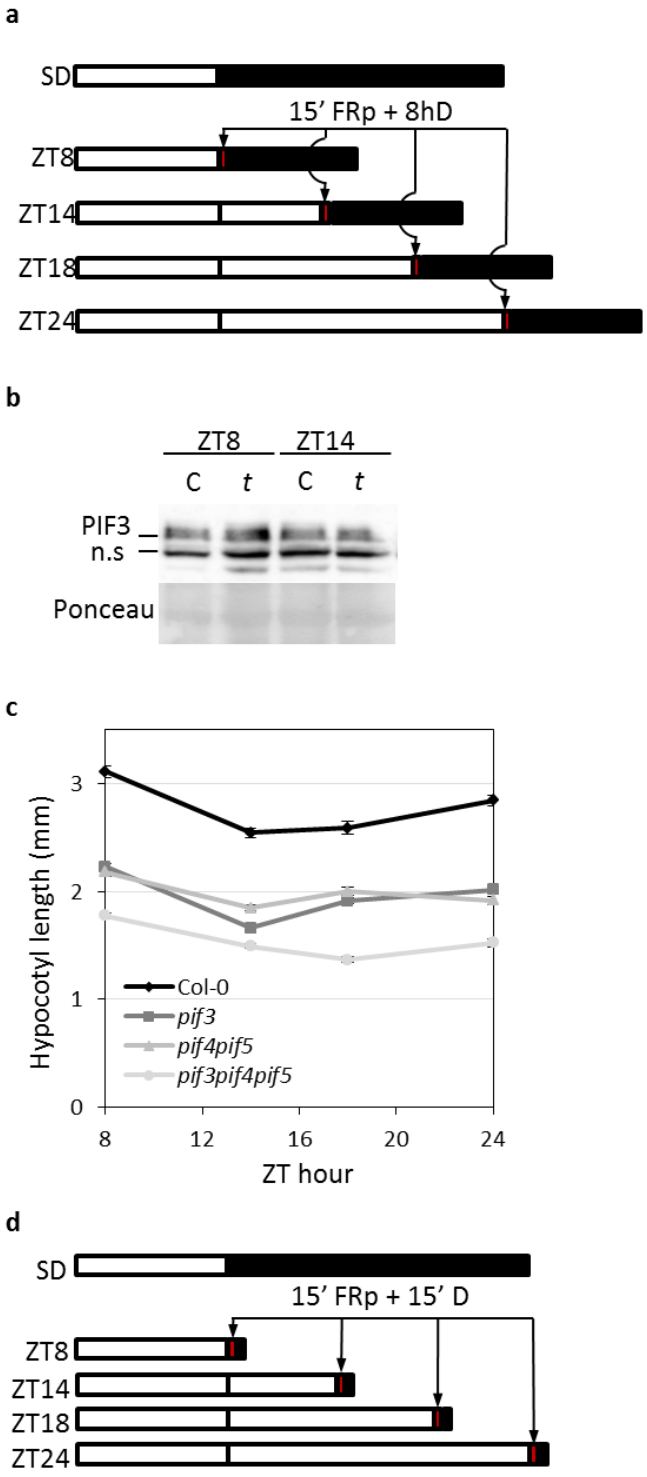
For quantitative RT-PCR, RNA extraction, cDNA synthesis and qRT-PCR were done as described [39]. Gene expression was measured in at least two technical replicates for each biological sample. *PP2A* (AT1G13320) gene was used for normalization as described [40]. Table 6 contains a list of primer sequences.

Protein extraction and Immunoblots

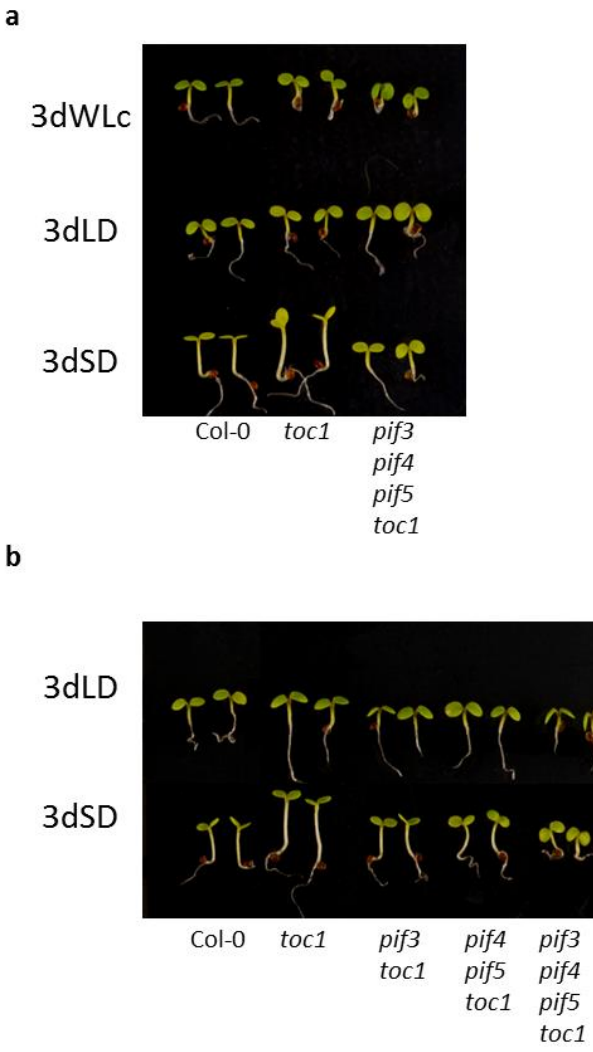
Protein extracts were prepared from 2 and 3 day-old seedlings grown under short-dayconditions. Total protein extracts were obtained by resuspending tissue samples previously grinded in frozen conditions. Extraction buffer and protein quantification were done as described in [32]. Immunoblots to detect endogenous PIF3 and YFP-PIF3 were done as described in [4]. For immunoblots to detected both TMG-YFP and MYC-TOC1, 80µg of total protein were loaded to a 7.5% SDS-PAGE. For YFP immunodetection, anti-GFP polyclonal (Invitrogen) was use and for MYC, monoclonal anti-cMYC (SIGMA). As secondary antibody, peroxidase-linked anti-rabbit antibody (Amersham Biosciences) for YFP and anti-mouse antibody for MYC (Amersham Biosciences) were used. Detection was performed as described in [4].

Chromatin Immunoprecipitation (ChIP) Assays

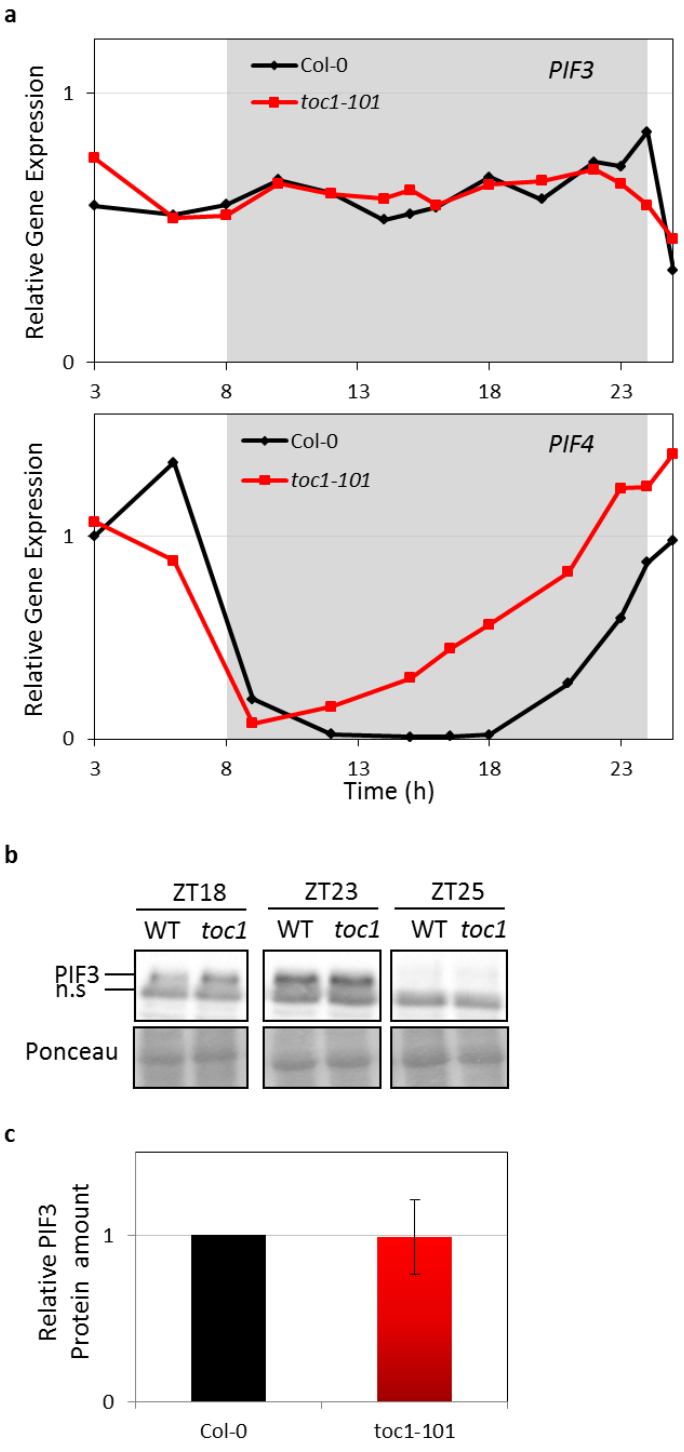
ChIP assays were performed using from 2 to 3 day-old seedling grown in short-day conditions as previously described in [4]. For YFP-PIF3 ChIP assays, immunoprecipitation of antibody samples was done by an overnight incubation with GFP antibody-bound resin (GFP Agarose Beads, MBL). Mock ChIP reactions were performed without antibody to measure non-specific binding to target sequences was done using a For TMG and TOC1ox/YFP-PIF3 ChIP, both mock and antibody samples were incubated overnight with rProtein A-Sepharose (Bionova) and 2.5 µl of anti-GFP polyclonal antibody or 2.5 µl of anti-cMYC antibody respectively (Amersham Biosciences) were added to antibody samples. Purified DNA obtained at the end of the ChIP procedure was subjected to quantitative RT-PCR using promoter-specific primers for each gene (Table 6).



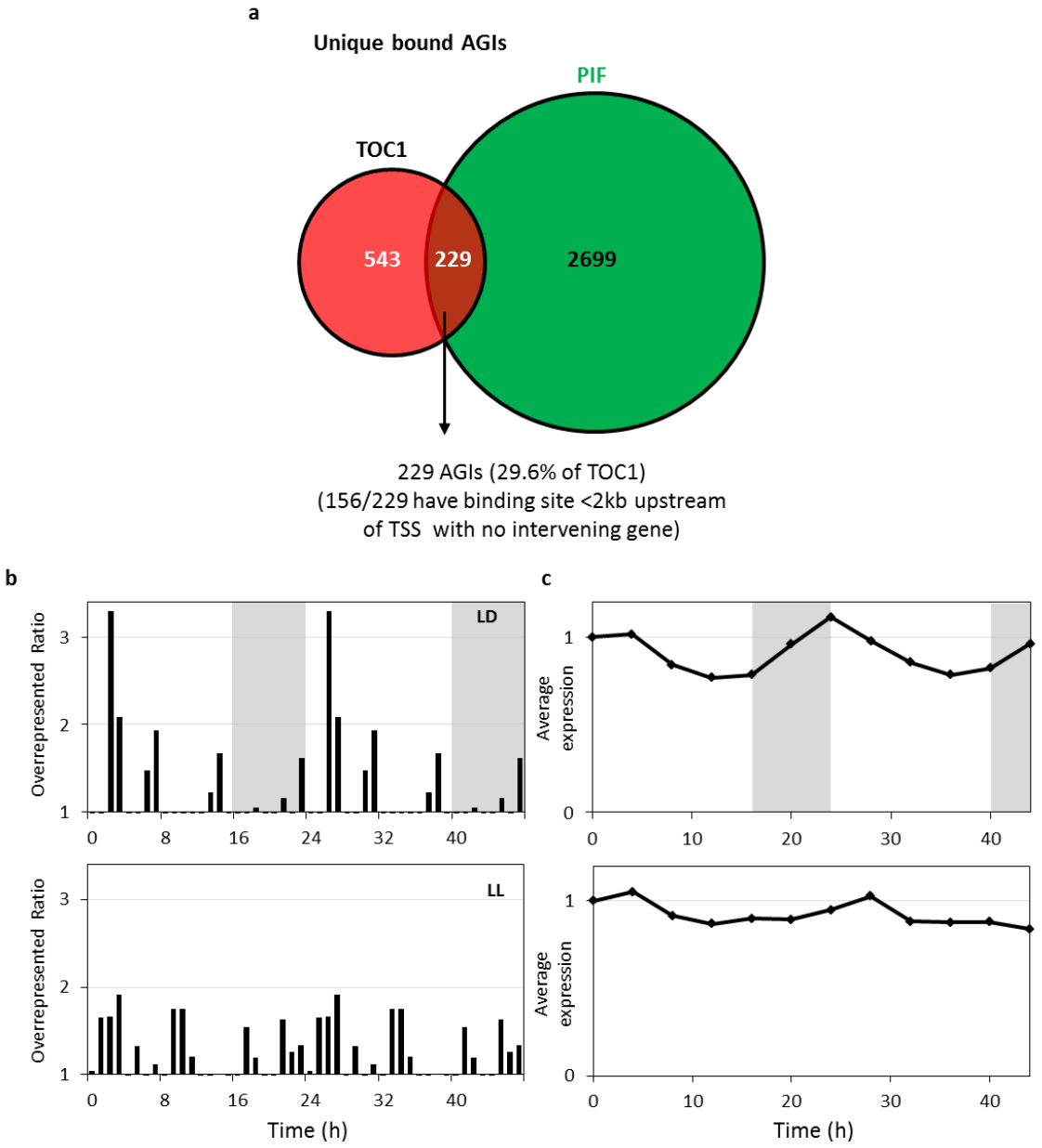
Supplementary Figure 1
a) and c) Scheme of the experimental design. Seedlings were grown for 2 days in SD conditions and during the third day of growth a FRp treatment of 15 min was done at different times. After the FRp seedlings were kept in dark. White rectangles represent light, red for the FRp and black for dark. a) For phenotypic studies a picture was taken just before the FRp and a second picture after 8h of dark. b) For gene expression samples were harvested just before the FRp and after 15 min of dark period.
b) Immunoblots of protein extracts from 3-day-old wild-type (C) and *toc1-101* mutant (t). Seedlings were grown under SD conditions for 2 days and at ZT8 and ZT14 were treated with a 15 minutes FRp and then kept in dark for 15 minutes. Extracts were probed using PIF3-specific polyclonal antibody (top). Ponceay staining was used as a loading control (bottom)



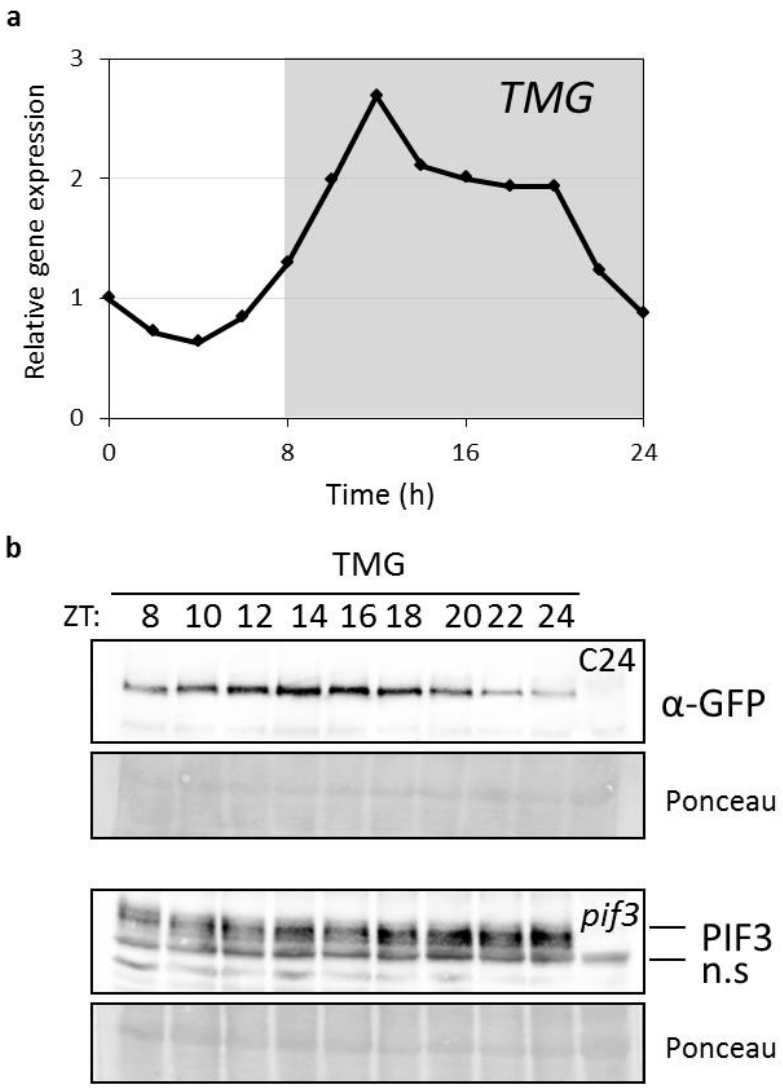
Supplementary Figure 2
a)Visual phenotype of 3-day-old Col-0 wild-type, *toc1-101* and *pif3pif4pif5toc1* seedlings grown for 3 days in continous white light (3dWLc), 3 days in long-day conditions (3dLD) or 3 days in short-day conditions (3dSD).
b) Visual phenotype of 3-day-old Col-0 wild-type, *toc1-101*, *pif3toc1*, *pif4pif5toc1* and *pif3pif4pif5toc1* seedlings grown for 3 days in long-day conditions (3dLD) or 3 days in short-day conditions (3dSD).



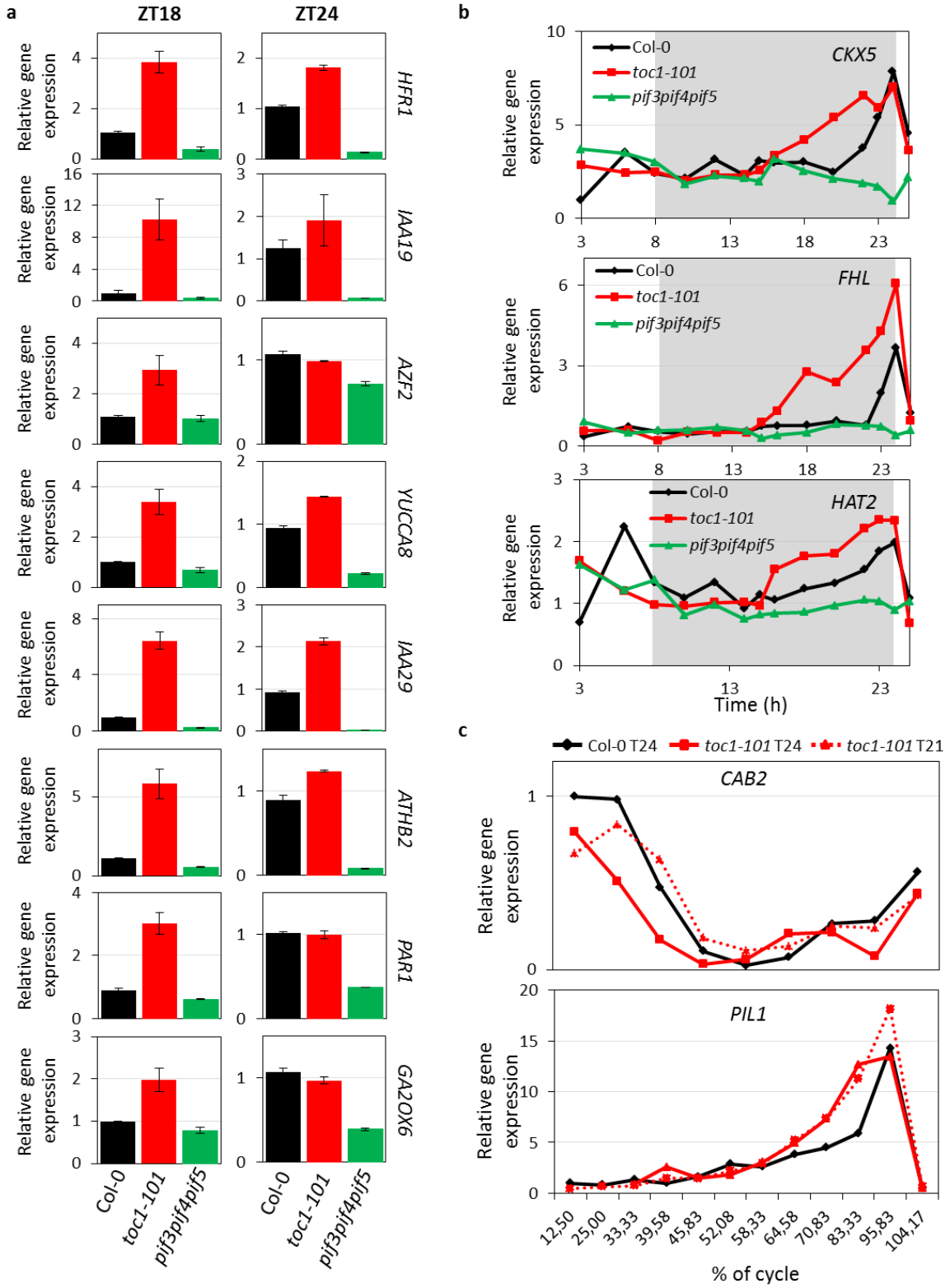
Supplementary Figure 3
a) Expression of *PIF3* and *PIF4* analyzed by qRT-PCR in 3-day-old SD-grown Col-0 wild-type and *toc1-101* seedlings. Seedlings were grown for 2 days in SD conditions and samples were harvested during the third day of growth. Values were normalized by *PP2A*.
b) Immunoblots of protein extracts from 3-day-old wild-type (C) and *toc1-101* mutant (t). Seedlings were grown under SD conditions for 2 days and samples were harvested during the third day of growth at the indicated time-points (ZT18, ZT23 and ZT25). Extracts were probed using PIF3-specific polyclonal antibody (top). Ponceau staining was used as a loading control (bottom).
c) Quantification of PIF3 protein levels in *toc1-101* mutant relative to levels of PIF in Col-0 wild-type at ZT23. Data is the mean of 3 biological replicates. Error bars indicate s.e.



Supplementary Figure 4
a) Comparison ofbound genes of TOC1 (Red) and PIFs (green). 229 AGIs are common between TOC1 and PIFs.
b) Overrepresentation ratio obtained using Phaser Website of the 133 predicted co-target genes of TOC1 and PIFs in long-day conditions (LD) or in continuous whitelight conditions (LL).
c) Average expression of the 52 predicted co-target genes of TOC1 and PIFs in long-day (LD) and continuous white light (LL) conditions. For both light conditions, expression data of each gene (obtained from Diurnal Website) was used to calculate fold change values relative to ZT0. Average expression value represented in the graphs was calculated using the fold change data of all 52 genes.



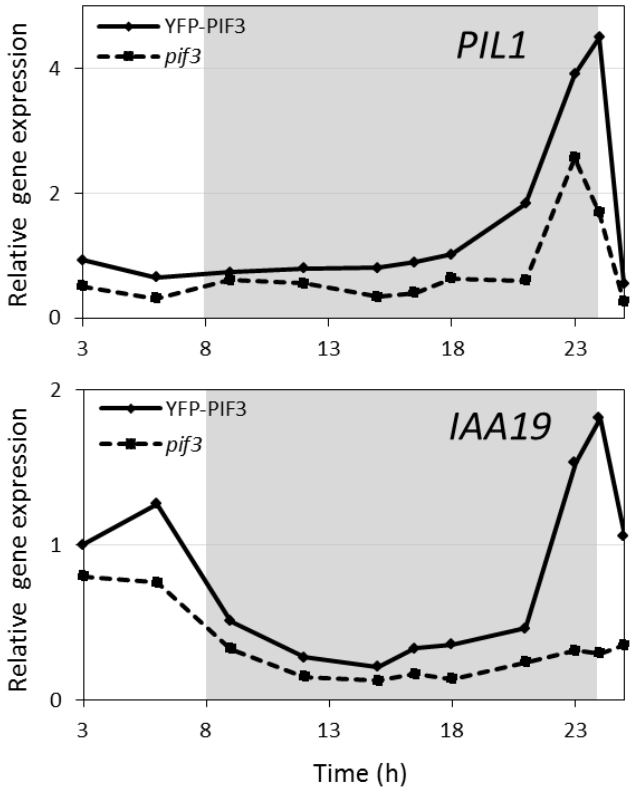
Supplementary Figure 5
a) Expression of TMG analyzed by qRT-PCR. Seedlings were grown for 2 days in SD conditions and samples were harvested at the indicated times during the third day of growth. Values were normalized by *PP2A*.
b) Immunoblots of protein extracts from 3-day-old TMG seedlings. Seedlings were grown under SD conditions for 2 days and samples were harvested during the third day of growth at the indicated time-points. Samples were probed with anti-GFP monoclonal antibody or anti-PIF3 polyclonal antibody. Ponceau staining was used as a loading control.



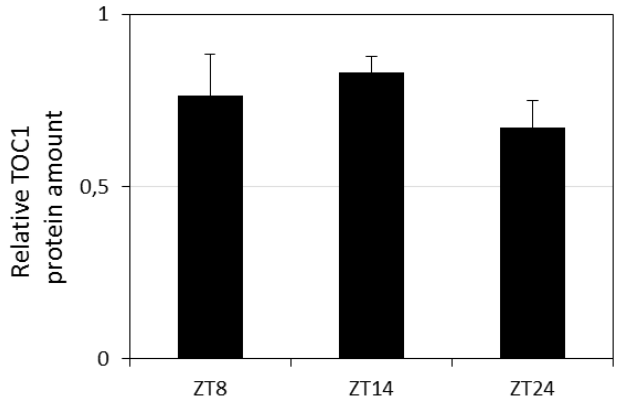
Supplementary Figure 6

a) and b) Gene expression data analyzed by qRT-PCR of Col-0 wild-type, *toc1-101* and *pif3pif4pif5* 3-day-old seedlings. Seedlings were grown for 2 days in SD conditions and samples were harvested at the indicated times. Values were normalized by *PP2A*. a) Gene expression was analyzed at ZT18 (graphs on the left) and ZT24 (graphs on the right). b) Expression of *CKX5*, *FHL* and *HAT2* was analyzed during the third day of growth in SD.

c) Expression of *CAB2* and *PIL1* genes was analyzed in Col-0 and *toc1-101* seedlings grown for 3 days in 24h short-day cycles (T24, 8h light and 16h dark) or 21h short-day cycles (T21, 7h light and 14h dark). Data is plotted as function of % of the diurnal cycle. Values were normalized by *PP2A*.



Supplementary Figure 7
Expression of *PIL1* and *IAA19* genes was analyzed in YFP-PIF3 and *pif3-3* seedlings grown for 3 days in SD. Samples were harvested during the third-day of growth. Values were normalized by *PP2A*.



Supplementary Figure 8
TOC1 protein levels in TOC1oxYFP-PIF3. Seedlings were grown for 2 days in SD and samples were harvested at the indicated times. Data is the mean of 3 biological replicates. Error bars indicate s.e.

Table 1. List of 154 common TOC1 and PIFs targets genes.

AGI	Gene Name
AT1G01520	
AT1G02060	
AT1G02065	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 8 (SPL8)
AT1G02340	LONG HYPOCOTYL IN FAR-RED (HFR1)
AT1G02350	
AT1G02391	
AT1G02400	GIBBERELLIN 2-OXIDASE 6 (GA2OX6)
AT1G06570	PHYTOENE DESATURATION 1 (PDS1)
AT1G09260	
AT1G09350	GALACTINOL SYNTHASE 3 (GolS3)
AT1G09520	
AT1G13080	CYTOCHROME P450 71B2 (CYP71B2)
AT1G17230	
AT1G18740	
AT1G19210	
AT1G19490	
AT1G19870	IQ-DOMAIN 32 (iqd32)
AT1G20890	
AT1G31410	
AT1G31420	FEI 1 (FEI1)
AT1G33580	
AT1G68020	(ATTPS6)
AT1G69260	ABI FIVE BINDING PROTEIN (AFP1)
AT1G73480	
AT1G73670	MAP KINASE 15 (MPK15)
AT1G75060	
AT1G75070	
AT1G75450	CYTOKININ OXIDASE 5 (CKX5)
AT1G76080	CHLOROPLASTIC DROUGHT-INDUCED STRESS PROTEIN OF 32 KD (CDSP32)
AT1G76170	
AT1G76590	
AT1G77450	NAC DOMAIN CONTAINING PROTEIN 32 (NAC032)
AT2G02710	PAS/LOV PROTEIN B (PLPB)
AT2G02720	
AT2G03550	
AT2G18780	
AT2G18790	PHYTOCHROME B (PHYB)
AT2G27500	
AT2G27505	
AT2G28130	
AT2G30520	ROOT PHOTOTROPISM 2 (RPT2)
AT2G37170	PLASMA MEMBRANE INTRINSIC PROTEIN 2 (PIP2B)
AT2G37180	RESPONSIVE TO DESICCATION 28 (RD28)
AT2G41870	
AT2G41880	GUANYLATE KINASE 1 (GK-1)
AT2G43010	PHYTOCHROME INTERACTING FACTOR 4 (PIF4)
AT2G44660	
AT2G45730	
AT2G45740	PEROXIN 11D (PEX11D)

AGI	Gene Name
AT2G45820	
AT2G45830	DOWNSTREAM TARGET OF AGL15 2 (DTA2)
AT2G46330	ARABINOGALACTAN PROTEIN 16 (AGP16)
AT2G46410	CAPRICE (CPC)
AT2G46420	
AT2G46780	
AT2G47000	ATP BINDING CASSETTE SUBFAMILY B4 (ABCB4)
AT2G47010	
AT3G02140	TWO OR MORE ABRES-CONTAINING GENE 2 (TMAC2)
AT3G02410	ISOPRENYLCYSTEINE METHYLESTERASE-LIKE 2 (ICME-LIKE2)
AT3G03456	
AT3G07350	
AT3G07360	PLANT U-BOX 9 (PUB9)
AT3G12300	
AT3G12320	
AT3G12920	
AT3G18060	
AT3G19170	PRESEQUENCE PROTEASE 1 (PREP1)
AT3G19180	PARALOG OF ARC6 (PARC6)
AT3G19580	ZINC-FINGER PROTEIN 2 (ZF2)
AT3G19590	BUB (BUDDING UNINHIBITED BY BENZYMIDAZOL) 3.1 (BUB3.1)
AT3G20250	PUMILIO 5 (PUM5)
AT3G20820	
AT3G22380	TIME FOR COFFEE (TIC)
AT3G24840	
AT3G27060	TSO MEANING 'UGLY' IN CHINESE (TSO2)
AT3G27210	
AT3G46630	
AT3G46640	
AT3G50060	MYB DOMAIN PROTEIN 77 (MYB77)
AT3G52880	MONODEHYDROASCORBATE REDUCTASE 1 (MDAR1)
AT3G53810	
AT3G53830	
AT3G55430	
AT3G55440	TRIOSEPHOSPHATE ISOMERASE (TPI)
AT3G55580	
AT3G60190	DYNAMIN-LIKE 1E (DL1E)
AT3G60200	
AT3G60400	
AT3G62640	
AT3G62650	
AT3G63010	GA INSENSITIVE DWARF1B (GID1B)
AT4G00810	
AT4G00820	IQ-DOMAIN 17 (iqd17)
AT4G04210	LANT UBX DOMAIN-CONTAINING PROTEIN 4 (PUX4)
AT4G12423	
AT4G16490	
AT4G17550	
AT4G17560	
AT4G18700	CBL-INTERACTING PROTEIN KINASE 12 (CIPK12)

AGI	Gene Name
AT4G18890	BES1/BZR1 HOMOLOG 3 (BEH3)
AT4G19040	ENHANCED DISEASE RESISTANCE 2 (EDR2)
AT4G22590	
AT4G23040	
AT4G23050	
AT4G25470	C-REPEAT/DRE BINDING FACTOR 2 (CBF2)
AT4G25490	C-REPEAT/DRE BINDING FACTOR 1 (CBF1)
AT4G25500	ARGININE/SERINE-RICH SPLICING FACTOR 35 (RSP35)
AT4G27300	
AT4G27310	
AT4G27657	
AT4G28240	
AT4G30350	
AT4G32800	
AT4G33240	FORMS APLOID AND BINUCLEATE CELLS 1A (FAB1A)
AT4G33905	
AT4G35750	
AT4G35850	
AT4G36030	ARMADILLO REPEAT ONLY 3 (ARO3)
AT4G38430	RHO GUANYL-NUCLEOTIDE EXCHANGE FACTOR 1 (ROPGEF1)
AT4G38470	
AT4G39400	BRASSINOSTEROID INSENSITIVE 1 (BRI1)
AT4G39800	MYO-INOSITOL-1-PHOSTPATE SYNTHASE 1 (MIPS1)
AT4G40011	
AT5G02200	FAR-RED-ELONGATED HYPOCOTYL1-LIKE (FHL)
AT5G02580	
AT5G05250	
AT5G05430	
AT5G05440	PYRABACTIN RESISTANCE 1-LIKE 5 (PYL5)
AT5G10740	
AT5G10745	
AT5G11260	ELONGATED HYPOCOTYL 5 (HY5)
AT5G13090	
AT5G13100	
AT5G13760	
AT5G18910	
AT5G18920	
AT5G24090	CHITINASE A (CHIA)
AT5G24100	
AT5G24470	PSEUDO-RESPONSE REGULATOR 5 (PRR5)
AT5G24810	
AT5G24820	
AT5G41590	
AT5G41600	VIRB2-INTERACTING PROTEIN 3 (BTI3)
AT5G44220	
AT5G47370	(HAT2)
AT5G51460	(ATTPPA)
AT5G54470	
AT5G54480	
AT5G58070	TEMPERATURE-INDUCED LIPOCALIN (TIL)

AGI	Gene Name
AT5G58530	
AT5G61970	
AT5G64430	
AT5G64440	FATTY ACID AMIDE HYDROLASE (FAAH)
AT5G65300	

Table 2. Phase information of the 133 out of 154 that appear in Phaser Website

AGI	Gene Name	PHASE in SD	pvalue in SD	PHASE in LD	pvalue in LD	PHASE in LL (LDHH)	pvalue in LL
AT1G01520		4	0,611	4	0,849	22	0,394
AT1G02060		20	0,509	21	0,381	5	0,258
AT1G02340	HFR1	23	0,003	0	0,625	2	0,153
AT1G02400	GA2OX6	18	0,018	2	0,001	3	0,038
AT1G06570	PDS1	23	0,003	3	0,023	23	0,320
AT1G09350	GolS3	6	0,938	7	0,092	3	0,038
AT1G09520		16	0,546	6	0,250	23	0,320
AT1G13080	CYP71B2	3	0,447	2	0,001	22	0,394
AT1G17230		15	0,357	13	0,385	17	0,148
AT1G18740		5	0,660	2	0,001	2	0,153
AT1G19210		8	0,738	4	0,849	10	0,089
AT1G19870	iqd32	18	0,018	23	0,106	16	0,700
AT1G20890		18	0,018	22	0,555	7	0,433
AT1G31410		10	0,605	18	0,532	12	0,985
AT1G31420	FEI1	16	0,546	23	0,106	17	0,148
AT1G33580		8	0,738	19	0,736	4	0,597
AT1G68020	ATTPS6	19	0,443	3	0,023	6	0,911
AT1G69260	AFP1	22	0,236	3	0,023	18	0,388
AT1G73480		4	0,611	5	0,568	3	0,038
AT1G73670	MPK15	2	0,284	5	0,568	21	0,141
AT1G75450	CKX5	23	0,003	3	0,023	1	0,159
AT1G76080	CDSP32	5	0,660	10	0,584	4	0,597
AT1G76170		15	0,357	16	0,897	15	0,914
AT1G76590		9	0,780	13	0,385	10	0,089
AT1G77450	NAC032	23	0,003	21	0,381	17	0,148
AT2G02710	PLPB	23	0,003	7	0,092	5	0,258
AT2G02720		0	0,832	14	0,109	0	0,513
AT2G03550		8	0,738	14	0,109	13	0,589
AT2G18780		23	0,003	10	0,584	20	0,906
AT2G18790	PHYB	23	0,003	8	0,802	21	0,141
AT2G27500		18	0,018	21	0,381	15	0,914
AT2G28130		14	0,489	21	0,381	18	0,388
AT2G30520	RPT2	23	0,003	0	0,625	0	0,513
AT2G37180	RD28	1	0,417	2	0,001	2	0,153
AT2G41870		3	0,447	5	0,568	22	0,394
AT2G41880	GK-1	18	0,018	3	0,023	0	0,513
AT2G43010	PIF4	4	0,611	7	0,092	5	0,258
AT2G44660		11	0,973	19	0,736	16	0,700
AT2G45730		14	0,489	15	0,601	16	0,700
AT2G45740	PEX11D	8	0,738	14	0,109	13	0,589
AT2G45820		22	0,236	22	0,555	8	1,000
AT2G45830	DTA2	22	0,236	2	0,001	11	0,397
AT2G46330	AGP16	18	0,018	22	0,555	5	0,258
AT2G46410	CPC	3	0,447	13	0,385	21	0,141
AT2G46420		17	0,898	15	0,601	19	0,563
AT2G46780		2	0,284	23	0,106	8	1,000
AT2G47000	ABCB4	12	0,853	0	0,625	17	0,148
AT2G47010		8	0,738	20	0,990	19	0,563
AT3G02140	TMAC2	20	0,509	23	0,106	20	0,906

AGI	Gene Name	PHASE in SD	pvalue in SD	PHASE in LD	pvalue in LD	PHASE in LL (LDHH)	pvalue in LL
AT3G07350		0	0,832	3	0,023	3	0,038
AT3G07360	PUB9	2	0,284	4	0,849	23	0,320
AT3G12300		21	0,136	3	0,023	3	0,038
AT3G12320		1	0,417	3	0,023	1	0,159
AT3G12920		18	0,018	2	0,001	21	0,141
AT3G18060		9	0,780	10	0,584	9	0,195
AT3G19170	PREP1	7	0,999	9	0,805	19	0,563
AT3G19580	ZF2	21	0,136	23	0,106	3	0,038
AT3G19590	BUB3.1	10	0,605	10	0,584	7	0,433
AT3G20250	PUM5	19	0,443	2	0,001	18	0,388
AT3G20820		19	0,443	5	0,568	5	0,258
AT3G22380	TIC	16	0,546	18	0,532	11	0,397
AT3G24840		3	0,447	6	0,250	20	0,906
AT3G27060	TSO2	10	0,605	11	0,892	13	0,589
AT3G27210		10	0,605	12	0,992	10	0,089
AT3G46630		6	0,938	10	0,584	6	0,911
AT3G46640		9	0,780	13	0,385	10	0,089
AT3G50060	MYB77	4	0,611	22	0,555	3	0,038
AT3G52880	MDAR1	5	0,660	10	0,584	22	0,394
AT3G53810		1	0,417	22	0,555	2	0,153
AT3G55430		0	0,832	0	0,625	7	0,433
AT3G55440	TPI	12	0,853	11	0,892	15	0,914
AT3G55580		3	0,447	4	0,849	23	0,320
AT3G60190	DL1E	14	0,489	15	0,601	10	0,089
AT3G60200		17	0,898	12	0,992	10	0,089
AT3G60400		13	0,977	7	0,092	12	0,985
AT3G62640		19	0,443	17	0,974	0	0,513
AT3G62650		18	0,018	22	0,555	7	0,433
AT3G63010	GID1B	18	0,018	1	0,787	17	0,148
AT4G00810		12	0,853	23	0,106	17	0,148
AT4G00820	iqd17	0	0,832	1	0,787	4	0,597
AT4G04210	PUX4	1	0,417	8	0,802	3	0,038
AT4G16490		20	0,509	15	0,601	18	0,388
AT4G17550		0	0,832	3	0,023	20	0,906
AT4G17560		15	0,357	15	0,601	11	0,397
AT4G18700	CIPK12	21	0,136	0	0,625	2	0,153
AT4G18890	BEH3	4	0,611	16	0,897	23	0,320
AT4G19040	EDR2	5	0,660	6	0,250	11	0,397
AT4G23040		15	0,357	16	0,897	10	0,089
AT4G23050		1	0,417	14	0,109	0	0,513
AT4G25470	CBF2	2	0,284	7	0,092	7	0,433
AT4G25490	CBF1	8	0,738	10	0,584	1	0,159
AT4G25500	RSP35	18	0,018	16	0,897	9	0,195
AT4G27300		4	0,611	5	0,568	1	0,159
AT4G27657		4	0,611	16	0,897	17	0,148
AT4G28240		21	0,136	6	0,250	4	0,597
AT4G30350		15	0,357	17	0,974	13	0,589
AT4G32800		14	0,489	14	0,109	16	0,700
AT4G33240	FAB1A	22	0,236	8	0,802	10	0,089
AT4G33905		20	0,509	4	0,849	20	0,906
AT4G35750		18	0,018	21	0,381	9	0,195

AGI	Gene Name	PHASE in SD	pvalue in SD	PHASE in LD	pvalue in LD	PHASE in LL (LDHH)	pvalue in LL
AT4G35850		16	0,546	10	0,584	6	0,911
AT4G36030	ARO3	12	0,853	22	0,555	0	0,513
AT4G38430	ROPGEF1	4	0,611	16	0,897	16	0,700
AT4G38470		19	0,443	22	0,555	9	0,195
AT4G39400	BRI1	22	0,236	23	0,106	4	0,597
AT4G39800	MIPS1	5	0,660	8	0,802	18	0,388
AT5G02200	FHL	23	0,003	18	0,532	5	0,258
AT5G02580		23	0,003	4	0,849	1	0,159
AT5G05250		16	0,546	14	0,109	19	0,563
AT5G05430		20	0,509	20	0,990	4	0,597
AT5G05440	PYL5	16	0,546	21	0,381	4	0,597
AT5G10740		18	0,018	23	0,106	4	0,597
AT5G11260	HY5	20	0,509	14	0,109	18	0,388
AT5G13090		21	0,136	2	0,001	2	0,153
AT5G13100		21	0,136	23	0,106	21	0,141
AT5G13760		18	0,018	3	0,023	1	0,159
AT5G18910		23	0,003	21	0,381	4	0,597
AT5G18920		19	0,443	8	0,802	3	0,038
AT5G24090	CHIA	20	0,509	2	0,001	17	0,148
AT5G24100		23	0,003	19	0,736	0	0,513
AT5G24470	PRR5	8	0,738	8	0,802	7	0,433
AT5G24810		4	0,611	15	0,601	19	0,563
AT5G24820		15	0,357	13	0,385	21	0,141
AT5G41600	BTI3	6	0,938	8	0,802	5	0,258
AT5G47370	HAT2	21	0,136	2	0,001	5	0,258
AT5G51460	ATTPPA	22	0,236	22	0,555	19	0,563
AT5G54470		2	0,284	6	0,250	3	0,038
AT5G54480		3	0,447	18	0,532	11	0,397
AT5G58070	TIL	5	0,660	7	0,092	4	0,597
AT5G58530		17	0,898	22	0,555	16	0,700
AT5G61970		18	0,018	21	0,381	7	0,433
AT5G64440	FAAH	16	0,546	14	0,109	8	1,000
AT5G65300		9	0,780	11	0,892	21	0,141

Table 3. List of 52 gene that have a phase peak between ZT18 and ZT23

AGI	Gene Name	PHASE in SD	pvalue in SD	PHASE in LD	pvalue in LD	PHASE in LL (LDHH)	pvalue in LL
AT1G02060		20	0,795	21	0,155	5	0,300
AT1G02340	HFR1	23	0,005	0	0,558	2	0,047
AT1G02400	GA2OX6	18	0,005	2	0,002	3	0,098
AT1G06570	PDS1	23	0,005	3	0,060	23	0,495
AT1G19870	iqd32	18	0,005	23	0,135	16	0,825
AT1G20890		18	0,005	22	0,182	7	0,464
AT1G68020	ATTPS6	19	0,693	3	0,060	6	0,964
AT1G69260	AFP1	22	0,594	3	0,060	18	0,344
AT1G75450	CKX5	23	0,005	3	0,060	1	0,100
AT1G77450	NAC032	23	0,005	21	0,155	17	0,253
AT2G02710	PLPB	23	0,005	7	0,187	5	0,300
AT2G18780		23	0,005	10	0,585	20	0,659
AT2G18790	PHYB	23	0,005	8	0,598	21	0,322
AT2G27500		18	0,005	21	0,155	15	0,580
AT2G30520	RPT2	23	0,005	0	0,558	0	0,562
AT2G41880	GK-1	18	0,005	3	0,060	0	0,562
AT2G45820		22	0,594	22	0,182	8	0,859
AT2G45830	DTA2	22	0,594	2	0,002	11	0,590
AT2G46330	ATAGP16	18	0,005	22	0,182	5	0,300
AT3G02140	AFP4	20	0,795	23	0,135	20	0,659
AT3G12300		21	0,111	3	0,060	3	0,098
AT3G12920		18	0,005	2	0,002	21	0,322
AT3G19580	ZF2	21	0,111	23	0,135	3	0,098
AT3G20250	PUM5	19	0,693	2	0,002	18	0,344
AT3G20820		19	0,693	5	0,345	5	0,300
AT3G62640		19	0,693	17	0,998	0	0,562
AT3G62650		18	0,005	22	0,182	7	0,464
AT3G63010	GID1B	18	0,005	1	0,932	17	0,253
AT4G16490		20	0,795	15	0,913	18	0,344
AT4G18700	CIPK12	21	0,111	0	0,558	2	0,047
AT4G25500	RSP35	18	0,005	16	0,966	9	0,449
AT4G28240		21	0,111	6	0,033	4	0,864
AT4G33240	FAB1A	22	0,594	8	0,598	10	0,086
AT4G33905		20	0,795	4	0,814	20	0,659
AT4G35750		18	0,005	21	0,155	9	0,449
AT4G38470		19	0,693	22	0,182	9	0,449
AT4G39400	BRI1	22	0,594	23	0,135	4	0,864
AT5G02200	FHL	23	0,005	18	0,827	5	0,300
AT5G02580		23	0,005	4	0,814	1	0,100
AT5G05430		20	0,795	20	0,990	4	0,864
AT5G10740		18	0,005	23	0,135	4	0,864
AT5G11260	HY5	20	0,795	14	0,030	18	0,344
AT5G13090		21	0,111	2	0,002	2	0,047
AT5G13100		21	0,111	23	0,135	21	0,322
AT5G13760		18	0,005	3	0,060	1	0,100
AT5G18910		23	0,005	21	0,155	4	0,864
AT5G18920		19	0,693	8	0,598	3	0,098
AT5G24090	CHIA	20	0,795	2	0,002	17	0,253
AT5G24100		23	0,005	19	0,510	0	0,562

AGI	Gene Name	PHASE in SD	pvalue in SD	PHASE in LD	pvalue in LD	PHASE in LL (LDHH)	pvalue in LL
AT5G47370	HAT2	21	0,111	2	0,002	5	0,300
AT5G51460	ATTPPA	22	0,594	22	0,182	19	0,556
AT5G61970		18	0,005	21	0,155	7	0,464

Table 4. PIF-bound genes with a peak phase at the end of the night

AGI	Gene Name	PHASE in SD	PHASE in LD	PHASE in LL
AT2G42870	PAR1	23	22	5
AT2G46970	PIL1	ND	ND	ND
AT3G15540	IAA19	1	1	0
AT4G14130	XTR7	22	0	3
AT4G16780	ATHB2	23	0	2
AT4G28720	YUC8	23	4	2
AT4G32280	IAA29	23	0	5
AT5G53980	ATHB52	18	0	6

Table 5. Phase in SD of 441 out of 543 TOC1 Unique bound AGIs

AGI	PHASE in SD	pvalue in SD
AT1G01060	23	0,147
AT1G01070	23	0,147
AT1G01420	16	0,698
AT1G01430	19	0,123
AT1G01460	3	0,036
AT1G01470	7	0,415
AT1G01510	19	0,123
AT1G01550	18	0,120
AT1G04990	9	0,767
AT1G06040	2	0,400
AT1G06050	23	0,147
AT1G06580	20	0,718
AT1G07000	16	0,698
AT1G07010	1	0,648
AT1G07880	12	0,934
AT1G07890	5	0,292
AT1G09070	21	0,442
AT1G09080	8	0,849
AT1G09960	18	0,120
AT1G10020	19	0,123
AT1G10030	10	0,819
AT1G10950	19	0,123
AT1G10960	4	0,050
AT1G10970	14	0,150
AT1G13440	5	0,292
AT1G15340	1	0,648
AT1G15350	20	0,718
AT1G15800	1	0,648
AT1G15810	12	0,934
AT1G16150	19	0,123
AT1G17220	5	0,292
AT1G17370	5	0,292
AT1G17420	5	0,292
AT1G17620	5	0,292
AT1G18210	14	0,150
AT1G18310	0	0,928
AT1G18990	4	0,050
AT1G19000	3	0,036
AT1G19190	20	0,718
AT1G19200	15	0,882
AT1G19500	18	0,120
AT1G19880	18	0,120
AT1G20816	0	0,928
AT1G21000	21	0,442
AT1G21310	4	0,050
AT1G21400	18	0,120
AT1G21590	6	0,678
AT1G21600	14	0,150
AT1G22200	16	0,698
AT1G22760	4	0,050
AT1G22770	8	0,849
AT1G22810	8	0,849
AT1G22830	1	0,648
AT1G23490	9	0,767
AT1G25400	13	0,115
AT1G25540	23	0,147
AT1G25550	18	0,120
AT1G27720	8	0,849
AT1G27770	4	0,050
AT1G28360	0	0,928
AT1G29390	9	0,767
AT1G29630	5	0,292
AT1G32700	19	0,123
AT1G35140	4	0,050
AT1G44510	23	0,147
AT1G45145	21	0,442
AT1G45150	23	0,147
AT1G55450	16	0,698
AT1G55460	4	0,050
AT1G56510	22	0,966
AT1G56660	0	0,928
AT1G57990	8	0,849
AT1G59870	13	0,115
AT1G60950	2	0,400
AT1G62180	4	0,050
AT1G62870	7	0,415
AT1G68010	3	0,036
AT1G70310	16	0,698
AT1G70570	17	0,980
AT1G70580	5	0,292
AT1G70700	21	0,442
AT1G71040	23	0,147
AT1G72520	4	0,050
AT1G73010	2	0,400
AT1G73050	2	0,400
AT1G73060	11	0,835
AT1G73390	4	0,050
AT1G73470	7	0,415
AT1G73530	11	0,835
AT1G74440	0	0,928
AT1G74450	1	0,648
AT1G74920	20	0,718
AT1G74930	5	0,292
AT1G74940	12	0,934
AT1G75080	18	0,120
AT1G75800	16	0,698
AT1G75810	21	0,442
AT1G76180	7	0,415
AT1G76580	13	0,115
AT1G76640	20	0,718
AT1G76650	9	0,767
AT1G77440	9	0,767
AT1G78270	13	0,115
AT1G78280	19	0,123
AT1G80600	10	0,819
AT1G80610	7	0,415
AT1G80850	5	0,292
AT2G01180	21	0,442
AT2G01830	7	0,415
AT2G01850	19	0,123
AT2G02670	0	0,928
AT2G10070	4	0,050
AT2G17710	18	0,120
AT2G17830	0	0,928
AT2G17840	8	0,849
AT2G18115	8	0,849
AT2G18420	20	0,718
AT2G20580	10	0,819
AT2G20670	19	0,123
AT2G20980	8	0,849
AT2G20990	8	0,849
AT2G21300	1	0,648
AT2G22070	14	0,150
AT2G22080	15	0,882
AT2G22500	10	0,819
AT2G22660	17	0,980
AT2G22850	3	0,036
AT2G23810	20	0,718
AT2G24600	3	0,036
AT2G25240	21	0,442
AT2G25250	18	0,120
AT2G25490	17	0,980
AT2G26190	20	0,718
AT2G27080	4	0,050
AT2G27090	18	0,120
AT2G28120	18	0,120
AT2G28360	14	0,150
AT2G29180	8	0,849
AT2G29190	5	0,292
AT2G30040		0,036
AT2G30050	21	0,442
AT2G30360	23	0,147
AT2G31370	21	0,442
AT2G31380	22	0,966
AT2G32160	11	0,835
AT2G33120	14	0,150
AT2G33470	10	0,819

AGI	PHASE in SD	pvalue in SD
AT2G33480	18	0,120
AT2G34480	13	0,115
AT2G34720	1	0,648
AT2G36710	7	0,415
AT2G37580	8	0,849
AT2G37585	15	0,882
AT2G39990	17	0,980
AT2G40010	16	0,698
AT2G40030	21	0,442
AT2G40050	20	0,718
AT2G40080	8	0,849
AT2G40350	8	0,849
AT2G41000	23	0,147
AT2G41010	21	0,442
AT2G41080	18	0,120
AT2G41090	12	0,934
AT2G41110	6	0,678
AT2G41660	9	0,767
AT2G42580	18	0,120
AT2G42750	0	0,928
AT2G42760	12	0,934
AT2G44200	12	0,934
AT2G44940	6	0,678
AT2G45160	18	0,120
AT2G45320	22	0,966
AT2G46790	4	0,050
AT2G46820	5	0,292
AT2G46830	23	0,147
AT2G47260	8	0,849
AT2G47490	23	0,147
AT3G01380	14	0,150
AT3G01390	10	0,819
AT3G01400	23	0,147
AT3G02420	16	0,698
AT3G02460	0	0,928
AT3G02468	23	0,147
AT3G03450	4	0,050
AT3G03860	9	0,767
AT3G03870	23	0,147
AT3G04420	4	0,050
AT3G04630	13	0,115
AT3G04640	7	0,415
AT3G04650	9	0,767
AT3G05500	21	0,442
AT3G05510	14	0,150
AT3G07150	4	0,050
AT3G07160	3	0,036
AT3G08860	20	0,718
AT3G08870	1	0,648

AGI	PHASE in SD	pvalue in SD
AT3G10920	9	0,767
AT3G10930	15	0,882
AT3G13510	18	0,120
AT3G13640	7	0,415
AT3G13650	8	0,849
AT3G13690	16	0,698
AT3G14200	4	0,050
AT3G15020	6	0,678
AT3G16720	14	0,150
AT3G16860	15	0,882
AT3G17390	12	0,934
AT3G17410	19	0,123
AT3G17790	4	0,050
AT3G17800	3	0,036
AT3G17810	0	0,928
AT3G18050	15	0,882
AT3G19680	0	0,928
AT3G19690	0	0,928
AT3G19700	23	0,147
AT3G20260	9	0,767
AT3G21560	7	0,415
AT3G21970	16	0,698
AT3G23090	5	0,292
AT3G24560	23	0,147
AT3G24820	12	0,934
AT3G24830	9	0,767
AT3G26960	5	0,292
AT3G26980	19	0,123
AT3G27050	3	0,036
AT3G27200	7	0,415
AT3G29030	7	0,415
AT3G32360	16	0,698
AT3G44250	4	0,050
AT3G44260	2	0,400
AT3G45140	7	0,415
AT3G45640	13	0,115
AT3G46540	6	0,678
AT3G48050	22	0,966
AT3G49530	5	0,292
AT3G50480	18	0,120
AT3G51090	6	0,678
AT3G51100	17	0,980
AT3G51950	0	0,928
AT3G55770	20	0,718
AT3G55780	7	0,415
AT3G56880	8	0,849
AT3G57020	3	0,036
AT3G57450	13	0,115
AT3G57460	8	0,849

AGI	PHASE in SD	pvalue in SD
AT3G57530	0	0,928
AT3G57990	3	0,036
AT3G59060	1	0,648
AT3G60120	10	0,819
AT3G60130	17	0,980
AT3G61430	2	0,400
AT3G61620	14	0,150
AT3G61630	19	0,123
AT4G00200	5	0,292
AT4G00370	6	0,678
AT4G01330	22	0,966
AT4G01810	6	0,678
AT4G02380	18	0,120
AT4G03070	6	0,678
AT4G03080	16	0,698
AT4G03490	3	0,036
AT4G05050	22	0,966
AT4G05100	3	0,036
AT4G08950	20	0,718
AT4G11220	4	0,050
AT4G11230	8	0,849
AT4G11280	4	0,050
AT4G11510	0	0,928
AT4G11530	2	0,400
AT4G12720	4	0,050
AT4G16960	2	0,400
AT4G17070	17	0,980
AT4G17080	19	0,123
AT4G17670	8	0,849
AT4G19045	13	0,115
AT4G20250	3	0,036
AT4G20260	12	0,934
AT4G21560	17	0,980
AT4G22592	6	0,678
AT4G22756	9	0,767
AT4G22760	15	0,882
AT4G22950	4	0,050
AT4G22960	4	0,050
AT4G23180	7	0,415
AT4G23620	10	0,819
AT4G23630	23	0,147
AT4G24370	18	0,120
AT4G24390	11	0,835
AT4G24560	23	0,147
AT4G24570	3	0,036
AT4G24580	4	0,050
AT4G25240	10	0,819
AT4G25250	5	0,292
AT4G26080	5	0,292

AGI	PHASE in SD	pvalue in SD
AT4G27250	20	0,718
AT4G27270	8	0,849
AT4G27510	1	0,648
AT4G27520	6	0,678
AT4G27652	5	0,292
AT4G27654	2	0,400
AT4G27930	12	0,934
AT4G27940	3	0,036
AT4G28230	11	0,835
AT4G28310	7	0,415
AT4G28320	16	0,698
AT4G29660	8	0,849
AT4G29670	6	0,678
AT4G29680	7	0,415
AT4G29780	3	0,036
AT4G30430	20	0,718
AT4G30440	7	0,415
AT4G31550	9	0,767
AT4G31570	15	0,882
AT4G31890	16	0,698
AT4G32030	19	0,123
AT4G32180	16	0,698
AT4G32190	3	0,036
AT4G32940	4	0,050
AT4G32950	10	0,819
AT4G33250	19	0,123
AT4G33650	16	0,698
AT4G33660	5	0,292
AT4G33666	3	0,036
AT4G33910	10	0,819
AT4G33920	12	0,934
AT4G33925	9	0,767
AT4G34135	19	0,123
AT4G34150	3	0,036
AT4G34160	16	0,698
AT4G34480	5	0,292
AT4G34490	6	0,678
AT4G35760	1	0,648
AT4G35780	18	0,120
AT4G35840	4	0,050
AT4G36000	4	0,050
AT4G36710	9	0,767
AT4G37170	16	0,698
AT4G38620	4	0,050
AT4G38700	3	0,036
AT4G38710	12	0,934
AT4G39100	21	0,442
AT4G39890	16	0,698
AT4G40010	20	0,718
AT4G40020	4	0,050
AT5G01340	19	0,123
AT5G01350	22	0,966
AT5G01510	15	0,882
AT5G01520	5	0,292
AT5G01710	18	0,120
AT5G01715	18	0,120
AT5G01810	19	0,123
AT5G01820	4	0,050
AT5G02230	4	0,050
AT5G02270	6	0,678
AT5G02280	4	0,050
AT5G02290	18	0,120
AT5G02810	6	0,678
AT5G02820	11	0,835
AT5G03200	15	0,882
AT5G03280	18	0,120
AT5G03290	16	0,698
AT5G04340	4	0,050
AT5G06290	7	0,415
AT5G06300	13	0,115
AT5G06320	11	0,835
AT5G07940	14	0,150
AT5G11070	12	0,934
AT5G11270	14	0,150
AT5G13190	4	0,050
AT5G13630	2	0,400
AT5G13640	3	0,036
AT5G13750	13	0,115
AT5G14700	7	0,415
AT5G18280	18	0,120
AT5G18390	1	0,648
AT5G18400	14	0,150
AT5G18820	20	0,718
AT5G18830	0	0,928
AT5G19240	3	0,036
AT5G19250	7	0,415
AT5G20170	17	0,980
AT5G20180	0	0,928
AT5G22250	13	0,115
AT5G22940	20	0,718
AT5G23120	18	0,120
AT5G23720	15	0,882
AT5G23730	2	0,400
AT5G24590	20	0,718
AT5G35735	8	0,849
AT5G40470	19	0,123
AT5G40850	17	0,980
AT5G42050	18	0,120
AT5G42060	8	0,849
AT5G46440	4	0,050
AT5G46450	19	0,123
AT5G47230	2	0,400
AT5G47650	4	0,050
AT5G47910	23	0,147
AT5G49270	6	0,678
AT5G49280	13	0,115
AT5G49690	22	0,966
AT5G49700	19	0,123
AT5G50950	6	0,678
AT5G51180	16	0,698
AT5G51190	4	0,050
AT5G54930	14	0,150
AT5G54940	4	0,050
AT5G57550	18	0,120
AT5G57560	4	0,050
AT5G58060	18	0,120
AT5G58080	5	0,292
AT5G58520	5	0,292
AT5G58710	11	0,835
AT5G58720	21	0,442
AT5G58730	13	0,115
AT5G59440	19	0,123
AT5G59450	15	0,882
AT5G59540	6	0,678
AT5G59550	12	0,934
AT5G59810	2	0,400
AT5G59820	4	0,050
AT5G60760	13	0,115
AT5G61380	14	0,150
AT5G62000	20	0,718
AT5G62130	3	0,036
AT5G62520	23	0,147
AT5G62530	13	0,115
AT5G62570	3	0,036
AT5G63120	18	0,120
AT5G63130	5	0,292
AT5G63135	20	0,718
AT5G63770	14	0,150
AT5G63780	7	0,415
AT5G64840	1	0,648
AT5G64850	3	0,036
AT5G66200	19	0,123
AT5G66210	18	0,120
AT5G66430	7	0,415
AT5G66680	11	0,835
AT5G66770	13	0,115
AT5G67420	21	0,442

Table 6. Gene primer sequences

Gene Name	AGI number	Primer		
		Gene Region	Primer Name	Sequence (5' to 3')
PIL1	AT2G46970	Coding region	EMP372	TGCCTTCGTGTGTTTCTCAG
			EMP373	AACTAAAACCGTTGCTTCCTC
		Promoter	EMP407 (Y3)	ACAAGAAAGAAGGGAGGGAGACA
			EMP408 (Y3)	TTCTCTTTAAATGGGACCCACAAT
			EMP550 (TMG)	GGACGCTTTGTCATTGCATAG
			EMP551 (TMG)	GATGCTCCAACAATAATGCAAC
HFR1	AT1G02340	Coding region	EMP448	GATGCGTAAGCTACAGCAACTCGT
			EMP449	AGAACCGAAACCTTGTCCGTCTTG
		Promoter	EMP444	ACGTGATGCCCTCGTGATGGAC
			EMP445	GTCGCTCGCTAAGACACCAAC
AT5G02580	AT5G02580	Coding region	EMP582	CAAAGAGTTCTTCGAGGCATACG
			EMP583	TAGTTCGTGAAAGCAAATCACGG
		Promoter	EMP618	ACAGATTTTAACTACGTAGTGTGGG
			EMP619	TGCTACTGCTAGTATCAGTTGCTG
IAA19	AT3G15540	Coding region	EMP689	AGATGAATATGACGTCGTCGG
			EMP690	CTCAACCTCTTGCATGACTCTAG
YUCCA8	AT4G28720	Coding region	EMP709	ATCAACCCTAAGTTCAACGAGTG
			EMP710	CTCCCGTAGCCACCACAAG
ATHB2	AT4G16780	Coding region	EMP713	GAGGTAGACTGCGAGTTCTTACG
			EMP714	GCATGTAGAACTGAGGAGAGAGC
FHL	AT5G02200	Coding region	EMP 584	TGGAGAACACAAAAACCAGCGATGA
			EMP 585	TCAATGGTTGGTTTCGTGGTAGCTT
BRI1	AT4G39400	Coding region	EMP721	GTAAACGGCCAACGGATTCACC
			EMP722	TTGCGTGCTGTTTCACCCATCC
BEH4	AT1G78700	Coding region	EMP725	GTAGAGCGTATGGAGATAGGTGGT
			EMP726	AAGATTAGCAAATGAGGATGAAGC
PAR1	AT2G42870	Coding region	EMP715	CACCGTCATGCTCAGCCA
			EMP716	TCGGTCTTCACGTACGCTTG
CKX5	AT1G75450	Coding region	EMP586	ACTCGAGCACGAATCTCTCTCGAAC
			EMP587	CGAGTCCTTCGTCCACAATCACAA
HAT2	AT5G47370	Coding region	EMP592	GACTCCCATGGAACCAAACATTCG
			EMP593	CTCTTCCCGCTAATGGTGCTTGA
GA2O6x	AT1G02400	Coding region	EMP783	GGTTGAATCACTATCCACCAGC
			EMP784	TAACGGTGGAGCTGCAAAGTAC
AZF2	AT3G26810	Coding region	EMP701	CAAGTATGAAACAATGCGATCCCTTTG
			EMP702	TTCTTCCATCCGGTTATTATCATTCTCG
BIM2	AT1G69010	Coding region	EMP723	CCAGAATGCAGGCATTGATCTGTC
			EMP724	CTCAACGCTTGAATCCCTTCCTTG

HB52	AT5G53980	Coding region	EMP697	ATCATGATGATCAAGTGGTGGT
			EMP698	TCAGACCCATGAGGTTGATG
IAA29	AT4G32280	Coding region	EMP711	ATCACCATCATTGCCCGTAT
			EMP712	ATTGCCACACCATCCATCTT
PP2A	AT1G13320	Coding region	EMP338	TATCGGATGACGATTCTTCGTGCAG
			EMP339	GCTTGGTCGACTATCGGAATGAGAG
PIF3	AT1G09530	Coding region	EMP417	GGTATGGG ATGCCTTATGCA
			EMP418	TGGAACTGTGGTCCGTGGTTA
PIF4	AT2G43010	Coding region	EMP419	GCG GCTTCGGCTCCGATGAT
			EMP420	AGTCGCGGCCTGCATGTGTG
CAB2	AT1G29920	Coding region	EMP474	CTATTTCTACAATCGAGCAACGTGA
			EMP475	TGTACCCATTGCCTTAATATGTTCAA

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IV. GENERAL DISCUSSION

"Sometimes it's not enough to know what things mean,
sometimes you have to know what things don't mean."
Bob Dylan

In the last recent years, works done by several research groups have considerably increased the knowledge of regulation of hypocotyl growth of SD-grown seedlings (some references: [62,63,64,74,148,150,163,164]). Together, the studies have shown that growth in SD diurnal cycles is controlled basically by the combined action of light, circadian clock and hormone signaling, although other factors also have an impact on stem growth, such as temperature or metabolic pathways. The concerted action of the different pathways ensures that, in SDs, hypocotyl growth rate changes along the day-night cycle, presenting a maximum peak at the end of the dark period [64,165]. PIF4 and PIF5 growth-promoting factors contribute to the implementation of this rhythmic growth, acting just before dawn as a consequence of a combined light and circadian clock regulation [64,72]. The experiments presented in this thesis examine the role of other members of the PIF family in the regulation of hypocotyl growth in SDs. Results obtained uncovered PIF1 and PIF3 as novel growth-promoting regulators in SD-grown seedlings and that all members of PIF quartet act together to induce growth at the end of the night in SD conditions. Moreover, considering that circadian clock component TOC1 had previously showed altered hypocotyl length in SDs, genetic and molecular studies were performed to address the question of a possible novel interplay between clock and light in the regulation of hypocotyl growth in SD conditions. Together, results provide evidence that clock gates growth towards the end of the night in SDs through a repressive action done by TOC1 clock protein over PIF3 transcriptional activity.

Results presented in Publication 1 and 2 [165,166] revealed that PIF1 and PIF3 transcription factors participate in the regulation of hypocotyl elongation in SD; the absence of these proteins results in seedlings with shorter hypocotyl than wild-type. Our results unmask that PIF1 and PIF3, together with PIF4 and PIF5, function as promoters of hypocotyl growth in SD. Moreover, all phenotypic studies performed in very young seedlings (3-day-old) grown under regular short-day conditions showed that PIF3 seems to have a more prominent role than the other members of the PIF quartet because its absence in comparison to the absence of other single members has a greater effect in hypocotyl growth [169,170]. Interestingly, the relative role of PIFs seems to change when seedlings are grown under modified SD conditions in which a far red pulse is included. In these growing conditions, a more evident effect was seen on *pif1* mutant, underscoring a more prominent role of PIF1 [166]. These findings are in concordance with the fact that different PIF proteins have a changing relative importance in comparison with the other members depending of the light conditions. For example, PIF4 protein have been shown to have a very important role in temperature-dependent flowering responses [58], whereas its role in the repression of photomorphogenesis seems to be relatively less important [60].

Additionally, phenotypic studies of hypocotyl elongation together with western blotting analysis of PIF3 protein done in different light conditions showed a clear correlation between the amount of PIF3 protein and the level of lengthening of the hypocotyl [166]. These findings reinforce the evidence that PIFs transcription factors play a crucial role in the regulation of stem growth and highlight how important is for plants to fine control PIFs protein levels.

At a level of transcriptional and protein accumulation regulation of PIFs, the experiments presented in Publication 1 and 2 reveal that *PIF1* and *PIF3* transcription is not controlled by the circadian clock; their transcription is relatively constant during all the diurnal cycle. At a protein level, our results indicate that for PIF3, and probably also for PIF1, protein oscillations are generated by the action of phytochromes along the day-night cycle. Analysis of PIF3 protein accumulation in single and double mutant of phyA and phyB showed that both phytochromes are in charge of PIF3 degradation in the early morning dark-to-light transition in SD. After some hours of light, at dusk and also beginning of dark period, phyB seems to have a more prominent role in PIF3 degradation [165]. These results are in concordance with other works in which the relative importance of different phys were analyzed and that reported that both phyA and phyB equally participate in the degradation of PIFs in rapid light-induced degradation [44,75,76] whereas phyB have a more prominent role in longer light conditions [80]. In the case of *PIF4* and *PIF5,* it was reported that, the transcription of these genes is directly regulated by the circadian clock, presenting clear oscillations already at a transcriptional level. Indeed, our results of gene expression of these genes showed exactly the same oscillating expression pattern. Again, the action of phytochromes is what restricts PIF4 and PIF5 protein accumulation to the end of the dark period, process known as the coincidence model [64]. Our new findings on how the transcription of *PIF1* and *PIF3* is in SD, and how their protein accumulation oscillations are achieved, have revealed that plants have developed at least two different strategies to ensure that these transcription factors oscillate at a protein level and that the peak of protein accumulation takes place towards the end of the night period.

The analysis of gene expression of known growth-related genes (done in single and multiple deficient *PIF* mutants) show a clear decrease in the expression of those genes when one or several PIFs are absent [165,166](Draft 1 of the thesis). These results provide evidence that, a part from PIF4 and PIF5 as regulators of gene expression under these diurnal conditions [62,163], PIF1 and PIF3 also participate in the process and that the PIF quartet act together to induce the expression of growth-related genes to promote hypocotyl elongation at the end of the night cycle. Gene expression analysis done in seedlings grown under regular SD conditions in comparison to seedlings grown in modified SD conditions that included a FRp treatment showed that the role of the different PIFs changes depending on the light treatment. In normal SD, PIF3 seems to have a more prominent role as a bigger effect is seen in the expression of the analyzed target genes while in SD+FRp, it seems that PIF1 acquires a more important role. Nicely, these results correlate with the results seen at the hypocotyl growth level in our phenotypic studies.

Interestingly, gene expression analysis also revealed that in diurnal conditions PIFs directly induce the expression of well-known growth repressors, for example *HFR1* and *PAR1* [165] (Draft 1 of the thesis). This could be seen as a contradiction but it actually shows the high level of regulation of the process; it can be understood as a regulatory mechanism to give more robustness to the system to avoid an over-response. This phenomenon is evident in the work done in the dark-to-light transition of etiolated seedlings in which PIF3 repress the expression of genes that are actually repressors of photomorphogenesis [167] (See Annex of this thesis). In the absence of PIF3, and even

more accentuated when *PIF4* and *PIF5* are also mutated, there is a clear over-reponse in both hypocotyl elongation and cotyledon aperture. As mentioned, in SD some genes induced by the PIFs are actually inhibitors of growth and, additionally, phenotypic studies done in *pifq* showed that cotyledon aperture is clearly bigger than that of the wild-type (see pictures in figure 5 of Publication 2, measures not shown), which suggest that an avoiding over-response mechanism could be also happening in SD.

In our studies, no PIF-repressed genes were analyzed, but transcriptomic analysis using *PIFs* mutants done in different growing conditions have revealed that PIFs can act also as repressors of gene expression (some references [39,48,66,92,88,167,168]). Considering the fact that the action of PIFs as transcriptional repressors have been seen in several growing conditions (including mature plants grown in diurnal cycles [93] and seedlings grown in modified SD conditions [168]), it is likely that also in SD PIFs could be repressing the expression of other groups of genes, for example inhibitors of growth.

The different ChIP assays performed in both Publication 1 and Draft 1 of this thesis make apparent that PIF3 is able to bind to the promoter of its target genes [165](Draft 1of the thesis). These results, together with other works that reported that the other PIFs are also able to bind to the promoter of their target genes in SD [62,92], indicate that the way PIFs regulate hypocotyl growth at dawn is by regulating genes expression of their targets genes through direct binding to their promoters.

Phenotypic studies presented in Publication 2 showed that a Far Red pulse (FRp) treatment causes an induction of hypocotyl elongation that depends on the presence of PIFs. Seedlings treated with a FRp have a higher accumulation of PIF3 protein, and probably also the other PIF members, which increases the expression of growth-promoting genes and it induces a lengthening of the hypocotyl. In Draft 1, the same experimental approach was used to compare growth induction at different moments of the subjective night period. Results showed that in WT seedlings, hypocotyl elongation is different depending on the moment of the subjective night the FRp treatment is done. This observation allowed us to conclude that it might exist a gating mechanism regulating hypocotyl growth in SD. The same experiments done in the *toc1* deficient mutant indicate that TOC1 clock protein is responsible of that gating mechanism, acting as a repressor of growth during approximately the first half of the dark period and restricting hypocotyl elongation to the end of the dark period. Interestingly, another gating process in hypocotyl elongation was already reported in diurnal conditions [164], but the work done by Sellaro and colleagues was focused on the day part of the diurnal cycle while our work is done during the dark period of the cycle. Considering the results obtained in the two works it seems that two different mechanisms of gating could be present during the diurnal cycle, one gating event that takes place during the day period [164] and another gating event done by TOC1 during the first half of the night period.

Moreover, hypocotyl growth induction after a FRp treatment, together with hypocotyl length measurements of seedlings grown in diurnal cycles of different dark length period, showed that the absence of PIFs in the *toc1* mutant (induced either by long light periods or by genetic removal) results in shorter hypocotyls. Those results gave evidence that the repressive role of TOC1 in hypocotyl growth relies on the presence of PIFs proteins. Importantly, considering the fact that the mutation of other clock

components have been shown to also result in longer hypocotyls than WT, for example the triple deficient mutant *prr9prr7prr5* [130], we cannot discard the clock gating mechanism is not only achieved by the action of TOC1 but for the combination of several clock components.

Importantly, TOC1 gating seen in growth was also observable at a transcriptional level after treating seedlings to FRp, consistent with a previous work that reported that *PIL1* induction in response to shade events is gated by the clock [169]. Different levels of *PIL1* and *HFR1* gene expression induction was observed in the WT depending the moment of the subjective night while in *toc1* the increase in gene expression after the FRp treatment was similar at all time-points. These results are in accordance with the recently reported TOC1 repressive transcriptional activity [116,117,170], suggesting that TOC1 could be using this activity to regulate gene expression to gate growth at the end of the dark period. Also at this level, results showed that the presence of PIFs is necessary to see the function of TOC1, as gene expression deregulation seen in *toc1* is additively reduced as a major number of PIFs is mutated.

Both phenotypic and molecular data obtained by the use of different *pifs* and *toc1* mutant combinations, suggests the existence of an unknown regulatory mechanism between TOC1 and PIF3. In the case of *PIF4* and *PIF5*, transcription is directly regulated by the clock [64,74]. Because of this clock regulation, a clear deregulation in their expression is seen in the *toc1* mutant, having considerable increased levels than the wild-type. This overexpression could explain the fact that hypocotyl elongation, and also gene expression induction of growth-related genes, are reduced in the *pif4pif5toc1* in comparison to *toc1*. Importantly, in *toc1* neither *PIF3* expression levels nor PIF3 protein accumulation are different than in the WT, but there is a significant reduction in hypocotyl length and *PIL1* and *HFR1* expression in the *pif3toc1* mutant compared to the single *toc1* mutant. These results give evidence that there must be a regulatory mechanism, different from the already known circadian regulation of *PIF4* and *PIF5* transcription factor, which relates TOC1 and PIF3 in the regulation of hypocotyl growth. We cannot discard this alternative mechanism is also happening for PIF4 and PIF5.

Direct-binding targets of both TOC1 and PIFs had previously been studied separately [90,117]. Here, we decided to explore the possibility that both types of proteins share some of their target genes to control hypocotyl growth, as the obtained phenotypic results together with gene expression data, indicate a connection between TOC1 protein and PIFs. Indeed, the comparison of the two gene target lists indicate that they seem to bind to common subset of genes and allowed us to create a list of putative shared genes. The study of the phase of the putative co-targets showed that a considerable amount of them peak at the end of the dark period specifically in SD conditions. Further gene expression analysis of the common target genes done in *toc1* and *pif3pif4pif5* mutants, together with DNA-binding assays done with PIF3 and TOC1 proteins indicate that, indeed, the two proteins directly bind to the promoter of the genes and regulate their expression in an antagonistic way: PIFs acting as inductors and TOC1 as repressor. Importantly, among the shared genes between PIFs and TOC1, we find several well-known growth-promoting genes as for example the hormone biosynthesis and signaling

genes *IAA19*, *YUCCA8* and *BRI1*, displaying that the co-target genes of PIFs and TOC1 have an important relevance on the growth process. Our findings that PIFs and TOC1 share a common set of target genes, unmasks an additional layer of co-regulation of hypocotyl growth between light and clock pathways in SD. And, importantly, together with other works that had been performed to understand the different crosstalk existing a transcriptional level between light, circadian clock together and hormones [88,168] it helps to decipher the complex network that exists between the three pathways (light, clock and hormones) that are acting together to control hypocotyl elongation.

Moreover, ChIP assays indicated that TOC1 is able to bind to the promoter of the co-target genes. Although the capacity of TOC1 to directly bind to DNA was recently reported [116], in some other cases it has been shown that TOC1 requires the presence of another protein to bind to DNA promoter, as it happens with protein CHE (CCA1 Hiking Expedition) in the regulation of the clock component *CCA1* expression [171]. Additional experiments are needed to determine whether TOC1 binding to PIF-TOC1 co-targets is direct or mediated by and additional factor.

Given that direct interaction between TOC1 and PIF3 proteins had been documented previously [113,124] (studies done using the yeast-two hybrid system), it is likely that these two proteins physically interact in short-day conditions to regulate gene expression. Interestingly, a previous work gave evidence that TOC1 can directly interact to another member of the PIF family, PIF7, also having a role as a repressor of gene expression [78]. However, the mechanism reported in that publication was different from the one we are proposing for TOC1 and PIF3. In that case both proteins seem to have the same transcriptional repressing activity (TOC1 enhances the repressive action that PIF7 have on the expression of the gene *DREB1*) while we propose a mechanism in which TOC1 and PIF3 act in an antagonistic manner.

Furthermore, the analysis of TOC1 and PIF3 binding to the promoters of the co-targets genes along the dark period suggest that in the middle of the night both protein can bind to the promoter of these genes. Intriguingly in the middle of the night and at the end of the night a similar amount of PIF3 is bound to the promoter of the genes but there are big differences of expression of the target genes between the two time points. There is a peak of expression at the end of the night while almost no expression is seen at ZT14, coinciding with the time-point in which a high amount of TOC1 is present on the promoter of those same genes. Such results may indicate that TOC1 could be repressing the activity of PIF3 in the middle of the night. The use of a TOC1 overexpressor line (that have similar amounts of TOC1 in the middle and at the end of the night) helped to demonstrate that TOC1 acts as a repressor. In the TOC1ox line in which there is a high level of binding of TOC1 at the end of the night, the expression of the genes is highly recuded. Also, it showed that the repressive action of TOC1 over PIF3 is achieved by repressing the transcriptional activity of PIF3 rather than affecting the binding levels of PIF3 to the promoter of the target genes because a significant amount of PIF3 is still able to bind.

Therefore we propose a model in which hypocotyl growth in SD-grown seedlings is gated towards the end of the night by the repressive action that TOC1 protein realize over PIF3 transcriptional activity during the rest of the dark period. As a consequence,

expression of growth-related genes is restricted to dawn, generating an induction of hypocotyl growth at that precise moment of the diurnal cycle.

Results obtained during the thesis have helped to gain a better insight into how hypocotyl elongation is regulated in seedlings grown in short-day conditions. In Figure 11 there is a scheme of the current knowledge of PIF proteins in the regulation of hypocotyl elongation.

In conclusion, our findings, together with the work done by other research groups, allow us to summarize that PIF quartet act collectively to promote hypocotyl growth in very young seedlings grown in SDs, PIF3 having a more prominent role, at the very end of the night period. In order to ensure that PIFs act precisely at that moment of the diurnal SD cycle, plants have evolved different regulatory mechanisms. Two alternative mechanisms are in charge to restrict the accumulation of PIFs proteins to the end of the dark period: direct transcriptional regulation in the case of PIF4 and PIF5 [64,74] or posttranslational regulation in PIF3 and, probably, PIF1 [165]. Moreover, as a significant amount of PIF3 (and probably also the other PIFq members) is present in the middle of the dark period a third regulatory mechanism have been developed to assure that the action of PIFs proteins is restricted to the end of the night. This mechanism is based on the regulation of the transcriptional activity of PIF proteins. One example is the reported inhibition that DELLA proteins do over PIFs [62,63]. A second example hormone-related mechanism is the combined action of PIF activity (at least for PIF4) with brassionesteroid-related transcription factors BES1 and BZR. Finally, a novel regulatory mechanism is the proposed inhibition done by the circadian clock, concretely by the protein TOC1, to PIF3 (and maybe also other PIF members) activity.

The several layers of regulation and multiple crosstalk points between the different pathways that regulate hypocotyl elongation highlights how important is for the plant that this biological process takes place at the end of the diurnal cycle in SD. An open question for the future will be to determine whether additional regulatory mechanisms such as alternative gene splicing, tissue and organ specific gene expression or histone modification-associated transcriptional regulation play a role in hypocotyl elongation of seedlings grown in short-day conditions.

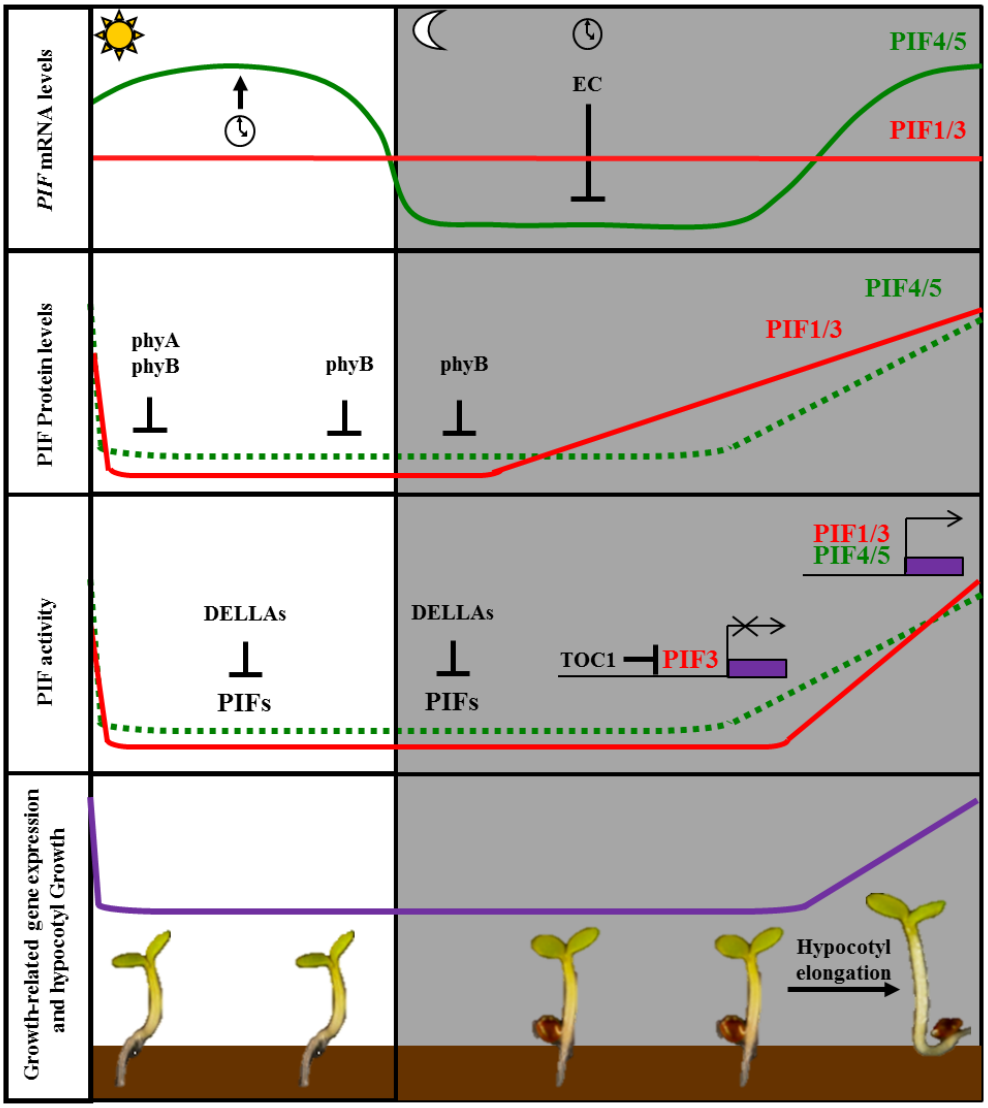

Figure 11. Simplified model depicting current knowledge about PIFs function regulating hypocotyl growth in SDs. Acronym EC is for Evening Complex. Drawing of a clock represents the Circadian clock. Already confirmed data is depicted in a solid line. Predicted endogenous PIF4 and PIF5 protein oscillation and PIF4 and PIF5 protein activity is depicted in a dashed line.

V. CONCLUSIONS

"We must not forget that when radium was discovered no one knew that it would prove useful in hospitals. The work was one of pure science. And this is a proof that scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science, and then there is always the chance that a scientific discovery may become like the radium a benefit for mankind. "

Marie Curie

The conclusions of this thesis are:

1.- Role of PIF3 in diurnal growth is:

1.1 PIF3 acts as positive regulator of hypocotyl growth.

1.2. In short-day conditions, *PIF3* transcript levels are relatively constant during all the day cycle. At a protein level, PIF3 presents a clear oscillatory pattern with a peak of accumulation at the end of the night period. These oscillations are imposed by the action of phytochromes.

1.3. PIF3 promotes growth by regulating the expression of growth-related genes. It directly binds to the promoter of these genes and induces their expression at the end of the night period.

1.4. PIF3 seems to have a more prominent role in the regulation of hypocotyl elongation in young seedlings (3d) grown in short-day conditions in comparison with PIF4 and PIF5. Even so, the three proteins act together to induce stem elongation in this photoperiodic condition.

2. The relevance of PIFs levels and involvement of PIF1 in hypocotyl elongation in short-day conditions are:

2.1. Hypocotyl elongation directly correlates with the levels of PIF3 protein (and probably also the other PIFs) present in the cell. In light treatments that promote the degradation of PIF3, a clear arrest in the hypocotyl growth is seen. On the contrary, light treatments that induce the accumulation of PIF3 clearly result in an induction of growth and a much longer hypocotyl length of the seedlings.

2.2. PIF1 protein also acts as a positive regulator of hypocotyl growth in SD. Its pattern of transcription is similar to that of PIF3 and we hypothesize that protein levels are regulated by the phytochromes as it is the case of PIF3.

3. The relationship between PIF3 (and other PIFs) with the circadian clock is:

3.1. Circadian clock protein TOC1 participates in the regulation of stem growth, having a more evident function as the number of dark hours increase. It acts as a negative regulator of hypocotyl growth, acting as a repressor of growth during the first hours of the dark period in short-day conditions.

3.2. TOC1 and PIFs share common targets genes. They bind to the promoter region of targets genes and regulate their expression in an antagonistic way: TOC1 acting as a transcriptional repressor and PIFs as transcriptional activators.

3.3. TOC1 inhibits the activity of PIF3. The inhibiting effect seems to be done mostly on the intrinsic transcriptional activity of PIF3 rather than affecting its binding to gene promoter.

VI. REFERENCES

"When we try to pick out anything by itself,
we find it is tied to everything else in the universe."
John Muir

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VII. ANNEX

"What we see depends mainly on what we look for."
John Lubbock

Publication 3

Functional profiling identifies genes involved in organ-specific branches of the PIF3 regulatory network in Arabidopsis.

Maria Sentandreu, Guiomar Martín, Nahuel González-Schain, Pablo Leivar, **Judit Soy**, James M. Tepperman, Peter H. Quail and Elena Monte.

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Functional Profiling Identifies Genes Involved in Organ-Specific Branches of the PIF3 Regulatory Network in *Arabidopsis* [C] [W]

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The phytochrome (phy)-interacting basic helix-loop-helix transcription factors (PIFs) constitutively sustain the etiolated state of dark-germinated seedlings by actively repressing deetiolation in darkness. This action is rapidly reversed upon light exposure by phy-induced proteolytic degradation of the PIFs. Here, we combined a microarray-based approach with a functional profiling strategy and identified four PIF3-regulated genes misexpressed in the dark (*MIDAs*) that are novel regulators of seedling deetiolation. We provide evidence that each one of these four *MIDA* genes regulates a specific facet of etiolation (hook maintenance, cotyledon appression, or hypocotyl elongation), indicating that there is branching in the signaling that PIF3 relays. Furthermore, combining inferred *MIDA* gene function from mutant analyses with their expression profiles in response to light-induced degradation of PIF3 provides evidence consistent with a model where the action of the PIF3/MIDA regulatory network enables an initial fast response to the light and subsequently prevents an overresponse to the initial light trigger, thus optimizing the seedling deetiolation process. Collectively, the data suggest that at least part of the phy/PIF system acts through these four MIDAs to initiate and optimize seedling deetiolation, and that this mechanism might allow the implementation of spatial (i.e., organ-specific) and temporal responses during the photomorphogenic program.

INTRODUCTION

The phytochrome (phy) family of photoreceptors (phyA through phyE in *Arabidopsis thaliana*) plays a central role in the regulation of seedling deetiolation, the developmental transition from skotomorphogenesis to photomorphogenesis that dark-germinated seedlings undergo upon exposure to light (Rockwell et al., 2006; Schäfer and Nagy, 2006; Quail, 2010). After germination in the dark, etiolated seedlings grow heterotrophically on seed reserves and follow a skotomorphogenic strategy of development, characterized by fast hypocotyl elongation and maintenance of an apical hook and appressed cotyledons, to rapidly reach for sunlight at the soil surface. Upon reaching the surface, light triggers seedling deetiolation, the developmental switch to photomorphogenesis, which involves the coordinated inhibition of hypocotyl elongation, unfolding of the apical hook, separation and expansion of the cotyledons, and activation of functional chloroplast and pigment biosynthesis to initiate photosynthesis. Photoactivation of the Pr form of the phy molecule during deetiolation results in rapid translocation of the Pfr form from the cytoplasm into the nucleus (Yamaguchi et al., 1999; Nagatani, 2004). Nuclear photoactivated phy molecules associate with phy-interacting factors (PIFs). The PIFs are a subset of basic helix-loop-helix transcription factors (PIF1, PIF3, PIF4, PIF5, PIF6, and PIF7 in *Arabidopsis*) that accumulate in the nucleus in the dark and interact conformer-specifically and photoreversibly with the phy-Pfr molecules in the light (Toledo-Ortiz et al., 2003; Duek and Fankhauser, 2005; Castillon et al., 2007; Monte et al., 2007). This phy-Pfr/PIF interaction initiates the gene expression changes that orchestrate the deetiolation response (Quail, 2002; Jiao et al., 2007; Bae and Choi, 2008). Nuclear interaction between active phyA and/or phyB and several of these transcription factors (including PIF1, PIF3, PIF4, and PIF5) has also been shown to induce rapid phosphorylation and degradation (within minutes) of the PIF proteins (Bauer et al., 2004; Park et al., 2004; Shen et al., 2005; Al-Sady et al., 2006; Oh et al., 2006; Nozue et al., 2007; Shen et al., 2007; Lorrain et al., 2008; Shen et al., 2008).

Recent studies with *Arabidopsis* seedlings deficient in one or multiple PIF proteins have established that progressive genetic removal of PIFs results in additive or synergistic effects in the dark that culminate in a partial *constitutively photomorphogenic* (*cop*)-like phenotype exhibited by the *pif* quadruple mutant *pif1*

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[C] Some figures in this article are displayed in color online but in black and white in the print edition.

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pif3 pif4 pif5 (*pifq*), which is deficient in PIF1, PIF3, PIF4, and PIF5 (Bae and Choi, 2008; Josse and Halliday, 2008; Leivar et al., 2008b). These results provide evidence that the PIF proteins function in the dark in a partially redundant manner, independently of phy action, to repress photomorphogenesis and promote skotomorphogenesis. Upon light exposure, active phys reverse this action by interacting with and inducing rapid degradation of the PIF proteins, allowing deetiolation to proceed.

The phy-mediated degradation of PIFs in dark-grown seedlings first exposed to light triggers the reduction of PIF protein levels to new steady state levels that represent ~10% of their dark levels (Monte et al., 2004; Shen et al., 2005; Nozue et al., 2007). *pif* mutant seedlings growing in continuous red light (Rc) display a hypersensitive phenotype that was initially interpreted as indicative of the PIFs having a negative role in phyB signaling in Rc (Huq and Quail, 2002; Kim et al., 2003; Fujimori et al., 2004; Monte et al., 2004; Khanna et al., 2007; de Lucas et al., 2008; Leivar et al., 2008a). However, more recent studies have shown that this phenotype is the result of elevated phyB levels in the absence of PIF proteins, an additive effect that correlates with increasing hypersensitivity to Rc with progressive genetic removal of multiple PIFs (Leivar et al., 2008a). Recently, Jang et al. (2010) have shown that the mechanism underlying the regulation of phyB levels (and other light-stable phys) during deetiolation involves direct interaction with the COP1 E3 ligase and that PIFs promote this interaction and the polyubiquitination of phyB by COP1.

Genome-wide expression analyses have started to provide some insight into the transcriptional network regulated by the PIFs. In dark-grown seedlings, transcriptomic profiling of single and double *pif1* (Moon et al., 2008), *pif3* (Leivar et al., 2009), and *pif4 pif5* (Lorrain et al., 2009) mutants have identified a small number of genes that are statistically and significantly deregulated in the mutants compared with their respective wild-type controls by at least twofold (Statistically Significantly and Two Fold [SSTF] genes). By contrast, microarray analysis of the *pifq* mutant compared with the wild type has resulted in the identification of a large subset of SSTF genes (~1000) that depend on PIF1, PIF3, PIF4, and PIF5 for their expression in the dark (Leivar et al., 2009; Shin et al., 2009). These results suggest redundancy at the molecular level between different members of the PIF family, similarly to their redundant contribution in establishing the *cop*-like visible phenotype of dark-grown *pifq* seedlings as explained above. The PIFq-regulated genes represent ~5% of the total genome and largely overlap with the transcriptome of wild-type seedlings grown under prolonged light, in accordance with the partial photomorphogenic phenotype of the *pifq* mutant in the dark (Leivar et al., 2009; Shin et al., 2009).

Some of these PIF-regulated genes are key regulators of pigment biosynthesis. PIF involvement in the regulation of chlorophyll biosynthesis became apparent upon transfer of 2-d-old or older dark-grown *pif* mutant seedlings to light, which failed to green (Huq et al., 2004; Stephenson et al., 2009). Microarray analysis identified the chlorophyll-biosynthesis-related genes *GLUTAMYL-tRNA REDUCTASE 1* (*HEMA1*), *Mg-CHELATASE H SUBUNIT* (*CHLH*), *GENOMES UNCOUPLED 4* (*GUN4*), and *PROTOCHLOROPHYLLIDE OXIDOREDUCTASE C* (*PORC*) to present altered levels in *pif* mutants (Moon et al., 2008; Stephenson et al., 2009). Misregulation of these genes in the dark results in exaggerated accumulation of the photooxidizing chlorophyll precursor protochlorophyllide in etiolated PIF-deficient seedlings, which causes photo bleaching upon transfer to light (Huq et al., 2004; Stephenson et al., 2009). PIFs also regulate the expression of the *PSY* gene encoding for the key carotenoid biosynthesis enzyme, which is upregulated during deetiolation to induce carotenoid accumulation (Toledo-Ortiz et al., 2010). In addition, many photosynthetic genes and genes associated with chloroplast biogenesis and function, like *LIGHT HARVESTING COMPLEX* (*LHC*) and *CHLOROPHYLL A/B BINDING PROTEIN* (*CAB*) genes, are also regulated by the PIFs in the dark (Moon et al., 2008; Leivar et al., 2009; Lorrain et al., 2009; Shin et al., 2009). This molecular phenotype is consistent with the partial conversion of etioplasts into chloroplasts exhibited by *pifq* seedlings in the dark (Leivar et al., 2009).

With the exception of pigment biosynthesis and chloroplast function, detailed analysis of the functional relevance of identified PIF-regulated genes in implementing the deetiolation program is still largely lacking (Leivar and Quail, 2011). Here, we identified an expanded set of genes that are regulated by PIF3 in the dark and examined their role in implementing seedling deetiolation by functional profiling of mutants. Integration of this information with the light-responsiveness of these genes is consistent with a model whereby the rapid initial deetiolation response is branched through PIF3-regulated genes and is subsequently counteracted to prevent an overresponse to light that could be detrimental for the emerging seedling.

RESULTS

PIF3 Represses Seedling Photomorphogenesis in the Dark by Regulating Gene Expression Both Positively and Negatively

Previous results have shown a role for PIF3 as negative regulator of photomorphogenesis in seedlings grown at specific time points in the dark (Leivar et al., 2008b; Stephenson et al., 2009). To characterize the role of PIF3 in more detail during extended periods of skotomorphogenic growth, we examined the morphological phenotype of the null *pif3-3* mutant (Monte et al., 2004) compared with the wild-type control during dark development for 4 d after germination (Figures 1A and 1B). During this period of dark growth, the wild-type hypocotyl elongates to ~12 mm, the hook partially and progressively unfolds to ~80°, and the cotyledons remain appressed. Compared with the wild type, *pif3* mutants are indistinguishable during germination and initial dark growth in the first 1.5 d (Figure 1A). By contrast, 2 d after germination, *pif3* mutants start displaying a partial photomorphogenic phenotype with more open hooks and cotyledons and marginal differences in hypocotyl elongation. These differences are maintained with increasing dark growth time up to 4 d of dark development (Figures 1A and 1B), in accordance with and expanding upon previous results by Leivar et al. (2008b) and Stephenson et al. (2009). Altogether, these results indicate that in the wild-type seedling growing in the dark for 4 d, cotyledons remain appressed, whereas there is a progressive elongation of the hypocotyl and

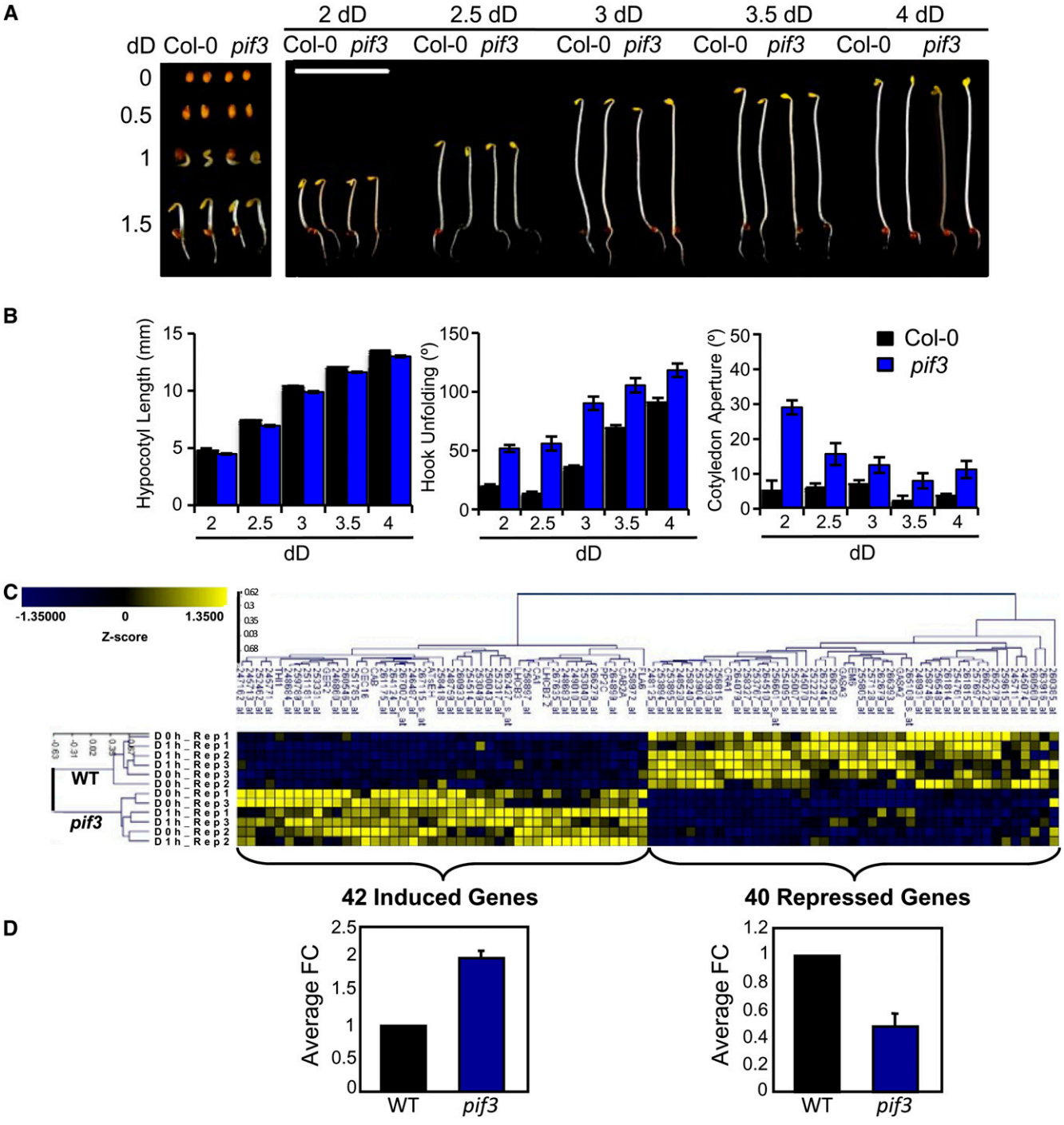

Figure 1. PIF3 Negatively Regulates Seedling Photomorphogenesis in the Dark from 2 d Onward after Germination.

(A) and **(B)** Characterization of the time of action of PIF3 during seedling etiolation in the dark. dD indicates days in the dark.

(A) Visual phenotype of representative seeds, embryos and seedlings for *Arabidopsis* wild-type Col-0 and *pif3-3* mutant seedlings in the dark at the indicated time points after germination.

(B) Quantification of hypocotyl length, hook unfolding, and cotyledon separation angle of *pif3-3* mutants compared with the wild-type Col-0 in the dark at the indicated time points after germination. Data represent the mean and SE of at least 30 seedlings.

(C) and **(D)** Regulation of gene expression in the dark by PIF3. Microarray expression profiling identified 82 HC target genes that are statistically significantly deregulated in the absence of PIF3 in the dark and by a FC greater than 1.5 (SS1.5F-HC).

(C) Two-dimensional-cluster diagram depicting the identified 82 SS1.5F-HC genes in 4-d-old dark-grown *pif3-3* seedlings compared with the wild-type

partial opening of the hook with increasing growth time in the dark. During this developmental process, PIF3 functions to repress photomorphogenesis from 2 d onward after germination and up to 4 d, with a role in promoting hypocotyl elongation and maintaining the hook and the cotyledons appressed, an effect that is sustained over time.

To identify downstream components that mediate PIF3 function as a repressor of photomorphogenesis in the dark, we first aimed to determine putative candidates by defining the PIF3-regulated transcriptome in the dark. To do so, we took advantage of a previous microarray study using Affymetrix ATH1 GeneChips, in which we analyzed the role of PIF3 in the regulation of phy-mediated gene expression in Rc (Monte et al., 2004). In that early work, our focus was to define the contribution of PIF3 in the regulation of the phy-mediated early transcriptional network in Rc. Here, given the current evidence that PIF3 and other PIF proteins act in the dark to sustain the skotomorphogenic state independently of phy activation (Bae and Choi, 2008; Leivar et al., 2008b), we analyzed the same raw microarray data (Monte et al., 2004), focusing now on the expression profiles in the dark (which were previously used exclusively to identify Rc responsive genes). In our current analysis, we took into consideration that, despite the obvious (although subtle) phenotypes observed for dark-grown *pif3* (Figures 1A and 1B) (Leivar et al., 2008b; Shin et al., 2009; Stephenson et al., 2009) and *pif1* (Leivar et al., 2008b; Shin et al., 2009; Stephenson et al., 2009) single mutant seedlings, previous microarray analysis of these mutants in the dark only identified 14 PIF3-regulated genes (Leivar et al., 2009) that were statistically and significantly expressed differently and by twofold (SSTF genes), and did not identify any SSTF genes regulated by PIF1 (Moon et al., 2008). Possible redundancy among PIFs in the regulation of gene expression (in accordance with their proposed redundant function as repressors of seedling deetiolation in the dark [Bae and Choi, 2008; Leivar et al., 2008b]) might translate into gene expression changes in single *pif* mutants smaller than SSTF. For this reason, we have decided to use a 1.5-fold cutoff in our new analysis presented here, a strategy that allowed Moon and colleagues to identify bona fide PIF1 targets (Moon et al., 2008).

Using the Rosetta Resolver platform (Rosetta Biosoftware), we analyzed two data sets of 4-d-old dark-grown seedlings (D0 h and D1 h) harvested 1 h apart and each including three biological replicates for wild type and three for *pif3-3* (Monte et al., 2004) (see Methods and Supplemental Figure 1A online). The complete analysis is presented in Supplemental Analysis 1 and associated Supplemental Figure 1 online; see also Supplemental References 1 online. This analysis identified a set of 121 PIF3-regulated genes (see Supplemental Figure 1A online) that are statistically and significantly expressed differently and by 1.5-fold in *pif3* compared with the wild type (SS1.5F; see Supplemental Data Set 1 online), and a subset of 82 high-confidence (HC) PIF3 targets (SS1.5F-HC; see Supplemental Figure 1A and Supplemental Data Set 2 online). The gene list containing the 39 SS1.5F genes that did not make the HC cutoff is presented in Supplemental Data Set 3 online.

A two-dimensional cluster diagram representing the z-score–normalized signal intensities for the 82 SS1.5F-HC genes is shown in Figure 1C. The diagram contains the expression data for each of the six (three D0 h and three D1 h) wild-type and *pif3* biological replicates used in the analysis, and shows clustering of the 82 SS1.5F-HC genes in two subsets (induced and repressed) that have opposite expression patterns in their dependence on PIF3: approximately one-half of the 82 genes (40 genes) are repressed in *pif3* compared with the wild type, whereas the other one-half (42 genes) are induced (Figures 1C and 1D). The mean fold change (FC) for the up- and downregulated subset of genes is approximately twofold (Figure 1D). Further distribution of the 82 genes by FC is presented in Supplemental Analysis 1 and Supplemental Figure 1 online. It can be concluded that PIF3 represses photomorphogenesis in the dark, at least in part, by positively and negatively regulating the expression of the identified 40 and 42 genes, respectively (Figure 1D), and that, conversely, the misregulation of these genes in dark-grown *pif3* mutant seedlings might contribute to the observed phenotypes (Figures 1A and 1B). Functional classification of the 82 SS1.5F-HC genes is detailed in Supplemental Analysis 2 and the associated Supplemental Figure 2 online; see also Supplemental References 1 online. Notably, 25% of the annotated genes in the induced group in *pif3* relative to the wild type were photosynthesis-/chloroplast-related genes, indicating a degree of photomorphogenesis derepression in *pif3* consistent with its phenotype in the dark.

These expression patterns detected by microarray analysis were validated for selected genes by quantitative RT-PCR (qRT-PCR) analysis (see Supplemental Figure 3 online). Interestingly, the fold difference in expression between the wild type and the *pif3* mutant was more robust after 2 d of dark growth compared with 4 d for some of the tested genes (*AT5G16030*, *AT3G05730*, and *AT5G02760*) (see Supplemental Figure 3B online). These results suggest the existence of a developmentally regulated expression program during seedling growth in the dark. Similar observations were reported by Stephenson et al. (2009) for the behavior of three chlorophyll-biosynthesis genes (*HEMA1*, *GUN4*, and *CHLH*) in dark-grown *pif1*, *pif3*, and *pif1 pif3* mutants. In addition, seed batch variation could also account for some of the data variability, especially when differences are small, as previously reported (Leivar et al., 2008b).

To provide a broader molecular framework for the PIF3-regulated transcriptome in the dark defined here (Figures 1C and 1D; see Supplemental Figure 1 online), we compared it with

Figure 1. (continued).

(WT) Col-0. A total of 42 genes are upregulated (induced) in the absence of PIF3, whereas 40 correspond to genes that are downregulated (repressed), suggesting that PIF3 can act both as repressor and activator of gene expression in the dark.

(D) Mean FC for the 42 upregulated genes (left) and the 40 downregulated genes (right) in the *pif3-3* mutant in the dark relative to the wild-type dark value set at unity. Bars indicate SE for the genes averaged for each group.

Bar in **(A)** = 10 mm.

previous genome-wide studies on *pif4 pif5* (Lorrain et al., 2009) and *pifq* (Leivar et al., 2009). This comparative analysis is presented in Supplemental Analysis 3 online and is associated with Supplemental Figures 4 and 5 and Supplemental Data Set 4 online; see also Supplemental References 1 online. Consistent with the described phenotypic data (Leivar et al., 2008b, Shin et al., 2009; Stephenson et al., 2009), the comparative analysis suggests that PIF3 regulates gene expression in the dark in a partially redundant manner with other PIF factors, including PIF1, and that some specificity might exist among the genes targeted by PIF3 and PIF4/PIF5 in the presence of other PIFs.

Selection of PIF3-Regulated *MISREGULATED IN DARK* Genes and Functional Characterization of *Arabidopsis mida* Mutants

The 82 PIF3-regulated genes identified by microarray analysis were considered good candidate genes to encode regulators of plant growth and development during the deetiolation process. To begin to determine whether some of them have functionally relevant roles in photomorphogenesis, we selected a subset of 10 genes functionally categorized as having potential transcription (*AT4G10240* and *AT5G04340*), signaling (*AT1G48260* and *AT5G02760*), growth and development (*AT4G37300*), stress and defense (*AT3G05730*), or hormone-related (*AT5G50600* and *AT4G10020*) activity, as well as two annotated as unknown (*AT3G47250* and *AT1G02470*), for systematic functional analysis using mutants. To this list, we have added three genes (*AT2G46070* encoding a MAPK kinase, and *AT1G05510* and *AT5G45690* of unknown function) from our SS1.5F gene set for their potential interest based on the predicted function (see Supplemental Analysis 2 online) and/or robust difference in expression in the *pifq* mutant (see Supplemental Analysis 3 online). Most of these genes show a response with respect to the wild type substantially more robust in the *pifq* mutant (Leivar et al. 2009) compared with *pif3* (see Supplemental Figure 6A online). Given that the two gene expression profile experiments were done using samples grown under different conditions (Monte et al., 2004; Leivar et al., 2009), we have validated these differences by qRT-PCR in *pif3* and *pifq* dark-grown seedlings grown at the same time and under the same conditions (see Supplemental Figure 6B online). These results suggest that these genes are targeted by PIF3 and possibly other PIFs during postgerminative growth in the dark.

These 13 genes were named *MISREGULATED IN DARK* (*MIDA*) genes. Table 1 contains a summary of these 13 *MIDA* genes, indicating for each one: *Arabidopsis* Gene Identification (AGI) number, previously ascribed name and reference (if published), FC in *pif3* with respect to the wild type, assigned functional group, our designated *MIDA* name, and the corresponding insertional mutant line isolated or the mutant line obtained if already available. The available mutants include one overexpressor line for *AT5G50600* (Li et al., 2007) and two RNA interference (RNAi) lines for *AT2G46070* (Lee et al., 2009). For *AT5G50600*, T-DNA insertional mutants were available; however, because the gene exists in two copies located in a large duplicated region, it is not possible to distinguish between homozygous and heterozygous lines, because the gene-specific primers cross-hybridize with the intact copy of the duplicated gene (not carrying the T-DNA insertion) during the genotyping process, and thus prevent the identification of *AT5G50600* mutants that are suitable for characterization.

For the T-DNA insertional *mida* mutants, we identified homozygous mutant lines together with corresponding wild-type siblings for the phenotypic studies. For *mida6*, we were unable to find homozygous plants, even after analyzing the progeny of several heterozygous lines, indicating that the mutation might be lethal in homozygosity. All the *mida* mutant lines are in the ecotype Columbia (Col-0) background. Any phenotypes observed in the homozygous lines compared with their wild-type siblings were further confirmed by comparisons with Col-0 seedlings. The 12 mutated loci investigated over here were analyzed for statistically significant differences from the wild type in hypocotyl, cotyledon, and hook phenotypes in 2-, 3-, and 4-d-old dark-grown seedlings. Given the observed wild-type phenotypes during this period of dark development (Figures 1A and 1B), we reasoned that possible photomorphogenic phenotypes of the *mida* mutants might include deviations in both directions in hypocotyl growth (shorter or longer compared with the wild type) and/or in hook opening (decreased or increased angle with respect to the wild type), and deviations in cotyledon separation only in the direction of enhanced opening, because cotyledons remain essentially appressed in the wild type throughout dark development (Figures 1A and 1B). *mida* loss-of-function mutants showing a derepression of photomorphogenesis in the dark (i.e., displaying a shorter hypocotyl and/or a more open hook and cotyledons) would correspond to MIDA factors that potentially function as repressors of photomorphogenesis, whereas those showing enhanced skotomorphogenesis in the dark (i.e., displaying a longer hypocotyl and/or a closer hook) would correspond to MIDA factors with a potential role as inducers of photomorphogenesis.

Figure 2 and Supplemental Data Set 5 online show the functional characterization of *Arabidopsis mida* mutants in the dark, with the quantitative data and statistical analysis for hypocotyl length, hook unfolding, and cotyledon aperture. For comparison, data from multiple experiments are compiled in Figure 2, whereas the complete primary data and statistical analysis for each *mida* line are presented in Supplemental Data Set 5 online. For simplicity, data from each *mida* mutant line in Figure 2 are shown relative to their respective wild-type sibling set at unity, and a horizontal black dashed line set at 1 is included as the wild-type reference. An asterisk indicates the *mida* lines displaying statistically significant differences (see Methods) compared with their respective wild-type sibling in 2-, 3-, and 4-d-old dark-grown seedlings (see Supplemental Data Set 5 online for the associated P values). Even where statistically significant differences were detected (Figure 2; see Supplemental Data Set 5 online), the phenotypic differences between the wild type and *mida* lines ranged in magnitude from marginal to moderate. To define which lines display bona fide phenotypes, we applied a FC criterion, comparing the magnitude of the phenotype to their respective wild-type sibling (Figure 2; see Supplemental Data Set 5 online). Based on the phenotypes displayed by single and double PIF-deficient mutants (Leivar et al., 2008b), we set a FC cutoff at 40% for the hook, 80% for the cotyledon, and 20% for

Table 1. List of the 13 *MIDA* Genes Analyzed, Including the AGI Loci, the Designated Protein Names, the FC in Expression in *pif3* Mutant in the Dark Relative to the Wild Type, and Their Functional Category

MIDA	AGI No.	Protein Name	FC at D0 h *pif3* versus Wild Type	Functional Category	Reported Function	Mutant Line	*Mida Line*
MIDA1	AT5G50600	HSD1	−1.61226	H	Li et al. (2007)	AOHSD16 (Li et al., 2007)	*mida1-OX*
MIDA2	AT3G05730	DEFL	2.78217	S/D	ND	SALK_031670	*mida2*
MIDA3	AT4G37300	MEE59	1.54716	G/D	ND	SALK_040468	*mida3*
MIDA4	AT1G02470	UNKNOWN	2.33482	UNK	ND	SALK_123221	*mida4*
MIDA5	AT3G47250	UNKNOWN	1.568	UNK	ND	SALK_099356	*mida5*
MIDA6	AT5G04340	ZN FINGER	−2.04231	TXN	ND	SALK_140448	*mida6*
MIDA7	AT1G48260	CIPK17	−1.76389	S	ND	SALK_130764	*mida7*
MIDA8	AT4G10020	HSD5	−1.50981	H	ND	SAIL_129B11	*mida8*
MIDA9	AT5G02760	PP2C	1.76423	S	ND	SAIL_764H11	*mida9-1*
MIDA9	AT5G02760	PP2C	1.76423	S	ND	SALK_672093	*mida9-2*
MIDA10	AT4G10240	BBX23	−1.50432	TXN	ND	SALK_053389C	*mida10*
MIDA11	AT2G46070	MPK12	1.679	S	Lee et al. (2009)	MPK12RNAi-9 (Lee et al., 2009)	*mida11-1*
MIDA11	AT2G46070	MPK12	1.679	S	Lee et al. (2009)	MPK12RNAi-17 (Lee et al., 2009)	*mida11-2*
MIDA12	AT1G05510	UNKNOWN	−2.5	UNK	ND	SALK_117754	*mida12*
MIDA13	AT5G45690	UNKNOWN	−1.705	UNK	ND	SALK_145109	*mida13*

The corresponding mutant lines isolated from SALK or SAIL, and the previously identified mutants are indicated together with their *mida* nomenclature. Functional categories: G/D, growth/development; H, hormone; S, signaling; S/D, stress/defense; TXN, transcription; UNK, unknown.
ND, not determined.

the hypocotyl (represented by horizontal red dashed dotted lines in Figure 2). In addition, given the variation in gene expression during dark development (see Supplemental Figures 3 and 4E online), which suggests that the action of PIF3-regulated genes might have variable relevance during the process of skotomorphogenesis, we required that the statistically significant differences and FC cutoffs had to be met in at least 2 d. Together, based on these three defined criteria (P value, FC, and time of action), mutations in four genes caused apparent photomorphogenic seedling phenotypes in the dark (Figure 2): *mida9* and *mida10* showed enhanced hook unfolding, whereas *mida11* displayed shorter hypocotyls, and *mida1-OX* had more separated cotyledons. These results suggest branching of the signal that PIF3 relays through the MIDAs to regulate specific aspects of the deetiolation response. Figures 3 and 4 show a more detailed characterization of these *mida* mutants (see below).

MIDA9 and MIDA10 Are Novel Repressors of Hook Unfolding

Figure 3 shows the *mida9* and *mida10* phenotypes, together with a more detailed characterization of the *mida* mutants, a diagram of the *MIDA* gene that indicates the position of the T-DNA insertion, and an RNA gel blot that confirms the disruption of the transcript in the *mida* mutant. A bar graph showing the FC difference in expression in the *pif3* mutant compared with the wild type in the dark is also included.

For *MIDA9*, a PIF3-repressed gene (Figure 3C), we identified a T-DNA insertional allele, designated *mida9-1*, that carries a T-DNA insertion in the first exon, from the Syngenta Arabidopsis Insertion Library (SAIL) collection (Figure 3A, Table 1). The *mida9-1* allele produced no detectable *MIDA9* transcript and is therefore likely a null (Figure 3B). Hook unfolding phenotypes of two mutant siblings compared with a wild-type sibling and with Col-0 showed that the *mida9* mutant exhibited enhanced hook unfolding after 2, 3, and 4 d in the dark (Figures 3D and 3E). Similar results were obtained for a second null mutant allele of *MIDA9* (*mida9-2*) (Table 1; see Supplemental Figure 7 online). *MIDA9* encodes a previously uncharacterized type 2C phosphatase, predicted to be nuclear, belonging to the D clade of type 2C phosphatases in *Arabidopsis* (Schweighofer et al., 2004). Based on these results, we conclude that MIDA9 is a PIF3-repressed repressor of photomorphogenesis in the dark with a specific role in hook unfolding.

For *MIDA10*, a PIF3-induced gene (Figure 3H), we identified a T-DNA insertional allele designated *mida10-1* from the SALK collection (Alonso et al., 2003; http://signal.salk.edu) that carries a T-DNA insertion in the second exon (Figure 3F, Table 1). The *mida10-1* allele produced no detectable *MIDA10* transcript and is therefore likely a null (Figure 3G). Hook unfolding phenotypes of a wild-type sibling and two mutant siblings compared with Col-0 show the enhanced hook unfolding of the *mida10* mutant after 3 and 4 d in the dark (Figures 3I and 3J). *MIDA10* encodes B-BOX CONTAINING PROTEIN 23 (BBX23) (Datta et al., 2008; Khanna et al., 2009). *BBX23*/*MIDA10* belongs to a clade among the B-Box family of proteins that consists of eight genes, with several of its related members previously implicated in light-dependent development (Datta et al., 2008 and references therein; Khanna et al., 2009). Based on these results, we conclude that BBX23/MIDA10 is a PIF3-induced repressor of photomorphogenesis in the dark with a specific role in hook unfolding. For simplicity, we refer to BBX23/MIDA10 as MIDA10 hereafter.

MIDA11 Is a Novel Regulator of Hypocotyl Elongation

mida11 is a previously published dexamethasone (DEX)-inducible RNAi line (Table 1) (Lee et al., 2009). It was originally shown to have a phenotype in root elongation under continuous

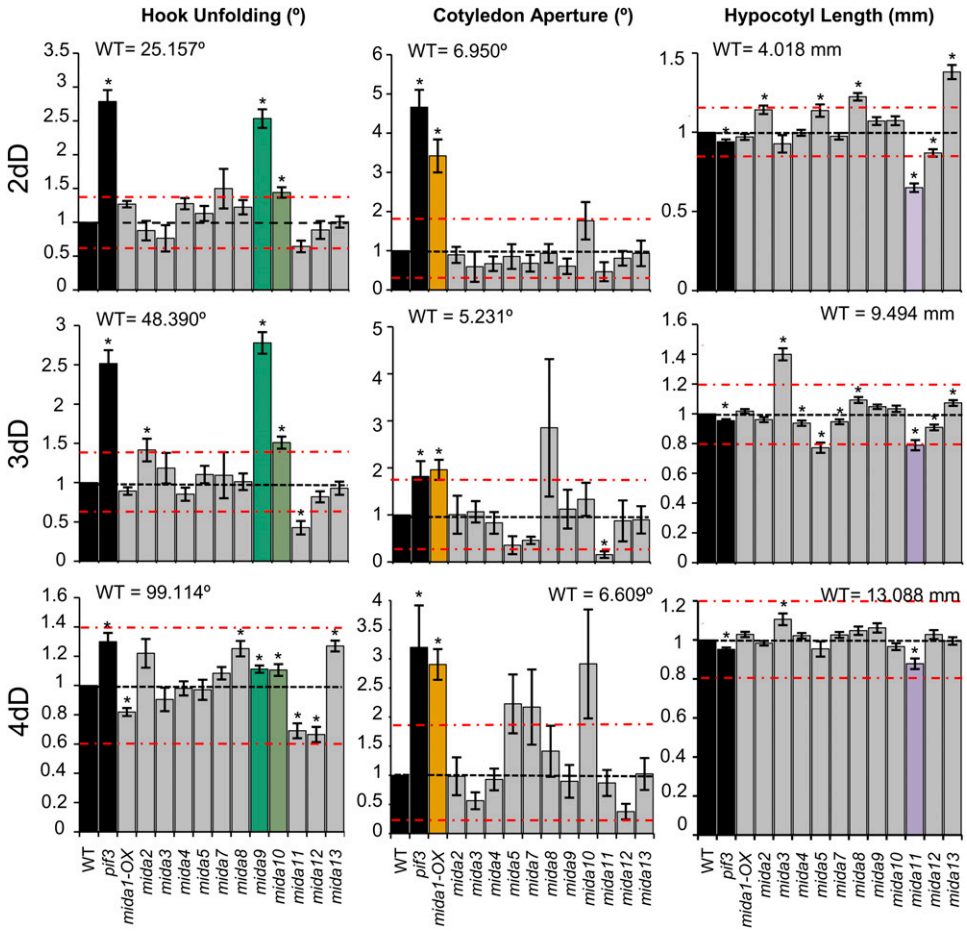

Figure 2. Functional Characterization of *Arabidopsis mida* Mutants Defective in PIF3 Target Genes Identifies Four Novel Regulators of Seedling Deetiolation.

Hook unfolding angle (left), cotyledon separation angle (middle), and hypocotyl length (right), displayed by 2- (top), 3- (middle), and 4-d-old (bottom) *mida* mutant lines. A total of 30 seedlings were used for measurements, and values were normalized to the corresponding wild-type (WT) sibling (see Supplemental Data Set 5 online for primary data and statistical analysis). For each *mida* line, a corresponding wild-type sibling was used as control to calculate the P value and FC difference (see Methods and Supplemental Data Set 5 online for further details). In the bar graph, measurements for *mida* mutant lines are expressed as a FC with respect to their wild-type sibling, whereas error bars represent the variation (SE) of this FC response of at least 30 seedlings (see Supplemental Data Set 5 online). For comparison purposes, a wild type set at unity is shown as reference (shown as horizontal dashed line). The *pif3* mutant is also included as reference. Based on statistical difference (P value < 0.05) (marked with an asterisk in the graph) together with a FC relative to the corresponding wild type greater than 40% for hook, and/or 80% for cotyledon, and/or 20% for hypocotyl (these cutoff percentage values are indicated by a dashed dotted line) in at least two of the 3 d assayed, four *mida* lines were determined to display a partial photomorphogenic phenotype in the dark: *mida9* and *mida10* display partially open hooks, *mida11* displays short hypocotyls, and *mida1-OX* displays partially separated cotyledons. The actual degrees of aperture or the length of the hypocotyl of an average wild-type response from the multiple experiments is indicated as reference on the top of each graph (see Supplemental Data Set 5 online for the calculation). The *mida9* and *mida11* mutant alleles used were *mida9-1* and *mida11-2*, respectively. [See online article for color version of this figure.]

white light (WLc) (Lee et al., 2009). Figure 4B shows the effect of DEX on the amount of *MIDA11* transcript in the wild type and two independent *mida11* (*mida11-1* and *mida11-2*) dark-grown seedlings, indicating that *mida11* has reduced levels in the dark (a 60 to 80% reduction compared with the wild type) in the presence of DEX. DEX application induced a hypocotyl phenotype in both *mida11* RNAi lines compared with the control Col-0 treated with DEX (Figures 4C and 4D). *MIDA11*, a PIF3-repressed gene (Figure 4A), encodes a MAP kinase (MPK12) that has been proposed to regulate auxin signaling (Lee et al., 2009). Based on these results, we conclude that MPK12/MIDA11 is a PIF3-repressed repressor of photomorphogenesis in the dark with a specific role in hypocotyl elongation. For simplicity, we refer to MPK12/MIDA11 as MIDA11 hereafter.

MIDA1 Is a Novel Regulator of Cotyledon Separation

mida1-OX is a previously published *HYDROXYSTEROID DEHYDROGENASE 1* (*HSD1*) overexpressor line (Table 1) (Li et al., 2007). It was originally shown to exhibit a growth phenotype in

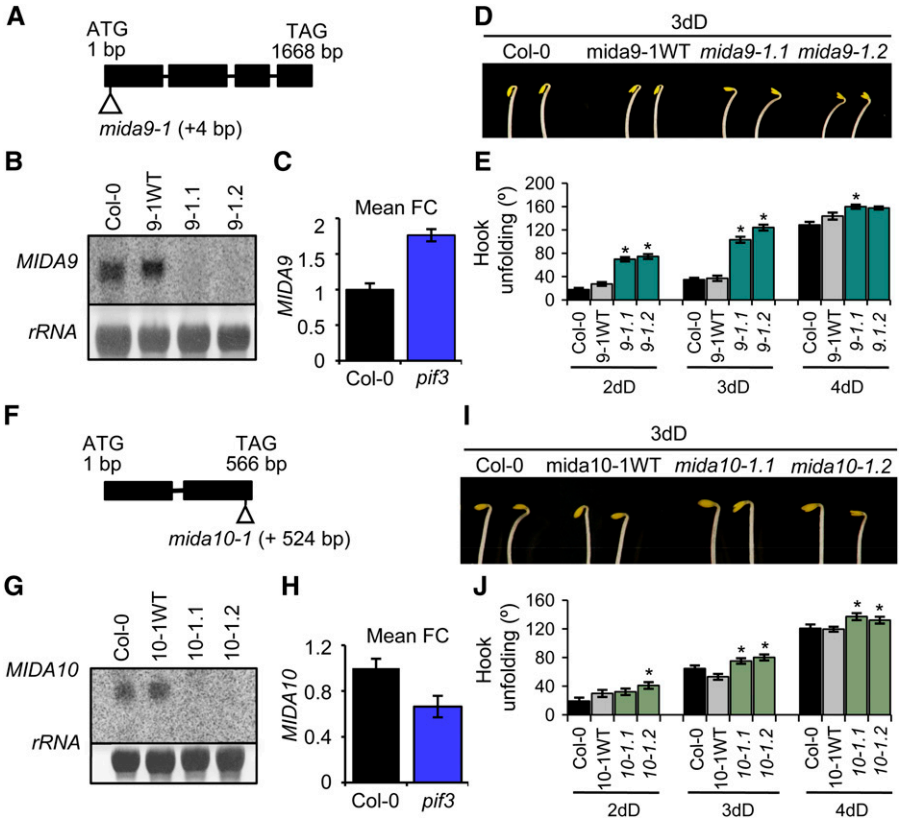

Figure 3. MIDA9 and MIDA10 Are Novel Repressors of Hook Unfolding in the Dark.

(A) The mutation identified in *Arabidopsis MIDA9*. The T-DNA insert in *mida9-1* is indicated at position +4 bp relative to the ATG.
(B) RNA gel blots of 2-d-old, dark-grown Col-0, *mida9-1.1*, and *mida9-1.2* mutant seedlings, and a corresponding *mida9-1* wild-type (WT) sibling. No *MIDA9* transcript was detected in *mida9-1*, indicating that it is likely a functional knockout mutant.
(C) Bar graph of microarray data showing the FC in *MIDA9* expression in *pif3* relative to the wild-type in the dark. Data correspond to biological triplicates, and bars indicate SE.
(D) Visual hook phenotype of 3-d-old, dark-grown Col-0, wild-type sibling, and *mida9-1* mutant seedlings.
(E) Quantification of hook angle in *mida9-1* compared with Col-0 and a wild-type sibling line after 2, 3, and 4 d of growth in the dark (dD) after germination. Data represent the mean and SE of at least 30 seedlings, and asterisks indicate statistically different mean values compared with their corresponding wild type.
(F) The mutation identified in *Arabidopsis MIDA10*. The T-DNA insert in *mida10-1* is indicated at position +524 bp relative to the ATG.
(G) RNA gel blot of 2-d-old, dark-grown Col-0, *mida10-1.1*, and *mida10-1.2* mutant seedlings, and a corresponding *mida10-1* wild-type sibling. No *MIDA10* transcript was detected in *mida10-1*, indicating that it is likely a functional knockout mutant.
(H) Bar graph of microarray data showing the FC in *MIDA10* expression in *pif3* relative to the wild type in the dark. Data correspond to biological triplicates and bars indicate SE.
(I) Visual hook phenotype of 3-d-old dark-grown Col-0, a wild-type sibling, and *mida10-1* seedlings.
(J) Quantification of hook angle in *mida10* compared with Col-0 and a wild-type sibling line after 2, 3, and 4 d of growth in the dark after germination. Data represent the mean and SE of at least 30 seedlings, and asterisks indicate statistically different mean values compared with their corresponding wild type.
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adult plants grown in WLc conditions (Li et al., 2007). Figure 4F shows the expression levels of *HSD1*/*MIDA1* in two overexpressor lines grown in the dark, indicating that *mida1-OX* exhibits increased levels of *HSD1*/*MIDA1* in the dark (between 1.5-fold to fourfold compared with the wild type). Enhanced cotyledon separation in these two overexpressor lines compared with Col-0 after 2, 3, and 4 d in the dark is shown in Figures 4G and 4H. *HSD1*/*MIDA1*, a PIF3-induced gene (Figure 4E), has been proposed to encode an enzyme involved in brassinosteroid (BR) synthesis (Li et al., 2007). Based on these results, we conclude that HSD1/MIDA1 is a PIF3-induced inducer of photomorphogenesis in the dark with a specific role in cotyledon separation. For simplicity, we refer to HSD1/MIDA1 as MIDA1 hereafter.

Light-Responsiveness of PIF3-Regulated Genes in the Dark

The above data are summarized in Supplemental Table 1 online and suggest that PIF3 action in the dark involves the induction of *MIDA10* and *MIDA1*, a negative and a positive regulator of photomorphogenesis, respectively, and the repression of *MIDA9*

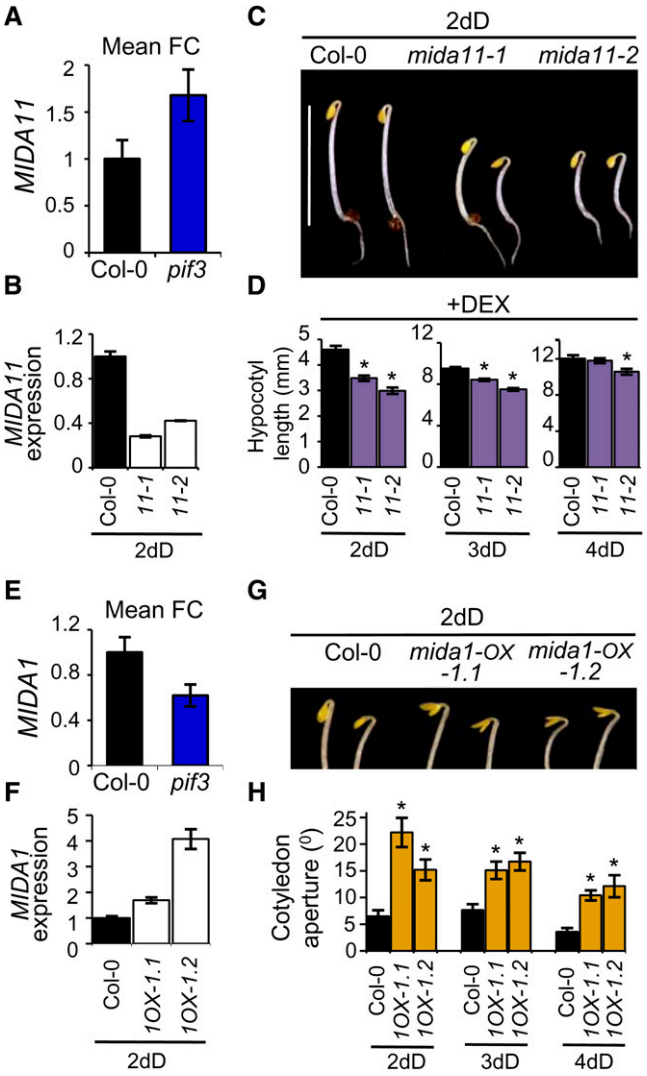

Figure 4. MIDA11 Is a Novel Inducer of Hypocotyl Length and MIDA1 Is a Novel Regulator of Cotyledon Separation in the Dark.

(A) Bar graph of microarray data showing the FC in *MIDA11* expression in *pif3* relative to the wild type in the dark. Data correspond to biological triplicates, and bars indicate SE.

(B) qRT-PCR analysis of 2-d-old, dark-grown Col-0 and *mida11-1* and *mida11-2* mutant seedlings grown in the presence of DEX. Expression levels were normalized to *PP2A* as described previously (Shin et al., 2007) and expressed relative to the wild-type value set at unity. *MIDA11* transcript levels were reduced ~80% in the two lines used, confirming that *MIDA11* expression is suppressed by the DEX-induced RNAi in dark conditions. *mida11-1* and *mida11-2*, two independent RNAi lines, were obtained from Lee et al. (2009) (Table 1). Error bars represent SE values of technical triplicates.

(C) Visual hypocotyl phenotype of 3-d-old dark-grown Col-0 and *mida11-1* and *mida11-2* seedlings in the presence of DEX.

(D) Quantification of hypocotyl length in *mida11* compared with Col-0 after 2, 3, and 4 d of growth in the dark (dD) after germination in the presence of DEX. Data represent the mean and SE of at least 30 seedlings, and asterisks indicate statistically different mean values compared with their corresponding wild type.

(E) Bar graph of microarray data showing the fold change in *MIDA1* expression in *pif3* relative to the wild type in the dark. Data correspond to biological triplicates and bars indicate SE.

(F) qRT-PCR analysis of 2-d-old, dark-grown wild type and *mida1-OX* mutant seedlings. Expression levels were normalized to *PP2A* as described previously (Shin et al., 2007) and expressed relative to the wild-type value set at unity. *MIDA1* transcript was overexpressed in *mida1-OX-1.1* and *mida1-OX-1.2*, confirming that the lines overexpress *MIDA1* in dark conditions. Overexpressor *mida1-OX-1.1* and *mida1-OX-1.2* lines (represented in the figure as 1OX-1.1 and 1OX-1.2, respectively) are two siblings from a transgenic line obtained from Li et al. (2007) (Table 1). Error bars represent SE values of technical triplicates.

(G) Visual cotyledon phenotype of 2-d-old, dark-grown Col-0 and *mida1-OX-1* seedlings.

(H) Quantification of cotyledon angle in *mida1-OX-1* (represented as 1OX-1 in the figure) compared with Col-0 after 2, 3, and 4 d of growth in the dark after germination. Data represent the mean and SE of at least 30 seedlings, and asterisks indicate statistically different mean values compared with their corresponding wild type.

Bar in **(C)** = 5 mm.

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and *MIDA11*, both negative regulators of photomorphogenesis in the dark (Figures 2 to 4). These data provide a complex and somewhat contradictory picture of how PIF3 might exert its function as a repressor of photomorphogenesis. To further analyze how this complex regulatory network might participate during seedling deetiolation, we next addressed the question of how the rapid phy-induced degradation of PIF3 (and other PIFs) upon illumination of dark-grown seedlings might affect the expression of the four identified *MIDAs*.

To do this, we reanalyzed the light data from the same microarray experiment (wild type after 1 h of Rc [R1 h], included in Monte et al., 2004, and the previously unpublished wild type [E. Monte and P. Quail, unpublished data] after 18 h of Rc [R18 h]), using the Rosetta Resolver software for consistency (see Methods). We defined early (R1 h) and late (R18 h) red light–responsive genes as genes that display SSTF alterations when comparing the wild type after 1 h of Rc (R1 h) versus the wild type kept in darkness for 1 h (D1 h) and the wild type after 18 h of Rc (R18 h) versus the wild type kept in darkness for 18 h (D18 h), respectively. We identified 546 R1 h SSTF genes and 2764 R18 h SSTF genes in our experiment. Supplemental Data Sets 6 and 7 online show the gene lists containing R1 h SSTF and R18 h SSTF genes, respectively. We then compared the genes displaying SS1.5F-HC alterations in *pif3* after 4 d in darkness (*pif3*-D) with genes displaying SSTF alterations in the wild type after R1 h and after R18 h. This comparative analysis is presented in Supplemental Analysis 4, the associated Supplemental Figure 8, and Supplemental Data Set 8 online; see also Supplemental References 1 online. Notably, 67% of *pif3*-D genes were light-responsive at R1 h and/or R18 h, with 83.6% of these responding to Rc later than 1 h after illumination (see Supplemental Figure 8A and Supplemental Analysis 4 online).

To establish the light-responsive kinetics of the four *MIDA* genes identified to have a role in deetiolation (Figures 3 and 4), we combined the R1 h and R18 h microarray information for each gene (see Supplemental Figure 9 online) with a detailed time-

course qRT-PCR analysis of 2-d-old dark-grown wild-type seedlings exposed to Rc for increasing periods of time (Figure 5A). Our results show that light triggers an immediate early response of the *MIDA10* transcript, with a 10-fold light repression at 1 h compared with dark levels, and reaches almost nondetectable levels after 12 h of Rc exposure (Figure 5A). *MIDA1* also responds early with a sixfold induction after 2 h of Rc exposure in 2-d-old dark-grown seedlings (Figure 5A). This induction of *MIDA1* in light conditions is transient, and transcript levels return to dark levels after 6 h of irradiation (Figure 5A). Finally, Rc triggers a twofold induction of *MIDA9* and *MIDA11* transcripts relative to their dark control after 3 and 6 to 9 h, respectively (Figure 5A), an induction that decreases again after 18 h of Rc (see Supplemental Figure 9 online). For all four genes, expression levels in the *pif3* mutant kept in the dark during this time showed little variation (Figure 5A). These qRT-PCR results validate and expand on the microarray data at R1 h and R18 h for these genes (see Supplemental Figure 9 online), and together indicate that the rapid phy-induced degradation of PIF3 triggers a light response in all four *MIDA* genes in the wild type that is in the same direction as the alteration in expression caused by PIF3 deficiency in the dark: One is light-repressed (*MIDA10*), and three are light-induced (*MIDA9*, *MIDA11* and *MIDA1*). In addition, these results indicate that PIF3 degradation triggers an early light response in *MIDA10* and *MIDA1* and a late light response in *MIDA9* and *MIDA11* (Figure 5A). Altogether, these data suggest that the MIDA factors induced by light (MIDA9, MIDA11, and MIDA1) might not only have a role during skotomorphogenesis in the dark but also function during deetiolation either early (after 1 to 3 h of Rc) and/or late (after more than 3 h of Rc) once the seedling has been exposed to light.

Participation of the MIDAs in the Seedling Responses to Light

We examined the phenotypes of *mida9*, *mida10*, *mida11*, and *mida1-OX* in the dark-to-Rc transition. Figure 5B shows the results for each of the mutants. For *mida10*, 2-d-old etiolated seedlings show a weak unfolded hook phenotype in the dark (Figures 2, 3J, and 5B), and exposure to light accelerates the hook opening response compared with the corresponding wild type, resulting in an aperture of 40° after 3 h (Figure 5B). These results suggest that MIDA10 acts as a repressor of hook opening during the initial deetiolation response, consistent with its role as a hook repressor in the dark (Figures 2, 3I, and 3J) and its rapid degradation upon exposure to light (Figure 5A). For *mida1-OX*, the differences in cotyledon separation between the mutant and the wild type in the dark (Figures 2, 4G, 4H, and 5B) are larger in response to Rc (Figure 5B): Cotyledons in 2-d-old wild-type seedlings are basically appressed in the dark (10° aperture) and start responding to light 12 h after Rc exposure, to reach an aperture of 80° after 24 h of illumination. By contrast, the cotyledons of *mida1-OX* are partially separated in the dark (30°, threefold the wild-type aperture), start responding to Rc

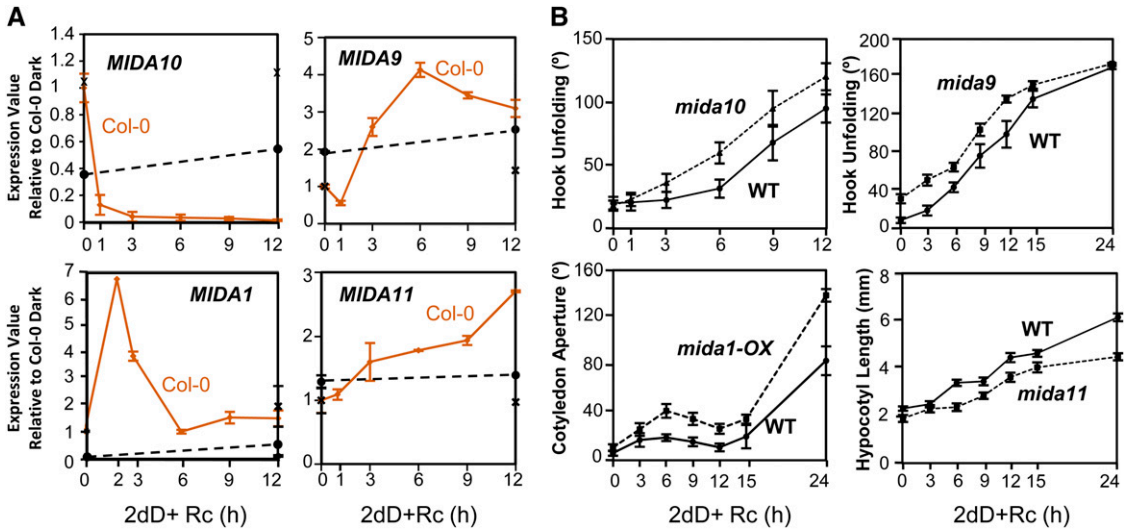

Figure 5. Light-Responsiveness of *MIDA* Gene Expression and Phenotypic Characterization of *mida* Mutants during the Dark-to-Light Transition.

(A) Light-responsiveness of selected PIF3-regulated *MIDA* genes in dark-grown wild-type (WT) seedlings exposed to Rc (8 μmol/m^2/s). Wild-type siblings were exposed to Rc for increasing periods from 0 (dark control) to 12 h, and expression levels were assayed by qRT-PCR, normalized to *PP2A* as described previously (Shin et al., 2007), and expressed relative to the Col-0 dark value set at unity. Expression levels in the *pif3* mutant in the dark are indicated with a dashed line for comparison. The expression level in Col-0 maintained in the dark for 12 h is indicated in the graph with an X. Error bars correspond to SE values of technical triplicates.

(B) Time-course quantification of hook opening (*mida9* and *mida10*), cotyledon separation (*mida1-OX*), and hypocotyl growth (*mida11*) (in the presence of DEX), of 2-d-old, dark-grown wild type (WT) (solid lines) and *mida* mutant seedlings (dashed lines) during the dark-to-red light transition. Data represent the mean and SE of at least 30 seedlings.

[See online article for color version of this figure.]

earlier than the wild type (after only 3 h of illumination), and reach an angle of 140° after 24 h of Rc. These results indicate that MIDA1 functions as an inducer of cotyledon separation during early deetiolation, consistent with the observed phenotype of *mida1-OX* in the dark (Figures 2, 4G, and 4H) and with the rapid *MIDA1* induction in response to Rc (Figure 5A). For *mida9*, our results showed more open hooks in *mida9* mutants compared with the wild type over the time-course analysis in response to light (Figure 5B). This effect is difficult to attribute specifically to light, given that *mida9* hooks are already opened in the dark (Figures 2, 3D, 3E, and 5B), similar to the hook response of *pif* mutants in the dark and in the dark-to-light response (Leivar et al., 2008b). Alternative evidence of a role for MIDA9 in hook repression in the light was obtained by growing seedlings continuously in low far-red light (FR) (see Supplemental Figure 10 online). In these conditions, the wild-type hooks are only partially opened after 4 d (aperture of 120°), and the hooks of *mida9* seedlings are wider open (160°) (see Supplemental Figure 10 online). These data suggest a role for MIDA9 as a repressor of hook unfolding in the dark (Figures 2, 3D, and 3E) and in the light, consistent with the observed phenotype of *mida9* in the dark (Figures 2, 3D, and 3E) and with the *MIDA9* induction in response to light (Figure 5A). Finally, for the DEX-inducible *mida11*, the differences in hypocotyl length between the mutant and the wild type in the dark (Figures 2, 4C, and 4D) increase in response to Rc in the presence of DEX (Figure 5B). Whereas the wild-type seedlings grow from 2.4 mm in the dark to 6.2 mm after 24 h of Rc, *mida11* seedlings grow from 1.9 mm in the dark (20% shorter than the wild type) to only 4.4 mm after 24 h of Rc (30% shorter than the wild type at the same time point) (Figure 5B). Hypocotyl elongation rate in *mida11* compared with the wild type seems to be progressively affected over time after the first 3 h of light exposure (Figure 5B). As a control, etiolated *mida11* seedlings grown in the absence of DEX showed no difference in hypocotyl length in the dark or in the transition to light compared with the control (see Supplemental Figure 11 online). These results indicate that MIDA11 functions as a repressor of hypocotyl elongation inhibition in the dark-to-light transition (Figure 5B), with a more prominent role after 3 h of light exposure, consistent with the observed phenotype of *mida11* in the dark (Figures 2, 4C, and 4D) and with the induction of *MIDA11* in response to Rc (Figure 5A).

Altogether, our data suggest that the apparent contradiction of having PIF3 in the dark induce *MIDA10* and *MIDA1*, a negative and a positive regulator of photomorphogenesis, respectively, and repress *MIDA9* and *MIDA11*, both negative regulators, can be explained if one considers the early or late light responsiveness of these MIDA factors as well as their time of action in the dark-to-light transition. A summary of the above data regarding light responsiveness of the four *MIDA* genes and light phenotypes of their *mida* mutants, integrated with the results of our previous analysis of the expression of each gene in seedlings grown in the dark, is shown in Supplemental Table 1 online. For MIDA10, these data suggest a simple scenario, where early PIF3/phy-mediated light repression allows the rapid removal of a dark hook repressor, which facilitates the rapid hook unfolding that occurs during the initiation of deetiolation. Likewise, for MIDA1, the early PIF3/phy-mediated induction upon exposure of the seedling to light allows for the rapid accumulation of a cotyledon separation inducer, which contributes to cotyledon separation during the initiation of deetiolation. Given that *mida1-OX* is an overexpressor mutant line (Figure 4F), the high levels of MIDA1 in this mutant in the dark compared with those of the wild type possibly mimic the levels reached in the wild type after light induction, and *mida1-OX* mutant seedlings display a phenotype of separated cotyledons in the absence of light. Also, the transient nature of its light induction suggests that after a few hours of illumination, the expression of *MIDA1* is repressed to stop its cotyledon separation action. MIDA10 and MIDA1 might therefore participate in the dark and/or the early (1 to 3 h of Rc) steps of deetiolation induction of hook unfolding and cotyledon separation. By contrast, MIDA9 and MIDA11 are both repressors of photomorphogenesis (specifically of hook opening and of the inhibition of hypocotyl elongation, respectively) that are late light-induced (after 3 to 6 h of Rc) and seem to function not only in the dark but also during deetiolation, once the seedling has been exposed to light (Figure 5B).

Interestingly, our unexpected finding that the seedlings possess photomorphogenesis repressors (MIDA9 and MIDA11) that are late light-induced (after 3 to 6 h of Rc), is consistent with the existence of a PIF3/phy-mediated regulatory response in the deetiolation process that might function after deetiolation is initiated. This late (after 3 to 6 h of Rc) regulatory response could represent a mechanism for the seedling to moderate the rapid initial response.

PIF3 Together with Other PIFs Prevent an Exaggerated Inhibition of Hypocotyl Elongation and Cotyledon Separation in Response to Light

PIFs have been previously reported to be negative regulators of hypocotyl elongation in Rc conditions, with PIF-deficient mutants showing hypersensitivity to Rc (Huq and Quail, 2002; Kim et al., 2003; Fujimori et al., 2004; Monte et al., 2004; Khanna et al., 2007; de Lucas et al., 2008; Leivar et al., 2008a). However, a possible role for the PIFs in the regulation of hypocotyl inhibition in the initial dark-to-light transition has not been explored. We examined the inhibition of hypocotyl elongation in *pif3* and *pif3 pif4 pif5* mutants. Figure 6A shows that dark-grown wild-type seedlings respond to the light trigger by inhibiting hypocotyl elongation and reducing the hypocotyl growth rate. Red light has been shown to induce inhibition of hypocotyl growth in dark-grown seedlings exposed to Rc during the first 3 h of illumination, effectively slowing down the hypocotyl growth rate (Parks and Spalding, 1999). This inhibition begins to decrease after 3 h of irradiation, and seedlings in red light keep growing at a reduced speed compared with seedlings maintained in darkness (Parks and Spalding, 1999). In accordance, our results show that the wild-type hypocotyls elongate from 3.8 mm to 8 mm 24 h after exposure to Rc, whereas seedlings kept in the dark maintain a more constant hypocotyl growth speed and reach 9.6 mm (Figure 6A). Strikingly, *pif3 pif4 pif5* seedlings almost completely stop elongating after exposure to light (Figure 6A). This phenotype suggests that there is an exaggerated inhibition of hypocotyl elongation during deetiolation in the absence of PIF3, PIF4, and PIF5. *pif3 pif4 pif5* mutants maintained in the dark during this

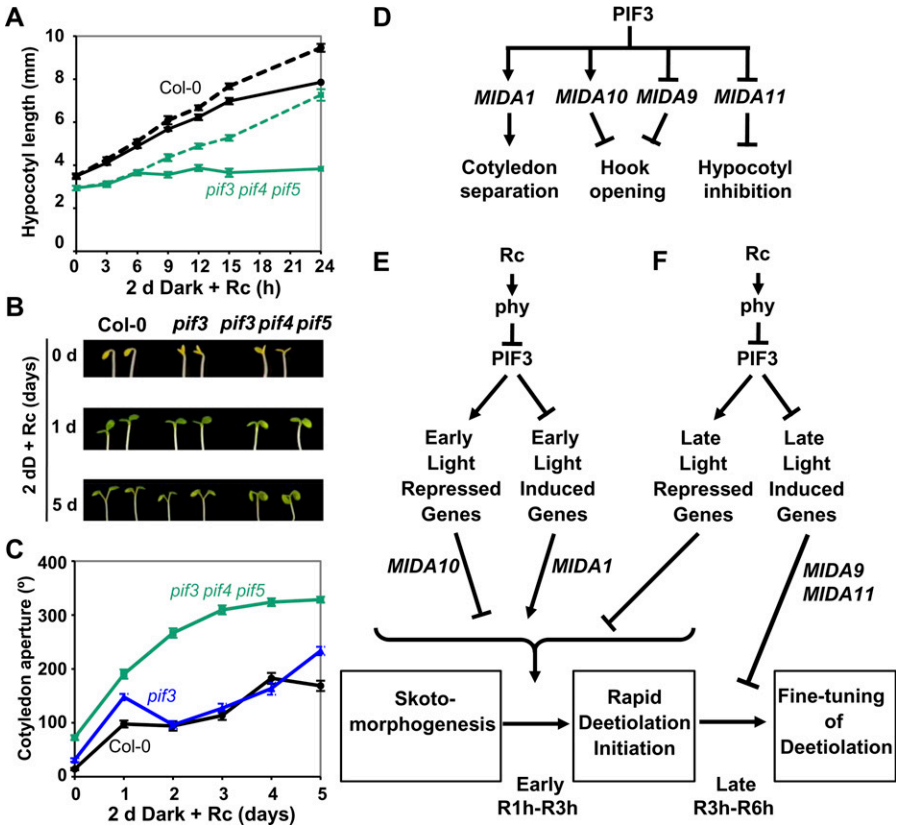

Figure 6. PIF-Regulated Transcriptional Network.

(A) to **(C)** Dark-grown PIF-deficient seedlings exhibit an exaggerated response to Rc (8 μmol/m^2/s).

(A) Time-course quantification of hypocotyl length of 2-d-old dark-grown Col-0 and *pif3 pif4 pif5* seedlings kept in the dark (dashed lines) or during the dark-to-light transition (solid lines) for 24 h. Data represent the mean and SE of at least 30 seedlings.

(B) Visual phenotype of 2-d-old, dark-grown Col-0, *pif3*, and *pif3 pif4 pif5* seedlings exposed to 0, 1, or 5 d of Rc.

(C) Time-course quantification of cotyledon separation of 2-d-old, dark-grown Col-0, *pif3*, and *pif3 pif4 pif5* seedlings during the transition to Rc light for 5 d. Data represent the mean and SE of at least 30 seedlings.

(D) Simplified schematic model depicting the branching in the signaling that PIF3 relays to regulate specific aspects of deetiolation, like cotyledon separation, hook opening, and hypocotyl inhibition through the MIDAs.

(E) and **(F)** Simplified schematic model depicting the PIF3-dependent *MIDA* transcriptional network that regulates seedling deetiolation in response to phy-mediated light signals. PIF3 acts constitutively in darkness as either a transcriptional repressor or activator, resulting in the regulation of *MIDA* gene expression. Phy-mediated, light-induced degradation of PIF3 triggers reversal of PIF3 action on *MIDA* genes that are early **(E)** or late **(F)** light-responsive. Early (1 h) light-responsive genes rapidly initiate deetiolation in response to phy-mediated PIF degradation **(E)**, acting either as light-induced inducers (such as *MIDA1*) or light-repressed repressors (such as *MIDA10*) of deetiolation. By contrast, late (3 to 6 h) light-responsive genes **(F)** have the opposite function to slow down and fine-tune the initial response and optimize seedling deetiolation, as exemplified here by *MIDA9* and *MIDA11*.

period kept growing at the same rate (Figure 6A). Single *pif3* mutants exhibit only a marginal phenotype after exposure to Rc (see Supplemental Figure 12A online), suggesting that PIF3 might be redundant to other PIFs, including PIF4 and PIF5, in the regulation of hypocotyl elongation during the dark-to-light transition, as has previously been described for skotomorphogenesis in the dark (Bae and Choi, 2008; Leivar et al., 2008b).

PIF-deficient mutants have also been shown to have more separated cotyledons during the dark-to-light transition, a phenotype that is partially established in the dark, and reach a maximum angle of 180° during the first 24 h of illumination (Leivar et al., 2008b). Closer examination of *pif3 pif4 pif5* mutant seedlings during extended Rc exposure after 2 d of dark growth reveals a striking cotyledon overseparation in response to light. The cotyledons of the wild-type seedlings separate to ~100° after 24 h of exposure to Rc (Figures 6B and 6C). This fast response is followed by a slower response over the next 3 d of growth in Rc, when cotyledons reach a maximum angle of 185° (i.e., perpendicular to the hypocotyl), effectively maintaining an optimum angle for light perception (Figures 6B and 6C). The cotyledons of the wild-type seedlings kept in darkness for this time period remain appressed (see Supplemental Figure 12B online). Compared with the wild-type seedlings, *pif3 pif4 pif5* mutants exhibit partially separated cotyledons in the dark (60°), as previously described (Leivar et al., 2008b), and have a fast initial response during the first 24 h of light exposure that is similar

in magnitude to the wild-type response, reaching a cotyledon separation of 200° (Figures 6B and 6C). However, in contrast with the wild type, this fast response is maintained over the next 3 d of growth in Rc to reach a cotyledon separation of 310° (Figures 6B and 6C). The cotyledons of *pif3 pif4 pif5* mutants maintained in the dark during this time period open from 60° to 150° (see Supplemental Figure 12B online), a difference that was greatly amplified by light (Figures 6B and 6C). The response of *pif3* (which reaches a cotyledon angle of 240°) is also greater than the wild-type response (which reaches 185° of cotyledon aperture, as detailed above) (Figures 6B and 6C). These results indicate that, in the absence of PIF3, seedlings undergo exaggerated cotyledon separation in response to light, suggesting that PIF3 regulates the inhibition of cotyledon separation. A detailed examination of *pif3 pif4 pif5* also shows an overresponse during the first 24 h of exposure to light (see Supplemental Figure 12C online), as occurs to a lesser extent in *pif3* (see Supplemental Figure 12C online) (Leivar et al., 2008b). Together, our data indicate that the PIF proteins have an important role in preventing the overseparation of cotyledons during seedling establishment, with PIF3 acting in a partially redundant manner to PIF4 and PIF5.

DISCUSSION

Despite much progress in recent years, our understanding of how PIFs function during seedling deetiolation is incomplete, partly because the role of PIF target genes remains largely unknown. In this study, we have expanded on the morphological and molecular characterization of the *pif3* mutant to identify bona fide target genes of PIF3 action in the dark. Functional profiling of the identified PIF3-target genes suggests branching of the signaling that PIF3 relays to regulate specific facets of deetiolation, such as hypocotyl elongation, cotyledon separation, and hook opening. The regulation of these downstream organ-specific targets by light is consistent with a model of PIF3/MIDA action that enables an initial fast response to the light and subsequently prevents overresponses to the light trigger.

Branching of PIF3 Signaling through Four Novel PIF3-Regulated MIDA Factors to Regulate Different Facets of Seedling Development in the Dark

Our analysis of PIF3-regulated gene expression in etiolated seedlings shows that, in darkness, PIF3 regulates 82 genes (Figure 1; see Supplemental Figure 1 online). With the objective of determining to what extent these PIF3-regulated genes are necessary for transducing the PIF3 signal during seedling deetiolation, we selected 13 PIF3 target genes (*MIDA1* to *MIDA13*) based on their predicted function for systematic analysis of mutant phenotypes (Table 1). Our phenotypic data analysis determined that four of the *MIDA* genes mutagenized in this study (*MIDA9*, *MIDA10*, *MIDA11*, and *MIDA1*) exhibit significant perturbation of the etiolated phenotypes and represent novel regulators of seedling development in the dark (Figure 2). Expression analyses by qRT-PCR and microarray suggest that these MIDA factors are likely targeted by other PIFs in addition to PIF3 (see Supplemental Figure 6 and Supplemental Analysis 3 online), because their response is more robust in *pifq* than in *pif3*.

Because this study systematically characterizes the role of PIF3-regulated genes in the dark, it was of interest to determine whether the *mida* mutants would be affected in the complete seedling etiolation development, and/or whether we would detect organ-specific actions. Based on the phenotypes of these four *mida* mutants, our data indicate that there is branching in the regulation of seedling deetiolation that PIF3 relays. Indeed, MIDA9 and MIDA10 are necessary for hook maintenance in the dark, whereas MIDA11 regulates hypocotyl elongation, and MIDA1 is involved in cotyledon separation (Figures 2, 3, and 4), indicating that these MIDA factors have organ-specific activity. One of these MIDA factors, MIDA10, is a negative regulator of hook unfolding (Figures 2 and 3). *MIDA10* encodes BBX23, a previously uncharacterized member of the *Arabidopsis* B-box family of transcription factors. Within this family, BBX23 forms part of a clade of eight members, four of which (BBX21, BBX22, BBX24, and BBX25) were previously implicated in light signaling (Khanna et al., 2006; Datta et al., 2007; Indorf et al., 2007) and possibly form a large complex with COP1 (Datta et al., 2008). BBX23 might also interact directly or indirectly with COP1. *MIDA9*, the second *MIDA* gene that participates in the regulation of hook maintenance as a negative regulator of hook unfolding, encodes a type 2C-phosphatase (PP2C) (Figures 2 and 3). Out of the 76 PP2Cs identified in *Arabidopsis* (Schweighofer et al., 2004), MIDA9 is the only PP2C shown to be involved in seedling deetiolation. The third gene found to make a significant contribution to seedling deetiolation, specifically in the regulation of hypocotyl elongation, is *MIDA11* (Figures 2 and 4), a gene that encodes a MAP kinase. *MIDA11* has been recently reported to regulate auxin signaling in *Arabidopsis* roots (Lee et al., 2009). Interestingly, auxin participates in the induction of fast hypocotyl growth in dark-grown seedlings (De Grauwe et al., 2005). Also related to hormone signaling, the fourth gene, *MIDA1*, encodes HSD1, a hydroxysteroid dehydrogenase proposed to participate in the biosynthesis of BRs (Li et al., 2007). Adult *Arabidopsis* plants constitutively overexpressing HSD1 constitutively express BR response genes and display phenotypes similar to those of plants overproducing BR or the BR receptor, BRI1; that is, greater growth with increased branching and longer roots (Li et al., 2007). Based on the phenotype of BR-deficient mutants, BRs have also been shown to participate in seedling deetiolation (Li et al., 1996; Szekeres et al., 1996). Although more investigation is required, both MIDA11 and MIDA1 might contribute to the interplay between light and hormone signaling pathways, an integration that is essential for the coordination of seedling development (Halliday, 2004; Alabadí and Blázquez, 2009; Lau and Deng, 2010). Altogether, our data indicate that PIF3 signaling branches at a point where MIDA9, MIDA10, MIDA11, and MIDA1 regulate different organ-specific pathways that might involve COP1 and hormone biosynthesis and/or signaling to coordinate the deetiolation response (see model in Figure 6D). Branching of the PIF3 signal might be achieved through differential spatial expression patterns of these MIDA factors in specific tissues or organs. More detailed analyses are required to assess this possibility (Bou-Torrent et al., 2008).

Eight out of the 12 tested loci seem not to have a significant role in regulating the hypocotyl, cotyledon, or hook responses downstream of PIF3 in the dark (Figure 2). Possible explanations for this lack of phenotype include: First, the expression changes detected in *pif3* for these *MIDA* genes might be functionally insignificant for the etiolated seedling, and thus irrelevant for the *pif3* phenotype in the dark. Although most of these genes are also targets of PIFq (see Supplemental Figures 4 and 6 online) and their expression is more robustly affected in *pifq*, correlating with the stronger phenotype, this remains a possibility. Second, some of these *MIDA* genes might cause a detectable phenotype when mutated, but this phenotype is not strong enough and/or sustained for long enough along dark development to meet our cutoff requirements for a bona fide phenotype and thus was not considered further (e.g., *mida11* and *mida12* in hook opening) (Figure 2; see Supplemental Data Set 5 online). Third, these genes might be relevant for PIF3-imposed seedling deetiolation, but functional redundancy with other factors ensures that disruption of a single gene does not have any phenotypic relevance. Functional redundancy is the most common explanation for lack of apparent phenotype, and the PIFs themselves exemplify this possibility (Leivar et al., 2008b; Shin et al., 2009). For the *MIDA* genes that lack an apparent phenotype, a search of the *Arabidopsis* databases reveals that two (*MIDA7* and *MIDA8*) belong to gene families (to the *CBL-INTERACTING PROTEIN KINASE* [*CIPK*] and the *HSD* gene families, respectively), and that *MIDA8* has another family member (*MIDA1*) that is also a PIF3 target (Gene Set 2) (Table 1). An assessment of possible functional redundancy in these cases would require the construction of higher-order combinations of the candidate genes. Finally, another possibility is that these MIDA factors might specifically affect deetiolation aspects that were not scored in our phenotypic analysis, such as chloroplast development or cotyledon expansion. More detailed analyses are needed to determine why mutation of each of these *MIDA* genes does not result in a dark seedling phenotype.

Given that PIF3 binds specifically to the G-box motif (Martínez-García et al., 2000; Shin et al., 2007), we inspected the 3-kb region upstream of the transcription start site of *MIDA* genes for the presence of the G-box motif CACGTG (See Methods) to determine whether functionally relevant *MIDA*s could potentially be directly regulated by PIF3. We found that of the four *MIDA* genes displaying a phenotype in the dark when mutated (*MIDA9*, *MIDA10*, *MIDA11*, and *MIDA1*) (Figures 3 and 4), only *MIDA9* had a G-box in its promoter sequence. Three other *MIDA* genes (*MIDA6*, *MIDA8*, and *MIDA13*) had G-boxes in their promoter sequences, but their mutants did not display a phenotype when examined in the dark (Figure 2), suggesting a lack of correlation in *MIDA* genes between the presence of a G-box in their promoters and the phenotypic effect in the dark when mutated.

Light Regulation of PIF3 Signaling through the Organ-Specific MIDA Factors

Our data indicate that two of the *mida* mutants (*mida9* and *mida10*) exhibiting a similar phenotype in the dark (failure to maintain an apical hook) correspond to genes that are both negative regulators of hook opening and are regulated by PIF3 in opposite directions in the dark: whereas *MIDA9* is repressed, *MIDA10* is induced by PIF3 (Figure 3, Table 1). This finding prompted us to hypothesize that this apparent contradiction might reflect the scenario played out once the wild-type etiolated seedling is exposed to light and PIF3 is degraded, rather than being a dark-specific phenomenon. A combination of Rc microarray data and detailed time courses analyzed by qRT-PCR (Figure 5; see Supplemental Figures 8 and 9 online) indicated that *MIDA10* is an early (1 h) light-repressed gene whose repression is maintained after 18 h of Rc, whereas *MIDA1* is early and transiently induced by light, and *MIDA9* and *MIDA11* show late light-induction after 3 to 6 h of Rc illumination (Figure 5; see Supplemental Figures 8 and 9 online). Our data show that these *MIDA* genes do not respond to light exposure simultaneously but rather in at least two temporally separated responses: one early (after 1 to 3 h of Rc) (*MIDA10* and *MIDA1*), and one late (after 3 to 6 h of Rc) (*MIDA9* and *MIDA11*). These data suggest that these MIDA factors that have a role in organ-specific seedling deetiolation might exert their function at different times, with those induced by light (*MIDA9*, *MIDA11*, and *MIDA1*) possibly extending their action beyond the dark period. Indeed, when we examined these *mida* mutants phenotypically in dark-to-red time courses, we detected that they also have defects in the deetiolation response upon Rc exposure (Figure 5B). Our data indicate that MIDA11 is a negative regulator of hypocotyl elongation inhibition both in the dark and upon illumination, MIDA1 is a positive regulator of cotyledon separation in the dark and during the first hours of red light illumination, and MIDA10 is a negative regulator of hook opening in the dark and in the early initiation of deetiolation. Furthermore, MIDA9 is a negative regulator of hook opening in the dark and during deetiolation, with a role that might be more prominent after 6 h of irradiation.

PIFs have been described as repressors of photomorphogenesis in the dark (Bae and Choi, 2008; Leivar et al., 2008b). The current model proposes that PIF action in the dark is exerted through the regulation of the expression of hundreds of genes by inducing presumptive repressors and by repressing presumptive inducers of photomorphogenesis, a function that is reversed by phy-induced PIF-degradation in response to light (Leivar et al., 2009; Shin et al., 2009). The functional profiling of PIF3-induced and -repressed genes presented here suggests an additional layer of complexity by which the PIF-phy system regulates deetiolation. Our data indicate that, in the dark, PIF3 both up- and downregulates inducers as well as repressors of photomorphogenesis, inducing the repressor MIDA10 and the inducer MIDA1, and repressing the repressors MIDA9 and MIDA11 (see Supplemental Table 1 online). A model for the phy/PIF/MIDA mode of action is shown in Figures 6E and 6F. Given the partially deetiolated phenotype of *pif3*- in the dark, these findings suggest that the PIF system maintains a balance of inducer and repressor factors in the dark, with a preponderance of photomorphogenesis repressor activity, to maintain the etiolated state of the seedling in darkness. This action would be rapidly reversed upon light-induced degradation of the PIFs, shifting this balance to a dominance of photomorphogenesis inducer activity to initiate deetiolation. Accordingly, during this early and rapid initiation of seedling deetiolation (after 1 to 3 h of Rc), our data show that the repressor MIDA10 is repressed in response to light, whereas the

inducer MIDA1 is induced by light. Furthermore, some of these MIDA regulators (MIDA9 and MIDA11) are late light-induced (after 3 to 6 h of Rc) (Figure 5A), suggesting that they act beyond the dark state and beyond the initial deetiolation trigger. Given that MIDA9 and MIDA11 correspond to repressors of photomorphogenesis (Figures 2, 3, and 5B) and that their induction takes place simultaneously with the late light repression of early inducers, such as MIDA1 (Figure 5A), our findings suggest that, after a few hours of illumination, once deetiolation is underway, the seedling again accumulates repressors of photomorphogenesis. These results are consistent with a scenario in which PIF3 regulates not only the rapid initial deetiolation trigger but also a subsequent counteractive response to prevent overresponses to light. In accordance, our data reveal that *pif3* and, to a greater extent, *pif3 pif4 pif5* are affected in the moderation of the initial light trigger and exhibit exaggerated cotyledon separation and inhibition of hypocotyl elongation, effects that are apparent after 1 to 2 d of Rc for cotyledon separation or after a few hours of illumination for hypocotyl response (Figures 6A to 6C). These data suggest that PIF3, together with other PIFs, such as PIF4 and PIF5, signal beyond the initial light trigger and exert a late repressive action to avoid excessive cotyledon separation and hypocotyl elongation inhibition. This late action is in apparent discrepancy with the rapid degradation of PIF3 in the light (Bauer et al., 2004; Monte et al., 2004; Park et al., 2004; Al-Sady et al., 2006). The late action of PIF3 could occur indirectly through secondary downstream targets and/or be exerted by the remaining light-stable pool of PIF3 (~10% of the levels in the dark) after the initial degradation (Monte et al., 2004). This late PIF-mediated process seems likely to be fundamental for seedling survival during the initial exposure to light. For example, it ensures that the cotyledons separate rapidly and are maintained at an angle parallel to the soil, optimal for light perception (Figures 6B and 6C). The existence of mechanisms that prevent overresponsiveness to the initial stimulus is an emerging theme in the regulation of responses to light, as has been described in the shade avoidance syndrome (Sessa et al., 2005) and, more recently, in responses to FR light (Li et al., 2010).

In conclusion, this study identifies downstream branching of PIF3 signaling as a means to optimize seedling deetiolation. We show that regulation of novel MIDA factors by the phy/PIF system enables the seedling to repress photomorphogenesis in the dark and respond optimally to light by regulating the abundance of positive and negative regulators of specific facets of photomorphogenesis, such as hypocotyl elongation, hook unfolding, and cotyledon separation. It will be of interest to determine how this regulation is achieved in the seedling by identifying additional PIF3-regulated components and the direct targets of PIF3 that orchestrate these organ-specific responses.

METHODS

Plant Material, Seedling Growth, and Measurements

T-DNA lines in the ecotype Col-0 background were identified by searching the Salk Institute Genomic Analysis Laboratory database (Alonso et al., 2003) (http://signal.salk.edu/cgi-bin/tdnaexpress). When possible, insertions within the promoter or in the 5′-region of the gene were favored as specified in Table 1. Homozygous T-DNA insertion lines and wild-type siblings were identified using PCR with T-DNA- and gene-specific primers designed using the iSct Primers tool available in the Salk Institute Genomic Analysis Laboratory website. The primer sequences for each line can be found in Supplemental Table 2 online. For phenotypic analyses, two sibling mutant lines were compared with a wild-type sibling line and with the Col-0 controls. Wild-type and mutant seedlings were plated on GM medium without Suc as previously described (Monte et al., 2003). Seedlings were then stratified for 4 d at 4°C in darkness, induced to germinate with 3 h of WLc, and then placed in the dark for the indicated period of time. For hypocotyl, hook, and cotyledon measurements, seedlings grown for 2, 3, and 4 d were arranged horizontally on a plate and photographed using a digital camera (Nikon D80). Measurements were performed using NIH Image software (Image J, National Institutes of Health), as described before (Leivar et al., 2008b). Hook angle was measured as the angle between the hypocotyl and an imaginary line between the cotyledons, and cotyledon angle was measured as the angle between the central axes of the two cotyledons. Measurements of at least 30 seedlings for each mutant line were tested using Excel (Microsoft) for statistically significant differences with the wild-type sibling controls. P values were determined by Student's *t* test (equal variance, two-tailed distribution), and values below P = 0.05 were considered statistically significant for differences in hypocotyl length, hook angle, or cotyledon angle between the wild-type and mutant lines. Mean values were used to calculate relative differences between the mutant and wild-type sibling, and phenotypes were expressed relative to the wild-type sibling value set at unity. Representative lines for each mutant were used in Figure 2 and Supplemental Data Set 5 online, whereas Figures 3 and 4 show all lines used in the analysis of the selected genes. For the red light treatments shown in Figure 5, seedlings were transferred after dark growth to Rc (8 μmols/m^2/s) for the time indicated. For the cotyledon separation experiment shown in Figure 6, cotyledon angle was calculated as specified above except for angles exceeding 180°, where outer angles were measured and corrections applied, because Image J only measures angles between 0° and 180°. For the FR treatments shown in Supplemental Figure 10 online, seedlings were transferred after 21 h of dark growth to continuous FR (0.01 μmols/m^2/s) at 21°C for 3 d. Control seedlings were kept in darkness. The DEX treatment shown in Figure 4 was performed using DEX (Sigma-Aldrich) diluted in HPLC-grade ethanol (minimum 98%) at a concentration of 5 μM.

Microarray-Based Expression Profiling: Samples and Data Analysis

Samples for microarray experiments in the dark correspond to samples in Monte et al. (2004), with the exception of R18 h and D18 h, which were part of the same experiment but were not included in the original analysis. Briefly, three biological replicates of wild-type and *pif3-3* seedlings were grown separately in GM medium without Suc for 4 d (96 h) in the dark (D0 h time point) as previously described (Monte et al., 2003). For dark treatments, D0 h (D96 h) and D1 h (D97 h) samples were harvested 1 h apart and were used in this work to identify PIF3-regulated genes in the dark. For red light treatments, 4-d-old wild-type seedlings were transferred to Rc (8 μmols/m^2/s) at D0 h, and samples were collected after 1 h (R1 h) and 18 h (R18 h), together with controls at D1 h and D18 h. These red light-treated samples and their dark controls were used in this work to identify early (R1 h) and late (R18 h) red light-responsive genes.

Dark data analysis was performed using the Rosetta Resolver Gene Expression Analysis System, version 7.0 (Rosetta Biosoftware). A gene list of transcripts whose expression is significantly altered by the *PIF3* mutation in 4-d-old, dark-grown seedlings was calculated by performing a two-group, two-way, error-weighted, Benjamini-Hochberg false discovery rate error-corrected analysis of variance comparing D0 h and D1 h samples for the wild type and *pif3*, with a P-value cutoff of 0.05, resulting

in 1402 significant transcripts. This statistical significance test was combined with experimental consistency by further reducing the statistically significant transcript list to only those transcripts exhibiting an absolute FC of greater than 1.5-fold in both D0 h and D1 h conditions. This resulted in a nonredundant list of 122 transcripts (statistically and significantly different by an absolute FC of 1.5, SS1.5F). Next, a ratio error model (Weng et al., 2006) that reduced the transcript list to 82 HC PIF3 target genes in the dark (SS1.5F-HC) was applied.

To identify early and late red light-responsive genes, wild-type red (R1 h and R18 h) and wild-type dark samples (D1 h and D18 h) were analyzed at each time point using the Rosetta Resolver Gene Expression Analysis System, version 7.0 (Rosetta Biosoftware). A list of Rc-responsive transcripts was calculated by performing a ratio analysis applying a ratio error model cutoff of 0.05 (Weng et al., 2006) and an absolute FC of greater than twofold. These analyses resulted in 546 significant transcripts (statistically and significantly different by an absolute FC of 2; SS2F) for R1 h, and 2764 SS2F genes for R18 h.

Gene Expression Analysis

For the RNA gel blot analyses in Figure 3, total RNA was isolated from 2-d-old, dark-grown seedlings using the RNeasy Plant Mini Kit (Qiagen), according to a previously described procedure (Monte et al., 2003). Gene-specific probes were amplified by PCR and labeled by random priming (Roche). Primer sequences can be found in Supplemental Table 2 online. Hybridization signal was detected with a Storm 860 PhosphorImager (Molecular Dynamics).

For qRT-PCR analysis, seedlings were grown in the dark for the indicated times (for Figure 4; see Supplemental Figures 2 and 6 online) or subsequently treated with red light (8 μmol/m^2/s) for up to 12 h for the analysis shown in Figure 5B. qRT-PCR analysis was performed as described previously (Khanna et al., 2007) with variations. Briefly, 10 μg of total RNA extracted using the RNeasy Plant Mini Kit (Qiagen) were treated with DNase (Ambion) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using the SuperScript III reverse transcriptase (Invitrogen) and oligo dT as a primer (dT30). cDNA was then treated with RNase Out (Invitrogen) before 1:20 dilution with water, and 20 μL was used for real-time PCR (Light Cycler 480; Roche) using SYBR Premix Ex Taq (Takara) and primers at a 300 nM concentration. Each PCR was repeated at least two times, and the mean expression values from these technical replicates were used for further calculations. Gene expression was measured from at least two biological replicates, and *PP2A* was used as a normalization control as described previously (Shin et al., 2007). Normalized gene expression is represented relative to the dark-grown wild-type set at unity. Primer sequences for qRT-PCR can be found in Supplemental Table 2 online.

Promoter Analysis for Presence of G-Box Motifs

Promoter analysis was performed using the "Motif Analysis" tool available at The Arabidopsis Information Resource (http://*Arabidopsis*.org/tools/bulk/motiffinder/index.jsp) to look for the CACGTG G-box motif in the 3-kb region upstream of the start codon of each of the *MIDA* genes.

Accession Numbers

The microarray data reported in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under the GEO Series accession number GSE30030. Sequence data can be found in the *Arabidopsis* Genome Initiative database under accession numbers AT5G50600 (MIDA1/HSD1), AT3G05730 (MIDA2), AT4G37300 (MIDA3), AT1G02470 (MIDA4), AT3G47250 (MIDA5), AT5G04340 (MIDA6), AT1G48260 (MIDA7/CIPK17), AT4G10020 (MIDA8/HSD5), AT5G02760 (MIDA9), AT4G10240 (MIDA10/BBX23), AT2G46070 (MIDA11/MPK12), AT1G05510 (MIDA12), AT5G45690 (MIDA13), and PP2A (AT1G13320).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. PIF3 Regulation of Gene Expression in the Dark.

Supplemental Figure 2. Distribution of PIF3-Regulated Genes among Functional Categories.

Supplemental Figure 3. qRT-PCR Validation of Microarray Data.

Supplemental Figure 4. Comparative Expression Analysis of PIF3 Function with Other PIF Factors in the Dark.

Supplemental Figure 5. Comparison of *pif3*-D SSTF-HC Genes versus *pifq*-D and *pif4pif5*-D.

Supplemental Figure 6. Expression of *MIDA* Genes in *pif3* and *pifq*.

Supplemental Figure 7. Characterization of *mida9-2*.

Supplemental Figure 8. Light-Responsiveness of PIF3-Regulated Genes in the Dark.

Supplemental Figure 9. Microarray Data Displaying the Mean Expression Level of *MIDA10*, *MIDA9*, *MIDA11*, and *MIDA1* after 1 h and 18 h of Rc Treatment.

Supplemental Figure 10. Hook Angle Phenotype Displayed by *mida9-1* in Continuous FR.

Supplemental Figure 11. Quantification of Hypocotyl Length Displayed by *mida11* in the Dark-to-Light Transition in the Absence of DEX.

Supplemental Figure 12. Quantification of Hypocotyl Length in *pif3* and Cotyledon Separation Angle in *pif3* and *pif3 pif4 pif5* after Dark-to-Light Transition.

Supplemental Table 1. Summary of *MIDA9*, *MIDA10*, *MIDA11*, and *MIDA1* Gene Regulation by PIF3 and by Red Light, as well as the Phenotype of Their Respective *Arabidopsis* Mutants.

Supplemental Table 2. Primer Sequences Used for PCR Amplification.

Supplemental Analysis 1. Definition of the PIF3-Regulated Transcriptome in the Dark.

Supplemental Analysis 2. Functional Classification of PIF3-Regulated Genes in the Dark.

Supplemental Analysis 3. Comparative Analysis of PIF3-, PIF4PIF5-, and PIFq-regulated Transcriptomes in the Dark.

Supplemental Analysis 4. Comparative Analysis of PIF3-Regulated Transcriptome in the Dark with Genes Displaying SSTF Alterations in the Wild Type after 1 h and 18 h of Red Light Treatment.

Supplemental References 1. The Supplemental References for Supplemental Analyses 1 to 4.

Supplemental Data Set 1. Expression Data and Statistical Analysis for the SS1.5F Genes at D0 h and D1 h Reported in Figure 1 and Supplemental Figure 1 online.

Supplemental Data Set 2. Expression Data and Statistical Analysis for the SS1.5F-HC Genes at D0 h and D1 h Reported in Figure 1 and Supplemental Figure 1 online.

Supplemental Data Set 3. List of SS1.5F Genes That Do Not Meet the Requirements to be Designated as SS1.5F-HC Genes as Reported in Supplemental Figure 1 online.

Supplemental Data Set 4. List of Class P3, P3/PQ, P3/P4P5, and P4P5/PQ Genes Reported in Supplemental Figure 4 online.

Supplemental Data Set 5. Primary Data and Statistical Analysis for *mida* Mutant Phenotypic Characterization Shown in Figure 2.

Supplemental Data Set 6. List of Early Light-Responsive Genes in Dark-Grown Wild-Type Seedlings after 1 h of Red Light Treatment (8 μmol/m^2/s) Reported in Supplemental Figure 8 online.

Supplemental Data Set 7. List of Late Light-Responsive Genes in Dark-Grown Wild-Type Seedlings after 18 h of Red Light Treatment (8 μmol/m^2/s) Reported in Supplemental Figure 8 online.

Supplemental Data Set 8. List of Induced Class 1 to 3 Genes (Ind-Class) and Repressed Class 1 to 4 Genes (Rep-Class) as Defined in Supplemental Figure 8 online.

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AUTHOR CONTRIBUTIONS

M.S., P.L., P.Q., and E.M. designed the research; M.S., G.M., N.G., J.S., J.T., and E.M. performed research. M.S. and E.M. analyzed data; and M.S., P.L., P.Q., and E.M. wrote the article.

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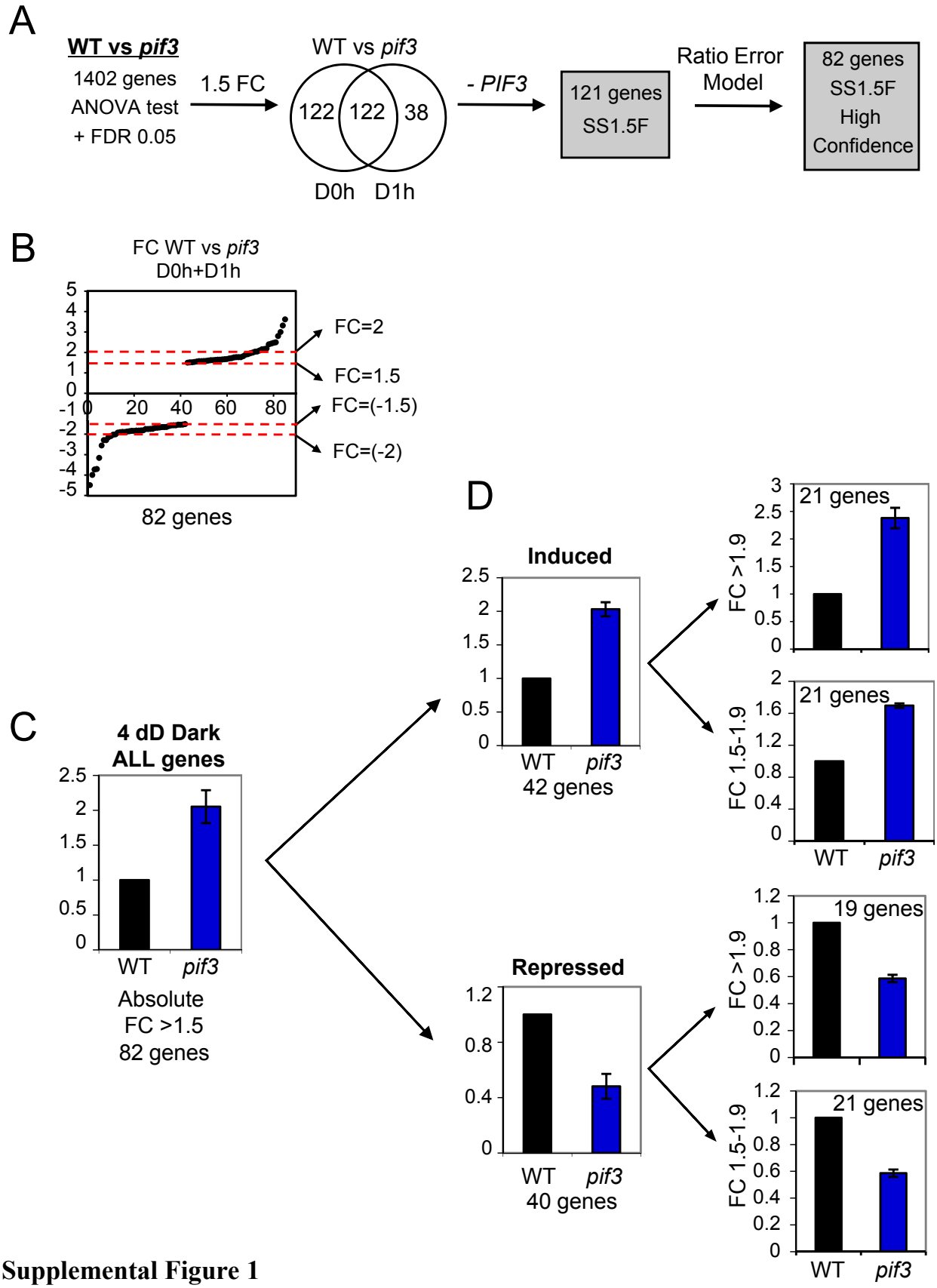


Supplemental Figure 1

Supplemental Figure 1. PIF3 regulation of gene expression in the dark.

(A) Microarray expression profiling identified 82 high-confidence (HC) target genes that are statistically significantly deregulated in the absence of PIF3 in the dark and by a fold-change (FC) greater than 1.5 (SS1.5F-HC).

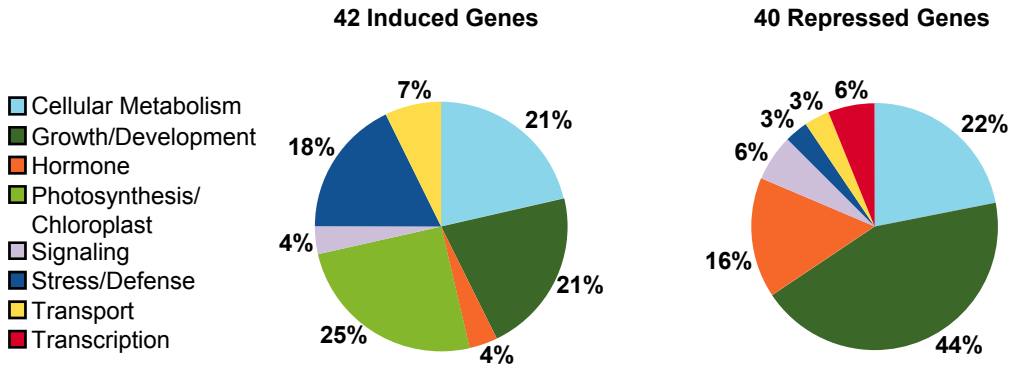
Flow chart of the analysis process to compare the expression profiles of WT and *pif3* seedlings in the dark. Statistical Anova test identified 1402 genes with a false discovery rate (FDR) set at 5%. The Venn diagram shows pairwise comparison between genes differentially expressed in WT seedlings compared to *pif3* at Dark 0 h (D0h) and Dark 1 h (D1h) time points with a FC greater than 1.5. The number of shared genes (SS1.5F gene list) is indicated in the intersection and contained *PIF3*, which was removed. The subsequent application of a ratio error model (Weng et al., 2006) yielded 82 high-confidence PIF3 target genes (SS1.5F-HC). Lists of each class of genes are in Supplemental Datasets 1 and 2.

(B) Scatter plot of FC values (*pif3*/WT) for the 82 genes showing altered SS1.5F-HC expression in *pif3* relative to WT.

(C) and (D). Sorting of PIF3 regulated gene expression in the dark according to FC difference. Mean FC for PIF3-regulated genes is expressed relative to the wild type dark value set at unity.

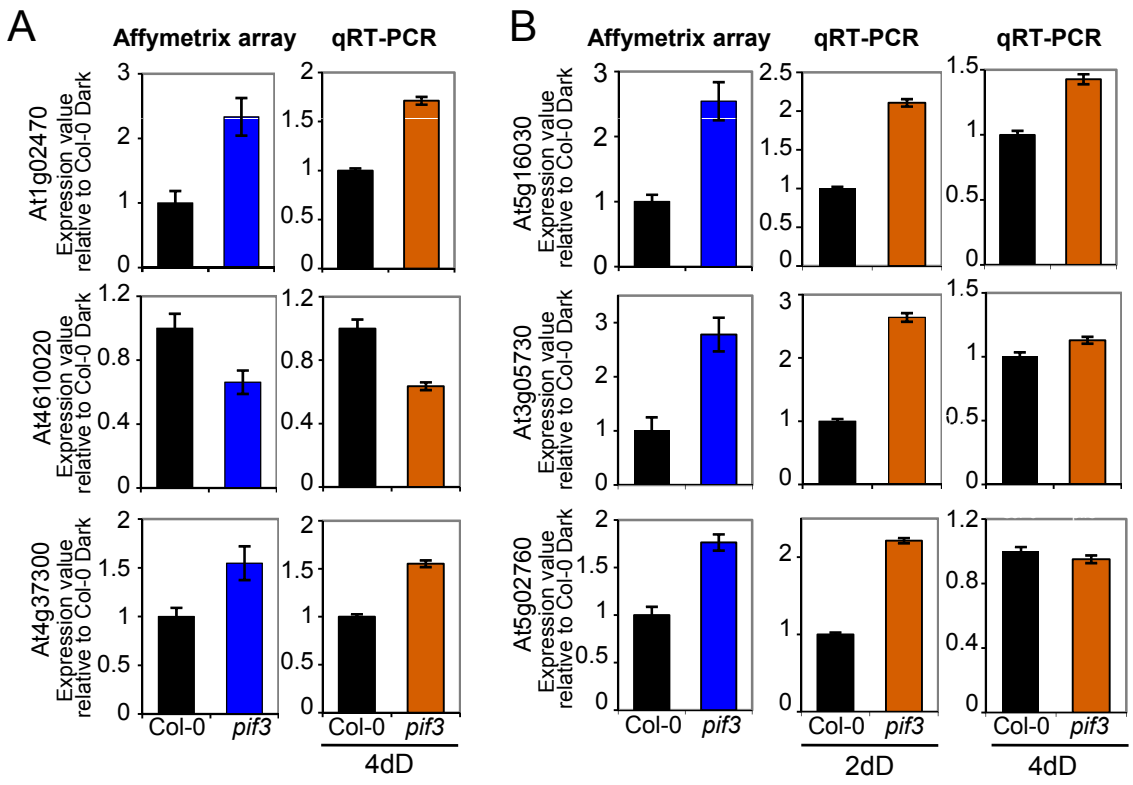
(C) Absolute mean FC for the 82 genes that are statistically significantly deregulated in the absence of PIF3 in the dark and by a FC greater than 1.5. Bars indicate SE for the genes averaged for each group.

(D) Mean FC for 42 up-regulated genes (induced) and the 40 down-regulated genes (repressed) in the *pif3-3* mutant in the dark. Induced and repressed genes were further classified according to their FC into two classes: FC >1.9 and FC 1.5-1.9. Bar graphs show the mean FC of each class of genes, and the number of genes falling into each category is specified. Bars indicate SE for the genes averaged for each group.



Supplemental Figure 2. Distribution of PIF3-regulated genes among functional categories.

Distribution of PIF3-regulated genes among functional categories, expressed as a percentage, for up- (left) and down- (right) regulated genes in the dark in the absence of PIF3. The assignment of functional categories was based on Gene Ontology annotations for biological and/or molecular function available at TAIR (www.Arabidopsis.org). Genes with unknown biological or molecular function (representing 34.8% of up-regulated and 19.5% of down-regulated genes) are not included.

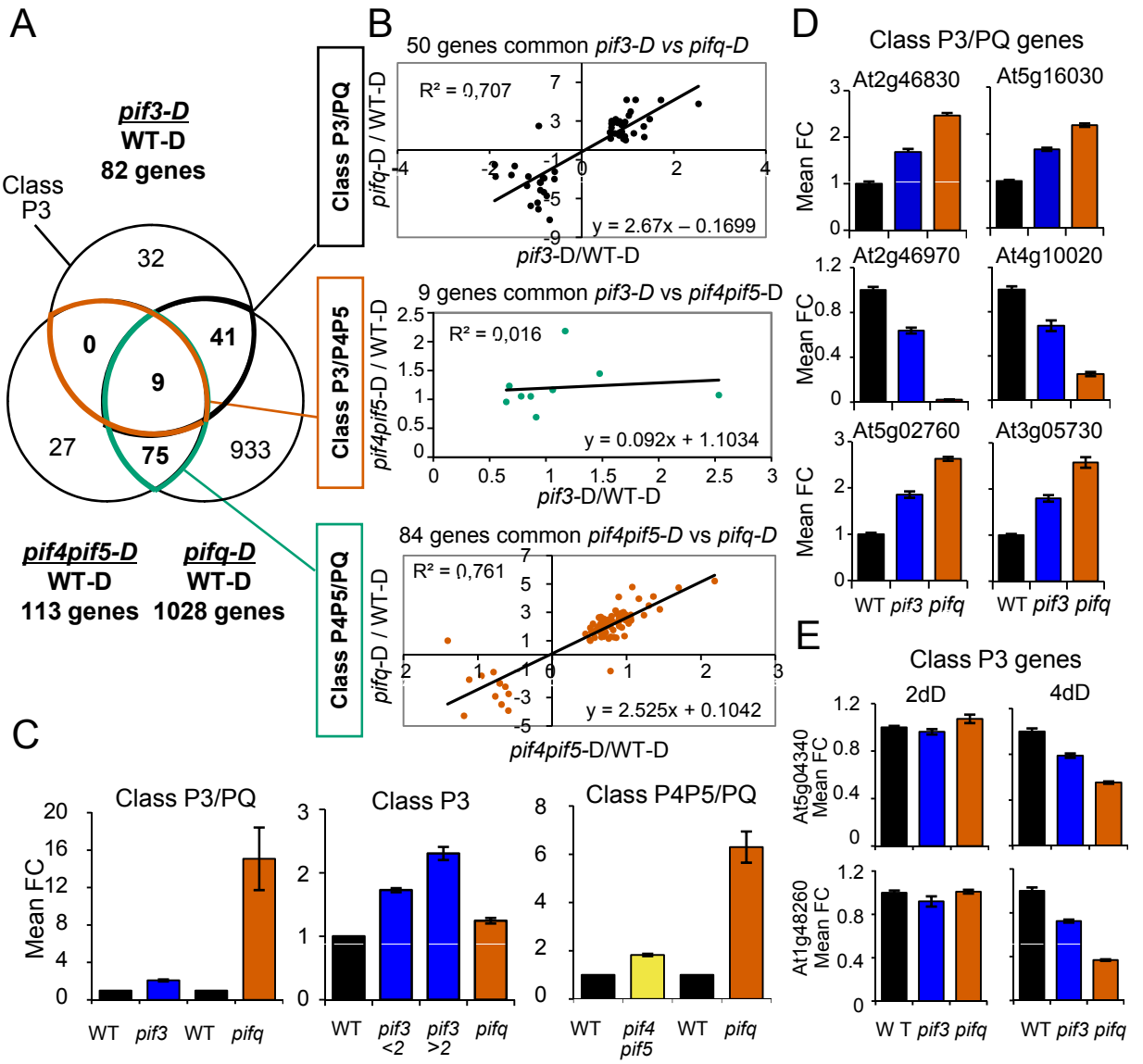


Supplemental Figure 3. qRT-PCR validation of microarray data.

(A) (B). Expression analysis of PIF3-dependent genes in WT Col-0 and *pif3-3* (*pif3*) mutant seedlings in the dark. For each gene, comparison of microarray and qRT-PCR data are shown. Microarray data displays the mean expression levels obtained by microarray analysis of three biological replicates relative to the Col-0 Dark value set at unity (bar graphs in blue). Validation of these results for each *MIDA* gene was performed by qRT-PCR analysis (bar graphs in vermillion). Levels were normalized to *PP2A* as described (Shin et al., 2007) and expressed relative to the Col-0 Dark value set at unity. Bars indicate SE of technical triplicates.

(A) Comparison of microarray data and qRT-PCR data performed on 4-d-old dark-grown seedlings (4dD).

(B) Comparison of microarray data and qRT-PCR data performed on 2-d-old dark-grown seedlings (2dD) and 4-d-old dark-grown seedlings (4dD).



Supplemental Figure 4. Comparative expression analysis of PIF3 function with other PIF factors in the dark.

Three-way comparison of genes responding to the *pif3* mutation (*pif3*-D versus *WT*-D), the *pif4 pif5* mutation (pif4pif5-D versus WT-D) (Lorrain et al., 2009), and the *pifq* mutation (*pifq*-D versus WT-D) (Leivar et al., 2009) in darkness.

(A) Venn diagram showing comparison of all genes in the three different sets of differentially PIF-regulated genes. This comparison between genes responding to *pif3-D*, *pif4pif5-D* and *pifq-D* resulted in the identification of four classes of genes responsive to: *pif3-D* and *pifq-D* (Class P3/PQ, 50 genes)(corresponding sections are indicated in black), *pif3-D* and *pif4pif5-D* (Class P3/P4P5, 9 genes)(indicated in vermillion), and *pif4pif5-D* and *pifq-D* (Class P4P5/PQ, 84 genes)(indicated in green), and to only *pif3-D* (Class P3, 32 genes). Lists for each class of genes are in Supplemental Dataset 4.

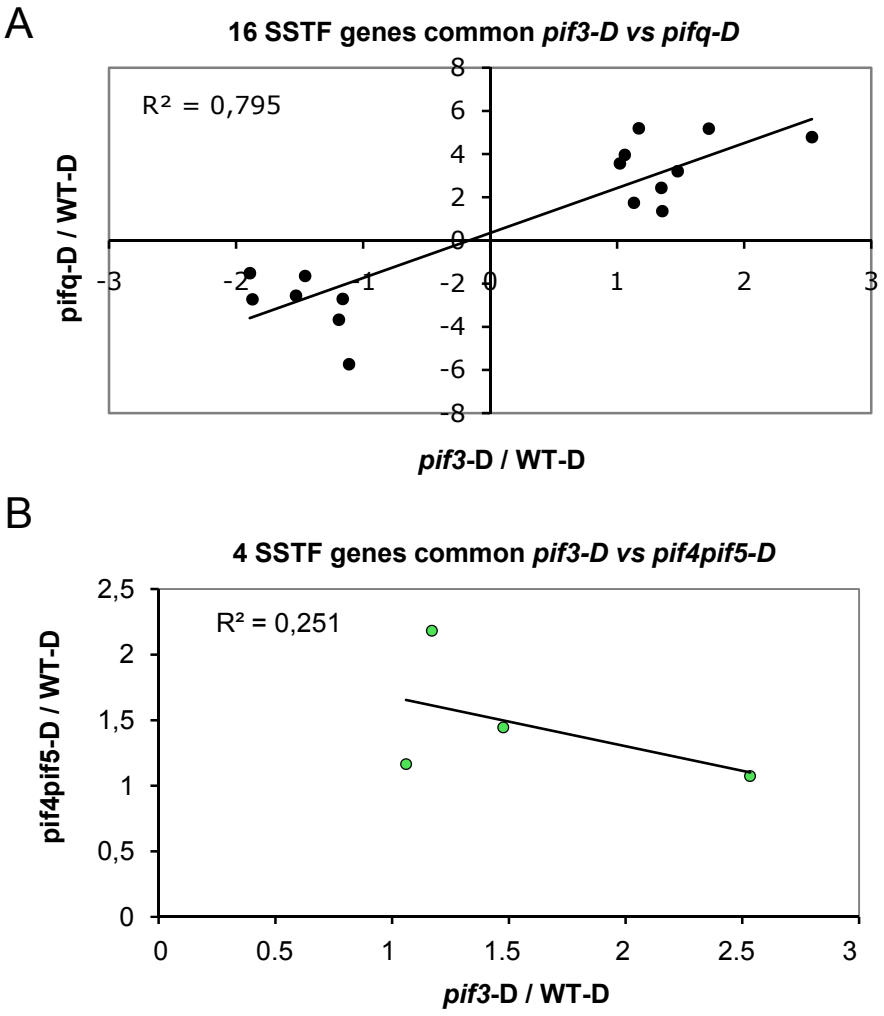
(B) Scatterplots of log2 fold change values for the three classes of genes identified in (A). The top plot corresponds to Class P3/PQ, the middle plot to Class P3/P4P5, and the bottom plot to Class P4P5/PQ. Dots in each plot represent genes that are shared between both genotypes as shown in the Venn diagram in (A). Correlation coefficient for the genes, the trendline and the regression equation are indicated.

Supplemental Figure 4 (Cont.)

(C) Mean fold change (FC) in WT, *pif3* and *pifq* for the 50 Class P3/PQ genes (left), the 32 Class P3 genes (middle), and the 84 Class P4P5/PQ genes (right) relative to the WT dark value set at unity. Class P3 genes are divided between FC<2 (17 genes, pif3<2 in legend) and FC>2 (15 genes, pif3>2 in legend). Bars indicate SE for the genes averaged for each group.

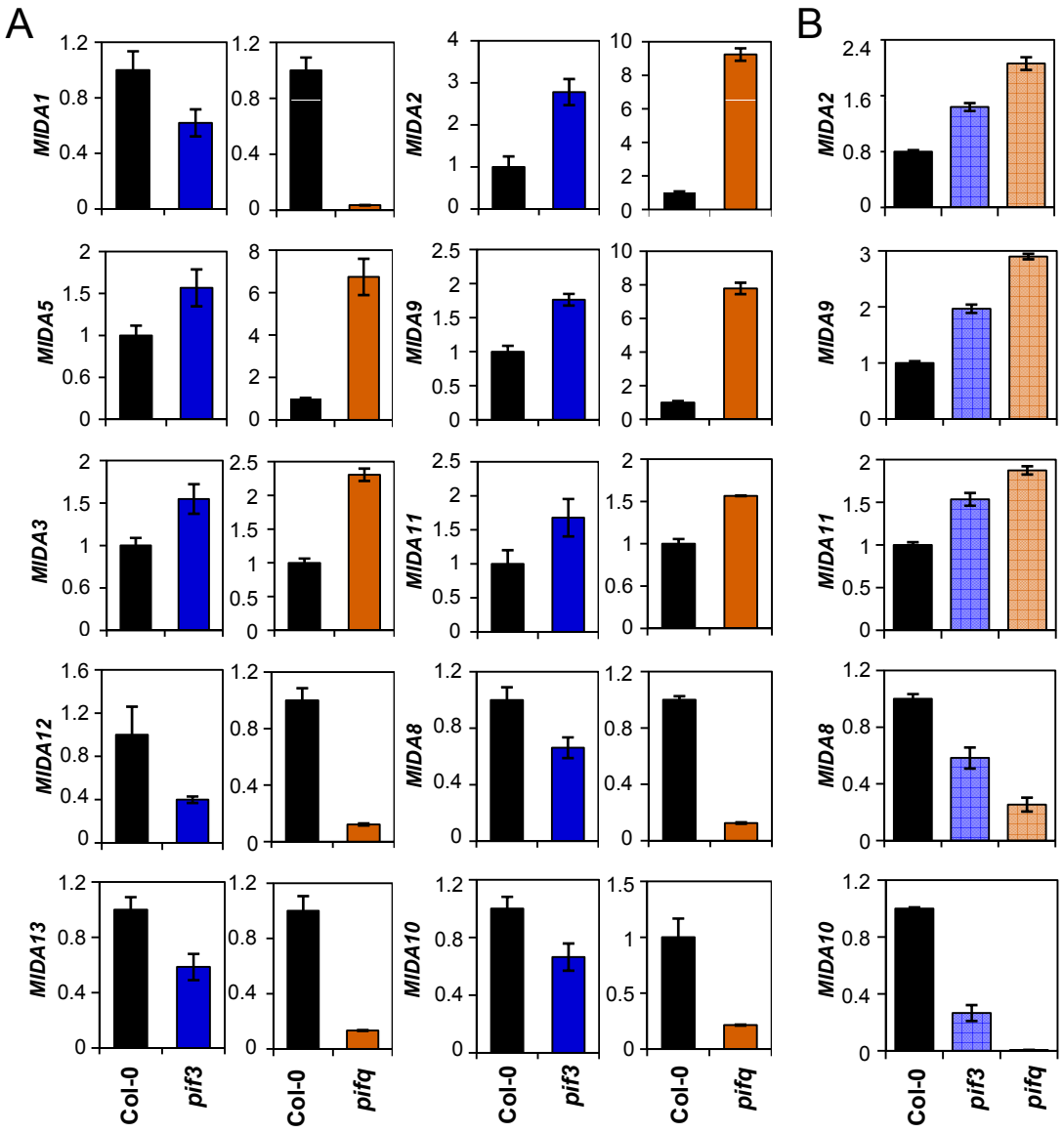
(D) Expression analysis by qRT-PCR of Class P3/PQ genes in 2-day-old dark-grown Col-0 wild-type (WT), *pif3-3* (*pif3*), and *pifq* mutant seedlings. *AT2G46830* and *AT2G46970* were selected as controls based on Leivar et al., 2009. Expression levels were normalized to *PP2A* as described (Shin et al., 2007) and expressed relative to the WT value set at unity. Error bars represent SE values of technical triplicates.

(E) Expression analysis by qRT-PCR of Class P3 genes *AT1G48260* and *AT5G04340,* in 2- (2dD) and 4-day-old (4dD) dark-grown Col-0 wild-type (WT), *pif3-3* (*pif3*), and *pifq* mutant seedlings. Expression levels were normalized to *PP2A* as described (Shin et al., 2007), and expressed relative to the 2dD WT value set at unity. Error bars correspond to SE values of technical triplicates.

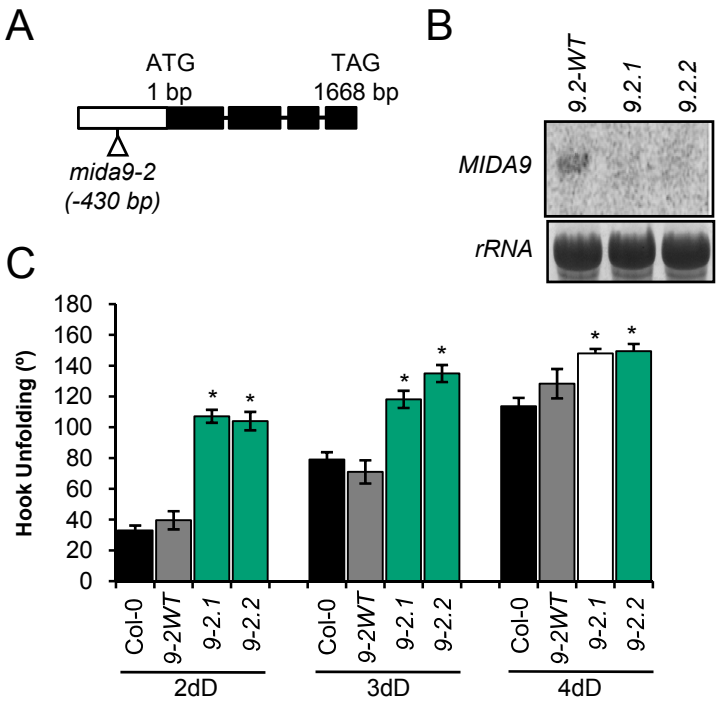


Supplemental Figure 5. Comparison of *pif3*-D SSTF-HC genes versus *pifq*-D and *pif4pif5*-D.

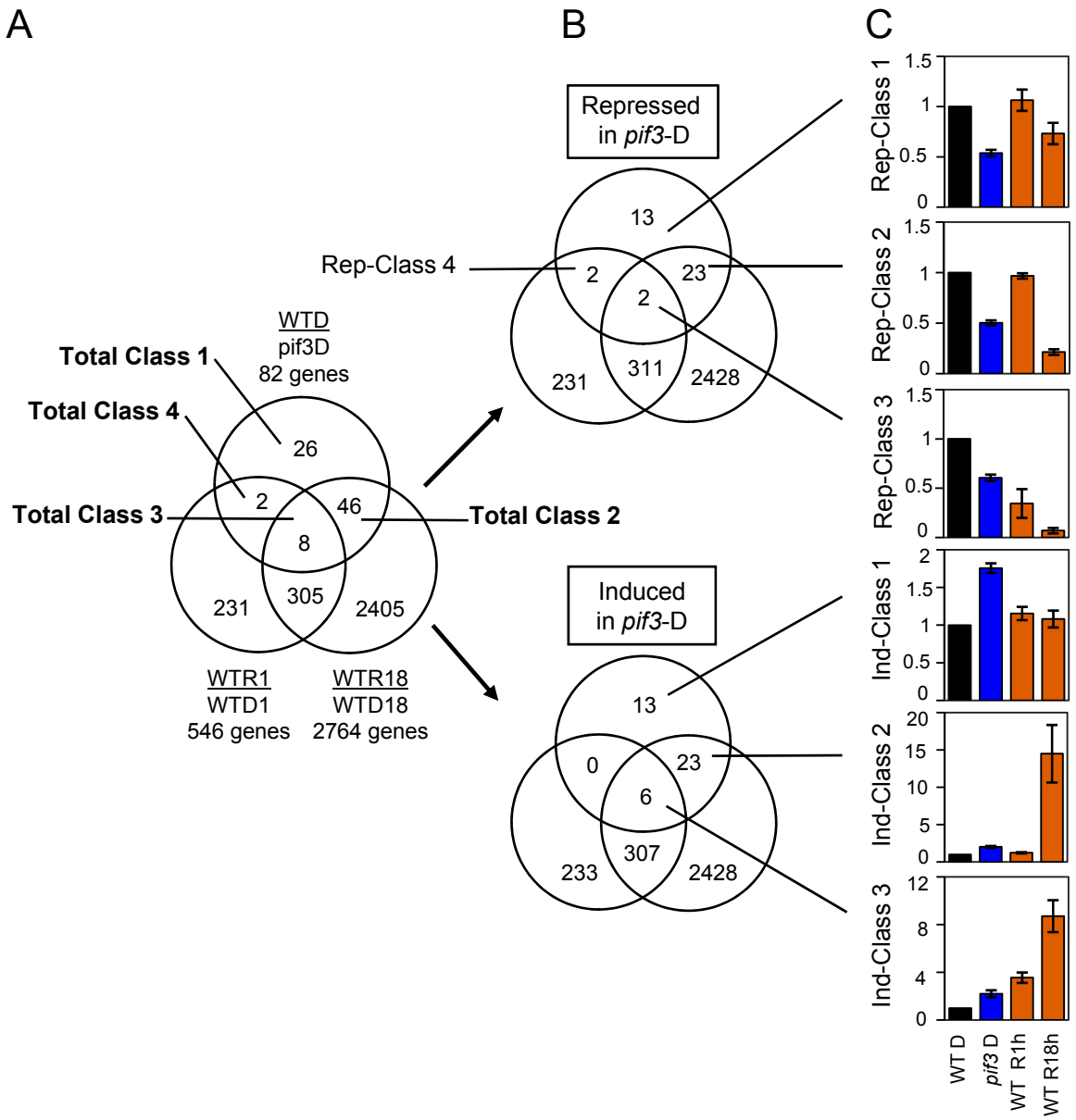
Scatterplots of log2 fold change values for *pif3*-D SSTF-HC genes shared with *pifq*-D (A) and *pif4pif5*-D (B). Dots in each plot represent genes that are shared between both genotypes. Correlation coefficient for the genes and the trendline are indicated.



Supplemental Figure 6. **Expression of *MIDA* genes in *pif3* and *pifq*.**
(A) Microarray expression analysis of *MIDA* genes in WT Col-0, *pif3-3* (*pif3*) (in blue), and *pifq* (in vermillion) mutant seedlings in the dark. For each gene, comparison of *pif3* (this work) and *pifq* (Leivar et al., 2009) microarray data are shown displaying the mean expression levels obtained by microarray analysis of three biological replicates relative to the Col-0 Dark value set at unity. Bars indicate SE.
(B) qRT-PCR expression analysis of selected *MIDA* genes in wild-type Col-0, *pif3-3* (*pif3*) (in light blue), and *pifq* (in light vermillion) in 2-day-old dark-grown seedlings. Expression levels assayed by qRT-PCR were normalized to *PP2A* as described (Shin et al., 2007). Data correspond to technical triplicates and error bars indicate SE.



Supplemental Figure 7. Characterization of *mida9-2*.
(A) The mutation identified in Arabidopsis *MIDA9*. The T-DNA insert in *mida9-2* is indicated at position -430 bp relative to the ATG.
(B) RNA blot of 2-day-old, dark-grown wild-type and *mida9-2* mutant seedlings. No *MIDA9* transcript was detected in *mida9-2,* indicating that the line is likely a functional knock-out mutant.
(C) Quantification of hook angle in *mida9-2.1* and *mida9-2.1* compared to Col-0 and a WT sibling line after 2, 3 and 4 days of growth in the dark (dD) after germination. Bars correspond to SE of at least 30 seedlings and asterisks indicate statistically different mean values compared to their corresponding WT.



Supplemental Figure 8. Light-responsiveness of PIF3-regulated genes in the dark.
(A) (B) and (C). Three-way comparison of genes responding to the *pif3* mutation in darkness (SS1.5F-HC of WT-D versus *pif3*-D) (Supplemental Dataset 2), 1 h Rc in the WT (genes displaying statistically significant differences in gene expression by at least two fold (SSTF) of WT-R1 versus WT-D1) (Supplemental Dataset 6), and to 18 h Rc in the WT (SSTF of WT-R18 versus WT-D18) (Supplemental Dataset 7). Classification of genes as induced or repressed (B) is based on the direction of the response of *pif3*-D relative to the WT-D. This classification includes all genes shown in A except those that are not light responsive in *pif3* (Total Class 1), which are divided between induced or repressed accordingly.
(A) Venn diagram showing comparison of all genes in the three sets of differentially regulated genes. This comparison resulted in the identification of four classes of genes responsive to: only *pif3-D* (Total-Class 1, 26 genes), *pif3-D* and WT-R18h only (Total-Class 2, 46 genes), *pif3-D* and WT-R1h and R18h (Total-Class 3, eight genes), and *pif3-D* and WT-R1h only (Total-Class 4, two genes).

Supplemental Figure 8. (Cont)

(B) (Top) Venn diagram showing comparison of repressed genes in PIF3-deficient seedlings in the dark with light responsive genes in the WT at R1h and R18h. This comparison resulted in the identification of four classes of repressed genes responsive to: only *pif3-D* (Rep-Class 1, 13 genes), *pif3-D* and WT-R18h only (Rep-Class 2, 23 genes), *pif3-D* and WT-R1h and R18h (Rep-Class 3, two genes), and *pif3-D* and WT-R1h only (Rep-Class 4, two genes).

(Bottom) Venn diagram showing comparison of induced genes in PIF3-deficient seedlings in the dark with light responsive genes in the WT at R1h and R18h. This comparison resulted in the identification of three classes of induced genes responsive to: only *pif3-D* (Ind-Class 1, 13 genes), *pif3-D* and WT-R18h only (Ind-Class 2, 23 genes), and *pif3-D* and WT-R1h and R18h (Ind-Class 3, six genes). Gene lists for each class of genes are presented in Supplemental Dataset 8.

(C) Bar graphs showing the mean fold change in expression relative to WT-D (set at unity) for all genes in each class as defined in (B). Error bars represent the mean SE for the genes averaged for each genotype and treatment combination defined in (B). Excluded from this analysis are a few ambiguous genes that respond in a different direction in *pif3-D*, WT-R1h and/or WT-R18h relative to WT-D: two genes in Repressed Class 4 and five genes in Induced Class 2 (Supplemental Dataset 8).

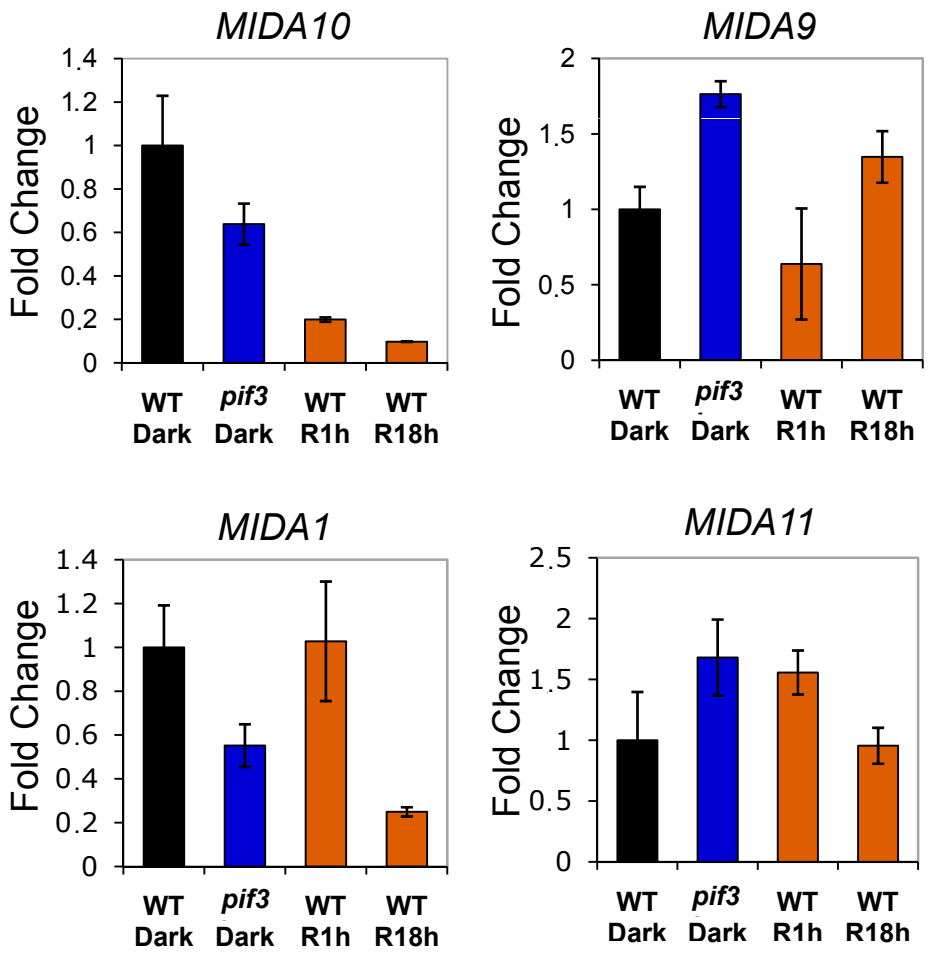
pif3-D, genes misexpressed in 4-day-old, dark-grown seedlings in the absence of PIF3 compared to the wild type.

WTD, wild type after 4 days in darkness

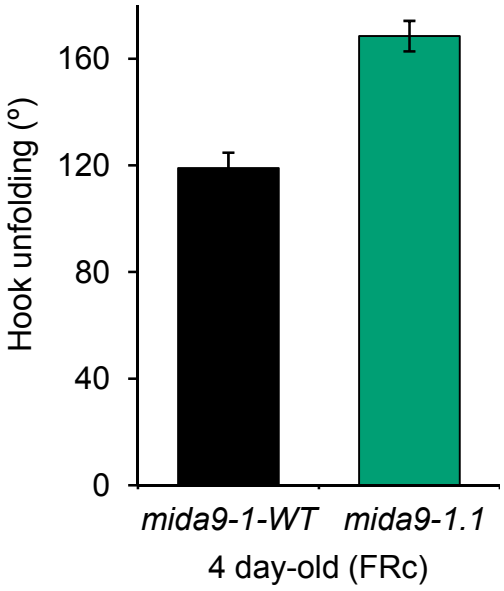
WTR1, wild type after 1 h of Rc relative to WTD

WTR18, wild type after 18 h of Rc relative to WTD

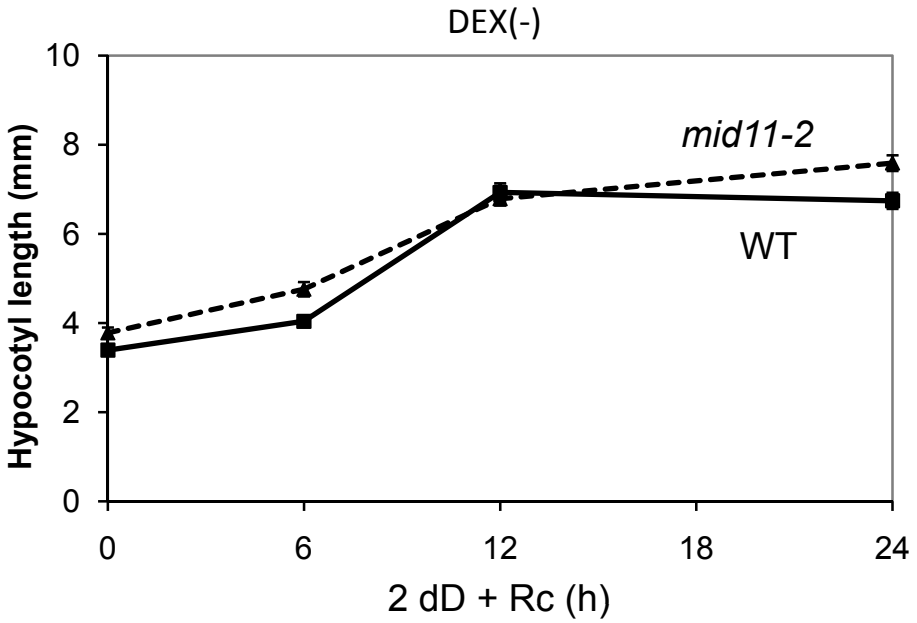
pif3D, *pif3* after 4 days in darkness



Supplemental Figure 9. Microarray data displaying the mean expression level of *MIDA10, MIDA9, MIDA11* and *MIDA1* after 1 h and 18 h of Rc treatment.
Microarray data displaying the mean expression level obtained for *MIDA10, MIDA9, MIDA11* and *MIDA1* replicates relative to the Col-0 Dark value set at unity after 1 h (WT R1h) or 18 h (WT R18h) of Rc (8 umol/m^2/s). Dark levels in the *pif3* mutant are also shown for comparison (*pif3* Dark). Dark, WT level is set at unity (WT Dark). Error bars correspond to SE values of three biological replicates.

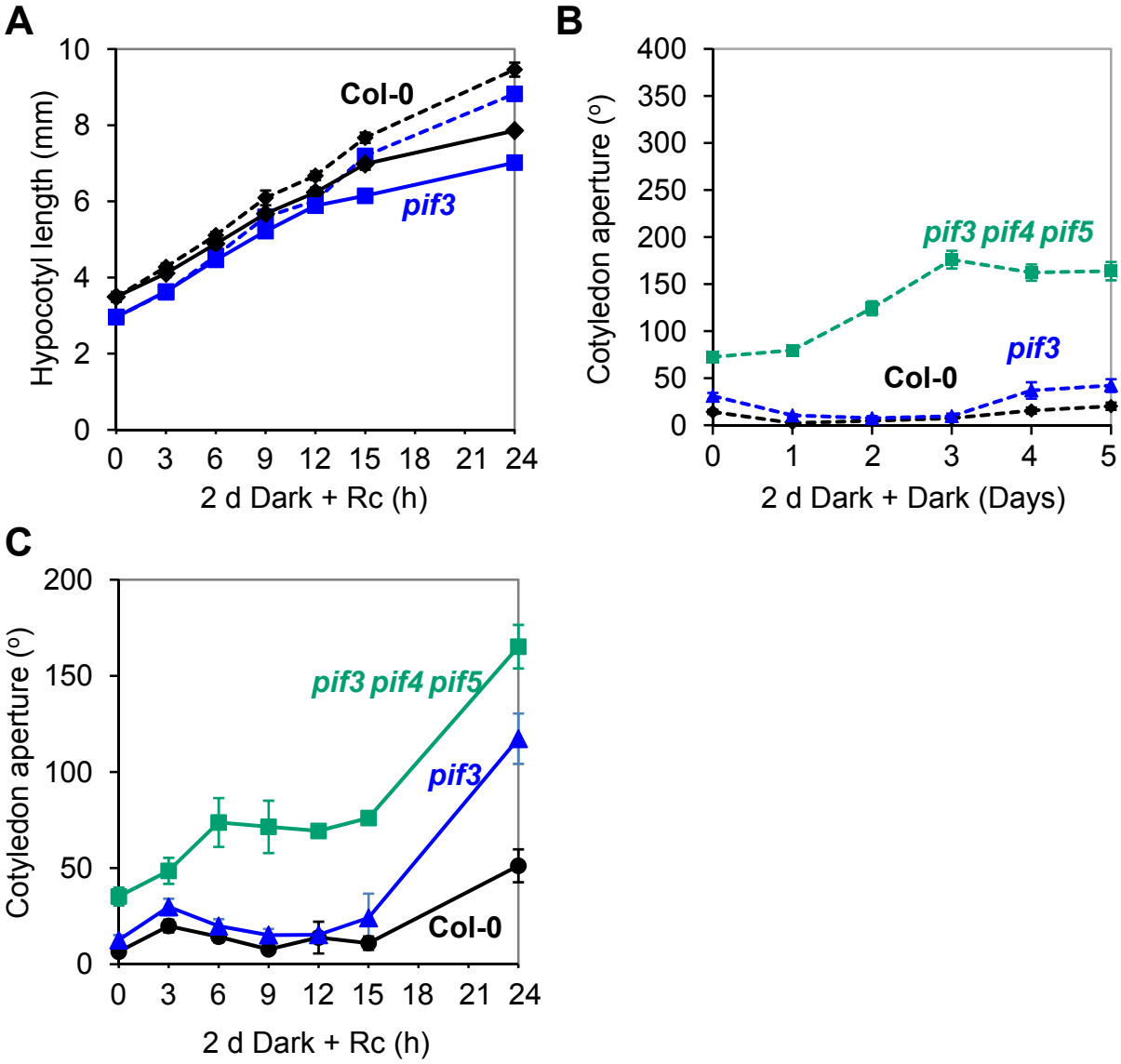


Supplemental Figure 10. Hook angle phenotype displayed by *mida9-1* in FRc
Quantification of the hook angle exhibited by 4-day-old *mida9-1.1* mutant seedlings compared to WT control siblings *mida9-1-WT* after growth in continuous far-red light (FRc) (0.01 umol/m^2/s). Bars indicate SE of at least 30 seedlings.



Supplemental Figure 11. Quantification of hypocotyl length displayed by *mida11* in the dark-to-light transition in the absence of DEX.

Time-course quantification of hypocotyl growth in the WT (solid line) and *mida11-2* (dashed line), of 2-day-old dark-grown seedlings during the dark-to-Rc transition (Rc = 8 umol/m^2/s) in the absence of dexamethasone (DEX). Bars indicate SE of at least 30 seedlings.



Supplemental Figure 12. Quantification of hypocotyl length in *pif3* and cotyledon separation angle in *pif3* and *pif3 pif4 pif5* after dark-to-light transition.

(A) Time-course quantification of hypocotyl length of 2-day-old, dark-grown Col-0 and *pif3* seedlings kept in the dark (dashed lines) or during the dark-to-light transition (solid lines) for 24 h.

(B) Time-course quantification of cotyledon separation of 2-day-old, dark-grown Col-0, and *pif3* and *pif3 pif4 pif5* seedlings kept in the dark for 5 days.

(C) Time-course quantification of cotyledon separation of 2-day-old, dark-grown Col-0, *pif3* and *pif3 pif4 pif5* seedlings during the dark-to-light transition for 24 h.

(A) (B) (C). Bars indicate SE of at least 30 seedlings.

MIDA	**Regulation by PIF3 in the Dark**	**Response in *pif3*-D**	**Response to Red Light**	**Dark K.O. phenotype**	**Dark OX phenotype**	**Role in Deetiolation**
MIDA9	Repressed	Induced	Induced (Late)	Open hooks		Repressor
MIDA10	Induced	Repressed	Repressed (Early)	Open hooks		Repressor
MIDA11	Repressed	Induced	Induced (Late)	Short hypocotyl		Repressor
MIDA1	Induced	Repressed	Induced (Early) Repressed (Late)		Open Cotyledons	Inducer

Supplemental Table 1. Summary of *MIDA9*, *MIDA10*, *MIDA11* and *MIDA1* gene regulation by PIF3 and by red light, as well as the phenotype of their respective *Arabidopsis* mutants.

Newly identified MIDA factors MIDA9, MIDA10, MIDA11 and MIDA1 are novel regulators of photomorphogenesis in the dark. This table summarizes their gene regulation by PIF3 and by red light (8 umol/m^2/s), as well as the phenotype of the respective *Arabidopsis mida* mutants and the inferred role of each MIDA factor as Inducer or Repressor in deetiolation. "Early" indicates a response within 1-3h, whereas "Late" indicates a response after 3h. K.O. and OX indicate loss-of-function and overexpressor mutants respectively.

	Reference			Sequence from 5' to 3'
Use	**Gene**	**AGI number**	**Code**	
Genotyping	*MIDA2*	*AT3G05730*	EMP33	TATCACAATTAGCCTCAGCCG
			EMP34	ACCACCCCACCCTTGTACTAC
	MIDA3	*AT4G37300*	EMP67	TTTTGCTAGAAGGTCTGCTGG
			EMP68	AAAGCTGTGGACAGAGACGAC
	MIDA4	*AT3G47250*	EMP27	TGGCAAGAAACTTAAATTTGGAG
			EMP28	TGGCAACGAGAATGAGGTATC
	MIDA5	*AT1G02470*	EMP23	CTCCATAAACGGTTTCATTGC
			EMP24	CCAAATCGACTCACCGTTAAC
	MIDA6	*AT5G04340*	EMP314	TGCCCAATACAAATTTGTCAAC
			EMP315	AGTAAGCGAAAAGCTTTTCCG
	MIDA7	*AT1G48260*	EMP307	AAACATGCATCCATCTTGGAG
			EMP308b	CTTCTCGATGATTTTGATGG
	MIDA8	*AT4G10020*	EMP318	TGGGCTTGCGGTATAATGAGG
			EMP319	AATTCGATGCAGTGGATCATC
	MIDA9	*AT5G02760*	EMP69	AAGAATGGTGGGGTCATTAGG
			EMP70	GACAGAGAATCATCATCGAACAG
	MIDA9	*AT5G02760*	EMP223	AATGTGCCTTGAACTGTCGG
			EMP105	ACGAGAGACTGAGAAAAGGGC
	MIDA10	*AT4G10240*	EMP305	TATGATCCCACCACACATGTG
			EMP306	TGGTCAAATCCAACAAGGTC
	MIDA12	*AT1G05510*	EMP9	ATTTCCGGATAAAGTTGTCCG
			EMP10	GTCATAGTCCATGCAAATGCC
	MIDA13	*AT5G45690*	EMP7	CCCCTGAAATTACCAAAACATAAC
			EMP8	CCTTCTCAAATCATCCACGTC
qRT-PCR	*MIDA1*	*AT5G50600*	EMP378	GATTGAGTGGGGTTGTCGG
			EMP379	TACAGAGTACTACTACGTACACC
	MIDA2	*AT3G05730*	EMP346	CGAAGTCACAGTGTATTACCC
			EMP347	AATGCTCTTCTTCGTTGTCATG
	MIDA3	*AT4G37300*	EMP350	GAAGGAAGACAACGGTGAAG
			EMP351	CCGGATTGCTTCTGTAAACC
	MIDA5	*AT1G02470*	EMP340	TTCAGACCCGTTATGCAATGG
			EMP341	GCGTATAACTTGTAAGCCACG
	MIDA6	*AT5G04340*	EMP354	TTCGCTTACTCAATCTGCCG
			EMP355	ACGTGCGACTTCACACTTCC
	MIDA7	*AT1G48260*	EMP342	AATGAGCTGGGCTCATCATCAC
			EMP343	GAAAATGTCTCTAGCATCCCG
	MIDA8	*AT4G10020*	EMP348	CCACCTCGAGTTCCTGCAAG
			EMP349	GCTTGCAGGATACCGTGGTG
	MIDA9	*AT5G02760*	EMP352	TCATGTTGCTTGGCAGGAGTG
			EMP353	TAACTGAACAGCTCTCACTCC
	MIDA10	*AT4G10240*	EMP426	TCCAAAGACATCACCGAGTCG
			EMP427	GTACCCTTTTCTCTCCTGGCAG
	MIDA11	*AT2G46070*	EMP344	CCAGTGATCAATGCCGTTTCC
			EMP345	TCGAGTTAAGTAGCACGTTGC
	CCA1	*AT2G46830*	EMP368	CCGCAACTTTCGCCTCAT
			EMP369	GCCAGATTCGGAGGTGAGTTC
	PIL1	*AT2G46970*	EMP370	AAATTGCTCTCAGCCATTCGTGG
			EMP371	TTCTAAGTTTGAGGCGGACGCAG
	UNK	*AT5G16030*	EMP356	CTCATGGGTGAGATCAAGAC
			EMP357	AGATGAGGAACACAAATAGGG
	PP2A	*AT1G13320*	EMP338	TATCGGATGACGATTCTTCGTGCAG
			EMP339	GCTTGGTCGACTATCGGAATGAGAG
Probes for RNA Blots	*MIDA9*	*AT5G02760*	EMP104	ACAACCAGCACTGCTACTAC
			EMP105	AATGTGCCTTGAACTGTCGG
	MIDA10	*AT4G10240*	EMP326	GACATCACCGAGTCGCC
			EMP327	CTCCGGAACCATGATGTTG

Supplemental Table 2. Primer sequences used for PCR amplification.